Aus dem
Institut für translationale Pharmakologie
(Direktor: Prof. Dr. med. Maria Grandoch)
der Medizinischen Fakultät
der Heinrich-Heine-Universität Düsseldorf

Insights into the Regulation of Vascular Function by Reactive Oxygen and Nitrogen Species in Health and Disease

Habilitationsschrift
zur Erlangung der *Venia legendi*
für das Fach Pharmakologie
an der Heinrich-Heine-Universität
Düsseldorf

vorgelegt von
Tatsiana Suvorava, PhD
aus Minsk, Belarus
Düsseldorf, 2022

Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass diese Habilitationsschrift selbstständig und ohne unzulässige fremde Hilfe erstellt worden ist.

Bei den wissenschaftlichen Untersuchungen die Gegenstände der schriftlichen Habilitationsleistung sind, wurden die ethischen Grundsätze und die jeweils gültigen Empfehlungen zur Sicherung guter wissenschaftlicher Praxis beachtet.

Diese Habilitationsschrift wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Außerdem erkläre ich, dass ich bisher noch keine weiteren Habilitationsverfahren eingeleitet oder erfolglos beendet habe.

Düsseldorf, den 11.11.2022 Tatsiana Suvorava, PhD

CONTENT

LIST OF PAPERS SELECTED FOR HABILITATION

This work is based on the following peer-reviewed research papers (the order is reverse chronological, * denotes equal contribution):

1. Leo F*, **Suvorava T***, Heuser SK, Li J, LoBue A, Barbarino F, Piragine E, Schneckmann R, Hutzler B, Good ME, Fernandez BO, Vornholz L, Rogers S, Doctor A, Grandoch M, Stegbauer J, Weitzberg E, Feelisch M, Lundberg JO, Isakson BE, Kelm M, Cortese-Krott MM. (2021) Red Blood Cell and Endothelial eNOS Independently Regulate Circulating Nitric Oxide Metabolites and Blood Pressure. *Circulation*;144(11): 870-889.
2. Schneckmann R, **Suvorava T**, Hundhausen C, Schuler D, Lorenz C, Freudenberger T, Kelm M, Fischer JW, Flögel U, Grandoch M. (2021) Endothelial Hyaluronan Synthase 3 Augments Postischemic Arteriogenesis through CD44/eNOS Signaling. *Arterioscler Thromb Vasc Biol*; 41(10): 2551-2562.
3. **Suvorava T***, Pick S*, Kojda G. (2017) Selective Impairment of Blood Pressure Reduction by Endothelial Nitric Oxide Synthase Dimer Destabilization in Mice. *J Hypertens*. 2017 Jan; 35(1): 76-88.
4. Kiene LS, Homann S, **Suvorava T**, Rabausch B, Müller J, Kojda G, Kretschmer I, Twarock S, Dai G, Deenen R, Hartwig S, Lehr S, Köhrer K, Savani RS, Grandoch M*, Fischer JW*. (2016) Deletion of Hyaluronan Synthase 3 Inhibits Neointimal Hyperplasia in Mice. *Atheroscler Thromb Vas Biol*; 36(2): e9-16.
5. Dao VT, Medini S, Bisha M., Balz V, **Suvorava T**, Bas M, Kojda G. (2016) Nitric Oxide Up-regulates Endothelial Expression of Angiotensin II Type 2 Rreceptors. Biochem Pharmacol. 2016 Jul 15;112:24-36.
6. **Suvorava T**, Nagy N, Pick S, Lieven O, Ruether U, Dao VT, Fischer JW, Weber M, Kojda G. (2015) Impact of eNOS-dependent Oxidative Stress on Endothelial Function and Neointima Formation. *Antioxidants & Redox Signaling*; 23(9): 711-23.
7. **Suvorava T**, Stegbauer J, Thieme M, Pick S, Friedrich S, Rump LC, Hohlfeld T, Kojda G. (2015) Sustained Hypertension despite Endothelial-Specific eNOS Rescue in eNOS-deficient mice. *Biochemical and Biophysical Research Communications*; 458: 576–583.
8. Oppermann M*, **Suvorava T***, Freudenberger T, Dao VT, Fischer JW, Weber M, Kojda G. (2011) Regulation of Vascular Guanylyl Cyclase by Endothelial Nitric Oxide-Dependent Posttranslational Modification. *Basic Res Cardiol*; 106(4): 539-549.
9. **Suvorava T**, Kumpf S, Rauch BH, Dao VT, Adams V, Kojda G. (2010) Hydrogen Peroxide Inhibits Exercise-Induced Increase of Circulating Stem Cells with Endothelial Progenitor Capacity. *Free Radic Res*; 44(2):199-207.
10. Dao VT, Floeren M, Kumpf S, Both C, Peter B, Balz V, **Suvorava T**, Kojda G. (2010) Catalase Activity Prevents Exercise-Induced Upregulation of Vasoprotective Proteins in Venous Tissue *J Cell Mol Med*; 15(11):2326-34
11. Oppermann M*, Balz V*, Adams V, Bas M, Dao VT**, Suvorava T**, Kojda G. (2009). Pharmacologic Induction of Vascular Extracellular Superoxide Dismutase Expression *in vivo*. *J Cell Mol Med*; 13(7): 1271-8.
12. Oppermann M, Dao VT, **Suvorava T**, Bas M, Kojda G. (2008) Effect of Oral Organic Nitrates on Expression and Activity of Vascular Soluble Guanylyl Cyclase. *Br J Pharmacol*; 155(3):335-42.
13. **Suvorava T**, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G. (2005) Endogenous Vascular Hydrogen Peroxide Regulates Arteriolar Tension *in vivo*. *Circulation*; 112, 2487-2495.
14. Lauer N, **Suvorava T**, Rüther U, Jacob R, Meyer M, Harrison DG, Kojda G. (2005) Critical Involvement of Hydrogen Peroxide in Exercise-Induced Upregulation of Endothelial NO-synthase. *Cardiovascular Research*; 65:254-262.
15. **Suvorava T**, Lauer N, Kojda G. (2004) Physical Inactivity Causes Endothelial Dysfunction in Healthy Young Mice. *Journal of the American College of Cardiology***;** 44(6): 1320-1327.

SUMMARY/ ZUSAMMENFASSUNG

This work was based on the hypothesis that vascular endogenous reactive oxygen and nitrogen species (ROS/RNS) are not only involved in the development of oxidative stress, but have distinct signaling properties and serve important regulatory functions. To test this hypothesis, the steady-state levels of hydrogen peroxide, superoxide and NO were modified *in vivo* by a vascular specific overexpression of proteins regulating their availability (catalase, wild-type endothelial NO-synthase (eNOS) and destabilized eNOS).

Studies in a mouse model with vascular-specific overexpression of catalase revealed that endogenous hydrogen peroxide contributes to maintenance of vascular tone and blood pressure but does not influence NO-bioavailability (§3.1). While singularization impairs endothelial function and reduces vascular eNOS expression in healthy mice (§3.2), in exercise training vascular hydrogen peroxide is crucial for the upregulation of arterial eNOS protein (§3.3) and inhibits the exercise-induced mobilization of endothelial progenitor cells from the bone marrow (§3.4). In striking difference to arterial vessels, in venous tissues the exercise-induced upregulation of vasoprotective proteins is prevented by physiological activity of catalase (§3.5).

Experiments in mice and rabbits treated with NO-donors and mice with an endothelial specific overexpression of eNOS showed that endogenous and exogenous NO regulates soluble guanylyl cyclase (sGC) activity by its posttranslational S-nitrosylation, without changing sGC protein level (§3.6-3.7) and increases expression of important proteins of antioxidant defense and RAAS (§3.8, §3.9). Studies in double transgenic mice re-expressing wild-type eNOS in eNOS-deficient endothelium surprisingly revealed that endothelial-specific eNOS rescue normalized vascular reactivity in conduit and resistance arteries but did not lower blood pressure (§3.10). This paradox finding suggested the existence of a previously unrecognized but likely important role for non-endothelial eNOS in the regulation of blood pressure and put forward a new hypothesis that impairment of non-endothelial NO generation might contribute to the development of hypertension. More recently, the importance of the red blood cell eNOS-dependent pathway in the regulation of NO metabolism and blood pressure has been demonstrated using endothelial cell- and red blood cell-specific transgenic mice (§3.11).

As there was ongoing intensive scientific interest on the role of eNOS itself as a source of oxidant stress in hypertension and atherosclerosis (“uncoupled” eNOS), several mutant eNOS transgenic mouse models were generated. A plasmid with a cysteine 101 alanine mutant (C101A) was used to express C101A-eNOS in cells and mice. This mutation destabilizes the normal dimer configuration of eNOS which results in reduced NO and increased superoxide production in a close proximity to each other. This resembles the *in vivo* situation where the parts of eNOS molecules are uncoupled while the others function normally. Examination of mutant young mice overexpressing C101A-eNOS in the endothelium revealed no apparent abnormalities despite selective impairment of blood pressure reducing activity of vascular eNOS (§3.12).

Introduction of mutant C101A-eNOS in eNOS knockout mice (C101A-eNOS-Tg/KO) caused an increase in superoxide and peroxynitrite formation in the aorta and myocardium which was completely blunted by pharmacological inhibition of NOS. Surprisingly, despite greatly reduced eNOS expression, endothelial function in the mutant C101A-eNOS/KO mice was almost normal while blood pressure was similar to eNOS deficient mice (§3.13). Further detailed investigations showed correct localization and preserved vascular signaling in the conduit arteries and negligible effects of destabilized eNOS on vascular remodeling and neointima formation. These data suggest that increased ROS/RNS generation by destabilized eNOS is unlikely a pathologic factor promoting endothelial dysfunction and intima hyperplasia as long as concomitant production of NO is present.

This research also identified a novel modulator of NO signaling, namely hyaluronan (HA). HA and specifically the HA synthase 3 (HAS3) was shown to critically interconnect NO and HA-mediated signaling pathways in mechanosensing and collateral vessels growth. HA, an unbranched glycosaminoglycan and major component of extracellular matrix, synthesized by HAS3 in medial vascular smooth muscle in the course of inflammatory disease has been found to enhance intimal hyperplasia (§3.14), whereas the HA matrix within endothelial glycocalyx is vasoproprotective (§3.15). The vascular protective effects of HAS3-derived HA are mediated via endothelial HA/eNOS axis: HAS3-derived HA appears to function as mechanosensor and contributes to acute (flow-mediated dilation) and chronic (hindlimb ischemia) changes in blood flow by hyaluronan/CD44-mediated stimulation of eNOS phosphorylation at Ser1177.

Taken together, these studies provided novel insights into the control of vascular function by endogenous hydrogen peroxide, NO, superoxide and HA in health and disease.

ZUSAMMENFASSUNG

Diese Arbeit basiert auf der Hypothese, dass vaskuläre endogene reaktive Sauerstoff- und Stickstoffverbindungen (*reactive oxygen species*, ROS/*reactive nitrogen species*, RNS) nicht nur an der Entstehung von oxidativem Stress beteiligt sind, sondern darüber hinaus unterschiedliche Signaltransduktionseigenschaften besitzen und auf diese Weise wichtige regulatorische Funktionen erfüllen. Um diese Hypothese zu testen, wurden die endogenen *Steady-State*-Spiegel von Wasserstoffperoxid, Superoxidradikalanion und Stickstoffmonoxid (NO) modifiziert, indem wichtige, die Bioverfügbarkeit dieser ROS/RNS regulierenden Proteine (Katalase, endotheliale NO-Synthase (eNOS) und destabilisierte eNOS), gefäßspezifisch überexprimiert wurden.

In einem Mausmodell mit gefäßspezifischer Überexpression der Katalase konnte gezeigt werden, dass endogenes Wasserstoffperoxid zur Aufrechterhaltung des Gefäßtonus und des Blutdrucks beiträgt, aber die NO Bioverfügbarkeit nicht beeinflusst (§3.1). Während eine Einzelhaltung gesunder Mäuse die Endothelfunktion beeinträchtigt und Expression vaskulärer eNOS reduziert (§3.2), wird diese bei körperlichem Training durch Wasserstoffperoxid hochreguliert (§3.3). Weiterhin konnte gezeigt werden, dass Wasserstoffperoxid die durch Bewegung induzierte Mobilisierung endothelialer Vorläuferzellen aus dem Knochenmark hemmt (§3.4). Im Gegensatz zu arteriellen Gefäßen wird in venösen Geweben die bewegungsinduzierte Hochregulation vasoprotektiver Proteine durch die physiologische Aktivität der Katalase verhindert (§3.5).

Untersuchungen an Mäusen und Kaninchen, die mit NO-Donatoren behandelt wurden, sowie an Mäusen mit endothelspezifischer Überexpression der eNOS zeigten, dass exogenes und endogenes NO die Aktivität der löslichen Guanylylcyclase (*soluble GC*, sGC) durch posttranslationale S-Nitrosylierung reguliert, ohne den sGC-Proteinspiegel zu verändern (§§ 3.6-3.7). Zudem wurde die Expression wichtiger Proteine der antioxidativen Abwehr und des Renin-Angiotensin II-Systems erhöht (§§3.8-3.9). Experimente an transgenen Mäusen, die Wildtyp-eNOS in eNOS-defizientem Endothel reexprimierten, zeigten überraschenderweise, dass eine Wiederherstellung der endothelspezifischen eNOS die vaskuläre Reaktivität in Leitungs- und Widerstandsarterien normalisierte ohne jedoch den Blutdruck zu senken (§3.10). Dieser paradoxe Befund deutete auf die Existenz einer bisher unbekannten, aber wichtigen Rolle für nicht-endotheliale eNOS bei der Regulierung des Blutdrucks hin und führte zu der Hypothese, dass eine Beeinträchtigung der nicht-endothelialen NO Synthese zu Hypertonie beitragen könnte. Tatsächlich wurde vor kurzem die Existenz eines erythrozytären eNOS-abhängigen Signalweges bei der Regulation des NO-Metabolismus und des Blutdrucks unter Verwendung von endothelzell- und erythrozytenspezifischen transgenen Mäusen nachgewiesen (§3.11).

Um die Forschung zur Bedeutung der eNOS-Entkopplung als unabhängige Quelle oxidativen Stresses im Kontext von Bluthochdruck und Atherosklerose voranzutreiben, wurden weitere transgene eNOS-Mausmodelle entwickelt. Ein Plasmid mit einer Cystein-101-Alaninmutante (C101A) wurde verwendet um C101A-eNOS in Zellen und Mäusen zu exprimieren. Diese Mutation destabilisiert die normale Dimerkonfiguration der eNOS was zu einer reduzierten NO- und erhöhten Superoxidproduktion in unmittelbarer räumlicher Nähe zueinander führt. Dies ähnelt der Situation *in vivo*, in der ein Teil der eNOS-Moleküle entkoppelt ist, während andere normal funktionieren. Die Untersuchung

junger mutierter Mäuse, die C101A-eNOS im Endothel überexprimierten, ergab keine offensichtlichen phänotypischen Unterschiede trotz selektiver Beeinträchtigung der blutdrucksenkenden Aktivität der vaskulären eNOS (§3.12).

Die Expression der mutierten C101A-eNOS im Endothel von eNOS-*Knockout*-Mäusen (C101A-eNOS-Tg/KO) verursachte einen Anstieg der Superoxid- und Peroxynitritbildung, welche durch pharmakologische NOS-Hemmung vollständig inhibiert werden konnte. Überraschenderweise war die Endothelfunktion in C101A-eNOS/KO-Mäusen trotz stark reduzierter eNOS-Expression fast normal, während der Blutdruck dem von eNOS-defizienten Mäusen ähnelte (§ 3.13). Weitere detaillierte Untersuchungen zeigten sowohl eine korrekte Lokalisation als auch eine erhaltene vaskuläre Signaltransduktion in den Leitungsarterien und geringfügige Auswirkungen von destabilisierter eNOS auf vaskuläres Remodelling und Neointimabildung. Diese Daten deuten darauf hin, dass eine erhöhte ROS/RNS-Generierung durch destabilisierte eNOS wahrscheinlich kein pathologischer Faktor ist, der endotheliale Dysfunktion und Intimahyperplasie fördert, zumindest solange die NO-Produktion erhalten bleibt.

Im Weiteren konnte ein neuer Modulator des NO Signalweges identifiziert und im kardiovaskulären Kontext genauer charakterisiert werden. Hyaluronsäure (*hyaluronic acid*, HA) und spezifisch die HA-Synthase (HAS)3 wurde hier als wichtiges Molekül im Zusammenspiel von NO- und HA-vermittelten Signalwegen beim *Mechanosensing* identifiziert, was die Ausbildung von Kollateralen modulierte. HA ist eine wichtige Komponente der extrazellulären Matrix und wird in arteriellen Leitgefäßen insbesondere im Rahmen inflammatorischer Erkrankungen durch HAS3 in der medialen glatten Muskulatur synthetisiert. In einem Modell der Karotisstenose trägt die durch HAS3 synthetisierte HA-Matrix zur Ausbildung einer Intimahyperplasie bei (§ 3.14), während die HA-Matrix innerhalb der endothelialen Glykokalyx vasoprotektive Eigenschaften besitzt (§3.15). Untersuchungen konnten zeigen, dass die vaskulären Schutzwirkungen von HA, welche durch HAS3 synthetisiert wird, über eine endotheliale HA/eNOS-Achse vermittelt werden: HAS3-assoziierte HA, scheint als Mechanosensor zu fungieren und trägt durch HA/CD44-vermittelte Stimulation der eNOS-Phosphorylierung an Ser1177 zu akuten (flussvermittelte Vasodilatation) und chronischen (Hinterlaufischämiemodell) Veränderungen des Blutflusses bei.

Zusammengefasst liefern diese Studien neue Erkenntnisse zur Regulation der Gefäßfunktion durch endogenes Wasserstoffperoxid, NO, Superoxid und HA im Kontext physiologischer und pathophysiologischer Bedingungen.

1. INTRODUCTION

"Truth in science can be defined as the working hypothesis best suited to open the way to the next better one".

Konrad Lorenz, *1973*

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) summarize several small highly reactive molecules including 1) radicals such as superoxide anion radical and hydroxyl radical, 2) non-radical species hydrogen peroxide and adducts such as hypoclorous acid and 3) nitrogen-containing species including peroxynitrite and nitrogen dioxide form by the reaction of superoxide and nitric oxide (NO). The main sources of vascular ROS are NAD(P)H oxidase (NOX) (1), mitochondrial-derived superoxide (2), uncoupled nitric oxide synthase (uncNOS) (3) and to a lesser extent xanthine oxidase (4), cyclooxygenase (5), myeloperoxidase (6), and some other enzymes (reviewed in (7)) while three forms of superoxide dismutases convert superoxide to hydrogen peroxide and oxygen (8). Catalase, thioredoxin peroxidase and glutathione peroxidases regulate steady state levels of hydrogen peroxide by conversion to water. Antioxidant defence system (SOD, peroxidases, antioxidants and vitamins) maintain oxidation-reduction (redox) state by preventing ROS accumulation (9). The stoichiometry of the chemical reactions underlying generation and metabolism of ROS is a subject of tight enzymatic regulation resulting in well balanced steady-state concentrations throughout the healthy organism (10, 11).

Based on the oxidative stress theory of disease (12, 13), an increase of vascular ROS production was considered as important pathogenic factor. According to this theory aberrant ROS formation may trigger disease state in the condition of oxidative stress, when ROS/RNS production exceeds the capacity of cellular antioxidant defense mechanisms (SOD, peroxidases, low molecular weight antioxidants etc.). Although clinical evidence for associations (but not causality) of oxidative stress with cardiovascular disease are strong (14-16), antioxidant therapies have provided disappointing results (reviewed in (11, 17)). It became evident recently that the oxidative stress theory of disease has been over-simplified, in fact, ROS/RNS have a complex metabolism and functions and are generated by different enzymes at diverse cites and at different times (9, 11, 18, 19).

Within the vascular wall a delicate ROS balance can be either beneficial or deleterious, depending on the source of ROS or the mechanisms of ROS capture or quenching (20). ROS-associated proteins and their expression profiles vary depending on vessel location within the vascular tree and tissue origin (20). Vascular smooth muscle cells, endothelial cells, immune cells, and other cell types have different expression patterns for the various ROS-related proteins which can be also changed in disease state (21-24).

Similar to ROS, RNS, specifically NO and its derivatives, play pivotal roles in vascular function. Nitric oxide (NO), a liable free radical gas, is a structurally simple diatomic molecule with a highly diverse biological profile (25, 26) including vasodilation, antiaggregation, antiapoptosis, antiantiadhesion, antiproliferation and antioxidation (27). As with ROS, excess NO production can have harmful effects on the vasculature and promote cardiovascular imbalance (20, 28). This depends on the microenvironment in which NO is produced and on the chemical reaction

partners (20), e.g. reaction between NO and pathological superoxide. Similar to ROS production, NO generation requires tight regulation to avoid pathologies of insufficient or excess RNS for homeostasis (20, 28, 29).

Accumulating evidence has provided a basis for a paradigm shift from the roles of ROS as pathological detriments to physiological signaling molecules (7, 30, 31). ROS/RNS and redox signaling have fundamental role in pathophysiology of hypertension, vascular dysfunction, endothelial damage, immune cell activation and cardiovascular remodelling. Recent advances in proteomics delivered new informations on the role of redox regulation, defined as oxidation-reduction posttranslational moifications of downstream signaling molecules induced by ROS/RNS, with recent studies focusing on cysteine oxidation of proteins (32). This also led to a definition of "oxidative stress" to include the importance of redox regulation, which is now described as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling, and control and molecular damage" (9, 33). The results of the studies presented here contributed to the different stages of development of the current view on ROS/RNS as important redox regulators of vascular homeostasis.

1.1. Vascular Effects of Hydrogen Peroxide

Hydrogen peroxide is a non-radical uncharged oxidant produced in many different cell types in the human body including vascular endothelial and smooth muscle cells (34). For many years, hydrogen peroxide was viewed as the inevitable but unwanted by-product of an aerobic existence. Given the damage inflicted by hydrogen peroxide, it was assumed that the faster the elimination of this toxic waste, the better for the cell (35). Hydrogen peroxide is chemically more stable than other ROS and can permeate through the vascular wall. It was suggested that hydrogen peroxide can also accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions, even in more distant cells. Under normal conditions, the level of hydrogen peroxide is maintained at steady-state level in the vasculature by constitutive oxidase activities and endogenous scavenger systems. While hydrogen peroxide is mainly formed by superoxide dismutases, it is rapidly degraded by catalase, glutathione peroxidase and thioredoxin peroxidases. These antioxidant enzymes play important roles as molecular sensors and biological modulators of the effects exerted by hydrogen peroxide on cellular signaling events (10, 35). Recent revision of the oxidative stress theory supports the role of hydrogen peroxide as the major redox metabolite operative in redox sensing, signaling and redox regulation (36) and the concept of constitutive cellular low-level hydrogen peroxide steady-states has gained increased recognition (10, 37).

In vascular smooth muscle cells hydrogen peroxide is known to play an important role in cellular proliferation, migration, and differentiation (reviewed in (38)). A number of early reports demonstrated the role of hydrogen peroxide in regulation of vasomotor function (39-43). For instance, Gao et al. showed that in rat mesenteric arteries hydrogen peroxide caused a biphasic effect, a transient constriction followed by the relaxation response (43). In human coronary arterioles, shear stress increases endothelial cell-derived hydrogen peroxide resulting in smooth muscle hyperpolarization and relaxation (42). Furthermore, hydrogen peroxide has been considered as a possible candidate for endothelium-derived hyperpolarizing factor (EDHF (44))(28). However, there was no general agreement on the mechanisms involved in the vascular effects of hydrogen peroxide. It was suggested that

hydrogen peroxide more likely indirectly affect the other pathways responsible for relaxation by interactions with other vascular mediators such as NO and/or arachidonic acid (41, 45).

The steady state concentration of hydrogen peroxide in human plasma, blood cells and vascular cells is unknown. Basal hydrogen peroxide level in extracellular fluids is most likely in the lower micromolar range (1-5 µM) (34, 46) or less (47). Spatial distribution of hydrogen peroxide in cells is not uniform (48) with physiological intracellular pools in the nanomolar range (37). The vast majority of early studies investigated vasomotor effects of exogenous hydrogen peroxide at much larger concentrations (up to 10 mM) and both vasodilator and vasoconstrictor effects were reported (40, 41, 49, 50). Vasodilator effects of hydrogen peroxide have been suggested to be mediated by activation of K^+-channels (44), activation of endothelial NO production (51, 52), decreased myosin phosphorylation (53) and inhibition of myosin ATPase (54), while vasoconstriction was attributed to an increase of intracellular Ca^{2+} (55), generation of arachidonic acid metabolites with vasoconstrictor activity (43) and direct Ca^{2+}-independent tonic effects on the smooth muscle contractile apparatus (56, 57). Thus, the vasomotor responses to hydrogen peroxide *ex vivo* were complex and depended on different conditions such as the type of vessel tested, the species studied and the concentration range used. These data stimulated interest to the role of endogenous hydrogen peroxide in vascular tone regulation and led to a hypothesis that the vasomotor effects of endogenously produced hydrogen peroxide may contribute to blood pressure homeostasis and NO bioavailability (Ref. § 3.1).

1.2. Molecuar Mechanisms of Vascular Adaptation to Exercise Training

Regular physical activity is a promising inexpensive approach for prevention of cardiovascular disease (58-60). Human epidemiological studies demonstrated that regular exercise reduces the risk of death after acute myocardial infarction (reviewed in (59, 61)). According to the current guidelines of the American Heart Association (62) and the European Society of Cardiology (63) regular exercise training along with weight loss and healthy diet belongs to the best proven nonpharmacological interventions for prevention and treatment of hypertension.

Regular exercise induces a reduction of heart rate and blood pressure, increases maximal myocardial oxygen uptake and leads to several physiological adaptations involving skeletal muscle, cardiac muscle, circulating blood volume and a variety of metabolic and anti-inflammatory modifications (59, 64-67). One of the main vascular adaptation to exercise training is an upregulation of eNOS (68, 69). Shear stress induced eNOS-activation by exercise is known to increase NO production (68, 70-73) and this process requires two intact eNOS alleles (74). In humans exercise-induced physiological stimulation of eNOS activity is mirrored by the increased levels of circulating NO metabolites: Plasma nitrite/nitrate levels were reported to be significantly increased after acute and chronic exercise training (75-80).

Furthermore, shear stress was shown to increase the generation of ROS by an endothelium-dependent mechanism (81, 82). Analysis of this phenomenon revealed an activation of endothelial NADPH oxidase as a possible underlying cause (82, 83). A hypothesis to explain beneficial effects of regular exercise training in the vasulature has been put forward (84). Accordingly, each bout of exercise induces a considerable but transient

increase of ROS levels which lasts not much longer than exercise itself. These peaks of oxidant stress induce the expression of antioxidant enzymes such as eNOS, CuZnSOD or ecSOD in the vascular cells. If the time span between each bout of exercise is shorter than the duration of increased expression of these enzymes, e.g. 24–48 hours, the total antioxidative capacity of the vascular wall likely increases. Thus, while acute exercise training transiently increases ROS/RNS levels, regular exercise presumably provides protection against permanent oxidative stress as present in atherosclerosis, hypertension, diabetes and heart failure.

Another important vascular adaptation to exercise is a mobilization of endothelial progenitor cells (EPCs) (85-87). Asahara and colleagues first published that circulating $CD34^+$-angioblasts within human peripheral blood are able to differentiate to endothelial phenotype (88). EPCs were reported to participate in endothelial homeostasis by contribution to re-endothelialization and improve organ blood flow by homing into ischemic regions and forming entirely new vessels or by releasing angiogenic factors in a paracrine manner (89-91). These beneficial effects are impaired when the number and/or functional activities of EPCs are reduced. Clinical trials revealed that the number of circulating EPCs is decreased in subjects with cardiovascular risk factors, such as age, hypercholesterolemia, diabetes and smoking (92) and a similar situation likely holds true for EPC homing (93). Since these pathologies are associated with increased ROS/RNS, depletion of circulating EPCs was considered as a marker of the progression of cardiovascular disease (94). PI3K/Akt/eNOS pathway is reported to be important for EPC mobilization, migration and homing (87, 95, 96). It is also known that proliferative EPCs showed a decreased clonogenic capacity after treatment with oxidants including hydrogen peroxide (97) and an increase of ROS is crucially involved in the regulation of vessel growth (98). Therefore, ROS/RNS may directly affect stem cell-induced vascular repair mechanisms by influencing the EPCs mobilisation and functions in response to exercise training.

Our understanding of the mechanisms and mediators leading to the positive effects of exercise (and on the opposite, negative effects of sedentary lifestyle) in the vasculature is incomplete. The effects of exercise training on eNOS expression and phosphorylation profile are influenced by a number of factors such as training time, exercise intensity, basal level of physical (in)activity, and eNOS gene polymorphisms (58, 64, 65, 99, 100). Furthermore, since blood flow and hemodynamic shear forces strongly depend on the localization and morphology of the vessels along the vascular tree (101, 102), the effect of exercise on vascular eNOS may differ in various regions. Previous investigations, showing that physical activity increases the expression of eNOS in animals and humans have been performed almost exclusively in large conduit arteries such as aorta or coronary arteries. In striking contrast, little is known about the effect of exercise in veins and/or low-pressure tissues such as the vena cava and lung.

1.3. Vascular Effects of Nitric Oxide

Under physiological conditions vascular NO is mainly produced by endothelial NO-synthase (eNOS, EC 1.14.13.39), a major isoform of NOS in the cardiovascular system (103, 104). eNOS is a heme-containing protein, which utitilize L-arginine and molecular oxygen as substrates and require the cofactors reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and

(6R-)5,6,7,8-tetrahydrobiopterin (BH_4) to catalyse the five-electron oxidation of the guanidine-nitrogen of L-arginine to NO and citrulline (26). Structurally, eNOS is a homodimer with a C-terminal reductase and N-terminal oxygenase domain. NADPH binds to the reductase domain and electrons are transferred to the heme in the oxygenase domain, catalazyd by FAD, FMN and calmodulin. After reduction of the heme-iron, oxygen is able to bind and the products NO and L-citrulline are formed from the substrate L-arginine (105).

Vasodilatory effects of NO for the most part are mediated by activation of soluble guanylyl cyclase (sGC), formation of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG) and phosphorylation of various downstream cellular proteins regulating calcium homeostasis (25, 106, 107). The sGC enzyme is a heterodimeric protein composed of two subunits, α and β, and a prosthetic heme group. The majority of vascular sGC is formed by the subunits α_1 (sGC-α_1) and β_1 (sGC-β_1) (108, 109). The heme-free form of sGC is unresponsive to NO and prone to ubiquitin-mediated degradation (Stasch et al. 2006; Schmidt 2009).

Studies with NO-donors in cultured aortic smooth muscle cells have provided evidence for a NO-dependent downregulation of sGC protein expression suggesting a negative feedback loop, where NO acts as a signaling molecule regulating sGC expression (110). In contrast, overexpression of eNOS in mice with endothelil preproendothelin-1 promoter showed partial resistance to endothelium-dependent and NO-induced vasodilation but no decrease of sGC expression (111). Inhibition of NOS also had no effect on vascular sGC expression, but potentiated the aortic cGMP response to the exogenous NO (112). Likewise, no changes of aortic sGC expression (113) but increased sensitivity of sGC to activation by exogenous NO was found in eNOS deficient mice (113, 114). In addition to classical NO/sGC/cGMP signaling through binding to heme (as in the case of cGMP) NO can also modulate hundreds of proteins (including sGC itself) through S-nitrosylation occurring in a spatially confined manner (115, 116). In view of controversial data described above and the complexity of NO-signaling, effects of endogenously formed NO vs NO donors on sGC regulation remained largely unclear.

NO has also important antioxidative properties in the vasculature. Previous investigations have shown that NO is an essential stimulus of ecSOD expression in large conductance vessels, and that an increase of eNOS expression by exercise results in a consecutive overexpression of ecSOD (72). ecSOD is expressed in vascular smooth muscle cells and is subsequently secreted to the interstitium (117). It binds to polyanionic sites such as heparin sulphates at the outer membrane of endothelial cells and is suggested to be protective for NO while it traverses to the smooth muscle cell layer of the vascular wall (118). The highest ecSOD concentrations are found between the endothelial cell layer and the smooth muscle, and 99% of ecSOD is tissue-bound (118). Hence, driving the expression of ecSOD appears to be an important mechanism underlying the antioxidative effects of NO.

Increasing vascular NO levels following up-regulation of endothelial nitric oxide synthase (eNOS) is considered beneficial in cardiovascular disease. Whether such beneficial effects exerted by increased NO levels include the vascular renin–angiotensin II-aldosterone (RAAS) system was not elucidated. Activation of Ang II type 2 (AT2) receptors is known to counter-regulate many effects mediated by Ang II type 1 (AT1) receptors and is vasculoprotective (119). For example, stimulation of AT2 was shown to induce the prekallikrein activator prolylcarboxypeptidase (120, 121) and thereby increases generation of bradykinin and subsequently endothelial

NO. AT2 was also shown to inhibit degradation of bradykinin by the angiotensin converting enzyme (ACE) (122). However, it remained uninvestigated whether NO impacts the AT2 signaling and what is an underlying mechanism of these interactions.

1.4. eNOS on the Guard against Hypertension: Role for Non-endothelial eNOS?

Among all other functions, NO-dependent regulation of vascular tone is the most important. Based on findings following intrabrachial infusion of NOS-inhibitor L-monomethyl arginine (L-NMMA) Vallance et al. concluded as early as 1989 that NO is likely to be a key determinant of arterial blood pressure (123). In accordance, i.v. L-NMMA application increased mean arterial blood pressure in healthy volunteers (124). Forte et al. showed that basal NO synthesis is reduced in patients with essential hypertension accentuating the importance of NO synthesized by the vascular endothelium for the control of blood pressure in humans (125).

Based on the high expression of eNOS in endothelial cells, role of eNOS in vascular homeostasis was assessed using several independently generated eNOS deficient (eNOS KO) models. The most obvious phenotype in 4 different strains of eNOS KO mice is increased blood pressure (126-129) suggesting that other homeostatic mechanisms do not compensate for the absence of eNOS. Hypertension in eNOS KO is assumed to be caused by "the lack of vasorelaxing pathway mediated by endothelium-derived NO on smooth muscle cells" or by "the absence of inhibitory effects of NO on renin release from the kidney into blood stream" (127). Alternatively, diminution in activity of RAAS and autonomic nervous system, which serve as a defence against hypertension and/or involvement of NOS in establishing the baroreceptor setpoint were proposed as an explanation of hypertensive phenotype (126). However, studies on nNOS-deficient mice revealed that they are normotensive (130, 131). Furthermore, triple e/i/nNOS knockouts have hypertension similar to that in the eNOS-gene disrupted single and double e/nNOS KO (132). Taken together, these results demonstrate that among NO-synthases eNOS plays the major role for blood pressure regulation. Thus, it is frequently assumed that hypertension in eNOS KO mice is caused by the lack of endothelium-derived NO and a resulting increase in peripheral resistance.

However, other observations suggest that this explanation is too simple. There was virtually no evidence from knockout models clearly linking eNOS activity in endothelial cells to blood pressure. In contrast to large arteries of eNOS KO, where no compensation for the lack of eNOS is observed, other endothelium-dependent vasodilators compensate for the lack of eNOS in resistance arteries. For example, while endothelium-dependent vasodilation in isolated conductance arteries such as the aorta (126, 133) or the carotid artery (134) is completely inhibited in eNOS KO, the dilatory response of coronary arteries to acetylcholine is preserved by compensatory generation of cyclooxygenase products (128, 135). Furthermore, preservation of endothelium-dependent vasodilation has been demonstrated in femoral and mesenteric vessels (136), in cerebral arteries (137, 138) and in small skeletal muscle arterioles (139) of eNOS KO mice. These data indicate that different vascular-bed specific mechanisms may be activated to regulate arterial tone in the absence of vascular eNOS and raise the question if or to what extend hypertension in eNOS KO is caused by the lack of endothelial eNOS expression. Furthermore, non-endothelial eNOS expression was suggested in RBCs (140), kidney (141) and astrocytes of the brain stem (142).

Development of Cre/LoxP technology made possible tissue-specific knocking-out and knocking-in of different proteins incuding eNOS. First floxed eNOS mouse line ($eNOS^{flox/flox}$) was generated by Jiang et al. in 2012 (143). The targeting vector was designed to allow conditional deletion of exons 9-12 of the mouse eNOS gene, removing the calmodulin-binding site and resulted in non-functional eNOS protein. As a proof of concept, this flox allele was globally deleted by mating $eNOS^{flox/flox}$ mice to Sox2-Cre (Cre Deleter) mouse strain, which express Cre recombinase under the control of the mouse Sox2 (SRY-box containing gene 2) promoter and drives Cre recombinase in all epiblast cells at an early embryonic stage (144). These experiments demonstrated that floxed eNOS allele behaves as wild type allele and it can be converted to deleted allele through Cre-loxP recombination, completely abolishing eNOS activity and exhibiting significant hypertension comparable to hypertension of global eNOS KO strains (126-128).

This mouse model was used to elucidate a role of non-vascular eNOS localized in the kidney. To accomplish this, $eNOS^{flox/flox}$ mice harbouring the floxed eNOS allele were bred with mice transgenic for aquaporin-2 Cre recombinase to obtain collecting duct-specific knockout of eNOS, however blood pressure measured via telemetry was not affected by this collecting duct-specific deletion of eNOS (145). In contrast, mice with doxycycline-inducible nephron-wide deletion of eNOS had significantly elevated blood pressure as compared with control mice and impaired urinary Na^+-excretion during salt loading (146). These data demonstrate that renal eNOS-derived NO in the distal tubule appears to be involved in the regulation of blood pressure. Unfortunately, systemic NO-bioavailability and endothelial function were not assessed in the nephron-specific eNOS KO model. Likewise, the role of eNOS expressed in other cell types remained unknown.

1.5. Uncoupled eNOS and eNOS-generated ROS/RNS: The Proverbial Chicken or the Egg?

NO produced by eNOS represents a key vasoprotective regulator of endothelial function under physiological conditions. However, for several reasons, eNOS may become dysfunctional under pathophysiological conditions. A popular term for dysfunctional eNOS has emerged to describe a reduced enzymatic production of NO is “uncoupled” eNOS. This term describes early findings with purified NOS protein showing increased production of superoxide/hydrogen peroxide as a consequence of uncoupling of the electron transfer to O_2 yielding superoxide rather than hydroxy-L-arginine, which is the most important intermediate for NO-synthesis. The result is a generation of ROS primarily within the eNOS oxygenase domain which may further promote endothelial dysfunction and vascular pathology. Likewise, this reduces NO production and facilitates pre-existing oxidative stress (147, 148). Many oxidative enzymes have been implicated in vascular pathologies (21, 149-151) and this makes it difficult to define the relative contribution of eNOS-dependent formation of superoxide and peroxynitrite.

A number of mechanisms of eNOS uncoupling has been reported including 1) oxidative depletion of (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4), 2) reduced L-arginine transport and/or competition of eNOS with other arginine-utilizing enzymes such as arginase, 3) oxidative disruption of the dimeric eNOS complex, 4) S-glutathionylation and adverse phosphorylation at Thr495/Tyr657 as well as 5) ROS and RNS triggered increases in levels of asymmetric dimethylarginine (ADMA) (reviewed in (152)).

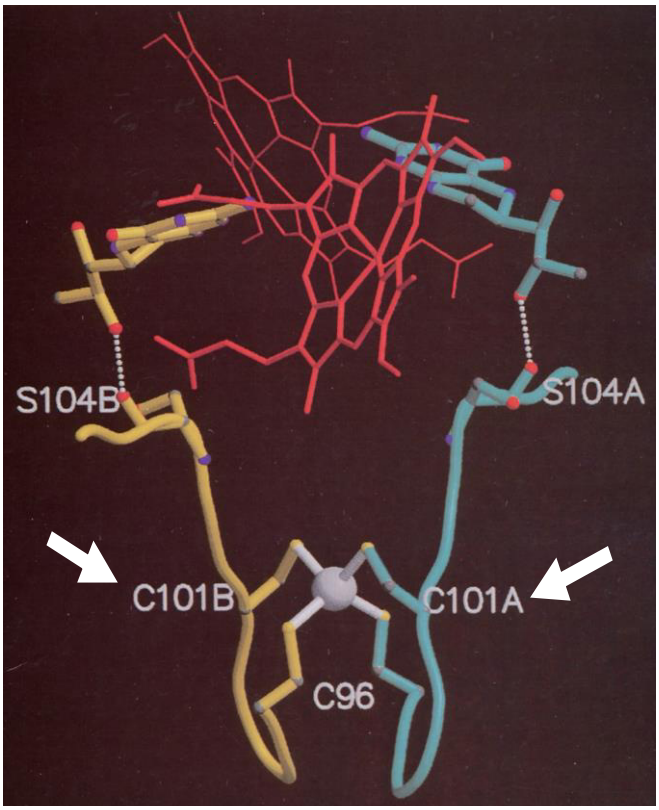


Figure 1: The ZnS_4 metal center and its relationship with tetrahydrobiopterin (red). The central zinc ion (grey) coordinates the dimerization two eNOS monomers (yellow and green) via the binding cysteines 96 and 101. Reproduced from (153) with permission from Elsevier.

According to the crystal structure of the heme domain (oxygenase domain) of bovine eNOS (153), binding and correct orientation of BH_4 is critically dependent on the three-dimensional structure of the homodimeric protein. Dimerization itself depends on a zinc ion tetrahedrally coordinated to pairs of cysteine residues, i.e. Cys96 and Cys101 of bovine eNOS in each monomer. Thus, genetic disruption of the Zn ion complex would be expected to decrease dimer stability, impair correct BH_4 orientation and NO synthesis and increase eNOS-dependent superoxide and peroxynitrite formation. For example, replacement of cysteine 99 to alanine in human eNOS resulted in a reduction of BH_4 affinity, enzyme stability and citrulline formation and an irreversible loss of heme (154). Therefore, an impairment of the correct dimer formation likely results in diminished NO synthesis and favours generation of superoxide. Whether this impairment of eNOS function had a pathophysiological significance in the absence of the concomitant complex disease processes occurring in hypertension, atherosclerosis and/or diabetes – under otherwise healthy conditions, was important to estimate.

1.6. NO and Hyaluronan: Interrelated Signaling?

Endothelial dysfunction and reduced bioavailability of NO is a hall-mark of many cardiovascular risk factors and diseases including peripheral artery disease (PAD), restenosis and atherosclerosis. The marked upregulation of hyaluronan (HA) production and accumulation in the neointima of restenotic vessels and atherosclerotic lesions generated an interest to the role of HA matrix in these diseases (155-157). Likewise, inhibition of HA synthesis leads to disturbance of endothelial glycocalyx, endothelial dysfunction, hypertension and the reduction of vascular NO-bioavailability (158). In contrast, interstitial HA has been shown to promote neointima hyperplasia and atherosclerosis (155, 157), affect the phenotype of immune cells (159-161) and had profound effect on phenotypic switching of SMCs and plaque stability in an animal model of atherosclerosis (162).

HA is a linear glucosaminoglycan which is synthesized by three hyaluronan synthase (HAS) isoforms (HAS1-3) that assemble UDP (uridinediphosphate)-glucuronic acid and UDP-N-glucosamine at the plasma membrane. Afterward, HA is extruded to the extracellular space where it exerts an important role for tissue structural integrity,

contributes to the matrix microenvironment of cells and acts as a direct signaling agent via HA-binding receptors (163, 164).

In PAD, HA appears to be a critical player in mechanosensing and collateral artery growth. *Ex vivo* studies suggested a role for HA in endothelial NO production in response to shear stress (Mochizuki *et al.*, 2003; Kumagai *et al.*, 2009). For instance, enzymatic degradation of hyaluronan in isolated canine (165) and porcine femoral arteries (166) coincided with impaired NO production. Likewise, interaction of HA receptor cluster of differentiation 44 (CD44) with ankyrin and the IP_3 receptor promoted Ca^{2+} signaling and NO production *in vitro* (167). Modification of glycocalyx with 4-methylesculetin, which inhibits all 3 HAS isoenzymes renders the endothelium unresponsive to altered hemodynamic conditions (induced by femoral artery ligation) (168) suggesting that HA synthesis likely interferes with its mechanosensory properties. However, whether this also reflects the *in vivo* situation in health and disease context and which HAS isoenzyme(s) is/are crucial for mechanosensing, mechanotransduction and NO-release was largely unknown.

Defining a role of HA in vascular disease is difficult in view of the multitude of HA synthases and hyaluronidases involved in its metabolism (159, 164). Although each HAS isoform produces structurally identical HA, the expression pattern of each isoform and their differential regulation in vascular health and disease states may differ. Furthermore, the molecular weight of HA appears to be important for vascular function: Longer high molecular weight HA supports vascular homeostasis and immune health, while under inflammatory conditions, HA is degraded into smaller fragments in a context-dependent manner (159, 169, 170). Of interest, HA breakdown is mediated not only by special enzymes hyaluronidases, but also non-enzymatically by ROS/RNS and UV radiation (171) and this might be of particular importance after tissue damage and during inflammation (169, 170).

With its simple structure, HA regulates biological responses in a highly complex manner. This attributes to its high abundance and rapid turnover, the generation of different-sized HA fragments, due to its localization outside and inside the cell, binding to different surface receptors and offers insights to divergent, and in part, opposing roles of hyaluronan in different cell types. Particularly, understanding of cellular origin and specific role of different HA synthesis as well as interconnections between HA and NO-signaling in the development of neointimal hyperplasia and collateral arteries growth promise important informations in the future. Keeping in mind the well-established antioxidative, antithrombotic, antiproliferative, antrihypertensive properties of NO, the involvement of endothelial glycocalyx HAS/HA/eNOS axis in vascular protection seemed to be reasonable and indeed a close interrelationship between both signaling pathways was identified (Ref §3.15).

1.7 Open Questions

"To know that we know that we know, and to know that we do not know that we do not know, that is true knowledge"

Nicolaus Copernicus (1473-1543)

Previous studies were mainly focused on the examination of chemically reactive oxygen and nitrogen species and their interactions with other molecules and enzymes that lead to vascular pathology. Likewise, HA was thought to have a mere structural role. However, it became obvious that ROS/RNS and HA have also important signaling properties in the vasculature. Among others, several open questions remained:

1. What are the effects of endogenous steady state levels of hydrogen peroxide on vascular tone and blood pressure?

2. What is a role of ROS/RNS in vascular adaptation to exercise training?

3. How endogenous and exogenous NO regulates its receptor sGC and other vasoprotective proteins of antioxidative defence and RAAS?

4. What is a specific contribution of endothelial and non-endothelial eNOS to the regulation of vascular tone, NO-bioavailability, and blood pressure?

5. How endothelial ROS/RNS generation by destabilized eNOS influences endothelial function, expression and activity of vasoprotective proteins, arterial remodeling and blood pressure?

6. What is a role for HAS3-mediated HA synthesis in the development of neointimal hyperplasia and collateral arteries growth and whether and how HA-mediated effects interreact with changes of vascular NO-signaling?

2. AIMS AND RESEARCH APPROACHES

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them".

William Bragg, (1862-1942)

This work was based on the hypothesis that ROS and RNS are not only involved in the development of oxidative stress and disease states, but have distinct signaling properties and serve important regulatory functions in the vasculature. The overall purpose of this study was to further explore the roles of endogenous hydrogen peroxide, NO and superoxide in the regulation of vascular function in health and disease state. Another focus of this study was on the significance of HA synthesis in neointima formation and possible interconnection of NO and HA-mediated signaling pathways in collateral vessels growth.

To test these hypotheses, the vascular levels of endogenous hydrogen peroxide, superoxide and NO were modified by Tie-2 driven overexpression of vasoactive proteins regulating their bioavailability (catalase, destabilized C101A-eNOS and wild-type eNOS) (Figure 2).

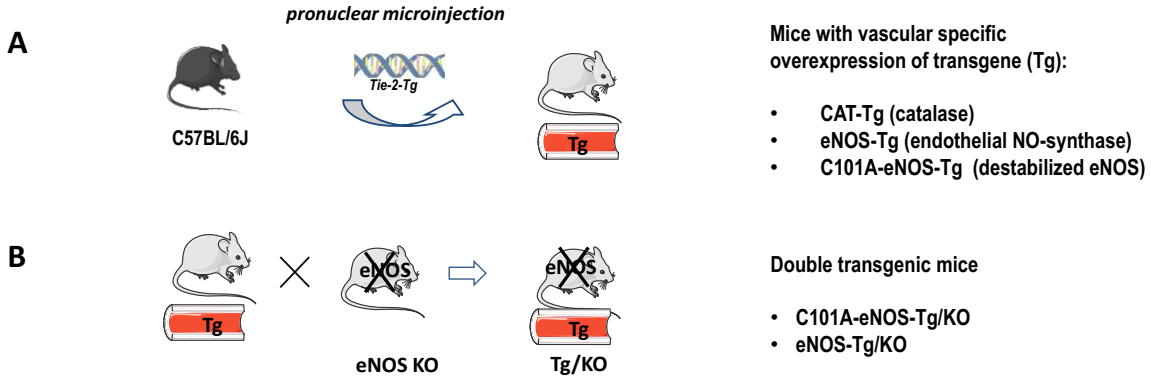


Figure 2: Transgenic mouse models with the Tie-2 driven overexpression of key vasoprotective proteins. (**A**) Transgenic mice with vascular specific overexpression of human catalase (CAT-Tg), eNOS (eNOS-Tg) and destabilized C101A-eNOS (C101A-eNOS-Tg). (**B**) Breeding strategy to obtain double transgenic mice with expression of the transgene (eNOS and C101A-eNOS) exclusively in vascular endothelial cells, mice overexpressing normal eNOS and its destabilized form (C101-eNOS) were crossed with global eNOS knock-out mice (127) (eNOS KO). Figure created by author.

The Cre/LoxP technology was used to create endothelial cell (EC) and red blood cell (RBC) specific eNOS KO and knock-in (KI) mice by deleting exon 2 of eNOS (Figure 3). Cell-specific KO or KI of eNOS was achieved using a hemoglobin β-chain (Hbb-Cre) or Cdh5 expressing Cre-ERT2 recombinase for erythrocyte or endothelial cell-specific eNOS KO and KI, respectively.

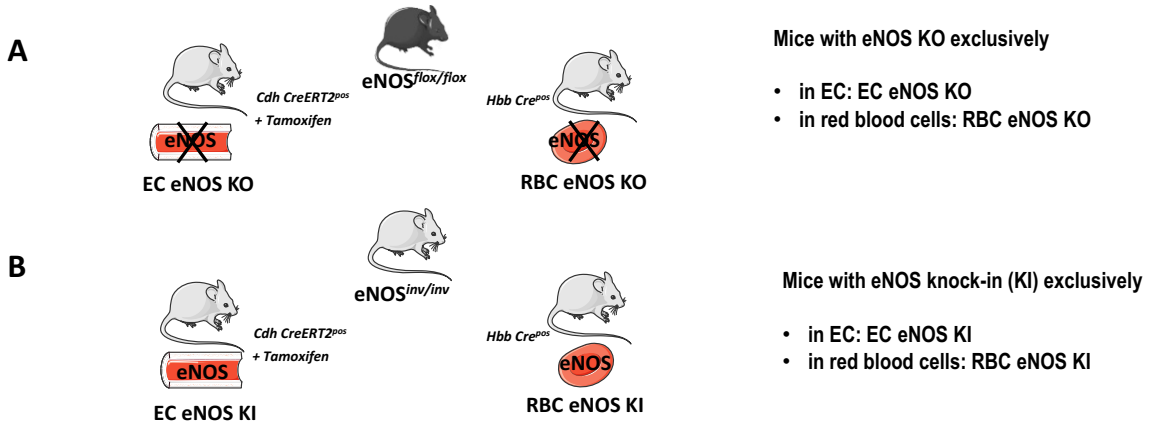


Figure 3: Endothelial cell (EC) and red blood cell (RBC) eNOS KO and knock-in (KI) mice. (**A**) Transgenic mice with EC and RBC specific deletion of eNOS and (**B**) EC and RBC knock-in (KI) eNOS. To generate EC eNOS KO mice and RBC eNOS KO, the founder eNOS$^{flox/flox}$ mice were crossed with endothelial-specific tamoxifen-inducible Cdh5-Cre/ERT2^{pos} or erythroid-specific Hbb-Cre^{pos} mice. To create EC eNOS KI mice, eNOS$^{inv/inv}$ mice = conditional eNOS KO were crossed with endothelial-specific tamoxifen-inducible Cdh5-Cre/ERT2^{pos} or erythroid-specific HbbCre^{pos} mice. Figure created by author.

To study a role of HAS3-mediated HA synthesis in vascular remodeling, mechanisensing and collateral athery growth, mice with global deletion of HAS3 and EC specific deletion of HAS3 KO were used (Figure 3).

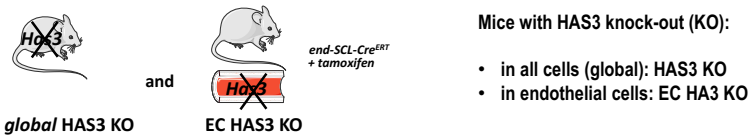


Figure 4: Global and endothelial cell specific HAS3 defieient mice. Transgenic mice expressing Cre recombinase under control of the tamoxifen-inducible 5' endothelial enhancer of the stem cell leukemia locus (*end-SCL-Cre^{ERT}*) were used to generate EC-specific HAS3-deficient mice. Figure created by author.

In details, the following experimental approaches were applied:

- The effects of endogenous hydrogen peroxide on vascular tone, blood pressure, adaptation to exercise in arterial and venous vasculature and exercise-induced EPCs mobilization were studied in mice with vascular specific overexpression of catalase (CAT-Tg mice) using voluntary running and forced exercise training.

- Effects of exogenous and endogenous NO on its receptor sGC and other important vasoprotective proteins of antioxidative and RAAS were studied in murine, bovine and human cells, mice and rabbits treated with long-acting NO-donors and mice with endothelial specific overexpression of eNOS (eNOS-Tg).

- The specific role of endothelial and non-endothelial eNOS in the regulation of NO-bioavailability, vascular tone and blood pressure was investigated in mice with endothelial-specific overexpression of eNOS (eNOS-Tg), with

endothelial specific rescue of eNOS (eNOS Tg/KO) and in EC- and RBC eNOS KO and KI mice.

- The role of destabilized eNOS and eNOS-dependent oxidative stress in the regulation of endothelial function, blood pressure, expression of vasoprotective proteins was investigated in mice with endothelial overexpression of mutated C101A-eNOS (C101A-eNOS-Tg) and mice expressing only destabilized form of eNOS in the endothelium (C101A-eNOS-Tg/KO mice).

- Significance of eNOS-dimer dependent oxidative stress and HAS3-mediated HA synthesis for neointima hyperplasia were studied in wild types, eNOS-knockouts (eNOS KO), C101A-eNOSTg/KO and HAS3-deficient mice (HAS3 KO) subjected to the carodid ligation. Hind limb ischemia model was used for unraveling the role of HA and HAS3 for collateral arteries growth in HAS3 KO and EC HAS3 KO mice.

3. SELECTED RESEARCH PAPERS

The order of selected research papers reflects the flow in Introduction and Discussion

3.1. Endogenous Vascular Hydrogen Peroxide Regulates Arteriolar Tension *in vivo*

Published in Circulation 2005, 112, 2487-2495

by **Suvorava T**, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G.

Objective: The reactive oxygen species hydrogen peroxide (H_2O_2) showed vasoconstrictor and vasodilator effects *in vitro* but little is known about vascular H_2O_2 effects *in vivo*.

Methods and Results: We have generated mice overexpressing human catalase driven by the Tie-2 promoter to specifically target this transgene to the vascular tissue. Vessels of these mice (CAT-Tg) expressed significantly higher levels of catalase mRNA, protein and activity. The overexpression was selective for vascular tissue as evidenced by immunohistochemistry in specimens of aorta, heart, lung, and kidney. Quantitation of reactive oxygen species by fluorescence signals in CAT-Tg vs. wild-types showed a strong decrease in aortic endothelium and left ventricular myocardium but not in leucocytes. Awake male CAT-Tg at 3-4 months of age had a significantly lower systolic blood pressure (sBP, 102.7±2.2 mmHg, n=10) compared to their transgene- negative littermates (WT, 115.6±2.5 mmHg, P=0.0211) and C57BL/6 (118.4±3.06 mmHg, n=6). Treatment with the catalase inhibitor aminotriazole increased sBP of CAT-Tg to 117.3±4.3 mmHg (P=0.0345), while having no effect in WT (118.4±2.4 mmHg, n=4, P>0.05). In contrast, treatment with the NO-synthase inhibitor nitro-L-argininemethylester (100 mg/kg BW/day) increased sBP in CAT-Tg and C57Bl/6 to a similar extent. Likewise, phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in skeletal muscle, left ventricular myocardium and lung was identical in CAT-Tg and WT. Endothelium- and NO-dependent aortic vasodilations were unchanged in CAT-Tg. Aortic KCl-contractions were significantly lower in CAT-Tg and exogenous H_2O_2 (10 µmol/L) induced vasoconstriction.

Conclusions and Significance: These data suggest that endogenous H_2O_2 is a vasoconstrictor in resistance vessels *in vivo* and contributes to the regulation of blood pressure.

3.2. Physical Inactivity Causes Endothelial Dysfunction in Healthy Young Mice

Published in *Journal of the American College of Cardiology*; 2004, 44(6), 1320-1327.

by **Suvorava T**, Lauer N, Kojda G.

Objective: Recent studies have linked exercise training to increased bioavailability of vascular nitric oxide (NO) and to improved endothelial function in patients with cardiovascular disorders. The effects of physical inactivity on normal vascular endothelial function are not known. We sought to determine if physical inactivity affects endothelial function in young healthy individuals.

Methods and Results: Healthy young male C57Bl/6 mice living in groups of five in large cages, where they were running, climbing, and fighting during their active cycle, were randomly assigned to stay there or to live alone in small cages where they were predominantly resting. After five and nine weeks citrate synthase activity, heart weight/body weight ratio, vascular reactivity, and protein expression of endothelial nitric oxide synthase (eNOS) were assessed.

Singularized mice showed a reduction of citrate synthase activity ($p < 0.05$), of endothelium-dependent vasorelaxation (to 65 +/- 5% of control levels; $p < 0.001$), and of eNOS protein expression (to 53 +/- 8% of control levels; $p < 0.01$). In striking contrast, vascular responses to potassium chloride, phenylephrine, and the NO-donor racemic S-nitroso-N-acetylpenicillamine were unchanged. The alterations of vascular eNOS-activity were completely reversible when singularized mice underwent exercise. In mice living in groups, exercise showed only a small effect on aortic eNOS expression.

Conclusions and Significance: In young healthy individuals physical inactivity induces endothelial dysfunction, which is completely reversible by a short period of moderate exercise training. We suggest that physical inactivity, the so-called sedentary lifestyle, increases cardiovascular risk in young healthy individuals by inducing endothelial dysfunction.

3.3. Critical Involvement of Hydrogen Peroxide in Upregulation of eNOS Induced by Exercise

Published in *Cardiovascular Research,* 2005, 65, 254-262.

by Lauer N, **Suvorava T**, Rüther U, Jacob R, Meyer M, Harrison DG, Kojda G**.**

Objective: Recent studies from our groups have indicated that endothelial nitric oxide synthase (eNOS) expression is increased in cell culture by both shear stress and by hydrogen peroxide (H_2O_2). *In vivo*, exercise training, known to increase both endothelial shear stress and oxidative stress, also increases eNOS expression. It is unclear if H_2O_2 contributes to an increase in eNOS expression in response to exercise training.

Methods and Results: To address this question, we generated mice overexpressing human catalase (CAT) driven by the murine Tie-2 promoter to specifically target this transgene to the vasculature (CAT-Tg). Vessels of CAT-Tg expressed significantly higher levels of catalase mRNA and catalase protein and activity but normal levels of eNOS. Exercise alone had no effect on catalase expression in C57BL/6. Wild-type littermates of CAT-Tg showed an increase in eNOS expression with 3 weeks of exercise (2.53+/-0.42-fold) comparable to C57BL/6 (2.93+/-0.45-fold). In striking contrast, 3 weeks of exercise had no effect on aortic (1.33+/-0.32-fold) and myocardial (1.1+/-0.2-fold) eNOS expression in catalase transgenic mice.

Conclusions and Significance: These data suggest that endogenous H_2O_2 plays a key role in the endothelial adaptation to exercise training by stimulating an up-regulation of eNOS.

.

3.4. Hydrogen Peroxide Inhibits Exercise-Induced Increase of Circulating Stem Cells with Endothelial Progenitor Capacity

Published in *Free Radic Res*; 2010, 44(2):199-207

by **Suvorava T**, Kumpf S, Rauch BH, Dao VT, Adams V, Kojda G.

Objective: The number of circulating stem cells with endothelial progenitor capacity (EPCs) inversely correlates with the number of cardiovascular risk factors. In this study we sought to investigate the effects of vascular H_2O_2 on circulating EPC levels.

Methods and Results: In C57BL/6 mice 3 weeks of freely moving or forced physical activity or voluntary exercise failed to increase circulating EPCs defined as double positive for Flk-1 and CD34, CD133 or Sca-1. Likewise, neither vascular overexpression of catalase (CAT-Tg) or eNOS (eNOS-Tg) nor eNOS knock-out changed EPCs in resting mice. In striking contrast, inhibition of catalase by aminotriazole strongly reduced circulating EPCs in sedentary CAT-Tg and their transgen-negative littermates, while forced or voluntary exercise training of C-Tg mice significantly increased the number of circulating EPCs. The latter effect was completely inhibitable by aminotriazole.

Conclusions and Significance: These data suggest that endogenous vascular H_2O_2 likely contributes to the impairment of important stem cell-induced vascular repair mechanisms in cardiovascular disease.

3.5. Catalase Activity Prevents Exercise-Induced Up-regulation of Vasoprotective Proteins in Venous Tissue

Published in *J Cell Mol Med*; 2010, 15(11):2326-34.

by Dao VT, Floeren M, Kumpf S, Both C, Peter B, Balz V, **Suvorava T**, Kojda G.

Objective: Physical activity induces favourable changes of arterial gene expression and protein activity, although little is known about its effect in venous tissue. Although our understanding of the initiating molecular signals is still incomplete, increased expression of endothelial nitric oxide synthase (eNOS) is considered a key event. This study sought to investigate the effects of two different training protocols on the expression of eNOS and extracellular superoxide dismutase (ecSOD) in venous and lung tissue and to evaluate the underlying molecular mechanisms.

Methods and Results: C57Bl/6 mice underwent voluntary exercise or forced physical activity. Changes of vascular mRNA and protein levels and activity of eNOS, ecSOD and catalase were determined in aorta, heart, lung and vena cava. Both training protocols similarly increased relative heart weight and resulted in up-regulation of aortic and myocardial eNOS. In striking contrast, eNOS expression in *vena cava* and lung remained unchanged. Likewise, exercise up-regulated ecSOD in the aorta and in left ventricular tissue but remained unchanged in lung tissue. Catalase expression in lung tissue and vena cava of exercised mice exceeded that in aorta by 6.9- and 10-fold, respectively, suggesting a lack of stimulatory effects of hydrogen peroxide. In accordance, treatment of mice with the catalase inhibitor aminotriazole for 6 weeks resulted in significant up-regulation of eNOS and ecSOD in vena cava.

Conclusions and Significance: These data suggest that physiological venous catalase activity prevents exercise-induced up-regulation of eNOS and ecSOD. Furthermore, therapeutic inhibition of vascular catalase might improve pulmonary rehabilitation.

3.6. Regulation of Vascular Guanylyl Cyclase by Endothelial Nitric Oxide-Dependent Posttranslational Modification

Published in *Basic Res Cardiol; 2011, 106(4):539-49.*

by Oppermann M*, **Suvorava T***, Freudenberger T, Dao VT, Fischer JW, Weber M, Kojda G. (2011).

*contributed equally

Objective: In isolated cells soluble guanylyl cyclase (sGC) activity is regulated by exogenous nitric oxide (NO) via downregulation of expression and post-translational S-nitrosylation. The aim of this study was to investigate whether such regulatory mechanism impact on endothelium-dependent vasodilation in a newly developed mouse strain carrying an endothelial-specific overexpression of eNOS (eNOS-Tg).

Methods and Results: Compared to transgene negative controls (eNOS-n), eNOS-Tg mice showed a 3.3-fold higher endothelial specific aortic eNOS expression, increased vascular cGMP and VASP phosphorylation, a L-nitro-arginine(L-NA)-inhibitable decrease of systolic blood pressure but normal levels of peroxynitrite and nitrotyrosine formation, endothelium-dependent aortic vasodilation and vasodilation to NO donors. Western blot analysis for sGC showed similar protein levels of sGC-α1 and sGC-β1 subunits in eNOS-n and eNOS-Tg. In striking contrast, the activity of isolated sGC was strongly decreased in lungs of eNOS-Tg. Semiquantitative evaluation of sGC-β1-S-nitrosylation demonstrated that this loss of sGC activity is associated with increased nitrosylation of the enzyme in eNOS-Tg, a difference that disappeared after L-NA-treatment.

Conclusions and Significance: Our data suggest the existence of a physiologic NO-dependent posttranslational regulation of vascular sGC in mammals involving S-nitrosylation as a key mechanism. Since this mechanism can compensate for reduction of vascular NO-bioavailability, it may mask the development of endothelial dysfunction.

.

3.7. Effect of Oral Organic Nitrates on Expression and Activity of Vascular Soluble Guanylyl Cyclase

Published in *Br. J. Pharmacol 2008, 115(3): 335-42.*

by Oppermann M, Dao VT, **Suvorava T**, Bas M, Kojda G.

Objective: The regulation of vascular soluble guanylyl cyclase (sGC) expression by nitric oxide (NO) is still under discussion. In vitro, NO has been shown to downregulate the expression of sGC but it is unclear if this mechanism is operative *in vivo* and occurs during nitrate treatment.

Methods and Results: We investigated whether high dose isosorbide mononitrate (ISMN) or pentaerythrityl tetranitrate (PETN) treatment changes vascular sGC expression and activity *in vivo*. New Zealand white rabbits received a standard diet, 2 or 200 mg ISMN kg(-1) d(-1) for 16 weeks, and C57Bl/6 mice received a standard diet, 6, 60 or 300 mg PETN kg(-1) d(-1) for four weeks. Absorption was checked by measuring the plasma levels of the drug/metabolite. Western blots of rabbit aortic rings showed similar protein levels of sGC alpha1- (P=0.2790) and beta1-subunits (P=0.6900) in all groups. Likewise, ANOVA showed that there was no difference in the expression of sGC in lungs of PETN-treated mice (P=0.0961 for alpha1 and P=0.3709 for beta1). The activities of isolated sGC in response to SNAP (1 microM -1 mM) were identical in aortae of ISMN-treated rabbits (P=0.0775) and lungs of PETN-treated mice (P=0.6348). The aortic relaxation response to SNAP slightly decreased at high ISMN but not at high PETN.

Conclusions and Significance: These data refute the hypothesis that therapeutic treatment with long acting NO donors has a significant impact on the regulation of vascular sGC expression and activity *in vivo*.

3.8. Pharmacological Induction of Vascular Extracellular Superoxide Dismutase Expression *in vivo*.

Published in *J Cell Mol Med. 2009 Jul;13(7):1271-8.*

by Oppermann M, Balz V, Adams V, Dao VT, Bas M, **Suvorava T**, Kojda G.

Objective: Pentaerythritol tetranitrate (PETN) treatment reduces progression of atherosclerosis and endothelial dysfunction and decreases oxidation of low-density lipoprotein (LDL) in rabbits. These effects are associated with decreased vascular superoxide production, but the underlying molecular mechanisms remain unknown. Previous studies demonstrated that endogenous nitric oxide could regulate the expression of extracellular superoxide dismutase (ecSOD) in conductance vessels *in vivo*.

Methods and Results: We investigated the effect of PETN and overexpression of endothelial nitric oxide synthase (eNOS-Tg) on the expression and activity of ecSOD. C57BL/6 mice were randomized to receive placebo or increasing doses of PETN for 4 weeks and eNOS-Tg mice with a several fold higher endothelial-specific eNOS expression were generated. The expression of ecSOD was determined in the lung and aortic tissue by real-time PCR and western blot. The ecSOD activity was measured using inhibition of cytochrome C reduction. There was no effect of PETN treatment or eNOS overexpression on ecSOD mRNA in the lung tissue, whereas ecSOD protein expression increased from 2.5-fold to 3.6-fold ($P < 0.05$) by 6 mg PETN/kg body weight (BW)/day and 60 mg PETN/kg BW/day, respectively. A similar increase was found in aortic homogenates. eNOS-Tg lung cytosols showed an increase of ecSOD protein level of 142 +/- 10.5% as compared with transgene-negative littermates ($P < 0.05$), which was abolished by N(omega)-nitro-L-arginine treatment. In each animal group, the increase of ecSOD expression was paralleled by an increase of ecSOD activity.

Conclusions and Significance: Increased expression and activity of microvascular ecSOD are likely induced by increased bioavailability of vascular nitric oxide. Up-regulation of vascular ecSOD may contribute to the reported antioxidative and anti-atherosclerotic effects of PETN.

3.9. Nitric Oxide Up-regulates Endothelial Expression of Angiotensin II type 2 Receptors

Published in *Biochemical Pharmacology; 2016, 112:24-36.*

by Dao VT, Medini S, Bisha M, Balz V, **Suvorava T**, Bas M, Kojda G.

Objective: Increasing vascular NO levels following up-regulation of endothelial nitric oxide synthase (eNOS) is considered beneficial in cardiovascular disease. Whether such beneficial effects exerted by increased NO levels include the vascular renin–angiotensin system remains elucidated.

Methods and Results: Exposure of endothelial cells originated from porcine aorta, mouse brain and human umbilical veins to different NO-donors showed that expression of the angiotensin-II-type-2-receptor (AT2) mRNA and protein is up-regulated by activation of soluble guanylyl cyclase, protein kinase G and p38 mitogen-activated protein kinase without changing AT2 mRNA stability. In mice, endothelial-specific overexpression of eNOS stimulated, while chronic treatment with the NOS-blocker L-nitroarginine inhibited AT2 expression. The NO-induced AT2 up-regulation was associated with a profound inhibition of angiotensin-converting enzyme (ACE)-activity. In endothelial cells this reduction of ACE-activity was reversed by either the AT2 antagonist PD 123119 or by inhibition of transcription with actinomycin D. Furthermore, in C57Bl/6 mice an acute i.v. bolus of L-nitroarginine did not change AT2-expression and ACE-activity suggesting that inhibition of ACE-activity by endogenous NO is crucially dependent on AT2 protein level. Likewise, three weeks of either voluntary or forced exercise training increased AT2 expression and reduced ACE-activity in C57Bl/6 but not in mice lacking eNOS suggesting significance of this signaling interaction for vascular physiology. Finally, aortic AT2 expression is about 5 times greater in female as compared to male C57Bl/6 and at the same time aortic ACE activity is reduced in females by more than 50%.

Conclusions and Significance: Taken together, these findings imply that endothelial NO regulates AT2 expression and that AT2 may regulate ACE-activity.

.

3.10. Sustained Hypertension despite Endothelial-Specific eNOS Rescue in eNOS-Deficient Mice

Published in *Biochemical and Biophysical Research Communications*; 2015, 458: 576–583.
by **Suvorava T,** Stegbauer J, Thieme M, Pick S, Friedrich S, Rump LC, Hohlfeld T, Kojda G.

Objective: The aim of the study was to evaluate the possible contribution of non-endothelial eNOS to the regulation of blood pressure (BP).

Methods and Results: To accomplish this, a double transgenic strain expressing eNOS exclusively in the vascular endothelium (eNOS-Tg/KO) has been generated by endothelial-specific targeting of bovine eNOS in eNOS-deficient mice (eNOS-KO). Expression of eNOS was evaluated in aorta, myocardium, kidney, brain stem and skeletal muscle. Organ bath studies revealed a complete normalization of aortic reactivity to acetylcholine, phenylephrine and the NO-donors in eNOS-Tg/KO. Function of eNOS in resistance arteries was demonstrated by acute i.v. infusion of acetylcholine and the NOS-inhibitor L-NAME. Acetylcholine decreased mean arterial pressure in all strains but eNOS-KO responded significantly less sensitive as compared eNOS-Tg/KO and C57BL/6. Likewise, acute i.v. L-NAME application elevated mean arterial pressure in C57BL/6 and eNOS-Tg/KO, but not in eNOS-KO. In striking contrast to these findings, mean, systolic and diastolic BP in eNOS-Tg/KO remained significantly elevated and was similar to values of eNOS-KO. Chronic oral treatment with L-NAME increased BP to the level of eNOS KO only in C57BL/6, but had no effect on hypertension in eNOS-KO and eNOS-Tg/KO.

Conclusions and Significance: Taken together, functional reconstitution of eNOS in the vasculature of eNOS KO not even partially lowered BP. These data suggest that the activity of eNOS expressed in non-vascular tissue might play a role in physiologic BP regulation.

3.11. Red Blood Cell and Endothelial eNOS Independently Regulate Circulating Nitric Oxide Metabolites and Blood Pressure

Published in *Circulation. 2021; 144(11):870-889.*

by Leo F*, **Suvorava T***, Heuser SK, Li J, LoBue A, Barbarino, F, Piragline E, Schneckmann R, Hutzler B, Good ME, Fernandez BO, Vornholz L, Rogers S, Doctor A, Grandoch M, Stegbauer J, Weitzberg E, Feelisch M, Lundberg JO, Isakson BE, Kelm M, Cortese-Krott MM.

*contributed equally

Objective: Current paradigms suggest that nitric oxide (NO) produced by endothelial cells (ECs) through endothelial nitric oxide synthase (eNOS) in the vessel wall is the primary regulator of blood flow and blood pressure. However, red blood cells (RBCs) also carry a catalytically active eNOS, but its role is controversial and remains undefined. This study aimed to elucidate the functional significance of RBC eNOS compared with EC eNOS for vascular hemodynamics and nitric oxide metabolism.

Methods and Results: We generated tissue-specific loss- and gain-of-function models for eNOS by using cell-specific Cre-induced gene inactivation or reactivation. We created 2 founder lines carrying a floxed eNOS ($eNOS^{flox/flox}$) for Cre-inducible knockout (KO), and gene construct with an inactivated floxed/inverted exon ($eNOS^{inv/inv}$) for a Cre-inducible knock-in (KI), which respectively allow targeted deletion or reactivation of eNOS in erythroid cells (RBC eNOS KO or RBC eNOS KI mice) or in ECs (EC eNOS KO or EC eNOS KI mice). Vascular function, hemodynamics, and nitric oxide metabolism were compared *ex vivo* and *in vivo*.

The EC eNOS KOs exhibited significantly impaired aortic dilatory responses to acetylcholine, loss of flow-mediated dilation, and increased systolic and diastolic blood pressure. RBC eNOS KO mice showed no alterations in acetylcholine-mediated dilation or flow-mediated dilation but were hypertensive. Treatment with the nitric oxide synthase inhibitor Nγ-nitro-L-arginine methyl ester further increased blood pressure in RBC eNOS KOs, demonstrating that eNOS in both ECs and RBCs contributes to blood pressure regulation. Although both EC eNOS KOs and RBC eNOS KOs had lower plasma nitrite and nitrate concentrations, the levels of bound NO in RBCs were lower in RBC eNOS KOs than in EC eNOS KOs. Reactivation of eNOS in ECs or RBCs rescues the hypertensive phenotype of the eNOSinv/inv mice, whereas the levels of bound NO were restored only in RBC eNOS KI mice.

Conclusions and Significance: These data reveal that eNOS in ECs and RBCs contribute independently to blood pressure homeostasis.

3.12. Selective Impairment of Blood Pressure Reduction by Endothelial Nitric Oxide Synthase Dimer Destabilization in Mice

Published in *J. Hypertens. 2017 Jan; 35(1):76-88.*

by **Suvorava T***, Pick S*, Kojda G.

*contributed equally

Objective: Endothelial dysfunction and oxidative stress are associated with hypertension but whether endothelial superoxide may play a role in the early development of essential hypertension remains uncertain.

The aim of this study was to elucidate whether eNOS-derived endothelial oxidative stress is involved in the regulation of blood pressure.

Methods and Results: Wild-type eNOS (eNOS-Tg) or a novel dimer-destabilized eNOS-mutant harboring a partially disrupted zinc-finger (C101A-eNOS-Tg) was introduced in C57BL/6 in an endothelial-specific manner. Mice were monitored for aortic endothelium-dependent relaxation, systolic blood pressure, levels of superoxide and several post-translational modifications indicating activity and/or increased vascular oxidative stress. Some groups of mice underwent voluntary exercise training for 4 weeks or treatment with the SOD mimetic Tempol.

C101A-eNOS-Tg showed significantly increased superoxide generation, protein- and eNOS-tyrosine-nitration, eNOS-S-glutathionylation, eNOS$^{1176/79}$ phosphorylation and AMP kinase (AMPKα) phosphorylation at Thr172 in aorta, skeletal muscle, left ventricular myocardium and lung as compared to eNOS-Tg and wildtype (WT)-controls. Exercise training increased phosphorylation of eNOS at Ser$^{1176/79}$ and AMPKα in WT. These physiologic adaptations were absent in C101A-eNOS-Tg. Aortic endothelium-dependent relaxation was similar in all strains. C101A-eNOS-Tg displayed normal blood pressure despite higher levels of eNOS, while eNOS-Tg showed significant hypotension. Tempol completely reversed the occurring protein modifications and significantly reduced blood pressure in C101A-eNOS-Tg but not in WT.

Conclusions and Significance: Oxidative stress generated by endothelial-specific expression of genetically destabilized C101A-eNOS selectively prevents BP reducing activity of vascular eNOS, while having no effect on aortic endothelial-dependent relaxation. These data suggest that oxidative stress in microvascular endothelium may play a role for the development of essential hypertension.

3.13. Impact of eNOS-dependent Oxidative Stress on Endothelial Function and Neointima Formation

Published in *Antioxidants & Redox Signaling 2015; 23(9):711-23.*

by **Suvorava T**, Nagy N, Pick S, Lieven O, Ruether U, Dao VT, Fischer JW, Weber M, Kojda G.

Objectives: Vascular oxidative stress generated by uncoupled endothelial NO-synthase (eNOS) was observed in experimental and clinical cardiovascular disease but its relative importance for vascular pathologies is unclear. We investigated the impact of eNOS-dependent vascular oxidative stress on endothelial function and on neointimal hyperplasia.

Methods and Results: A dimer-destabilized mutant of bovine eNOS where cysteine 101 was replaced by alanine (C101A-eNOS-Tg) was cloned and introduced into an eNOS-deficient mouse strain (eNOS-KO) in an endothelial-specific manner (C101A-eNOS-Tg/KO). Destabilization of mutant eNOS in cells and eNOS-KO was confirmed by the reduced dimer/monomer ratio. Purified mutant eNOS and transfected cells generated less citrulline and NO, respectively, while superoxide generation was enhanced. In eNOS-KO introduction of mutant eNOS caused a significant increase in superoxide and peroxynitrite formation in aorta and myocardium. This was completely blunted by a NOS-inhibitor. Nevertheless, expression of mutant eNOS in eNOS-KO completely restored maximal aortic endothelium-dependent relaxation to acetylcholine. Neointimal hyperplasia induced by carotid binding was much larger in eNOS-KO than in mutant C101A-eNOS-Tg/KO and C57BL/6, while the latter strains showed comparable hyperplasia. Likewise, vascular remodeling was blunted in eNOS-KO only.

Conclusions and Significance: Our results provide the first *in vivo* evidence that eNOS-dependent oxidative stress is unlikely to be an initial cause of impaired endothelium-dependent vasodilation and/or a pathologic factor promoting intimal hyperplasia. These findings highlight the importance of other sources of vascular oxidative stress in cardiovascular disease. eNOS-dependent oxidative stress is unlikely to induce functional vascular damage as long as concomitant generation of NO is preserved. This underlines the importance of current and new therapeutic strategies in improving endothelial NO generation.

3.14. Deletion of Hyaluronan Synthase 3 Inhibits Neointimal Hyperplasia in Mice

Published in *Atheroscler Thromb Vas Biol. 2016;36(2):e9-16.*

by Kiene LS, Homann S, **Suvorava T**, Rabausch B, Müller J, Kojda G, Kretschmer I, Twarock S, Dai G, Deenen R, Hartwig S, Lehr S, Köhrer K, Savani RS, Grandoch M*, Fischer JW*

*contributed equally

Objective: Hyaluronan (HA) is a polymeric glucosaminoglycan that forms a provisional extracellular matrix in diseased vessels. HA is synthesized by 3 different HA synthases (HAS1, HAS2, and HAS3). Aim of this study was to unravel the role of the HAS3 isoenzyme during experimental neointimal hyperplasia.

Methods and Results: Neointimal hyperplasia was induced in HAS3-deficient mice by ligation of the carotid artery. HA in the media of HAS3-deficient mice was decreased 28 days after ligation, and neointimal hyperplasia was strongly inhibited. However, medial and luminal areas were unaffected. Cell density, proliferation, and apoptosis were not altered, suggesting a proportional decrease of both, the number of cells and extracellular matrix. In addition, endothelial function as determined by acetylcholine-induced relaxation of aortic rings, immunoblotting of endothelial nitric oxide synthase, and arterial blood pressure were not affected. Furthermore, the oxidative stress response was not affected as determined in total protein extracts from aortae. Transcriptome analysis comparing control versus ligated carotid arteries hinted toward a mitigated differential regulation of various signaling pathways in Has3-deficient mice in response to ligation that were related to vascular smooth muscle cell (VSMC) migration, including focal adhesions, integrins, mitogen-activated protein kinase, and phosphatidylinositol signaling system. Lentiviral overexpression of HAS3 in VSMC supported the migratory phenotype of VSMC in response to platelet-derived growth factor BB in vitro. Accordingly, knockdown of HAS3 reduced the migratory response to platelet-derived growth factor BB and in addition decreased the expression of PDGF-B mRNA.

Conclusions and Significance: HAS3-mediated HA synthesis after vessel injury supports seminal signaling pathways in activation of VSMC, increases platelet-derived growth factor BB-mediated migration, and in turn enhances neointimal hyperplasia *in vivo*.

3.15. Endothelial Hyaluronan Synthase 3 Augments Postischemic Arteriogenesis through CD44/eNOS Signaling

Published in *Atheroscler Thromb Vasc Biol.* 2021;41(10):2551-2562.

Schneckmann R., **Suvorava T**, Hundhausen C, Schuler D, Lorenz C, Freudenberger T, Kelm M, Fischer JW, Flögel U, Grandoch M.

Objective: The dominant driver of arteriogenesis is elevated shear stress sensed by the endothelial glycocalyx thereby promoting arterial outward remodeling. Hyaluronan, a critical component of the endothelial glycocalyx, is synthesized by 3 HAS isoenzymes (hyaluronan synthases 1-3) at the plasma membrane. Considering further the importance of HAS3 for smooth muscle cell and immune cell functions we aimed to evaluate its role in collateral artery growth

Methods and Results: Male HAS3-deficient (HAS3 KO) mice were subjected to hindlimb ischemia. Blood perfusion was monitored by laser Doppler perfusion imaging and endothelial function was assessed by measurement of flow-mediated dilation *in vivo*. Collateral remodeling was monitored by high resolution magnetic resonance angiography. A neutralizing antibody against CD44 (clone KM201) was injected intraperitoneally to analyze hyaluronan signaling *in vivo*. After hindlimb ischemia, HAS3 KO mice showed a reduced arteriogenic response with decreased collateral remodeling and impaired perfusion recovery. While postischemic leukocyte infiltration was unaffected, a diminished flow-mediated dilation pointed towards an impaired endothelial cell function. Indeed, endothelial AKT (protein kinase B)-dependent eNOS (endothelial nitric oxide synthase) phosphorylation at Ser1177 was substantially reduced in HAS3 KO thigh muscles. Endothelial-specific HAS3 KO mice mimicked the hindlimb ischemia-induced phenotype of impaired perfusion recovery as observed in global HAS3-deficiency. Mechanistically, blocking selectively the hyaluronan binding site of CD44 reduced flow-mediated dilation, thereby suggesting hyaluronan signaling through CD44 as the underlying signaling pathway.

Conclusions and Significance: In summary, HAS3 contributes to arteriogenesis in hindlimb ischemia by hyaluronan/CD44-mediated stimulation of eNOS phosphorylation at Ser1177. Thus, strategies augmenting endothelial HAS3 or CD44 could be envisioned to enhance vascularization under pathological conditions.

4. CONCLUSIONS

"A conclusion is simply the place where someone got tired of thinking."

Albert Einstein (1879-1955)

The main results of these studies are summarized graphically in Figure 5 and in the following chapers. The full texts are attached to this document.

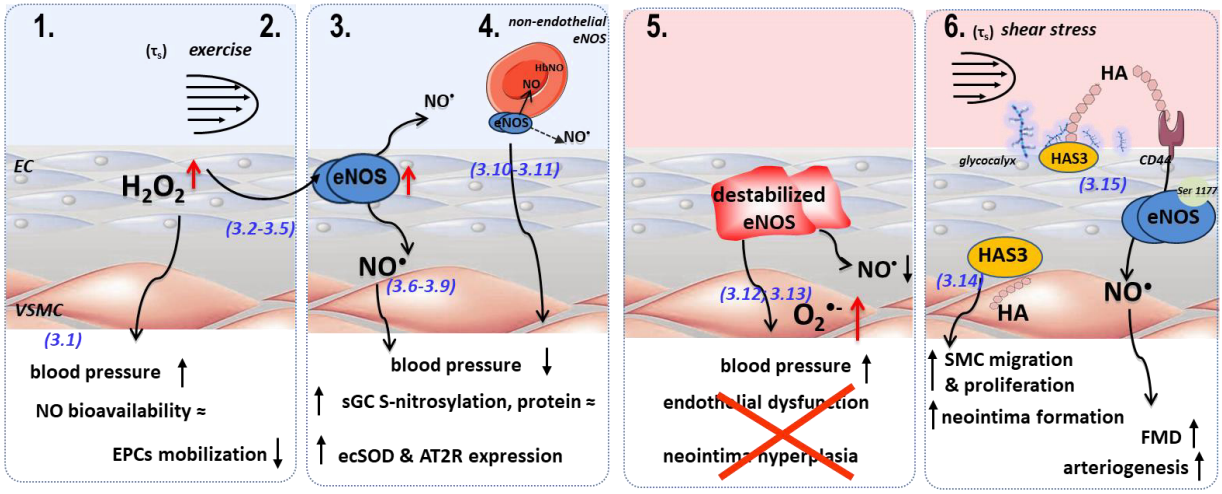


Figure 5: Graphic summary of the main results. 3.1 – 3.15 selected research papers as listed in Chapter 3. EC - endothelial cells, VSMC – vascular smooth muscle cells, EPC – endothelial progenitor cells, eNOS – endothelial NO-synthase, ecSOD – extracellular superoxide dismutase, AT2R – angiotensin II type 2 receptor, HA – hyaluronan, HAS3 – hyaluronan synthase 3, FMD – flow-mediated dilation, CD44 – cluster of differentiation 44. Figure created by author.

The main findings of these studies are:

1. Endogenous steady state levels of hydrogen peroxide contribute to maintenance of arteriolar tone and blood pressure.

2. Vascular hydrogen peroxide is a crucial player in exercise-induced mobilization of EPCs, upregulation of eNOS and other vasoprotective proteins in arterial but not venous tissue.

3. Endogenous and exogenous NO increases vascular ecSOD, angiotensin type 2 receptors expression and regulates sGC activity by its posttranslational S-nitrosylation.

4. Non-endothelial eNOS in red blood cells is an important regulator of NO metabolism and blood pressure homeostasis.

5. Increased generation of vascular superoxide and peroxynitrite by destabilized eNOS plays is unlikely a pathological factor promoting endothelial dysfunction and intima hyperplasia.

6. The vascular smooth muscle cell-associated HA synthesyzed by HAS3 enhances neointima hyperplasia; endothelial HAS3-derived HA contributes to mechanosensing and arteriogenesis in hindlimb ischemia by HA/CD44-mediated stimulation of eNOS phosphorylation at Ser1177.

4.1. Endogenous Hydrogen Peroxide and Blood Pressure Homeostasis

The main finding of the study (Ref. § 3.1) is that a reduction of steady-state concentrations of vascular hydrogen peroxide induced by a vascular-specific overexpression of catalase resulted in a marked reduction of systolic blood pressure in mice. This hypotension was completely reversible by the catalase inhibitor aminotriazole. In contrast to the reports in unspecific catalase overexpressing mice, which showed normal blood pressure (172), specific targeting of catalase overexpression to the vasculature was important to unmask a vasotonic effect of endogenous hydrogen peroxide.

It is known that vascular hydrogen peroxide has many associations to the vascular NO/cGMP pathway (41, 173, 174). However, profound hypotension in CAT-Tg mice was not dependent on endogenous nitric oxide bioavailability. There was no difference in vascular eNOS protein content, eNOS activity and the efficiency of the NO/cGMP pathway between CAT-Tg and wild type mice. Thus, these data did not support the hypothesis that vascular hydrogen peroxide impairs endogenous NO production and bioavailability in blood pressure-regulating skeletal resistance vessels.

Investigation of endothelial function and vascular tone in aortic tissue of CAT-Tg and WT mice revealed no difference in concentration-dependent vasoconstriction to phenylephrine. However, inhibition of catalase activity unmasked a potentiation of adrenergic vasoconstriction by phenylephrine in aortic rings of WT mice which was attenuated in CAT-Tg. When exogenous hydrogen peroxide was applied, small vasoconstrictor effects were observed in wild type mice only, while higher concentrations induced strong and irreversible vasodilator effects in both WT and CAT-Tg, suggesting that concentrations of hydrogen peroxide which likely resemble *in vivo* conditions potentiate adrenergic vasoconstrictor effects. Interactions of hydrogen peroxide with some proteins of α1A/C-receptors signal transduction pathway such as phospholipase C, protein kinase C and phosphoinositide 3-kinase (175) might explain the molecular mechanisms of hydrogen peroxide-induced increases of vascular tone. These results are consistent with a previous report showing that increases of blood pressure in response to vasoconstrictor agents such as norepinephrine and angiotensin II were less pronounced in mice with an unspecific overexpression of catalase (172).

Early studies suggested that elevated hydrogen peroxide levels may contribute to pathologic events resulting in hypertension. Lacy et al. showed that in hypertension the plasma concentration of hydrogen peroxide is increased and is positively correlated to plasma renin activity and systolic blood pressure, while negatively correlated to cardiac contractility and renal function (46). The direct demonstration of hypotension in mice carrying a vascular-

specific overexpression of catalase argued for the vasoconstrictor effects of endogenous hydrogen peroxide on resistance vessels and possible contribution of these effects to the development of hypertension.

In recent years, the view on the signaling properties of hydrogen peroxide has been greatly changed by the implication of redox signaling (10, 35, 37, 176, 177) in regulation of different physiological and pathological processes. Hydrogen peroxide is now undoubtly considered as an important redox regulatory molecule. Biological reactions catalysed by hydrogen peroxide typically involve the reversible oxidation of cysteine residues of the key signaling proteins, which may affect vascular proteins function to enable homeostatic regulation (10, 35, 176-178). For instance, an importance of hydrogen peroxide for vascular redox signaling was shown in a study using mice with "redox dead" version of protein kinase G I-alpha. The inability of *resistance* vessels to sense, transduce and relax in response to hydrogen peroxide resulted in a hypertensive phenotype (179). Activation/deactivation cycles of hydrogen peroxide production coordinate the spatiotemporal organization of redox signaling (10, 178). Future studies are emerged to define the components and mechanisms involved in performing vascular redox chemistry as well as how the cells spatially and temporally channel hydrogen peroxide into specific signaling pathways to achieve the desired cellular outcomes.

4.2. Endogenous Hydrogen Peroxide, eNOS and Exercise Training

The studies described here (Ref. §3.2-3.5) were focused on the effects of endogenous hydrogen peroxide and NO in the vascular adaptation to exercise training. Activation of redox sensitive regulatory by transient ROS/RNS generation in acute exercise may stimulate the longer lasting expression of certain antioxidants enzymes and thereby increases antioxidant capacity of the vascular wall. More extended periods of exercise results in a stimulation of radical scavenging systems that include copper-zinc-containing superoxide dismutase (180), extracellular superoxide dismutase (72), and glutathione peroxidase (181) as well as reduced expression of NADPH oxidase (99). These effects contribute to improved endothelial function and are beneficial for vascular health.

In contrast to moderate exercise, sedentary lifestyle decerases NO-bioavailability and induces endothelial dysfunction (Ref. § 3.2, (67, 182)). In general, the mechanism of endothelial dysfunction is multifactorial and depends on the underlying pathological process (183-185). Here (§ 3.2.) the development of endothelial dysfunction was found in healthy mice that had been subjected to singularization. At the same time, the protein expression of eNOS was reduced by one-half, whereas activation of the NO/cGMP pathway was equally effective in singularized and control mice. Downregulation of eNOS expression appears to be a key mechanism underlying the impairment of endothelial function in sedentary mice. In accordance, we demonstrate that exercise reversed endothelial dysfunction induced by singularization. However, other mechanisms decreasing the bioavailability of endothelial NO in sedentary mice, such as post-translational modification of eNOS activity or other proteins might also play a role.

Regarding the wildlife habits, singularization of mice is an artificial situation. This holds also true for mice being caged in groups. Interestingly, we found identical endothelium-dependent vasodilation in exercised and group living mice, although there was a large and significant difference concerning citrate synthase activity, which was shown to correlate with physical activity (186, 187). This suggests that low-intensity physical activity may be sufficient to maintain normal endothelial function in young healthy individuals. Furthermore, vigorous exercise has no further impact on endothelium-dependent vasodilation and eNOS expression in normally active mice. Thus, it seems likely that the differences in physical activity in the experimental setup described above mimic to a certain extent the situation in humans. For example, it was shown that there is an identical rate of cardiovascular events between women who simply walked for exercise and those who underwent vigorous exercise (188). Interestingly, only leisure time but not occupational physical activity is shown to improve cardiorespiratory and metabolic fitness and health (189).

Using the transgenic mouse model with vascular-specific overexpression of catalase, contribution of endogenously produced hydrogen peroxide in eNOS upregulation by exercise training was established supporting the concept that ROS/RNS play an important role as second messengers for the adaptations to exercise (Ref. §3.3). The mechanism of exercise-induced up-regulation of eNOS activity appears to be multifactorial (65, 66, 190, 191). Regular bouts of increased laminar flow along the endothelium increase eNOS expression and eNOS phosphorylation at Ser1177 and improve antioxidative protection. Experimental evidence suggested that activation of c-Src (192) and vascular hydrogen peroxide (Ref. §3.3, (193) are critically involved. The increase of eNOS activity in response to hydrogen peroxide comes along with an Akt-dependent eNOS phosphorylation at Ser 1177 and is thought to be an acute cellular adaptation to an increase in oxidant stress (52, 99, 194). Likewise, exercise increases expression of vascular ecSOD, which could enhance formation of hydrogen peroxide (72), which could, in its turn, also contribute to the upregulation of eNOS expression. Physical training also results in activation of AMP-activated protein kinase (AMPK) as global (195) and endothelial-specific deletion of AMPK (196) prevented eNOS upregulation by exercise.

Furthermore, exercise increases not only oxygen consumption but also the generation of ROS such as superoxide and hydrogen peroxide (81, 197) and shear stress has been shown to increase the vascular generation of ROS by an endothelium-dependent mechanism (81, 82). In a physiological context, increased generation of ROS by acute bout of exercise could be important to avoid excessive dilation. Interestingly, antioxidants appear to prevent health-promoting effects of physical exercise in humans (198). Thus, ROS generation by acute exercise could be part of a hormetic response that is known to mediate benefits to physical training, in which redox signaling prevention by improved antioxidant capacity abrogates the favourable health effects.

Mobilization of EPCs from the bone marrow is another important adaptation to exercise. Numerous studies provided evidence of the exercise-induced mobilization of EPCs from the bone marrow to the circulation (199) or improved functional capacities of EPCs after exercise (200). Experimental data have shown that mobilization of EPCs *in vivo* from the bone marrow is substantially eNOS dependent (201, 202), and is due to activation of matrix

metalloproteinases 2 and 9 (203). Using training protocols of different intensities and several approaches to assess peripheral EPCs levels, we found that 4 week exercise apparently doesn't change circulating EPCs in young healthy wild type mice (Ref. § 3.4). In striking contrast, exercise strongly increased EPCs in mice with vascular specific overexpression of catalase and this was completely abolished by a catalase inhibitor. Likewise, inhibition of catalase decreased circulating EPCs in both CAT-Tg and their wild type littermates independent of exercise. Our data provide a new pathophysiologic role for endogenous hydrogen peroxide, i.e. a reduction of the number of circulating EPCs and inhibition of exercise-induced EPCs mobilization from the bone marrow.This finding was supported by other studies showing involvement of ROS, especially hydrogen peroxide and the ROS-regulated bone marrow microenvironment in the regulation of stem and progenitor cell functions such as self-renewal, differentiation, survival/apoptosis, proliferation, and mobilization (204).

Little was known about the exercise-induced expression and activity of eNOS in low-pressure vasculature. In one investigation there was a small increase of eNOS after 1 week of exercise in pulmonary arteries of miniature pigs (205) and another study showed similar effects after an acute bout of exercise in rats (206). To understand the importance of exercise in venous vascular biology, effects of exercise on the expression of eNOS and ecSOD in low pressure vasculature such as vena cava or the lung circulation were investigated in Ref. § 3.5 using forced and voluntary exercise approaches. The major new finding is that – in contrast to arterial tissue – exercise had no effect on the expression of eNOS and ecSOD in venous tissue, however, inhibition of catalase resulted in up-regulation of these proteins in *vena cava* after exercise training.

These data might be helpful to explain why pulmonary rehabilitation programs do not improve lung mechanics and gas exchange, while exercise improves arterial functions such as endothelium dependent vasodilation and organ perfusion (73, 207). In view of the significance of lung perfusion for blood arterialization, exercise induced up-regulation of eNOS in the pulmonary circulation could theoretically improve pulmonary gas exchange. Furthermore, upregulation of eNOS might reduce the transmural pressure of pulmonary blood vessels by shear-induced endothelium-dependent vasodilation and this might have a protective effect on the blood gas barrier (208). Taking together, the results of Ref. §3.5 suggest that physiological venous catalase activity prevents exercise-induced up-regulation of eNOS and ecSOD and that the effect of exercise on these protein expression does not occur in a similar manner in the venous vessels.

4.3. NO-dependent Regulation of Vasoprotective Proteins *in vivo*

NO-dependent regulation of sGC protein and activity In the study (Ref. §3.6) a new mouse strain with endothelial-specific overexpression of eNOS (eNOS-Tg) was applied to unravel the effects of endogenous NO on the expression and activity of vascular sGC. In contrast to another existing mouse model for endothelial specific eNOS overexpression (209), eNOS-Tg showed a relative moderate increase of vascular eNOS protein level. The overexpressed eNOS protein was functionally active as indicated by a significant reduction of blood pressure,

which was inhibited by treatment with the NOS-inhibitor. Moreover, there was no evidence of nitrosative stress in these mice as vascular levels of superoxide and peroxynitrite measured by electron spin resonance as well as the abundance of protein nitrotyrosine residues were identical to the transgene negative littermates. Likewise, no evidence for a resistance to vasodilator effects of endogenous NO and NO-donors were found.

The major new finding in eNOS-Tg is that endothelial NO-bioavailability triggers S-nitrosylation of its key receptor sGC and thereby negatively regulates sGC activity *in vivo* in a reversible manner. Despite significant changes of aortic NO-bioavailability, the functional efficacy of the vascular NO/cGMP pathway is maintained over a considerable range of vascular NO-levels. This study demonstrates that S-nitrosylation of sGC is an important physiologic posttranslational modification, which regulates sGC activity in an NO-dependent manner and provide experimental evidence that downregulation of sGC expression, which can be shown by treating isolated cells or arteries with NO (110, 210), appears not to occur *in vivo* as also found in PETN-treated mice and ISMN-treated rabbits (Ref. §3.7).

S-nitrosylation of sGC is likely a physiologic response to changes of vascular NO-bioavailability in both directions. Thus, S-nitrosylation of sGC by endothelial NO likely compensates for limited changes of vascular NO-bioavailability. For example, acetylcholine-dependent vasodilation was identical in eNOS-Tg and WT although one would have expected that a moderate increase in endothelial eNOS expression would potentiate thisresponse. In accordance, this compensatory mechanism might contribute to explain why exercise training, another approach to increase endothelial eNOS expression and vascular NO-bioavailability, is not consistently associated with improved endothelium-dependent vasodilation in healthy mammals (Ref. §3.2). On the other hand, a reduction or lack of vascular NO-bioavailability as accomplished by NOS-inhibition or eNOS depletion, respectively potentiates vascular sGC activity (113, 133). The reduction of NO-bioavailability might be masked and therefore not be detected until the NO-levels fall beyond the potentiator effect on sGC activity induced by reversal of sGC S-nitrosylation (Ref. §3.6).

The results of this research indicate that downstream of eNOS, the NO-cGMP pathway has a great capacity which ensures full functional activity even in the setting of strong desensitization of the central enzyme sGC. These results are in line with reports demonstrating that the majority of NO-sensitive sGC is not required for cGMP-forming activity (106, 211). In these studies, a complete NO-dependent vasodilation was achieved in aortic rings of sGC-α_1 deficient mice. This vasodilation occurred despite a low increase in vascular cGMP and was mediated by the sGCα_2/sGC-β_1 heterodimer which accounts for only 6% of total vascular sGC.

In summary, our results (Ref. §3.6-3.7) do not support the hypothesis that therapeutic and endogenous NO play a significant role in regulation of vascular sGC protein expression. Feedback signaling by NO on vascular sGC-expression did not occur *in vivo*, not even at continuous treatment with long-acting NO-donors (Ref. §3.7) or increased endogenous NO production (in eNOS-Tg, Ref. § 3.6). The activity of the key enzyme of the NO-cGMP pathway, the sGC is a subject of redox regulation via S-nitrosylation.

Regulation of vascular ecSOD by NO in vivo The study (Ref. §3.8) aimed to investigate whether vascular expression of an important antioxidant ecSOD is affected by NO. Applying genetic and pharmacological approaches to increase NO levels it was found that an exogenous NO donor, the organic nitrate PETN increases the expression and activity of microvascular and macrovascular ecSOD in mice *in vivo*, and a similar increase was observed in untreated eNOS-transgenic mice. Our data extend previous observations that increased bioavailability of vascular NO upregulates ecSOD expression, and that this effect might contribute to the antioxidative and anti-atherosclerotic effects of PETN observed previously (212, 213).

This is also the first study demonstrating that NO donor the organic nitrate PETN can induce the expression of ecSOD. There was some debate whether NO is indeed the pharmacologically active principle of organic nitrates (214). This study (Ref. § 3.8) demonstrated that neither PETN nor its trinitrate metabolite (PETriN) appears in blood plasma, suggesting that PETN and PETriN undergo extensive hepatic metabolism following intestinal absorption, the so-called first-pass effect (215) and that vascular NO formation from pentaerythritol dinitrate (PEDN) and pentaerythritol mononitrate (PEMN) is a prerequisite for the therapeutical efficacy of PETN. This was further substantiated using the eNOS-Tg mouse model were a significant up-regulation of ecSOD protein expression and activity has been found, suggesting that NO is the active mediator. In line with this findings, a strong down-regulation of ecSOD protein expression was measured after NOS-inhibition in eNOS-Tg and wild-type mice.

In human vascular smooth muscle cells, NO was shown to increase the transcription but not the half-life of ecSOD mRNA, and the effect on transcription was critically dependent on the activity of p38 mitogen activated protein (MAP) kinase (72). In contrast to these findings, in the present study (Ref. §3.8) no detectable changes in mRNA levels of the enzyme were found in murine lung and aortic tissue after PETN treatment and in eNOS-Tg mice as well. ecSOD is the only extracellular scavenger of superoxide anion that is secreted from the producing cells into the extracellular space and is bound to cell surface and extracellular matrix through its heparin-binding domain. One alternative pathway is that ecSOD is produced in other organs or tissues and reaches the lungs by travelling through the circulation. In this case, one would expect an increase of circulating ecSOD protein, which was indeed measurable (Ref. §3.8). However, it remains unclear whether this moderate increase of circulating ecSOD is sufficient to account for the increase of ecSOD found in the aorta and lungs.

Alternatively, a yet unresolved, solely post-translational *in vivo* regulation of ecSOD protein expression by NO might occur. ecSOD may undergo various posttranslational modifications through the secretory pathway, including N-glycosylation (216), disulfide bond formation (217), proteolytic cleavage (218, 219), cathepsin A-mediated degradation of EC-SOD protein (220) and a recent proteomics study showed ecSOD S-gluthathionylation (221). Peng *et al.* found an NO-induced inhibition of the ubiquitin–proteasome system in murine primary cortical neurons (222). Given this, one could speculate that the increase of ecSOD protein expression and activity *in vivo* could be a yet undiscovered effect of NO that needs to be substantiated further. However, other studies on this matter have suggested that NO rather increases proteasomal degradation of proteins (223). Taken together, established mechanisms of ecSOD regulation like the influence of proteolytic removal of the heparin-binding domain (218) and

inflammatory cytokines (117) might extend to a NO-dependent mechanism that deserves further interest and investigation.

The upregulation of AT2 expression by NO Among vascular mediator systems, the NO-cGMP, the kallikrein-kinin and RAAS are of major importance. The upregulation of AT2 expression by NO is predominantly mediated by the classical NO-cGMP pathway, i.e. activation of sGC, generation of cGMP and activation of PKG as was shown *in vitro* in mouse, porcine and human endothelial cell lines (Ref. § 3.9). The increase of AT2 by NO was found to be completely dependent on transcription suggesting that posttranscriptional regulation of AT2 expression, which has been described previously (224), is not altered by NO in endothelial cells. Importantly, the NO-induced AT2 up-regulation was associated with a profound inhibition of ACE-activity. In addition, study on the effects of exercise revealed a new vasoprotective pathway contributing to beneficial effects of physical training. Using two different training protocols, e.g. high-intensity forced exercise or low-intensity (voluntary running) protocols it was shown that exercise NO-dependently increases AT2 expression (Ref. § 3.9).

Taken together, NO increases the expression of AT2 in cells and mice by a transcriptional mechanism involving the activation of PKG and p38 MAPK and up-regulation of AT2 was associated with a reduced activity of ACE. This sequence of molecular events describing a feed-forward mechanism likely contributes to vasoprotective mechanisms of endothelial NO and the beneficial effects of exercise. In addition, it might play a role to the pathophysiology of angioedema induced by AT1-blockers, as these drugs increase vascular NO levels in cardiovascular patients (225), which might contribute to elevated AT2 expression levels with subsequent reduction of ACE. However, future studies are necessary to substantiate this hypothesis.

4.4. Non-endothelial eNOS in Blood Pressure Homeostasis

The relative importance of endothelium-derived NO for the regulation of blood pressure was first questioned in the study in which the bovine eNOS was re-expressed in the endothelium of eNOS-KO mice (Ref. §3.10 (226). The endothelium-dependent relaxation in aortic rings was completely rescued in these transgenic mice demonstrating a functionally active enzyme in the conduit arteries. Likewise, vasodilation in resistance arteries displayed a NOS inhibitor-sensitive component. Surprisingly, not even a partial reduction of blood pressure in eNOS-KO has been found. The mice with reconstituted eNOS had sustained hypertension as evidenced by elevated mean, systolic and diastolic blood pressure that was similar to hypertension recorded in the eNOS KO (126-128). These data pointed to the importance of eNOS expressed in non-endothelial cells for physiological regulation of blood pressure.

Non-endothelial eNOS expression was found in kidney (141), red blood cells (140) and astrocytes of the brain stem (142). A study, using cross-transplant chimera models suggested a role for a blood-borne eNOS in the regulation of blood pressure and nitrite homeostasis (227). However, the use of bone marrow chimeric mice present serious methodological limitations such as irradiation-dependent activation of inflammatory pathways, the

possibility of protein transfer from the blood to the endothelium (through the EPCs) or the lack of erythroid lineage targeting specificity. This maked it difficult to discriminate the blood cell population(s) carrying eNOS that might affect the tone of resistance arteries in chimeric mice (227).

More recently, red blood cell (RBC) and or endothelial cell (EC) specific eNOS KO and KI mice were created using Cre/LoxP technology by deleting exon 2 of eNOS (Ref § 3.11). Cell-specific deletion or re-introduction of eNOS was achieved using a hemoglobin β-chain or Cdh5 expressing Cre recombinase for RBC or EC-specific eNOS KO and KI, respectively. As expected, mice lacking eNOS only in ECs were hypertensive suggesting the central role of vascular endothelium in eNOS-mediated control of vascular function and blood pressure. Surprisingly, also RBC eNOS KO mice had a mild hypertension, although eNOS activity and function at the level of conduit and proximal resistance arteries were fully preserved. When eNOS was re-introduced into either ECs or RBCs of conditional global eNOS KO, it successfully rescued the hypertensive phenotype in both cases. These data strongly affirm the role of eNOS in both cell types being essential for blood pressure regulation. Likewise, both eNOS and RBC eNOS contribute to the levels of systemic NO metabolites, especially NO-heme, which was proposed to be an indicator of systemic NO-bioavailability (228, 229), but RBC eNOS and not EC eNOS was the major contributor to the level of NO-heme.

It is well known that eNOS protein expression and activity are significantly lower in RBCs than in ECs (140, 230). However, according to a recent estimate of the total number of cells in human body, RBCs were the largest contributor to the overall cell number (231). Thus, total RBC numbers with their extensive surface area may provide sufficient NO-bioavailability to contribute to BP regulation, especially at the distal sites of the arterial circulation, where eNOS expression is low (232). The contact of RBCs with the vessel wall there becomes closer and the smaller distance to the endothelial surface may facilitate the eNOS-dependent export of NO-bioactivity from erythrocytes.

In vascular endothelium eNOS is present on the luminal side of the EC and the basal side of the EC. NO is released from EC towards the vascular smooth muscle in immediate proximity and, to a lesser extent as a spillover into the blood at its luminal site. In resistance arteries NO transfer from the endothelium to the SMC occurs via myoendothelial junctions and is controlled by the redox state of hemoglobin alpha (29, 233). Thus, the contribution of ECs or RBCs to the circulating NO and regulation of vascular tone may differ substantially along the arterial circulation, and RBC-mediated release of NO bioactivity and its vasodilatory potential may critically depend on the diameter of the respective arterial segment.

The data present here (Ref. §3.11) also show that EC eNOS and RBC eNOS contribute to the level of systemic NO metabolites in different ways. In EC eNOS KO the amount of NO metabolites was decreased mainly in plasma, lung and heart, whereas in the RBC eNOS KO mice clear changes were detected in the blood. The data also show that the lack of eNOS in the RBCs and concomitant decrease of NO levels in RBCs (in form of NO-heme) and plasma correspond to an increase in blood pressure without affecting functional activity of eNOS in the endothelium.

These findings demonstrate the existence of RBC eNOS-dependent pathway in regulation of NO metabolism and blood pressure homeostasis. However, how exactly does RBC eNOS export NO-bioactivity and contribute to blood pressure regulation is yet to be determined.

4.5. Effects of Destabilized eNOS on Endothelial Function, Blood Pressure and Vascular Remodeling

Although essential hypertention is known to be associated with impairment of endothelium-dependent vasodilation in many different animal models, experimental hypertension was usually observed in coincidence with vascular ROS/RNS formation (150, 185, 234, 235). The initial formation of superoxide (for instance from NADPH-oxidase) and subsequent formation of peroxynitrite represent a mechanism of ROS-induced eNOS uncoupling. In contrast, little was known about the specific effects of eNOS-dependent formation of ROS (e.g. superoxide) on endothelial function and blood pressure in healthy state.

The studies Ref. § 3.12-3.13 were focused on the impact of the impairment of eNOS-activity induced by dimer-destabilization (and subsequent increase in superoxide production) on endothelial function, blood pressure and neointima formation. Genetic destabilization of eNOS in mutant mice and the alteration of activity of C101A-eNOS resemble the features of uncoupled eNOS (236). Mutant C101A-eNOS produces less NO and more superoxide (measured by electron spin resonance) which is likely the result of impairment of binding and correct orientation of BH_4 (153).

The novel transgenic mouse model C101A-eNOS-Tg is characterized by the coexistence of normal and dimer-destabilized eNOS and increased generation of ROS in the endothelium (Ref. § 3.12). Increase of ROS levels induced by dimer destabilization of eNOS in an otherwise healthy, without any signs of cardiovascular disease mouse strain had no effect on endothelial function but prevented the expected blood pressure reduction by eNOS. C101A eNOS transgenic mice display normal systolic blood pressure despite higher levels of eNOS whereas mice overexpressing wild type eNOS are hypotensive. However, a significant and reversible reduction of blood pressure was observed during treatment with the antioxidant Tempol in C101A-eNOS-Tg but not in their controls. Thus, dimer destabilization of eNOS and the resulting increase of endothelium-specific superoxide levels in microvascular endothelium may play a role in the development of essential hypertension.

Increased vascular ROS/RNS levels induced by eNOS dimer destabilization cause nitration and S-glutathionylation of vascular proteins, as well as Ser1177-hyper-phosphorylation of eNOS which is presumably mediated by AMP-activated protein kinase α (AMPKα). The post-translational protein modifications were associated with the resistance of eNOS to Ser1177 -phosphorylation by physical forces, such as shear stress in exercise training. These finding suggest that shear-dependent activation of eNOS is diminished by either dimer-destabilization itself, subsequently increased endothelial superoxide or both. Antioxidant treatment with Tempol completely corrected hyperphosphorylation of eNOS at Ser 1177 and AMPKα pointing to a close relationship between superoxide,

hyper-phosphorylation at Ser1177 and eNOS-mediated blood pressure reduction. However, a diminished eNOS activity may play a role as well, since eNOS S-githathionylation and tyrosine-nitration of the eNOS was observed in C101A eNOS mutant with impaired zunk-sulfur complex forming ability.

A somewhat surprising finding of a study using double transgenic mice obtained by re-introduction of destabilized C101A-eNOS in endothelium of eNOS deficient mice was a normal endothelial function despite reduced NO-formation, decreased eNOS expression and increased levels of vascular ROS/RNS (Ref. § 3.13). The relaxation developed at tissue levels of superoxide and/or peroxynitrite which resemble those occurring in various animal models with the clearly detectable impairment of eNOS function (237-241). However, in each of these studies, concomitant conditions such as atherosclerosis, severe hypertension, diabetes or disruption of certain genes were present which might have contributed to endothelial dysfunction beside oxidative and/or nitrosative stress. It might be argued that the lack of effect of C101A-eNOS on mild hypertension in eNOS KO demonstrates an effect of increased ROS/RNS levels on resistance vessels and blood pressure. One would expect a blood pressure lowering effect of endothelial expression of functional eNOS as reported previously (209) However, this data are in line with the results of our study, which have shown that even endothelial-specific expression of normal eNOS does not reduce the hypertension of eNOS-KO (Ref. 3.10). Thus, the persistence of hypertension in C101A-eNOS-Tg/KO is probably not provoked by C101A-eNOS-dependent redox dysregulation and unlikely represents a specific phenotype caused by pronounced vascular oxidative stress in this mouse model. On the other hand, our data certainly don't exclude that increased ROS levels due to an impairment of eNOS activity influence blood pressure, e.g. in more severe hypertensive cardiovascular disease state (242).

Expression of C101A-eNOS-Tg in the endothelium of eNOS KO mice has been found to correct the accelerated neointimal hyperplasia and the abnormal vascular remodeling observed in eNOS-deficient mice (Ref. §.3.13). Smooth muscle proliferation is an obvious event during the development of atherosclerosis and restenosis (243-245) and ROS/RNS play a crucial role in this process (151, 246). This pathologic role is closely linked to a downregulation of vascular NO bioavailability (247). In the study (Ref. §3.13) neointima formation was significantly more pronounced in eNOS KO than in wild type mice and C101A-eNOS-Tg/KO confirming the protective effects of vascular eNOS in the setting of carotid ligation (248). Another important role of NO is to mediate vascular remodeling in response to changes in flow, however the vascular remodeling was similar in the wild type and mutant mice with the destabilized eNOS. These data obtained from the carotid ligation experiments indicate that the increased neointima formation in eNOS KO is caused by the lack of endothelial NO formation and not by the presence of mild hypertension.

Taken together the results of this study (Ref. §3.13) provided the first *in vivo* evidence that vascular ROS/RNS generated by an impairment of eNOS activity is unlikely an initial cause of impaired endothelium-dependent vasodilation and/or a pathologic factor promoting intimal hyperplasia and highlight the contribution of other enzymatic sources of ROS, such as vascular NADPH-oxidases to vascular pathologies (21, 149, 234, 243, 249).

4.6. Interrelationships of HA and NO Signaling: Role for HAS3

HA-matrix has a strategically important position in the vasculature and complex and context-specific biological functions. In healthy vessels the HA is mainly limited to the endothelial glycocalyx and adventitia and is protective against adhesion of platelets at the endothelium, leukocytes rolling, adhesion and extravasation and thus contributes to vasoprotection and vascular homeostasis. HA is shed from the glycocalyx in response to pathological factors such as ROS, hyperglycemia, smoking and inflammatory cytokines (250-252) and our previous study has shown that systemic inhibition of HA synthesis induces endothelial dysfunction (158). On the contrary, the marked upregulation of HA production and its accumulation are observed during neointima thickening in the atherosclerotic plaques and restenotic lesions (155, 157), where HA contributes to volume expansion and supports proliferative and secretory phenotype of VSMCs. The expression pattern of HA synthesis differs and HAS 1-3 isoforms are differently regulated in homeostasis and pathological states. Here (Ref. §3.14 - §3.15), we hypothesized an interconnection between HAS3-mediated HA- and NO-signaling pathways and tested the significance of HAS3/HA/eNOS axes for the development of neointimal hyperplasia and collateral arteries growth.

Hyaluronan Synthase 3-mediated HA Synthesis Enhances Neointima Hyperplasia Consistent with the HA-driven promotion of hyperplastic VSMC phenotypes, substantial reduction of neointima hyperplasia was found in mice lacking HAS3 isoform after carotid artery ligation (Ref. §3.14). No differences in medial and intimal cell density, proliferation and apoptosis were found, however, HAS3 deletion resulted in a dramatical reduction of vascular HA content in the media rather than neointima, suggesting that HAS3 is involved in the induction of provisional HA-rich extracellular matrix in the intima of ligated artery and thus promotes VSMC migration. Various regulatory pathways including integrins, focal adhesion, mitogen-activated protein kinases and phosphatidylinositol signaling were identified to be related to VSMCs proliferation in HAS3-deficient mice, especially migratory response to platelet-derived growth factor BB and decreased expression of its mRNA (Ref. §3.14). These data clearly demonstrate a role of HAS3 in VSMC phenotypic changes. Interestingly, inflammatory response was not affected in HAS3-deficient mice. Expression of the genes representing the inflammatory responses were upregulated in both genotypes as compared to unligated controls, but there was no effect of HAS3-deletion.

Previous study showed that systemic inhibition of HA synthesis with 4-methylumbelliferone significantly reduced endothelial gycocalyx, induced endothelial dysfunction and mild hypertension pointing out to the reduction of NO-bioavailability and/or impaired NO-signaling after perturbance of glycocalyx. To clarify whether global deletion of HAS3 isoform affects vascular functions vascular NO-bioavailability, aortic endothelium- and NO-dependent relaxation, constriction to phenylephrine, blood pressure and eNOS expression were assessed in healthy HAS3-KO mice and their controls and no difference was found in all measured parameters. Likewise, evaluation of the markers of oxidative protein modification and genes, related to oxidative-stress response revealed no effect of global HAS3 deletion. These data suggest that endothelial dysfunction and altered inflammatory response is unlikely the mechanism underlying reduced neointima formation in HAS3 deficiency and highlight the role of HAS3 in the phenotypic activation of medial VSMC during neointimal hyperplasia.

HAS3 Mediated HA Signaling in Endothelial Glycocalyx: Mechanosensing, FMD and Collateral Artery Growth The endothelial glycocalyx, building the top surface layer of endothelial cells is a highly dynamic hub for intra- and extracellular signals (253, 254) is perturbated in several disease states including hypertension, diabetes and atherosclerosis (159, 160, 255, 256) and close interconnection between damaged endothelial glycocalyx and progression of endothelial dysfunction has progressively been recognized (158, 252, 254).

HA is the second most abundant glycosaminoglycan in the glycocalyx and lack of HA synthesis appears to interfere with its mechanosensory properties. Enzymatic degradation of HA *ex vivo* in isolated femoral arteries reduced shear-induced NO-production in canine (165) and porcine (166) femoral arteries (166). Potter et al. (168) showed that complete depletion of the endothelial hyaluronan matrix by 4-methylesculetin suppressed mechanotransduction as well as arteriogenesis in mice. However, 4-methylesculetin inhibits all 3 isoenzymes and in addition might exhibit pleiotropic effects as described for the HAS-inhibitor 4-methylumbelliferone (257). In this study (Ref. § 3.15) an important question whether and how global and EC specific deletion of HAS3-mediated HA synthesis affects mechanosensing/mechanoransduction, *in vivo* endothelial function and collateral artery growth was addressed.

Endothelial HAS3 deficiency has been shown to impair shear stress induced activation of eNOS measured *in vivo* by flow-mediated dilation (FMD), a clinical method used in patients to evaluate endothelial (dys)function. FMD is an endothelium-dependent dilation of the conduit artery in response to increased blood flow that activates eNOS, increases NO production and subsequent relaxation of vascular smooth muscle cells (258). A decreased AKT (protein kinase B)-mediated phosphorylation of eNOS at Ser1177 was determined as underlying mechanism of impaired FMD in global and EC-specific HAS3 deficient mice (Ref. §3.15). While total eNOS content was unchanged, eNOS phosphorylation at Ser1177 and AKT phosphorylation at Ser473 were substantially reduced giving rise to a massive decline in peak FMD. Importantly, treatment of wild type mice with anti-CD44 antibody impaired vascular response to acute increase in blood flow suggesting that CD44 represents a link between HAS3-derived HA and eNOS signaling.

Consequently, flow-mediated AKT/eNOS phosphorylation at Ser1177 is a major trigger for collateral artery growth and exactly this pathway was found to be strongly impaired in global and endothelial-specific HAS3-deficient mice. Similar to impairement of blood recovery after femoral artery ligation in eNOS-deficient mice (259), lack of endothelial HAS3 (Ref. § 3.15) caused disturbed collateral growth and worsened perfusion recovery despite unaltered leukocyte recruitment in a mouse model of PAD. Strikingly, blocking of CD44 also mimicked the FMD phenotype of eNOS-deficient mice (258) pointing towards a high relevance of HA/CD44-dependent signaling for endothelial function and flow-induced mechanosignaling *in vivo* as well as arteriogenesis post-femoral artery occlusion.

HA mediates its effects via certain receptors such as RHAMM (receptor of HA-mediated motility) or CD44. In the context of hyaluronan-dependent eNOS signaling, CD44 was attributed a specific role due to its high abundance in plasmalemmal caveolae (260). These cholesterol rich scaffolds are mechanosensitive microdomains enriched

in signaling molecules such as G protein coupled receptors,(tyrosine) kinases, phosphatases, ion channels and, most importantly, eNOS (261). Since clustering of these molecules facilitates signal transduction, it is possible that hyaluronan-mediated clustering of CD44 gives rise to enhanced activation of kinases involved in eNOS phosphorylation at Ser1177. CD44 itself lacks intrinsic kinase activity (262) needed for AKT phosphorylation at Ser473. However, CD44 is known to recruit intracellular kinases and adaptor molecules upon shear stress-mediated receptor clustering. The cytoplasmic tail of CD44 was reported to contain crucial binding motifs for PI3K (263), a major activator of AKT. Thus, enhanced HA-dependent clustering of CD44 might activate the PI3K/AKT pathway which gives rise to eNOS phosphorylation at Ser1177 and its activation.

Although it is widely accepted that elevated shear stress is a major trigger of collateral arteries growth after arterial occlusion, it is still a matter of debate how flow is exactly sensed. Here it is demonstrated that (Ref. §3.15) endothelial HAS3-derived HA functions as mechanosensor mediating its beneficial effects via a modulation of NO-signaling pathways with functional significance *in vivo* for acute (FMD) and chronic (hindlimb ischemia) changes in blood flow. Furthermore, this is the first study reporting the CD44's role in endothelial function *in vivo* highlighting its specific function as a mechanotransducer for HA-derived signals.

5. SIGNIFICANCE

"The important thing is not to stop questioning"

Albert Einstein (1879-1955)

The studies presented here provided novel insights into the role of endothelial hydrogen peroxide, NO, superoxide and HA as important mediators of vascular function in health and disease. Experimental approaches such as exercise training and singularization allowed not only to reveal new mechanisms of exercise-induced upregulation of vasoprotective proteins, but also suggested that unfavourable change of endothelial function by sedentary lifestyle can be prevented or remarkably delayed by either a daily short-lasting forced exercise or continuous voluntary physical activity. These findings are important for the use of antioxidants and drugs that suppress the production of ROS *in vivo*: suppression of cellular production of ROS might lead to a loss of important vascular signaling events, e.g. an inability to increase eNOS and other vasoprotective proteins in response to exercise.

Demonstration of the previously unrecognized role of non-endothelial eNOS in the blood pressure homeostasis opens a perspective that non-endothelial eNOS impairment may contribute to the pathogenesis of hypertension. Along with endothelial eNOS, red cell eNOS was found to be an important regulator of NO metabolism, systemic hemodynamics, and blood pressure homeostasis. These findings might have significant implications in understanding of the interrelationship between hematological and cardiovascular disease, might enable us to refine the criteria for blood banking and transfusion and reveal novel therapeutic approaches to improve tissue perfusion.

Findings on the limited importance of eNOS-dependent oxidative stress for functional vascular damage suggest that other enzymatic sources of vascular oxidative stress such as the many known oxidases appear to be more important. Concomitant generation of NO by eNOS which is a fundamental difference to oxidases, substantiated the importance of vasoprotection conferred by endothelial NO, which appears to be able to cope with redox dysbalance. These data support previous observations on beneficial effects of therapeutic interventions associated with increased eNOS protein expression and improvement of endothelial NO production such as regular exercise training, pharmacotherapy with statins and treatment with inhibitors of the RAAS.

Unravelling dual and opposite roles of hyaluronan matrix in vascular pathologies, such as vascular remodelling and peripheral artery disease significantly contributed to our knowledge on regulation of vascular function by HA and HAS3. The intimal vascular smooth muscle cells - associated HA matrix enhances neointima hyperplasia, whereas the HA matrix of endothelial cells is vasoprotective. Importantly, for the first time the direct interrelation between HA, HAS3 and NO could be demonstrated *in vivo* identifying HAS3-derived HA as a novel modulator of NO signaling. Activation of the HAS3-HA-CD44-NO signaling pathway contributes to arteriogenesis in hindlimb ischemia, thus, strategies augmenting endothelial HAS3 (e.g. using anti-sense oligonucleotide approach) or CD44 could be envisioned to enhance vascularization under pathological conditions.

Our understanding of how ROS/RNS serve as signals or modulators of vascular function in health and disease continues to evolve, as it was an active research topic at the time of performimg this work. Instead of interpreting oxidation events merely as damage that culminates in disease the data provided here contributed to the development of the current view on the role of ROS/RNS and HA in vascular biology.

6. ACKNOWLEDGEMENTS

I wish to express my deep gratitude to

… Prof. Dr. med. Maria Grandoch, Institute of Translational Pharmacology for giving me an opportunity to submit this work, encouragement, support and constructive criticism, for the cheerful spirit, and for critical revision of this work.

… Prof. Georg Kojda, who welcomed me to Germany, for comprehensive introduction to the field of pharmacology and vascular biology by sharing expansive knowledge, for helpful discussions we had, and especially that you had always time for the details.

… to the heads of the Institute of Pharmacology and clinical Pharmacology, HHU Düsseldorf Prof. Jens Fischer and Prof. Karsten Schrör and Department of Cardiology, Pneumology and Angiology HHU Düsseldorf Prof. Dr. Malte Kelm for giving me the opportunity to work at their research facilities and Prof. Dr. Miriam Cortese-Krott (Myocardial Infarction Research Laboratory, HHU Düsseldorf) for encouragement and intresting discussions during our fruitful collaborations.

… to Prof. Dr. Ulrich Rüther and Dr. Oliver Lieven, Institute for Animal Developmental and Molecular Biology, Heinrich-Heine-University, Düsseldorf, Germany for their help with microinjections and generation of transgenic mice.

… Prof. Dr. Volker Adams (Heart Center Dresden, University Hospital at the Technische Universität Dresden), Prof. Dr. Johanes Stegbauer (Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany), Prof. Dr. Sergeeva (Institute of Clinical Neurosciences and Medical Psychology, Medical Faculty of Heinrich-Heine-University, Düsseldorf, Germany) for support during our cooperations and insightfull comments about our manuscripts.

… to my friend and colleague Dr. Stephanie Pick, to my collegues Dr. Rebekka Schneckmann, Dr. Thao-Vi Dao, Dr. Oktay Kocgirli, Dr. Marc Oppermann and to Mrs. Erika Lohmann and Mrs. Karen Montag for their valuable help with Uni-administration.

…to my colleagues and students in the Institute of Translational Pharmacology, Institute of Pharmacology, Cardiovascular Research Lab, both past and present.

...to Prof. Leanid Lobanok (Belarusian State Medical University, Minsk) and Prof. Dr. Klavdya Bulanova (International Sakharov Environmental Institute, Belarusian State University, Minsk) for getting me interested in cardiovascular research and and for introducing me to the “world of endothelium”.

…to my family for their support and encouragement.

…to Eduard, who saw me through all the enjoyments and frustrations of the research for helping me to stay focused on the live issues, for patience, love and inspiration.

The studies were supported by the German Research Council Foundation (research grant and principle investigator position SU-783 to TS), by the Forschungskommission of the Universitätsklinikum Düsseldorf (9772 345 and 9772 446 to TS), and by DAAD and FEBS research scholarships to TS.

7. REFERENCES

1. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nature reviews Immunology.* 2004;4(3):181-9.
2. Cadenas E, Boveris A, Ragan CI, and Stoppani AO. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Arch Biochem Biophys.* 1977;180(2):248-57.
3. Xia Y, Tsai AL, Berka V, and Zweier JL. Superoxide generation from endothelial nitric-oxide synthase - A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *JBiolChem.* 1998;273:25804-8.
4. Kelley EE, Khoo NK, Hundley NJ, Malik UZ, Freeman BA, and Tarpey MM. Hydrogen peroxide is the major oxidant product of xanthine oxidase. *Free radical biology & medicine.* 2010;48(4):493-8.
5. Retailleau K, Belin de Chantemele EJ, Chanoine S, Guihot AL, Vessieres E, Toutain B, et al. Reactive oxygen species and cyclooxygenase 2-derived thromboxane A2 reduce angiotensin II type 2 receptor vasorelaxation in diabetic rat resistance arteries. *Hypertension.* 2010;55(2):339-44.
6. Nicholls SJ, and Hazen SL. Myeloperoxidase and cardiovascular disease. *ArterioscIerThrombVascBiol.* 2005;25:1102-11.
7. Knaus UG. Oxidants in Physiological Processes. *Handbook of experimental pharmacology.* 2021;264:27-47.
8. Liochev SI, and Fridovich I. The effects of superoxide dismutase on H2O2 formation. *Free RadicBiol Med.* 2007;42:1465-9.
9. Griendling KK, Camargo LL, Rios FJ, Alves-Lopes R, Montezano AC, and Touyz RM. Oxidative Stress and Hypertension. *Circ Res.* 2021;128(7):993-1020.
10. Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox biology.* 2017;11:613-9.
11. Schmidt HH, Stocker R, Vollbracht C, Paulsen G, Riley D, Daiber A, et al. Antioxidants in Translational Medicine. *Antioxidants & redox signaling.* 2015;23(14):1130-43.
12. Harman D. Aging: a theory based on free radical and radiation chemistry. *Journal of gerontology.* 1956;11(3):298-300.
13. Paniker NV, Srivastava SK, and Beutler E. Glutathione metabolism of the red cells. Effect of glutathione reductase deficiency on the stimulation of hexose monophosphate shunt under oxidative stress. *Biochim Biophys Acta.* 1970;215(3):456-60.
14. Khaw KT, Bingham S, Welch A, Luben R, Wareham N, Oakes S, et al. Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. European Prospective Investigation into Cancer and Nutrition. *Lancet.* 2001;357(9257):657-63.
15. Griendling KK, and FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation.* 2003;108(17):2034-40.
16. Egea J, Fabregat I, Frapart YM, Ghezzi P, Gorlach A, Kietzmann T, et al. European contribution to the study of ROS: A summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS). *Redox biology.* 2017;13:94-162.
17. Forman HJ, and Zhang H. Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. *Nat Rev Drug Discov.* 2021;20(9):689-709.
18. Ghezzi P, Jaquet V, Marcucci F, and Schmidt H. The oxidative stress theory of disease: levels of evidence and epistemological aspects. *Br J Pharmacol.* 2017;174(12):1784-96.
19. Ghezzi P, and Mooradian AD. Demystifying Oxidative Stress. *Handbook of experimental pharmacology.* 2021;264:3-26.
20. Galley JC, and Straub AC. Redox Control of Vascular Function. *Arterioscler Thromb Vasc Biol.* 2017;37(12):e178-e84.
21. Lassegue B, San MA, and Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *CircRes.* 2012;110:1364-90.
22. Reglero-Real N, Colom B, Bodkin JV, and Nourshargh S. Endothelial Cell Junctional Adhesion Molecules: Role and Regulation of Expression in Inflammation. *Arterioscler Thromb Vasc Biol.* 2016;36(10):2048-57.
23. Yin K, You Y, Swier V, Tang L, Radwan MM, Pandya AN, et al. Vitamin D Protects Against Atherosclerosis via Regulation of Cholesterol Efflux and Macrophage Polarization in Hypercholesterolemic Swine. *Arterioscler Thromb Vasc Biol.* 2015;35(11):2432-42.
24. Kratofil RM, Kubes P, and Deniset JF. Monocyte Conversion During Inflammation and Injury. *Arterioscler Thromb Vasc Biol.* 2017;37(1):35-42.
25. Ignarro LJ, Cirino G, Casini A, and Napoli C. Nitric oxide as a signaling molecule in the vascular system: An overview. *Journal of Cardiovascular Pharmacology.* 1999;34:879-86.

26. Moncada S. Nitric oxide in the vasculature: Physiology and pathophysiology. *AnnNY AcadSci.* 1997;811:60-9.

27. Ignarro LJ, Freeman, B. A. *Nitric Oxide Biology and Pathobiology* 2015.

28. Godo S, and Shimokawa H. Divergent roles of endothelial nitric oxide synthases system in maintaining cardiovascular homeostasis. *Free radical biology & medicine.* 2017;109:4-10.

29. Biwer LA, Taddeo EP, Kenwood BM, Hoehn KL, Straub AC, and Isakson BE. Two functionally distinct pools of eNOS in endothelium are facilitated by myoendothelial junction lipid composition. *Biochim Biophys Acta.* 2016;1861(7):671-9.

30. Taniyama Y, and Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension.* 2003;42(6):1075-81.

31. Burgoyne JR, Oka S, Ale-Agha N, and Eaton P. Hydrogen peroxide sensing and signaling by protein kinases in the cardiovascular system. *Antioxidants & redox signaling.* 2013;18(9):1042-52.

32. Mannaa A, and Hanisch FG. Redox Proteomes in Human Physiology and Disease Mechanisms. *Journal of proteome research.* 2020;19(1):1-17.

33. Lushchak VI. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-biological interactions.* 2014;224:164-75.

34. Halliwell B, Clement MV, and Long LH. Hydrogen peroxide in the human body. *FEBS Lett.* 2000;486:10-3.

35. Rhee SG. Cell signaling. H2O2, a necessary evil for cell signaling. *Science.* 2006;312(5782):1882-3.

36. Marinho HS, Real C, Cyrne L, Soares H, and Antunes F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox biology.* 2014;2:535-62.

37. Sies H, Belousov VV, Chandel NS, Davies MJ, Jones DP, Mann GE, et al. Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. *Nat Rev Mol Cell Biol.* 2022;23(7):499-515.

38. Byon CH, Heath JM, and Chen Y. Redox signaling in cardiovascular pathophysiology: A focus on hydrogen peroxide and vascular smooth muscle cells. *Redox biology.* 2016;9:244-53.

39. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *CircRes.* 2000;86:494-501.

40. Wolin MS. Interactions of oxidants with vascular signaling systems. *ArterioscIerThrombVascBiol.* 2000;20:1430-42.

41. Ellis A, and Triggle CR. Endothelium-derived reactive oxygen species: their relationship to endothelium-dependent hyperpolarization and vascular tone. *CanJ Physiol Pharmacol.* 2003;81:1013-28.

42. Liu Y, Bubolz AH, Mendoza S, Zhang DX, and Gutterman DD. H2O2 is the transferrable factor mediating flow-induced dilation in human coronary arterioles. *Circ Res.* 2011;108(5):566-73.

43. Gao YJ, Hirota S, Zhang DW, Janssen LJ, and Lee RM. Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. *BrJ Pharmacol.* 2003;138:1085-92.

44. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J ClinInvest.* 2000;106:1521-30.

45. Satoh K, Godo S, Saito H, Enkhjargal B, and Shimokawa H. Dual roles of vascular-derived reactive oxygen species--with a special reference to hydrogen peroxide and cyclophilin A. *J Mol Cell Cardiol.* 2014;73:50-6.

46. Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, and Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. *Hypertension.* 2000;36:878-84.

47. Stocker S, Van Laer K, Mijuskovic A, and Dick TP. The Conundrum of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs. *Antioxidants & redox signaling.* 2018;28(7):558-73.

48. Marinho HS, Cyrne L, Cadenas E, and Antunes F. The cellular steady-state of H2O2: latency concepts and gradients. *Methods Enzymol.* 2013;527:3-19.

49. Wolin MS, Gupte SA, and Oeckler RA. Superoxide in the vascular system. *J VascRes.* 2002;39:191-207.

50. Jones RD, and Morice AH. Hydrogen peroxide--an intracellular signal in the pulmonary circulation: involvement in hypoxic pulmonary vasoconstriction. *PharmacolTher.* 2000;88:153-61.

51. Drummond GR, Cai H, Davis ME, Ramasamy S, and Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circulation Research.* 2000;86:347-54.

52. Cai H, Li Z, Davis ME, Kanner W, Harrison DG, and Dudley SC, Jr. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *MolPharmacol.* 2003;63:325-31.

53. Lorenz RR, Warner DO, and Jones KA. Hydrogen peroxide decreases Ca(2+) sensitivity in airway smooth muscle by inhibiting rMLC phosphorylation. *AmJ Physiol.* 1999;277:L816-L22.

54. Perkins WJ, Lorenz RR, Bogoger M, Warner DO, Cremo CR, and Jones KA. A novel mechanism by which hydrogen peroxide decreases calcium sensitivity in airway smooth muscle. *AmJ Physiol Lung Cell MolPhysiol.* 2003;284:L324-L32.

55. Yang ZW, Zheng T, Wang J, Zhang A, Altura BT, and Altura BM. Hydrogen peroxide induces contraction and raises [Ca2+]i in canine cerebral arterial smooth muscle: participation of cellular signaling pathways. *Naunyn Schmiedebergs ArchPharmacol.* 1999;360:646-53.

56. Pelaez NJ, Braun TR, Paul RJ, Meiss RA, and Packer CS. H(2)O(2) mediates Ca(2+)- and MLC(20) phosphorylation-independent contraction in intact and permeabilized vascular muscle. *AmJ Physiol Heart CircPhysiol.* 2000;279:H1185-H93.

57. Nowicki PT, Flavahan S, Hassanain H, Mitra S, Holland S, Goldschmidt-Clermont PJ, et al. Redox signaling of the arteriolar myogenic response. *CircRes.* 2001;89:114-6.

58. Gielen S, Schuler G, and Adams V. Cardiovascular effects of exercise training: molecular mechanisms. *Circulation.* 2010;122:1221-38.

59. Schuler G, Adams V, and Goto Y. Role of exercise in the prevention of cardiovascular disease: results, mechanisms, and new perspectives. *Eur Heart J.* 2013;34(24):1790-9.

60. Eckel RH, Jakicic JM, Ard JD, de Jesus JM, Houston Miller N, Hubbard VS, et al. 2013 AHA/ACC guideline on lifestyle management to reduce cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol.* 2014;63(25 Pt B):2960-84.

61. Zucker IH, and Musch TI. Benefits of exercise training on cardiovascular dysfunction: molecular and integrative. *American journal of physiology Heart and circulatory physiology.* 2018;315(4):H1027-H31.

62. Whelton PK, Carey RM, Aronow WS, Casey DE, Jr., Collins KJ, Dennison Himmelfarb C, et al. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Hypertension.* 2018;71(6):1269-324.

63. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J.* 2018;39(33):3021-104.

64. Adams V, Reich B, Uhlemann M, and Niebauer J. Molecular effects of exercise training in patients with cardiovascular disease: focus on skeletal muscle, endothelium, and myocardium. *American journal of physiology Heart and circulatory physiology.* 2017;313(1):H72-H88.

65. Kojda G, and Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *CardiovascRes.* 2005;67:187-97.

66. Erkens R, Suvorava T, Kramer CM, Diederich LD, Kelm M, and Cortese-Krott MM. Modulation of Local and Systemic Heterocellular Communication by Mechanical Forces: A Role of Endothelial Nitric Oxide Synthase. *Antioxidants & redox signaling.* 2017;26(16):917-35.

67. Frodermann V, Rohde D, Courties G, Severe N, Schloss MJ, Amatullah H, et al. Exercise reduces inflammatory cell production and cardiovascular inflammation via instruction of hematopoietic progenitor cells. *Nat Med.* 2019;25(11):1761-71.

68. Sessa WC, Pritchard KA, Jr., Seyedi N, Wang J, and Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *CircRes.* 1994;74:349-53.

69. Woodman CR, Muller JM, Laughlin MH, and Price EM. Induction of nitric oxide synthase mRNA in coronary resistance arteries isolated from exercise-trained pigs. *AmJPhysiolHeart CircPhysiol.* 1997;273:H2575-H9.

70. Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, and Hintze TH. Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. *MedSciSports Exerc.* 1995;27:1125-34.

71. Kojda G, Cheng Y, Burchfield J, and Harrison DG. Dysfunctional Regulation of eNOS Expression in Response to Exercise in Mice Lacking One eNOS Gene. *Circulation.* 2001;103:2839-44.

72. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, and Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *Journal of Clinical Investigation.* 2000;105:1631-9.

73. Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *NEnglJMed.* 2000;342:454-60.

74. Kojda G, Cheng YC, Burchfield J, and Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation.* 2001;103:2839-44.

75. Rassaf T, Heiss C, Mangold S, Leyendecker T, Kehmeier ES, Kelm M, et al. Vascular formation of nitrite after exercise is abolished in patients with cardiovascular risk factors and coronary artery disease. *J Am Coll Cardiol.* 2010;55(14):1502-3.

76. Rassaf T, Lauer T, Heiss C, Balzer J, Mangold S, Leyendecker T, et al. Nitric oxide synthase-derived plasma nitrite predicts exercise capacity. *Br J Sports Med.* 2007;41(10):669-73; discussion 73.

77. Lauer T, Heiss C, Balzer J, Kehmeier E, Mangold S, Leyendecker T, et al. Age-dependent endothelial dysfunction is associated with failure to increase plasma nitrite in response to exercise. *Basic Res Cardiol.* 2008;103(3):291-7.

78. Wang JS. Effects of exercise training and detraining on cutaneous microvascular function in man: the regulatory role of endothelium-dependent dilation in skin vasculature. *European journal of applied physiology.* 2005;93(4):429-34.

79. Higashi Y, Sasaki S, Kurisu S, Yoshimizu A, Sasaki N, Matsuura H, et al. Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as well as hypertensive subjects - Role of endothelium-derived nitric oxide. *Circulation.* 1999;100:1194-202.

80. Krause M, Rodrigues-Krause J, O'Hagan C, Medlow P, Davison G, Susta D, et al. The effects of aerobic exercise training at two different intensities in obesity and type 2 diabetes: implications for oxidative stress, low-grade inflammation and nitric oxide production. *European journal of applied physiology.* 2014;114(2):251-60.

81. Laurindo FRM, De Almeida Pedro M, Barbeiro HV, Pileggi F, Carvalho MHC, Augusto O, et al. Vascular free radical release: Ex vivo and in vivo evidence for a flow-dependent endothelial mechanism. *CircRes.* 1994;74:700-9.

82. Tanaka LY, Bechara LR, dos Santos AM, Jordao CP, de Sousa LG, Bartholomeu T, et al. Exercise improves endothelial function: a local analysis of production of nitric oxide and reactive oxygen species. *Nitric oxide : biology and chemistry.* 2015;45:7-14.

83. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, and Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *CircRes.* 1998;82:1094-101.

84. Suvorava T, and Kojda G. Prevention of transient endothelial dysfunction in acute exercise: a friendly fire? *ThrombHaemost.* 2007;97:331-3.

85. Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, et al. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *J AmCollCardiol.* 2004;43:2314-8.

86. Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, et al. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. *ArterioscIerThrombVascBiol.* 2004;24:684-90.

87. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation.* 2004;109:220-6.

88. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* 1999;18:3964-72.

89. Dimmeler S, Zeiher AM, and Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *JClinInvest.* 2005;115:572-83.

90. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation.* 2001;104(9):1046-52.

91. Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, et al. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. *Blood.* 2006;107(9):3546-54.

92. Zampetaki A, Kirton JP, and Xu Q. Vascular repair by endothelial progenitor cells. *CardiovascRes.* 2008;78:413-21.

93. Urbich C, Dernbach E, Rossig L, Zeiher AM, and Dimmeler S. High glucose reduces cathepsin L activity and impairs invasion of circulating progenitor cells. *JMolCell Cardiol.* 2008;45:429-36.

94. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *NEnglJ Med.* 2005;353:999-1007.

95. Everaert BR, Van Craenenbroeck EM, Hoymans VY, Haine SE, Van Nassauw L, Conraads VM, et al. Current perspective of pathophysiological and interventional effects on endothelial progenitor cell biology: focus on PI3K/AKT/eNOS pathway. *Int J Cardiol.* 2010;144(3):350-66.

96. De Biase C, De Rosa R, Luciano R, De Luca S, Capuano E, Trimarco B, et al. Effects of physical activity on endothelial progenitor cells (EPCs). *Frontiers in physiology.* 2013;4:414.

97. Ingram DA, Krier TR, Mead LE, McGuire C, Prater DN, Bhavsar J, et al. Clonogenic endothelial progenitor cells are sensitive to oxidative stress. *Stem Cells.* 2007;25:297-304.

98. Roy S, Khanna S, and Sen CK. Redox regulation of the VEGF signaling path and tissue vascularization: Hydrogen peroxide, the common link between physical exercise and cutaneous wound healing. *Free radical biology & medicine.* 2008;44(2):180-92.

99. Adams V, Linke A, Krankel N, Erbs S, Gielen S, Möbius-Winkler S, et al. Impact of Regular Physical Activity on the NAD(P)H Oxidase and Angiotensin Receptor System in Patients With Coronary Artery Disease. *Circulation.* 2005;111(5):555-62.

100. Suvorava T, Metry S, Pick S, and Kojda G. Alterations in endothelial nitric oxide synthase activity and their relevance to blood pressure. *Biochem Pharmacol.* 2022;205:115256.

101. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, and Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J Am Coll Cardiol.* 2007;49(25):2379-93.

102. Davies PF, Civelek M, Fang Y, and Fleming I. The atherosusceptible endothelium: endothelial phenotypes in complex haemodynamic shear stress regions in vivo. *Cardiovascular research.* 2013;99(2):315-27.

103. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, et al. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *ProcNatlAcadSciUSA.* 1991;88(23):10480-4.

104. Moncada S, and Higgs A. Mechanisms of disease: The L-arginine-nitric oxide pathway. *NEnglJMed.* 1993;329:2002-12.

105. Gorren AC, and Mayer B. Nitric-oxide synthase: a cytochrome P450 family foster child. *BiochimBiophysActa.* 2007;1770:432-45.

106. Koesling D, Mergia E, and Russwurm M. Physiological Functions of NO-Sensitive Guanylyl Cyclase Isoforms. *Current medicinal chemistry.* 2016;23(24):2653-65.

107. Feil R, Lohmann SM, de Jonge H, Walter U, and Hofmann F. Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ Res.* 2003;93(10):907-16.

108. Buechler WA, Nakane M, and Murad F. Expression of soluble guanylate cyclase activity requires both enzyme subunits. *BiochemBiophysResCommun.* 1991;174:351-7.

109. Russwurm M, and Koesling D. NO activation of guanylyl cyclase. *EMBO J.* 2004;23(22):4443-50.

110. Filippov G, Bloch DB, and Bloch KD. Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *JClinInvest.* 1997;100:942-8.

111. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Rikitake Y, Inoue N, et al. Mechanisms of reduced nitric oxide/cGMP-mediated vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *Hypertension.* 2000;36:97-102.

112. Mullershausen F, Russwurm M, Koesling D, and Friebe A. The enhanced NO-induced cGMP response induced by long-term L-NAME treatment is not due to enhanced expression of NO-sensitive guanylyl cyclase. *VasculPharmacol.* 2003;40:161-5.

113. Brandes RP, Kim DY, Schmitz-Winnenthal FH, Amidi M, Gödecke A, Mülsch A, et al. Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice - Role of soluble guanylyl cyclase. *Hypertension.* 2000;35:231-6.

114. Hussain MB, Hobbs AJ, and MacAllister RJ. Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta. *British Journal of Pharmacology.* 1999;128:1082-8.

115. Lima B, Forrester MT, Hess DT, and Stamler JS. S-nitrosylation in cardiovascular signaling. *CircRes.* 2010;106:633-46.

116. Schulman IH, and Hare JM. Regulation of cardiovascular cellular processes by S-nitrosylation. *Biochim Biophys Acta.* 2012;1820(6):752-62.

117. Strålin P, Karlsson K, Johansson BO, and Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *ArteriosclerThrombVascBiol.* 1995;15:2032-6.

118. Jung O, Marklund SL, Geiger H, Pedrazzini T, Busse R, and Brandes RP. Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice. *CircRes.* 2003;93:622-9.

119. Kaschina E, Namsolleck P, and Unger T. AT2 receptors in cardiovascular and renal diseases. *Pharmacol Res.* 2017;125(Pt A):39-47.

120. Zhu L, Carretero OA, Liao TD, Harding P, Li H, Sumners C, et al. Role of prolylcarboxypeptidase in angiotensin II type 2 receptor-mediated bradykinin release in mouse coronary artery endothelial cells. *Hypertension.* 2010;56:384-90.

121. Shariat-Madar Z, Mahdi F, and Schmaier AH. Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator. *JBiolChem.* 2002;277:17962-9.

122. Hunley TE, Tamura M, Stoneking BJ, Nishimura H, Ichiki T, Inagami T, et al. The angiotensin type II receptor tonically inhibits angiotensin- converting enzyme in AT2 null mutant mice. *Kidney Int.* 2000;57:570-7.

123. Vallance P, Collier J, and Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet.* 1989;334:997-1000.

124. Haynes WG, Noon JP, Walker BR, and Webb DJ. L-NMMA increases blood pressure in man. *Lancet.* 1993;342:931-2.

125. Forte P, Copland M, Smith LM, Milne E, Sutherland J, and Benjamin N. Basal nitric oxide synthesis in essential hypertension. *Lancet.* 1997;349:837-42.

126. Huang PL, Huang ZH, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature.* 1995;377:239-42.

127. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, et al. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *ProcNatlAcadSciUSA.* 1996;93:13176-81.

128. Gödecke A, Decking U, Ding Z, Hirchenhain J, Bidmon HJ, Godecke S, et al. Coronary hemodynamics in endothelial NO synthase knockout mice. *CircRes.* 1998;82:186-94.

129. Gregg AR, Schauer A, Shi O, Liu Z, Lee CG, and O'Brien WE. Limb reduction defects in endothelial nitric oxide synthase-deficient mice. *AmJPhysiol.* 1998;275:H2319-H24.

130. Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, et al. Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature.* 1995;378:383-6.

131. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, et al. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature.* 2002;416:337-40.

132. Tsutsui M, Shimokawa H, Otsuji Y, and Yanagihara N. Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases. *PharmacolTher.* 2010;128(3):499-508.

133. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, et al. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *CardiovascRes.* 1999;42:206-13.

134. Scotland RS, Morales-Ruiz M, Chen Y, Yu J, Rudic RD, Fulton D, et al. Functional reconstitution of endothelial nitric oxide synthase reveals the importance of serine 1179 in endothelium-dependent vasomotion. *CircRes.* 2002;90(8):904-10.

135. Lamping KG, Nuno DW, Shesely EG, Maeda N, and Faraci FM. Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice. *AmJPhysiol Heart CircPhysiol.* 2000;279:H1906-H12.

136. Waldron GJ, Ding H, Lovren F, Kubes P, and Triggle CR. Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. *British Journal of Pharmacology.* 1999;128:653-8.

137. Meng W, Ayata C, Waeber C, Huang PL, and Moskowitz MA. Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice. *AmJPhysiolHeart CircPhysiol.* 1998;274:H411-H5.

138. Drouin A, Thorin-Trescases N, Hamel E, Falck JR, and Thorin E. Endothelial nitric oxide synthase activation leads to dilatory H2O2 production in mouse cerebral arteries. *CardiovascRes.* 2007;73(1):73-81.

139. Sun D, Huang A, Smith CJ, Stackpole CJ, Connetta JA, Shesely EG, et al. Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circulation Research.* 1999;85:288-93.

140. Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, et al. Red blood cells express a functional endothelial nitric oxide synthase. *Blood.* 2006;107(7):2943-51.

141. Ortiz PA, and Garvin JL. Cardiovascular and renal control in NOS-deficient mouse models. *AmJPhysiol RegulIntegrComp Physiol.* 2003;284:R628-R38.

142. Lin LH, Taktakishvili O, and Talman WT. Identification and localization of cell types that express endothelial and neuronal nitric oxide synthase in the rat nucleus tractus solitarii. *Brain Res.* 2007;1171:42-51.

143. Jiang R, Wang S, Takahashi K, Fujita H, Fruci CR, Breyer MD, et al. Generation of a conditional allele for the mouse endothelial nitric oxide synthase gene. *Genesis.* 2012;50(9):685-92.

144. Hayashi S, Lewis P, Pevny L, and McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Gene expression patterns : GEP.* 2002;2(1-2):93-7.

145. Gao Y, Stuart D, Pollock JS, Takahishi T, and Kohan DE. Collecting duct-specific knockout of nitric oxide synthase 3 impairs water excretion in a sex-dependent manner. *American journal of physiology Renal physiology.* 2016;311(5):F1074-F83.

146. Gao Y, Stuart D, Takahishi T, and Kohan DE. Nephron-Specific Disruption of Nitric Oxide Synthase 3 Causes Hypertension and Impaired Salt Excretion. *Journal of the American Heart Association.* 2018;7(14).

147. Forstermann U, and Sessa WC. Nitric oxide synthases: regulation and function. *EurHeart J.* 2012;33:829-37d.

148. Gielis JF, Lin JY, Wingler K, Van Schil PE, Schmidt HH, and Moens AL. Pathogenetic role of eNOS uncoupling in cardiopulmonary disorders. *Free RadicBiolMed.* 2011;50:765-76.

149. Radermacher KA, Wingler K, Langhauser F, Altenhofer S, Kleikers P, Hermans JJ, et al. Neuroprotection after stroke by targeting NOX4 as a source of oxidative stress. *AntioxidRedoxSignal.* 2013;%20;18:1418-27.

150. Harrison DG, and Gongora MC. Oxidative stress and hypertension. *MedClinNorth Am.* 2009;93:621-35.

151. Li H, Horke S, and Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis.* 2014;237:208-19.

152. Daiber A, Xia N, Steven S, Oelze M, Hanf A, Kroller-Schon S, et al. New Therapeutic Implications of Endothelial Nitric Oxide Synthase (eNOS) Function/Dysfunction in Cardiovascular Disease. *International journal of molecular sciences.* 2019;20(1).

153. Raman CS, Li H, Martasek P, Kral V, Masters BS, and Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell.* 1998;95:939-50.

154. Chen PF, Tsai AL, and Wu KK. Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *BiochemBiophysResCommun.* 1995;215:1119-29.

155. Riessen R, Wight TN, Pastore C, Henley C, and Isner JM. Distribution of hyaluronan during extracellular matrix remodeling in human restenotic arteries and balloon-injured rat carotid arteries. *Circulation.* 1996;93(6):1141-7.

156. Evanko SP, Angello JC, and Wight TN. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1999;19(4):1004-13.

157. Chai S, Chai Q, Danielsen CC, Hjorth P, Nyengaard JR, Ledet T, et al. Overexpression of hyaluronan in the tunica media promotes the development of atherosclerosis. *Circ Res.* 2005;96(5):583-91.

158. Nagy N, Freudenberger T, Melchior-Becker A, Rock K, Ter Braak M, Jastrow H, et al. Inhibition of hyaluronan synthesis accelerates murine atherosclerosis: novel insights into the role of hyaluronan synthesis. *Circulation.* 2010;122(22):2313-22.

159. Grandoch M, Bollyky PL, and Fischer JW. Hyaluronan: A Master Switch Between Vascular Homeostasis and Inflammation. *Circ Res.* 2018;122(10):1341-3.

160. Fischer JW. Role of hyaluronan in atherosclerosis: Current knowledge and open questions. *Matrix biology : journal of the International Society for Matrix Biology.* 2019;78-79:324-36.

161. Homann S, Grandoch M, Kiene LS, Podsvyadek Y, Feldmann K, Rabausch B, et al. Hyaluronan synthase 3 promotes plaque inflammation and atheroprogression. *Matrix biology : journal of the International Society for Matrix Biology.* 2018;66:67-80.

162. Hartmann F, Gorski DJ, Newman AAC, Homann S, Petz A, Owsiany KM, et al. SMC-Derived Hyaluronan Modulates Vascular SMC Phenotype in Murine Atherosclerosis. *Circ Res.* 2021;129(11):992-1005.

163. Jiang D, Liang J, and Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiological reviews.* 2011;91(1):221-64.

164. Garantziotis S, and Savani RC. Hyaluronan biology: A complex balancing act of structure, function, location and context. *Matrix biology : journal of the International Society for Matrix Biology.* 2019;78-79:1-10.

165. Mochizuki S, Vink H, Hiramatsu O, Kajita T, Shigeto F, Spaan JA, et al. Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. *American journal of physiology Heart and circulatory physiology.* 2003;285(2):H722-6.

166. Kumagai R, Lu X, and Kassab GS. Role of glycocalyx in flow-induced production of nitric oxide and reactive oxygen species. *Free radical biology & medicine.* 2009;47(5):600-7.

167. Singleton PA, and Bourguignon LY. CD44 interaction with ankyrin and IP3 receptor in lipid rafts promotes hyaluronan-mediated Ca2+ signaling leading to nitric oxide production and endothelial cell adhesion and proliferation. *Exp Cell Res.* 2004;295(1):102-18.

168. Potter DR, van Teeffelen J, Vink H, and van den Berg BM. Perturbed mechanotransduction by endothelial surface glycocalyx modification greatly impairs the arteriogenic process. *American journal of physiology Heart and circulatory physiology.* 2015;309(4):H711-7.

169. Ruppert SM, Hawn TR, Arrigoni A, Wight TN, and Bollyky PL. Tissue integrity signals communicated by high-molecular weight hyaluronan and the resolution of inflammation. *Immunologic research.* 2014;58(2-3):186-92.

170. Cowman MK. Hyaluronan and Hyaluronan Fragments. *Advances in carbohydrate chemistry and biochemistry.* 2017;74:1-59.

171. Lee-Sayer SS, Dong Y, Arif AA, Olsson M, Brown KL, and Johnson P. The where, when, how, and why of hyaluronan binding by immune cells. *Frontiers in immunology.* 2015;6:150.

172. Yang H, Shi M, VanRemmen H, Chen X, Vijg J, Richardson A, et al. Reduction of pressor response to vasoconstrictor agents by overexpression of catalase in mice. *AmJ Hypertens.* 2003;16:1-5.

173. Griffith TM. Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? *BrJ Pharmacol.* 2004;141:881-903.

174. Neo BH, Kandhi S, and Wolin MS. Roles for soluble guanylate cyclase and a thiol oxidation-elicited subunit dimerization of protein kinase G in pulmonary artery relaxation to hydrogen peroxide. *American journal of physiology Heart and circulatory physiology.* 2010;299(4):H1235-41.

175. Griendling KK, Sorescu D, Lassegue B, and Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *ArterioscIerThrombVascBiol.* 2000;20:2175-83.

176. Jones DP, and Sies H. The Redox Code. *Antioxidants & redox signaling.* 2015;23(9):734-46.

177. Godecke A, and Haendeler J. Intra- and Interorgan Communication in the Cardiovascular System: A Special View on Redox Regulation. *Antioxidants & redox signaling.* 2017;26(12):613-5.

178. Cuello F, and Eaton P. Cysteine-Based Redox Sensing and Its Role in Signaling by Cyclic Nucleotide-Dependent Kinases in the Cardiovascular System. *Annu Rev Physiol.* 2019;81:63-87.

179. Prysyazhna O, Rudyk O, and Eaton P. Single atom substitution in mouse protein kinase G eliminates oxidant sensing to cause hypertension. *Nat Med.* 2012;18(2):286-90.

180. Inoue N, Ramasamy S, Fukai T, Nerem RM, and Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *CircRes.* 1996;79:32-7.

181. Takeshita S, Inoue N, Ueyama T, Kawashima S, and Yokoyama M. Shear stress enhances glutathione peroxidase expression in endothelial cells. *Biochemical and Biophysical Research Communications.* 2000;273:66-71.

182. Laufs U, Wassmann S, Czech T, Munzel T, Eisenhauer M, Bohm M, et al. Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2005;25(4):809-14.

183. Harrison DG, Guzik TJ, Lob HE, Madhur MS, Marvar PJ, Thabet SR, et al. Inflammation, immunity, and hypertension. *Hypertension.* 2011;57:132-40.

184. Ghiadoni L, Taddei S, and Virdis A. Hypertension and endothelial dysfunction: therapeutic approach. *CurrVascPharmacol.* 2012;10:42-60.

185. Kojda G, and Harrison DG. Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *CardiovascRes.* 1999;43:562-71.

186. Siu PM, Donley DA, Bryner RW, and Alway SE. Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. *J ApplPhysiol.* 2003;94:555-60.

187. Vigelso A, Andersen NB, and Dela F. The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training. *International journal of physiology, pathophysiology and pharmacology.* 2014;6(2):84-101.

188. Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A, et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women. *NEnglJ Med.* 2002;347:716-25.

189. Holtermann A, Schnohr P, Nordestgaard BG, and Marott JL. The physical activity paradox in cardiovascular disease and all-cause mortality: the contemporary Copenhagen General Population Study with 104 046 adults. *Eur Heart J.* 2021;42(15):1499-511.

190. Balligand JL, Feron O, and Dessy C. eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. *Physiological reviews.* 2009;89(2):481-534.

191. Gielen S, Sandri M, Erbs S, and Adams V. Exercise-induced modulation of endothelial nitric oxide production. *Curr Pharm Biotechnol.* 2011;12(9):1375-84.

192. Davis ME, Cai H, Drummond GR, and Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *CircRes.* 2001;89:1073-80.

193. Cai H, Davis ME, Drummond GR, and Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. *ArterioscIerThrombVascBiol.* 2001;21:1571-6.

194. Thomas SR, Chen K, and Keaney JF, Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J BiolChem.* 2002;277:6017-24.

195. Chen X, An X, Chen D, Ye M, Shen W, Han W, et al. Chronic Exercise Training Improved Aortic Endothelial and Mitochondrial Function via an AMPKalpha2-Dependent Manner. *Frontiers in physiology.* 2016;7:631.

196. Jansen T, Kvandova M, Schmal I, Kalinovic S, Stamm P, Kuntic M, et al. Lack of Endothelial alpha1AMPK Reverses the Vascular Protective Effects of Exercise by Causing eNOS Uncoupling. *Antioxidants.* 2021;10(12).

197. Powers SK, and Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiological reviews.* 2008;88(4):1243-76.

198. Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M, et al. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A.* 2009;106(21):8665-70.

199. Lenk K, Uhlemann M, Schuler G, and Adams V. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. *J Appl Physiol (1985).* 2011;111(1):321-8.

200. Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964-7.

201. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *NatMed.* 2003;9:1370-6.

202. Heiss C, Schanz A, Amabile N, Jahn S, Chen Q, Wong ML, et al. Nitric oxide synthase expression and functional response to nitric oxide are both important modulators of circulating angiogenic cell response to angiogenic stimuli. *Arterioscler Thromb Vasc Biol.* 2010;30(11):2212-8.

203. Iwakura A, Shastry S, Luedemann C, Hamada H, Kawamoto A, Kishore R, et al. Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced

neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. *Circulation.* 2006;113(12):1605-14.

204. Urao N, and Ushio-Fukai M. Redox regulation of stem/progenitor cells and bone marrow niche. *Free radical biology & medicine.* 2013;54:26-39.

205. Johnson LR, Rush JW, Turk JR, Price EM, and Laughlin MH. Short-term exercise training increases ACh-induced relaxation and eNOS protein in porcine pulmonary arteries. *JApplPhysiol.* 2001;90:1102-10.

206. Miyauchi T, Maeda S, Iemitsu M, Kobayashi T, Kumagai Y, Yamaguchi I, et al. Exercise causes a tissue-specific change of NO production in the kidney and lung. *JApplPhysiol.* 2003;94:60-8.

207. Casaburi R, and ZuWallack R. Pulmonary rehabilitation for management of chronic obstructive pulmonary disease. *NEnglJMed.* 2009;360:1329-35.

208. West JB. Comparative physiology of the pulmonary blood-gas barrier: the unique avian solution. *AmJPhysiol RegulIntegrComp Physiol.* 2009;297:R1625-R34.

209. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, et al. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase [see comments]. *JClinInvest.* 1998;102:2061-71.

210. Ferrero R, and Torres M. Prolonged exposure of chromaffin cells to nitric oxide down-regulates the activity of soluble guanylyl cyclase and corresponding mRNA and protein levels. *BMC biochemistry.* 2002;3:26.

211. Mergia E, Friebe A, Dangel O, Russwurm M, and Koesling D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *JClinInvest.* 2006;116:1731-7.

212. Kojda G, Stein D, Kottenberg E, Schnaith EM, and Noack E. In vivo effects of pentaerythrityl-tetranitrate and isosorbide- 5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. *JCardiovascPharmacol.* 1995;25:763-73.

213. Hacker A, Noack E, and Kojda G. In: Navaro-López F ed. *XXI Congress of the European Society of Cardiology*. Bologna, Italy: Monduzzi Editore; 2000:251-7.

214. Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E, et al. Does nitric oxide mediate the vasodilator activity of nitroglycerin? *CircRes.* 2003;93:e104-e12.

215. Weber W, Michaelis K, Luckow V, Kuntze U, and Stalleicken D. Pharmacokinetics and bioavailability of pentaerythrityl tetranitrate and two of its metabolites. *ArzneimForsch.* 1995;45:781-4.

216. Stromqvist M, Holgersson J, and Samuelsson B. Glycosylation of extracellular superoxide dismutase studied by high-performance liquid chromatography and mass spectrometry. *Journal of chromatography.* 1991;548(1-2):293-301.

217. Petersen SV, Kristensen T, Petersen JS, Ramsgaard L, Oury TD, Crapo JD, et al. The folding of human active and inactive extracellular superoxide dismutases is an intracellular event. *The Journal of biological chemistry.* 2008;283(22):15031-6.

218. Enghild JJ, Thogersen IB, Oury TD, Valnickova Z, Hojrup P, and Crapo JD. The heparin-binding domain of extracellular superoxide dismutase is proteolytically processed intracellularly during biosynthesis. *The Journal of biological chemistry.* 1999;274(21):14818-22.

219. Olsen DA, Petersen SV, Oury TD, Valnickova Z, Thogersen IB, Kristensen T, et al. The intracellular proteolytic processing of extracellular superoxide dismutase (EC-SOD) is a two-step event. *The Journal of biological chemistry.* 2004;279(21):22152-7.

220. Hohl M, Mayr M, Lang L, Nickel AG, Barallobre-Barreiro J, Yin X, et al. Cathepsin A contributes to left ventricular remodeling by degrading extracellular superoxide dismutase in mice. *The Journal of biological chemistry.* 2020;295(36):12605-17.

221. Srivastava D, Kukkuta Sarma GR, Dsouza DS, Muralidharan M, Srinivasan K, and Mandal AK. Characterization of residue-specific glutathionylation of CSF proteins in multiple sclerosis - A MS-based approach. *Anal Biochem.* 2019;564-565:108-15.

222. Peng ZF, Chen MJ, Yap YW, Manikandan J, Melendez AJ, Choy MS, et al. Proteasome inhibition: An early or late event in nitric oxide-induced neuronal death? *NitricOxide.* 2007.

223. Lee CM, Kim BY, Li L, and Morgan ET. Nitric oxide-dependent proteasomal degradation of cytochrome P450 2B proteins. *The Journal of biological chemistry.* 2008;283(2):889-98.

224. De Gasparo M, Catt KJ, Inagami T, Wright JW, and Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *PharmacolRev.* 2000;52:415-72.

225. Schiffrin EL, Park JB, Intengan HD, and Touyz RM. Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation.* 2000;101:1653-9.

226. Suvorava T, Stegbauer J, Thieme M, Pick S, Friedrich S, Rump LC, et al. Sustained hypertension despite endothelial-specific eNOS rescue in eNOS-deficient mice. *Biochem Biophys Res Commun.* 2015;458(3):576-83.

227. Wood KC, Cortese-Krott MM, Kovacic JC, Noguchi A, Liu VB, Wang X, et al. Circulating blood endothelial nitric oxide synthase contributes to the regulation of systemic blood pressure and nitrite homeostasis. *ArterioscIerThrombVascBiol.* 2013;33:1861-71.

228. Kleschyov AL. The NO-heme signaling hypothesis. *Free radical biology & medicine.* 2017;112:544-52.

229. Dei Zotti F, Lobysheva, II, and Balligand JL. Nitrosyl-hemoglobin formation in rodent and human venous erythrocytes reflects NO formation from the vasculature in vivo. *PloS one.* 2018;13(7):e0200352.

230. Cortese-Krott MM, Rodriguez-Mateos A, Sansone R, Kuhnle GG, Thasian-Sivarajah S, Krenz T, et al. Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. *Blood.* 2012;120(20):4229-37.

231. Sender R, Fuchs S, and Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biology.* 2016;14(8):e1002533.

232. Shu X, Keller TCSt, Begandt D, Butcher JT, Biwer L, Keller AS, et al. Endothelial nitric oxide synthase in the microcirculation. *Cellular and molecular life sciences : CMLS.* 2015;72(23):4561-75.

233. Straub AC, Lohman AW, Billaud M, Johnstone SR, Dwyer ST, Lee MY, et al. Endothelial cell expression of haemoglobin α regulates nitric oxide signalling. *Nature.* 2012;491(7424):473-7.

234. Harrison DG. The mosaic theory revisited: common molecular mechanisms coordinating diverse organ and cellular events in hypertension. *J AmSocHypertens.* 2013;7:68-74.

235. Landmesser U, and Harrison DG. Oxidative stress and vascular damage in hypertension. *CoronArtery Dis.* 2001;12:455-61.

236. Heinzel B, John M, Klatt P, Böhme E, and Mayer B. Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *BiochemJ.* 1992;281:627-30.

237. Zou MH, Shi C, and Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J ClinInvest.* 2002;109:817-26.

238. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, and Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation.* 1997;95:588-93.

239. Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, et al. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension.* 1995;26:854-7.

240. Didion SP, Kinzenbaw DA, Schrader LI, Chu Y, and Faraci FM. Endogenous interleukin-10 inhibits angiotensin II-induced vascular dysfunction. *Hypertension.* 2009;54:619-24.

241. Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, and Brandes RP. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation.* 2004;109:1795-801.

242. Lee MY, and Griendling KK. Redox signaling, vascular function, and hypertension. *AntioxidRedoxSignal.* 2008;10:1045-59.

243. Libby P, Ridker PM, and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature.* 2011;473:317-25.

244. Ross R. Atherosclerosis--an inflammatory disease. *NEnglJMed.* 1999;340:115-26.

245. Napoli C, De Nigris F, Williams-Ignarro S, Pignalosa O, Sica V, and Ignarro LJ. Nitric oxide and atherosclerosis: an update. *NitricOxide.* 2006;15:265-79.

246. Satoh K, Nigro P, and Berk BC. Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A. *AntioxidRedoxSignal.* 2010;12:675-82.

247. Tabima DM, Frizzell S, and Gladwin MT. Reactive oxygen and nitrogen species in pulmonary hypertension. *Free RadicBiolMed.* 2012;52:1970-86.

248. Yogo K, Shimokawa H, Funakoshi H, Kandabashi T, Miyata K, Okamoto S, et al. Different vasculoprotective roles of NO synthase isoforms in vascular lesion formation in mice. *ArteriosclerThrombVascBiol.* 2000;20:E96-E100.

249. Sugamura K, and Keaney JF, Jr. Reactive oxygen species in cardiovascular disease. *Free RadicBiolMed.* 2011;51:978-92.

250. Nieuwdorp M, Meuwese MC, Mooij HL, van Lieshout MH, Hayden A, Levi M, et al. Tumor necrosis factor-alpha inhibition protects against endotoxin-induced endothelial glycocalyx perturbation. *Atherosclerosis.* 2009;202(1):296-303.

251. Broekhuizen LN, Lemkes BA, Mooij HL, Meuwese MC, Verberne H, Holleman F, et al. Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus. *Diabetologia.* 2010;53(12):2646-55.

252. Dogne S, and Flamion B. Endothelial Glycocalyx Impairment in Disease: Focus on Hyaluronan Shedding. *The American journal of pathology.* 2020;190(4):768-80.

253. Cosgun ZC, Fels B, and Kusche-Vihrog K. Nanomechanics of the Endothelial Glycocalyx: From Structure to Function. *The American journal of pathology.* 2020;190(4):732-41.

254. Fels B, and Kusche-Vihrog K. It takes more than two to tango: mechanosignaling of the endothelial surface. *Pflugers Archiv : European journal of physiology.* 2020;472(4):419-33.

255. Dogne S, Flamion B, and Caron N. Endothelial Glycocalyx as a Shield Against Diabetic Vascular Complications: Involvement of Hyaluronan and Hyaluronidases. *Arterioscler Thromb Vasc Biol.* 2018;38(7):1427-39.

256. van den Berg BM, Spaan JA, Rolf TM, and Vink H. Atherogenic region and diet diminish glycocalyx dimension and increase intima-to-media ratios at murine carotid artery bifurcation. *American journal of physiology Heart and circulatory physiology.* 2006;290(2):H915-20.

257. Grandoch M, Flogel U, Virtue S, Maier JK, Jelenik T, Kohlmorgen C, et al. 4-Methylumbelliferone improves the thermogenic capacity of brown adipose tissue. *Nature metabolism.* 2019;1(5):546-59.

258. Schuler D, Sansone R, Freudenberger T, Rodriguez-Mateos A, Weber G, Momma TY, et al. Measurement of endothelium-dependent vasodilation in mice--brief report. *Arterioscler Thromb Vasc Biol.* 2014;34(12):2651-7.

259. Dai X, and Faber JE. Endothelial nitric oxide synthase deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis. *Circ Res.* 2010;106(12):1870-81.

260. Tarbell JM, and Pahakis MY. Mechanotransduction and the glycocalyx. *J Intern Med.* 2006;259(4):339-50.

261. Patel HH, Murray F, and Insel PA. Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu Rev Pharmacol Toxicol.* 2008;48:359-91.

262. Thorne RF, Wang Y, Zhang Y, Jing X, Zhang XD, de Bock CE, et al. Evaluating nuclear translocation of surface receptors: recommendations arising from analysis of CD44. *Histochemistry and cell biology.* 2020;153(2):77-87.

263. Ouhtit A, Rizeq B, Saleh HA, Rahman MM, and Zayed H. Novel CD44-downstream signaling pathways mediating breast tumor invasion. *International journal of biological sciences.* 2018;14(13):1782-90.

8. CURRICULUM VITAE

Tatsiana Suvorava, PhD

EDUCATION

October, 2006	PhD in Biology (high distinction) "Role of nitric oxide in post-radiational changes of heart contractility and coronary circulation", Department of Cardiovascular Physiology, Institute of Radiobiology, National Academy of Science, Minsk, Republic of Belarus
May, 1997	Master of Science in Biology (with honor) "Post-radiational changes in adenylate and guanylate cyclase activity in rat cardiomyocytes and aortic smooth muscle cells" Graduate group of Animal and Human Physiology, Biological Faculty, Belarusian State University, Minsk, Republic of Belarus

CURRENT POSITION

since 07/2019	Staff Scientist, Institute of translational Pharmacology, Heinrich-Heine-University, Düsseldorf

PREVIOUS POSITIONS

2016-2019	Principle Investigator, Cardiovascular Research Laboratory, Clinic of Cardiology, University Hospital Düsseldorf
2013 - 2015	Principle Investigator (extramural funding DFG&PI position), Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf
2006 - 2012	Postdoctoral Researcher, Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf
2004 – 2006	Lecturer in Physiology, Belarusian State Medical University, Physiology Department, Minsk, Belarus
2003 – 2004	DAAD Exchange Fellow, Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf, Germany
1997 – 2003	PhD student, Department of Cardiovascular Physiology, Institute of Radiobiology, National Academy of Sciences, Minsk, Belarus

FELLOWSHIPS AND AWARDS

2022	Joseph Loscalzo Award for the best basic/translational article published in Circulation in the preceding twelve months. Basic Cardiovascular Sciences (BCVS) Scientific Sessions, July 25-28, 2022 in Chicago, Illinois
2015	Walter-Clawiter-Preis 2015, Heinrich-Heine-University Düsseldorf, Germany
2015	Best Poster Presentation Award, Antioxidants Word Congress 2015, Paris, France
2013	Best Poster Prize, European Society of Cardiology, Amsterdam, Netherlands
2012	American Heart Association (AHA) Travel Award for Young Investigators; one of the top five abstracts submitted from Germany, LA, USA
2012	1st Poster Prize, DGPT (German Society for Pharmacology and Toxicology), Mainz, Germany
2010	Young Investigator Award, Council of Basic Cardiovascular Science for the best original scientific work, European Society of Cardiology (ESC), Charité, Berlin
2010	1st Schmiedeberg's Archives of Pharmacology Poster Prize, DGPT, Mainz, Germany
2008	EuroPRevent, ESC, Best Poster Prize in Basic Science, Paris, France
2007	Angioedema Forum, Poster Prize, Cologne, Germany
2006 &	FEBS (Federation of European Biochemical Societies) Collaborative Scholarship, project "Effect

2005 of Endogenous Hydrogen Peroxide On Circulating Cells with Endothelial Progenitor Capacity"
2003-2004 DAAD (German Academic Exchange Service) research fellowship, project "Exercise and Vascular Oxidative Stress: The Role of Hydrogen Peroxide"
2002- 2015 Travel awards of ESC, AHA, DAAD, ISANH and NASA
2002-2010 Several Youth Travel Funds, FEBS

RESARCH SUPPORT

2018-2020 Principal investigator, project "RBC and EC NOS3 in exercise-dependent cardioprotection: focus on mitochondria", DAAD-MIUR Joint Research Program.

2016- 2019 Principal investigator, project "Role of red blood cells signaling in exercise-dependent cardiovascular protection" funded by the Forschungskommission of the Medical Faculty, Heinrich Heine University.

2013-2014 Principal Investigator Position and DFG research grant "Potential role of brain stem extra-endothelial activity of eNOS in the regulation of blood pressure"

2011-2013 Principal Investigator, project: "Role of non-endothelial eNOS in blood pressure regulation" funded by the Forschungskommission of the Medical Faculty, Heinrich-Heine-University.

2008-2010 Principal Investigator, project: "Is Endothelial Bradykinin Receptor Type 2 Agonism a Cardiovascular Protective Intervention?" funded by the Forschungskommission of the Medical Faculty, Heinrich-Heine-University.

2001-2003 Young Scientist Research Grant "Role of nitric oxide in regulation of coronary flow under hypoxia and reoxygenation: influence of low-density ionizing radiation" funded by Belarusian Foundation for Fundamental Research, National Academy of Sciences, Minsk, Belarus.

MEMBERSHIPS OF SCIENTIFIC SOCIETIES

2012 - present American Heart Association, USA

2009 - present European Association for Cardiovascular Prevention & Rehabilitation, ESC

2000 - present Federation of European Biochemical Societies & Ukranian Biochemical Society

PUBLICATIONS

Original articles - 38, Reviews - 10, Editorials - 2, Book chapter - 1

OTHER ACTIVITIES

Member of the Research Committee, Faculty of Medicine, Heinrich-Heine-University, Düsseldorf

Radiation Protection Representative, Institute of translational Pharmacology, HHU Düsseldorf

Deputy Head of Animal Experiments, Institute for translational Pharmacology, HHU Düsseldorf

Ad hoc Reviewer:
European Heart Journal, Biochemical Pharmacology, Journal of Cellular Physiology, Nitric Oxide etc.

Reviewer for grant proposals of the Research Foundation Flanders (FWO, Belgium).

9. ORIGINAL PAPERS

The following original papers on which this cumulative habilitation thesis is based are reproduced reverse chronologically either under the terms of a Creative Creative Commons Licence or with the kind permission of the publishers.

Circulation

ORIGINAL RESEARCH ARTICLE

Red Blood Cell and Endothelial eNOS Independently Regulate Circulating Nitric Oxide Metabolites and Blood Pressure

Francesca Leo, MSc*; Tatsiana Suvorava, PhD*; Sophia K. Heuser, MSc; Junjie Li, MSc; Anthea LoBue, MSc; Frederik Barbarino, PhD; Eugenia Piragine, PhD; Rebekka Schneckmann, PhD; Beate Hutzler, PhD; Miranda E. Good, PhD; Bernadette O. Fernandez, PhD; Lukas Vornholz, PhD; Stephen Rogers, PhD; Allan Doctor, MD; Maria Grandoch, MD; Johannes Stegbauer, MD; Eddie Weitzberg, MD, PhD; Martin Feelisch, PhD; Jon O. Lundberg, MD, PhD; Brant E. Isakson, PhD; Malte Kelm, MD; Miriam M. Cortese-Krott, PhD

BACKGROUND: Current paradigms suggest that nitric oxide (NO) produced by endothelial cells (ECs) through endothelial nitric oxide synthase (eNOS) in the vessel wall is the primary regulator of blood flow and blood pressure. However, red blood cells (RBCs) also carry a catalytically active eNOS, but its role is controversial and remains undefined. This study aimed to elucidate the functional significance of RBC eNOS compared with EC eNOS for vascular hemodynamics and nitric oxide metabolism.

METHODS: We generated tissue-specific loss- and gain-of-function models for eNOS by using cell-specific Cre-induced gene inactivation or reactivation. We created 2 founder lines carrying a floxed eNOS ($eNOS^{flox/flox}$) for Cre-inducible knockout (KO), and gene construct with an inactivated floxed/inverted exon ($eNOS^{inv/inv}$) for a Cre-inducible knock-in (KI), which respectively allow targeted deletion or reactivation of eNOS in erythroid cells (RBC eNOS KO or RBC eNOS KI mice) or in ECs (EC eNOS KO or EC eNOS KI mice). Vascular function, hemodynamics, and nitric oxide metabolism were compared ex vivo and in vivo.

RESULTS: The EC eNOS KOs exhibited significantly impaired aortic dilatory responses to acetylcholine, loss of flow-mediated dilation, and increased systolic and diastolic blood pressure. RBC eNOS KO mice showed no alterations in acetylcholine-mediated dilation or flow-mediated dilation but were hypertensive. Treatment with the nitric oxide synthase inhibitor N^{γ}-nitro-L-arginine methyl ester further increased blood pressure in RBC eNOS KOs, demonstrating that eNOS in both ECs and RBCs contributes to blood pressure regulation. Although both EC eNOS KOs and RBC eNOS KOs had lower plasma nitrite and nitrate concentrations, the levels of bound NO in RBCs were lower in RBC eNOS KOs than in EC eNOS KOs. Reactivation of eNOS in ECs or RBCs rescues the hypertensive phenotype of the $eNOS^{inv/inv}$ mice, whereas the levels of bound NO were restored only in RBC eNOS KI mice.

CONCLUSIONS: These data reveal that eNOS in ECs and RBCs contribute independently to blood pressure homeostasis.

Key Words: blood circulation ■ blood pressure ■ hypertension ■ models, animal ■ nitric oxide synthase

Editorial, see p 890

Nitric oxide (NO) produced by the endothelial isoform of nitric oxide synthase (eNOS) in endothelial cells (ECs) is considered to be the central regulator of vascular tone and systemic hemodynamics.[1] All strains of global eNOS knockout (KO) mice are hypertensive,[2–4] and some show decreased levels of the

Correspondence to: Miriam M. Cortese-Krott, PhD, Myocardial Infarction Research Laboratory, Department of Cardiology, Pulmonology, and Vascular Medicine, Medical Faculty, Heinrich-Heine-University of Düsseldorf, Postfach 128, Universitätsstrasse 1, 40225 Düsseldorf, Germany. Email Miriam.cortese@hhu.de

*F. Leo and T. Suvorava contributed equally.

The Data Supplement, podcast, and transcript are available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.120.049606.

For Sources of Funding and Disclosures, see page 887.

Clinical Perspective

What Is New?

- We generated endothelial cell– and red blood cell (RBC)-specific endothelial nitric oxide synthase (eNOS) knockout and eNOS knock-in mice
- Endothelial cell eNOS knockout mice show hypertension, endothelial dysfunction, and increased systemic vascular resistance, whereas reactivation of eNOS in endothelial cells restores endothelial function and normotension.
- RBC eNOS knockout mice show hypertension, a preserved arterial endothelial function, and reduced levels of bound nitric oxide in RBCs, whereas reactivation of eNOS in RBCs restores nitric oxide bioavailability in RBCs and rescues the hypertensive phenotype.

What Are the Clinical Implications?

- Both endothelial cells and RBCs are important regulators of blood pressure through eNOS.
- RBC eNOS contributes to the regulation of nitric oxide metabolism, systemic hemodynamics, and blood pressure.
- These findings may have important pathophysiological implications in our understanding of the interrelationship between hematologic and cardiovascular disease and may reveal novel therapeutic approaches to improve tissue perfusion.

Nonstandard Abbreviations and Acronyms

BP	blood pressure
CondKO	conditional global eNOS knockout mice
DBP	diastolic blood pressure
EC	endothelial cell
EC eNOS KI	endothelial-specific eNOS knockin mice
EC eNOS KO	endothelial-specific eNOS knockout mice
eNOS	endothelial nitric oxide synthase
KO	knockout
L-NAME	Nγ-nitro-l-arginine methyl ester
MAP	mean arterial pressure
NO	nitric oxide
NO-heme	nitrosylheme
NorNOHA	*N*-hydroxy-nor-L-arginine
PCR	polymerase chain reaction
RBC	red blood cell
RBC eNOS KI	RBC-specific eNOS knock-in mice
RBC eNOS KO	RBC-specific eNOS knockout mice
SBP	systolic blood pressure

circulating nitric oxide (NO) oxidation products, nitrite and nitrate.[5–7]

However, eNOS is also expressed in other cell types, including red blood cells (RBCs).[8,9] The functional significance of eNOS in RBC physiology, systemic NO metabolism, and tissue protection remains controversial.[10–16] There are indications that eNOS in the blood may participate in the regulation of circulating nitrite levels and blood pressure (BP) homeostasis,[9] but its specific contribution is unknown. One way to test the physiological effect directly would be through genetic manipulation of eNOS in ECs versus RBCs.

This study aimed at elucidating the specific role of eNOS in RBCs in direct comparison with the role of eNOS in ECs in controlling systemic NO metabolism and BP regulation. To accomplish this, we generated tissue-specific loss- and gain-of-function models for eNOS by using tissue-specific Cre-induced gene inactivation or reactivation. To our knowledge, these studies are the first performed on tissue-specific eNOS KO/knock-in (KI) mice providing compelling evidence that eNOS in RBCs contributes to the regulation of systemic NO bioavailability and systemic hemodynamics, independently of eNOS in the endothelium. Altogether, these findings suggest the existence of a noncanonical RBC eNOS-dependent pathway for regulation of BP homeostasis independent of the eNOS expressed in the vessel wall.

METHODS

A detailed description of the methods is available in the Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

Materials

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich Co LLC.

Animals

All experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123). Animal care was provided according to the institutional guidelines. Tamoxifen-inducible endothelial-specific Cre mice (Tg(Cdh5-Cre/ERT2)1Rha; MGI:3848982)[17] were kindly provided by Prof Dr E. Lammert (Heinrich-Heine-University of Düsseldorf). Mice expressing Cre recombinase in erythroid cells under the control of the promoter of the hemoglobin β-chain (C57BL/6-Tg(Hbb-Cre)12Kpe/J; MGI: J:89725)[18] were obtained by Jackson Laboratory (JAX stock No. 008314) and crossed for >10 generations with C57BL/6J. DeleterCre (C57Bl/6.C-Tg(CMVCre)1Cgn/J)[19] mice expressing Cre in all tissues were kindly provided by Prof Claus Pfeffer (Heinrich Heine University of Düsseldorf). Experimental planning and execution followed the ARRIVE recommendations (Animal Research: Reporting

of In Vivo Experiments).[20] For experiments, 2- to 6-months-old male mice up to 30 g were used. Mice of the same genotype and age were randomly assigned to the experimental groups. The evaluation of data obtained by ultrasound was performed by a blinded researcher.

Generation of EC/RBC eNOS KO and EC/RBC eNOS KI Mice

We generated 2 independent founder lines carrying a floxed eNOS ($eNOS^{flox/flox}$) or a gene construct with an inactivated floxed/inverted exon ($eNOS^{inv/inv}$) for a Cre-inducible KI. Phenotypically $eNOS^{flox/flox}$ are wild-type (WT) mice, and $eNOS^{inv/inv}$ are conditional global eNOS KO (CondKO) mice. These founder lines respectively allow targeted removal or reactivation of eNOS in either ECs or RBCs, or all cells. To generate $eNOS^{flox/flox}$ mice, we designed a loxP eNOS targeting construct by simultaneously inserting an orphan loxP site and an FRT-neo-FRT-loxP resistance cassette inserted into the Nos3 genomic locus to target exon 2 of Nos3 by Cre-mediated excision. To generate $eNOS^{inv/inv}$ mice, we inserted an inverted exon 2 of Nos3 and 2 additional Lox511 sites in the loxP-eNOS construct, which allowed the Cre-induced reactivation of eNOS in a cell type of interest. The plasmids were sequenced, linearized, and electroporated in A9 embryonic stem cells (hybrid C57/129), 300 clones were picked, and positive clones were screened by Southern blot at the 5′ arm and by long-range polymerase chain reaction (PCR). Homozygous $eNOS^{flox/flox}$ mice or $eNOS^{inv/inv}$ mice were crossed with Cdh5-Cre/$ERT2^{pos}$ mice to obtain $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ and $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$ mice or $eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{pos}$ and $eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{neg}$ mice, respectively. To induce EC-specific activation of the Cre recombinase, we treated Cre-positive and Cre-negative mice of each line with tamoxifen (33 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for 5 consecutive days and allowed a 21-day waiting period after the last injection, which generated EC eNOS KO ($eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$+TAM) mice and EC eNOS KI ($eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{pos}$+TAM) mice and their respective Cre-negative controls. Homozygous $eNOS^{flox/flox}$ mice or $eNOS^{inv/inv}$ mice were also crossed with erythroid-specific Hbb-Cre^{pos} mice to obtain erythroid-specific eNOS KO mice ($eNOS^{flox/flox}$ Hbb-Cre^{pos}=RBC eNOS KO) and their respective WT littermate control ($eNOS^{flox/flox}$ Hbb-Cre^{neg}) or erythroid-specific eNOS KI mice ($eNOS^{inv/inv}$ Hbb-Cre^{pos}=RBC eNOS KI) and their WT littermate control ($eNOS^{inv/inv}$ Hbb-Cre^{neg}). In addition, $eNOS^{flox/flox}$ mice were crossed with DelCre mice to create global eNOS KO (gKO). For a structured list of the lines and nomenclature used in the figures and the text please refer to Table 1. For further details on the genetic strategy and selection see the Methods and Figures I and II in the Data Supplement Methods.

Analysis of Tissue-Specific Loss-of-Function or Gain-of-Function

The Cre recombinase–dependent genetic locus recombination was determined in targeted and nontargeted tissues by real-time PCR with specific primers and probes designed to recognize the floxed allele and the allele with targeted deletion (Transnetyx). The eNOS and Cre recombinase expression was analyzed by TaqMan real-time reverse transcriptase PCR in EC ($CD31^{+}$ $CD45^{-}$) magnetically isolated from lung homogenates or in erythroid cells ($Ter119^{+}$ $CD71^{+}$ $CD45^{-}$) magnetically isolated from the bone marrow of the mice, and in targeted or nontargeted tissues, as explained in detail in the figures and Data Supplement. The expression of eNOS in RBCs, in RBC membrane preparations (ghosts), and in targeted and nontargeted tissues, was analyzed by immunotransmission electronic microscopy,[21] Western blot analysis, and quantitative ELISA according to the manufacturer's protocol (Abcam). The expression of eNOS in RBC lysates was also measured by immunoprecipitation and Western blot analysis according to published procedures.[8]

Measurements of BP and Systemic Hemodynamics

Invasive assessment of hemodynamic parameters was performed by using a 1.4F Millar pressure-conductance catheter (SPR-839, Millar Instrument) placed into the left ventricle through the right carotid artery according to the closed chest method as described.[22] Transthoracic echocardiography was performed as previously described.[22] Left ventricular end-systolic and end-diastolic volumes, left ventricular ejection fraction, fractional shortening, cardiac output, stroke volume, and systemic vascular resistance were calculated. Left ventricular diastolic function was assessed by analysis of the characteristic flow profile of the mitral valve Doppler, which was visualized in an apical 4-chamber view, as described.[22] For the assessment of hemodynamic responses in awake mice, we used radiotelemetry with a microminiaturized electronic monitor (PA-C10; Data Sciences International) attached to an indwelling aortic catheter. After 3 days of baseline measurements, we measured hemodynamic responses to nitric oxide synthase (NOS) inhibition by the administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mg/mL in drinking water for 3 days; we determined that mice drink 5 mL/d independently of the presence of L-NAME, thus resulting in a dose of 166 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$); these were followed by hemodynamic responses to increased L-arginine bioavailability achieved by administration of the arginase inhibitor *N*-hydroxy-nor-L-arginine (NorNOHA, 10 mg/kg intraperitoneally for 3 days).

Measurement of Endothelial Function/Vascular Reactivity Ex Vivo and In Vivo

Thoracic aortas were excised and their functional reactivity analyzed in an organ bath as previously described.[23] Vascular function in vivo was measured as flow-mediated dilation with a Vevo 2100 with a 30 to 70 MHz linear array microscan transducer (VisualSonics) as described.[22]

Determination of NO Metabolites in Blood and Tissues

Nitrosated (*S*-nitroso and *N*-nitroso) products (RXNO), and nitrosylheme (NO-heme) were quantified by gas phase chemiluminescence as described.[24] For nitrite and nitrate analysis, samples were deproteinized with ice-cold methanol (1:1 v/v), cleared by centrifugation, and subjected to analysis by high-performance liquid chromatography using a dedicated nitrite/nitrate analyzer (ENO20, Eicom).[25]

Table 1. Blood Pressure and Heart Rate in All Strains Investigated

No.	Strain	Genotype	Litter size	Body-weight, g	Systolic blood pressure, mm Hg	Diastolic blood pressure, mm Hg	Mean arterial pressure, mm Hg	Heart rate, bpm	Rate pressure product, mm Hg×bpm	n
1	WT	$eNOS^{flox/flox}$	7±2	30.3±2.5	92±8	62±9	71±1	543±67	49591±5512	14
2	WT	$eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$ +TAM	6±2	29.3±3.6	86±5	57±8	67±7	564±53	48413±5018	12
	EC eNOS KO	$eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ +TAM		29.4±3.1	104±8	72±7	83±7	535±58	55696±6929	19
3	WT	$eNOS^{flox/flox}$ $HbbCre^{neg}$	6±2	29.1±2.1	89±9	61±1	70±1	528±56	46622±5306	15
	RBC eNOS KO	$eNOS^{flox/flox}$ $HbbCre^{pos}$		29.7±2.6	103±1	69±7	80±8	476±93	48506±9392	16
4	gKO	$eNOS^{flox/flox}$ $DelCre^{pos}$	4±2	30.2±2.6	115±1	79±1	91±1	516±76	56275±20504	10
5	CondKO	$eNOS^{inv/inv}$	5±3	27.8±1.8	127±1	84±1	98±1	465±44	59046±8299	11
6	CondKO	$eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{neg}$ +TAM	4±2	28.7±2.2	122±6	81±1	94±9	547±49	66562±5285	11
	EC eNOS KI	$eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{pos}$ +TAM		28.3±2.2	94±7	58±8	70±7	540±48	50849±5346	13
7	CondKO	$eNOS^{inv/inv}$ $HbbCre^{neg}$	4±2	28.7±1.2	119±1	75±1	90±1	494±45	58845±5542	18
	RBC eNOS KI	$eNOS^{inv/inv}$ $HbbCre^{pos}$		31.1±2.5	95±8	56±9	69±8	469±67	47022±7322	10

CondKO indicates conditional global eNOS knockout mice; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; KO, knockout; RBC, red blood cell; and WT, wild type.

Statistical Analysis

Sample size was calculated a priori by using G-Power V.3.1 (Heinrich Heine University of Düsseldorf). Statistical analysis was performed with GraphPad Prism 9 for macOS (Version 9.0.2(134)). Unless stated otherwise, the results are reported as means±SD. Normal distribution was tested by the D'Agostino-Pearson test. Comparisons among multiple groups were performed using 1-way and 2-way ANOVA or 2-way repeated measures ANOVA, as appropriate, followed by Tukey or Sidak post hoc analysis, as indicated. Where indicated, unpaired Student *t* test with Welch correction was used to determine if 2 groups of data were significantly different. The Mann-Whitney *U* test was performed when data were not normally distributed. $P<0.05$ was considered statistically significant.

RESULTS

Generation of Endothelial and Erythroid-Specific eNOS KO Mice

To obtain mice specifically lacking eNOS in ECs (EC eNOS KO) or erythroid cells (RBC eNOS KO), we applied the loxP-Cre approach to delete exon 2 of the Nos3 gene. Therefore, an orphan loxP site and an FRT-neo-FRT-loxP resistance cassette were simultaneously inserted into the Nos3 genomic locus to target exon 2 of Nos3 for Cre-mediated excision (Figure 1A). Sequencing confirmed base pair–precise modification of the Nos3 genomic locus and integrity, whereas stable transfection of the construct in embryonic stem cells was assessed by Southern blotting/long-range PCR (see Figure I in the Data Supplement). Two male mice fully derived from A9 embryonic stem cells (hybrid C57/129) were obtained and backcrossed with C57/BL6j mice for at least 10 generations. The $eNOS^{flox/flox}$ mice were crossed with tamoxifen-inducible EC-specific Cre-expressing mice (Cdh5-Cre/$ERT2^{pos}$ mice)[17]; once activated by tamoxifen, the Cre recombinase expressed in $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ mice removed the floxed segment (Figure 1A), creating EC-specific eNOS KO mice (EC eNOS KO; Figure 1B). In WT ($eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$) littermate mice lacking the Cre recombinase, tamoxifen does not lead to genetic recombination.

Tamoxifen-induced Cre expression, DNA recombination, and the resulting genetic deletion of exon 2 of Nos3 in $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ mice (EC eNOS KO) and the lack thereof in $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$ mice (control) were confirmed by real-time PCR analysis of DNA extracted from vascular tissue (aorta) of the mice (Figure 1C, blue). This resulted in a lack of eNOS expression in lung EC ($CD31^+$ $CD45^-$; Figure 1D, blue), and in the aorta, as assessed by real-time PCR (Figure 1E, blue) and immunoblot analysis of aortic lysates (Figure 1F). In EC eNOS KO mice, we also found that eNOS expression was nondetectable in highly vascularized tissues such as the lungs, the heart, and the kidney, as assessed by immunoblot analysis (Figure III in the Data Supplement) and quantitative ELISA (Figure 1J).

To create erythroid-specific eNOS KO mice (RBC eNOS KO), $eNOS^{flox/flox}$ mice were crossed with mice constitutively expressing Cre recombinase in erythroid cells under the control of the promoter for the hemoglobin β-chain (Hbb-Cre^{pos}[18]; Figure 1B). Cre-dependent deletion of exon 2 was confirmed by real-time PCR in DNA extracted from the bone marrow of the mice (Figure 1C, orange) but was not found in the aorta or other tissues. Cre expression was found in the bone marrow

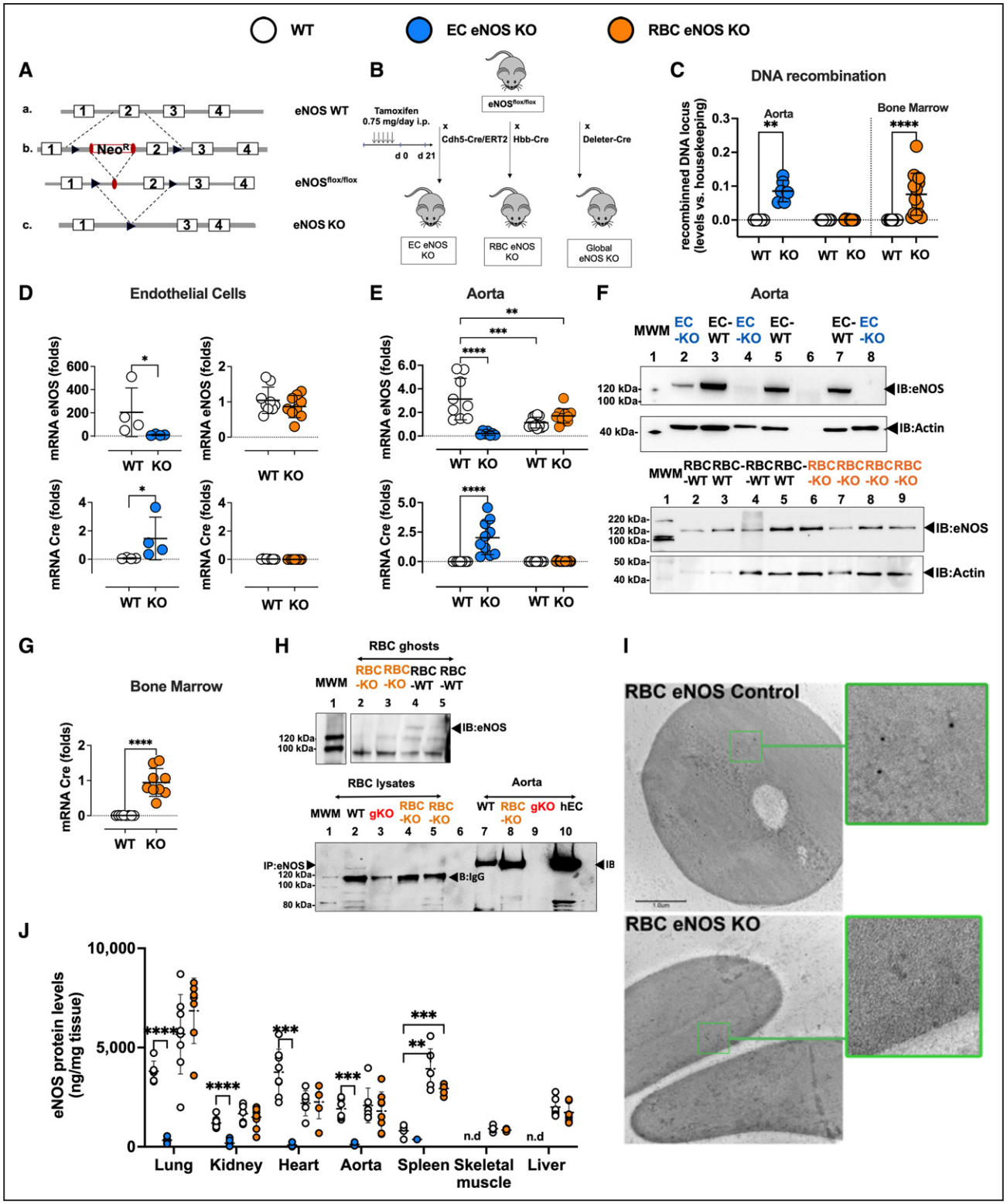


Figure 1. Generation and characterization of EC eNOS KO or RBC eNOS KO mice.

A, Scheme describing the gene-targeting strategy showing the position of the loxP sequences (black) within the gene-targeting construct before and after exon 2 of Nos3 was used to generate the founder eNOS$^{flox/flox}$ mice. **B**, To generate EC eNOS KO mice and their respective WT control (eNOS$^{flox/flox}$ Cdh5-Cre/ERT2^{neg}), the founder eNOS$^{flox/flox}$ mice were crossed with endothelial-specific tamoxifen (TAM)-inducible Cdh5-Cre/ERT2^{pos} mice to obtain eNOS$^{flox/flox}$ Cdh5-Cre/ERT2$^{pos/neg}$ mice; these were treated with TAM for 5 days and analyzed after 21 days. To create RBC eNOS KO mice or global eNOS KO mice and their respective WT littermate controls, the founder eNOS$^{flox/flox}$ was crossed with erythroid-specific (Hbb-Cre^{pos}) mice or with Deleter-Cre^{pos} mice (expressing Cre in all tissues). **C**, Real-time PCR analysis shows that tissue-specific DNA recombination occurs in the aorta of EC eNOS KO mice (blue), whereas in RBC eNOS KO mice, DNA recombination occurs in the bone marrow, (*Continued*)

Figure 1 Continued. but not in the aorta (orange). No recombination is observed in WT littermate control mice (white). *t* test ***P*<0.01, *****P*<0.0001 vs respective WT control. **D**, **Top**, Real-time reverse transcriptase PCR analysis shows that endothelial cells ($CD31^+$ $CD45^-$) extracted from the lung of EC eNOS KO mice (blue) have a significant loss of eNOS expression compared with WT controls. *Mann-Whitney *U* test *P*=0.0286. Instead, the expression of eNOS in lung EC ($CD31^+$ $CD45^-$) from RBC eNOS KO (orange) is not different from WT control. It is notable that TAM increased the expression of eNOS in lung EC from WT ($eNOS^{flox/flox}$Cdh5-Cre/$ERT2^{neg}$) mice**. Bottom**, Cre recombinase is expressed in lung endothelial cells from EC eNOS KO (blue) but not in endothelial cells from WT (white) or RBC eNOS KO (orange) mice. *Mann-Whitney *U* test *P*=0.0286. **E**, **Top**, Real-time reverse transcriptase PCR analysis shows loss of eNOS expression and Cre recombinase expression in the aorta of EC eNOS KO (blue), but not in WT littermate control mice (white). TAM increased mRNA eNOS expression in the aorta of the WT ($eNOS^{flox/flox}$Cdh5-Cre/$ERT2^{neg}$) mice, but the protein levels are not different from WT mice (see also **I**, ELISA). One-way ANOVA *P*<0.0001; Tukey multiple comparison test ***P*<0.01,*** *P*<0.001, *****P*<0.0001 vs respective WT control. **F**, **Top**, Immunoblot (IB) analysis shows loss of eNOS (135 kDa) expression in the aorta of EC eNOS KO ($eNOS^{flox/flox}$Cdh5-Cre/$ERT2^{pos}$+TAM) mice but not in littermate controls ($eNOS^{flox/flox}$Cdh5-Cre/$ERT2^{neg}$+TAM) after treatment with TAM. Loading control actin (45 kDa). Note: Sample in the first lane shows a residue of eNOS expression in that particular mouse because the knockdown efficiency of this model is ≈90% to 95%. **Bottom**, Immunoblot of aorta of RBC eNOS KO mice and WT littermate controls, showing that eNOS expression is not different in these 2 groups. **G**, Real-time reverse transcriptase PCR analysis shows eNOS expression and Cre recombinase expression in the bone marrow of RBC eNOS KO mice. Cre-recombinase is expressed in the bone marrow of RBC eNOS KO mice, but not in the aorta. Note that, although eNOS deletion was specifically detected in the bone marrow of RBC eNOS KO mice, we determined similar eNOS expression levels in the RBC eNOS KO mice compared with WT littermates; this is attributable to the high abundance of endothelial cells in the bone marrow. *****P*<0.0001 Mann-Whitney *U* test vs WT mice. **H**, **Upper**, Immunoblot of membrane preparations of RBCs (ghosts) showing the presence of eNOS in WT RBCs and its absence in RBCs from RBC eNOS KO mice. **Bottom**, Immunoprecipitation (IP) of eNOS (135 KDa) from RBC lysates shows eNOS expression in WT mice and lack of eNOS in global eNOS KO mice (gKO) and RBC eNOS KO mice. The IgG (120 kDa) is seen in the IP samples. **I**, Electron scanning microscopy with immunogold staining of eNOS in WT ($eNOS^{flox/flox}$ $HbbCre^{pos}$) mice (**Upper**) and RBC eNOS KO (**Bottom**). **J**, The quantification of eNOS protein expression in multiple organs by ELISA shows a significant loss of eNOS in multiple tissues of EC eNOS KO mice compared with WT littermate controls. The differences between the WT control groups are not significant, except in the spleen. Data were analyzed according to mixed-effect model with Geisser-Greenhouse correction (variables: strain, tissue); *P*<0.0001; multiple comparisons of eNOS levels among the groups within the same tissue were assessed by a Tukey test ***P*<0.01; ****P*<0.001; *****P*<0.000. Lines represent means± SD. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KO, knockout; MWM, molecular weight marker; RBC, red blood cell; and WT, wild type.

of RBC eNOS KO (Figure 1G, orange), but not in the aorta or other tissues. In RBC eNOS KO we did not detect any eNOS in membrane preparations of RBCs (ghosts) by immunoblotting, in RBC lysates by immunoprecipitation + immunoblotting (Figure 1H), and by scanning electron microscopy and immunogold staining (Figure 1I). On the other hand, in RBC eNOS KO mice, we found that eNOS expression was fully preserved in lung EC ($CD31^+$ $CD45^-$; Figure 1D, orange), aorta (Figure 1E, orange; Figure 1F, Bottom), and all other tissues of RBC eNOS KO analyzed, including lung, kidney, heart, spleen, skeletal muscle, and liver (Figure 1J; Figure III in the Data Supplement).

Hematologic Characterization of EC eNOS KO and RBC eNOS KO Mice

Blood counts and rheological parameters remained unchanged in all strains (Table I in the Data Supplement). However, hemoglobin oxygen affinity was significantly higher and oxygen-binding cooperativity significantly lower in RBC eNOS KO mice compared with their WT littermate controls; this difference progressed as pH was lowered within the physiological range (from 7.6 to 7.2; Figure IV in the Data Supplement).

Vascular Endothelial Function and eNOS Expression Are Compromised in EC eNOS KO but Fully Preserved in RBC eNOS KO Mice

As expected, we found a loss of NO-dependent vascular endothelial function in EC eNOS KO mice, as demonstrated by the lack of acetylcholine (ACh)-induced vasodilation of aortic rings ex vivo (Figure 2A and 2E; Figure V in the Data Supplement), and an abrogated flow-mediated dilation response in the iliac artery as determined by ultrasound in vivo (Figure 2B; Figure VI in the Data Supplement). RBC eNOS KO mice, on the other hand, showed no changes in ACh-mediated vasodilation of aortic rings (Figure 2C and 2E) and a normal flow-mediated dilation (Figure 2D and 2F; Figure VI in the Data Supplement). These results are consistent with fully preserved eNOS expression and activity in the vasculature (Figure 1F and 1J; Figure III in the Data Supplement). Preservation of eNOS activity and function in conductance vessels of RBC eNOS KO mice also indicates that there were no off-target effects of genetic knockdown in vascular tissue. The responses to the constrictor phenylephrine and the NO donor sodium nitroprusside were fully preserved in the aorta of both mouse strains (Figure V in the Data Supplement). In EC eNOS KO, we observed a significant leftward shift in the sodium nitroprusside–mediated response, which indicates an increased sensitivity to NO donors. This response has been observed on inhibition of NOS activity and in different strains of global eNOS KO mice.[26–28]

Loss of RBC eNOS Leads to Hypertension and an Increase in Systemic Vascular Resistance

In line with the general knowledge on the role of vascular endothelial eNOS in the regulation of BP, we found that EC eNOS KO mice have significantly higher mean

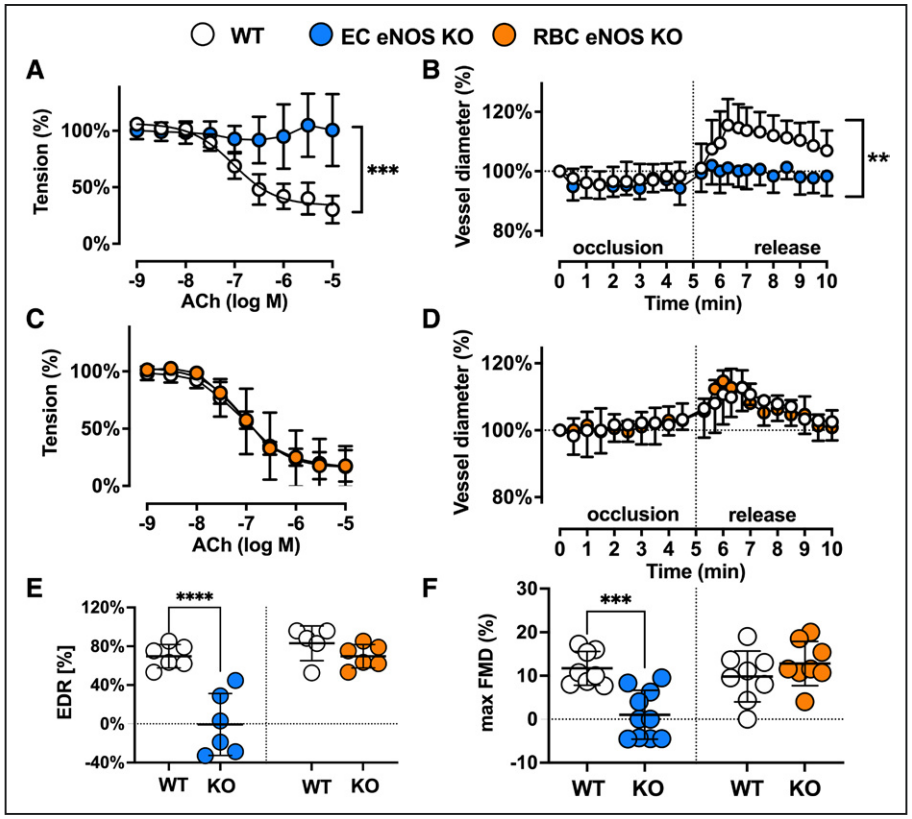


Figure 2. Vascular endothelial dilator function is lost in EC eNOS KO mice and preserved in RBC eNOS KO mice.
Nitric oxide–dependent vascular endothelial function is fully abolished in EC eNOS KO mice and fully preserved in RBC eNOS KO mice compared with the respective littermate controls. **A**, Preconstricted aortic rings from EC eNOS KO lack acetylcholine (ACh)-induced vasodilation (2-way repeated measurement [RM]-ANOVA $P<0.0001$; Sidak ***$P<0.001$ vs WT control for concentrations of ACh>$10^{-6.5}$ mol/L (n= 6 per group). **B**, Flow-mediated dilation (FMD) of the iliac artery assessed in vivo by ultrasound is abolished in EC eNOS KO (blue; 2-way RM-ANOVA $P<0.0001$; Sidak **$P<0.01$ vs WT control [white] for $t>6.3$ minutes; n=10 per group). **C**, In aortic rings from RBC eNOS KO (orange), ACh-induced vasodilation was not different from WT littermates (white). Two-way RM-ANOVA $P<0.0001$. n=5 per group. **D**, FMD of the iliac artery is fully preserved in RBC eNOS KO mice, 2-way RM-ANOVA $P<0.0001$. n=8 per group. **E**, Endothelium-dependent relaxation (EDR) in response to ACh (calculated as the percentage of the maximal ACh response) is significantly impaired in EC eNOS KO and fully preserved in RBC eNOS KO compared with their respective WT controls. One-way ANOVA $P<0.0001$; Tukey test ****$P<0.0001$. **F**, Maximal FMD (corresponding to the percentage of maximal flow-mediated dilator response) is significantly decreased in EC eNOS KO mice and fully preserved in RBC eNOS KO mice, compared with their respective WT controls. Lines represent means±SD. One-way ANOVA $P<0.0001$; Tukey test ***$P<0.001$. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KO, knockout; RBC, red blood cell; PCR, polymerase chain reaction; and WT, wild type.

arterial pressure (MAP; Figure 3A, blue) compared with their WT littermates, which is characterized by a difference of 15 to 18 mm Hg in both systolic blood pressure (SBP) and diastolic blood pressure (DBP), whereas heart rate (HR) was similar in both groups (Table 1). RBC eNOS KO mice also had significantly higher MAP than their WT littermates (Figure 3A, orange), characterized by a 14 mm Hg increase in SBP and 8 mm Hg in DBP and no differences in HR (Table 1). In global eNOS KO mice, we measured a 17 to 23 mm Hg increase in SBP and DBP compared with WT mice (Figure 3A, gKO, red). The effect size of eNOS deletion on BP (calculated as Δ% changes in BP versus the respective WT controls) was more prominent in the global eNOS KO (28%–31% increase) and the EC eNOS KO (20%–26% increase) compared with RBC eNOS KOs (13%–16%). Thus, the knockout of eNOS in RBCs modulates vascular tone at the level of smaller resistance arteries, whereas endothelial function in the large conduit arteries is fully preserved.

In a subcohort of mice, we characterized systemic hemodynamics by measuring in the same mouse both cardiac function by echocardiography and BP by Millar measurements. We did not observe changes in cardiac output or HR in EC eNOS KO or RBC eNOS KO mice, as assessed by echocardiography (Figure 3B, Inset). HR assessed invasively by a Millar catheter was also not different between groups (Table 2). However, systemic vascular resistance, which was estimated as the ratio between MAP and cardiac output, was higher in both strains than in their respective WT controls (Figure 3B). These results clearly demonstrate that both RBC eNOS and EC eNOS contribute independently to BP homeostasis.

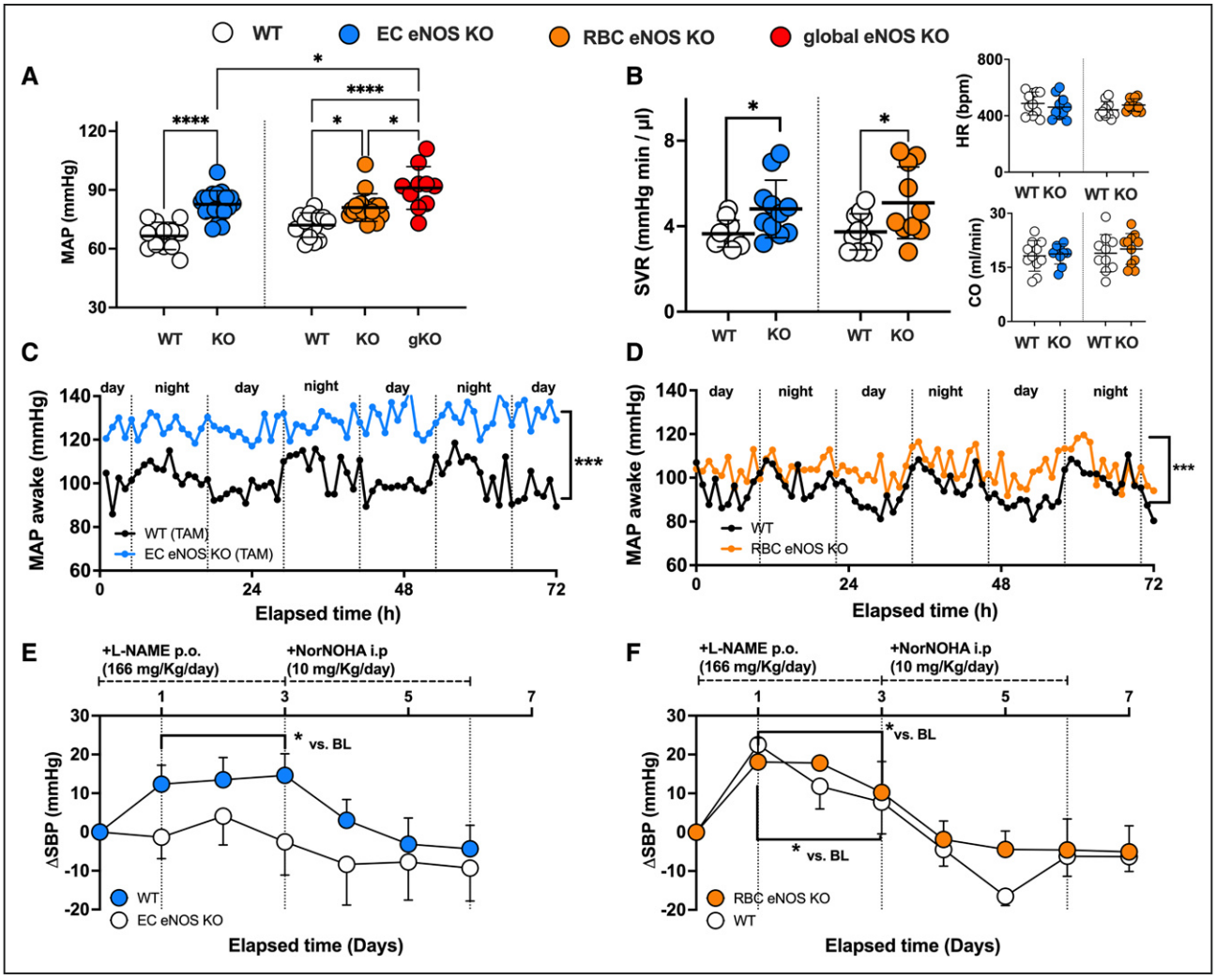


Figure 3. RBC eNOS and EC eNOS both contribute to blood pressure homeostasis.

A, Invasive measurements of blood pressure (BP) in anesthetized mice show that both EC eNOS KO and RBC eNOS KO mice have increased mean arterial pressure (MAP) compared with their respective WT littermate controls. Global eNOS KO mice (gKO=eNOS$^{flox/flox}$ Deleter Cre) are hypertensive and show significantly higher MAP than EC eNOS KO and RBC eNOS KO mice. One-way ANOVA $P<0.001$; Tukey $^{*}P<0.05$; $^{****}P<0.0001$. **B**, Systemic vascular resistance (SVR) was estimated in a subcohort of mice by measuring MAP and cardiac output (CO) by ultrasound in the same animal (SVR ≈ MAP/CO) and was significantly increased in both EC eNOS KO and RBC eNOS KO mice compared with their respective controls. One-way-ANOVA $P=0.0149$; Welch t test $P<0.05$. **Inset Top**, The heart rate (HR) depicted here represents the values measured by cardiac ultrasound. **Inset Bottom**, Cardiac output. See also Table 2 for other cardiac parameters. **C**, Mean of MAP measurements performed in awake EC eNOS KO (n=7) and WT littermates (n=5) by radiotelemetry showing the diurnal and nocturnal variation of blood pressure with highest values during the night. KO (SD = ±10 mm Hg) vs WT (SD = ±8 mm Hg); Welch t test $^{*}P<0.001$. Refer also to Figure VII in the Data Supplement for mean diurnal values. **D**, Mean of MAP measurements performed in awake RBC eNOS KO (n=8) and WT littermates (n=8) by radiotelemetry. KO (SD = ±15 mm Hg) vs WT (SD = ±13 mm Hg); Welch t test correction $^{*}P<0.001$. Refer also to Figure VII in the Data Supplement for mean diurnal values. **E**, Telemetric measurements of changes in systolic BP (SBP) in awake EC eNOS KO mice (n=7) show that NOS inhibition by L-NAME or increase in arginine availability by the administration of the arginase inhibitor NorNOHA did not affect SBP in awake EC eNOS KO mice (blue) compared with WT mice (n=5). Two-way RM ANOVA $P<0.001$; Holm-Sidak $^{*}P<0.05$ vs baseline. See Figure IV in the Data Supplement for data on diastolic BP and HR. **F**, Radiotelemetric measurements of changes in SBP in a subcohort of awake RBC eNOS KO mice (n=5) show that treatment with L-NAME further increases SBP in RBC eNOS KO and WT littermate (n=3) to the same extent as in the WT controls (although the baseline levels between RBC eNOS KO and WT controls were significantly different, see **C** and **D**, this figure); increase of arginine bioavailability by the administration of NorNOHA rapidly restored BP to the baseline levels. Two-way RM ANOVA $P<0.001$; Holm-Sidak $^{*}P<0.05$ vs baseline. BL indicates baseline; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; i.p., intraperitoneally; KO, knockout; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NorNOHA, N-hydroxy-nor-L-arginine; p.o., orally; RBC, red blood cell; RM, repeated measures; TAM, tamoxifen; and WT, wild type.

Hypertension in Awake RBC eNOS KO Mice Can Be Further Increased by NOS Inhibition

Next, we aimed to confirm the BP/HR phenotype in awake mice and analyze the effects of pharmacological regulation of eNOS activity/arginine availability. Therefore, we monitored BP and HR with radiotelemetry in awake mice under untreated conditions for 3 days (Figure 3C and 3D; Figure VII in the Data Supplement). Mice are nocturnal animals, and, as expected, baseline radio-

Table 2. Systemic Hemodynamics as Assessed in a Subcohort of Mice by Measurements of Echocardiography and Millar in the Same Individual

Systemic hemodynamics	WT eNOS$^{flox/flox}$ Cdh5-Cre/ERT2^{neg}	EC eNOS KO eNOS$^{flox/flox}$ Cdh5-Cre/ERT2^{pos}	*P* value	WT eNOS$^{flox/flox}$ HbbCre ^{neg}	RBC eNOS KO eNOS$^{flox/flox}$ HbbCre ^{pos}	*P* value
n	10	11		11	9	
Echocardiography						
Heart rate, bpm	461±8	487±82	0.4743	476±44	448±59	0.2514
Cardiac output, mL/min	19±3	18±4	0.7426	20±4	19±5	0.4979
Stroke volume, µL	41±8	37±5	0.1274	42±7	41±11	0.8748
End-diastolic volume, µL	64±2	66±14	0.8169	79±17	83±33	0.7287
End-systolic volume, µL	23±1	29±15	0.3386	37±16	42±25	0.6154
Ejection fraction, %	67±1	58±15	0.1740	55±14	52±11	0.5152
Millar						
Heart rate, bpm	555±55	537±56	0.4586	511±53	487±101	0.5381
Systolic blood pressure, mm Hg	86±4	103±10	<0.0001§	91±6	105±13	0.0107*
Diastolic blood pressure, mm Hg	57±7	71±9	0.0007‡	63±8	71±8	0.0464*
Mean arterial pressure, mm Hg	67±6	82±9	0.0002‡	73±7	82±9	0.0176*
Systemic vascular resistance, mm Hg/mL per min	3.7±0.6	4.8±1.3	0.0220*	3.7±0.8	4.8±1.5	0.0797
Rate pressure product, mm Hg×bpm	47 418±4152	55 248±7061	0.0063†	46 302±4099	50 951±10 560	0.2412

t test vs respective control group. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KO, knockout; RBC, red blood cell; and WT, wild type.
*P<0.05.
†P<0.01.
‡P<0.001.
§P<0.0001.

telemetry traces show higher BP levels during the night; it is important to note that diurnal BP levels in awake mice (Figure VII in the Data Supplement) are higher than in anesthetized mice (which were measured during the day), but in awake mice differences between groups remained overall comparable to the one measured in anesthesia (Figure 3C, EC=ΔMAP=20 mm Hg; Figure 3D Δ%=20%; RBC ΔMAP=10 mm Hg; Δ%=10%), confirming hypertension in both EC eNOS KO and RBC eNOS KO mice.

The effects of regulation of eNOS activity on BP in these mice were determined by the administration of a NOS inhibitor (L-NAME) in the drinking water for the next 3 days, which was followed by daily intraperitoneal administration of the arginase inhibitor NorNOHA for the last 3 days of the protocol; NorNOHA was given to the mice to increase the availability of L-arginine to restore eNOS activity after NOS inhibition (Figure 3E and 3F; Figure VIII in the Data Supplement). In EC eNOS KOs, the administration of L-NAME was lethal in 5 of 11 mice tested, but in the mice that survived, it did not significantly affect BP (but decreased HR, see Figure VIII in the Data Supplement) compared with baseline measurements (Figure 3E). In RBC eNOS KO mice, the administration of L-NAME increased SBP by 20 mm Hg (Figure 3F) and decreased HR, whereas DBP was not significantly affected (Figure VII in the Data Supplement). In the WT controls (Figure 3E, white, tamoxifen-treated control; Figure 3D, white, untreated control) L-NAME administration increased SBP by 12 to 20 mm Hg and decreased HR without affecting DBP (Figure VIII in the Data Supplement). Administration of NorNOHA decreased BP by 5 to 10 mm Hg in all mice, thereby restoring BP levels to baseline levels, before L-NAME administration. These data further corroborate that both EC eNOS and RBC eNOS contribute independently to BP regulation in a manner dependent on the availability of the substrate L-arginine.

Reactivation of eNOS Specifically in RBCs Rescues Global eNOS KO Mice From Hypertension

To induce cell type–specific reactivation of eNOS in RBCs or ECs in a global eNOS KO mouse, we created a Cre-inducible eNOS knock-in (KI) gene construct (eNOS$^{inv/inv}$) by inserting an inverted exon 2 and 2 additional Lox511 sites in the loxP-eNOS construct (Figure 4A and Figure VIII in the Data Supplement) This construct allows a Cre-induced reactivation of eNOS in any cell type of interest; therefore, these mice were defined as conditional global eNOS KO mice (CondKO).

As shown in Figure 4B, by breeding eNOS$^{inv/inv}$ with Cdh5-Cre/ERT2^{pos} mice and after tamoxifen treatment, we obtained EC eNOS KI (eNOS$^{inv/inv}$ Cdh5-Cre/ERT-2^{pos}) with tissue-targeted reactivation of eNOS in ECs

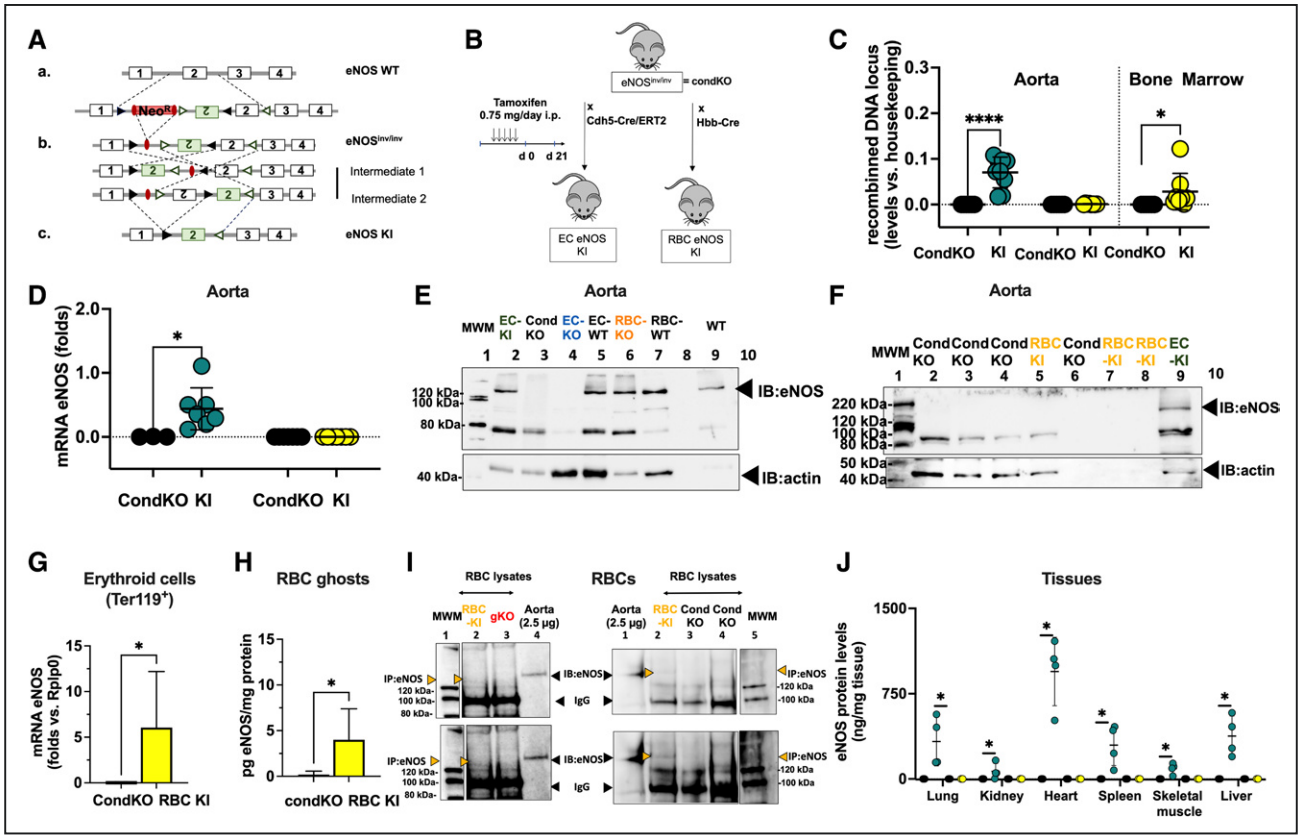


Figure 4. Reactivation of eNOS expression in either ECs or RBCs rescues global eNOS KO mice from hypertension.
A, Scheme describing the gene-targeting strategy showing the position of a loxP (black) and loxP511 (green) sequences within the gene-targeting construct used to generate eNOS$^{inv/inv}$ mice, which are conditional eNOS KO mice (CondKO). **B**, Schematic representation of the crossing strategy. To create EC eNOS KI mice (green), eNOS$^{inv/inv}$ mice=CondKO mice (black) were crossed with endothelial-specific tamoxifen-inducible Cre mouse (Cdh5-Cre/ERT2^{pos}) and treated with tamoxifen for 5 days and analyzed after 21 days. To create RBC eNOS KI mice (yellow), eNOS$^{inv/inv}$ mice=CondKO mice (black) were crossed with erythroid-specific (HbbCre^{pos}) mice. **C**, Real-time polymerase chain reaction analysis shows that tissue-specific DNA recombination occurs in the aorta of EC eNOS KI mice (blue), whereas in RBC eNOS KI mice (yellow), DNA recombination occurs in the bone marrow, but not in the aorta. No DNA recombination is observed in CondKO littermate control mice (white). *t* test **P*<0.05, *****P*<0.0001 vs respective CondKO control. **D**, Real-time reverse transcriptase polymerase chain reaction analysis eNOS expression in the aorta of EC eNOS KI mice (green) but not in CondKO littermate control mice (black). One-way ANOVA *P*<0.0001; Tukey multiple comparisons test **P*<0.05 vs respective CondKO control. **E**, Representative immunoblot analysis demonstrating eNOS expression after Cre-dependent reactivation of eNOS expression in aorta EC eNOS KI mice, compared with CondKO, EC eNOS KO, RBC KO, and WT controls. Refer also to **J** (eNOS expression by ELISA) and Figure IX in the Data Supplement for immunoblot analysis from other tissues. **F**, Representative Western blot analysis demonstrating lack of eNOS expression in the aorta of RBC eNOS KI mice and CondKO mice. See Figure IX in the Data Supplement for immunoblot analysis from other tissues. **G**, Real-time reverse transcriptase polymerase chain reaction analysis shows that erythroid cells (Ter119^{+}CD71^{+}CD45^{-}) extracted from the bone marrow of RBC eNOS KI mice (yellow, n=3) express eNOS mRNA compared with CondKO controls, where we could not detect any eNOS mRNA (n=3) **P*=0.05 (exact) Mann-Whitney *U* test. **H**, ELISA detection of eNOS expression in RBC membrane preparations (ghosts) from RBC eNOS KI mice (n=4) compared with CondKO controls (n=4), where we could not detect any eNOS protein. **P*=0.0286 (exact) Mann-Whitney *U* test. **I**, Low (**Upper**) and high (**Lower**) contrast images representing immunoprecipitation (IP) and immunoblot (IB) analysis of eNOS (135 kDa) from RBC lysates show eNOS expression in RBC eNOS KI mice and lack of eNOS in global eNOS KO mice (gKO) and CondKO mice. The IgG (120 kDa) is seen in the IP samples. **J**, The quantification of eNOS protein expression in multiple organs by ELISA shows a significant eNOS expression in multiple tissues of EC eNOS KI mice, whereas no eNOS was detected in RBC eNOS KI or littermate CondKO mice. Data were analyzed according to the mixed-effect model with Geisser-Greenhouse correction; *P*<0.0001; multiple comparisons of eNOS levels among the groups within the same tissue were assessed by a Tukey test; *P*<0.0001; Tukey **P*<0.001. Lines represent means± SD. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; KO, knockout; MWM, molecular weight marker; RBC, red blood cell; and WT, wild type.

only or their littermate global eNOS KO controls (eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{neg}). Tamoxifen-induced DNA recombination and the resulting genetic inversion of exon 2 of Nos3 in eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{pos} mice (EC eNOS KI), and the lack thereof, in eNOS$^{inv/inv}$ Cdh5-Cre/ERT-2^{neg} mice (CondKO control), were all confirmed by real-time PCR analysis of DNA extracted from vascular tissue (aorta) of these mice (Figure 4C, green). This resulted in the reactivation of eNOS expression in the aorta and other tissues, as assessed by real-time reverse transcriptase PCR (Figure 4D), immunoblot (Figure 4E; Figure IX in the Data Supplement), and ELISA of multiple tissues (Figure 4J). In CondKO littermate mice, eNOS expression was nondetectable in any of the tissues analyzed.

In addition, by crossing eNOS$^{inv/inv}$ with Hbb-Cre^{pos} mice, we created RBC eNOS KI (eNOS$^{inv/inv}$ Hbb-Cre^{pos}) mice (Figure 4B). These mice are characterized by a targeted eNOS reactivation in bone marrow erythroid cells, as determined by analyzing DNA recombination in the bone marrow (Figure 4C), and eNOS expression in isolated erythroid cells (Ter119$^+$ CD71$^+$ CD45$^-$; Figure 4G), RBC ghosts (Figure 4H), and RBC lysates (Figure 4I), as well, whereas the littermate CondKO mice did not show DNA recombination (Figure 3C, black) or express any eNOS in bone marrow erythroid cells or RBCs (Figure 4G, 4H, and 4I). In all other tissues and cells analyzed, we did not detect any eNOS expression (Figure 4F and 4J; Figure IX in the Data Supplement).

Of note, in EC eNOS KI mice, reactivation of eNOS specifically in ECs fully restored vascular endothelial dilator function as determined ex vivo (Figure 5A; Figure X in the Data Supplement) and in vivo (Figure 5B; Figure VI in the Data Supplement), and decreased BP by 23 to 28 mm Hg (corresponding to a 25% decrease in MAP; Table 1) and increased systemic vascular resistance (Figure 5D, Table 3) with no significant differences in HR. An important finding was that reactivation of eNOS specifically in erythroid cells fully rescued the global eNOS KO (eNOS$^{inv/inv}$) from hypertension with a significant decrease in BP of 19 to 24 mm Hg (23% decrease in MAP) in the RBC eNOS KI, as compared with their CondKO littermate controls (Table 1 and Table 3). These data provide additional evidence for our finding that RBC eNOS and EC eNOS contribute independently to BP homeostasis.

Circulating Nitrite Is Decreased in Both EC eNOS KO and RBC eNOS KO and the Levels of NO-Heme Are Changed in RBC eNOS KO/KI Mice

Next, we aimed to analyze the impact of cell-specific eNOS deletion on the concentrations of NO metabolites in blood and tissues of all KO and KI lines. In EC eNOS KO

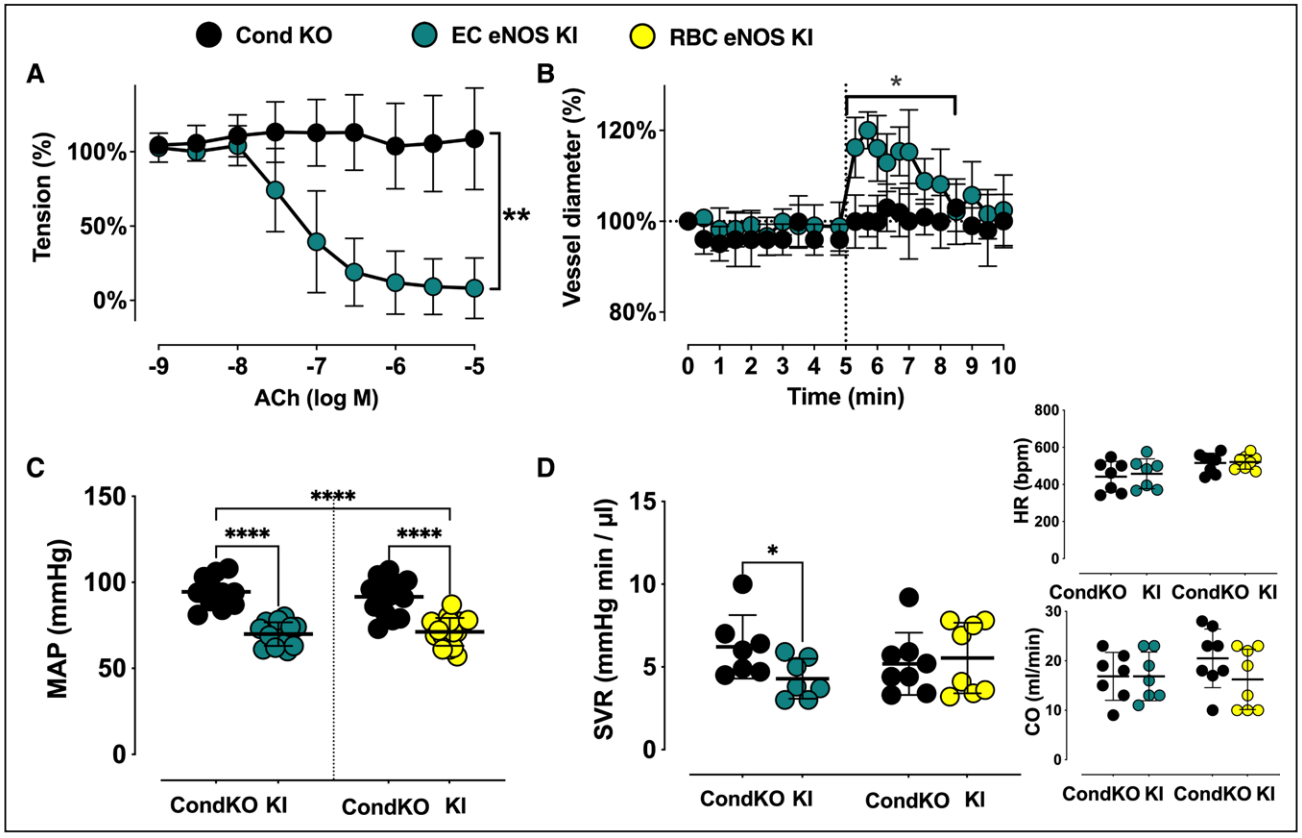


Figure 5. Reactivation of eNOS expression in either ECs or RBCs rescues global eNOS KO mice from hypertension.
A and **B**, Vascular endothelial dilator function is fully restored in EC eNOS KI. **A**, Preconstricted aortic rings from CondKO mice (black, n=2) lack acetylcholine (ACh)-induced vasodilation, whereas reactivation of eNOS fully restores ACh response in EC eNOS KI (green, n = 3). Two-way repeated-measures ANOVA concentration $P<0.0001$; KI vs KO $P=0.0461$; Sidak $^{**}P<0.01$ vs CondKO control for concentrations of ACh>10^{-7} mol/L. **B**, Flow-mediated dilation of the iliac artery assessed in vivo by ultrasound is abolished in CondKO mice (black; 2-way repeated-measures ANOVA time $P=0.0007$ KI vs KO $P=0.329$; Fisher least significant difference test $^{**}P<0.01$ vs CondKO control (black) for $t>6.3$ minutes); and restored in EC eNOS KI mice (green). **C**, Invasive blood pressure analysis shows that reactivation of eNOS expression in ECs in EC eNOS KI mice significantly decreases MAP compared with their littermate conditional eNOS KO mice (CondKO). One-way ANOVA $P<0.001$; Tukey $^{****}P<0.0001$. **D**, Systemic vascular resistance (SVR) was estimated in a subcohort of mice by measuring MAP and cardiac output (CO) by ultrasound in the same animal (SVR ≈ MAP/CO). The heart rate depicted here represents the values measured by cardiac ultrasound. SVR is decreased in EC eNOS KI compared with CondKO mice; *t* test Welch $^{*}P<0.05$. See also Table 3 for other cardiac parameters. CondKO, conditional global eNOS knockout mice; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; KO, knockout; MAP, mean arterial pressure; RBC, red blood cell; and WT, wild type.

Table 3. Hemodynamics of EC eNOS KI and RBC eNOS KI mice and Their respective Controls

Systemic hemodynamics	CondKO $eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{neg}$ + TAM	EC eNOS KI $eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{pos}$ + TAM	*P* value	CondKO $eNOS^{inv/inv}$ $HbbCre^{neg}$	RBC eNOS KI $eNOS^{inv/inv}$ $HbbCre^{pos}$	*P* value
n	7	7		8	8	
Echocardiography						
Heart rate, bpm	442±83	458±81	0.7227	516±52	521±38	0.8430
Cardiac output, mL/min	17±5	17±5	0.9999	21±6	16±6	0.1791
Stroke volume, µL	38±8	37±7	0.8177	40±1	31±1	0.1368
End-diastolic volume, µL	59±1	64±1	0.5246	62±2	51±2	0.2668
End-systolic volume, µL	21±1	27±1	0.4061	22±1	20±1	0.7158
Ejection fraction, %	67±2	59±1	0.3514	67±2	63±1	0.6033
Millar						
Heart rate, bpm	531±56	514±55	0.5795	508±40	507±80	0.9663
Systolic blood pressure, mm Hg	125±4	95±8	<0.0001§	120±7	98±5	<0.0001§
Diastolic blood pressure, mm Hg	84±1	57±8	0.0009‡	85±5	67±6	<0.0001§
Mean arterial pressure, mm Hg	98±9	70±8	<0.0001§	97±5	77±5	<0.0001§
Systemic vascular resistance, mm Hg/mL per min	6.2±2	4.3±1.2	0.0475*	5.2±1.9	5.5±2.1	0.7331
Rate pressure product, mm Hg×bpm	66 292±6396	48 793±6236	0.0002‡	60 589±4805	49 439±6941	0.0027†

t test. CondKO indicates conditional global eNOS knockout mice; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; RBC, red blood cell; and WT, wild type.

*$P<0.05$.

†$P<0.01$

‡$P<0.001$.

§$P<0.0001$.

mice, we found a decrease in circulating nitrite and nitrate levels in plasma (Figure 6A and 6B; Table 4), without significant changes of these 2 metabolites in RBCs or aorta (Table 4). We also found a decrease in total NO species (ie, the sum of all NO species assessed in a specific compartment) in plasma, aorta, and lung of EC eNOS KO mice, compared with their WT littermates (Table 4); however, decreases in the aorta and plasma were not statistically significant. In the liver, on the other hand, we found an increase in nitrate, contributing to an overall increase in total NO species (Table 4). This is consistent with the observation that the liver may act as a reservoir of nitrate.[29] Similar changes as observed in EC eNOS KO were observed in global eNOS KO mice by us and others before.[5,6,24,30]

RBC eNOS KOs showed alterations in nitrite and nitrate concentrations in plasma and aorta. Specifically, we found a significant decrease in nitrite and nitrate in plasma (Figure 6A, Table 4), together with an increase in nitroso species (Figure 6C) compared with WT control mice. In the RBC eNOS KO mice, we observed a significant decrease in total NO species in plasma (Table 4), but no other significant changes in any other tissues, except for an increase in nitrite in the aorta compared with WT controls (Table 4).

CondKO mice showed an overall decrease in NO metabolites in plasma and tissues compared with WT mice (compare Table 4, WT with Table 5, Cond KO), with an almost 50% decrease in circulating nitrite levels (Figure 6B, black). However, nitrite levels in the plasma of EC eNOS KI or RBC eNOS KI were not different from their littermate global eNOS KO mice (Figure 6B, green and yellow triangles). These data show that plasma nitrite levels depend on the presence of eNOS in both the ECs and the RBCs and decrease in the global absence of eNOS, but the reintroduction of eNOS in ECs or RBCs does not restore nitrite levels (as it does with BP; see also Figure XII in the Data Supplement).

The levels of bound NO (measured as NO-heme concentrations) were fully preserved in RBCs from EC eNOS KO mice (Figure 6C, blue) but significantly lower in RBCs from RBC eNOS KOs (Figure 6C, Right, orange) and CondKO mice (Figure 6C, black). Accordingly, levels of NO-heme were not altered in RBCs from EC eNOS KI mice (Figure 4C, green), but elevated in RBCs from RBC eNOS KI mice (Figure 4C, yellow), compared with the respective CondKO littermates. These data show that the levels of bound NO in RBCs depend on the presence/absence of eNOS in RBCs.

DISCUSSION

In this work, we were able to determine the independent contribution of vascular ECs and RBCs to NO metabolism, vascular function, and BP homeostasis by creating and comparing tissue-specific eNOS KO and eNOS KI mice in ECs or RBCs. As expected, we find that eNOS in

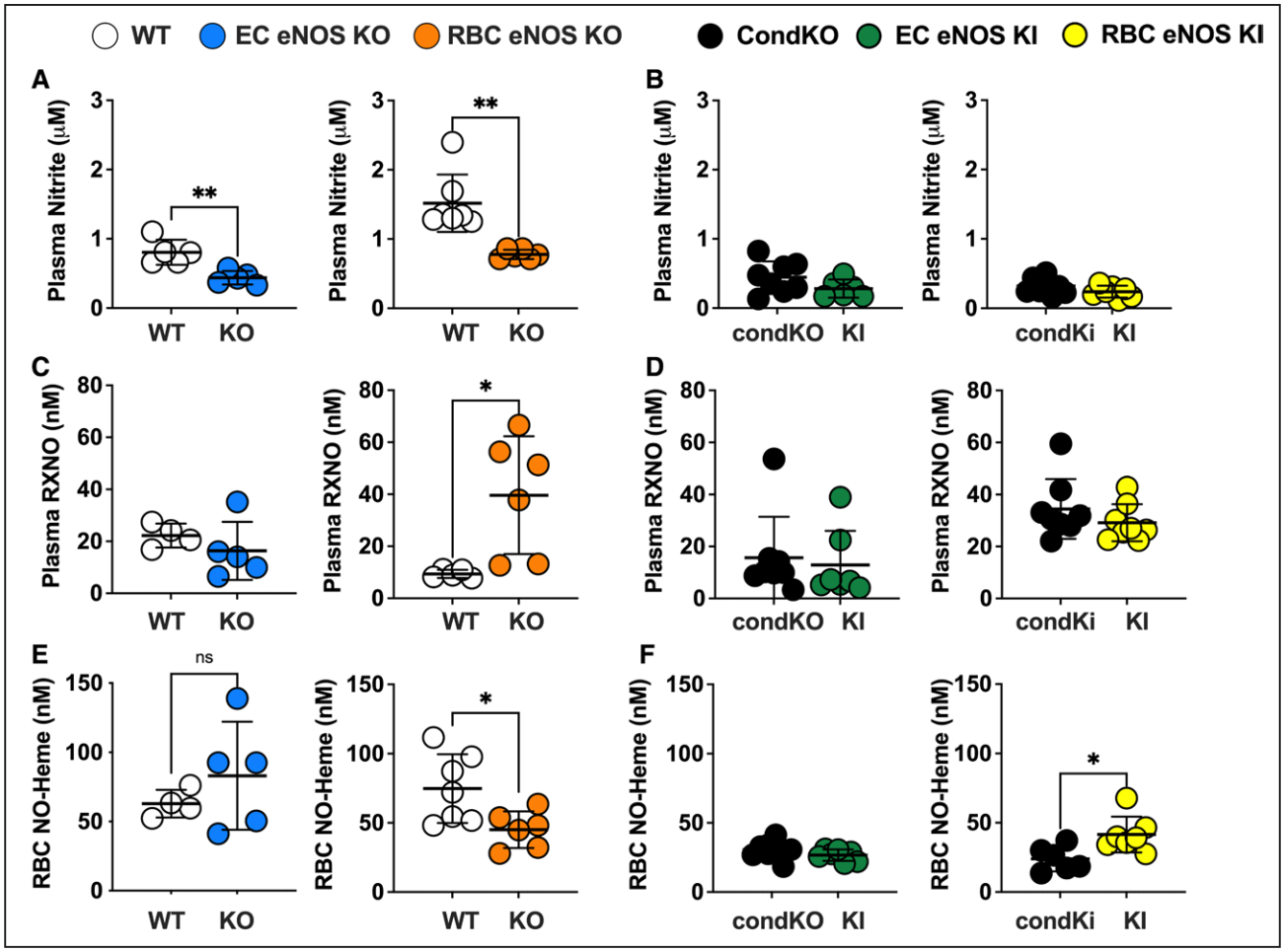


Figure 6. RBC eNOS and EC eNOS both contribute to plasma nitrite, but red cell eNOS is the major determinant of circulating NO-heme.

A, A significant decrease in plasma nitrite is observed in both EC eNOS KO (blue) and RBC eNOS KO (orange), compared with their respective WT controls (white). Welch *t* test **P*<0.05; ***P*<0.01. **B**, Plasma nitrite in EC eNOS KI (green) or RBC eNOS KI (yellow) is not different from CondKO mice (black), but CondKO mice show lower plasma nitrite levels than WT mice (white; see also Table 4). **C**, The levels of nitrosospecies (RXNO=RSNO+RNNO) were unchanged in EC eNOS KO (blue) and significantly higher in the plasma of RBC eNOS KO (orange) mice, compared with their respective WT control (white). Welch *t* test **P*<0.05. **D**, The levels of RXNO in plasma of EC eNOS KI, RBC eNOS KI, and CondKO mice are comparable. **E**, NO-heme concentrations in RBCs were unchanged in EC eNOS KO mice and decreased in RBC eNOS KO mice. Welch *t* test **P*<0.05. **F**, Accordingly, concentrations of NO-heme were unchanged in EC eNOS KI mice (green) but higher in RBC eNOS KI mice (yellow) compared with CondKO mice (black), indicating that RBC eNOS is the major determinant of circulating NO-heme. Welch *t* test **P*<0.05. CondKO, conditional global eNOS knockout mice; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; KO, knockout; NO, nitric oxide; RBC, red blood cell; RXNO, nitrosospecies (nitrosothiols+nitrosamines); and WT, wild type.

the vascular wall is the main determinant of endothelial function in conduit and resistance arteries. It is surprising that eNOS in RBCs effectively contributes to BP homeostasis, tissue perfusion, and systemic circulatory NO bioavailability independently of vascular eNOS.

Previous work relied solely on the use of global eNOS KOs or pharmacological inhibition with drugs targeting all 3 NOS isoforms. By creating mice lacking eNOS only in ECs, we can now ultimately confirm the suggested central role of these cells in eNOS-mediated control of cardiovascular function as originally proposed when endothelium-derived relaxing factor was discovered.[31] The fact that RBC eNOS KO mice were also hypertensive was definitely more surprising, although a role for blood cell eNOS had been suggested in 1 study using chimeric mice obtained by transplanting bone marrow from eNOS KO mice into irradiated WT mice, and vice versa.[9] The functional significance of eNOS in RBCs has been a matter of intense debate for some time.[10,11,14,32,33]

We previously demonstrated that eNOS is present and active in RBCs.[8,34] The data presented here show that genetic deletion of eNOS specifically in erythroid cells leads to significant increases in SBP and DBP. These changes were not accompanied by changes in HR and cardiac output, but by increases in systemic vascular resistance. In RBC eNOS KO mice, eNOS activity and function at the level of conduit and proximal resistance arteries were fully preserved. In line with these observa-

Table 4. NO Metabolites in Blood and Organs of EC eNOS KO, RBC eNOS KO, and Corresponding WT Littermate Controls

Metabolites	WT $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$	EC eNOS KO $eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$	*P* value	WT $eNOS^{flox/flox}$ $HbbCre^{neg}$	RBC eNOS KO $eNOS^{flox/flox}$ $HbbCre^{pos}$	*P* value
n	5	5		7	6	
Heart						
Nitrite, µmol/L	1.10±0.30	1.27±0.25	0.3557	0.69±0.15	0.92±0.60§	0.4463
Nitrate, µmol/L	93.86±26.51	77.84±14.97	0.2818	65.16±19.11	86.82±54.70	0.3914
RXNO, nmol/L	81.94±26.31	102.74±37.20	0.3406	54.00±19.06	43.81±10.47	0.2528
NO-heme, nmol/L	475.21±320.85	97.74±24.20	0.0579*	125.04±37.83	38.82±20.11	0.0005‡
Total NO species, µmol/L	95.09±26.43	79.31±14.91	0.2871	66.02±19.14	87.66±54.33	0.3890
Lung						
Nitrite, µmol/L	0.88±0.51§	0.71±0.11	0.5100	0.71±0.24	0.50±0.13§	0.0801
Nitrate, µmol/L	35.62±6.74§	19.49±5.52	0.0035†	44.01±13.20	40.10±22.66	0.7202
RXNO, nmol/L	94.01±19.94§	79.43±20.31	0.3173	62.99±29.43	122.80±106.3	0.2331
NO-heme, nmol/L	228.44±92.18§	463.51±121.30	0.0131*	282.16±166.87	104.77±61.72	0.0316*
Total NO species, µmol/L	34.25±5.57§	20.74±5.62	0.0096†	45.06±13.40	33.78±16.50§	0.2450
Liver						
Nitrite, µmol/L	0.63±0.34	0.83±0.15	0.2798	0.20±0.06	0.16±0.04§	0.2483
Nitrate, µmol/L	38.47±22.34	71.02±20.84	0.0445*	57.69±17.70	33.88±7.05§	0.0116*
RXNO, nmol/L	1378.28±749.29	1038.64±283.33	0.3857	366.42±71.71	1836.38±1541.77	0.0666
NO-heme, nmol/L	1190.08±722.98	310.85±104.09	0.0523	1149.31±323.81	494.14±573.76	0.0396*
Total NO species, µmol/L	40.65±22.39	73.20±21.08	0.0456*	59.41±17.54	36.68±8.85§	0.0159*
Aorta						
Nitrite, µmol/L	138.12±62.10	141.36±39.21	0.9243	57.57±16.44§	118.71±42.34	0.0147*
Nitrate, µmol/L	10191.95±7764.94	5473.15±4015.51	0.2730	2931.00±1184.00§	3408.00±1888.00	0.6357
RXNO, nmol/L	2058.42±1653.39	1870.34±1002.49	0.8344	2103.00±980.10§	3067.00±1737.00	0.2712
Total NO species, µmol/L	10331.13±7805.42	5616.38±4052.67	0.2758	3020.34±1181.83§	2455.27±1651.94	0.5126
Plasma						
Nitrite, µmol/L	0.81±0.18	0.44±0.10	0.0065†	1.52±0.42	0.78±0.07	0.0031†
Nitrate, µmol/L	41.35±15.75	20.65±4.25	0.0402*	57.63±9.22	25.40±10.93	0.0002‡
RXNO, nmol/L	22.24±4.58	16.34±11.15	0.3268	9.39±1.57‖	39.65±22.64	0.0220*
Total NO species, µmol/L	36.66±11.28	21.11±4.30	0.0642	59.96±9.07‖	26.22±10.91	0.0003‡
RBCs						
Nitrite, µmol/L	0.49±0.16	0.43±0.16	0.6088	0.94±0.14§	1.09±0.35§	0.4063
Nitrate, µmol/L	35.23±14.27	17.64±3.14	0.0880	67.95±15.61	68.43±29.82	0.9727
RXNO, nmol/L	475.06±158.27	225.04±76.99	0.0201*	196.32±123.29	125.32±28.68	0.1846
NO-heme, nmol/L	62.92±9.99§	83.12±39.12	0.3209	74.76±24.84	45.06±13.31	0.0220*
Total NO species, µmol/L	32.02±21.79§	18.38±3.33	0.2999	56.34±31.65§	75.91±28.53§	0.3097

t test between the groups. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KO, knockout; NO, nitric oxide; RBC, red blood cell; RXNO, nitrosospecies (nitrosothiols+nitrosamines); and WT, wild type.

*$P<0.05$.

†$P<0.01$.

‡$P<0.001$.

§One value excluded as outlier according to the Tukey test or not determined/available.

‖Two values were not determined/available, or one was excluded as an outlier according to the Tukey test.

tions, the administration of a NOS inhibitor increased BP further in RBC eNOS KO mice. Moreover, the administration of an arginase inhibitor to restore arginine availability counteracted the effect of NOS inhibition and quickly restored baseline conditions, showing that, in RBC eNOS KO mice, both regulatory components are active and can be modulated by pharmacological intervention. Last, when eNOS was reintroduced into either ECs or RBCs of conditional global eNOS KO mice, it successfully rescued the hypertensive phenotype in both cases.

Table 5. NO Metabolites in Blood and Organs of EC eNOS KI, RBC eNOS KI, and Corresponding Conditional Global eNOS Knockout Mouse Littermate Controls

Metabolite	CondKI	EC eNOS KI	*P* value	WT	RBC eNOS KI	*P* value
	eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{neg}	eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{pos}		eNOS$^{inv/inv}$ HbbCre^{neg}	eNOS$^{inv/inv}$ HbbCre^{pos}	
n	8	7		8	8	
Heart						
Nitrite, μmol/L	2.27±0.14‡	2.24±0.48	0.8745	0.64±0.23	0.67±0.18§	0.7817
Nitrate, μmol/L	ND	ND		13.86±6.27	13.98±6.08§	0.9715
RXNO, nmol/L	34.86±16.40	32.50±3.38	0.7043	15.54±4.38	20.65±6.23	0.0810
NO-heme, nmol/L	26.57±12.00	32.67±8.46	0.2865	6.94±1.49	8.40±2.09	0.1303
Total NO species, μmol/L	ND	ND		14.52±6.31	14.68±6.09§	0.9628
Lung						
Nitrite, μmol/L	2.42±0.52‡	3.05±0.62	0.0626	0.70±0.26	0.70±0.65§	0.9796
Nitrate, μmol/L	ND	ND		28.75±14.78	21.31±9.03§	0.2674
RXNO, nmol/L	71.73±35.40	54.26±12.34	0.2237	3783.51±2023.74§	3666.83±2476.96	0.9244
NO-heme, nmol/L	27.62±14.30	29.86±7.76	0.7090	425.90±238.14‡§	443.89±88.30§	0.8792
Total NO species, μmol/L	ND	ND		39.21±16.11‡§	25.28±9.53§	0.1378
Liver						
Nitrite, μmol/L	3.32±1.44	2.72±0.36‡	0.2921	0.65±0.52	0.31±0.08	0.1050
Nitrate, μmol/L	ND	ND		17.56±9.23	14.68±5.38	0.4606
RXNO, nmol/L	617.11±336.52	496.28±193.14	0.4046	672.09±412.26	377.99±47.68§	0.0842
NO-heme, nmol/L	118.74±46.90‡	79.44±3.42§	0.0686	342.06±207.87	353.56±136.61	0.8981
Total NO species, μmol/L	ND	ND		19.23±9.47	14.52±4.20§	0.2389
Aorta						
Nitrite, μmol/L	9.56±4.74	6.20±2.06	0.0995	2.90±1.05	2.28±1.21§	0.3407
Nitrate, μmol/L	ND	ND		126.67±104.88	100.97±97.25§	0.6451
RXNO, nmol/L	79.10±46.81	55.80±47.50	0.3579	141.72±106.74	191.45±106.22‡	0.3834
NO-heme, nmol/L	11.49±3.97§	21.28±5.55	0.0037†	129.71±105.59	67.44±48.22§	0.1780
Total NO species, μmol/L	ND	ND				
Plasma						
Nitrite, μmol/L	0.45±0.23	0.28±0.13‡	0.1218	0.32±0.12	0.24±0.09‡	0.1542
Nitrate, μmol/L	ND	ND		55.72±48.50	43.47±21.12	0.5279
RXNO, nmol/L	15.67±15.77	12.89±13.12	0.7160	34.46±11.52	29.16±7.14	0.2909
Total NO species, μmol/L	ND	ND		56.06±48.53	44.71±22.62‡	0.5668
RBCs						
Nitrite, μmol/L	0.15±0.04	0.21±0.10	0.2011	3.29±1.24‡	2.94±1.51‡	0.6435
RXNO, nmol/L	88.78±47.89	103.57±72.60	0.6562	1.26±0.52	0.72±0.25	0.0253*
NO-heme, nmol/L	3.06±0.66	2.67±0.41	0.1904	23.89±8.89§	41.54±12.94‡	0.0150*
Total NO species, μmol/L	ND	ND		4.66±1.43‡	3.78±1.72§	0.3425

t test between the groups. EC indicates endothelial cell; eNOS, endothelial nitric oxide synthase; KI, knock-in; ND, nondetermined; NO, nitric oxide; RBC, red blood cell; RXNO, nitrosospecies (nitrosothiols+nitrosamines); and WT, wild type.

*$P<0.05$.

†$P<0.01$.

‡One value excluded as outlier according to the Tukey test, or not determined/available.

§Two values were not determined/available, or one was excluded as an outlier according to Tukey test.

These data strongly affirm the role of eNOS in both cell types as being essential to BP regulation.

BP homeostasis is primarily regulated at the level of distal conduit, proximal and distal resistance, and precapillary sphincter arteries. Our findings underscore unequivocally that an L-arginase-eNOS signaling cascade is effectively expressed in ECs and RBCs and, by comparing the phenotype of the 4 mice lines presented here, it becomes clear that, depending on its localization, this pathway exerts distinct and complementary physi-

ological effects on NO metabolism and BP control. This may be because of the specific (sub)compartmentalization of eNOS within the vessel wall and its cellular components,[35–38] the unique anatomy and function of the vascular tree[39] and its relationship with the circulating blood cells,[14] and the different half-life and distribution kinetics of the NO metabolites within the blood and the tissues.[9,16,24,40–42] The functional differences of the pathway in RBCs or the vasculature may become prominent under specific pathophysiological conditions such as endothelial dysfunction or blood disorders.

It is well known that eNOS protein expression and activity in RBCs are both significantly lower than in the endothelium.[8] However, according to a recent estimate of the total number of cells in the human body, RBCs were the largest contributor to the overall number.[43] Thus, total RBC numbers may compensate for lower eNOS activity per cell. Therefore, the high density of RBCs and extensive surface area might provide sufficient eNOS-derived NO signaling/export of NO bioactivity to contribute to blood flow and BP regulation where arterial vessel size decreases.

Moreover, it has been shown previously that endothelial eNOS expression declines from proximal to distal sites of the arterial and coronary circulation.[39] Conversely, RBCs carry eNOS uniformly along the vascular tree, and their contact with the vessel wall becomes more intimate as they travel from proximal to distal sites of the arterial circulation. The smaller distance to the endothelial surface may facilitate the effectiveness of the eNOS-dependent export of NO bioactivity from RBCs. This may explain in part the distinct size effects of induction and rescue of arterial hypertension using our cell-specific eNOS KO/KI mice in the absence and presence of NOS/Arg inhibitors (L-NAME and NorNOHA) and, in particular, the apparent lack of effect of L-NAME in EC eNOS KO mice (which was accompanied by increased mortality in this group only). This points to the existence of 2 fundamental and complementary eNOS-dependent pathways controlling BP hemostasis.

Further regulatory components are the nature, distribution, and kinetics of the several NO metabolites found in plasma, RBCs, and vascular tissues.[24,40,44] Under normal physiological conditions, the main enzymatic source of NO in the body is thought to be the endothelial eNOS,[6,41] with lesser contributions of the neuronal NOS and the inducible NOS (in monocytes/macrophages). It was suggested that eNOS-derived NO may exert endocrine effects that are thought to be mediated mainly by nitrite and other semistable circulating metabolites; these include nitrate, nitroso compounds, and possibly nitro fatty acids,[24,40,44] which can be formed in blood and tissues by several reactions. Based on ex vivo experiments, RBC eNOS is proposed to control the release of NO metabolites from RBCs.[9,10,34,45]

The data presented here show that both EC eNOS and RBC eNOS contribute to the levels of systemic NO metabolites, albeit in different ways. In EC eNOS KO mice, the overall amount of NO metabolites was decreased mainly in plasma, lung, and heart, whereas in the RBC eNOS KO mice clear changes are observed only in the blood, which further supports the notion of differential regulation of the circulating NO pool and NO bioactivity affecting vascular tone as generated from either EC or RBC eNOS. The levels of RXNO in plasma or RBCs show some variability (which was not observed in the other groups) and show no correlation with the presence/absence of eNOS in these models.

When looking specifically at the levels of NO-heme in RBCs, which has been proposed to be a good indicator of EC eNOS-derived NO and systemic NO bioavailability,[46] we surprisingly found that RBC eNOS rather than EC eNOS was the major contributor, as clearly demonstrated by comparing the levels of NO-heme in the 4 mouse lines analyzed. This indeed suggests that eNOS in the RBCs is functional and is 1 important source of NO bound in RBCs.

Signaling by eNOS in both erythroid and endothelial bone marrow cells has been proposed to control the differentiation of erythroid cells and hematopoiesis.[47] We did not observe any changes in the hematologic phenotype in our mice: blood count, hematocrit, and blood RBC deformability were not significantly different in RBC eNOS KO mice or EC eNOS KO mice, limiting the role of rheological changes in BP phenotype in those mice. However, oxygen affinity was significantly higher and oxygen-binding cooperativity was significantly lower in the RBC eNOS KO mice; this difference progressed when pH was lowered within the physiological range (from 7.6 to 7.2), indicating a role of RBC eNOS in the Bohr effect, which had earlier been proposed to occur by *S*-nitrosation of Band 3/Anion exchanger 1.[48]

The specificity of gene-targeting deletion or reactivation of eNOS in 1 specific cell type relies on the specificity of the promoter regulating the Cre recombinase. Both the cadherin-5 promoter and the promoter for the hemoglobin β-chain were previously characterized as being highly specific for the endothelial[17] and erythroid[18] compartment, respectively. It is important to point out that some chimerism was observed for the expression of Cre recombinase in megakaryocytes,[18] the precursors of platelets. Although the presence and role of eNOS in megakaryocytes and platelets is still highly controversial and its role in BP regulation unknown, it cannot be excluded a priori. Nevertheless, the efficiency and specificity of EC or RBC targeting were verified here by multiple independent techniques ranging from detection of DNA recombination to cell- and tissue-specific expression of eNOS and Cre recombinase in isolated cells and tissues.

How then does RBC eNOS contribute to BP regulation? At the moment, this question can only be speculated on. Numerous studies suggest that RBCs are capable of exporting NO bioactivity under hypoxic conditions,[45,48,49] although the identity of the species

released responsible for carrying and exerting NO-like bioactivity is yet to be determined.

The finding of decreased NO-heme levels in RBC eNOS KO mice and increased NO-heme levels in RBC eNOS KI mice is very interesting. The main heme protein in RBCs is hemoglobin (Hb) and nitrosyl-Hb (heme iron-bound NO, Fe^{2+}-NO) is the central precursor in 2 hypotheses related to the export of NO bioactivity from RBCs; the one involving nitrosation of Cys-β93 in Hb[50] and the other one involving deoxyhemoglobin-mediated reduction of nitrite to NO.[49] The key point of the controversy is how nitrosyl-Hb is formed and whether nitrosyl-Hb is simply an inert biomarker for NO levels in the RBCs, or rather it is a reservoir of NO, if it could be released.[51]

During NO inhalation, NO-heme forms in the pulmonary vasculature and is rapidly metabolized from artery to vein, suggesting its ability to undergo an oxidative denitrosylation along the vascular tree (ie, its conversion into Fe^{2+}-NO to Fe^{3+} and release of NO). The proposed mechanism consists of concerted reactions of oxyHb and nitrite generating oxidative intermediates (H_2O_2 and NO_2) that convert Fe^{2+}-NO to Fe^{3+}+NO and N_2O_3 that can escape Hb autocapture.[52] As RBCs deoxygenate, nitrosyl-Hb and nitrite participate in reactions that generate NO, NO_2, and N_2O_3. A further mechanism for bioactivity related to nitrosyl-Hb is the recently proposed release of the entire NO-heme complex to function as a signaling entity,[53] but its functional significance is still under investigation.

Nevertheless, here, we presented genetic evidence linking the presence of eNOS in RBCs with the levels of NO bound to RBCs. If one considers that eNOS-derived NO formation from L-arginine requires O_2 and the formation of nitrosyl-Hb may result from RBC eNOS activity, these data indicate that in RBCs an interesting interplay of eNOS-dependent NO formation, formation of nitrosyl-Hb, and export of NO bioactivity related to P_{O_2} may occur.

Another possibility is that the RBC is a net exporter of nitrite, which has been shown to act as a vasodilator and regulator of vascular tone independently of EC eNOS[49] through mechanisms including myoglobin- or xanthine oxidase–mediated nitrite reduction in the vessel wall.[44,54] Nitrite and iron-nitrosyl-Hb likely relate inversely to BP changes, as suggested by independent groups[7,49,55,56] and confirmed with recent translational human studies.[57,58]

The data presented here show that the lack of eNOS in the RBCs and concomitant decrease in NO levels in RBCs (in form of NO-heme) and plasma correspond to an increase in BP, without affecting the activity or function of eNOS in the endothelium. They could indicate a paracrine role of nitrite/NO metabolites in the regulation of BP[49] and demonstrate a central role of eNOS-dependent signaling in RBCs. Further confirmation of this hypothesis is the observation that conditional global eNOS KO mice show a ≈50% decrease of circulating nitrite compared with the WT mice. However, eNOS reactivation in either compartment did not restore circulating nitrite levels in these models, although BP was comparable to that in WT mice. These findings suggest that in a global eNOS KO the mechanism governing the regulation of plasma nitrite is only in part dependent on vascular/RBC eNOS–derived NO production.

A common uncertainty when trying to explain RBC export of NO bioactivity relates to where the NO is originating from. Most authors would suggest that the endothelium is the major source and that NO is first picked up by the RBCs.[1,46] Despite being present at very low levels, the data presented here actually show that RBC eNOS plays a role both in regulating circulating levels of nitrite/nitrate and NO-heme levels in the RBCs, and BP homeostasis, as well. It is most important that we could demonstrate that NO bioactivity exported from RBCs is effective in the regulation of vascular function at the level of resistance arteries, although it does not affect conduit arteries, because RBC eNOS KO mice have increased MAP but preserved function of conduit arteries.

These distinct effects of eNOS on arterial macro- and microcirculation are in line with the concept that multiple layers of compartmentalization are in place to regulate eNOS-derived NO bioactivity, as presented by independent authors.[35–39] In the bloodstream, RBCs are located closer to the endothelium downstream in the circulation, whereas, in the large elastic conduit arteries, RBCs are aligned mainly in the central bloodstream far away from the endothelial lining, thus limiting NO scavenging upstream and allowing the release of NO bioactivity downstream.[14,16]

In the vessel wall, eNOS is found both at the luminal side of ECs (apical EC) and the basal side of ECs. Endothelial NO is released abluminally from ECs toward the vascular smooth muscle, and, to a lesser extent, as a spillover into the blood at its luminal site. In addition, specifically in resistance arteries, NO goes through myoendothelial junctions, and its transfer from ECs to smooth muscle cells depends on the redox state of hemoglobin alpha.[37,38] Accordingly, the contribution of ECs or RBCs to the circulating NO pool and regulation of vascular tone may differ substantially along the arterial circulation, and RBC-mediated release of NO bioactivity and its vasodilatory potential may critically depend on the diameter of the respective arterial segment.

A further aspect that contributes to the complexity of the interplay between ECs and RBCs is the nature and stability of NO metabolites carrying NO bioactivity, their kinetics, and their longitudinal and transversal gradients within arterial segments that may affect their vasodilatory bioactivity. The complexity of these 3 components controlling arterial NO bioactivity (the blood, the vessels, and the NO metabolites) underscores the need for biochemical and functional assessment in vivo of cell-specific eNOS activity from either ECs or RBCs, which, according to our data, may represent the 2 major cell types involved in the regulation of tissue perfusion.

In summary, we created a series of novel mouse models for tissue-specific gene targeting of eNOS. The comparison of mice lacking eNOS specifically in the endothelium or erythroid cells demonstrates that eNOS plays independent roles in these 2 cellular compartments. We present here, for the first time, compelling evidence demonstrating that RBC eNOS directly contributes to systemic NO bioavailability and BP homeostasis, independently of vascular endothelial eNOS. The data provided herein, and the availability of the mice models, will allow for a better understanding of the specific role that RBC eNOS plays in normal physiology and in disease conditions, as well. These include coronary artery disease and chronic kidney disease, where RBC eNOS is decreased,[8,59,60] hematologic diseases and hemoglobinopathies, which are characterized by a systemic decrease in NO bioavailability and defects in eNOS signaling, like in sickle cell disease.[61] Moreover, our data and models may also help understanding how RBC eNOS signaling affects RBC function, NO scavenging, NO/sulfide cross-talk, and oxygen transport[62] and may enable us to refine the criteria for blood banking and transfusion.[63]

ARTICLE INFORMATION

Received August 29, 2020; accepted June 22, 2021.

Affiliations

Myocardial Infarction Research Laboratory, Department of Cardiology, Pulmonology, and Angiology (F.L., T.S., S.K.H., J.L., A.L.B., F.B., E.P., B.H., L.V., M.M.C.-K.), Department of Pharmacology and Clinical Pharmacology (R.S., M.G.), Department of Nephrology (J.S.), Department of Cardiology Pneumology and Angiology (T.S., M.K., M.M.C.-K.), and CARID, Cardiovascular Research Institute Düsseldorf (M.K.), Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany. Department of Pharmacy, University of Pisa, Italy (F.P.). Robert M. Berne Cardiovascular Research Center, Department of Molecular Physiology and Biophysics, University of Virginia School of Medicine, Charlottesville (M.E.G., B.E.I.). Molecular Cardiology Research Institute, Tufts Medical Center, Boston, MA (M.E.G.). Clinical & Experimental Sciences, Faculty of Medicine, University of Southampton, United Kingdom (B.O.F.). Department of Pediatrics, Center for Blood Oxygen Transport and Hemostasis, University of Maryland School of Medicine, Baltimore (S.R., A.D.). Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden (E.W., J.O.L., M.M.C.-K.).

Acknowledgments

The authors thank S. Becher, E. Bruns, J. Hocks, M. Mikus-Lelinska, W. Lückstadt, C. Nihlén, A. Olsson, S. Thasian-Sivarajah, and N. Thomas for their skillful assistance in the experiments. The authors are indebted to S. Münch, C. Gourgoula, and P. Benten (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben [ZETT]; Heinrich-Heine-Universität Düsseldorf [HHU]) for the help with the breeding and maintenance of the mice lines, and to Dr M. Hoffmann and all the colleagues of the Research Laboratory of the Clinic for Urology (Universitätsklinikum Düsseldorf [UKD]) for the use of their gel documentation system ChemiDoc (BioRad) and the flow cytometer MACS quant (Miltenyi Biotech). Moreover, they thank Dr M. Carlström (Department of Physiology and Pharmacology, Karolinska Institutet) for the inspiring discussion about the role of NO metabolites in hemodynamics, Drs A. Heinen and A. Gödecke (Institute of Physiology, HHU) for the discussions about the determination of systemic vascular resistance, the use of their Vevo. and the invaluable and expert help.

Sources of Funding

This work was supported by the German Research Council (DFG CO 1305/2-1 to Dr Cortese-Krott; DFG SFB 1116 TP B06 to Drs Cortese-Krott and Kelm; TP B10 to Dr Grandoch; the IRTG1902 TP9 to Drs Cortese-Krott and Kelm; TP10 to Dr Stegbauer; TP12 to Dr Grandoch) and by independent research grants of the Forschungskommission, Medical Faculty, Heinrich-Heine University Düsseldorf (to Dr Cortese-Krott; to Dr Suvorava; to Drs Cortese-Krott and Stegbauer). Dr Leo is a scholar of the IRTG1902. Mr Li, Mrs LoBue, Drs Hutzler, and Barbarino are scholars of the SPP1710. Dr Feelisch acknowledges funding from the Medical Research Council and the Wellcome Trust. Dr Isakson was supported by National Institutes of Health HL088554. Dr Cortese-Krott is a visiting professor and Wenner-Gren research fellow in the Department of Physiology and Pharmacology, Karolinska Institutet.

Disclosures

None.

Supplemental Materials

Expanded Methods

Data Supplement Table I

Data Supplement Figures I–XII

REFERENCES

1. Farah C, Michel LYM, Balligand JL. Nitric oxide signalling in cardiovascular health and disease. *Nat Rev Cardiol*. 2018;15:292–316. doi: 10.1038/nrcardio.2017.224
2. Gödecke A, Decking UK, Ding Z, Hirchenhain J, Bidmon HJ, Gödecke S, Schrader J. Coronary hemodynamics in endothelial NO synthase knockout mice. *Circ Res*. 1998;82:186–194. doi: 10.1161/01.res.82.2.186
3. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239–242. doi: 10.1038/377239a0
4. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA*. 1996;93:13176–13181. doi: 10.1073/pnas.93.23.13176
5. Erkens R, Suvorava T, Sutton TR, Fernandez BO, Mikus-Lelinska M, Barbarino F, Flögel U, Kelm M, Feelisch M, Cortese-Krott MM. Nrf2 deficiency unmasks the significance of nitric oxide synthase activity for cardioprotection. *Oxid Med Cell Longev*. 2018;2018:8309698. doi: 10.1155/2018/8309698
6. Milsom AB, Fernandez BO, Garcia-Saura MF, Rodriguez J, Feelisch M. Contributions of nitric oxide synthases, dietary nitrite/nitrate, and other sources to the formation of NO signaling products. *Antioxid Redox Signal*. 2012;17:422–432. doi: 10.1089/ars.2011.4156
7. Park JW, Piknova B, Huang PL, Noguchi CT, Schechter AN. Effect of blood nitrite and nitrate levels on murine platelet function. *PLoS One*. 2013;8:e55699. doi: 10.1371/journal.pone.0055699
8. Cortese-Krott MM, Rodriguez-Mateos A, Sansone R, Kuhnle GG, Thasian-Sivarajah S, Krenz T, Horn P, Krisp C, Wolters D, Heiß C, et al. Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. *Blood*. 2012;120:4229–4237. doi: 10.1182/blood-2012-07-442277
9. Wood KC, Cortese-Krott MM, Kovacic JC, Noguchi A, Liu VB, Wang X, Raghavachari N, Boehm M, Kato GJ, Kelm M, et al. Circulating blood endothelial nitric oxide synthase contributes to the regulation of systemic blood pressure and nitrite homeostasis. *Arterioscler Thromb Vasc Biol*. 2013;33:1861–1871. doi: 10.1161/ATVBAHA.112.301068
10. Cortese-Krott MM, Kelm M. Endothelial nitric oxide synthase in red blood cells: key to a new erythrocrine function? *Redox Biol*. 2014;2:251–258. doi: 10.1016/j.redox.2013.12.027
11. Benz PM, Fleming I. Can erythrocytes release biologically active NO? *Cell Commun Signal*. 2016;14:22. doi: 10.1186/s12964-016-0145-y
12. Gambaryan S, Subramanian H, Kehrer L, Mindukshev I, Sudnitsyna J, Reiss C, Rukoyatkina N, Friebe A, Sharina I, Martin E, et al. Erythrocytes do not activate purified and platelet soluble guanylate cyclases even in conditions favourable for NO synthesis. *Cell Commun Signal*. 2016;14:16. doi: 10.1186/s12964-016-0139-9
13. Tziakas DN, Chalikias G, Pavlaki M, Kareli D, Gogiraju R, Hubert A, Böhm E, Stamoulis P, Drosos I, Kikas P, et al. Lysed erythrocyte membranes promote vascular calcification. *Circulation*. 2019;139:2032–2048. doi: 10.1161/CIRCULATIONAHA.118.037166
14. Cortese-Krott MM, Kramer CM, Kelm M. NOS, NO, and the red cell. In: Ignarro LJ, Freeman BA, eds. *Nitric Oxide*. 3rd ed. Academic Press; 2017:185–194.
15. Diederich L, Suvorava T, Sansone R, Keller TCS 4th, Barbarino F, Sutton TR, Kramer CM, Lückstädt W, Isakson BE, Gohlke H, et al. On the effects of reactive oxygen species and nitric oxide on red blood cell deformability. *Front Physiol*. 2018;9:332. doi: 10.3389/fphys.2018.00332

16. Helms CC, Gladwin MT, Kim-Shapiro DB. Erythrocytes and vascular function: oxygen and nitric oxide. *Front Physiol.* 2018;9:125. doi: 10.3389/fphys.2018.00125
17. Sörensen I, Adams RH, Gossler A. DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. *Blood.* 2009;113:5680–5688. doi: 10.1182/blood-2008-08-174508
18. Peterson KR, Fedosyuk H, Zelenchuk L, Nakamoto B, Yannaki E, Stamatoyannopoulos G, Ciciotte S, Peters LL, Scott LM, Papayannopoulou T. Transgenic Cre expression mice for generation of erythroid-specific gene alterations. *Genesis.* 2004;39:1–9. doi: 10.1002/gene.20020
19. Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* 1995;23:5080–5081. doi: 10.1093/nar/23.24.5080
20. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 2010;8:e1000412. doi: 10.1371/journal.pbio.1000412
21. Johnstone SR, Kroncke BM, Straub AC, Best AK, Dunn CA, Mitchell LA, Peskova Y, Nakamoto RK, Koval M, Lo CW, et al. MAPK phosphorylation of connexin 43 promotes binding of cyclin E and smooth muscle cell proliferation. *Circ Res.* 2012;111:201–211. doi: 10.1161/CIRCRESAHA.112.272302
22. Erkens R, Kramer CM, Lückstädt W, Panknin C, Krause L, Weidenbach M, Dirzka J, Krenz T, Mergia E, Suvorava T, et al. Left ventricular diastolic dysfunction in Nrf2 knock out mice is associated with cardiac hypertrophy, decreased expression of SERCA2a, and preserved endothelial function. *Free Radic Biol Med.* 2015;89:906–917. doi: 10.1016/j.freeradbiomed.2015.10.409
23. Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G. Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo. *Circulation.* 2005;112:2487–2495. doi: 10.1161/CIRCULATIONAHA.105.543157
24. Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, Rodriguez JR, Feelisch M. Cellular targets and mechanisms of nitros (yl) ation: an insight into their nature and kinetics in vivo. *Proc Natl Acad Sci USA.* 2004;101:4308-4313. doi: 10.1073/pnas.0306706101
25. Levett DZ, Fernandez BO, Riley HL, Martin DS, Mitchell K, Leckstrom CA, Ince C, Whipp BJ, Mythen MG, Montgomery HE, et al; Caudwell Extreme Everest Research Group. The role of nitrogen oxides in human adaptation to hypoxia. *Sci Rep.* 2011;1:109. doi: 10.1038/srep00109
26. Hussain MB, MacAllister RJ, Hobbs AJ. Reciprocal regulation of cGMP-mediated vasorelaxation by soluble and particulate guanylate cyclases. *Am J Physiol Heart Circ Physiol.* 2001;280:H1151–H1159. doi: 10.1152/ajpheart.2001.280.3.H1151
27. Brandes RP, Kim D, Schmitz-Winnenthal FH, Amidi M, Gödecke A, Mülsch A, Busse R. Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice: role of soluble guanylyl cyclase. *Hypertension.* 2000;35(1 pt 2):231–236. doi: 10.1161/01.hyp.35.1.231
28. Moncada S, Rees DD, Schulz R, Palmer RM. Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. *Proc Natl Acad Sci USA.* 1991;88:2166–2170. doi: 10.1073/pnas.88.6.2166
29. Eriksson KE, Yang T, Carlström M, Weitzberg E. Organ uptake and release of inorganic nitrate and nitrite in the pig. *Nitric Oxide.* 2018;75:16–26. doi: 10.1016/j.niox.2018.02.001
30. Peleli M, Zollbrecht C, Montenegro MF, Hezel M, Zhong J, Persson EG, Holmdahl R, Weitzberg E, Lundberg JO, Carlström M. Enhanced XOR activity in eNOS-deficient mice: effects on the nitrate-nitrite-NO pathway and ROS homeostasis. *Free Radic Biol Med.* 2016;99:472–484. doi: 10.1016/j.freeradbiomed.2016.09.004
31. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. *FASEB J.* 1989;3:2007–2018.
32. Böhmer A, Beckmann B, Sandmann J, Tsikas D. Doubts concerning functional endothelial nitric oxide synthase in human erythrocytes. *Blood.* 2012;119:1322–1323. doi: 10.1182/blood-2011-11-393124
33. Cortese-Krott MM. Response to commentary. *Redox Biol.* 2015;5:411–412. doi: 10.1016/j.redox.2015.08.004
34. Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozüyaman B, et al. Red blood cells express a functional endothelial nitric oxide synthase. *Blood.* 2006;107:2943–2951. doi: 10.1182/blood-2005-10-3992
35. Fulton D, Fontana J, Sowa G, Gratton JP, Lin M, Li KX, Michell B, Kemp BE, Rodman D, Sessa WC. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem.* 2002;277:4277–4284. doi: 10.1074/jbc.M106302200
36. García-Cardeña G, Oh P, Liu J, Schnitzer JE, Sessa WC. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci USA.* 1996;93:6448–6453. doi: 10.1073/pnas.93.13.6448
37. Biwer LA, Taddeo EP, Kenwood BM, Hoehn KL, Straub AC, Isakson BE. Two functionally distinct pools of eNOS in endothelium are facilitated by myoendothelial junction lipid composition. *Biochim Biophys Acta.* 2016;1861:671–679. doi: 10.1016/j.bbalip.2016.04.014
38. Straub AC, Lohman AW, Billaud M, Johnstone SR, Dwyer ST, Lee MY, Bortz PS, Best AK, Columbus L, Gaston B, et al. Endothelial cell expression of haemoglobin α regulates nitric oxide signalling. *Nature.* 2012;491:473–477. doi: 10.1038/nature11626
39. Shu X, Keller TC IV, Begandt D, Butcher JT, Biwer L, Keller AS, Columbus L, Isakson BE. Endothelial nitric oxide synthase in the microcirculation. *Cell Mol Life Sci.* 2015;72:4561–4575. doi: 10.1007/s00018-015-2021-0
40. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* 2008;7:156–167. doi: 10.1038/nrd2466
41. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, et al. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med.* 2003;35:790–796. doi: 10.1016/s0891-5849(03)00406-4
42. Lauer T, Kleinbongard P, Preik M, Rauch BH, Deussen A, Feelisch M, Strauer BE, Kelm M. Direct biochemical evidence for eNOS stimulation by bradykinin in the human forearm vasculature. *Basic Res Cardiol.* 2003;98:84–89. doi: 10.1007/s003950300000
43. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* 2016;14:e1002533. doi: 10.1371/journal.pbio.1002533
44. Jansson EA, Huang L, Malkey R, Govoni M, Nihlén C, Olsson A, Stensdotter M, Petersson J, Holm L, Weitzberg E, et al. A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat Chem Biol.* 2008;4:411–417. doi: 10.1038/nchembio.92
45. Yang J, Gonon AT, Sjöquist PO, Lundberg JO, Pernow J. Arginase regulates red blood cell nitric oxide synthase and export of cardioprotective nitric oxide bioactivity. *Proc Natl Acad Sci USA.* 2013;110:15049–15054. doi: 10.1073/pnas.1307058110
46. Dei Zotti F, Lobysheva II, Balligand JL. Nitrosyl-hemoglobin formation in rodent and human venous erythrocytes reflects NO formation from the vasculature in vivo. *PLoS One.* 2018;13:e0200352. doi: 10.1371/journal.pone.0200352
47. Cokic VP, Andric SA, Stojilkovic SS, Noguchi CT, Schechter AN. Hydroxyurea nitrosylates and activates soluble guanylyl cyclase in human erythroid cells. *Blood.* 2008;111:1117–1123. doi: 10.1182/blood-2007-05-088732
48. Pawloski JR, Hess DT, Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature.* 2001;409:622–626. doi: 10.1038/35054560
49. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med.* 2003;9:1498–1505. doi: 10.1038/nm954
50. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-Nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* 1996;380:221–226. doi: 10.1038/380221a0
51. Gladwin MT, Schechter AN, Shelhamer JH, Pannell LK, Conway DA, Hrinczenko BW, Nichols JS, Pease-Fye ME, Noguchi CT, Rodgers GP, et al. Inhaled nitric oxide augments nitric oxide transport on sickle cell hemoglobin without affecting oxygen affinity. *J Clin Invest.* 1999;104:937–945. doi: 10.1172/JCI7637
52. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, Ringwood LA, Jiang A, Hogg N, Kim-Shapiro DB, et al. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. *J Biol Chem.* 2007;282:12916–12927. doi: 10.1074/jbc.M700546200
53. Kleschyov AL. The NO-heme signaling hypothesis. *Free Radic Biol Med.* 2017;112:544–552. doi: 10.1016/j.freeradbiomed.2017.08.025
54. Totzeck M, Hendgen-Cotta UB, Luedike P, Berenbrink M, Klare JP, Steinhoff HJ, Semmler D, Shiva S, Williams D, Kipar A, et al. Nitrite regulates hypoxic vasodilation via myoglobin-dependent nitric oxide generation. *Circulation.* 2012;126:325–334. doi: 10.1161/CIRCULATIONAHA.111.087155
55. Cannon RO 3rd, Schechter AN, Panza JA, Ognibene FP, Pease-Fye ME, Waclawiw MA, Shelhamer JH, Gladwin MT. Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. *J Clin Inv.* 2001;108:279-287. doi: 10.1172/JCI12761

56. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med.* 2003;348:1483–1485. doi: 10.1056/NEJMcibr023045
57. Hughan KS, Levine A, Helbling N, Anthony S, DeLany JP, Stefanovic-Racic M, Goodpaster BH, Gladwin MT. Effects of oral sodium nitrite on blood pressure, insulin sensitivity, and intima-media arterial thickening in adults with hypertension and metabolic syndrome. *Hypertension.* 2020;76:866–874. doi: 10.1161/HYPERTENSIONAHA.120.14930
58. Bailey DM, Rasmussen P, Overgaard M, Evans KA, Bohm AM, Seifert T, Brassard P, Zaar M, Nielsen HB, Raven PB, et al. Nitrite and S-nitrosohemoglobin exchange across the human cerebral and femoral circulation: relationship to basal and exercise blood flow responses to hypoxia. *Circulation.* 2017;135:166–176. doi: 10.1161/CIRCULATIONAHA.116.024226
59. Di Pietro N, Giardinelli A, Sirolli V, Riganti C, Di Tomo P, Gazzano E, Di Silvestre S, Panknin C, Cortese-Krott MM, Csonka C, et al. Nitric oxide synthetic pathway and cGMP levels are altered in red blood cells from end-stage renal disease patients. *Mol Cell Biochem.* 2016;417:155–167. doi: 10.1007/s11010-016-2723-0
60. Eligini S, Porro B, Lualdi A, Squellerio I, Veglia F, Chiorino E, Crisci M, Garlaschè A, Giovannardi M, Werba JP, et al. Nitric oxide synthetic pathway in red blood cells is impaired in coronary artery disease. *PLoS One.* 2013;8:e66945. doi: 10.1371/journal.pone.0066945
61. Kim-Shapiro DB, Gladwin MT. Nitric oxide pathology and therapeutics in sickle cell disease. *Clin Hemorheol Microcirc.* 2018;68:223–237. doi: 10.3233/CH-189009
62. Cortese-Krott MM. Red blood cells as a "central hub" for sulfide bioactivity: scavenging, metabolism, transport, and cross-talk with nitric oxide. *Antioxid Redox Signal.* 2020;33:1332–1349. doi: 10.1089/ars.2020.8171
63. Kanias T, Gladwin MT. Nitric oxide, hemolysis, and the red blood cell storage lesion: interactions between transfusion, donor, and recipient. *Transfusion.* 2012;52:1388–1392. doi: 10.1111/j.1537-2995.2012.03748.x

Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Endothelial Hyaluronan Synthase 3 Augments Postischemic Arteriogenesis Through CD44/eNOS Signaling

Rebekka Schneckmann, Tatsiana Suvorava, Christian Hundhausen, Dominik Schuler, Christin Lorenz, Till Freudenberger, Malte Kelm, Jens W. Fischer, Ulrich Flögel, Maria Grandoch

OBJECTIVE: The dominant driver of arteriogenesis is elevated shear stress sensed by the endothelial glycocalyx thereby promoting arterial outward remodeling. Hyaluronan, a critical component of the endothelial glycocalyx, is synthesized by 3 HAS isoenzymes (hyaluronan synthases 1-3) at the plasma membrane. Considering further the importance of HAS3 for smooth muscle cell and immune cell functions we aimed to evaluate its role in collateral artery growth.

APPROACH AND RESULTS: Male *Has3*-deficient (*Has3*-KO) mice were subjected to hindlimb ischemia. Blood perfusion was monitored by laser Doppler perfusion imaging and endothelial function was assessed by measurement of flow-mediated dilation in vivo. Collateral remodeling was monitored by high resolution magnetic resonance angiography. A neutralizing antibody against CD44 (clone KM201) was injected intraperitoneally to analyze hyaluronan signaling in vivo. After hindlimb ischemia, *Has3*-KO mice showed a reduced arteriogenic response with decreased collateral remodeling and impaired perfusion recovery. While postischemic leukocyte infiltration was unaffected, a diminished flow-mediated dilation pointed towards an impaired endothelial cell function. Indeed, endothelial AKT (protein kinase B)-dependent eNOS (endothelial nitric oxide synthase) phosphorylation at Ser1177 was substantially reduced in *Has3*-KO thigh muscles. Endothelial-specific *Has3*-KO mice mimicked the hindlimb ischemia-induced phenotype of impaired perfusion recovery as observed in global *Has3*-deficiency. Mechanistically, blocking selectively the hyaluronan binding site of CD44 reduced flow-mediated dilation, thereby suggesting hyaluronan signaling through CD44 as the underlying signaling pathway.

CONCLUSIONS: In summary, HAS3 contributes to arteriogenesis in hindlimb ischemia by hyaluronan/CD44-mediated stimulation of eNOS phosphorylation at Ser1177. Thus, strategies augmenting endothelial HAS3 or CD44 could be envisioned to enhance vascularization under pathological conditions.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: arteriogenesis ■ eNOS ■ flow-mediated dilation ■ perfusion

Peripheral artery disease (PAD) is a highly prevalent disease caused by atherosclerosis and stenosis of peripheral arteries in the leg.[1,2] Upon occlusion of a conduit artery, native collateral anastomoses remodel into functional arteries supplying blood to the ischemic tissue. Indeed, this arteriogenic response may explain why patients with early-stage disease have no or only mild symptoms of leg ischemia and barely any functional restrictions. However, already the presence of PAD is indicative for an overall increased atherosclerotic burden and increased cardiovascular risk.[3,4] Consequently, PAD is an independent and strong predictor of cardiovascular events such as myocardial infarction or stroke. Early diagnosis of PAD as well as novel strategies to improve therapeutic vessel growth are, therefore, highly desirable.

A clinical tool to identify patients with PAD long before symptoms appear is the measurement of flow-mediated

Correspondence to: Maria Grandoch, MD, Institute for Pharmacology and Clinical Pharmacology, University Hospital of the Heinrich-Heine-University, Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany. Email maria.grandoch@hhu.de
The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.121.315478.
For Sources of Funding and Disclosures, see page 2561.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

BW	body weight
CD	cluster of differentiation
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
FAL	femoral artery ligation
FMD	flow-mediated dilation
HAS	hyaluronan synthase
MR	magnetic resonance
NO	nitric oxide
PAD	peripheral artery disease
SMC	smooth muscle cell
WT	wild type

Highlights

- HAS3 (hyaluronan synthase 3) is involved in shear stress dependent AKT (protein kinase B)/eNOS (endothelial NO synthase) activation in endothelial cells giving rise to flow-mediated dilation in vivo.
- Hyaluronan-mediated effects on flow-mediated dilation are critically dependent on hyaluronan/CD44 interaction.
- Endothelial HAS3 promotes arteriogenesis after femoral artery occlusion.

dilation (FMD).[5] FMD is an endothelium-dependent dilation of the conduit artery in response to elevated shear stress that activates eNOS (endothelial nitric oxide [NO] synthase) with increased NO production and relaxation of vascular smooth muscle cells (SMCs).[6] Of note, PAD is often associated with reduced FMD as a surrogate for endothelial dysfunction related to altered eNOS signaling and impaired NO bioavailability.[7,8] Interestingly, clinical studies confirmed a strong correlation between FMD and flow-induced arterial remodeling suggesting that shear stress-mediated NO signaling is indispensable for collateral artery growth.[9]

The discovery of factors that are involved in shear stress-mediated NO signaling could help to (1) predict the underlying cardiovascular risk, (2) provide reliable biomarkers for PAD, and (3) identify novel therapeutic targets to enhance collateral artery growth in patients. In this context, the endothelial glycocalyx has been attributed a key role due to its function as a delicate shear stress sensor that is involved in mechanotransduction and NO-related pathways.[10] It is tempting to speculate that also hyaluronan, an integral component of the endothelial glycocalyx, is a critical player in endothelial mechanosignaling and collateral artery growth.

Hyaluronan is synthesized by 3 HAS (hyaluronan synthase) isoforms (HAS1, -2 and -3) at the plasma membrane and extruded into the extracellular space. Besides its association with hyaladherins like versican, hyaluronan signals via receptors such as cluster of differentiation (CD) 44 and RHAMM (receptor for hyaluronan-mediated motility).[11]

There are studies suggesting a role of hyaluronan and CD44 for endothelial NO production: enzymatic degradation of hyaluronan ex vivo in isolated femoral arteries coincided with impaired NO production[12] and interaction of CD44 with ankyrin and the IP3 receptor promoted endothelial NO release in vitro.[13,14] However, ex vivo isolated vessel preparations do not fully resemble the in vivo situation. Furthermore, glycocalyx formation in cultured endothelial cells (ECs) is either weak or even absent under static conditions, a fact that further complicates interpretation of the results obtained by in vitro techniques. A recent study reported on the importance of hyaluronan in response to shear stress conditions. Here, these effects in human umbilical vein ECs were reported to be mediated by HAS2.[15] However, whether this also reflects the in vivo situation in PAD and whether HAS isoenzymes might represent a promising and novel therapeutic target in this context, is currently unknown.

Of note, HAS3 has been reported to be critically involved in immune cell responses and SMC migration in cardiovascular diseases such as atherosclerosis or neointima formation.[16,17] Since both processes are also relevant for collateral artery growth, we hypothesized that HAS3 plays an important role in arteriogenesis. In the present study, we used high-resolution imaging techniques in vivo and HAS3-defcient mice to unravel the role of HAS3 in collateral artery growth as an attempt to identify novel therapeutic targets for PAD.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and in the Data Supplement.

Mice

For all experiments male *hyaluronan synthase 3 (Has3)*-deficient mice (*Has3*-KO) and wild-type (WT) littermate controls (on C57BL/6JRj background, Janvier Labs, Le Genest-Saint-Ile, France) at 16 to 18-weeks of age were used. Male 18-weeks old C57BL/6J WT mice from Janvier Labs were used to determine HAS expression in thigh and calf muscle. Transgenic mice expressing Cre recombinase under control of the 5′ endothelial enhancer of the stem cell leukemia locus were used to generate EC-specific *Has3*-deficient mice (EC *Has3*-KO) as follows. 5′ endothelial enhancer of the stem cell leukemia locus-Cre^{ErT} mice (cre-controls)[18] provided by PD Dr J.R. Göthert (University

Hospital Essen, Germany) were crossed with floxed HAS3 knockin mice, as described by Kiene et al,[17] and Cre recombination was induced via intraperitoneal injections of 2 mg tamoxifen, every second day for 2 weeks followed by a 3-week washout phase. Prof. Dr A. Gödecke (Heinrich-Heine-University of Düsseldorf, Germany) provided homozygous endothelial NOS-knockout mice. Mice were kept on a 12-hour dark/light cycle with access to normal laboratory diet (Ssniff, Soest, Germany) and water ad libitum. Starting at 12 weeks of age, *Apoe*$^{-/-}$/*Has3*$^{-/-}$ mice and respective littermate *Apoe*$^{-/-}$ control mice were fed a Western-type diet containing 21% fat and 0.15% cholesterol (S8200-E010; Ssniff Spezialdiäten GmbH, Soest, Germany). To avoid variations caused by fluctuating estrus cycle in female individuals, all experiments were performed in male mice. Animal experiments were performed according to the German Animal Welfare Law and were authorized by the local Animal Ethics Committee (LANUV; State Agency for Nature, Environment and Consumer Protection). Mice were excluded from the experiment when certain criteria of suffering were observed. These included weight loss >20% of body weight (BW), cessation of food and water ingestion or lack of voluntary movement. Blinded data collection and analysis were performed in following experiments: magnetic resonance (MR) angiography, Western blot analysis, flow cytometry of cardiac blood, CD44 blocking antibody test, and FMD measurements.

CD44 Blocking Experiments

C57BL6/J WT mice were administered 100 µg of low endotoxin/azide free anti-CD44 antibody (clone KM201, Southern Biotech, Birmingham, AL, No. 1500-14) or rat IgG1 Isotype Control (Southern Biotech, Birmingham, AL, No. 0116-14) by intraperitoneal injection at time points 0 and 24 hours (Table I in the Data Supplement). At 25 hours, FMD was measured. Following FMD measurements, mice were euthanized and cardiac blood was collected for flow cytometric analysis of CD44 blocking using the antibody combination depicted in Table II in the Data Supplement. An APC-labeled anti-CD44 antibody (clone KM201, Southern Biotech, Birmingham, AL, No. 1500-11) detected CD44^{+} cells. Two mice were excluded due to insufficient CD44 blocking as determined by flow cytometric analysis. To test the impact of temporary CD44 inhibition on long-term perfusion recovery, *Has3*-KO and WT mice were treated with Rat IgG isotype or anti CD44 clone KM201 one hour before, 24 and 48 hours after femoral artery ligation (FAL). Perfusion recovery was determined immediately and 21 days post-FAL.

Determining FMD and Flow Velocity

Endothelial function was assessed in vivo by measuring FMD of the femoral and iliac artery using Vevo 2100 with a 30 to 70 MHz linear array microscan transducer (Visual Sonics 2100, Toronto, Canada). The artery was identified by its characteristic blood flow pattern using the duplex ultrasound method. Distal hindlimb ischemia and subsequent reactive hyperemia were induced by placing an inflatable cuff around the lower limb for 5 minutes and deflating it for 3 minutes, as previously described.[19,20]

Hindlimb Ischemia Model

Mice were anesthetized by intraperitoneal injection of midazolam (8 mg/kg BW, Actavis, Muenchen, Germany), medetomidine (0.4 mg/kg BW, Orion, Hamburg, Germany), and fentanyl (0.08 mg/kg BW, Ratiopharm, Ulm, Germany). During surgery, mice were placed on a heating plate to maintain temperature at 37 °C. FAL was induced proximal to the superficial epigastric artery and the anterior tibialis artery branch by electrocoagulation.[21] After surgery, anesthesia was antagonized by subcutaneous injection of flumazenil (0.7 mg/kg BW, Ratiopharm, Ulm, Germany) and atipamezole (3.3 mg/kg BW, Orion, Hamburg, Germany). For postoperative analgesia, buprenorphine was administered (0.2 mg/kg BW, Temgesic, Indivior UK Ltd, Slough, United Kingdom).

Experimental Design

Hindlimb perfusion was monitored by laser Doppler perfusion imaging. After 14 days, mice were euthanized and muscles were harvested for histology. Twenty-four hours and 3 days after FAL, flow cytometric analyses were performed. To assess collateral growth, an additional group underwent MR angiography 21 days after FAL.

Statistical Analysis

Sample size estimation was based on previous results in comparable studies, assuming 80% power at a significance level of 0.05. Specific details on how many independent biological samples or mice were included in an experiment or how many times experiments were repeated independently are given in the corresponding figure legends. Sample size is given in the figure legends and data are presented as mean±SEM. For continuous variables, normality and homogeneity of variance were assessed by Shapiro-Wilk and Brown-Forsythe tests, respectively. After confirming homogeneous variances and normality, 2-group comparisons for means were performed by 2-sided unpaired Student *t* test. In case of inhomogeneous variances, Mann-Whitney *U* test was performed. Multi-group comparisons for means were performed by 2-way ANOVA with Sidak multiple comparison test. $P<0.05$ was considered statistically significant. Statistical analyses mentioned above were performed using Graph Pad Prism version 9.1.0.

RESULTS

Has3-Deficiency Impairs Shear Stress Induced Activation of eNOS

Considering the thigh muscle as the main compartment of collateral artery growth after FAL,[22] we first determined HAS isoforms' mRNA expression profile in male C57BL/6J WT mice. Baseline characterization revealed that expression of *Has3* mRNA accounted for ≈2/3 of the total *Has* expression in thigh muscle, while *Has1* and *Has2* expression were at comparably low levels (Figure IA and IB in the Data Supplement). Analysis of the calf muscle revealed similar expression patterns (Figure IC and ID in the Data Supplement). Since *Has3* turned out to be the main isoenzyme in thigh muscle at baseline and also in

response to FAL (Figure IE in the Data Supplement), 16 to 18-week-old *Has3*-deficient (*Has3*-KO) mice and WT littermate controls were used for further experiments. Importantly, there was no compensatory upregulation of *Has1* or *Has2* mRNA in *Has3*-KO mice in neither the thigh (Figure IIA and IIB in the Data Supplement) nor the calf muscle (Figure IIC through IIF in the Data Supplement).

To analyze the impact of hyaluronan and specifically HAS3-derived hyaluronan on vascular reactivity and endothelial function, we measured FMD of the femoral artery in vivo. Employing this clinical method frequently used in patients to evaluate vascular endothelial function, we did not observe any differences in flow velocity (Figure 1A). However, FMD was substantially impaired in *Has3*-KO mice (Figure 1B). This finding clearly demonstrates that the *Has3*-deficient endothelium was characterized by a compromised shear stress response. Importantly, reduced FMD coincided with impaired eNOS phosphorylation at Ser1177 in ischemic hind limbs of *Has3*-KO mice: While hindlimb shear stress resulted in an increased ratio of eNOS phosphorylation Ser1177 to total eNOS in WT controls, this response was strongly blunted in the mutants (Figure 1C and 1D). Similarly, upstream signaling cascades were affected in that AKT (protein kinase B) phosphorylation at Ser473 was substantially reduced in *Has3*-deficient mice (Figure 1E and 1F). In contrast, total eNOS protein expression was unaffected in thigh muscle (data not shown) in line with previously published data on

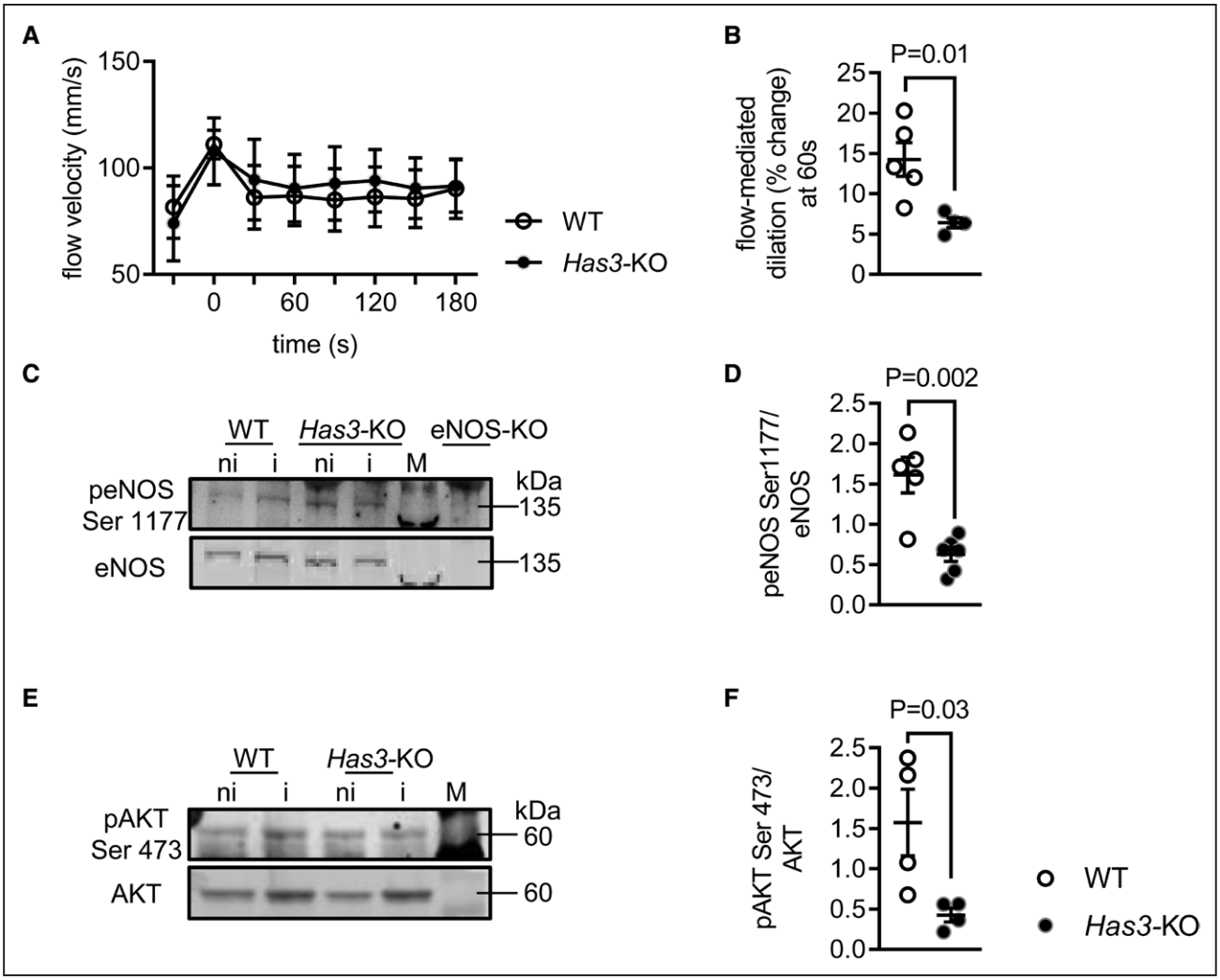


Figure 1. Has3 (hyaluronan synthase 3)-deficiency impairs vascular response to shear stress by decreased AKT (protein kinase B)-mediated phosphorylation of eNOS (endothelial NO synthase) at Ser1177.
A, Flow-velocity at baseline and over a time period of 180 seconds (s) during reactive hyperemia. **B**, Flow-mediated dilation (FMD) of the femoral artery 60s after cuff release following 5 min distal ischemia of the lower limb in *Has3*-deficient (*Has3*-KO) and wild-type (WT) mice; n=4–5. **C** and **D**, Representative Western blots showing the level of (**C**) peNOS (eNOS phosphorylation) at Ser1177 and total eNOS and (**E**) AKT phosphorylation (pAKT) at Ser473 and total AKT and in ischemic (i) and nonischemic (ni) thigh muscles and respective densitometric analyses, (**D**) phospho-Ser1177 eNOS (normalized to total eNOS) and **F**, phospho-Ser473 AKT (normalized to total AKT) in ischemic thigh muscles after vascular occlusion in *Has3*-KO and WT mice; n=5–6. In (**E**) *eNOS*-deficient thigh muscle lysate (*eNOS*-KO) was used as negative control for eNOS signal. Values are shown as fold of nonischemic thigh muscles. Data represent mean±SEM. Statistical significance was assessed by unpaired Student *t* test in **B**, **D**, and **F**. M indicates marker.

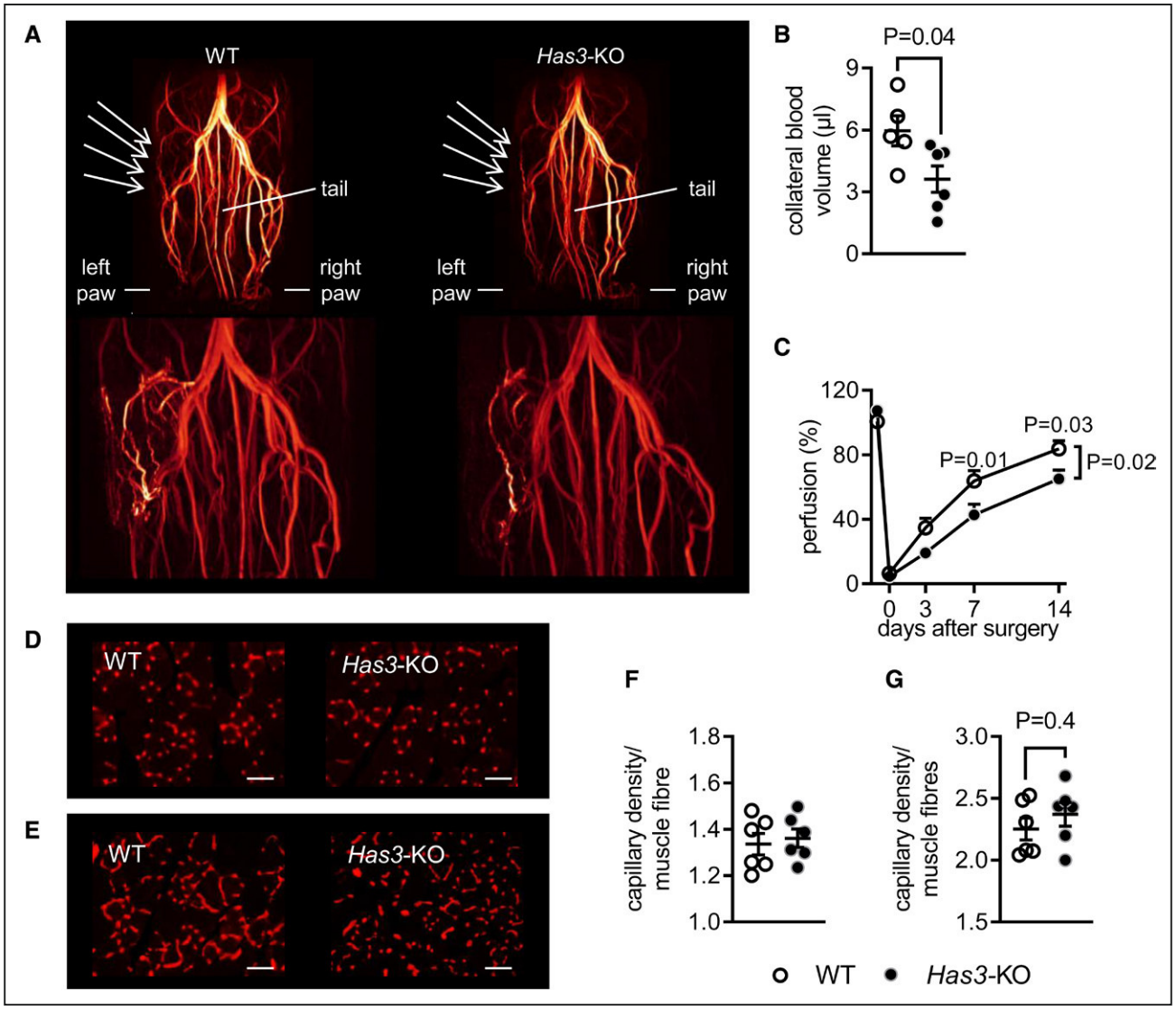


Figure 2. Impaired blood flow recovery and collateral growth in Has3 (hyaluronan synthase 3)-deficient mice.
A, Coronal maximum intensity projections (MIP) of ^{1}H time-of-flight (TOF) MR angiographies from wild-type (WT; **left**) and *Has3*-deficient (*Has3*-KO; **right**) mice. Arrows indicate the area of collateral formation (**top**), which is color-accentuated in the zoomed regions at the **bottom**. The reduced collateral formation in *Has3*-KO leads to an overall decreased blood supply of the left hindlimb as compared with WT mice, as can be clearly recognized in the overview images (**top**). **B**, Determination of collateral blood volume; n=5–6. **C**, Hindlimb perfusion determined by laser Doppler perfusion imaging (LDPI) before, directly after, and 3, 7, and 14 days after femoral artery ligation (FAL) in *Has3*-KO and WT mice. Perfusion was calculated as the percentage of ischemic over nonischemic hindlimb; n=7. Staining of capillaries in calf muscle sections of *Has3*-KO and WT mice using anti-CD31 (red). Representative images of capillaries (**D**) at baseline and (**E**) 14 days after FAL. Quantification of capillary density (**F**) at baseline and (**G**) 14 days after FAL shown as capillaries per muscle fiber; n=6. Bars represent 50 µm. Data represent mean±SEM. Statistical significance was assessed by unpaired Student *t* test in **B**, and 2-way ANOVA and Sidak post hoc test in **C**.

unchanged eNOS expression in aortas of *Has3*-deficient mice as compared with WT mice.[17]

Has3-Deficiency Impairs Collateral Formation After Hindlimb Ischemia

To confirm our hypothesis that impaired FMD and endothelial dysfunction in *Has3*-deficient mice correlates with poor collateral remodeling, we used a murine model of hindlimb ischemia. In this model, the chronic increase in flow induces outward arterial remodeling leading to collateral arteries growth, feeding arteries growth, and arteriogenesis. Twenty-one days after FAL, *Has3*-KO, and WT mice underwent 3-dimensional MR time-of-flight angiography to monitor collateral development in vivo. Notably, representative coronal MR angiograms of both groups' hind limbs are displayed in Figure 2A as maximum intensity projections, showing in great detail the entire vessel tree with the femoral arteries and its branches into the popliteal artery (Movies I and II in the Data Supplement). These images clearly show the origin, course, and diminished perfusion of the left hindlimb

as well as remodeled collateral vessels' re-entry zones (highlighted in the zoomed sections at the bottom). Collaterals are characterized by their corkscrew morphology, typical for arteriogenesis, during which growth in length outpaces increase in diameter.[23] The MR angiograms indicate reduced arteriogenesis and less collateralization in *Has3*-KO compared with WT mice. Collateral vessel volume quantification via surface rendering, segmentation, and voxel count demonstrated significantly less collateralization in *Has3*-KO mice (Figure 2B). Laser Doppler perfusion imaging confirmed that hindlimb perfusion recovery was substantially impaired in *Has3*-deficient as compared with WT mice underlining the functional relevance of this vessel recruitment deficit (Figure 2C). While basal perfusion did not differ, impaired blood flow recovery was already detectable after 3 days and persisted during the entire follow-up period. Notably, we also determined the angiogenic response in calf muscle sections 14 days after FAL. However, neither at baseline (Figure 2D and 2F) nor after FAL (Figure 2E and 2G), we could determine any differences in capillary density when comparing *Has3*-KO to WT mice.

Reduced Perfusion Recovery and Arteriogenic Response in *Apoe*$^{-/-}$ *Has3*-KO Mice

Since PAD is characterized by atherosclerosis and gradual narrowing of peripheral vessels in humans, we next sought to repeat our experiment in apolipoprotein E (*Apoe*$^{-/-}$)-deficient mice, a well-established mouse model of hypercholesterolemia and accelerated atherosclerosis. Therefore, male 12 weeks old *Apoe*$^{-/-}$*Has3*-KO and *Apoe*$^{-/-}$*Has3*-WT mice were fed a cholesterol rich Western diet for 4 weeks followed by FAL (Figure III in the Data Supplement).

Here, we show that *Apoe*$^{-/-}$*Has3*-KO mice respond in a similar way to FAL than *Has3*-deficient mice on C57BL6/J background. Despite similar perfusion immediately post-FAL, *Apoe*$^{-/-}$*Has3*-KO mice showed significantly and strongly reduced hindlimb perfusion after 3 days of FAL as compared with controls (Figure IIID in the Data Supplement). By trend, perfusion recovery remained compromised but converged at 20 days post-FAL and beyond. Furthermore, we assessed collateral blood volume via MR angiography at 21 days post-FAL (Figure IIIE in the Data Supplement). Similar to mice lacking *Has3* on C57BL/6J background, *Apoe*$^{-/-}$*Has3*-KO mice showed a significantly reduced collateral blood volume indicating an insufficient arteriogenic response as compared with control mice.

Endothelial and Not Immune Effectors Are Mediating *Has3* Effects After FAL

There is compelling evidence that immune cells accumulate in close proximity to growing collaterals where they release large amounts of proarteriogenic factors triggering both endothelial and SMC proliferation.[24] In this context, it was reported that the number of circulating monocytes correlates well with collateral artery remodeling after FAL.[25] To this end, we used flow cytometric analysis to reveal the effect of *Has3* deletion on immune cell recruitment to the thigh muscle after ischemia. Surprisingly, we did not detect any differences in the postischemic inflammatory response at 24 hours (Figure IV in the Data Supplement) and 72 hours post-FAL. Neutrophil (CD11b$^+$/Ly6G$^+$) as well as monocyte (CD11b$^+$/CD115$^+$)/macrophage (CD45$^+$/CD11b$^+$/F4/80$^+$) numbers did not differ between mutant and control mice, neither in the blood (Figure 3A through 3C, Figure IV in the Data Supplement) nor in thigh muscle (Figure 3D through 3G, data for neutrophils not shown, Figure IV in the Data Supplement). Also, circulating lymphocytes were not altered (Figure V in the Data Supplement) suggesting that mechanisms independent of the immune response were the predominant drivers of decreased collateral formation in *Has3*-KO mice as presented above.

Impaired FMD in *Has3*-deficient mice pointed towards an important role of endothelial HAS3 for the observed phenotype. To investigate the role of endothelial HAS3 in collateral vessel growth, we used a tamoxifen inducible *Has3*-KO model, recently generated by us (a validation of the mouse model is found in Figure VI in the Data Supplement). Using these mice, we aimed to study the impact of EC-specific *Has3*-deficiency in our FAL model (Figure 4). Of note, the hindlimb ischemia-induced phenotype of global *Has3*-KO mice could be mimicked in EC-specific *Has3*-deficient mice in that perfusion recovery was impaired to an almost identical extent in both strains (Figure 4A through 4E, [endothelial-specific *Has3*-KO] versus Figure 2C [global *Has3*-KO]). Furthermore, we assessed collateral blood volume via MR angiography at 21 days (Figure 4F) and found a strong correlation between impaired perfusion recovery, reduced collateral blood volume, and compromised arteriogenic response (Figure 4G) as indicated by reduced collateral artery growth in EC-specific *Has3*-KO mice. In contrast, the number of arterioles (Figure 4H) as well as capillary density were not affected (Figure 4I and 4K) confirming altered collateral artery remodeling rather than neovessel formation as the underlying mechanism for reduced perfusion in EC-*Has3*-KOs.

CD44 Is a Link Between HAS3-Derived Hyaluronan and eNOS Signaling

AKT-dependent eNOS phosphorylation at Ser1177 is the underlying mechanism for rapid shear responses,[19,26–28] and we found this pathway to be strongly impaired in global and EC-specific *Has3*-deficient mice (Figure 1

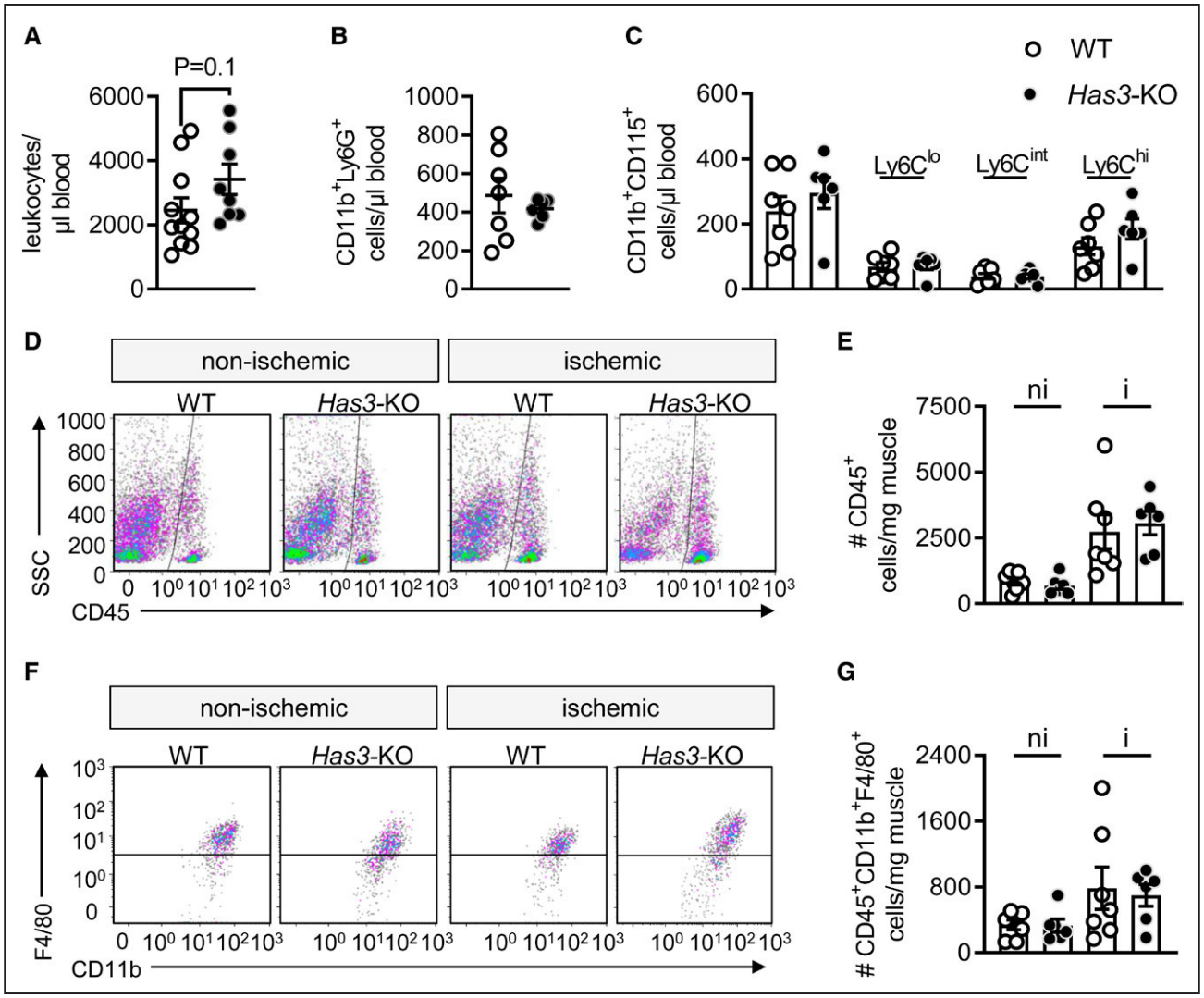


Figure 3. No differences in inflammatory response after femoral artery ligation (FAL).
Flow cytometric analysis of circulating leukocytes 72 h after electrocoagulation of the femoral artery in *Has3*-deficient (*Has3*-KO) and wild-type (WT) mice determined as cells per microliter blood with (**A**) leukocytes (CD45$^+$), (**B**) neutrophils (CD11b$^+$Ly6G$^+$), (**C**) monocytes (CD11b$^+$CD115$^+$) and monocyte subpopulations (CD11b$^+$CD115$^+$Ly6C^{lo}, CD11b$^+$CD115$^+$Ly6C^{int}, CD11b$^+$CD115$^+$Ly6C^{hi}); n=6–11. Flow cytometric analysis of leukocyte infiltration in nonischemic (ni) and ischemic (i) thigh muscles 72 h after electrocoagulation of the left femoral artery shown as cells per mg muscle tissue with (**D** and **E**), leukocytes (CD45$^+$) and (**F** and **G**) macrophages (CD45$^+$CD11b$^+$F4/80$^+$); n=6–7. Representative plots are gated on (**D**) living cells, (**E**) CD11b$^+$Ly6G$^-$ cells. Data represent mean±SEM. SSC indicates side scatter.

and Figure 5A and 5B, Figure VII in the Data Supplement). However, the exact mechanism by which extracellular HAS3-derived hyaluronan triggers intracellular AKT/eNOS signaling was still missing. Studies suggested enrichment of CD44 in plasmalemmal caveolae, one of the main compartments of eNOS phosphorylation at Ser1177.[29] Thus, we hypothesized that HAS3-derived hyaluronan needs CD44 as a signaling partner to stimulate AKT-dependent eNOS phosphorylation at Ser1177, the pathway which mediates rapid FMD responses.

To test this hypothesis, we administered a CD44 neutralizing antibody (clone KM201), designed to specifically block the hyaluronan binding epitope, to C57BL6/J mice followed by FMD measurements. Since CD44 was reported to be highly expressed on leukocytes,[30] we performed flow cytometric analyses on blood cells to test antibody efficiency and found that antibody injections were sufficient to almost completely block CD44 on leukocytes (Figure VIII in the Data Supplement) allowing us to investigate FMD in the context of CD44 inhibition: Despite unaltered flow-velocity (data not shown), the FMD response was completely abolished in C57BL6/J mice treated with the CD44 blocking antibody as compared with isotype-treated controls (Figure 5C and 5D). Notably, CD44 blocking resembled the phenotype of EC-specific *Has3*-KO and *eNOS*-deficiency in that FMD responses were also strongly inhibited in EC-*Has3*-KO (Figure 5A and 5B) and completely abolished in eNOS-deficient mice as compared with control mice (Figure 5E and 5F).

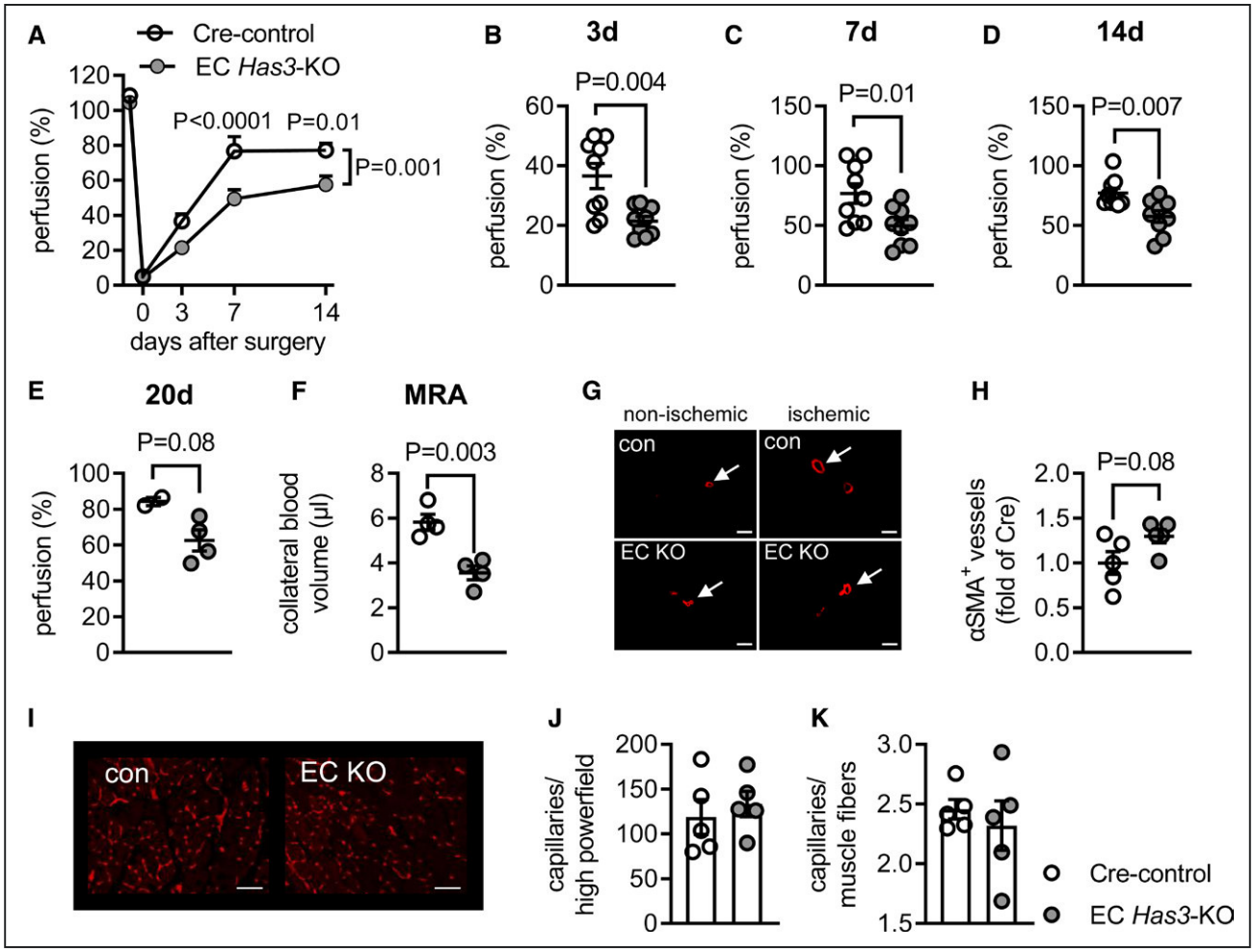


Figure 4. Endothelial hyaluronan synthase 3 is the major driver of blood flow recovery after femoral artery ligation (FAL). **A**–**E**, Hindlimb perfusion determined by laser Doppler perfusion imaging (LDPI; **A**) overtime and at (**B**) 3, (**C**) 7, (**D**) 14, and (**E**) 20 days after FAL in endothelial-specific *Has3*-deficient (EC *Has3*-KO) and respective cre-control mice. Perfusion was calculated as the percentage of ischemic over nonischemic hindlimb; n=9 (**A**–**D**); n=2–4 (**E**). **F**, Collateral blood volume determined by magnetic resonance angiography; n=4. **G**, Representative α-SMA staining of nonischemic and ischemic thigh muscle sections from cre-control (con) EC *Has3*-KO (EC KO) mouse showing arterioles. **H**, Quantification of α-SMA-positive vessels in whole ischemic thigh muscle sections. shown as fold of Cre-control mice; n=5. **I**, Representative image of CD31-staining (red) in ischemic calf muscle sections of cre-control and EC *Has3*-KO mice (EC KO). **J**, Number of capillaries per high power field. **K**, Number of capillaries normalized to muscle fibers; n=5. Bars represent 50 µm. Data represent mean±SEM. Statistical significance was assessed by 2-way ANOVA and Sidak post hoc test in **A**, and unpaired Student *t* test in **B**–**F**, **H**.

Role of the HAS3-Hyaluronan/CD44 Axis in the Arteriogenic Process

Since elevated shear stress is the dominant driver of arteriogenesis, intact endothelial flow-sensing is a prerequisite for induction of collateral artery remodeling after hindlimb ischemia. Having shown its crucial relevance in FMD we suggested a critical role of the endothelial HAS3-hyaluronan/CD44 axis for long-term perfusion recovery post-FAL.

Therefore, we treated *Has3*-KO and WT mice with Rat IgG isotype or anti CD44 clone KM201 one hour before, 24 and 48 hours after FAL (Figure 6A). Laser Doppler perfusion imaging measurements served to control perfusion. While we could not detect any differences immediately after FAL (Figure 6B), perfusion recovery was significantly impaired after 21 days in anti-CD44 treated WT mice as compared with isotype controls (mean: 63% versus 92%); Figure 6C). Thus, we confirmed the high relevance of early hyaluronan/CD44 signaling for long-term perfusion recovery in the setting of FAL.

Assuming this hyaluronan/CD44 interaction as the underlying mechanism for *Has3*-mediated effects, we repeated this experiment in *Has3*-KO mice: In line with our findings in WT mice, we did not find any differences in hindlimb perfusion immediately post-FAL (Figure 6D). Strikingly, *Has3*-KO deficiency reduced perfusion to a similar extent as CD44 blocking in WT mice (Figure 6E versus 6C): *Has3*-KO+iso: 68% and WT+anti-CD44: 63% versus WT+iso: 91%. Remarkably, we did not observe any additional reduction in blood flow recovery after blocking CD44 in *Has3*-KO mice (Figure 6E).

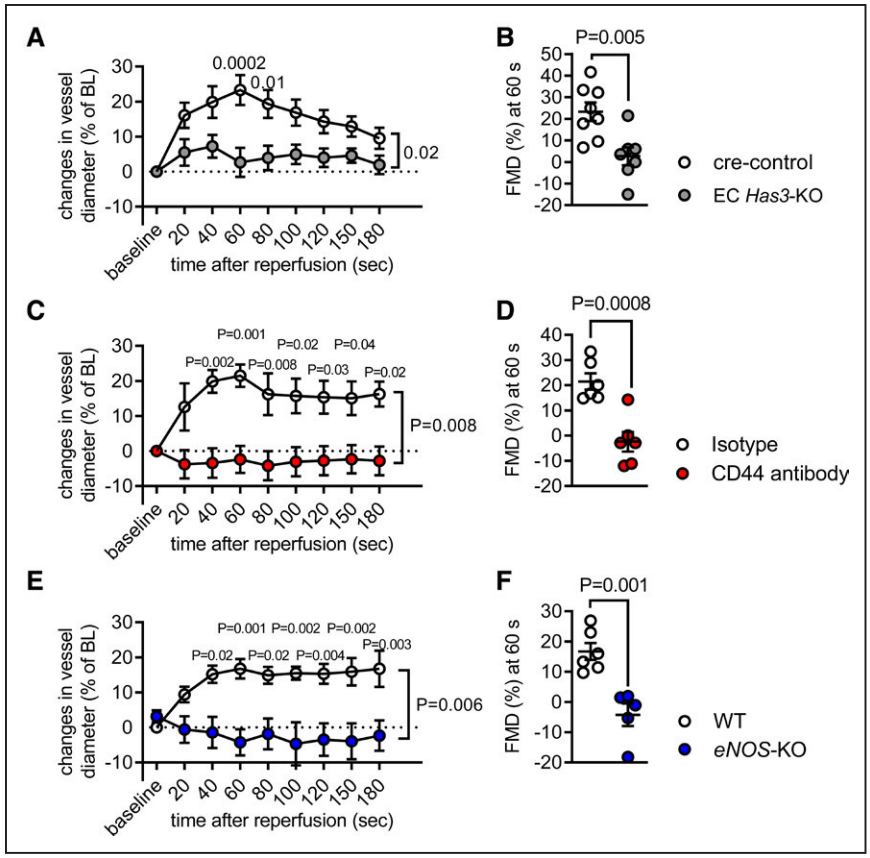


Figure 5. Treatment of C57BL6/J mice with anti-CD44 antibody clone KM201 impairs vascular response to acute increase in blood flow.
A and **B**, Flow-mediated dilation (FMD) of the iliac artery in endothelial-specific *Has3*-KO (EC *Has3*-KO) and respective cre-control mice. **A**, Overtime and (**B**) 60s after cuff release following 5-minute distal ischemia of the lower limb; n=7 and 8. C57BL6/J wild-type (WT) mice were injected with anti-CD44 antibody clone KM201 or isotype control at time points 0 and 24 h. At 25 h, FMD was measured. **C** and **D**, Flow-mediated dilation (FMD) of the iliac artery in CD44-antibody- and isotype-treated mice (**C**), overtime and (**D**) 60s after cuff release following 5 min distal ischemia of the lower limb; n=6. **E** and **F**, FMD of the iliac artery in *eNOS-knockout* (KO) mice and WT controls **E**, overtime and **F**, 60s after cuff release following 5 min distal ischemia of the lower limb; n=5 and 6. Data represent mean±SEM. Statistical significance was assessed by 2-way ANOVA and Sidak post hoc test in **A**, **C**, **E** and unpaired Student *t* test in **B**, **D**, and **F**.

DISCUSSION

To our knowledge, this is the first study elucidating the impact of HAS3 on endothelium-dependent FMD and collateral artery growth in vivo. Our data demonstrate that endothelial HAS3 is involved in shear stress dependent AKT/eNOS activation in ECs and contributes to FMD of the conduit arteries in mice. We determined that hyaluronan-mediated effects on FMD are critically dependent on hyaluronan/CD44 interaction. Furthermore, we provide evidence that endothelial the HAS3/hyaluronan-CD44 axis is a crucial determinant for collateral artery formation after hindlimb ischemia. Considering eNOS activation as a major driver for arteriogenesis, we conclude that HAS3 promotes collateral artery remodeling through endothelial eNOS signaling.

Under baseline conditions, native collaterals are only poorly perfused. However, after femoral artery occlusion, blood flow is redirected through these small anastomoses leading to a dramatic increase in fluid shear stress which marks the very first step in the arteriogenic cascade.[25] Following recognition via mechanosensory complexes, fluid shear stress activates intracellular kinases such as AKT (protein kinase B [PKB]), PKA (protein kinase A), or AMPK (adenosine monophosphate-activated protein kinase) leading to eNOS phosphorylation at Ser1177 and NO mediated SMC relaxation.[26] Among all kinases described to target eNOS at Ser1177, PI3K (phosphoinositol-3-kinase) dependent AKT is considered the most relevant for flow-mediated responses in vivo.[27–29,31,32]

Consequently, flow-mediated AKT/eNOS phosphorylation is a major trigger for collateral artery growth and exactly this pathway was found to be strongly impaired in global and endothelial-specific *Has3*-deficient mice: While total eNOS content was unchanged, eNOS phosphorylation at Ser1177 and AKT phosphorylation at Ser473 were substantially reduced giving rise to a massive decline in peak FMD as an expression of endothelial dysfunction.

Importantly, eNOS/NO signaling is not only responsible for shear stress-mediated vasodilation but also contributes to EC and SMC proliferation/migration during collateral artery growth. Therefore, impaired eNOS signaling shapes the SMC response in that expansion of the SMC-rich layer is strongly delayed.[33] Indeed, studies revealed severely impaired blood flow recovery after FAL in *eNOS*-deficient mice.[34] Mechanistically, impaired activation of cell cycle genes such as Ki67 in *eNOS*-deficient mice contributed to reduced EC and vascular smooth cell proliferation in growing collaterals. In contrast, leukocyte recruitment and inflammatory response genes were not affected. Similar to *eNOS*-deficient mice, lack of endothelial HAS3 caused disturbed collateral growth and impaired perfusion recovery despite unaltered leukocyte recruitment.

A possible explanation for the observed effects are changes in endothelial mechanosensing, which themselves are likely to be mediated by alterations of the endothelial glycocalyx, a highly dynamic meshwork

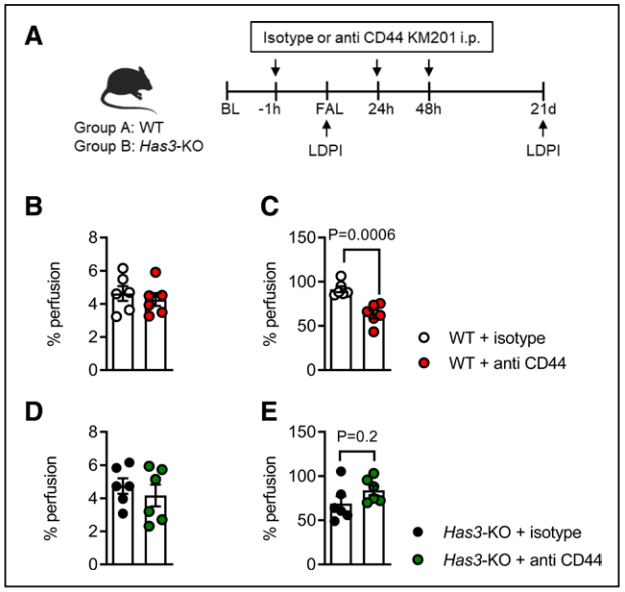


Figure 6. Impaired perfusion recovery in C57BL6/J mice treated with anti-CD44 antibody clone KM201.
A, Experimental scheme. C57BL6/J wild type (WT; group A) and *Has3*-deficient (*Has3*-KO) mice (group B) were injected with anti-CD44 antibody clone KM201 or isotype control one hour before, 24 and 48 h after femoral artery ligation (FAL). Hindlimb perfusion was measured via laser Doppler perfusion imaging (LDPI) immediately (**B**–**D**) and 21 days after FAL (**C**–**E**); n=6. Data represent mean±SEM. Statistical significance was assessed by unpaired Student *t* test in **C** and **E**.

consisting of glycoproteins, proteoglycans, and associated glycosaminoglycans.[35] Importantly, the glycocalyx not only serves as a protective coat against platelet adhesion and inflammation but is also involved in transmitting shear stress to the underlying endothelium. According to the so-called bumper car model even small changes in glycocalyx composition can have severe effects on mechanotransduction.[36] Considering hyaluronan as the second most abundant glycosaminoglycan in the glycocalyx following heparan sulfate, lack of hyaluronan synthesis likely interferes with its mechanosensory properties. Indeed, Potter et al[37] showed that complete depletion of the endothelial hyaluronan matrix by 4-methylesculetin suppressed mechanotransduction as well as arteriogenesis in mice. However, 4-methylesculetin inhibits all 3 isoenzymes and in addition, might exhibit pleiotropic effects as described for the HAS-inhibitor 4-MU.[38] Therefore, care should be taken when drawing conclusions on HAS-specific effects from this study.

A recent study reported on the role of HAS2-mediated biosynthesis of the endothelial glycocalyx in response to shear in the aortic arch as well as in human umbilical vein EC, a process crucially dependent on EC glycolysis.[15] Given the high heterogeneity of ECs,[39] it might be assumed that this process likely differs depending on the vascular bed as well as the stimulus itself. Further, since the stimulus differs in various pathological conditions (eg, acute versus chronic development of shear stress) also the response might diverge. In murine hindlimb muscles, HAS3 was shown to be the major isoenzyme suggesting a significant role in the adaptive response to arterial occlusion. Indeed, our study demonstrates for the first time that endothelial HAS3 is critically involved in mechanosignaling and flow-responses in vivo.

It is assumed that components of the glycocalyx such as syndecan facilitate mechanotransduction by their direct connectivity to both the endothelial glycocalyx and the cytoskeleton.[40] Based on the fact, that hyaluronan lacks this connectivity, hyaluronan needs to be attached to a membrane protein with associations to the cytoskeleton. As mentioned before, hyaluronan mediates its effects via certain receptors such as RHAMM or CD44. In the context of hyaluronan-dependent eNOS signaling, CD44 was attributed a specific role due to its high abundance in plasmalemmal caveolae.[41] These cholesterol rich scaffolds are mechanosensitive microdomains enriched in signaling molecules such as G protein coupled receptors, (tyrosine) kinases, phosphatases and, most importantly, eNOS.[42,43] Since clustering of these molecules facilitates signal transduction, it is possible that hyaluronan-mediated clustering of CD44 gives rise to enhanced activation of kinases involved in eNOS phosphorylation at Ser1177. Therefore, we concluded that elevated shear stress is sensed by HAS3-derived hyaluronan using CD44 as a signaling partner to mediate AKT and eNOS activation.

CD44 itself lacks intrinsic kinase activity[40] needed for AKT phosphorylation at Ser473. However, CD44 is known to recruit intracellular kinases and adaptor molecules upon shear stress-mediated receptor clustering. Of note, the cytoplasmic tail of CD44 has been reported to contain crucial binding motifs for PI3K,[44] a major activator of AKT. Thus, enhanced hyaluronan-dependent clustering of CD44 might activate the PI3K/AKT pathway which gives rise to eNOS activation.

To further investigate this potential mechanism, we blocked CD44 in vivo using a specific CD44 blocking antibody (clone KM201) and tested its impact on FMD. Of note, blocking of CD44 completely abolished FMD response as compared with isotype control, thereby mimicking the phenotype of global and endothelial *Has3*-KO. In contrast to other CD44 antibody clones such as IM7, clone KM201 is highly specific for the hyaluronan binding site of CD44. Thus, the observed antibody mediated effects on FMD were likely hyaluronan dependent and other effects such as CD44 receptor shedding as a possible confounder could be excluded. Strikingly, CD44 blocking also mimicked the phenotype of e*NOS*-deficient mice pointing towards a high relevance of hyaluronan/CD44-dependent signaling for endothelial function and flow-induced mechanosignaling in vivo as well as arteriogenesis post-FAL.

The significance of CD44 for arteriogenesis has been suggested in studies showing a strong increase in CD44 expression during collateral artery growth and impaired

arteriogenic response in CD44-deficient mice.[45] However, as glycoprotein with many interaction partners including hyaluronan, ECM proteins, growth factors or cytokines,[46] systemic lack of CD44 is likely to disrupt multiple signaling pathways in different cell types, making it challenging to draw conclusions about the underlying mechanism. Here, we provide novel mechanistic insight by identifying endothelial HAS3 derived hyaluronan as an important mediator for CD44's effects in arteriogenesis.

First, we showed that reduced blood flow recovery in *Has3*-deficient could not be further decreased by blocking CD44-hyaluronan interaction in these mice. This finding strongly suggests that the effects of Has3 on reperfusion recovery are fully mediated by the Has3-hyaluronan/CD44 axis and that other potential CD44 ligands such as osteopontin are not involved. In addition, also in FMD measurements similar responses to blocking CD44 and endothelial-specific *HAS3*-deficiency were observed thereby further highlighting the crucial role of CD44 in mediating HAS3 effects. Since an increase in FMD of only 1% decreases the risk for future cardiovascular events by already 13% in humans,[47] our findings of >20% change in FMD point towards a high translational relevance of the HAS3-hyaluronan/CD44 axis for endothelial function and vascular health.

Although it is widely accepted that elevated shear stress is a major trigger for collateral artery growth after arterial occlusion, it is still a matter of debate how flow is exactly sensed. Here, we report that endothelial HAS3 derived hyaluronan likely functions as mechanosensor and demonstrate that this has a functional significance in vivo for acute (FMD) and chronic (hindlimb ischemia) changes in blood flow. Furthermore, to our knowledge this is the first study reporting on CD44's role in endothelial function in vivo highlighting its specific function as a mechanotransducer for hyaluronan-derived signals.

In summary, our study demonstrates that endothelial HAS3 contributes to arteriogenesis in hindlimb ischemia by hyaluronan/CD44-mediated stimulation of eNOS phosphorylation at Ser1177. In the future, either hyaluronan application or pharmacological CD44 agonists might be considered in translational studies to improve therapeutic options in PAD.

ARTICLE INFORMATION

Received April 14, 2020; accepted July 26, 2021.

Affiliations

Institute for Pharmacology and Clinical Pharmacology, Medical Faculty (R.S., T.S., C.H., C.L., T.F., J.W.F., M.G.), Clinic for Cardiology, Pneumology and Angiology (D.S., M.K.), and Experimental Cardiovascular Imaging, Institute for Molecular Cardiology (U.F.), University Clinics and Heinrich-Heine University Düsseldorf, Germany. CARID, Cardiovascular Research Institute Düsseldorf, University Hospital Düsseldorf, Heinrich-Heine-University, Germany (M.K., J.W.F.).

Acknowledgments

We wish to acknowledge Petra Rompel, Kerstin Freidel, Martina Hübel, Beate Weyrauther, and Peggy Mara-Mann for technical support.

Sources of Funding

This study was supported by the Deutsche Forschungsgemeinschaft SFB 1116 (B10 to M. Grandoch and U. Flögel; B06 to M. Kelm; A08 to J.W. Fischer), IRTG 1902 (P7 to J.W. Fischer; P12 to M. Grandoch), Forschungskommission der Heinrich-Heine-Universität, Walter-Clawiter-Stiftung and Friede-Springer-Herzstiftung.

Disclosures

None.

Supplemental Materials

Supplemental Methods
Online Figures I–IX
Online Movies I and II
References[1–4]

REFERENCES

1. Shu J, Santulli G. Update on peripheral artery disease: Epidemiology and evidence-based facts. *Atherosclerosis*. 2018;275:379–381. doi: 10.1016/j.atherosclerosis.2018.05.033
2. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. *Circ Res*. 2015;116:1509–1526. doi: 10.1161/CIRCRESAHA.116.303849
3. Bhatt DL, Peterson ED, Harrington RA, Ou FS, Cannon CP, Gibson CM, Kleiman NS, Brindis RG, Peacock WF, Brener SJ, et al; CRUSADE Investigators. Prior polyvascular disease: risk factor for adverse ischaemic outcomes in acute coronary syndromes. *Eur Heart J*. 2009;30:1195–1202. doi: 10.1093/eurheartj/ehp099
4. Grenon SM, Vittinghoff E, Owens CD, Conte MS, Whooley M, Cohen BE. Peripheral artery disease and risk of cardiovascular events in patients with coronary artery disease: insights from the Heart and Soul Study. *Vasc Med*. 2013;18:176–184. doi: 10.1177/1358863X13493825
5. Xu Y, Arora RC, Hiebert BM, Lerner B, Szwajcer A, McDonald K, Rigatto C, Komenda P, Sood MM, Tangri N. Non-invasive endothelial function testing and the risk of adverse outcomes: a systematic review and meta-analysis. *Eur Heart J Cardiovasc Imaging*. 2014;15:736–746. doi: 10.1093/ehjci/jet256
6. Charakida M, Masi S, Lüscher TF, Kastelein JJ, Deanfield JE. Assessment of atherosclerosis: the role of flow-mediated dilatation. *Eur Heart J*. 2010;31:2854–2861. doi: 10.1093/eurheartj/ehq340
7. Kiani S, Aasen JG, Holbrook M, Khemka A, Sharmeen F, LeLeiko RM, Tabit CE, Farber A, Eberhardt RT, Gokce N, et al. Peripheral artery disease is associated with severe impairment of vascular function. *Vasc Med*. 2013;18:72–78. doi: 10.1177/1358863X13480551
8. Silvestro A, Scopacasa F, Ruocco A, Oliva G, Schiano V, Zincarelli C, Brevetti G. Inflammatory status and endothelial function in asymptomatic and symptomatic peripheral arterial disease. *Vasc Med*. 2003;8:225–232. doi: 10.1191/1358863x03vm503oa
9. Vita JA, Holbrook M, Palmisano J, Shenouda SM, Chung WB, Hamburg NM, Eskenazi BR, Joseph L, Shapira OM. Flow-induced arterial remodeling relates to endothelial function in the human forearm. *Circulation*. 2008;117:3126–3133. doi: 10.1161/CIRCULATIONAHA.108.778472
10. Ebong EE, Lopez-Quintero SV, Rizzo V, Spray DC, Tarbell JM. Shear-induced endothelial NOS activation and remodeling via heparan sulfate, glypican-1, and syndecan-1. *Integr Biol (Camb)*. 2014;6:338–347. doi: 10.1039/c3ib40199e
11. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell*. 1990;61:1303–1313. doi: 10.1016/0092-8674(90)90694-a
12. Mochizuki S, Vink H, Hiramatsu O, Kajita T, Shigeto F, Spaan JA, Kajiya F. Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. *Am J Physiol Heart Circ Physiol*. 2003;285:H722–H726. doi: 10.1152/ajpheart.00691.2002
13. Turley EA, Austen L, Vandeligt K, Clary C. Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. *J Cell Biol*. 1991;112:1041–1047. doi: 10.1083/jcb.112.5.1041
14. Singleton PA, Bourguignon LY. CD44 interaction with ankyrin and IP3 receptor in lipid rafts promotes hyaluronan-mediated Ca2+ signaling leading to nitric oxide production and endothelial cell adhesion and proliferation. *Exp Cell Res*. 2004;295:102–118. doi: 10.1016/j.yexcr.2003.12.025
15. Wang G, Kostidis S, Tiemeier GL, Sol WMPJ, de Vries MR, Giera M, Carmeliet P, van den Berg BM, Rabelink TJ. Shear stress regulation of endothelial glycocalyx structure is determined by glucobiosynthesis. *Arterioscler Thromb Vasc Biol*. 2020;40:350–364. doi: 10.1161/ATVBAHA.119.313399

16. Homann S, Grandoch M, Kiene LS, Podsvyadek Y, Feldmann K, Rabausch B, Nagy N, Lehr S, Kretschmer I, Oberhuber A, et al. Hyaluronan synthase 3 promotes plaque inflammation and atheroprogression. *Matrix Biol*. 2018;66:67–80. doi: 10.1016/j.matbio.2017.09.005
17. Kiene LS, Homann S, Suvorava T, Rabausch B, Müller J, Kojda G, Kretschmer I, Twarock S, Dai G, Deenen R, et al. Deletion of hyaluronan synthase 3 inhibits neointimal hyperplasia in mice. *Arterioscler Thromb Vasc Biol*. 2016;36:e9–16. doi: 10.1161/ATVBAHA.115.306607
18. Göthert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Göttgens B, Izon DJ, Begley CG. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood*. 2004;104:1769–1777. doi: 10.1182/blood-2003-11-3952
19. Schuler D, Sansone R, Freudenberger T, Rodriguez-Mateos A, Weber G, Momma TY, Goy C, Altschmied J, Haendeler J, Fischer JW, et al. Measurement of endothelium-dependent vasodilation in mice–brief report. *Arterioscler Thromb Vasc Biol*. 2014;34:2651–2657. doi: 10.1161/ATVBAHA.114.304699
20. Erkens R, Kramer CM, Lückstädt W, Panknin C, Krause L, Weidenbach M, Dirzka J, Krenz T, Mergia E, Suvorava T, et al. Left ventricular diastolic dysfunction in Nrf2 knock out mice is associated with cardiac hypertrophy, decreased expression of SERCA2a, and preserved endothelial function. *Free Radic Biol Med*. 2015;89:906–917. doi: 10.1016/j.freeradbiomed.2015.10.409
21. Hellingman AA, Bastiaansen AJ, de Vries MR, Seghers L, Lijkwan MA, Löwik CW, Hamming JF, Quax PH. Variations in surgical procedures for hind limb ischaemia mouse models result in differences in collateral formation. *Eur J Vasc Endovasc Surg*. 2010;40:796–803. doi: 10.1016/j.ejvs.2010.07.009
22. Aref Z, de Vries MR, Quax PHA. Variations in surgical procedures for inducing hind limb ischemia in mice and the impact of these variations on neovascularization assessment. *Int J Mol Sci*. 2019;20:E3704. doi: 10.3390/ijms20153704
23. Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W. Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol*. 2002;34:775–787. doi: 10.1006/jmcc.2002.2013
24. Fung E, Helisch A. Macrophages in collateral arteriogenesis. *Front Physiol*. 2012;3:353. doi: 10.3389/fphys.2012.00353
25. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res*. 2004;95:449–458. doi: 10.1161/01.RES.0000141145.78900.44
26. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605. doi: 10.1038/21224
27. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601. doi: 10.1038/21218
28. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, et al. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem*. 1999;274:30101–30108. doi: 10.1074/jbc.274.42.30101
29. Fulton D, Fontana J, Sowa G, Gratton JP, Lin M, Li KX, Michell B, Kemp BE, Rodman D, Sessa WC. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem*. 2002;277:4277–4284. doi: 10.1074/jbc.M106302200
30. Sherman L, Sleeman J, Herrlich P, Ponta H. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Curr Opin Cell Biol*. 1994;6:726–733. doi: 10.1016/0955-0674(94)90100-7
31. Figueroa XF, González DR, Puebla M, Acevedo JP, Rojas-Libano D, Durán WN, Boric MP. Coordinated endothelial nitric oxide synthase activation by translocation and phosphorylation determines flow-induced nitric oxide production in resistance vessels. *J Vasc Res*. 2013;50:498–511. doi: 10.1159/000355301
32. Fisslthaler B, Dimmeler S, Hermann C, Busse R, Fleming I. Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta Physiol Scand*. 2000;168:81–88. doi: 10.1046/j.1365-201x.2000.00627.x
33. Metaxa E, Meng H, Kaluvala SR, Szymanski MP, Paluch RA, Kolega J. Nitric oxide-dependent stimulation of endothelial cell proliferation by sustained high flow. *Am J Physiol Heart Circ Physiol*. 2008;295:H736–H742. doi: 10.1152/ajpheart.01156.2007
34. Dai X, Faber JE. Endothelial nitric oxide synthase deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis. *Circ Res*. 2010;106:1870–1881. doi: 10.1161/CIRCRESAHA.109.212746
35. Grandoch M, Bollyky PL, Fischer JW. Hyaluronan: a master switch between vascular homeostasis and inflammation. *Circ Res*. 2018;122:1341–1343. doi: 10.1161/CIRCRESAHA.118.312522
36. Thi MM, Tarbell JM, Weinbaum S, Spray DC. The role of the glycocalyx in reorganization of the actin cytoskeleton under fluid shear stress: a "bumper-car" model. *Proc Natl Acad Sci USA*. 2004;101:16483–16488. doi: 10.1073/pnas.0407474101
37. Potter DR, van Teeffelen J, Vink H, van den Berg BM. Perturbed mechanotransduction by endothelial surface glycocalyx modification greatly impairs the arteriogenic process. *Am J Physiol Heart Circ Physiol*. 2015;309:H711–H717. doi: 10.1152/ajpheart.00257.2015
38. Grandoch M, Flögel U, Virtue S, Maier JK, Jelenik T, Kohlmorgen C, Feldmann K, Ostendorf Y, Castañeda TR, Zhou Z, et al. 4-Methylumbelliferone improves the thermogenic capacity of brown adipose tissue. *Nat Metab*. 2019;1:546–559. doi: 10.1038/s42255-019-0055-6
39. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA*. 2003;100:10623–10628. doi: 10.1073/pnas.1434429100
40. Thorne RF, Wang Y, Zhang Y, Jing X, Zhang XD, de Bock CE, Oliveira CS. Evaluating nuclear translocation of surface receptors: recommendations arising from analysis of CD44. *Histochem Cell Biol*. 2020;153:77–87. doi: 10.1007/s00418-019-01835-y
41. Tarbell JM, Pahakis MY. Mechanotransduction and the glycocalyx. *J Intern Med*. 2006;259:339–350. doi: 10.1111/j.1365-2796.2006.01620.x
42. Patel HH, Murray F, Insel PA. Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu Rev Pharmacol Toxicol*. 2008;48:359–391. doi: 10.1146/annurev.pharmtox.48.121506.124841
43. Mineo C, Shaul PW. Regulation of eNOS in caveolae. *Adv Exp Med Biol*. 2012;729:51–62. doi: 10.1007/978-1-4614-1222-9_4
44. Ouhtit A, Rizeq B, Saleh HA, Rahman MM, Zayed H. Novel CD44-downstream signaling pathways mediating breast tumor invasion. *Int J Biol Sci*. 2018;14:1782–1790. doi: 10.7150/ijbs.23586
45. van Royen N, Voskuil M, Hoefer I, Jost M, de Graaf S, Hedwig F, Andert JP, Wormhoudt TA, Hua J, Hartmann S, et al. CD44 regulates arteriogenesis in mice and is differentially expressed in patients with poor and good collateralization. *Circulation*. 2004;109:1647–1652. doi: 10.1161/01.CIR.0000124066.35200.18
46. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003;4:33–45. doi: 10.1038/nrm1004
47. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *Int J Cardiovasc Imaging*. 2010;26:631–640. doi: 10.1007/s10554-010-9616-1

Original Article

Selective impairment of blood pressure reduction by endothelial nitric oxide synthase dimer destabilization in mice

Tatsiana Suvorava[a,b,*], Stephanie Pick[a,*], and Georg Kojda[a]

Objectives: Endothelial dysfunction and oxidative stress are associated with hypertension but whether endothelial superoxide may play a role in the early development of essential hypertension remains uncertain. We investigated whether endothelial nitric oxide synthase (eNOS)-derived endothelial oxidative stress is involved in the regulation of SBP.

Methods: Wild-type eNOS [mice with endothelium-specific overexpression of bovine endothelial NO-synthase (eNOS-Tg)] or a novel dimer-destabilized eNOS-mutant harboring a partially disrupted zinc-finger [mice with endothelium-specific overexpression of destabilized bovine eNOS destabilized by replacement of Cys 101 to Ala (C101A-eNOS-Tg)] was introduced in C57BL/6 in an endothelial-specific manner. Mice were monitored for aortic endothelium-dependent relaxation, SBP, levels of superoxide and several posttranslational modifications indicating activity and/or increased vascular oxidative stress. Some groups of mice underwent voluntary exercise training for 4 weeks or treatment with the superoxide dismutase mimetic Tempol.

Results: C101A-eNOS-Tg showed significantly increased superoxide generation, protein-tyrosine-nitration and eNOS-tyrosine-nitration, eNOS-*S*-glutathionylation, $eNOS^{1176/79}$ phosphorylation and AMP kinase-α phosphorylation at Thr172 in aorta, skeletal muscle, left ventricular myocardium and lung as compared with eNOS-Tg and wild-type controls. Exercise training increased phosphorylation of eNOS at $Ser^{1176/79}$ and AMP kinase-α in wild-type. These physiologic adaptations were absent in C101A-eNOS-Tg. Maximal aortic endothelium-dependent relaxation was similar in all strains. C101A-eNOS-Tg displayed normal SBP despite higher levels of eNOS, whereas eNOS-Tg showed significant hypotension. Tempol completely reversed the occurring protein modifications and significantly reduced SBP in C101A-eNOS-Tg but not in wild-type.

Conclusion: Oxidative stress generated by endothelial-specific expression of genetically destabilized C101A-eNOS selectively prevents SBP-reducing activity of vascular eNOS, while having no effect on aortic endothelium-dependent relaxation. These data suggest that oxidative stress in microvascular endothelium may play a role for the development of essential hypertension.

Keywords: blood pressure, endothelial oxidative stress, endothelial nitric oxide synthase, superoxide, transgenic mice

Abbreviations: C101A-eNOS, bovine eNOS destabilized by replacement of Cys 101 to Ala; C101A-eNOS-Tg, mice with endothelium-specific overexpression of destabilized C101A-eNOS; EDR, endothelium-dependent vasorelaxation; eNOS, endothelial NO-synthase; eNOS-KO, eNOS-deficient mice; eNOS-Tg, mice with endothelium-specific overexpression of bovine endothelial NO-synthase; L-NAME, *N*(G)-nitro-L-arginine methyl ester; sGC, soluble guanylyl cyclase; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine

INTRODUCTION

Vascular oxidative stress is considered as a pathophysiologic factor promoting cardiovascular diseases such as coronary artery disease, heart failure, diabetes and hypertension [1,2]. It has been suggested that an impairment of endothelial function at the level of resistance vessels may precede the development of cardiovascular disease and therefore is likely an additional risk factor [3]. For example, endothelial dysfunction at this level of vessels was identified in normotensive young adults with a family history of hypertension [4]. In another clinical study, it was shown that the magnitude of the impairment of endothelium function as measured by flow-dependent vasodilation in the right forearm may predict the onset of essential hypertension in postmenopausal women [5], although in this study the dilator response of the brachial artery to increased flow was assessed. However, as

Journal of Hypertension 2017, 35:76–88

[a]Institute of Pharmacology and Clinical Pharmacology and [b]Cardiovascular Research Laboratory, Division of Cardiology, Pulmonology and Vascular Medicine, University Hospital, Heinrich-Heine-University, Düsseldorf, Germany

Correspondence to Prof Dr Georg Kojda, PharmD, PhD, Institute of Pharmacology and Clinical Pharmacology, University Hospital, Heinrich-Heine-University, Moorenstr. 5, 40225 Düsseldorf, Germany. Tel: +49 211 81 12518; fax: +49 211 81 14781; e-mail: kojda@uni-duesseldorf.de

*Tatsiana Suvorava and Stephanie Pick contributed equally to the article.

Received 6 April 2016 **Revised** 21 July 2016 **Accepted** 26 August 2016

DOI:10.1097/HJH.0000000000001127

pointed out in a recent thorough review [6], hypertension also likely induces endothelial dysfunction associated with increased vascular oxidative stress. Therefore, the authors conclude that the interconnection between endothelial dysfunction and essential hypertension is two-sided, that is that each may worsen and/or improve the other.

In resistance vessels, endothelium-dependent vasorelaxation (EDR) to acetylcholine is different from that of conductance vessels such as aorta or coronary arteries. Resistance vessels possess many options to compensate for a loss of nitric oxide (NO) produced by endothelial NO synthase (eNOS) by other endothelium-dependent vasodilators such as prostaglandins, by neuronal NOS expression or by endothelium-derived hyperpolarizing factor [7–10]. Certainly, increased vascular oxidative stress is linked with a conversion of eNOS activity leading to a so-called uncoupled state in which eNOS produces more superoxide and less NO. In addition to deficiency of the cofactor tetrahydrobiopterin, mechanisms underlying this process include depletion of L-arginine, accumulation of endogenous asymmetrical dimethylarginine and eNOS-*S*-glutathionylation [11]. There are several sources of superoxide in vascular smooth muscle and endothelial cells [12] but the specific role of endothelial oxidative stress in the regulation of blood pressure (BP) before overt hypertension occurs, that is under otherwise healthy conditions, remains uncertain. Of note, one study showed that increased superoxide generation induced by a very strong eight-fold endothelial-specific overexpression of bovine eNOS and presumably caused by a shortage of the essential cofactor tetrahydrobiopterin does not change BP, as concomitant overexpression of guanosine triphosphate cyclohydrolase 1 to increase the endothelial generation of tetrahydrobiopterin in a double transgenic strain had no effect on BP [13]. However, in both transgenic mouse models, there is clear evidence for an impairment of vascular NO signaling, which might impact on BP as well. For example, the magnitude of hypotension observed in another mouse model characterized by endothelial-specific overexpression of eNOS, that is a reduction of SBP of about 15 mmHg, is achievable already by a 3.3-fold overexpression of bovine eNOS, and in this model no impairment of NO signaling and no increase of vascular oxidative stress is evident [14,15]. Therefore, it remains to be elucidated whether partial impairment of the catalytic function of eNOS and thus generation of endothelial oxidative stress in otherwise healthy conditions might support the development of hypertensive disease states such as essential hypertension.

We generated a mutant eNOS in which one of the two essential cysteines that are required for the coordination with the central Zn-ion, correct dimer formation and normal activity [16] is replaced by alanine [bovine eNOS destabilized by replacement of Cys 101 to Ala (C101A-eNOS)]. Expression of this mutant eNOS in human embryonic kidney cells 293 (HEK cells) resulted in a substantial reduction of citrulline formation in cell homogenates and similar data were obtained following investigation of purified C101A-eNOS, whereas at the same time superoxide generation detected by electron spin resonance was strongly increased [17]. This mutant eNOS was used to generate a novel transgenic mouse model [mice with endothelium-specific overexpression of destabilized C101A-eNOS (C101A-eNOS-Tg)] characterized by endothelial-specific overexpression of C101A-eNOS on a C57BL/6 background. In this novel transgenic mouse strain, we observed largely increased vascular oxidative stress but no impairment of vascular NO signaling suggesting that this transgenic model might help to clarify whether oxidative stress induced by impairment of the catalytic function of eNOS may impact on BP. To accomplish this, another newly generated mouse model characterized by an about 2.4-fold increased endothelial-specific overexpression of normal bovine eNOS was used for comparison purpose.

METHODS

Transgenic mice

Endothelial-specific overexpression of wild-type bovine eNOS (eNOS-Tg) [14,15] or a dimer-destabilized C101A-mutant eNOS [17] (C101A-eNOS-Tg) was achieved using the Tie-2 promoter. Mice were backcrossed to C57BL/6 for at least 13 generations. As for eNOS-Tg, lines from three founders were established [15]. In this study, mice from colony 5 were used. Transgene-negative littermates served as wild-type controls. Mice were sacrificed by inhalation of carbon dioxide, and tissues were either used for organ bath or lucigenin studies or immediately frozen in liquid nitrogen. Permission for the animal studies was provided by the regional Government of Germany (AZ 8.87-51.04.10.09.383 and AZ 84-02.04.2013.A213), and the experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' and the Directive 2010/63/EU of the European Parliament.

Western blot analysis

A total of 50–100 μg protein was loaded on discontinuous and denaturating 7.5–10% SDS-PAGE gels. After blotting on nitrocellulose membranes antibodies detecting total eNOS, Ser239-phospho-vasoactive stimulated protein (VASP), total VASP, Thr172-phospho-AMP kinase (AMPKα), total AMPKα, Ser473-phospho-protein kinase B (AKT), total AKT, α-actin and fluorescence dye-coupled secondary antibodies were used. Final detection and quantification was performed using Odyssey Infrared Imager 9120 (LI-COR Biosciences GmbH, Bad Homburg, Germany). As for eNOS dimer visualization, low-temperature native gels (6%) were used.

Real-time PCR

Quantification of murine and bovine eNOS-mRNA was carried out with species-specific primers using TaqMan (Thermo Fisher Scientific, Schwerte, Germany) technology. For murine eNOS, the comparative C_T-method was used for quantification, whereas for bovine expression no wild-type control was applicable. Here, individual data points were calculated [18].

Immunohistochemistry

Colocalization of eNOS and the endothelial-specific marker, CD31, in aortas of eNOS-deficient mice (eNOS-KO) and C101A-eNOS-Tg was performed as described previously [17,19].

Lucigenin-enhanced chemiluminescence

Superoxide was detected in tissue probes of aorta, left ventricular (LV) myocardium and skeletal muscle by lucigenin-enhanced chemiluminescence as described previously [20]. To ensure that superoxide is NOS-derived, the NOS blocker – *N*(G)-nitro-L-arginine methyl ester (L-NAME) – was applied additionally.

On-bead immunoprecipitation and fluorescence detection

By the use of Dynabeads M-280 Sheep anti-Rabbit IgG (Life Technologies GmbH, Darmstadt, Germany) loaded with a polyclonal total eNOS antibody, an immunoprecipitation of eNOS and related proteins was performed (Supplemental Fig. S1a and b, http://links.lww.com/HJH/A678). Detection and quantification were achieved by application of fluorescence dye-coupled secondary antibodies on Odyssey Infrared Imager 9120 (LI-COR Biosciences). A more detailed description is given in Supplementary material, http://links.lww.com/HJH/A678.

Vasorelaxation studies

After preconstriction of aortic rings with 0.2 μmol/l phenylephrine, accumulating concentrations (1 nmol/l–10 μmol/l) of acetylcholine were applied in an organ bath apparatus as described previously [21]. The contractile response of aorta to alpha1-agrenergic stimulation was assessed by a cumulative application of phenylephrine (0.01–10 μmol/l). As for the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 1 nmol/l–10 μmol/l), preconstriction was induced by 10 μmol/l phenylephrine.

Blood pressure and heart rate measurements

SBP and heart rate (HR) were obtained using the tail-cuff method as described previously [21].

Antioxidative treatment

The superoxide dismutase-mimetic Tempol was applied orally for 3 weeks in drinking water (1 mmol/l), which corresponds to a dosage of 80 μmol/kg body weight/day [22]. The drinking water was replaced daily.

Voluntary exercise training

Mice ($n = 6$ per group) underwent voluntary exercise training for 4 weeks on running wheels as described previously [23]. Briefly, mice were housed individually in cages equipped with running wheels (0.25 m in diameter, Tecniplast, Germany) and with counters to record the daily running distance. The efficacy of exercise training was ascertained by daily running distance and by heart weight/body weight ratio. The running distance in C101A-eNOS-Tg was 5596 ± 82.21 m/day and did not differ to the running distance of C57BL/6 (5421 ± 82.21 m/day).

Statistics

All results are presented as arithmetic mean ± SEM, n indicates the number of animals. Statistical comparisons between groups were performed by t tests, Newman–Keuls multiple comparisons post-hoc test following one-way analysis of variance (ANOVA, for more than two groups) or two-way ANOVA (concentration–response curves). For the comparison of pD2-values, that is half-maximal effective concentration (EC_{50}-value) in $-\log M$, and heart weight/body weight ratios, an unpaired two-tailed t test was used. For BP evaluation in one individual before and after intervention (Tempol treatment), a paired t test was used. All calculations and statistical evaluations were done using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, California, USA). A P value <0.05 was considered statistically significant.

Supplementary material, http://links.lww.com/HJH/A678

A more detailed description of Methods is provided in Supplementary material, http://links.lww.com/HJH/A678.

RESULTS

Expression of eNOS in eNOS-Tg and C101A-eNOS-Tg

Western blot analysis revealed a significantly increased eNOS protein expression in aorta, skeletal muscle, lung and LV myocardium of C101A-eNOS-Tg and, to a significantly greater extent, in eNOS-Tg (Fig. 1a and b; Supplemental Fig. S1c and d, http://links.lww.com/HJH/A678). Separation of aortic homogenates of C101A-eNOS-Tg on low-temperature gels demonstrated a considerable eNOS dimer destabilization, as evidenced by the strong monomer signal at 133 kDa (Fig. 1c) compared with transgene-negative littermates. Analysis of mRNA expression showed a significant downregulation of murine eNOS-mRNA in C101A-eNOS-Tg and eNOS-Tg and a stronger expression of bovine eNOS in eNOS-Tg than in C101A-eNOS-Tg, whereas there was no bovine signal in wild-type (Fig. 1d and e). The endothelial-specific localization of mutant eNOS is shown in Fig. 1f. There was a clearly visible endothelial-specific signal for CD31 [platelet endothelial cell adhesion molecule (PECAM-1)] but no signal for eNOS in eNOS-KO, whereas eNOS and CD31 were colocalized in C101A-eNOS-Tg.

Oxidative stress

Increased generation of superoxide quantified by lucigenin-enhanced chemiluminescence was evident in C101A-eNOS-Tg, and this was completely inhibited by L-NAME demonstrating NOS-dependency in aorta, skeletal muscle (Fig. 2a and b) and LV myocardium (Supplemental Fig. S2a, http://links.lww.com/HJH/A678). Likewise, we observed an increase of markers of oxidative stress such as eNOS-*S*-glutathionylation (Fig. 2c and d) and protein tyrosine-nitration in C101A-eNOS-Tg (Fig. 2e and f). Western blots using immune-precipitated eNOS protein revealed that eNOS itself is subject of tyrosine-nitration as evidenced by an overlay of the fluorescence signals generated with an eNOS antibody and a three-nitrotyrosine antibody (Supplemental Fig. S2b, http://links.lww.com/HJH/A678). Similar changes of eNOS-*S*-glutathionylation and protein tyrosine-nitration were observed in lung and myocardium of C101A-eNOS-Tg (Supplemental Fig. 2c–f, http://links.lww.com/HJH/A678). These data demonstrate significant endothelial oxidative stress in C101A-eNOS-Tg.

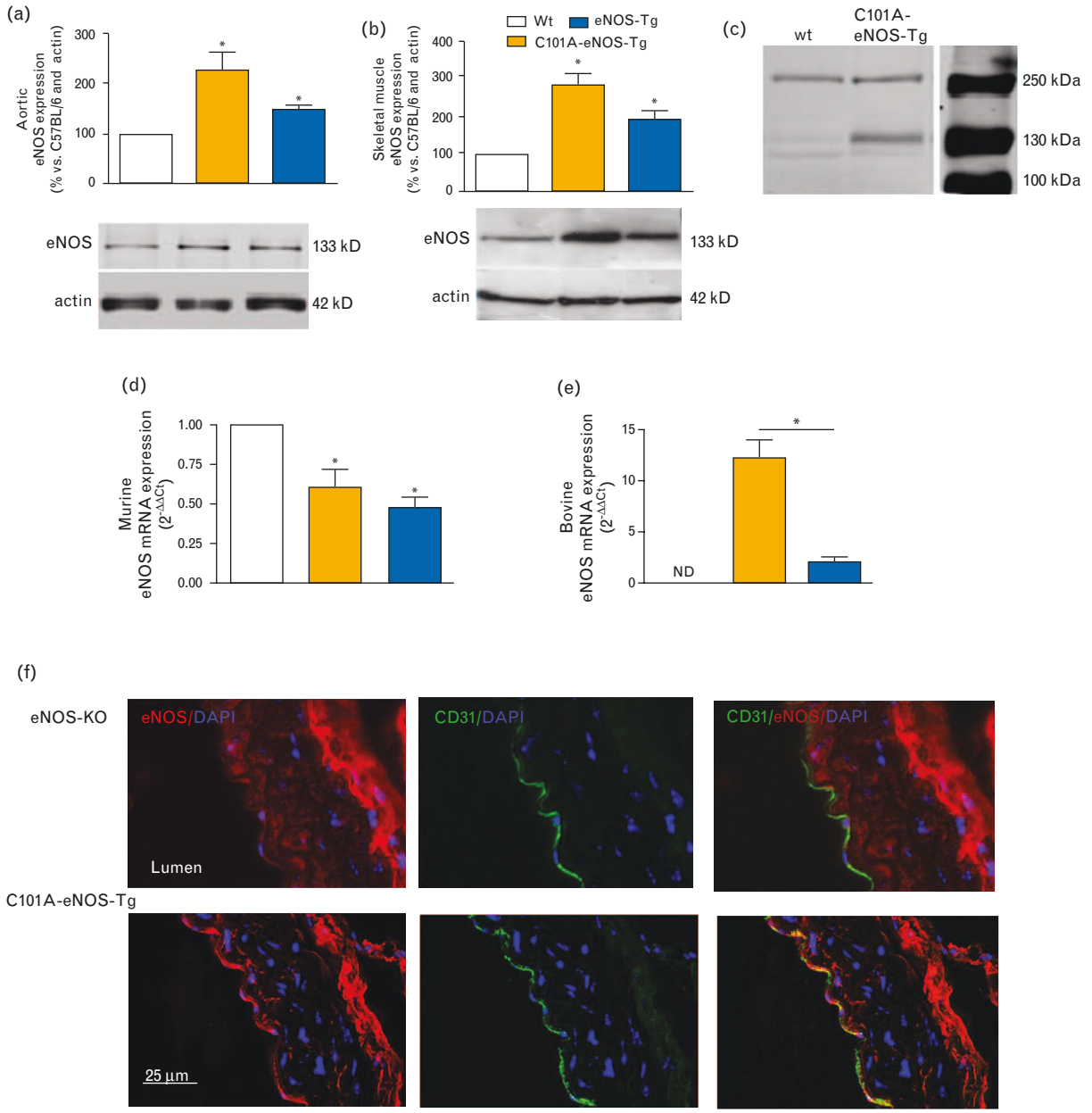


FIGURE 1 Expression of eNOS in eNOS-Tg and C101A-eNOS-Tg. eNOS protein expression in (a) aorta of wild-type, eNOS-Tg (*$P < 0.001$ vs. wild-type, $n = 6$ each, one-way analysis of variance) and C101A-eNOS-Tg (*$P < 0.05$ vs. wild-type, $n = 12$), and (b) in skeletal muscle of eNOS-Tg and C101A-eNOS-Tg (*$P < 0.01$ vs. wild-type, $n = 6$ each, one-way analysis of variance). Results are given as mean ± SEM percentage relative to wild-type (upper panel). The figure underneath shows representative Western blots for eNOS including β-actin expression as loading control. (c) Representative low-temperature gel showing a much more prominent monomer but similar dimer bands in aortic tissue of C101A-eNOS-Tg compared with wild-type. (d) Aortic murine eNOS mRNA-expression (fold change) in eNOS-Tg (*$P < 0.01$ vs. wild-type, $n = 6$ each, one-way analysis of variance) and C101A-eNOS-Tg ($P < 0.001$ vs. wild-type, $n = 6$ each, one-way analysis of variance) and (e) bovine eNOS mRNA-expression (individual data points) in eNOS-Tg ($n = 6$) and C101A-eNOS-Tg (*$P < 0.001$ vs. eNOS-Tg, t test, ND, not detectable, $n = 6$ each). (f) Images showing immunofluorescent staining for eNOS (red) and CD31 (pseudocolored in green) in the aorta of eNOS-KO and C101A-eNOS-Tg mice. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). There was no eNOS signal in the aorta of eNOS-deficient mice, whereas C101A-eNOS-Tg showed an endothelial-specific expression of eNOS as evident by its overlay with CD31 (yellow).

Functional studies

To evaluate whether oxidative stress in C101A-eNOS-Tg impacts on aortic EDR, concentration–response curves for acetylcholine were constructed in rings of thoracic aorta. The concentration-dependent dilatory response to acetylcholine was almost identical in all three strains studied (Fig. 3a and b), that is there was no detectable difference between eNOS-Tg, C101A-eNOS-Tg and their respective wild-types. However, a direct comparison of the half-maximal vasodilator concentration of acetylcholine expressed as pD_2 values revealed a small but significant difference between eNOS-Tg (7.006 ± 0.09) and C101A-eNOS-Tg (6.717 ± 0.09, $P = 0.047$), which was increased for the direct comparison of the respective transgene-negative littermates (wild-types) of eNOS-Tg (7.152 ± 0.09) and C101A-eNOS-Tg (6.595 ± 0.08, $P = 0.0011$). Therefore,

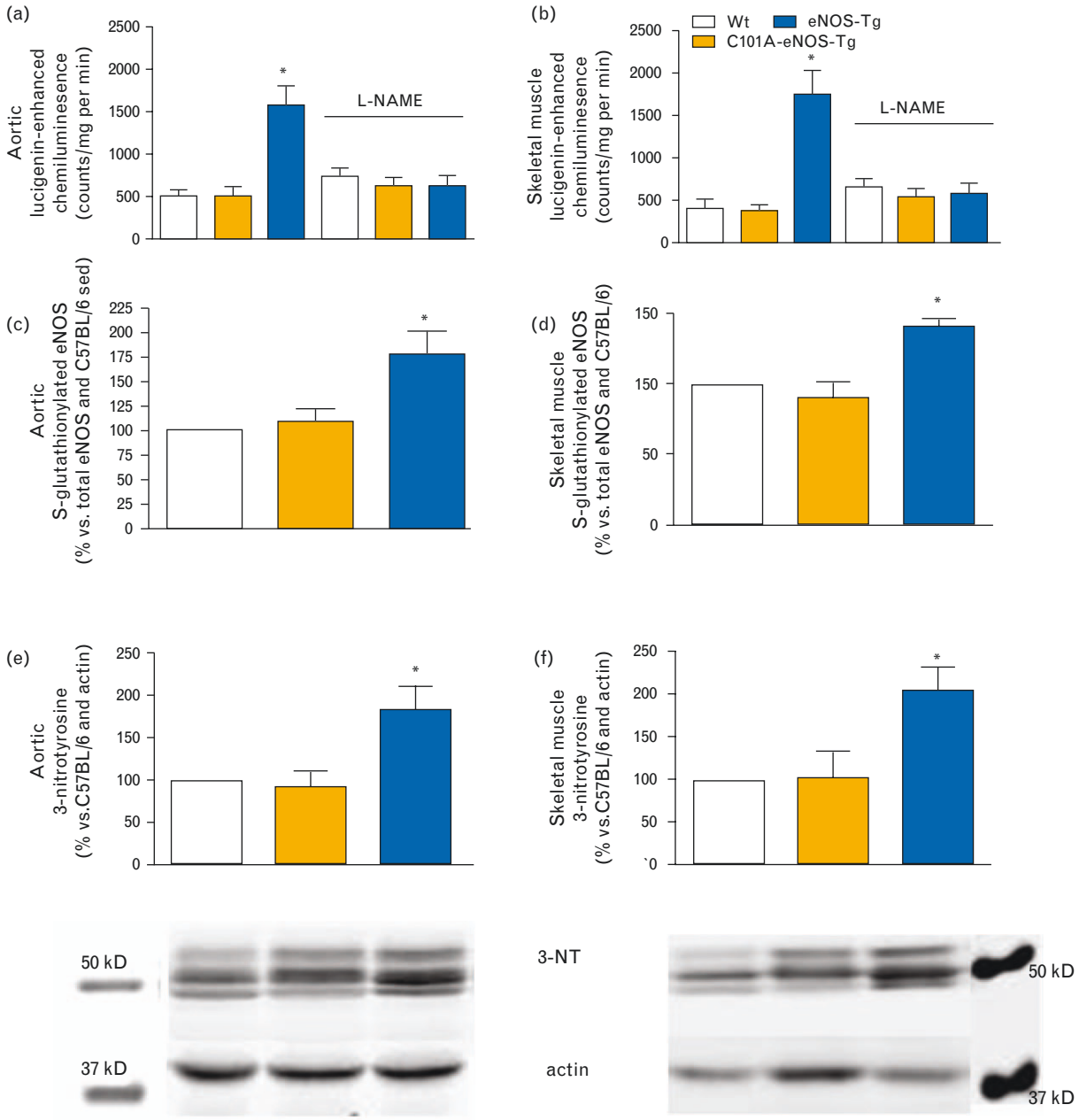


FIGURE 2 Oxidative stress and posttranslational protein modifications. Superoxide levels in (a) aorta and (b) skeletal muscle of wild-type, eNOS-Tg and C101A-eNOS-Tg ($P < 0.001$ vs. wild-type and eNOS-Tg, $n = 5$ each, one-way analysis of variance). *N*(G)-nitro-L-arginine methyl ester blunted the difference in both organs investigated indicating superoxide being produced by eNOS. (c) Aortic and (d) skeletal muscle eNOS-*S*-glutathionylation in eNOS-Tg and C101A-eNOS-Tg ($P < 0.01$ vs. wild-type and eNOS-Tg, $n = 6$ each, one-way analysis of variance) following on-bead immunoprecipitation and staining. Western blot analysis on 3-nitrotyrosine in (e) aorta and (f) skeletal muscle of eNOS-Tg and C101A-eNOS-Tg ($n = 5$–6 each, $P < 0.05$ vs. wild-type and eNOS-Tg, one-way analysis of variance). Results are given as mean ± SEM percentage relative to wild-type. The figure underneath shows representative Western blots for nitrotyrosine (all three bands at 55 kD were evaluated as described in our previous publication [15]) including β-actin expression as loading control.

increased superoxide in C101A-eNOS-Tg was obviously insufficient to induce an impairment of aortic EDR as the small differences between the two strains occurred in wild-types as well. Likewise, there was no difference between the concentration-dependent dilatory response to the NO-donor SNAP in eNOS-Tg, C101A-eNOS-Tg and their respective transgene littermates (Fig. 3c and d). Again, a direct comparison of the half-maximal vasodilator concentration expressed as pD_2 values revealed a small but significant difference between the respective transgene-negative littermates (wild-types) of eNOS-Tg (6.813 ± 0.058) and C101A-eNOS-Tg (6.448 ± 0.063, $P = 0.001$) but no significant difference was observed between eNOS-Tg (6.661 ± 0.123) and C101A-eNOS-Tg (6.389 ± 0.065, $P = 0.0609$). Taken together, these data suggest that neither transgene impacted on the sensitivity of the NO-cGMP pathway in these strains. This is in line with the results obtained for vasoconstrictor effects of phenylephrine which did not differ at all (Fig. 3e and f). However, we observed almost identical SBP in C101A-

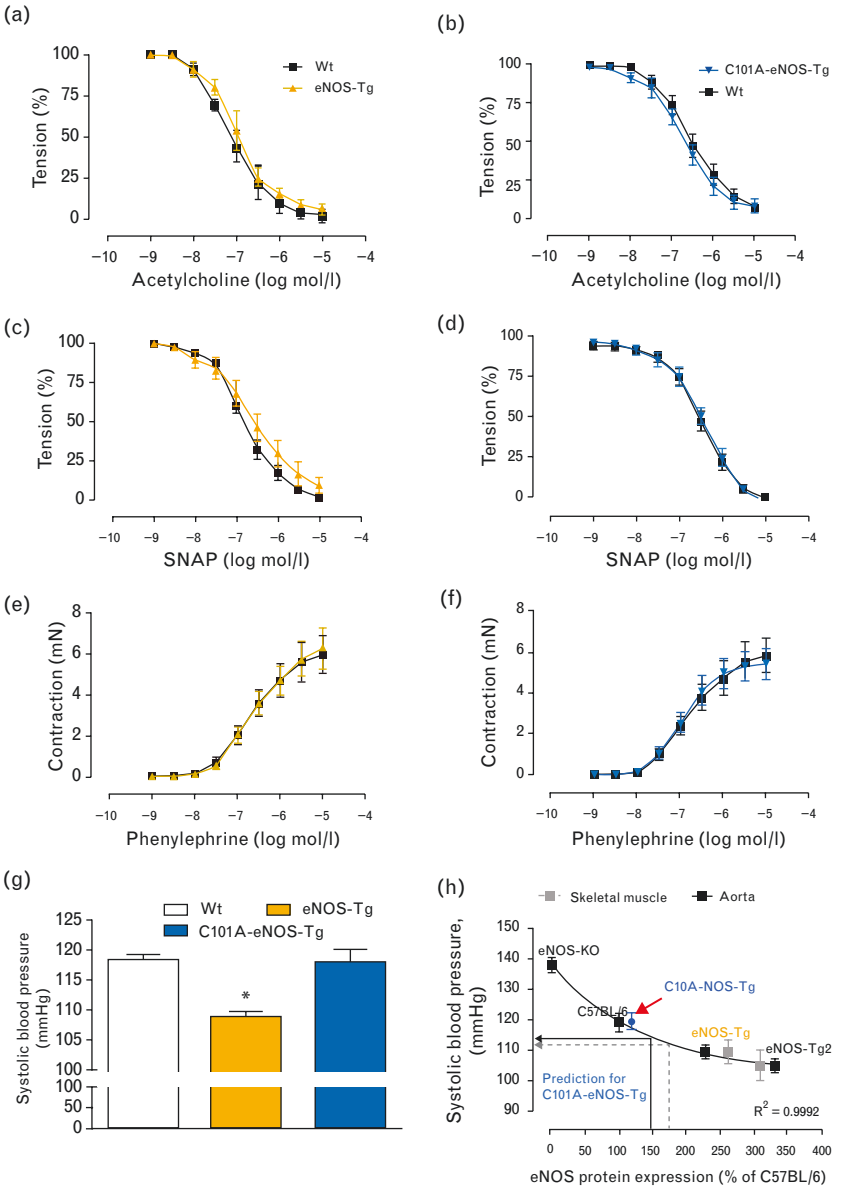


FIGURE 3 Functional studies on aortic reactivity in eNOS-Tg and C101A-eNOS-Tg. Neither aortic endothelium-dependent vasorelaxation in (a) eNOS-Tg ($n=5$ each) and (b) C101A-eNOS-Tg ($n=7$ each) nor sensitivity to exogenous NO by application of the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine differed in (c) eNOS-Tg ($n=5$ each) and (d) C101A-eNOS-Tg ($n=7$ each) compared with the respective wild-type and (e and f) similar results were obtained for vasoconstrictor effects of phenylephrine ($P>0.05$ for all comparisons, two-way analysis of variance). (g) Blood pressure measurements revealed a significant variation (*$P<0.0001$, $n=4$, one-way analysis of variance) that was dependent on a significant reduction of blood pressure in eNOS-Tg vs. wild-type ($P<0.0001$, $n=10$, Newman–Keuls) and vs. C101A-eNOS-Tg ($P<0.0001$, $n=6$, Newman–Keuls). Blood pressure values of the different transgene-negative littermates strains (wild-type) were combined. The mean blood pressure values for each wild-type strain were 118.3 ± 1.42 and 118.3 ± 2.8 mmHg. (h) Correlation of SBP with eNOS protein expression in aorta and skeletal muscle of eNOS-KO, C57BL/6, eNOS-Tg2 [15] and eNOS-Tg. The calculation of the expected blood pressure reduction was accomplished using the equation underlying the correlation [$R^2=0.9992$, 'blood pressure $=35.92\times e^{(-0.007267\times\chi)}+102.0$ mmHg'], for aortic eNOS expression, where χ is the percentage of aortic eNOS expression related to C57BL/6, and the equation underlying the correlation ($R^2=0.992$) for skeletal muscle eNOS expression [blood pressure $=35.56\times e^{(-0.007202\times\chi)}+102.3$ mmHg]. Given the aortic expression value for eNOS in C101A-eNOS-Tg of 147.3% and the skeletal muscle expression value 188.4% [Fig. 1a and b], the equations yielded a SBP value of 114.6 mmHg (angled black arrow) and of 112 mmHg (angled black dotted arrow), respectively. The red arrow indicates measured SBP in C101A-eNOS-Tg.

eNOS-Tg and wild-type, although overexpression of normal eNOS in eNOS-Tg resulted in a BP reduction (Fig. 3g), which confirms previous observations [15]. This difference appeared to be associated with a lower stimulation of protein kinase G in C101A-eNOS-Tg as estimated by VASP-Ser239-phosphorylation in aorta, skeletal muscle, lung and myocardium (Supplemental Fig. 3, http://links.lww.com/HJH/A678). We estimated the expected BP reduction induced by the magnitude of endothelial eNOS overexpression in C101A-eNOS-Tg. Significant correlations between resting SBP values and the expression of eNOS in skeletal ($R^2=0.992$) and aortic ($R^2=0.9992$) tissue of different transgenic strains with variations of eNOS expression were obtained (equation and plot are shown in Fig. 3h). The strains used for this comparison were eNOS-Tg, another eNOS-Tg colony (eNOS-Tg2) [15], eNOS-KO [24]

and C57BL/6. Calculation of the expected SBP based on the correlation with skeletal muscle values and the expression value for skeletal muscle eNOS in C101A-eNOS-Tg of 188.4% (Fig. 1b) yielded a value of 111.5 mmHg (Fig. 3h). Similarly, calculation of the expected SBP based on the correlation with aortic expression of eNOS and the expression value for aortic eNOS in C101A-eNOS-Tg of 147.3% (Fig. 1a) yielded a value of 114.3 mmHg (Fig. 3h).

Tempol treatment

As increased superoxide generation in C101A-eNOS-Tg appears to be the trigger for protein modifications (Fig. 2c–f), C101A-eNOS-Tg and wild-type mice received the superoxide dismutase mimetic Tempol by oral treatment. As shown in Fig. 4a and b, Tempol treatment had no effect on HR in all strains and significantly reduced the BP in C101A-eNOS-Tg but not in wild-type. The BP reduction observed in C101A-eNOS-Tg from 118.3 ± 0.71 to 113.3 ± 1.23 mmHg closely resembles the calculated values given above (Fig. 3h). Moreover, the cessation of Tempol treatment for 2 weeks restored the initial BP in C101A-eNOS-Tg confirming that the BP reduction was due to Tempol treatment. Lucigenin-enhanced chemiluminescence was no longer detectable in tissues of C101A-eNOS-Tg under Tempol treatment. In addition, the activity of Tempol was evident by inhibition of eNOS-*S*-glutathionylation (Fig. 4c and d) and eNOS-tyrosine-nitration (Fig. 4e and f). The extent of these posttranslational modifications induced by vascular oxidative stress was normalized in aorta and skeletal muscle, as well as in lung and myocardial tissue (Supplemental Fig. 4a–d, http://links.lww.com/HJH/A678). Likewise, Tempol treatment significantly increased VASP-Ser239 phosphorylation in aortic and skeletal muscle tissue of C101A-eNOS-Tg (Fig. 4g and h). None of these changes occurred in wild-type (Supplemental Fig. 4e and f, http://links.lww.com/HJH/A678).

Hyperphosphorylation of eNOS at Ser1176/79

Phosphorylation of eNOS at Ser1176/79 was increased in C101A-eNOS-Tg compared with wild-type but not in eNOS-Tg (Fig. 5a and b). Similar changes of eNOS-Ser1176/79-phosphorylation were observed in lung and myocardium of C101A-eNOS-Tg (Supplemental Fig. S5a and b, http://links.lww.com/HJH/A678). Based on the observation that eNOS in C101A-eNOS-Tg is hyperphosphorylated at Ser1176/79 and that this is sensitive to Tempol treatment (Fig. 5c and d), we sought to evaluate whether AKT (protein kinase B) or AMPK mediates this phosphorylation. Although the activity of AKT, as estimated by phosphorylation at Ser473, remained unchanged (Supplemental Fig. 5c and d, http://links.lww.com/HJH/A678), the activity of AMPKα, as estimated by phosphorylation at Thr172, was strongly increased in C101A-eNOS-Tg (Fig. 5e and f). This increase was completely abolished following Tempol treatment suggesting that activation of AMPKα is dependent on increased oxidative stress. Total AMPK was not changed in both strains (data not shown).

Exercise

To investigate whether increased eNOS Ser1176/79-phosphorylation in C101A-eNOS-Tg vs. C57BL/6 (Fig. 5a and b) could be further elevated at this site, we subjected both strains to 4 weeks of voluntary exercise as a model for increased shear stress *in vivo* as described previously [23]. This intervention significantly increased heart weight/body weight ratio in comparison with sedentary mice. In sedentary C57BL/6, heart weight/body weight ratio was 0.3884 ± 0.02157 mg/g, whereas in exercised C57BL/6, heart weight/body weight ratio was 0.4635 ± 0.01417 mg/g ($n = 6$ each, $P = 0.0032$). Likewise, in sedentary C101A-eNOS-Tg, heart weight/body weight ratio was 0.3827 ± 0.02697 mg/g, whereas in exercised C101A-eNOS-Tg, heart weight/body weight ratio was 0.4635 ± 0.02218 mg/g ($n = 6$, $P = 0.0148$). These data indicate the efficacy of the training protocol. Exercise strongly increased AMPKα-Thr172-phosphorylation and eNOS-Ser1176/79-phosphorylation in aortic and skeletal muscle tissue of wild-type, but not in C101A-eNOS-Tg (Fig. 6a–d). There was no difference in total AMPKα-expression (data not shown). In contrast, AKT-phosphorylation remained unchanged in both strains (Supplemental Fig. 6a and b, http://links.lww.com/HJH/A678). Exercise training also reduced eNOS-*S*-glutathionylation and eNOS-tyrosine-nitration in both wild-type and C101A-eNOS-Tg, and this reduction was much more pronounced in C101A-eNOS-Tg and not significant in wild-type (Supplemental Fig. 6e and f, http://links.lww.com/HJH/A678).

DISCUSSION

Essential hypertension is associated with an impairment of endothelium-dependent vasodilation, and in many different animal models, experimental hypertension was observed in coincidence with vascular oxidative stress [12]. In contrast, little is known about the specific role of eNOS-dependent formation of superoxide and thus the impact of endothelial oxidative stress on the regulation of BP. Here, we report that endothelial-specific 1.5-fold overexpression of a dimer-destabilized eNOS mutant in C57BL/6, proven by colocalization of CD31 (PECAM-1) and eNOS, generates endothelial oxidative stress as evidenced by increased formation of superoxide as well as posttranslational protein modifications such as nitration of tyrosine and *S*-glutathionylation in both aorta and in skeletal muscle tissue that primarily contains arterioles. Of note, mRNA expression of native murine eNOS was decreased, suggesting that the content of C101A-eNOS protein is greater than about one-third of total eNOS expression. Oxidative stress had no effect on aortic EDR but prevented the expected BP reduction by endothelial eNOS in an otherwise healthy and normotensive mouse strain. However, a significant and reversible reduction of BP was observed during treatment with the antioxidant Tempol indicating a causal role for dimer destabilization–induced oxidative stress, whereas Tempol showed no effect in C57BL6. These data strongly suggest that dimer destabilization of eNOS and the resulting increase of endothelium-specific superoxide generation is involved in the regulation of BP in C101A-eNOS-Tg.

Dimer destabilization of eNOS and oxidative stress

Mutation of eNOS at its Zn-binding cysteines has been shown to inhibit dimerization and generation of NO. For

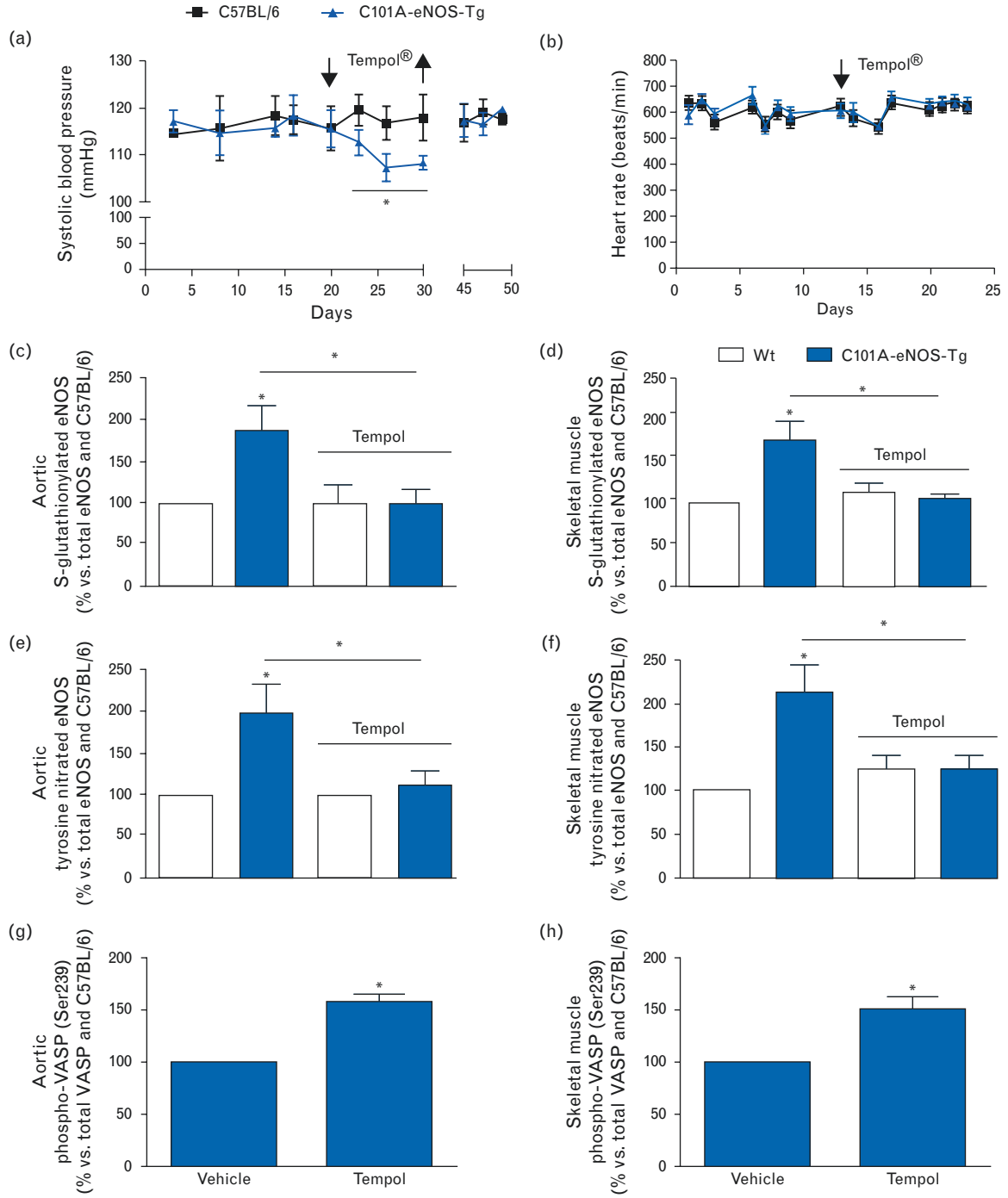


FIGURE 4 Tempol treatment. Time-dependent changes of (a) blood pressure and (b) heart rate in wild-type and in C101A-eNOS-Tg before, during and after Tempol treatment. Although there was no difference in heart rate between wild-type and C101A-eNOS-Tg before (day) and after Tempol treatment ($P > 0.05$, $n = 6$), a significant reduction of blood pressure occurred during Tempol treatment starting at the third day of treatment. The statistical analysis compared the mean blood pressure values of C101A-eNOS-Tg for the last 8 days before treatment with the mean blood pressure values during the last 8 days of Tempol treatment (*$P = 0.0088$, paired one-tailed *t* test, $n = 6$). The levels of eNOS-*S*-glutathionylation significantly differed both in (c) aorta ($P = 0.014$, $n = 6$, one-way analysis of variance) and (d) skeletal muscle ($P < 0.0001$, $n = 6$, one-way analysis of variance). Before Tempol treatment, there was a significant difference between C101A-eNOS-Tg and wild-type in both tissues (*$P < 0.01$, Newman–Keuls), which disappeared during Tempol treatment. The levels of eNOS-tyrosine-nitration significantly differed both in (e) aorta ($P = 0.003$, $n = 6$, one-way analysis of variance) and (f) skeletal muscle ($P = 0.003$, $n = 6$, one-way analysis of variance). Before Tempol treatment, there was a significant difference between C101A-eNOS-Tg and wild-type in both tissues (*$P < 0.01$, Newman–Keuls), which disappeared during Tempol treatment. The levels of vasoactive stimulated protein phosphorylation at Ser239-VASP significantly increased during Tempol treatment both in (e) aorta (*$P < 0.0001$, $n = 6$, two-tailed *t* test) and (f) skeletal muscle (*$P = 0.006$, $n = 6$, two-tailed *t* test) of C101A-eNOS-Tg.

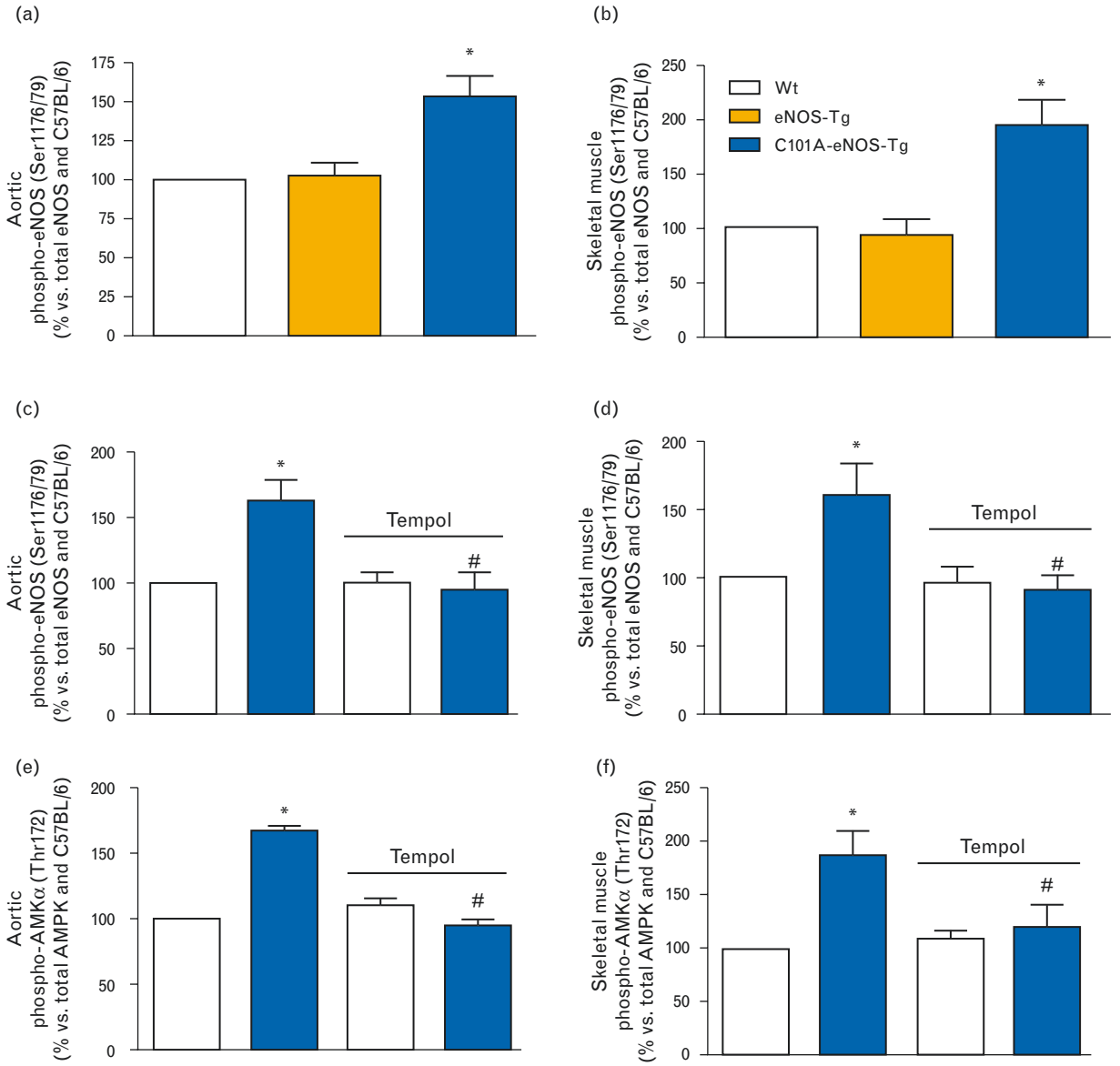


FIGURE 5 eNOS-Ser1176/79-hyperphosphorylation. Increased Ser1176/79 phosphorylation of eNOS in (a) aortic tissue ($P<0.0001$, $n=6$ each, one-way analysis of variance, $^{*}P<0.01$ vs. wild-type and eNOS-Tg, Newman–Keuls) and (b) skeletal muscle ($P<0.0001$, $n=6$ each, one-way analysis of variance, $^{*}P<0.001$ vs. wild-type and eNOS-Tg, Newman–Keuls) of C101A-eNOS-Tg. Treatment with Tempol had no effect in wild-type tissues, but significantly reduced eNOS Ser1176/79-phosphorylation in (c) aortic tissue ($P=0.001$, $n=6$ each, one-way analysis of variance, $^{\#}P<0.01$ vs. no treatment, Newman–Keuls) and (d) skeletal muscle tissue of C101A-eNOS-Tg ($P=0.0058$, $n=6$ each, one-way analysis of variance, $^{\#}P<0.05$ vs. no treatment, Newman–Keuls). Increased Tempol sensitive Thr172-phosphorylation of AMP kinase-α in (e) aortic tissue ($P<0.001$, $n=6$ each, one-way analysis of variance, $^{*}P<0.0001$ vs. wild-type and $^{\#}P<0.0001$ vs. Tempol treatment, Newman–Keuls) and (f) skeletal muscle tissue ($P=0.0053$, $n=6$ each, one-way analysis of variance, $^{*}P<0.01$ vs. wild-type and $^{\#}P<0.01$ vs. Tempol treatment, Newman–Keuls) AMP kinase-α-Thr172-phosphorylation of C101A-eNOS-Tg at baseline ($^{*}P<0.01$ vs. wild-type) and after Tempol treatment.

example, a C99A mutation of human eNOS resulted in a reduction of BH_4 affinity, enzyme stability and citrulline formation and an irreversible loss of heme [25]. The decreased stability of the human eNOS C99A mutant could be confirmed by gel filtration analysis in which the mutant enzyme appeared as a mixture of dimer and monomer [26]. Another investigation on C94A, C99A and C94A plus C99A mutations of human eNOS also confirmed the destabilization of dimer formation and showed a small catalytic activity (<10%) of the purified mutated enzymes [27]. In contrast, a C96S/C101S double mutant of bovine eNOS expressed in CV-1 (simian) in Origin and carrying the SV40 genetic material cells (COS-7 cells) showed about 50% of the catalytic activity of wild-type eNOS despite strong dimer destabilization [28]. These reports are consistent with our previous findings in stably transfected HEK cells in which the mutant C101A-eNOS activity was 45% lower compared with normal eNOS, whereas superoxide detected by electron-spin-resonance was doubled [17].

The novel transgenic mouse model, C101A-eNOS-Tg, is characterized by the coexistence of normal and dimer-destabilized eNOS in the endothelium of otherwise healthy mice without any signs of cardiovascular disease. Likewise, such concomitant generation of NO by wild-type enzyme and superoxide by C101A-eNOS itself might resemble the situation in endothelial cells of diseased vessels in which dysfunctional

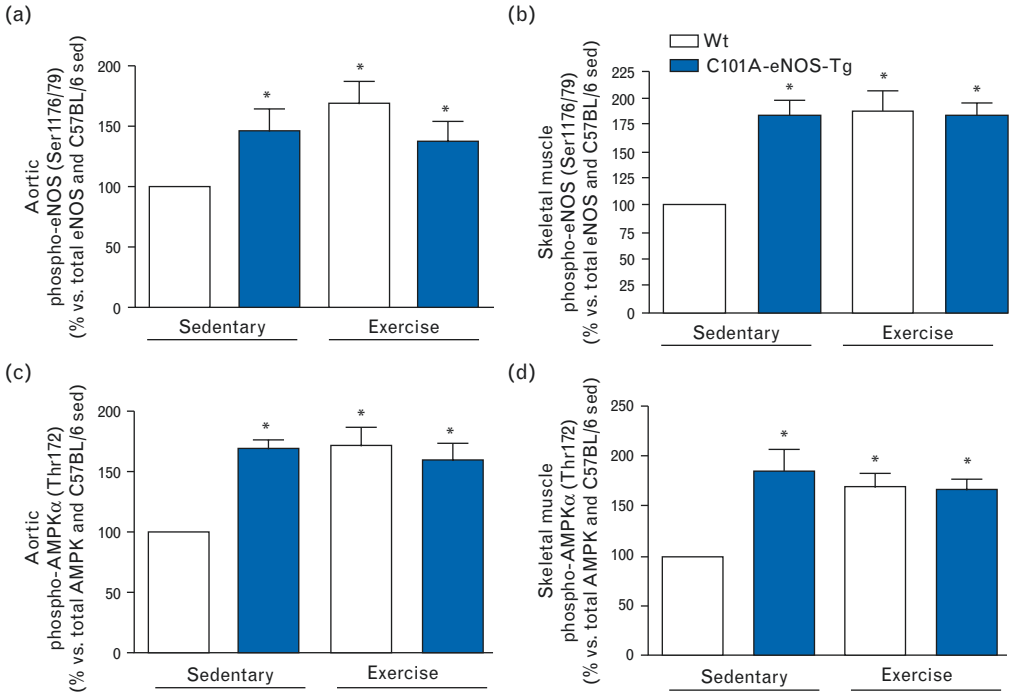


FIGURE 6 Voluntary exercise significantly increased eNOS-Ser1176/79 phosphorylation in (a) aortic tissues of wild-type but not of C101A-eNOS-Tg ($P=0.0195$, $n=6$ each, one-way analysis of variance, $^*P<0.05$ vs. sedentary wild-type, Newman–Keuls) and in (b) skeletal muscle tissue of wild-type but not of C101A-eNOS-Tg ($P=0.0003$, $n=6$ each, one-way analysis of variance, $^*P<0.01$ vs. sedentary wild-type, Newman–Keuls). Likewise, voluntary exercise significantly increased AMP kinase-α-Thr172-phosphorylation in (c) aortic tissues of wild-type but not of C101A-eNOS-Tg ($P=0.0004$, $n=6$ each, one-way analysis of variance, $^*P<0.001$ vs. sedentary wild-type, Newman–Keuls) and in (d) skeletal muscle tissue of wild-type but not of C101A-eNOS-Tg ($P=0.0032$, $n=6$ each, one-way analysis of variance, $^*P<0.01$ vs. sedentary wild-type, Newman–Keuls).

'uncoupled' eNOS molecules and coupled eNOS molecules can exist in the same cell at the same time [29]. Dimer destabilization of eNOS was evidenced by the increase of eNOS monomer appearance when separating aortic homogenates on low-temperature gels. Furthermore, an increase of superoxide formation by C101A-eNOS was evident in C101A-eNOS-Tg. This was abolished by L-NAME identifying C101A-eNOS as the underlying source. In addition, the strong increase of superoxide-dependent protein modifications such as tyrosine-nitration and *S*-glutathionylation in C101A-eNOS-Tg demonstrates the biological relevance of increased superoxide. In contrast, overexpression of wild-type eNOS in eNOS-Tg did not increase superoxide formation, tyrosine-nitration or *S*-glutathionylation, which further indicates that impairment of eNOS dimerization is the cause of oxidative stress in C101A-eNOS-Tg. Similar results, that is the absence of increased oxidative stress, were obtained by investigating another colony harboring a stronger endothelium-specific overexpression of wild-type eNOS [15].

Dimer destabilization of eNOS and aortic reactivity

Surprisingly, aortic EDR in C101A-eNOS-Tg was normal despite considerable oxidative stress, whereas EDR impairment was clearly detectable in various animal models with similar oxidative stress [30–37]. Although the reasons for this somewhat contradictory finding are not known, it appears likely that concomitant disease processes such as atherosclerosis, overt hypertension or diabetes likely contributed to endothelial dysfunction in these studies. For example, pharmacologic normalization of increased aortic superoxide generation in a rabbit model of atherosclerosis did only partially restore aortic EDR and the reduced sensitivity to NO-donors [38]. We also observed an unchanged vasodilator response to the NO-donor SNAP in aortic rings of C101A-eNOS-Tg. These data confirm that increased superoxide formation in C101A-eNOS-Tg had little impact on NO-induced vasodilation of conductance arteries in otherwise healthy adult mice.

Dimer destabilization of eNOS and blood pressure

The comparable BP reduction following endothelial-specific overexpression of normal eNOS has been demonstrated by different groups. In addition, expression levels of eNOS ranging between a three-fold [14,15] and a eight-fold increase [39] resulted in a comparable reduction of BP, suggesting that the effect of endothelial eNOS overexpression on BP regulation is limited. In striking contrast, neither BP nor VASP-Ser239-phosphorylation, a measure of protein kinase G activity and a surrogate for NO bioavailability [40], were altered in tissues of C101A-eNOS-Tg such as aorta, skeletal muscle, lung and myocardium. As the only difference between normal eNOS and C101A-eNOS is the generation of increased endothelial superoxide and oxidative protein modifications, we treated wild-type and C101A-eNOS-Tg with Tempol, a low molecular superoxide dismutase mimetic with known efficacy in hypertensive animal models [22]. The observed reversible BP reduction of approximately 5 mmHg induced by Tempol resembles

the calculated reduction and is consistent with the view that oxidative stress in C101A-eNOS-Tg inhibits hypotensive effects of NO. These data extend previous findings on impaired eNOS activity induced by dimer destabilization in overt experimental hypertension [41–43] to the physiologic BP regulation and suggest that dimer destabilization of eNOS, for example induced by known cardiovascular risk factors, might contribute to the pathophysiology of essential hypertension.

Posttranslational eNOS modification

Although there was no inhibition of aortic EDR, oxidative stress induced by dimer destabilization might impair activation of eNOS by physical forces including shear. This activation is largely mediated by eNOS-Ser1176/79-phosphorylation and is considered to be the most important physiologic stimuli for the continuous generation of vascular NO *in vivo* [44,45]. We observed a significantly increased phosphorylation of eNOS at Ser 1176/79 and of AMPK at Thr172 in sedentary C101A-eNOS-Tg. Moreover, exercise training, which has been shown to increase such phosphorylation of both enzymes in C57BL/6 [46], induced no change in C101A-eNOS-Tg, suggesting that shear-dependent activation of eNOS is diminished by either dimer destabilization itself, subsequently increased endothelial superoxide or both. Tempol treatment completely corrected hyperphosphorylation of eNOS and AMPKα, pointing to a close relationship between superoxide, hyperphosphorylation and eNOS-mediated BP reduction. In addition, a mouse model expressing a mutant phosphomimetic S1179D-eNOS on an eNOS-KO background showed almost normal EDR but hypertension similar to eNOS-KO, that is no reduction of BP [47]. These data are consistent with the observations reported here suggesting an inhibitory effect of eNOS-Ser1176/79-hyperphosphorylation on eNOS-mediated BP reduction. However, a diminished eNOS activity induced by the detected tyrosine-nitration of the enzyme may play a role as well.

Clinical aspects

Endothelial dysfunction in resistance vessels was already identified in normotensive young adults having hypertensive parents [4]. In addition, endothelial dysfunction in resistance vessels was demonstrated in other disease states such as in young adults with insulin-dependent diabetes mellitus despite normalization of plasma glucose [48] or might underlie the prolonged effect of passive smoking on endothelial progenitor cells [49]. Although there are no single-nucleotide-polymorphisms of eNOS-Zn-finger cysteines, eNOS-Ser1177-hyperphosphorylation by oxidative stress might contribute to endothelial dysfunction in resistance arteries of otherwise healthy humans, which is considered to be a risk factor for the development of overt hypertension in later life [49]. With respect to essential hypertension, our findings may provide another piece of mechanistic insight in this multifactorial disease.

In conclusion, taken together, our data suggest that vascular oxidative stress induced by eNOS dimer destabilization causes nitration and glutathionylation of several proteins as well as Ser1177-hyperphosphorylation of eNOS, which is presumably mediated by AMPKα. These posttranslational protein modifications were associated with resistance of eNOS to phosphorylation at Ser1177 induced by physical forces such as shear. Furthermore, this C101A-eNOS-dependent oxidative stress selectively prevented the BP-reducing activity of vascular eNOS. These data suggest that eNOS-dependent oxidative stress in microvascular endothelium may play a role for the development of essential hypertension.

ACKNOWLEDGEMENTS

The work was supported by the Research Commission of the Heinrich-Heine-University's Medical Faculty, 9772179 to G.K. and 9772446 to T.S., and partially by the DFG research grant SU 783/1-1 to T.S.

Reprints are not to be made available.

Parts of this work were presented during the American Heart Scientific Sessions 2015 in Orlando.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kojda G, Harrison DG. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res* 1999; 43:562–571.
2. Ghiadoni L, Taddei S, Virdis A. Hypertension and endothelial dysfunction: therapeutic approach. *Curr Vasc Pharmacol* 2012; 10:42–60.
3. Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, *et al.* The assessment of endothelial function: from research into clinical practice. *Circulation* 2012; 126:753–767.
4. Taddei S, Virdis A, Mattei P, Ghiadoni L, Sudano I, Salvetti A. Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. *Circulation* 1996; 94:1298–1303.
5. Rossi R, Chiurlia E, Nuzzo A, Cioni E, Origliani G, Modena MG. Flow-mediated vasodilation and the risk of developing hypertension in healthy postmenopausal women. *J Am Coll Cardiol* 2004; 44:1636–1640.
6. Mordi I, Mordi N, Delles C, Tzemos N. Endothelial dysfunction in human essential hypertension. *J Hypertens* 2016; 34:1464–1472.
7. Lamping KG, Nuno DW, Shesely EG, Maeda N, Faraci FM. Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice. *Am J Physiol Heart Circ Physiol* 2000; 279:H1906–H1912.
8. Waldron GJ, Ding H, Lovren F, Kubes P, Triggle CR. Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. *Br J Pharmacol* 1999; 128:653–658.
9. Meng W, Ayata C, Waeber C, Huang PL, Moskowitz MA. Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice. *Am J Physiol Heart Circ Physiol* 1998; 274:H411–H415.
10. Sun D, Huang A, Smith CJ, Stackpole CJ, Connetta JA, Shesely EG, *et al.* Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circ Res* 1999; 85:288–293.
11. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012; 33:829d–837d.
12. Lee MY, Griendling KK. Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal* 2008; 10:1045–1059.
13. Adlam D, Bendall JK, De Bono JP, Alp NJ, Khoo J, Nicoli T, *et al.* Relationships between nitric oxide-mediated endothelial function, eNOS coupling and blood pressure revealed by eNOS-GTP cyclohydrolase 1 double transgenic mice. *Exp Physiol* 2007; 92:119–126.
14. Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G. Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo. *Circulation* 2005; 112:2487–2495.
15. Oppermann M, Suvorava T, Freudenberger T, Dao VT, Fischer JW, Weber M, *et al.* Regulation of vascular guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification. *Basic Res Cardiol* 2011; 106:539–549.

16. Raman CS, Li H, Martasek P, Kral V, Masters BS, Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 1998; 95:939–950.
17. Suvorava T, Nagy N, Pick S, Lieven O, Ruther U, Dao VT, *et al.* Impact of eNOS-dependent oxidative stress on endothelial function and neointima formation. *Antioxid Redox Signal* 2015; 23:711–723.
18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3:1101–1108.
19. Suvorava T, Stegbauer J, Thieme M, Pick S, Friedrich S, Rump LC, *et al.* Sustained hypertension despite endothelial-specific eNOS rescue in eNOS-deficient mice. *Biochem Biophys Res Commun* 2015; 458:576–583.
20. Hacker A, Müller S, Meyer W, Kojda G. The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis. *Br J Pharmacol* 2001; 132:1707–1714.
21. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, *et al.* Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res* 1999; 42:206–213.
22. Wilcox CS, Pearlman A. Chemistry and antihypertensive effects of tempol and other nitroxides. *Pharmacol Rev* 2008; 60:418–469.
23. Suvorava T, Kumpf S, Rauch BH, Dao VT, Adams V, Kojda G. Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity. *Free Radic Res* 2010; 44:199–207.
24. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, *et al.* Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 1996; 93:13176–13181.
25. Chen PF, Tsai AL, Wu KK. Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem Biophys Res Commun* 1995; 215:1119–1129.
26. Ravi K, Brennan LA, Levic S, Ross PA, Black SM. *S*-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci U S A* 2004; 101:2619–2624.
27. Rafikov R, Fonseca FV, Kumar S, Pardo D, Darragh C, Elms S, *et al.* eNOS activation and NO function: structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity. *J Endocrinol* 2011; 210:271–284.
28. Erwin PA, Lin AJ, Golan DE, Michel T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 2005; 280:19888–19894.
29. Li H, Horke S, Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis* 2014; 237:208–219.
30. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* 1997; 95:588–593.
31. Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, *et al.* Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension* 1995; 26:854–857.
32. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, *et al.* Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; 103:1282–1288.
33. Zou MH, Shi C, Cohen RA. Oxidation of the zinc–thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002; 109:817–826.
34. Didion SP, Kinzenbaw DA, Schrader LI, Chu Y, Faraci FM. Endogenous interleukin-10 inhibits angiotensin II-induced vascular dysfunction. *Hypertension* 2009; 54:619–624.
35. Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, Brandes RP. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* 2004; 109:1795–1801.
36. Anea CB, Cheng B, Sharma S, Kumar S, Caldwell RW, Yao L, *et al.* Increased superoxide and endothelial NO synthase uncoupling in blood vessels of Bmal1–Knockout mice. *Circ Res* 2012; 111:1157–1165.
37. Morawietz H, Weber M, Rueckschloss U, Lauer N, Hacker A, Kojda G. Upregulation of vascular NAD(P)H oxidase subunit gp91phox and impairment of the nitric oxide signal transduction pathway in hypertension. *Biochem Biophys Res Commun* 2001; 285:1130–1135.
38. Muller S, Konig I, Meyer W, Kojda G. Inhibition of vascular oxidative stress in hypercholesterolemia by eccentric isosorbide mononitrate. *J Am Coll Cardiol* 2004; 44:624–631.
39. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, *et al.* Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest* 1998; 102:2061–2071.
40. Lohmann SM, Walter U. Tracking functions of cGMP-dependent protein kinases (cGK). *Front Biosci* 2005; 10:1313–1328.
41. Mollnau H, Wendt M, Szöcs K, Lassègue B, Schulz E, Oelze M, *et al.* Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. *Circ Res* 2002; 90:E58–E65.
42. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, *et al.* Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; 111:1201–1209.
43. Zheng JS, Yang XQ, Lookingland KJ, Fink GD, Hesslinger C, Kapatos G, *et al.* Gene transfer of human guanosine 5′-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation* 2003; 108:1238–1245.
44. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, *et al.* Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; 399:597–601.
45. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; 399:601–605.
46. Kojda G, Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res* 2005; 67:187–197.
47. Atochin DN, Wang A, Liu VW, Critchlow JD, Dantas AP, Looft-Wilson R, *et al.* The phosphorylation state of eNOS modulates vascular reactivity and outcome of cerebral ischemia in vivo. *J Clin Invest* 2007; 17:1961–1967.
48. Mäkimattila S, Virkamäki A, Groop PH, Cockcroft J, Utriainen T, Fagerudd J, *et al.* Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation* 1996; 94:1276–1282.
49. Heiss C, Amabile N, Lee AC, Real WM, Schick SF, Lao D, *et al.* Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. *J Am Coll Cardiol* 2008; 51:1760–1771.

Reviewers' Summary Evaluations

Reviewer 1

Does endothelial oxidative stress impair blood pressure regulation before overt hypertension occurs? In this study, blood pressure was lower in transgenic mice in which eNOS was overexpressed than in transgenic mice where eNOS was destabilized. In these, superoxide antagonism reversed the changes in eNOS-regulating proteins and blood pressure fell significantly.

The authors conclude that deficiencies in eNOS function may play a role in sustained high blood pressure through superoxide-mediated microvascular constriction, a view consistent with a facultative role for increased peripheral resistance following initially increased cardiac output, known to occur in animal and human hypertension.

Reviewer 2

This study tested effects of endothelial nitric oxide synthase (eNOS)-derived endothelial oxidative stress on

blood pressure. The model involved a mouse with endothelial-specific expression of a dimer-destabilized C101A-mutant eNOS (C101A-eNOS-Tg). In C101A-eNOS-Tg mice, hypotension - that is normally reported in mice overexpressing eNOS in the endothelium - was no longer present.

This was associated with increased aortic superoxide, although aortic endothelium-dependent relaxation was unchanged. The superoxide scavenger, tempol, reduced blood pressure in C101A-eNOS-Tg mice only. Investigating superoxide generation and endothelial function in a resistance artery would be an important subsequent study in this mouse model.

Deletion of Hyaluronan Synthase 3 Inhibits Neointimal Hyperplasia in Mice

Lena S. Kiene, Susanne Homann, Tatsiana Suvorava, Berit Rabausch, Julia Müller, Georg Kojda, Inga Kretschmer, Sören Twarock, Guang Dai, René Deenen, Sonja Hartwig, Stefan Lehr, Karl Köhrer, Rashmin C. Savani, Maria Grandoch,* Jens W. Fischer*

Objective—Hyaluronan (HA) is a polymeric glucosaminoglycan that forms a provisional extracellular matrix in diseased vessels. HA is synthesized by 3 different HA synthases (HAS1, HAS2, and HAS3). Aim of this study was to unravel the role of the HAS3 isoenzyme during experimental neointimal hyperplasia.

Approach and Results—Neointimal hyperplasia was induced in *Has3*-deficient mice by ligation of the carotid artery. HA in the media of *Has3*-deficient mice was decreased 28 days after ligation, and neointimal hyperplasia was strongly inhibited. However, medial and luminal areas were unaffected. Cell density, proliferation, and apoptosis were not altered, suggesting a proportional decrease of both, the number of cells and extracellular matrix. In addition, endothelial function as determined by acetylcholine-induced relaxation of aortic rings, immunoblotting of endothelial nitric oxide synthase, and arterial blood pressure were not affected. Furthermore, the oxidative stress response was not affected as determined in total protein extracts from aortae. Transcriptome analysis comparing control versus ligated carotid arteries hinted toward a mitigated differential regulation of various signaling pathways in *Has3*-deficient mice in response to ligation that were related to vascular smooth muscle cell (VSMC) migration, including focal adhesions, integrins, mitogen-activated protein kinase, and phosphatidylinositol signaling system. Lentiviral overexpression of *HAS3* in VSMC supported the migratory phenotype of VSMC in response to platelet-derived growth factor BB in vitro. Accordingly, knockdown of *HAS3* reduced the migratory response to platelet-derived growth factor BB and in addition decreased the expression of *PDGF-B* mRNA.

Conclusions—HAS3-mediated HA synthesis after vessel injury supports seminal signaling pathways in activation of VSMC, increases platelet-derived growth factor BB–mediated migration, and in turn enhances neointimal hyperplasia in vivo. **(*Arterioscler Thromb Vasc Biol*. 2016;36:e9-e16. DOI: 10.1161/ATVBAHA.115.306607.)**

Key Words: carotid arteries ■ extracellular matrix ■ hyaluronan ■ neointima ■ smooth muscle cells

Hyaluronan (HA) is considered to be a key constituent of permissive extracellular matrices (ECMs) that supports tissue remodeling in response to physiological and pathophysiological stimuli. HA is composed of repeating nonsulfated disaccharides [-β (1,4)-glucuronic acid -β (1,3)-*N*-acetylglucosamine-]$_n$ assembled into polymers of high molecular weight ranging from 1 to 10×10^6 Da by HA synthase (HAS1–3) isoenzymes.

See accompanying editorial on page 224

HA activates receptor for HA-mediated motility (RHAMM)– and CD44-mediated signaling[1] in vascular smooth muscle cells (VSMC), which in turn causes increased VSMC proliferation and migration in vitro and in vivo.[2,3] Recently, RHAMM has been attributed an important role in the control of division fidelity during neointimal hyperplasia.[4] Lack of RHAMM prevents constrictive vascular remodeling after vessel injury.[5] Therefore, inhibition of interstitial HA synthesis by VSMC seems to be a promising target to inhibit restenosis.[6–8]

Studying the regulation of HAS isoenzyme expression in vitro in human VSMC revealed that *HAS1* and *HAS2* transcription are strongly responsive to cAMP-activated signaling by prostaglandins/prostaglandin receptors.[9] Furthermore, *HAS2* is known to be responsive to platelet-derived growth factor BB (PDGF-BB), transforming growth factor β 1, and thrombin-mediated protease activated receptor 1 signaling.[10] In addition, *HAS1* is upregulated by adenosine A2A and A2B receptors in VSMC.[11]

Received on: October 24, 2014; final version accepted on: November 7, 2015.

From the Institut für Pharmakologie und Klinische Pharmakologie (L.S.K., S. Homann, B.R., G.K., S.T., G.D., M.G., J.W.F.), Cardiovascular Research Institute Düsseldorf (CARID) (L.S.K., S. Homann, B.R., J.M., I.K., S.T., G.D., M.G., J.W.F.), and Biologisch-Medizinisches Forschungszentrum (BMFZ) (R.D., K.K.), Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany; German Center for Diabetes Research (DZD), München-Neuherberg, Germany (S. Hartwig, S.L.); Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center at the Heinrich-Heine-University Duesseldorf, Leibniz Center for Diabetes Research, Duesseldorf, Germany (S. Hartwig, S.L.); and Divisions of Pulmonary and Vascular Biology and Neonatal-Perinatal Medicine, Department of Pediatrics, The University of Texas Southwestern Medical Center, Dallas (R.C.S.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.115.306607/-/DC1.

Correspondence to Jens W. Fischer, PhD, Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany. E-mail jens.fischer@uni-duesseldorf.de

Nonstandard Abbreviations and Acronyms	
ECM	extracellular matrix
HA	hyaluronan
HAS	hyaluronan synthase
PDGF-BB	platelet-derived growth factor BB
RHAMM	receptor for HA-mediated motility
VSMC	vascular smooth muscle cell
WT	wild-type

Functionally, it was shown in these studies that HAS2-mediated HA synthesis increases proliferation and migration of VSMC. Recently, it was discovered that HAS1 is involved in migratory responses of VSMC as well.[11] In addition, overexpression of *HAS1* promotes adhesion of monocytes to the pericellular HA matrix suggesting that the HA matrix formed by HAS1 is involved in inflammatory responses.[12] Interestingly, in the study by Wilkinson et al[12] overexpression of all HAS isoforms led to decreased proliferation and migration rates, which is in contrast with the results of knocking down endogenous *HAS1* and *HAS2* that also inhibited proliferation and migration.[9,10]

Has2 knockout mice die at midgestation due to disturbed cardiac and vascular morphogenesis and defects in endothelial mesenchymal transition, suggesting that HAS2 fulfills critical physiological functions.[13] A fascinating example of physiological HA-driven neointimal hyperplasia is the closure of the ductus arteriosus Botalli. Here, prostaglandin E–mediated activation of the prostaglandin E receptor 4 promotes induction of *Has2* and subsequently VSMC migration and the formation of an HA-rich intimal cushion.[14] However, the dilatory effect of prostaglandin E 2 keeps the ductus open. This remodeling process is essential in preparing the timely and rapid closure of the ductus arteriosus after cessation of prostaglandin E synthesis. In contrast to the lethal genetic deletion of *Has2*, deletion of *Has1* or *Has3* did not result in an obvious phenotype without pathophysiological challenge.

HAS2 probably represents an attractive target to inhibit neointimal hyperplasia because in vivo studies revealed that overexpression of *Has2* in VSMC led to increased atherosclerosis in apolipoprotein E–deficient mice and to increased cuff-mediated neointimal hyperplasia in C57BL/6 mice.[15,16] However, a limitation that needs to be considered is that those results reflect increased HA synthesis in medial VSMC because of *Has2* overexpression in differentiated VSMC, and do not specifically reflect the physiological function of HAS2 during neointimal hyperplasia.

Because of the embryonic lethality of *Has2*-deficient mice and the importance of HAS2 for proper development of the cardiovascular system as well as for endothelial mesenchymal transition, it is doubtful that HAS2 represents a suitable therapeutic target. Therefore, the other HAS isoenzymes are of great interest with respect to their specific role in vascular pathologies. Whether HAS1 and HAS3 play a role in

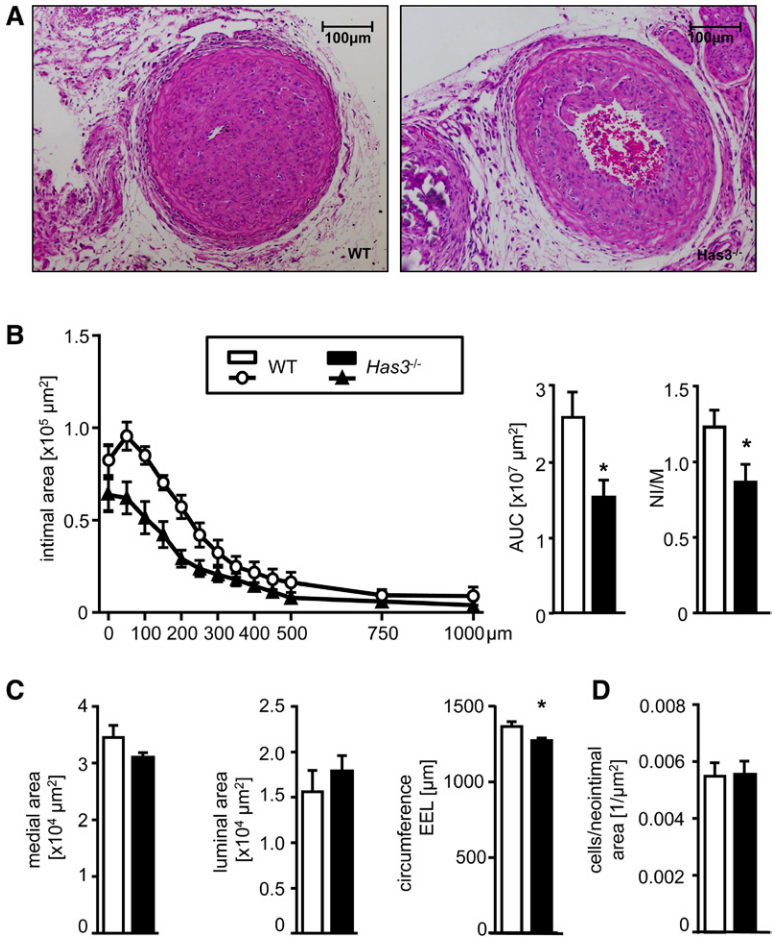


Figure 1. Neointimal hyperplasia is reduced in *Has3*-deficient mice. Neointimal hyperplasia was induced by carotid artery ligation in wild-type (WT) and *Has3*-deficient mice and analyzed after 4 weeks. **A**, Representative images of hematoxylin and eosin–stained sections of WT (**left**) and *Has3*-deficient (**right**) mice. **B**, Neointimal hyperplasia was characterized by morphometry. The neointimal area is plotted as function of the distance to ligation (**left**). Mean area under the curve (AUC) and the neointimal/medial (NI/M) ratio are given as well (**right**). **C**, Mean medial and luminal area, mean circumference of the external elastic lamina (EEL), and **D**, cellular density in the neointima. Data are mean±SEM, n=9, **P*<0.05 versus WT.

neointimal hyperplasia is not known. Similarly, other than the knowledge that *HAS3* is upregulated in aged VSMC and that oxidized low-density lipoprotein and cholesterol induce *HAS3* in human VSMC little is known about its regulation and function in VSMC.[17,18] Hence, the aim of this study was to test the effect of loss of HAS3 during neointimal hyperplasia in vivo using mice with targeted deletion of *Has3* and to study the underlying mechanisms in vitro.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Inhibition of Neointimal Hyperplasia in *Has3*-Deficient Mice

Has3-deficient mice displayed no differences with respect to body weight, blood glucose, and total cholesterol (Figure I in the online-only Data Supplement). To address the role of HAS3 for VSMC phenotype in vivo, left carotid arteries of *Has3*-deficient mice and wild-type (WT) mice were ligated to induce neointimal hyperplasia driven by VSMC proliferation and migration.[19] In *Has3*-deficient mice, the neointimal area and neointimal/medial ratio were strongly reduced compared with WT mice (Figure 1A and 1B). In contrast, the medial and luminal areas were not changed (Figure 1C). A small reduction of the circumference of the external elastic lamina of ligated carotid arteries was observed (Figure 1C), indicating constrictive remodeling. Determination of cell density in the neointima suggested that both cell number and ECM declined proportionally in *Has3*-deficient mice after ligation (Figure 1D). Analysis of HA staining in control carotid arteries versus ligated WT carotids revealed an accumulation of HA both in the media and in the neointima after 28 days (Figure 2A). Quantitative image analysis revealed less HA accumulation in the whole vessel (Figure 2A and 2B) in *Has3*-deficient mice after ligation. Interestingly, the difference was pronounced in the media (Figure 2B). In addition, after ligation expression of *Has1* and *Has2* were not further upregulated in *Has3*-deficient mice compared with WT, thereby excluding a compensatory counter regulation in response to *Has3* deficiency (Figure 2C and 2D). *Has3* mRNA was not detected in *Has3*-deficient mice (not shown).

To gain further mechanistic insight, ligated carotid arteries were investigated 5 days after ligation. As evidenced by 5-bromo-2′-deoxyuridine staining, the proliferation rate was not affected in the neointima of *Has3*-deficient compared with WT controls (Figure 3A). In addition, neither apoptotic rate (Figure 3B) nor immunostaining of α smooth muscle actin were affected (Figure 3C) 5 days after ligation. Furthermore, mRNA of *Acta2* and *SM22alpha* were determined. Both markers of differentiated VSMC were strongly downregulated in response to ligation as expected. However, the extent of this downregulation was not different between genotypes (Figure 3D).

Next, it was addressed if *Has3* deficiency might have general effects on endothelial function that might result in changes of ligand-induced vasodilatation and blood pressure. First, expression of endothelial nitric oxide synthase was found to be unaffected in aortas of WT versus *Has3*-deficient mice (Figure 4A). Likewise, acetylcholine-induced vasorelaxation was not affected (Figure 4B) as well as relaxation in response to an exogenous NO donor (*S*-nitroso-*N*-acetyl-$_{D,L}$-penicillamine) and contraction in response to phenylephrine (Figure 4C and 4D).

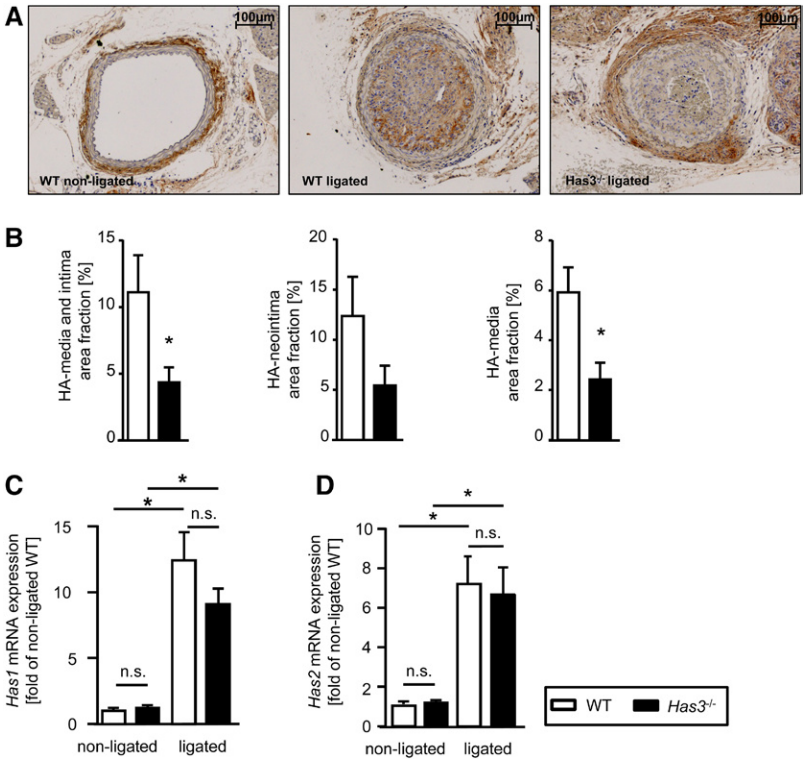


Figure 2. Carotid arteries from *Has3*-deficient mice show reduced hyaluronan (HA) accumulation and no compensatory regulation of *Has1* and *Has2*. Neointimal hyperplasia was induced by carotid artery ligation in wild-type (WT) and *Has3*-deficient mice. Histological sections were analyzed for HA content after 4 weeks. **A**, representative images of uninjured vessel (**left**) and WT (**middle**) and *Has3*-deficient (**right**) carotid arteries after ligation stained with hyaluronan-binding protein. **B**, Area fraction quantified in media and intimal layer, and in media and intima separately, n=9, **P*<0.05 versus WT. **C** and **D**, *Has1* and *Has2* mRNA expression were analyzed after 5 days. **C**, *Has1* gene expression. **D**, *Has2* mRNA expression; n=7 to 9, **P*<0.05 versus respective nonligated controls analyzed using Kruskal–Wallis test with Dunn post hoc test. Data are mean±SEM.

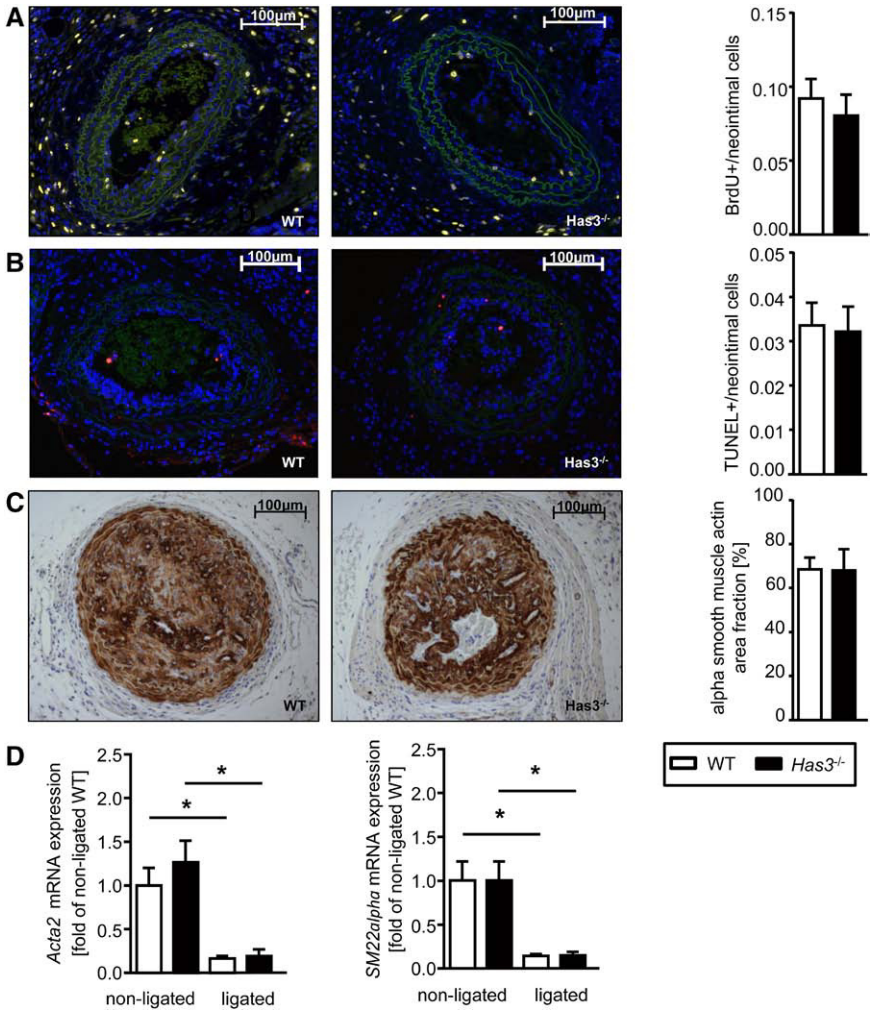


Figure 3. Characterization of vascular smooth muscle cell proliferation, apoptosis, and differentiation in the neointima of *Has3*-deficient mice. Five days after ligation 5-bromo-2′-deoxyuridine (BrdU) incorporation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and mRNA expression were analyzed. **A**, Representative images of immunostaining of incorporated BrdU (yellow), proliferative index calculated as the ratio of BrdU$^+$ nuclei to total nuclei within the neointimal layer; n=11. **B**, Representative images of TUNEL staining (red) and quantification of apoptosis as TUNEL–positive cells per total neointimal nuclei; n=13. Original magnification 200-fold, nuclei are stained in blue, elastic laminae are indicated by autofluorescence (FITC [fluorescein isothiocyanate] channel, green), pictures from wild-type (WT) are shown on the **left**, images from *Has3*-deficient mice on the **right**. **C**, Representative images of α smooth muscle actin–stained sections of WT (**left**) and *Has3*$^{-/-}$ (**right**) mice harvested 4 weeks after ligation and quantification of intimal area fraction, n=8 to 9. **D**, mRNA expression of α smooth muscle actin (*Acta2*) and *SM22alpha*; n=8 to 9, **P*<0.05 versus respective nonligated controls analyzed using Kruskal–Wallis test with Dunn post hoc test. Data are presented as mean±SEM.

Furthermore, arterial blood pressure of *Has3*-deficient mice was similar to WT mice (Figure 4E). To address the question whether the observed phenotype might involve changes in the oxidative stress response, oxyblot analysis was performed. No differences in oxidative protein modifications were observed between genotypes (Figure 4F).

All things considered, an effect of *Has3*-deletion on vascular tone and reagibility can be excluded as the mechanism underlying reduced neointimal hyperplasia in *Has3*-deficient mice.

Attenuated Transcriptional Response in *Has3*-Deficient Mice After Carotid Artery Ligation

In search of the underlying mechanisms, genome-wide transcriptome analysis of control and ligated carotids was performed 5 days after ligation. In WT mice, 8006 transcripts were differentially regulated when comparing ligated carotid arteries to nonligated control carotids (Figure II in the online-only Data Supplement). In contrast, using identical experimental settings in *Has3*-deficient mice only 3635 transcripts were affected by the ligation suggesting a strong influence of *Has3* deficiency on transcriptional responses to vessel injury. The dampened response in *Has3*-deficient mice can also be seen in the hierarchical clustering and the volcano plots shown in Figure III in the online-only Data Supplement. Next, the transcriptome data were analyzed for Kyoto Encyclopedia of Genes and Genomes pathways that were only significantly regulated in response to ligation in carotid arteries from WT and not in carotids from *Has3*-deficient mice (Table I in the online-only Data Supplement). Of note, various pathways related to the migratory SMC phenotype, ECM signaling, and general signaling leading to phenotypic activation were identified,

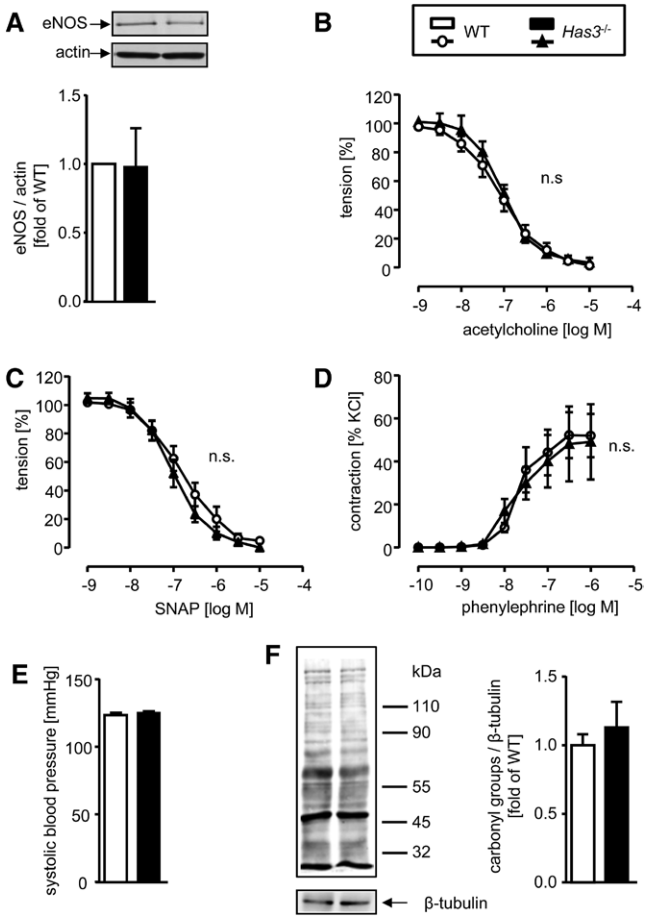


Figure 4. Endothelial nitric oxide synthase (eNOS) expression and endothelial function is not altered by *Has3* deficiency. Thoracic aortae were harvested from *Has3*-deficient mice and wild-type (WT) controls. **A**, eNOS protein expression in aortas of *Has3*-deficient and WT mice. **Top**, Representative Western blots for eNOS in aortic tissue. **Bottom**, Densitometric quantification of the signals normalized to actin; n=4 to 5. **B**, Endothelium-dependent relaxation to acetylcholine, **C**, endothelium-independent relaxation to the NO donor *S*-nitroso-*N*-acetyl-$_{D,L}$-penicillamine (SNAP) and **D**, contractile response to phenylephrine in aortic rings of *Has3*-deficient and WT mice, not significant (n.s.), n=3 to 4. **E**, Systolic blood pressure; n=5. **F**, Representative Western blot of carbonyl groups as a marker for oxidative protein modifications and densitometric quantification of the signals normalized to β-tubulin; n=4. Data are means±SEM.

such as regulation of actin cytoskeleton (mmu04810), vascular smooth muscle contraction (mmu04270), calcium-signaling pathway (mmu04020), focal adhesion (mmu04510), ECM–receptor interaction (mmu04512), phosphatidylinositol signaling system (mmu04070), inositol phosphate metabolism (mmu00562), and mitogen-activated protein kinase–signaling pathway (mmu04010). Expression of genes representing the oxidative stress response did not seem to be differentially regulated between the genotypes confirming the results from the Oxyblot analysis as indicated above (Figure 4F).

HAS3-Mediated HA Synthesis Promotes Migration and Proliferation

Analysis of the neointima as presented in Figures 1–3 revealed decreased neointimal hyperplasia in *Has3*-deficient mice because of proportional decrease in cells and matrix. Because neither 5-bromo-2′-deoxyuridine incorporation nor apoptosis were changed in *Has3*-deficient mice, it is likely that primarily migratory responses are affected. In line with this assumption was the finding that *Pdgf-b* mRNA, known to promote VSMC migration,[20,21] was upregulated in WT after carotid artery ligation but not in *Has3*-deficient mice as determined by gene array analysis (NCBI GEO accession number GSE70410). To complement the results obtained in *Has3*-deficient mice in vitro, lentiviral shRNA was used to specifically knockdown *HAS3* (sh*HAS3*, Figure 5A) in human VSMC. Lentiviral sh*HAS3* efficiently reduced *HAS3* mRNA expression and reduced HA secretion into the cell culture medium (Figure 5A). As observed in the gene array analysis, *PDGF-B* mRNA expression was significantly reduced in sh*HAS3* cells (Figure 5A). Importantly, migration was diminished in sh*HAS3* as determined in a microchemotaxis chamber (Figure 5B). Neither the mitogenic response to PDGF-BB

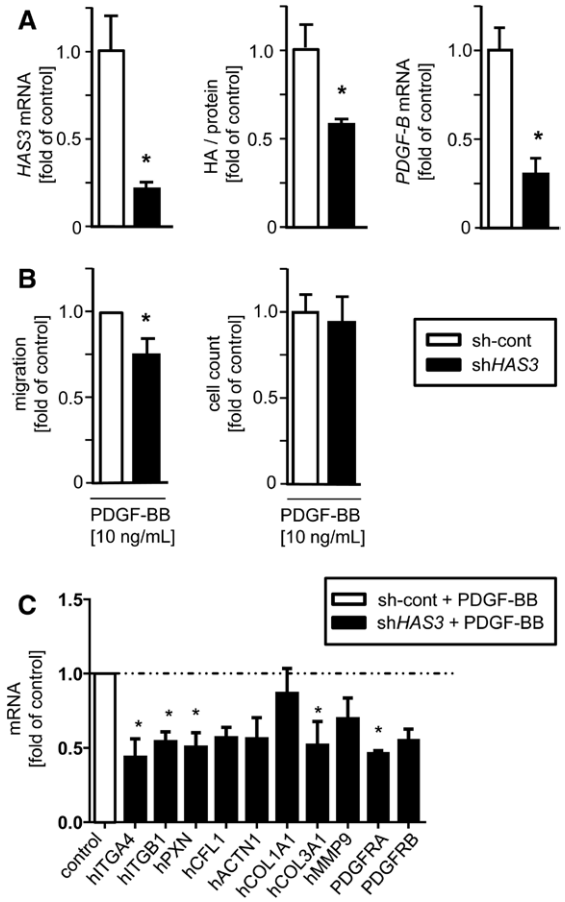


Figure 5. HAS3 supports the migratory and proliferative phenotype of human coronary vascular smooth muscle cell (VSMC). **A**, *HAS3* mRNA was reduced using lentiviral shRNA compared with nontargeting shRNA (sh-cont) in VSMC, leading to a diminished hyaluronan (HA) secretion, and a downregulation of *PDGF-B* mRNA. **B**, sh*HAS3* inhibited migration as determined by a modified Boyden-chamber microchemotaxis assay on collagen type 1-coated membranes and proliferation as determined by cell count. *P<0.05 versus control. **C**, sh*HAS3* reduced mRNA expression of a variety of migration-associated genes in VSMCs stimulated with platelet-derived growth factor BB (PDGF-BB; 10 ng/mL) for 24 hours. *P<0.05 versus sh-cont (control); 1-way ANOVA with Dunnett multiple comparison test; n=3 to 6. Data are mean±SEM.

(Figure 5B) nor the apoptotic rate (data not shown) was significantly altered in sh*HAS3*. The gene array and the in vitro migration data strongly suggested a role of HAS3-mediated HA synthesis in migration. In line with this assumption, the expression of a panel of genes involved in the migratory response was downregulated after knockdown of *HAS3* in VSMC as determined by quantitative polymerase chain reaction (Figure 5C). Accordingly, the migratory response of VSMC to PDGF-BB was strongly increased in *HAS3* overexpressing (*HAS3*oe) cells (Figure IV in the online-only Data Supplement). Proliferation was quantified by DNA-synthesis, as determined by [^{3}H]-thymidine incorporation, and was slightly increased in *HAS3*oe cells compared with controls (Figure IV in the online-only Data Supplement).

Finally, it was considered whether the inflammatory response was affected in *Has3*-deficient mice. Importantly, neointimal hyperplasia in normocholesteremic mice is considered mainly a model of VSMC-driven neointimal hyperplasia.[22]

In the gene array analysis, expression of genes representing the inflammatory response was upregulated in both genotypes and also inflammatory Kyoto Encyclopedia of Genes and Genomes pathways were regulated in both genotypes.

mRNA expression of *Tnfa*, *Ccl2* (monocyte chemotactic protein 1), and *Il1b* as determined by quantitative polymerase chain reaction in ligated carotid arteries, showed a strong increase compared with unligated controls. However, no differences between genotypes were observed (data not shown). Likewise, circulating cytokines were mostly unaffected except increased interleukin (IL)-13 and granulocyte–macrophage colony-stimulating factor (Figure 6). Importantly, IL-13-primed macrophages are characterized by a more anti-inflammatory M2 phenotype and suppress proinflammatory type 1 macrophages,[23] which in turn have been shown to substantially promote neointima formation via promotion of VSMC proliferation and migration.[24] In contrast, granulocyte–macrophage colony-stimulating factor is thought to promote inflammation so that both proinflammatory and primarily anti-inflammatory cytokines seem to be induced in *Has3* deficiency and the net outcome remains unknown. Taken together, reduced inflammation is unlikely the mechanism underlying reduced neointimal hyperplasia in *Has3*-deficient mice.

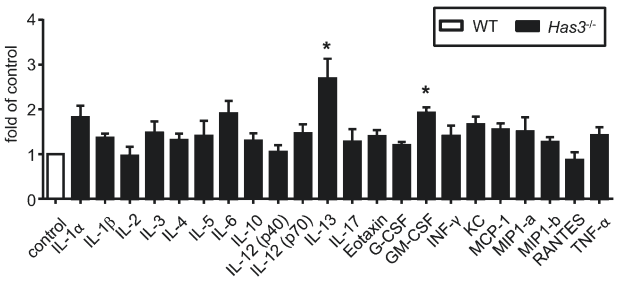


Figure 6. Multiplex cytokine immunoassay. Plasma samples were collected 5 days after carotid artery ligation. Determination of multiple cytokines in the plasma by a commercially available multiplex bead–based immunoassay. Expressed as fold of respective wild-type (WT) control; mean±SEM vs WT; n=4 to 5, **P*<0.05 Kruskal–Wallis test with Dunn post hoc test.

Discussion

This study is the first to address the specific role of HAS3 during experimental neointimal hyperplasia in vivo and shows a substantial reduction of neointimal hyperplasia in *Has3*-deficient mice. In response to the arterial injury, strong accumulation of HA was observed within the neointimal layer, in line with earlier results.[3,25] In healthy vessels, HA matrix is mainly limited to the adventitial layer and the endothelial glycocalyx. Because HAS2 is pivotal for development of the cardiovascular system as well as endothelial mesenchymal transition, it is likely not a suitable therapeutic target with favorable benefit/risk ratios. Therefore, therapeutic strategies specifically inhibiting HAS3-mediated HA expression might be considered in the prevention of restenosis after surgical intervention. On the basis of the unaltered cell density in the neointima in vivo, a proportional decrease of both cell number and ECM is suggested in *Has3*-deficient mice. Gene expression of *Acta2* and *SM22alpha* was reduced 5 days after ligation, indicating dedifferentiation of VSMC. However, *Has3* deficiency did not affect the expression of differentiation markers. In addition, immunostaining of α smooth muscle actin and determination of proliferation and apoptosis revealed no changes between the genotypes 5 days after ligation. In search of the underlying mechanisms, effects of *Has3*-deficiency on endothelial function, blood pressure, inflammation, and the oxidative stress response were excluded.

Importantly, HA accumulation in the ligated vessels was reduced in *Has3*-deficient mice. Analysis of medial and intimal HA accumulation revealed that reduction of HA occurred also in the media of ligated carotids from *Has3*-deficient mice. In this context, it is important to consider that migration of VSMC from the media into the intima is one of the first steps during neointimal hyperplasia. PDGF-BB has been shown to promote formation of pericellular HA matrix in VSMC that, in turn, strongly promotes migration.[2] Therefore, HAS3 may be involved in the induction of the provisional HA-rich ECM in the media and neointima of ligated carotid arteries and, in turn, promotes VSMC migration. In this context, it may be important that *Pdgf-b* mRNA expression as assessed by gene array analysis was significantly upregulated in ligated WT carotids but not in *Has3*-deficient carotids in response to ligation. Furthermore, *PDGF-B* was reduced in VSMC after lentiviral knockdown of *HAS3*. This may represent a feed forward mechanism that further inhibits migratory responses in the absence of HAS3. On the basis of these findings and the literature, it is likely that HAS3 expression in VSMC promotes neointimal hyperplasia as opposed to HAS3 expression by other cell types, such as endothelial cells. This is also supported by the differentially regulated Kyoto Encyclopedia of Genes and Genomes pathways that point toward VSMC but not endothelial cells, and the in vitro results. In future studies, VSMC-specific deletion of *Has3* may be used to further confirm the role of VSMC.

To further understand the underlying cellular mechanisms, mRNA expression in the uninjured and ligated carotids was analyzed in an unbiased genome-wide approach using gene array technology. This analysis revealed that in WT mice about twice as many transcripts are regulated after

ligation when compared with *Has3*-deficient mice (8006 versus 3635). Furthermore, a variety of Kyoto Encyclopedia of Genes and Genomes pathways were identified, which were only differentially regulated in WT mice in response to carotid ligation but not in *Has3*-deficient mice. Among these pathways those related to the migratory VSMC phenotype (contractility and actin cytoskeleton), ECM signaling (focal adhesion kinase and ECM–receptor interactions), and general signaling (mitogen-activated protein kinase and phosphatidylinositol signaling system) seemed particularly interesting because they could represent avenues of phenotypic activation of VSMC in WT that were dampened in *Has3*-deficient mice. In line with these effects on transcriptional programs in vivo, we detected corresponding functional differences in VSMC after knockdown of *HAS3* in vitro. Lentiviral knockdown of *HAS3* inhibited migration in VSMC and proliferation was not altered. Conversely, overexpression of *HAS3* also revealed that HAS3-mediated HA synthesis augmented mainly migration and to a lesser extent proliferation in human VSMC.

The in vivo role of RHAMM and CD44 in neointimal hyperplasia has been demonstrated before. With respect to RHAMM, a role in both migration but also proliferation and cell division fidelity has recently been proposed in vivo.[4,26] In *Rhamm*-deficient mice, constrictive artery wall remodeling was reduced after carotid artery ligation improving the lumen caliber, but surprisingly did not alter the neointimal area itself.[5] Similarly, a role of CD44 in promoting neointimal hyperplasia has been proposed.[27] Although, this has not directly been shown by the use of knockout mice. Strong evidence however suggests that CD44 orchestrates also inflammatory responses in vivo, for example, during atherosclerosis.[28,29] Interestingly, it is known that inflammatory stimuli induce CD44 expression in VSMC.[30,31] Toll-like receptors are also involved in neointimal hyperplasia.[32,33] However, from the data presented here it cannot be concluded which of the receptors CD44 or RHAMM or even alternative HA receptors, such as toll-like receptor 2, toll-like receptor 4, LYVE1 (lymphatic vessel endothelial hyaluronan receptor), and HARE (hyaluronan receptor for endocytosis) are involved in the in vivo response and the inhibition of neointimal hyperplasia. For this purpose, future in vivo studies using the respective receptor knockouts or pathway inhibitors are needed.

Collectively, it is demonstrated here (1) that *Has3* deletion inhibits neointimal hyperplasia and (2) that loss of HAS3-dependent synthesis of HA decreases PDGF-BB–mediated migration of VSMC. We conclude that HAS3 is a critical player in the phenotypic activation of medial VSMC during neointimal hyperplasia and may represent a suitable therapeutic target.

Acknowledgments

The excellent technical assistance by Petra Rompel and Annika Zimmermann is gratefully acknowledged.

Sources of Funding

This study was funded by Deutsche Forschungsgemeinschaft (DFG FI 682/3-1, IRTG 1902), National Institutes of Health grants HL073896 and HL075930, as well as the William Buchanan Chair in Pediatrics to R.C. Savani.

Disclosures

None.

References

1. Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. *J Biol Chem*. 2002;277:4589–4592. doi: 10.1074/jbc.R100038200.
2. Evanko SP, Angello JC, Wight TN. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1999;19:1004–1013.
3. Riessen R, Wight TN, Pastore C, Henley C, Isner JM. Distribution of hyaluronan during extracellular matrix remodeling in human restenotic arteries and balloon-injured rat carotid arteries. *Circulation*. 1996;93:1141–1147.
4. Silverman-Gavrila R, Silverman-Gavrila L, Bendeck MP. Cell division fidelity is altered during the vascular response to injury: its novel role in atherosclerosis progression. *Am J Pathol*. 2013;182:628–639. doi: 10.1016/j.ajpath.2012.11.007.
5. Ma X, Pearce JD, Wilson DB, English WP, Edwards MS, Geary RL. Loss of the hyaluronan receptor RHAMM prevents constrictive artery wall remodeling. *J Vasc Surg*. 2014;59:804–813. doi: 10.1016/j.jvs.2013.03.047.
6. Bot PT, Hoefer IE, Piek JJ, Pasterkamp G. Hyaluronic acid: targeting immune modulatory components of the extracellular matrix in atherosclerosis. *Curr Med Chem*. 2008;15:786–791.
7. Bot PT, Pasterkamp G, Goumans MJ, Strijder C, Moll FL, de Vries JP, Pals ST, de Kleijn DP, Piek JJ, Hoefer IE. Hyaluronic acid metabolism is increased in unstable plaques. *Eur J Clin Invest*. 2010;40:818–827. doi: 10.1111/j.1365-2362.2010.02326.x.
8. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res*. 2004;94:1158–1167. doi: 10.1161/01.RES.0000126921.29919.51.
9. Sussmann M, Sarbia M, Meyer-Kirchrath J, Nüsing RM, Schrör K, Fischer JW. Induction of hyaluronic acid synthase 2 (HAS2) in human vascular smooth muscle cells by vasodilatory prostaglandins. *Circ Res*. 2004;94:592–600. doi: 10.1161/01.RES.0000119169.87429.A0.
10. van den Boom M, Sarbia M, von Wnuck Lipinski K, Mann P, Meyer-Kirchrath J, Rauch BH, Grabitz K, Levkau B, Schrör K, Fischer JW. Differential regulation of hyaluronic acid synthase isoforms in human saphenous vein smooth muscle cells: possible implications for vein graft stenosis. *Circ Res*. 2006;98:36–44. doi: 10.1161/01.RES.0000199263.67107.c0.
11. Grandoch M, Hoffmann J, Röck K, Wenzel F, Oberhuber A, Schelzig H, Fischer JW. Novel effects of adenosine receptors on pericellular hyaluronan matrix: implications for human smooth muscle cell phenotype and interactions with monocytes during atherosclerosis. *Basic Res Cardiol*. 2013;108:340. doi: 10.1007/s00395-013-0340-6.
12. Wilkinson TS, Bressler SL, Evanko SP, Braun KR, Wight TN. Overexpression of hyaluronan synthases alters vascular smooth muscle cell phenotype and promotes monocyte adhesion. *J Cell Physiol*. 2006;206:378–385. doi: 10.1002/jcp.20468.
13. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest*. 2000;106:349–360. doi: 10.1172/JCI10272.
14. Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Misra S, Tamura K, Hori H, Yokota S, Toole BP, Sugimoto Y, Ishikawa Y. Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. *J Clin Invest*. 2006;116:3026–3034. doi: 10.1172/JCI28639.
15. Kashima Y, Takahashi M, Shiba Y, Itano N, Izawa A, Koyama J, Nakayama J, Taniguchi S, Kimata K, Ikeda U. Crucial role of hyaluronan in neointimal formation after vascular injury. *PLoS One*. 2013;8:e58760. doi: 10.1371/journal.pone.0058760.
16. Chai S, Chai Q, Danielsen CC, Hjorth P, Nyengaard JR, Ledet T, Yamaguchi Y, Rasmussen LM, Wogensen L. Overexpression of hyaluronan in the tunica media promotes the development of atherosclerosis. *Circ Res*. 2005;96:583–591. doi: 10.1161/01.RES.0000158963.37132.8b.
17. Vigetti D, Viola M, Karousou E, Rizzi M, Moretto P, Genasetti A, Clerici M, Hascall VC, De Luca G, Passi A. Hyaluronan-CD44-ERK1/2 regulate human aortic smooth muscle cell motility during aging. *J Biol Chem*. 2008;283:4448–4458. doi: 10.1074/jbc.M709051200.
18. Viola M, Bartolini B, Vigetti D, Karousou E, Moretto P, Deleonibus S, Sawamura T, Wight TN, Hascall VC, De Luca G, Passi A. Oxidized low

density lipoprotein (LDL) affects hyaluronan synthesis in human aortic smooth muscle cells. *J Biol Chem*. 2013;288:29595–29603. doi: 10.1074/jbc.M113.508341.
19. Kumar A, Hoover JL, Simmons CA, Lindner V, Shebuski RJ. Remodeling and neointimal formation in the carotid artery of normal and P-selectin-deficient mice. *Circulation*. 1997;96:4333–4342.
20. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science*. 1991;253:1129–1132.
21. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest*. 1992;89:507–511. doi: 10.1172/JCI115613.
22. Kumar A, Lindner V. Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol*. 1997;17:2238–2244.
23. El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol*. 2008;9:1399–1406. doi: 10.1038/ni.1671.
24. Lavin B, Gómez M, Pello OM, Castejon B, Piedras MJ, Saura M, Zaragoza C. Nitric oxide prevents aortic neointimal hyperplasia by controlling macrophage polarization. *Arterioscler Thromb Vasc Biol*. 2014;34:1739–1746. doi: 10.1161/ATVBAHA.114.303866.
25. Farb A, Kolodgie FD, Hwang JY, Burke AP, Tefera K, Weber DK, Wight TN, Virmani R. Extracellular matrix changes in stented human coronary arteries. *Circulation*. 2004;110:940–947. doi: 10.1161/01.CIR.0000139337.56084.30.
26. Savani RC, Wang C, Yang B, Zhang S, Kinsella MG, Wight TN, Stern R, Nance DM, Turley EA. Migration of bovine aortic smooth muscle cells after wounding injury. The role of hyaluronan and RHAMM. *J Clin Invest*. 1995;95:1158–1168. doi: 10.1172/JCI117764.
27. Jain M, He Q, Lee WS, Kashiki S, Foster LC, Tsai JC, Lee ME, Haber E. Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. *J Clin Invest*. 1996;97:596–603. doi: 10.1172/JCI118455.
28. Zhao L, Lee E, Zukas AM, Middleton MK, Kinder M, Acharya PS, Hall JA, Rader DJ, Puré E. CD44 expressed on both bone marrow-derived and non-bone marrow-derived cells promotes atherogenesis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol*. 2008;28:1283–1289. doi: 10.1161/ATVBAHA.108.165753.
29. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Puré E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J Clin Invest*. 2001;108:1031–1040. doi: 10.1172/JCI12455.
30. Foster LC, Wiesel P, Huggins GS, Pañares R, Chin MT, Pellacani A, Perrella MA. Role of activating protein-1 and high mobility group-I(Y) protein in the induction of CD44 gene expression by interleukin-1beta in vascular smooth muscle cells. *FASEB J*. 2000;14:368–378.
31. Foster LC, Arkonac BM, Sibinga NE, Shi C, Perrella MA, Haber E. Regulation of CD44 gene expression by the proinflammatory cytokine interleukin-1beta in vascular smooth muscle cells. *J Biol Chem*. 1998;273:20341–20346.
32. Shishido T, Nozaki N, Takahashi H, Arimoto T, Niizeki T, Koyama Y, Abe J, Takeishi Y, Kubota I. Central role of endogenous Toll-like receptor-2 activation in regulating inflammation, reactive oxygen species production, and subsequent neointimal formation after vascular injury. *Biochem Biophys Res Commun*. 2006;345:1446–1453. doi: 10.1016/j.bbrc.2006.05.056.
33. Karper JC, de Vries MR, van den Brand BT, Hoefer IE, Fischer JW, Jukema JW, Niessen HW, Quax PH. Toll-like receptor 4 is involved in human and mouse vein graft remodeling, and local gene silencing reduces vein graft disease in hypercholesterolemic APOE*3Leiden mice. *Arterioscler Thromb Vasc Biol*. 2011;31:1033–1040. doi: 10.1161/ATVBAHA.111.223271.

Significance

Intimal thickening causes luminal narrowing after stent implantation, balloon catheter–based revascularization, by-pass grafting, and during atherosclerosis. Accumulation of extracellular matrix is critically involved in these pathologies because it directly contributes to neointimal volume expansion and regulates smooth muscle cell responses. As therapeutic targets hyaluronan (HA) synthases seem attractive because 3 different isoenzymes exist, which rapidly generate the hyaluronan matrix and might be targeted specifically in the future. Here, we show that genetic deletion of *Has3* inhibits neointimal hyperplasia and that HA synthase 3–dependent HA synthesis increases platelet-derived growth factor BB–mediated migration. These results suggest that inhibition of HA synthase 3–mediated HA synthesis may be used to limit neointimal hyperplasia.

Biochemical Pharmacology 112 (2016) 24–36

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Nitric oxide up-regulates endothelial expression of angiotensin II type 2 receptors

Vu Thao-Vi Dao [a,d], Sawsan Medini [a], Marion Bisha [a], Vera Balz [b], Tatsiana Suvorava [a], Murat Bas [c,1], Georg Kojda [a,*,1]

[a] Institute for Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf, Germany
[b] Institute of Transplantation Diagnostics and Cell Therapeutics, University Hospital, Heinrich-Heine-University, Düsseldorf, Germany
[c] Otorhinolaryngology Department, University Hospital Rechts der Isar, Munich Technical University, Munich, Germany
[d] Maastricht University, Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands

ARTICLE INFO

Article history:
Received 11 March 2016
Accepted 24 May 2016
Available online 26 May 2016

Keywords:
Nitric oxide
Endothelial nitric oxide synthase
Exercise
Angiotensin II type 2 receptor
Angiotensin converting enzyme 1

ABSTRACT

Increasing vascular NO levels following up-regulation of endothelial nitric oxide synthase (eNOS) is considered beneficial in cardiovascular disease. Whether such beneficial effects exerted by increased NO-levels include the vascular renin–angiotensin system remains elucidated. Exposure of endothelial cells originated from porcine aorta, mouse brain and human umbilical veins to different NO-donors showed that expression of the angiotensin-II-type-2-receptor (AT2) mRNA and protein is up-regulated by activation of soluble guanylyl cyclase, protein kinase G and p38 mitogen-activated protein kinase without changing AT2 mRNA stability. In mice, endothelial-specific overexpression of eNOS stimulated, while chronic treatment with the NOS-blocker L-nitroarginine inhibited AT2 expression. The NO-induced AT2 up-regulation was associated with a profound inhibition of angiotensin-converting enzyme (ACE)-activity. In endothelial cells this reduction of ACE-activity was reversed by either the AT2 antagonist PD 123119 or by inhibition of transcription with actinomycin D. Furthermore, in C57Bl/6 mice an acute i.v. bolus of L-nitroarginine did not change AT2-expression and ACE-activity suggesting that inhibition of ACE-activity by endogenous NO is crucially dependent on AT2 protein level. Likewise, three weeks of either voluntary or forced exercise training increased AT2 expression and reduced ACE-activity in C57Bl/6 but not in mice lacking eNOS suggesting significance of this signaling interaction for vascular physiology. Finally, aortic AT2 expression is about 5 times greater in female as compared to male C57Bl/6 and at the same time aortic ACE activity is reduced in females by more than 50%. Together these findings imply that endothelial NO regulates AT2 expression and that AT2 may regulate ACE-activity.

1. Introduction

Clinical trials on the effects of exercise have provided a large body of evidence demonstrating a reduction of morbidity and mortality in cardiovascular disease [1–4]. A variety of underlying molecular mechanisms in response to exercise have been evaluated in cardiovascular tissues [1,5]. As for the vasculature, an increase of endothelial NO-synthase (eNOS) expression and activity, which has been demonstrated in many species including cardiovascular patients [6], appears to be a hallmark of regular physical activity [1]. Subsequently, NO increases the expression of putatively vasoprotective genes such as extracellular superoxide dismutase [7]. In striking contrast, little is known about the impact of exercise on the vascular renin–angiotensin-system. In coronary artery disease patients, Adams et al. observed an increase of angiotensin II type 2 receptor (AT2) mRNA levels following exercise training [8] but the underlying molecular mechanisms are unknown. Nevertheless, activation of AT2 is known to counter regulate many effects mediated by angiotensin II type 1 receptor (AT1) and thus is viewed as vasoprotective target for future therapeutic interventions [9,10]. For example, stimulation of AT2 was shown to induce the prekallikrein activator prolylcarboxypeptidase [11,12] and thereby increases generation of bradykinin and subsequently endothelial NO. AT2 was also shown to inhibit degradation of bradykinin by the angiotensin converting enzyme (ACE) [13]. Hence, activation of AT2 stimulates the kallikrein kinin as well as the NO system in vascular endothelial cells. In addition,

* Corresponding author at: Institute of Pharmacology and Clinical Pharmacology, University Hospital Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany.
E-mail address: kojda@uni-duesseldorf.de (G. Kojda).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bcp.2016.05.011
0006-2952/

Non-standard abbreviations and acronyms

ACE	angiotensin converting enzyme 1
ANGII	angiotensin II
AT2	angiotensin II type 2 receptor
$AT2^{-/-}$	angiotensin II type 2 receptor knock out mice
AT1	angiotensin II type 1 receptor
b.END3	mouse brain endothelial cells
cGMP	cyclic guanosine monophosphate
DEA/NO	diethylamine nonoate (PubChem CID: 9920715)
DETA/NO	diethylenetriamine nitric oxide adduct (PubChem CID: 4518)
eNOS	endothelial nitric oxide synthase
$eNOS^{tg}$	endothelial nitric oxide synthase transgene positive mice
$eNOS^{-/-}$	endothelial nitric oxide synthase knockout mice
$eNOS^{n}$	endothelial nitric oxide synthase transgene negative mice
HUVEC	human umbilical vein endothelial cells
L-NA	L-nitroarginine (PubChem CID: 28360916)
NO	nitric oxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (PubChem CID: 1456)
PAEC	porcine aortic endothelial cells
p38 MAPK	p38 mitogen-activated protein kinase
PKG	protein kinase G
SNAP	S-nitroso-N-acetyl-DL-penicillamine (PubChem CID: 5231)
sGC	soluble guanylate cyclase

increased levels of bradykinin [14] are most likely involved in the beneficial effects of drugs such as ACE-inhibitors [15] and AT1 blockers [16]. While these data likely provide a reasonable molecular basis to explain vasoprotective effects in response to AT2 stimulation, it remains unknown whether exercise increases AT2 signaling. We hypothesized that a link between exercise, NO and angiotensin receptors might present a possible molecular interaction underlying beneficial cardiovascular effects of exercise. The aim of this study was to investigate the role of NO and of exercise for AT2 expression.

2. Materials and methods

2.1. Compounds

All chemicals were purchased from Sigma–Aldrich (Munich, Germany) or Merck (Darmstadt, Germany), except otherwise stated in the text.

2.2. Cell culture

Porcine aortic endothelial cells (EC) were isolated from aortas of freshly slaughtered 7- to 9-month-old pigs, and cultured in M199 medium containing 10% FCS (PAA, Kölbe) until passage 3. Brain endothelial cells (b.END3) were provided from the department of Pharmaceutical Chemistry II of the Pharmaceutical Institute, Bonn, Germany (passage 32–35) and purchased from ATCC® (CRL2299™, Wesel, Germany, passage 24–28). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad CA) containing GlutaMAX™ (Gibco, Darmstadt, Germany). Human umbilical vein endothelial cells (HUVEC) were purchased from (ATCC® PCS-100-013, Wesel, Germany) and cultured in Vascular Cell Basal Medium (ATCC® PCS-100-030™, Wesel, Germany) supplemented with Endothelial Cell Growth Kit-BBE (ATCC® PCS-100-040™, Wesel, Germany) (passage 3–5). The rationale for selecting the time points used in cell culture studies was evaluated during the course of the study (please refer to Section 3).

2.3. Animals

C57Bl/6 mice (male, 3–4 months old, 24–28 g) were purchased from JANVIER LABS (France). Transgenic mice (male, 3–4 months old, 24–28 g) such as $eNOS^{tg}$ mice [17,18], $eNOS^{-/-}$ [19] and their transgenic negative littermates ($eNOS^{n}$), backcrossed> 20 times to C57Bl/6 background as well as male, 3–4 months old (24–28 g) mice deficient of AT2 ($AT2^{-/-}$, generously provided by Michael Bader, PhD, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), backcrossed >20 times to FVB/N background were bread and housed in the animal facility (ZETT, UKD Duesseldorf, Germany). The experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' (approval references: 50.05-230-18/06, 8.87-51.04.20.09.383) and the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health.

2.4. Quantitative real-time PCR experiments

Total RNA from mouse aortic tissue, HUVEC and bEND.3 was isolated using the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNAse digestion. Complementary DNA was synthesized from 1 μg total RNA by Turbo™ DNase (Ambion, Carlsbad, USA) according to the manufacturers' protocol. Quantitative real-time PCR experiments were performed with TaqMan® Gene Expression Assays (Applied Biosystems, Weiterstadt Germany) using Mm01341373 and Hs00169126 for mouse AT2 and human AT2 detection, respectively. For normalization purpose, co-amplification of hypoxanthine guanine phosphoribosyl transferase (hprt1) cDNA was performed with the TaqMan® Gene Expression Assay for mouse hprt1 (Mm00446968) and human hprt1 (Hs02800695). Quantitative real-time PCR experiments were carried out on a 7300 Real-Time PCR System (Applied Biosystems, Weiterstadt, Germany). AT2 mRNA expression levels relative to hprt1 were determined using the $^{\Delta\Delta}$Ct method and expressed in arbitrary units [a.u] relative to paired controls.

2.5. Western blot analysis

AT2, AT1, ACE1, and eNOS [20,21] protein expression as well as p38 MAPK phosphorylation and total p38 MAPK ratio were determined in membrane fractions of aortic mouse tissues prepared as described previously [20,22]. Membrane fractions from endothelial cells were obtained after lysis in RIPA buffer containing 150 mM NaCl, 50 mM Tris/HCl, 1% Nonidet-40, 0.1% SDS, and PIC™ (Roche, Mannheim, Germany) and centrifuged at 100×g at 4 °C to obtain the supernatant. 20 μg of total protein were loaded for immunoblotting. Western blotting was performed using commercially available polyclonal antibodies against AT2 (1:1000) (AB15554, Millipore, Darmstadt, Germany and sc-9040, Santa Cruz, Heidelberg, Germany), AT1 (1:1000) (sc-1173, Santa Cruz), p38 MAPK (1:500) (9212, Cell Signaling), phospho-p38 MAPK (1:500)

(9211, Cell Signaling, Frankfurt am Main, Germany) and monoclonal antibodies against ACE1 (1:300) (ab75762, Abcam, Cambridge, UK) and eNOS (1:1000) (610297, BD Transduction, Heidelberg, Germany). It was found that the Millipore antibody, which served to detect AT2 protein in mouse tissue homogenates, gave a signal related to actin which was 10.2% for $AT2^{-/-}$ mice as compared to wildtype mice (100%, $n = 4$, $P < 0.0001$) suggesting a useful specificity. In case of the sc-9040 Santa Cruz antibody, we weren't able to detect the expected signal for AT2 in aortic tissue of $AT2^{-/-}$ mice at all. Blots were subsequently challenged either with a horseradish peroxidase-conjugated anti-mouse antibody (Biorad, Munich, Germany), anti-rabbit antibody (Calbiochem, Darmstadt, Germany), or with IRDye® 800CW-conjugated anti-rabbit antibody and IRDye® 680LT-conjugated anti-mouse antibody (LI-COR, Bad Homburg, Germany), respectively. All blots of cell homogenates were standardized to polyclonal anti-β-actin (Sigma–Aldrich, Munich, Germany) following a stripping procedure. Blots of tissue homogenates were standardized with monoclonal anti-glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) (Sigma–Aldrich, Munich, Germany) or beta-tubulin (Abcam, Germany) to avoid a stripping procedure. Blots were developed using ECL (Roche, Mannheim, Germany) and exposed to X-ray film to quantify by densitometry (Gel Doc 1000; Bio-Rad, Munich, Germany) or by infrared fluorescence (Odyssee Imagine System CLx, LI-COR, Bad Homburg, Germany). Protein bands were quantified using the Imagine Studio software (LI-COR, Bad Homburg, Germany). Comparative quantitative evaluation was performed with signals appearing on the same blot only.

2.6. ACE-activity assay

ACE-activity was quantified by measuring the reduction of absorption in the presence of the specific ACE-substrate furylacryloyl-phenylalanyl-glycyl-glycine (FAPGG, Sigma–Aldrich, Munich, Germany), which was hydrolyzed time-dependently to FAP and GG [23]. Briefly, aortic mouse tissues were lysed in buffer containing 150 mM NaCl, 50 mM Borax 10 μg/ml protease inhibitors (antipain, benzamidine, leupeptin, aprotinin), 0.2 mM phenylmethylsulfonylfluoride (PMSF), $ZnCl_2$ (1 μM) and 0.1% triton X-100. Homogenates were centrifuged at 100×*g* for 10 min and subsequently at 10,000×*g* for 5 min. The supernatant was taken for spectrophotometrical measurements at 340 nm at 37 °C for 45 min. The reaction was started by addition of the specific ACE-substrate FAPGG to the reaction mix. To determine ACE-activity in HUVEC, cells were lysed in buffer containing 10 mM Tris/HCl, 150 mM NaCl; 1 μM $ZnCl_2$; 2 μg/ml protease inhibitors (antipain, leupeptin, aprotinin, Pepstatin A), 10 μg/ml trypsin inhibitor, 1% Nonidet-40 and 44 μg/ml PMSF for 10 min on ice. Samples were centrifuged at 100×*g* for 10 min and the supernatant was taken for ACE-activity measurements as described above. Mouse aortic tissues and endothelial cells were lysed and homogenates were centrifuged. The reaction was started at 37 °C by addition of FAPGG to the supernatant and ACE-activity was continuously monitored at 340 nm for 45 min.

2.7. Pharmacological treatment of C57BL/6 and transgenic mice

Transgenic $eNOS^{tg}$ mice and their negative littermates $eNOS^{n}$ (male, 3–4 months old) received L-nitroarginine (**1**-NA) in the drinking water (100 mg L-NA/kg BW/day) [24] or placebo for 3 weeks. The bottles were changed daily. After the experimental period mice were sacrificed and organs were snap frozen in liquid nitrogen. In another set of experiments C57BL/6 mice received a bolus intravenous injection of 32 mg L-NA/kg BW [25,26]. After this 30 min acute intervention mice were sacrificed and organs were taken for further experiments.

2.8. Exercise training

All mice were singularized for 8 weeks prior exercise training. Thereafter C57Bl/6 mice (male, 4 months old) were randomized into three groups, sedentary, forced exercise and voluntary running group. Mice underwent exercise training following two exercise training protocols, i.e. voluntary running and forced exercise, as described previously [20,21]. For control experiments $eNOS^{-/-}$ mice (male, 4 months old) were randomized into voluntary running group and sedentary group ($n = 6$ mice, each group). After three weeks of exercise training, mice were sacrificed and their organs were taken for further experiments. To quantify training efficacy their heart weight, body weight, soleus weight and tibia length were determined [20,27].

2.9. Statistical analyses

All data were analyzed by standard computer program (GraphPad Prism PC software, version 4.00) and are expressed as mean ± S.E.M. of *n* individual samples. Statistical calculations are based on a minimum of 4 experimental replications as for endothelial cells and a minimum of 5 mice per experimental set. Statistical comparisons between groups were performed by either Student's *t* tests or Newman–Keuls multiple comparison post-hoc test following one-way ANOVA (for more than two groups). To compare the time-course of three treatment groups two-way ANOVA was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. NO-dependent up-regulation of AT2 expression in endothelial cells

Treatment of porcine aortic endothelial cells (PAEC) and brain derived endothelial cells (bEND.3) with increasing concentrations of the NO-donor diethylamine nonoate (DEA/NO, 1–100 μM) for 3 h increased AT2 protein expression to about 2.0–2.5-fold as compared to vehicle and 1 μM DEA/NO (Fig. 1A). Likewise exposure of PAEC to diethylenetriamine nitric oxide adduct (DETA/NO) for 3 h, a NO-donor leading to a sustained release of NO over 24 h, increased AT2 protein in a concentration-dependent manner (Fig. 1B). Using the quick releasing NO-donor SNAP, up-regulation of AT2 protein was found in PAEC at 3 h as well (Fig. 1C). In addition, a time-dependent increase of AT2 protein could be observed with 10 μM DETA/NO in PAEC and bEND.3 (Fig. 1D) and a similar effect occurred in PAEC and bEND.3 at a higher concentration of DETA/NO (1 mM) (data not shown). To exclude species specific effects and to be able to study AT2 mRNA expression in different cell types human umbilical vein endothelial cells (HUVEC) were exposed to DEA/NO (10 μM) that resulted in an increased AT2 protein (Fig. 1E). In contrast, there was no effect of exogenous NO on the expression of angiotensin II type 1 receptor (AT1) expression (Fig. 1F) suggesting that NO selectively regulates the AT2 expression.

3.2. Effect of NO on AT2 mRNA expression and stability

AT2 protein up-regulation in response to exogenous NO was paralleled by an increase in AT2 mRNA expression at 3 h and 6 h in both HUVEC (Fig. 2A) and b.END 3 (Fig. 2B). To investigate whether transcriptional or translational mechanisms or both lead to an increased AT2 expression in response to NO, de novo transcription was blocked by exposing bEND.3 with actinomycin D and cells were subsequently incubated with DEA/NO (10 μM) to determine AT2 mRNA expression. In another set of experiments cells were treated with DEA/NO alone (10 μM). There was a rapid

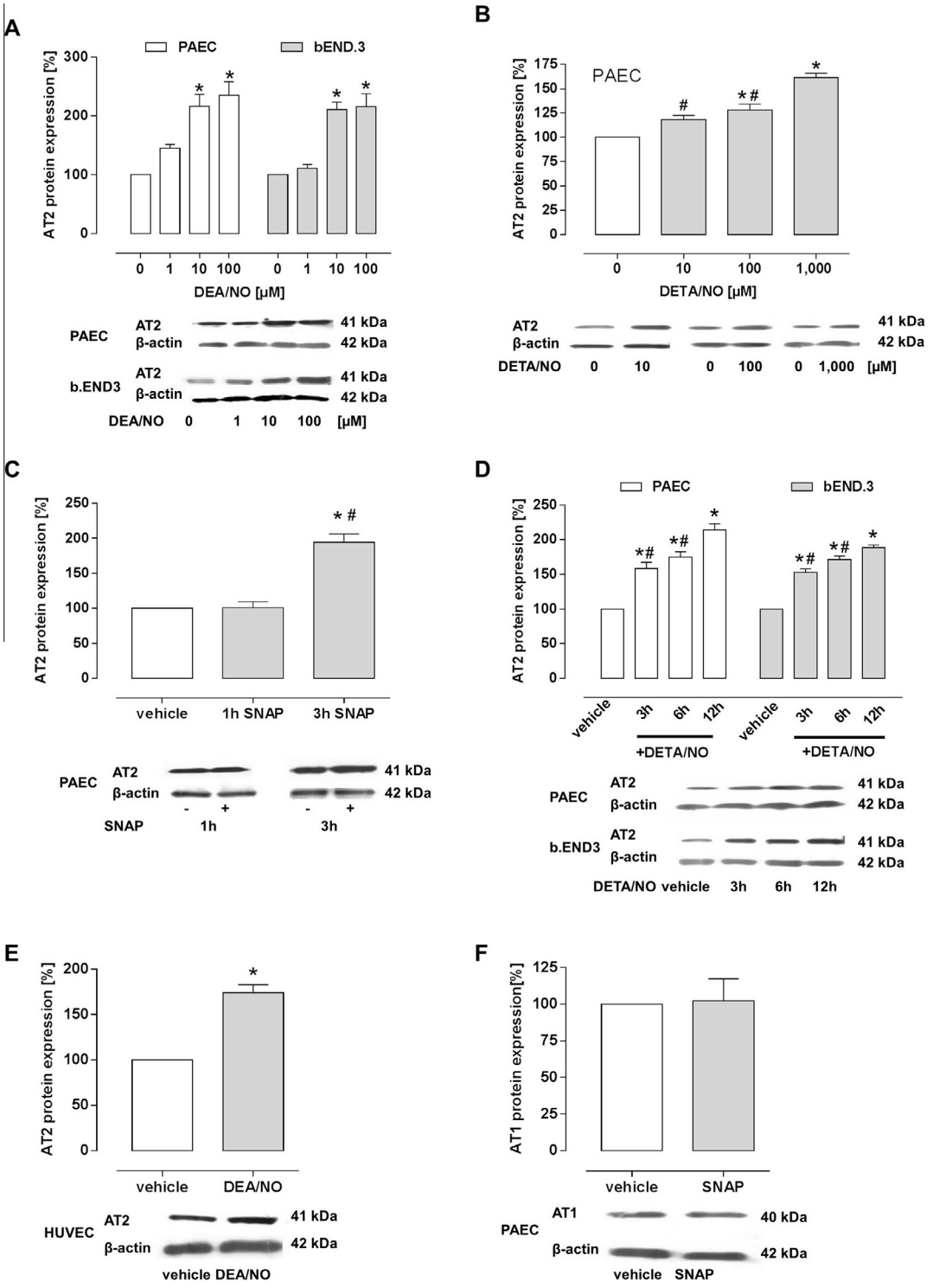


Fig. 1. Effect of NO on AT2 expression in endothelial cells. Concentration response of AT2 protein expression (A) in PAEC and b.END3 to a 3 h incubation with the NO-donor DEA/NO (1, 10, 100 μM) ($^{*}P < 0.05$ vs. vehicle, $n = 4$–6), and (B) in PAEC to a 3 h incubation with the NO-donor DETA/NO (0.01, 0.1, 1 mM) ($^{*}P < 0.05$ vs. vehicle, $^{\#}P < 0.05$ vs. 1 mM, $n = 4$). Time course of AT2 protein (C) in PAEC using the quick releasing NO-donor SNAP (10 μM) ($^{*}P < 0.05$ vs. vehicle and $^{\#}P < 0.05$ vs. 1 h, $n = 6$), (D) in PAEC using 1 mM DETA/NO ($^{*}P < 0.0001$ vs. vehicle $^{\#}P < 0.01$ vs. 12 h, $n = 6$) and in bEND.3 ($^{*}P < 0.0001$ vs. vehicle, $^{\#}P < 0.05$ vs. 24 h, $n = 6$). Up-regulation of (E) AT2 protein expression ($^{*}P < 0.001$ vs. vehicle, $n = 6$) induced by 10 μM DEA/NO in HUVEC at 3 h. (F) In contrast, 10 μM SNAP has no effect on AT1 protein expression in PAEC ($P > 0.05$, $n = 4$). Data are mean ± S.E.M. Statistical significance indicated as $^{*,\#}P < 0.05$ using a one-way-ANOVA and Newman–Keuls Multiple Comparison Test (A–D) and using Student's t test (E, F). Representative protein bands were cut from repetitive western blots and are depicted at the bottom of the corresponding graphs. AT2 and AT1 protein were detected at 41 kDa and standardized to β-actin (42 kDa) following stripping procedure; n indicates the individual number of experiments.

decay of AT2 mRNA to about 50% of the initial value within the first 3 h in the two groups treated with actinomyosin D (Fig. 2C). Actinomycin D completely inhibited the DEA/NO-induced elevation of AT2 mRNA level, while DEA/NO itself did not change the half-live of AT2 mRNA that was observed following inhibition of transcription with actinomycin. Furthermore, the increase of AT2

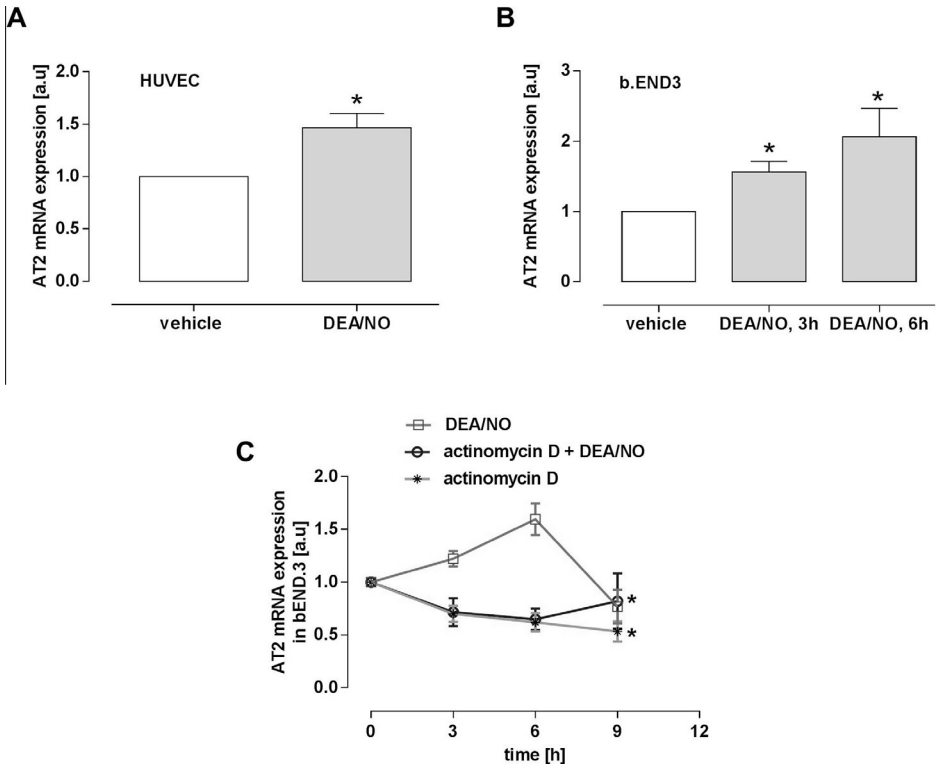


Fig. 2. Effect of NO on AT2 mRNA expression and stability in endothelial cells. (A) Induction of AT2 mRNA by 10 μM DEA/NO ($^{*}P < 0.05$ vs. vehicle, $n = 4$) in HUVEC. These data are well matched with the upregulation of AT2 protein shown in Fig. 1E. (B) Time course of AT2 mRNA expression in b.END.3 at 3 h and 6 h, ($^{*}P < 0.05$ vs. vehicle, $n = 6$). Again, these data are well matched with the upregulation of AT2 protein shown in Fig. 1D. (C) Messenger RNA levels were determined by quantitative real-time PCR in bEND.3 following inhibition of transcription with actinomycin D (10 μg/ml, 30 min) either alone or in presence of 10 μM DEA/NO. Treatment with actinomycin D significantly inhibited the time-dependent and reversible increase of AT2 mRNA content induced by DEA/NO ($P < 0.0001$ two-way ANOVA for treatment, $^{*}P < 0.05$ vs. DEA/NO, $n = 6$). DEA/NO did not change the decrease of AT2 mRNA following actinomycin D ($P > 0.05$ actinomycin D vs. actinomycin D + DEA/NO, $n = 6$). All data are mean ± S.E.M. and indicated *P*-values are calculated by using Student's *t* test (A), one-way-ANOVA and Newman–Keuls Multiple Comparison Test (B), and Newman–Keuls test following two-way ANOVA (C).

mRNA induced by DEA/NO rapidly declined between 6 h and 9 h of incubation time. These data suggest that the NO-induced increase of AT2 mRNA level in endothelial cells appears to result from an elevated transcription rate, whereas direct effects of NO on AT2 mRNA stability could not be observed.

3.3. NO-induced AT2 up-regulation is mediated by cGMP-signaling

To investigate the underlying mechanism mediating NO-dependent up-regulation of AT2 expression, PAEC were incubated with the soluble guanylyl cyclase (sGC)-inhibitor ODQ. While ODQ itself had no effect, it completely inhibited AT2 up-regulation induced by either DEA/NO or SNAP (Fig. 3A). Inhibition of cGMP degradation using the phosphodiesterase (PDE)-V inhibitor sildenafil (PubChem CID: 5212) showed only a trend for an increase of AT2 protein both in the presence and absence of SNAP (Fig. 3B). In contrast, direct stimulation of protein kinase G (PKG) in PAEC with the cGMP analog 8-pCPT-cGMP (Biolog, Bremen, Germany, PubChem CID: 123969) for 3 h significantly increased AT2 protein expression (Fig. 3C), while inhibition of PKG with the inhibitor RP-pCPT-cGMP (Biolog, Bremen, Germany, PubChem CID: 6426630) reversed the 2.0-fold up-regulation of AT2 protein level induced by DEA/NO (Fig. 3D). To study the involvement of downstream signaling molecules, which can be phosphorylated by PKG, PAEC were incubated with 8-pCPT-cGMP to stimulate directly PKG. This intervention increased phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) (Fig. 3E). Likewise, treatment of PAEC with DEA/NO increased phosphorylation of p38MAPK whereas co-incubation of PAEC with the PKG-inhibitor RP-pCPT-cGMP reversed this effect (Fig. 3F). Similar results were observed using the (MAPK)-inhibitor SB203580 (PubChem CID: 176155) following co-incubation with DEA/NO (Fig. 3G) emphasizing the crucial involvement of PKG mediated p38MAPK phosphorylation. To study a possible linkage of these signaling events to the up-regulation of AT2 upon stimulation with exogenous NO, PAEC were exposed to the (MAPK)-inhibitor SB203580 following co-incubation with SNAP (Fig. 3H). While SNAP increased AT2 protein level, inhibition of p38MAPK reversed this effect supporting the finding that up-regulation of AT2 by NO is mediated by the classical NO-sGC-cGMP-PKG pathway and involves phosphorylation of the transcription mediator p38 MAPK.

3.4. NO-dependent up-regulation of AT2 in transgenic mice

Transgenic mice overexpressing eNOS ($eNOS^{tg}$) [18,28] and their negative littermates ($eNOS^{n}$) were used to study the NO-dependent changes of AT2 expression *in-vivo*. The endothelial specific overexpression of eNOS was visualized by confocal microscopy and the 3.5-fold higher eNOS protein expression was associated with a strong blood pressure reduction indicating functional activity [18]. Quantification of aortic AT2 mRNA (Fig. 4A) showed a significant increase in $eNOS^{tg}$ as compared to $eNOS^{n}$ mice. To verify the NO-dependency of AT2 up-regulation, aortic AT2 expression in mice deficient of eNOS ($eNOS^{-/-}$) was determined. Surprisingly, AT2 mRNA (Fig. 4B) level was not altered in $eNOS^{-/-}$ as compared to their negative littermates ($eNOS^{n}$). A similar pattern was observed with AT2 protein expression which was increased in $eNOS^{tg}$ (Fig. 4C) and unchanged in $eNOS^{-/-}$ (Fig. 4D). To mimic a

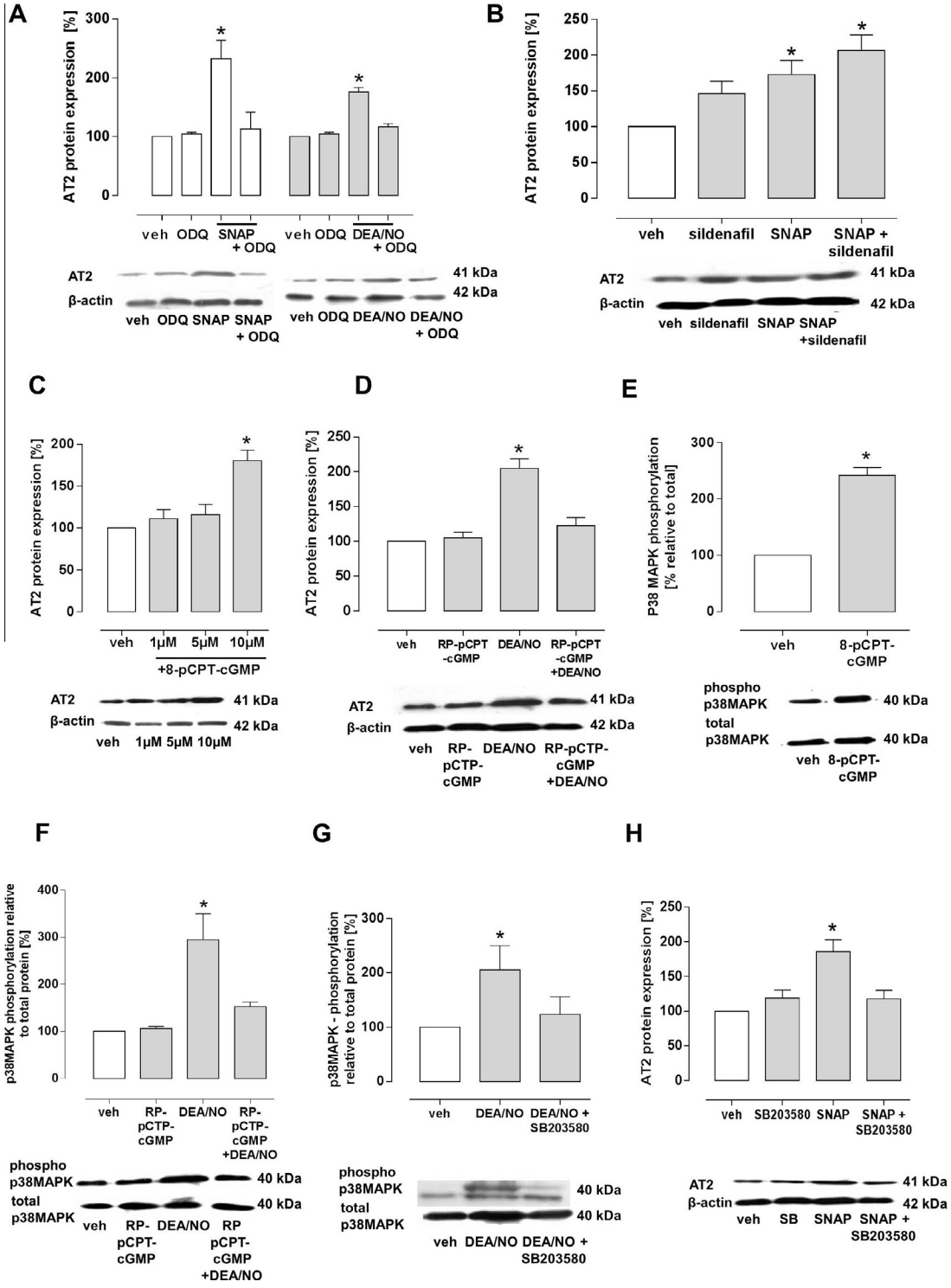


Fig. 3. NO dependent AT2 up-regulation is mediated by cGMP-signaling in PAEC. All experiments shown have been performed after 3 h of exposure. (A) Treatment with 10 μM SNAP ($^{*}P < 0.05$ vs. all other conditions, $n = 7$ each) or 10 μM DEA/NO for 3 h increased AT2 protein expression, while ODQ blunted this effect ($^{*}P < 0.05$ vs. all other conditions, $n = 6$ each). (B) Incubation of PAEC with both 10 μM SNAP and the PDE-V-inhibitor sildenafil (sild.) for 3 h increased AT2 protein ($^{*}P < 0.05$ vs. vehicle, $n = 5$ each). (C) Treatment with the cell permeable cGMP analog 8-pCPT-cGMP (10 μM) for 3 h increased AT2 protein expression ($^{*}P < 0.05$ vs. all other conditions, $n = 4$ each). (D) 10 μM RP-pCPT-cGMP, a cell permeable inhibitor of protein kinase G abolished the increase of AT2 protein expression induced by 10 μM DEA/NO in PAEC ($^{*}P < 0.05$ vs. all other conditions, $n = 6$ each). (E) Increased p38 MAPK phosphorylation in response to the PKG-mimetic 8-pCPT-cGMP (10 μM) ($^{*}P < 0.05$ vs. vehicle, $n = 6$). (F) DEA/NO (10 μM) induced increase in p38 MAPK phosphorylation was blunted by RP-pCPT-cGMP (10 μM) ($^{*}P < 0.05$ vs. all other conditions, respectively, $n = 8$) and (G) by the p38 MAPK-inhibitor SB203580 (SB) (10 μM) ($^{*}P < 0.05$ vs. all other conditions, respectively, $n = 6$). (H) Reversed effect of SB203580 (SB) on SNAP (10 μM)-induced AT2 protein up-regulation ($^{*}P < 0.05$ vs. all other conditions, $n = 5$ each). Data are mean ± S.E.M. Statistical significance indicated as $^{*}P < 0.05$ using a one-way-ANOVA and Newman–Keuls Multiple Comparison Test (A–D, F–H) and using Student's t test (E). Representative protein bands were cut from repetitive western blots and are depicted at the bottom of the corresponding graphs. AT2 and phospho p38MAPK were visualized at 41 kDa and 40 kDa and were standardized to β-actin (42 kDa) or total p38MAPK (40 kDa), respectively following stripping procedure; n indicates the individual number of experiments.

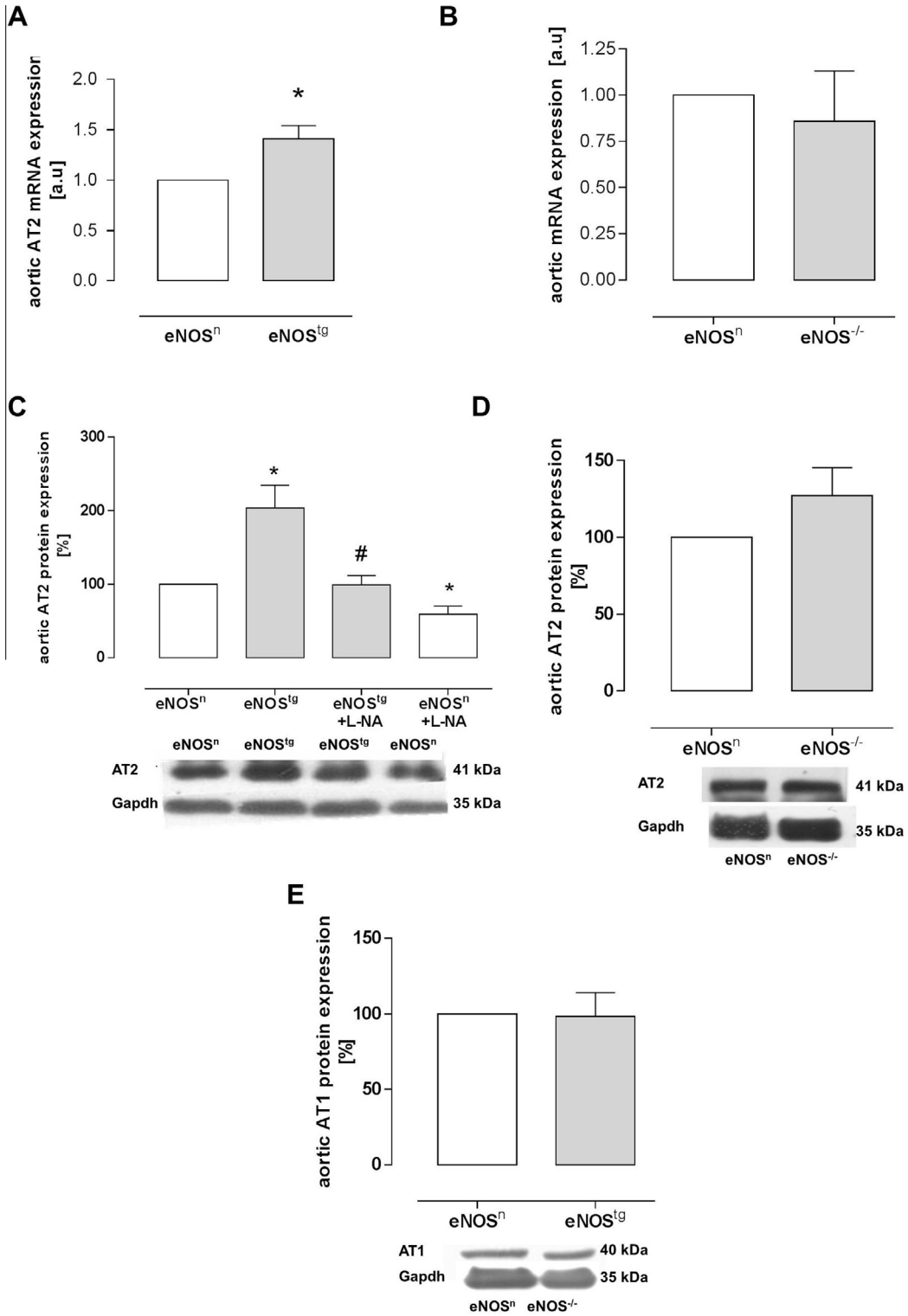


Fig. 4. Effect of NO on aortic AT2 expression in transgenic mice. (A) AT2 mRNA levels in aortic tissue homogenates of eNOS^{tg} mice as compared to eNOS^{n} mice ($^{*}P < 0.05$ vs. eNOS^{n}, $n = 6$) and (B) in eNOS$^{-/-}$ mice as compared to their negative littermates (eNOS^{n}) ($P > 0.05$, $n = 5$ each). (C) Increased aortic AT2 protein expression of eNOS^{tg} ($^{*}P < 0.05$ vs. all other conditions, $n = 6$) and the effect of the NOS inhibitor L-NA for 3 weeks, which reduced aortic AT2 protein of eNOS^{tg} ($^{\#}P < 0.05$ vs. eNOS^{tg}-L-NA, $n = 7$) and eNOS^{n} mice ($^{*}P < 0.05$ vs. eNOS^{n}-L-NA, One-sample t-test (two-tailed), $n = 7$). (D) No change of aortic protein expression in eNOS$^{-/-}$ mice as compared to their negative littermates (eNOS^{n}, $P > 0.05$, $n = 5$ each). (E) Unchanged AT1 protein in aortic homogenates of eNOS^{n} and eNOS^{tg} mice ($P > 0.05$, $n = 6$). Data are mean ± S.E.M. Statistical significance indicated as $^{\#,*}P < 0.05$ using Student's t test. Representative protein bands were cut from repetitive western blots and are depicted at the bottom of the corresponding graphs. AT2 (41 kDa), AT1 (41 kDa) and GAPDH (35 kDa) proteins are depicted at the bottom of the corresponding graphs; n indicates the individual sample number.

transient dysfunction of eNOS rather than a constitutive gene ablation, that may induce a battery of compensatory events, mice underwent pharmacologic inhibition of NOS by L-nitroarginine (L-NA) treatment (100 mg L-NA/kgBW/day) [24]. This three weeks intervention resulted in a decreased aortic AT2 protein expression in eNOS^{n} and eNOS^{tg} mice as compared to placebo-treated eNOS^{n}

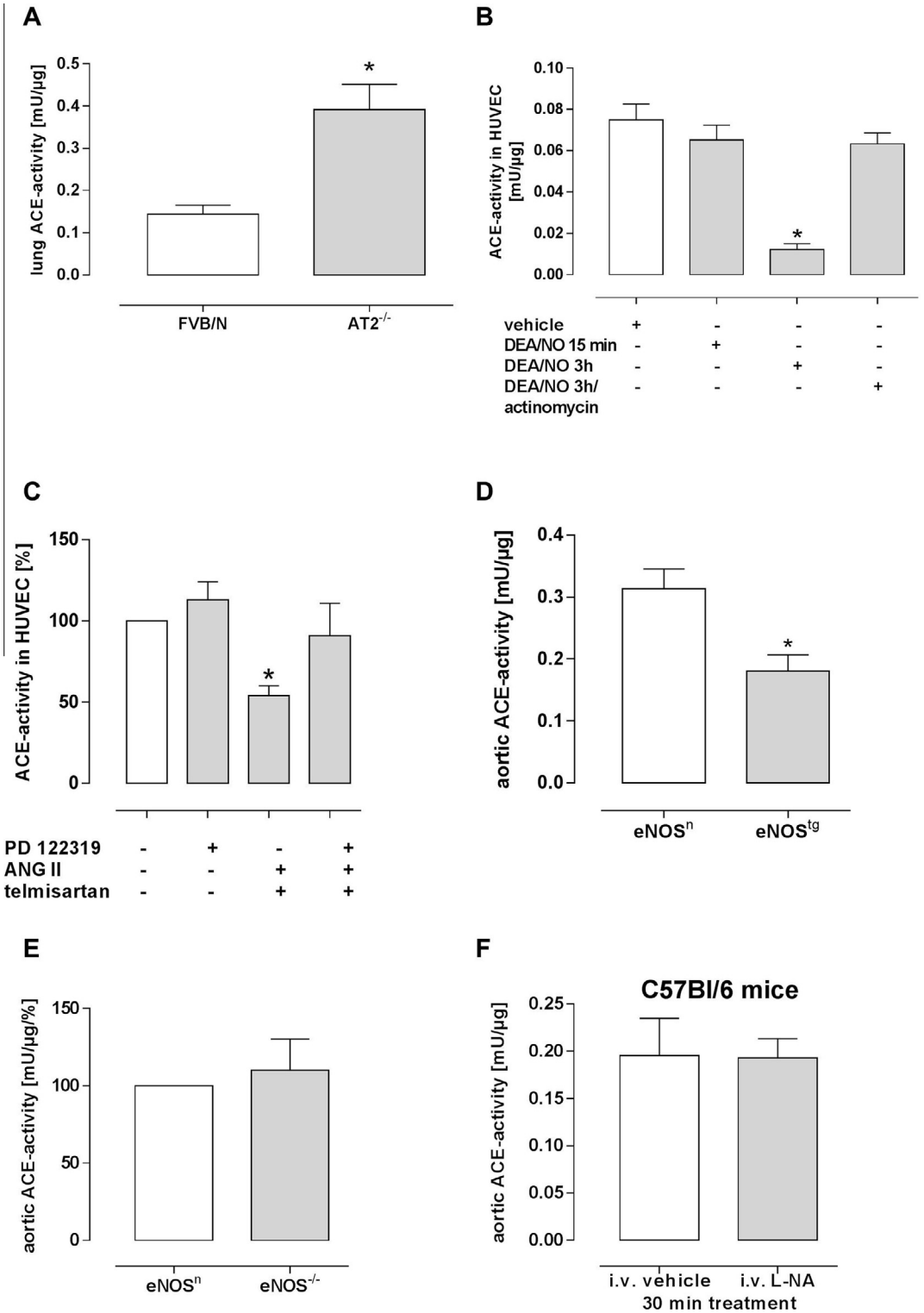


Fig. 5. Effect of NO-dependent AT2 up-regulation on ACE-activity. (A) Tonic inhibition of AT2 on lung ACE-activity measured in $AT2^{-/-}$ as compared to their negative littermates (FVB/N) ($^{*}P < 0.05$, $n = 6$). (B) ACE-activity in HUVEC treated with either 10 µM DEA/NO for 15 min, 10 µM DEA/NO for 3 h alone or in combination with actinomycin D (10 µg/ml). Only treatment of cells with DEA/NO reduced ACE-activity ($^{*}P < 0.05$ vs. all other conditions, $n > 4$, each). (C) Reduction of ACE-activity in HUVEC treated with angiotensin II and telmisartan (each 0.1 µmol/l) which was reversible by co-incubation with the AT2 antagonist 100 µM PD123319 ($^{*}P < 0.05$ vs. all other conditions, $n = 6$). (D) Reduced ACE-activity [mU/µg] in aortic homogenates of $eNOS^{tg}$ as compared to $eNOS^{n}$ mice ($^{*}P < 0.05$, $n = 8$), (E) unchanged aortic ACE-activity related to aortic ACE protein expression [mU/µg/%] in $eNOS^{-/-}$ as compared to their negative littermates ($P > 0.05$, $n = 8$) and (F) similar aortic ACE-activity [µg/mU] of C57BL/6 mice receiving an i.v. bolus of L-NA as compared to vehicle-treated mice ($P > 0.05$, $n = 8$). Data are mean ± S.E.M. ($^{*}P < 0.05$, one-way-ANOVA and Newman–Keuls Multiple Comparison Test (B and C) or Student's t test (A, D, E, F); n indicates the individual sample number.

and $eNOS^{tg}$ mice (Fig. 4C), respectively. According to the *in-vitro* results, increased bioavailability of NO selectively changed aortic AT2 but not AT1 protein level in $eNOS^{tg}$ as compared to $eNOS^{n}$ mice (Fig. 4E), although it was reported that AT1 expression is increased in $AT2^{-/-}$ [29]. Together, these data suggest that increased bioavailability of NO up-regulates vascular AT2 expression *in-vivo*.

Table 1
Quantitation of AT2 expression, ACE expression and ACE activity in aortic tissues of male and female C57Bl/6 mice. Data are mean ± S.E.M. (*P*-values were determined using Student's *t* test).

Measure	Male	Female	*P*-value
AT2 mRNA expression [%]	100.0	572.8 ± 108.4	=0.0120
AT2 protein expression [%]	100.0	420.2 ± 12.40	<0.0001
ACE-activity [%]	100.0	42.80 ± 6.608	<0.0001
ACE expression [%]	100.0	207.3 ± 19.63	=0.0016

3.5. Effect of NO-dependent AT2 signaling on ACE-activity

AT2 knock out ($AT2^{-/-}$) mice demonstrated a tonic inhibitory effect of AT2 on ACE-activity (Fig. 5A) and similar results have been reported previously [13]. To study whether augmentation of the NO-AT2 signaling interaction may alter ACE-activity, HUVEC, which have been previously evaluated for ACE expression (data not shown), were incubated with DEA/NO (10 μM) for 3 h. This intervention resulted in a strong inhibition of ACE-activity (Fig. 5B). To investigate a possible direct effect of NO on ACE-activity [30] HUVEC were incubated with DEA/NO (10 μM) for 15 min. At this time point the release of NO from DEA/NO is already completed [31]. There was no detectable change of ACE-activity (Fig. 5B) and ACE expression (95.50% ± 6.632%, $n = 6$, $P > 0.05$). Furthermore, HUVEC which were incubated with DEA/NO for 3 h and pretreated with actinomycin D to prevent an increase of AT2 protein expression showed also no change of ACE-activity (Fig. 5B), suggesting a minor, if any, direct effect of NO on ACE-activity. To further elucidate the involvement of AT2 in ACE-activity reduction in HUVEC, AT2 was directly stimulated using a combination of angiotensin II and the AT1-blocker telmisartan (Fig. 5C). In response to this treatment there was a strong reduction of ACE-activity that was blocked by the full AT2 antagonist PD123319 (PubChem CID: 176155) which itself had no effect suggesting a significant involvement of AT2 (Fig. 5C). In line with these data, NO-dependent AT2 up-regulation was associated with a reduction of aortic ACE-activity in $eNOS^{tg}$ as compared to $eNOS^{n}$ mice (Fig. 5D) while aortic ACE protein expression did not change (108.8% ± 23.42%, $n = 6$, $P > 0.05$). We have done additional experiments using another model of different AT2 expression levels. Briefly, aortic AT2 expression is about 5 times greater in female as compared to male C57Bl/6 and at the same time aortic ACE activity is reduced in females by more than 50%, despite a 2-fold increase in ACE expression (Table 1).

To further investigate whether changes in vascular ACE-activity are triggered by endogenous NO, ACE-activity was measured in mice lacking eNOS ($eNOS^{-/-}$) [32]. Surprisingly, but in accordance to previously mentioned unaltered aortic AT2 mRNA and protein expression, these mice showed no change of aortic ACE-activity (Fig. 5E) suggesting that changes of ACE-activity are closely linked with an AT2 up-regulation induced by NO *in-vivo*. To further verify this hypothesis NOS was pharmacologically inhibited by administering C57Bl/6 mice an intravenous bolus of L-NA (32 mg/kg BW) [25,26,33] or aqueous 0.9% saline solution. This 30 min treatment period had no effect on AT2 protein (125 ± 22.5%, $n = 6$, $P > 0.05$), ACE protein expression (138 ± 38.7%, $n = 6$, $P > 0.05$) and accordingly, ACE-activity remained unchanged (Fig. 5F). Together these data indicate that increased NO bioavailability leads to inhibition of ACE-activity via up-regulation of AT2.

3.6. Inhibition of ACE-activity by exercise training

Exercise training is known to promote up-regulation of arterial eNOS and subsequent elevated NO-release in C57Bl/6 [34]. To evaluate whether up-regulation of endogenous NO impact on AT2 expression and ACE-activity, mice underwent exercise training following two different protocols, i.e. voluntary running and forced exercise training [21]. Both training methods are known to up-regulate aortic eNOS expression and activity in C57Bl/6 mice [20,34,35]. This was confirmed in 6 mice which underwent 3 weeks of forced exercise training. In these mice the expression of eNOS increased to 235.6% ± 30.52%, $n = 6$ ($P < 0.05$). After 3 weeks of exercise, AT2 mRNA expression was significantly increased in aortic tissues of voluntarily running mice as compared to sedentary controls (Fig. 6A). Likewise, aortic AT2 protein expression of mice following both exercise training types was significantly increased as compared to sedentary controls (Fig. 6B). As there was no difference in upregulation of AT2 induced by voluntary running or forced exercise, expression of AT2 mRNA was measured following the voluntary exercise training protocol only. Moreover, this up-regulation was not present in $eNOS^{-/-}$ mice (Fig. 6C) despite efficient training (Table 2) demonstrating the importance of eNOS to mediate changes in AT2 expression. As shown already in endothelial cells (Fig. 1F), AT1 protein expression did not change in response to NO (Fig. 6D). In accordance to an increase of NO-induced AT2 expression by exercise training, aortic ACE-activity was significantly reduced following forced exercise as compared to sedentary controls (Fig. 6E).

4. Discussion

Among vascular mediator systems, the NO-cGMP, the kallikrein-kinin and the renin angiotensin aldosterone system are of major importance. To our knowledge this is the first finding demonstrating a crucial involvement of NO in the regulation of AT2 expression identifying NO as an indirect ACE-inhibitor *in-vivo*. Our data provide significant evidence to demonstrate a new interaction between these three players mediating vascular signaling cascades that may contribute to vasoprotective mechanisms of NO.

The up-regulation of AT2 expression by NO is predominantly mediated by the classical NO-cGMP pathway, i.e. activation of sGC, generation of cGMP and activation of PKG. Although the involvement of other pathways cannot be excluded, the complete inhibition of AT2 up-regulation by each inhibitor of this pathway including inhibition of p38 MAPK by SB203580 proofs the involvement of this proposed signaling pathway. It was shown that p38 MAPK is activated by Thr180/Tyr182 phosphorylation at the canonical dual phosphorylation site TGY [36,37] suggesting kinases other than PKG such as mitogen-activated protein kinase kinase (MKK) 3 and MKK 6, which are known to specifically phosphorylate p38 MAPK [38], are likely involved. In accordance, studies in mouse embryonic fibroblasts have shown that MKK3 and MKK6 are activated upon phosphorylation at serine 189 and serine 207, respectively [39] but whether activation of PKG results in phosphorylation of MKK3 and/or MKK6 is currently not known [37]. It has been further reported that p38 MAPK mediates nuclear signaling of NO via phosphorylation of the transcription factor Nrf2 [40] which is a representative of the antioxidant defense system and analysis of the AT2 promotor revealed the presence of this transcription factor binding site [41]. Further studies are needed to clarify whether Nrf2 may be one mechanism mediating up-regulation of AT2 by NO.

The increase of AT2 by NO was found to be completely dependent on transcription suggesting that posttranscriptional regulation of AT2 expression, which has been described previously [42], is not altered by NO in endothelial cells. The long half-life of AT2 mRNA reported in this study confirms previous observations in PC12 cells [43]. In line with *in-vitro* results, the effect of NO on AT2 expression was observed in mice as well. To determine the sig-

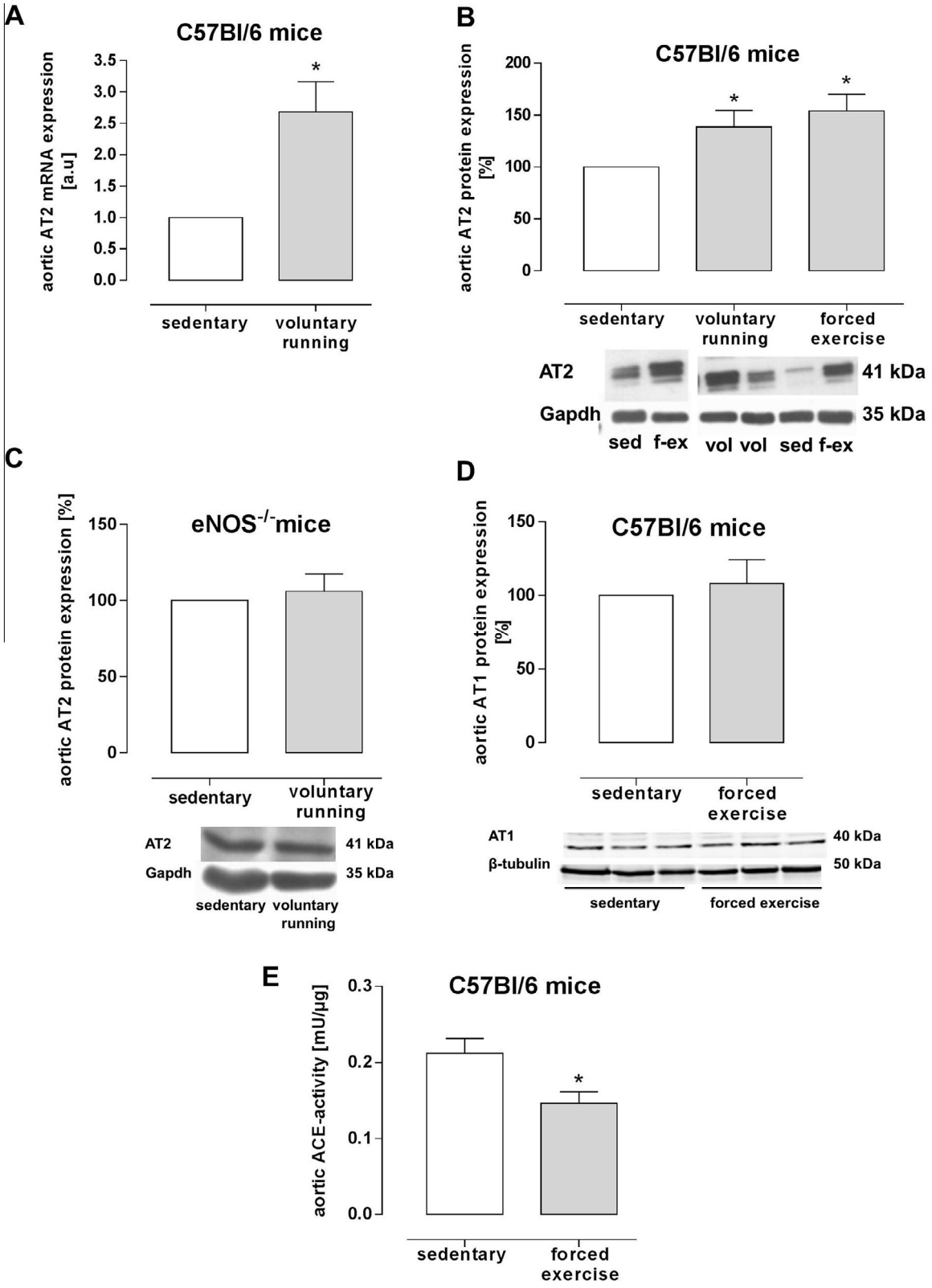


Fig. 6. Effect of exercise training on ACE-activity. (A) Up-regulation of AT2 mRNA expression in aortic tissue homogenates of voluntary running C57Bl/6 mice ($n = 7$) as compared to sedentary control mice ($^{*}P < 0.05$, $n = 6$). (B) Increased AT2 protein in aortic tissue homogenates of forced exercised (f-ex, $n = 6$), voluntary running (vol, $n = 6$) as compared to sedentary C57Bl/6 mice (sed, $n = 6$) ($^{*}P < 0.05$, $n = 6$). (C) In contrast, unchanged aortic AT2 protein expression in voluntary running eNOS$^{-/-}$ mice ($P > 0.05$ vs. sedentary eNOS$^{-/-}$, $n = 5$; one tissue sample dismissed) as compared to sedentary controls and (D) similar unchanged aortic AT1 protein expression in forced exercised C57Bl/6 mice ($P > 0.05$ vs. sedentary controls, $n = 4$). (E) Exercise-induced AT2 up-regulation was associated with a reduction of aortic ACE-activity [mU/µg] in forced exercise C57Bl/6 mice ($^{*}P < 0.05$ vs. sedentary controls, $n = 8$). Data are mean ± S.E.M. ($^{*}P < 0.05$, one-way-ANOVA and Newman–Keuls Multiple Comparison Test (B) or Student's t test (A, C, D, E); n indicates the individual sample number. Forced exercise training of C57Bl/6 mice has been performed twice. AT2 (41 kDa), AT1 (41 kDa) and GAPDH (35 kDa) β-tubulin (50 kDa) proteins are depicted at the bottom of the corresponding graphs; n indicates the individual sample number.

Table 2
Quantitation of training efficacy of voluntary running eNOS$^{-/-}$ mice ($n = 6$) as measured by heart weight (HW) body weight (BW) ratio, heart weight tibia length (TL) ratio, soleus weight (SW) vs. body weight and soleus weight tibia length. Data are mean ± S.E.M. ($^{*}P < 0.05$, using Student's t test).

Measure	Sedentary	Voluntary running	P-value
HW [g]	0.1506 ± .0063	0.1363 ± 0.0051	ns
BW [g]	29.65 ± 0.9599	25.65 ± 0.5942	*0.0069
SW [mg]	8.88 ± 0.6	10.1 ± 0.5	ns
TL [cm]	1.898 ± 0.0428	1.878 ± 0.0576	ns
HW/TL [mg/cm]	79.22 ± 2.380	72.60 ± 1.731	ns
SW/BW [mg/g]	0.2982 ± 0.0176	0.3942 ± 0.0176	*0.0026
SW/TL [mg/cm]	4.444 ± 0.2643	5.400 ± 0.2777	*0.0302

nificance of NO for the up-regulation of AT2 we assessed AT2 expression in eNOS^{tg} mice [18]. In contrast to a study reporting no change of AT2 mRNA expression after one week of oral L-NA treatment in rat adrenal glands [44], in the present study chronic pharmacological inhibition of NOS reversed the up-regulation of aortic AT2 expression in our eNOS^{tg} mouse strain. Moreover, this long-term but reversible intervention decreased aortic AT2 protein expression also in negative littermates (eNOS^{n}) indicating the importance of endogenous NO formation for AT2 expression. Surprisingly, eNOS$^{-/-}$ mice appear to maintain physiologic AT2 level similar to their transgene negative littermates. The reason for this discrepancy is unclear but might involve mechanisms occurring in vascular cells of eNOS$^{-/-}$ mice which might increase translation, e.g. by stabilizing AT2 mRNA. For example, it is well known that several endothelium-dependent vasodilators such as prostaglandins, nNOS or endothelium-derived hyperpolarizing factor compensate for lack of eNOS in coronary, femoral, mesenteric, cerebral and skeletal resistance arteries [45–50].

Activation of AT2 has been linked to a tonic inhibitory effect on ACE-activity [13]. Similar results were obtained in this study in HUVEC treated with a combination of telmisartan and angiotensin II demonstrating that AT2 activation was associated with ACE-activity inhibition that could be reversed by the AT2 antagonist PD123319. Likewise, cells and mice with increased AT2 protein expression showed a significant reduction of ACE-activity, while ACE-activity was largely increased in AT2$^{-/-}$. It has been reported that 0.1–100 μM of DEA/NO directly inhibits the activity of isolated rabbit ACE protein [30]. DEA/NO releases NO within 5 min and a peak concentration of about 3 μM [31]. Thus, ACE-activity was measured following incubation of HUVEC with DEA/NO for 15 min but this intervention had no effect. Similarly, a 3 h treatment with DEA/NO in the presence of actinomycin D to prevent AT2 up-regulation failed to change ACE-activity. These data are in good agreement with unchanged aortic ACE-activity in C57Bl/6 receiving a high dose i.v. bolus L-NA treatment for 30 min. This short-term intervention completely inhibits endogenous vascular NO formation [25,26,33] but had no effect on the expression of AT2 and ACE. Together, it appears that direct inhibition of ACE by NO, which has been shown *in-vitro*, does not occur in C57Bl/6 mice *in-vivo*. The signaling pathways initiating ACE-inhibition by AT2 remain unclear and are currently investigated further.

Exercise is well known to increase vascular expression of eNOS and generation of endothelial NO which is believed to improve organ blood flow by inducing angiogenesis [1]. It was demonstrated as well that up-regulation of eNOS drives the expression of other anti-oxidative proteins such as ecSOD [7]. This study shows that exercise NO-dependently increases AT2 expression and decreases ACE-activity. Thus, exercise training potentiates the activity of eNOS resulting in ACE-inhibition via NO-dependent up-regulation of AT2. This newly discovered pathway might contribute to the beneficial effects of exercise training, thereby extending the findings of this study to a clinically relevant situation, i.e. that endothelial NO initiates signaling cascades involving the AT2 and ACE which might contribute to maintain cardiovascular health.

NO-induced up-regulation of AT2 and subsequent inhibition of ACE may also have a pathophysiological role. Clinical studies have demonstrated that increased activation of the constitutively expressed bradykinin type 2 receptor (B2) induced by ACE inhibitors significantly contributes to both the reduction of blood pressure [51] and important side effects of these drugs such as cough [52] and angioedema [53,54]. In contrast, angioedema induced by AT1-blockers which occurs at a lower frequency are not fully explained mechanistically [55]. Regarding this, it has been reported that hypertensive patients treated with the AT1-blocker losartan showed increased plasma levels of bradykinin, while levels of bradykinin metabolites were reduced [14]. These data are consistent with an inhibitory effect of losartan treatment on ACE-activity, which the authors ascribed to increased stimulation of AT2 following blockade of AT1, because these very selective drugs inhibit AT1 mediated reduction of renin release and thereby increase circulating angiotensin II. In this context, it should be noted that activation of AT2 has also been shown to inhibit the amiloride-sensitive Na^{+}/H^{+} exchanger and to up-regulate the pre-kallikrein activator prolylcarboxypeptidase in cultured vascular smooth muscle and endothelial cells [11,12,56,57]. These activities likely increase the generation of bradykinin and subsequently the generation of endothelial NO [58], but an increase of bradykinin generation in response to AT1-blockers is not consistent with the clinical observation of a reduction of bradykinin metabolites [14]. In view of the clinical finding that AT1-blockers increase vascular NO levels in cardiovascular patients [59] and of the known increase of endothelial NO production by bradykinin [58], it appears possible that NO induced up-regulation of AT2 and subsequent inhibition of ACE might be involved in angioedema caused by AT1-blockers. However, further studies are necessary to substantiate this hypothesis.

Our study has some limitations. First, we used transgenic mice which overexpress eNOS protein by 3.5-fold that was associated with a strong blood pressure reduction indicating functional activity, while aortic endothelium-dependent was unchanged [17,18]. This overexpression might downregulate native murine eNOS expression. However, the exercise studies revealed an up-regulation of eNOS protein level as well, that was associated with higher AT2 expression and ACE activity inhibition, indicating that auto-inhibition of NO under these *in-vivo* conditions is rather unlikely and would have little effect on AT2 regulation. Another limitation is that the molecular mechanism of AT2 induced inhibition of ACE is not yet known. However, our results show clearly by using *in-vitro* and *in-vivo* models that NO can up-regulate AT2 expression that is associated with a reduction of ACE-activity.

Taken together, it was found that NO increases the expression of AT2 in cells and mice by a transcriptional mechanism involving the activation of PKG and p38 MAPK and up-regulation of AT2 was associated with a reduced activity of ACE. This sequence of molecular events describing a feed-forward mechanism might contribute to vasoprotective mechanisms of endothelial NO and the beneficial effects of exercise training. In addition, it might contribute as well to the pathophysiology of angioedema induced by AT1-blockers, as these drugs increase vascular NO levels in cardiovascular patients which might contribute to AT2 expression levels.

Sources of funding

This study was supported by the Forschungskommission of the Medical faculty of the Heinrich-Heine-Universität Düsseldorf – Germany (Project 9772504).

Disclosures

None.

Acknowledgements

The Authors wish to thank Martin Schlesinger, PharmD, PhD and Gerd Bendas, PhD, Department of Pharmaceutical Chemistry II of the Pharmaceutical Institute, Bonn, Germany for providing bEND.3. Furthermore, the authors wish to thank Mark Oppermann, PharmD, PhD and Bea Bialek, PhD for some technical assistance as well as Karin Kohlstedt, PhD and Ingrid Fleming, PhD for providing the protocol for ACE-activity assay and the ACE-plasmids and Michael Bader, PhD for providing AT2 knockout mice.

References

[1] G. Kojda, R. Hambrecht, Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? Cardiovasc. Res. 67 (2005) 187–197.
[2] S.J. Keteyian, E.S. Leifer, N. Houston-Miller, W.E. Kraus, C.A. Brawner, C.M. O'Connor, et al., Relation between volume of exercise and clinical outcomes in patients with heart failure, J. Am. Coll. Cardiol. 60 (2012) 1899–1905.
[3] R. Belardinelli, D. Georgiou, G. Cianci, A. Purcaro, 10-Year exercise training in chronic heart failure: a randomized controlled trial, J. Am. Coll. Cardiol. 60 (2012) 1521–1528.
[4] H. Noda, H. Iso, H. Toyoshima, C. Date, A. Yamamoto, S. Kikuchi, et al., Walking and sports participation and mortality from coronary heart disease and stroke, J. Am. Coll. Cardiol. 46 (2005) 1761–1767.
[5] S. Gielen, G. Schuler, V. Adams, Cardiovascular effects of exercise training: molecular mechanisms, Circulation 122 (2010) 1221–1238.
[6] R. Hambrecht, A. Wolf, S. Gielen, A. Linke, J. Hofer, S. Erbs, et al., Effect of exercise on coronary endothelial function in patients with coronary artery disease, N. Engl. J. Med. 342 (2000) 454–460.
[7] T. Fukai, M.R. Siegfried, M. Ushio-Fukai, Y. Cheng, G. Kojda, D.G. Harrison, Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training, J. Clin. Invest. 105 (2000) 1631–1639.
[8] V. Adams, A. Linke, N. Krankel, S. Erbs, S. Gielen, S. Möbius-Winkler, et al., Impact of Regular Physical Activity on the NAD(P)H Oxidase and Angiotensin Receptor System in Patients With Coronary Artery Disease, Circulation 111 (2005) 555–562.
[9] C.A. Lemarie, E.L. Schiffrin, The angiotensin II type 2 receptor in cardiovascular disease, J. Renin Angiotensin Aldosterone Syst. 11 (2010) 19–31.
[10] U.M. Steckelings, M. Larhed, A. Hallberg, R.E. Widdop, E.S. Jones, C. Wallinder, et al., Non-peptide AT2-receptor agonists, Curr. Opin. Pharmacol. 11 (2011) 187–192.
[11] L. Zhu, O.A. Carretero, T.D. Liao, P. Harding, H. Li, C. Sumners, et al., Role of prolylcarboxypeptidase in angiotensin II type 2 receptor-mediated bradykinin release in mouse coronary artery endothelial cells, Hypertension 56 (2010) 384–390.
[12] Z. Shariat-Madar, F. Mahdi, A.H. Schmaier, Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator, J. Biol. Chem. 277 (2002) 17962–17969.
[13] T.E. Hunley, M. Tamura, B.J. Stoneking, H. Nishimura, T. Ichiki, T. Inagami, et al., The angiotensin type II receptor tonically inhibits angiotensin- converting enzyme in AT2 null mutant mice, Kidney Int. 57 (2000) 570–577.
[14] D.J. Campbell, H. Krum, M.D. Esler, Losartan increases bradykinin levels in hypertensive humans, Circulation 111 (2005) 315–320.
[15] S. Yusuf, P. Sleight, J. Pogue, J. Bosch, R. Davies, G. Dagenais, Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators, N. Engl. J. Med. 342 (2000) 145–153.
[16] J.J. McMurray, R.R. Holman, S.M. Haffner, M.A. Bethel, B. Holzhauer, T.A. Hua, et al., Effect of valsartan on the incidence of diabetes and cardiovascular events, N. Engl. J. Med. 362 (2010) 1477–1490.
[17] M. Oppermann, V. Balz, V. Adams, V.T. Dao, M. Bas, T. Suvorava, et al., Pharmacologic induction of vascular extracellular superoxide dismutase expression in-vivo, J. Cell Mol. Med. 13 (2009) 1271–1278.
[18] M. Oppermann, T. Suvorava, T. Freudenberger, V.T. Dao, J.W. Fischer, M. Weber, et al., Regulation of vascular guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification, Basic Res. Cardiol. 106 (2011) 539–549.
[19] G. Kojda, Y. Cheng, J. Burchfield, D.G. Harrison, Dysfunctional regulation of eNOS expression in response to exercise in mice lacking one eNOS gene, Circulation 103 (2001) 2839–2844.
[20] V.T. Dao, M. Floeren, S. Kumpf, C. Both, B. Peter, V. Balz, et al., Catalase activity prevents exercise-induced up-regulation of vasoprotective proteins in venous tissue, J. Cell Mol. Med. 15 (2011) 2326–2334.
[21] T. Suvorava, N. Lauer, G. Kojda, Physical inactivity causes endothelial dysfunction in healthy young mice, J. Am. Coll. Cardiol. 44 (2004) 1320–1327.
[22] J.A. Gordon, Use of vanadate as protein-phosphotyrosine phosphatase inhibitor, Methods Enzymol. 201 (1991) 477–482.
[23] B.A. Murray, D.J. Walsh, R.J. Fitzgerald, Modification of the furanacryloyl-L-phenylalanylglycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity, J. Biochem. Biophys. Methods 59 (2004) 127–137.
[24] G. Kojda, J.B. Laursen, S. Ramasamy, J.D. Kent, S. Kurz, J. Burchfield, et al., Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control, Cardiovasc. Res. 42 (1999) 206–213.
[25] D.L. Mattson, C.J. Meister, Renal cortical and medullary blood flow responses to L-NAME and ANG II in wild-type, nNOS null mutant, and eNOS null mutant mice, Am. J. Physiol. Regul. Integr. Comp. Physiol. 289 (2005) R991–R997.
[26] T.J. Calvert, L.G. Chicoine, Y. Liu, L.D. Nelin, Deficiency of mitogen-activated protein kinase phosphatase-1 results in iNOS-mediated hypotension in response to low-dose endotoxin, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H1621–H1629.
[27] T. Suvorava, S. Kumpf, B.H. Rauch, V.T. Dao, V. Adams, G. Kojda, Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity, Free Radic. Res. 44 (2010) 199–207.
[28] T. Suvorava, N. Lauer, S. Kumpf, R. Jacob, W. Meyer, G. Kojda, Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo, Circulation 112 (2005) 2487–2495.
[29] M. Tanaka, S. Tsuchida, T. Imai, N. Fujii, H. Miyazaki, T. Ichiki, et al., Vascular response to angiotensin II is exaggerated through an upregulation of AT1 receptor in AT2 knockout mice, Biochem. Biophys. Res. Commun. 258 (1999) 194–198.
[30] A. Ackermann, M.S. Fernandez-Alfonso, D.R. Sanchez, T. Ortega, M. Paul, C. Gonzalez, Modulation of angiotensin-converting enzyme by nitric oxide, Br. J. Pharmacol. 124 (1998) 291–298.
[31] G. Kojda, M. Patzner, A. Hacker, E. Noack, Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate. A novel mechanism to explain preferential venodilation of organic nitrates, Mol. Pharmacol. 53 (1998) 547–554.
[32] E.G. Shesely, N. Maeda, H.S. Kim, K.M. Desai, J.H. Krege, V.E. Laubach, et al., Elevated blood pressures in mice lacking endothelial nitric oxide synthase, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 13176–13181.
[33] T. Wang, F.M. Inglis, R.G. Kalb, Defective fluid and HCO(3)(−) absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice, Am. J. Physiol. Renal Physiol. 279 (2000) F518–F524.
[34] G. Kojda, Y.C. Cheng, J. Burchfield, D.G. Harrison, Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene, Circulation 103 (2001) 2839–2844.
[35] N. Lauer, T. Suvorava, U. Rüther, R. Jacob, A. Meyer, D.G. Harrison, et al., Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase, Cardiovasc. Res. 65 (2005) 254–262.
[36] J. Raingeaud, S. Gupta, J.S. Rogers, M. Dickens, J. Han, R.J. Ulevitch, et al., Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine, J. Biol. Chem. 270 (1995) 7420–7426.
[37] F. Hofmann, D. Bernhard, R. Lukowski, P. Weinmeister, CGMP regulated protein kinases (cGK), Handb. Exp. Pharmacol. 137–62 (2009).
[38] A. Cuadrado, A.R. Nebreda, Mechanisms and functions of p38 MAPK signalling, Biochem. J. 429 (2010) 403–417.
[39] Y. Li, S. Batra, A. Sassano, B. Majchrzak, D.E. Levy, M. Gaestel, et al., Activation of mitogen-activated protein kinase kinase (MKK) 3 and MKK6 by type I interferons, J. Biol. Chem. 280 (2005) 10001–10010.
[40] G.E. Mann, B. Bonacasa, T. Ishii, R.C. Siow, Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones, Curr. Opin. Pharmacol. 9 (2009) 139–145.
[41] C. Lee, C.H. Huang, LASAGNA-search: an integrated web tool for transcription factor binding site search and visualization, Biotechniques 54 (2013) 141–153.
[42] M. De Gasparo, K.J. Catt, T. Inagami, J.W. Wright, T. Unger, International union of pharmacology. XXIII. The angiotensin II receptors, Pharmacol. Rev. 52 (2000) 415–472.
[43] M. Saito, Y. Shinohara, H. Sasaki, Y. Netsu, M. Yoshida, N. Nakahata, Type 1 angiotensin receptor (AT1-R)-mediated decrease in type 2 angiotensin receptor mRNA level is dependent on Gq and extracellular signal-regulated kinase 1//2 in AT1-R-transfected PC12 cells, J. Neuroendocrinol. 20 (2008) 299–308.
[44] M. Usui, T. Ichiki, M. Katoh, K. Egashira, A. Takeshita, Regulation of angiotensin II receptor expression by nitric oxide in rat adrenal gland, Hypertension 32 (1998) 527–533.
[45] A. Gödecke, U. Decking, Z. Ding, J. Hirchenhain, H.J. Bidmon, S. Godecke, et al., Coronary hemodynamics in endothelial NO synthase knockout mice, Circ. Res. 82 (1998) 186–194.
[46] K.G. Lamping, D.W. Nuno, E.G. Shesely, N. Maeda, F.M. Faraci, Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice, Am. J. Physiol. Heart Circ. Physiol. 279 (2000) H1906–H1912.
[47] G.J. Waldron, H. Ding, F. Lovren, P. Kubes, C.R. Triggle, Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase, Br. J. Pharmacol. 128 (1999) 653–658.
[48] W. Meng, C. Ayata, C. Waeber, P.L. Huang, M.A. Moskowitz, Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice, Am. J. Physiol. Heart Circ. Physiol. 274 (1998) H411–H415.

[49] D. Sun, A. Huang, C.J. Smith, C.J. Stackpole, J.A. Connetta, E.G. Shesely, et al., Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice, Circ. Res. 85 (1999) 288–293.
[50] A. Drouin, N. Thorin-Trescases, E. Hamel, J.R. Falck, E. Thorin, Endothelial nitric oxide synthase activation leads to dilatory H_2O_2 production in mouse cerebral arteries, Cardiovasc. Res. 73 (2007) 73–81.
[51] J.V. Gainer, J.D. Morrow, A. Loveland, D.J. King, N.J. Brown, Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects, N. Engl. J. Med. 339 (1998) 1285–1292.
[52] A.J. Fox, U.G. Lalloo, M.G. Belvisi, M. Bernareggi, K.F. Chung, P.J. Barnes, Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough, Nat. Med. 2 (1996) 814–817.
[53] M. Bas, J. Greve, K. Stelter, M. Havel, U. Strassen, N. Rotter, et al., A randomized trial of icatibant in ACE-inhibitor-induced angioedema, N. Engl. J. Med. 372 (2015) 418–425.
[54] M. Bas, J. Greve, K. Stelter, H. Bier, T. Stark, T.K. Hoffmann, et al., Therapeutic efficacy of icatibant in angioedema induced by angiotensin-converting enzyme inhibitors: a case series, Ann. Emerg. Med. 56 (2010) 278–282.
[55] M. Bas, J. Greve, U. Strassen, F. Khosravani, T.K. Hoffmann, G. Kojda, Angioedema induced by cardiovascular drugs: new players join old friends, Allergy 70 (2015) 1196–1200.
[56] Z. Shariat-Madar, F. Mahdi, A.H. Schmaier, Recombinant prolylcarboxypeptidase activates plasma prekallikrein, Blood 103 (2004) 4554–4561.
[57] Y. Tsutsumi, H. Matsubara, H. Masaki, H. Kurihara, S. Murasawa, S. Takai, et al., Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation, J. Clin. Invest. 104 (1999) 925–935.
[58] L.M. Leeb-Lundberg, F. Marceau, W. Muller-Esterl, D.J. Pettibone, B.L. Zuraw, International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences, Pharmacol. Rev. 57 (2005) 27–77.
[59] E.L. Schiffrin, J.B. Park, H.D. Intengan, R.M. Touyz, Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan, Circulation 101 (2000) 1653–1659.

Biochemical and Biophysical Research Communications 458 (2015) 576–583

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Sustained hypertension despite endothelial-specific eNOS rescue in eNOS-deficient mice

Tatsiana Suvorava [a], Johannes Stegbauer [b], Manuel Thieme [b], Stephanie Pick [a], Sebastian Friedrich [b], Lars C. Rump [b], Thomas Hohlfeld [a], Georg Kojda [a, *]

[a] Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Universitätsstr. 1, 40225 Düsseldorf, Germany
[b] Department of Nephrology, University Hospital, Heinrich-Heine-University, Universitätsstr. 1, 40225 Düsseldorf, Germany

ARTICLE INFO

Article history:
Received 22 January 2015
Available online 11 February 2015

Keywords:
eNOS
Blood pressure
Endothelium
Transgenic mice

ABSTRACT

The aim of the study was to evaluate the possible contribution of non-endothelial eNOS to the regulation of blood pressure (BP). To accomplish this, a double transgenic strain expressing eNOS exclusively in the vascular endothelium (eNOS-Tg/KO) has been generated by endothelial-specific targeting of bovine eNOS in eNOS-deficient mice (eNOS-KO). Expression of eNOS was evaluated in aorta, myocardium, kidney, brain stem and skeletal muscle. Organ bath studies revealed a complete normalization of aortic reactivity to acetylcholine, phenylephrine and the NO-donors in eNOS-Tg/KO. Function of eNOS in resistance arteries was demonstrated by acute i.v. infusion of acetylcholine and the NOS-inhibitor L-NAME. Acetylcholine decreased mean arterial pressure in all strains but eNOS-KO responded significantly less sensitive as compared eNOS-Tg/KO and C57BL/6. Likewise, acute i.v. L-NAME application elevated mean arterial pressure in C57BL/6 and eNOS-Tg/KO, but not in eNOS-KO. In striking contrast to these findings, mean, systolic and diastolic BP in eNOS-Tg/KO remained significantly elevated and was similar to values of eNOS-KO. Chronic oral treatment with L-NAME increased BP to the level of eNOS-KO only in C57BL/6, but had no effect on hypertension in eNOS-KO and eNOS-Tg/KO. Taken together, functional reconstitution of eNOS in the vasculature of eNOS-KO not even partially lowered BP. These data suggest that the activity of eNOS expressed in non-vascular tissue might play a role in physiologic BP regulation.

1. Introduction

Among NO-synthases (NOS) the endothelial isoform (eNOS) significantly contributes to the regulation of BP as hypertension is the most obvious phenotype in 4 different strains of eNOS-deficient mice (eNOS-KO) suggesting that other physiologic systems regulating BP cannot compensate for the absence of eNOS [1–4]. Likewise, hypertension develops following treatment with NOS-inhibitors such as monomethyl arginine (L-NMMA) or L-nitroarginine (L-NA) in rabbits [5], mice [1,6], and humans [5,7]. Based on these findings it is generally assumed that the lack of vasodilation by endothelial NO is an important underlying cause [1,2], i.e. that hypertension in eNOS-KO is caused by the lack of endothelium-dependent NO-induced vasodilation.

Anaesthetized mice of one eNOS-KO strain showed a paradoxical decrease in blood pressure (BP) in response to i.p. administration of the NOS-inhibitor L-NA suggesting a role for non-endothelial isoforms of NOS in maintaining BP [1]. Alternatively, diminution in the activity of the renin-angiotensin system and the autonomic nervous system, which serve as a defense against hypertension and/or involvement of NOS in establishing the baroreceptor setpoint were proposed as an explanation of hypertensive phenotype [1]. However, studies on nNOS-deficient mice revealed that they are normotensive [8]. Furthermore, triple e/i/nNOS knockouts have hypertension and the degree of hypertension is similar to that in the eNOS-gene disrupted single and double e/nNOS-KO [9]. These results clearly demonstrate that among NO-synthases eNOS plays by far the most important role in BP regulation. However, in contrast to large arteries of eNOS-KO where no compensation for the lack of eNOS is observed [1,6,10], other endothelium-dependent vasodilators such as prostaglandins, nNOS or endothelium-derived hyperpolarizing factor compensate for the lack of eNOS in coronary, femoral, mesenteric, cerebral and skeletal resistance arteries [11–16]. These data indicate that different vascular-bed specific mechanisms regulate arterial tone in

* Corresponding author. Fax: +49 211 81 14781.
E-mail address: kojda@uni-duesseldorf.de (G. Kojda).

http://dx.doi.org/10.1016/j.bbrc.2015.01.152
0006-291X/

the absence of vascular eNOS and raise the question to what extent hypertension in eNOS-KO is caused by the lack of endothelial eNOS expression.

The aim of this study was to evaluate the contribution of non-vascular eNOS expression and function in physiologic regulation of BP. To distinguish the relevance of endothelial and non-endothelial eNOS, we generated a novel double transgenic mouse model lacking eNOS in all organs except the vascular endothelium (eNOS-Tg/KO) and found that these mice were as hypertensive as eNOS-KO. These data establish the necessity of functional non-endothelial eNOS expression for BP regulation.

2. Methods

2.1. Transgenic mice

We generated a double transgenic mouse strain expressing wild-type eNOS only in vascular endothelial cells by crossing mice with an endothelial-specific overexpression of bovine eNOS driven by the Tie-2 promoter on a C57BL/6J background (eNOS-Tg) [17] with eNOS knockouts (eNOS-KO) [2] on a C57BL/6J background. All experiments were performed using 3–4 months old male mice. Permission for the animal studies was provided by the regional government of Germany (AZ 50.05-230-3-94/00, AZ 50.05-230-18/06, AZ 8.87-51.04.20.09.383), the experiments were performed according to the guidelines of German „Tierschutzgesetz“ and the „Guide for the Care and Use of Laboratory Animals“ of the U.S. National Institutes of Health.

2.2. Measurement of systolic blood pressure

Systolic blood pressure and heart rate was measured in awake C57BL/6, eNOS-KO, and C101A/eNOS-KO mice using an automated tail cuff system (Visitech Systems, Apex, North Carolina) as described [6]. In some experiments establishment of basal blood pressure was followed by L-NA treatment (100 mg L-NA/kg BW/day) with the drinking water and blood pressure recordings were continued for 3 weeks [6,17,19] (refer also to Fig. S1).

2.3. Acute infusion of L-NAME and acetylcholine

Measurements of mean arterial (MAP), systolic (sBP) and diastolic (dBP) blood pressure (BP) and L-NAME-induced changes of BP were performed invasively [18]. Briefly, eNOS-Tg/KO, eNOS-KO and C57BL/6 mice were anesthetized by i.p. injection with ketamine (100 mg/kg) and xylazine (5 mg/kg) and placed on a heating plate to ensure constant body temperature (36–37 °C). Right carotid artery was cannulated for continuous measurement of MAP, sBP and dBP. Right jugular vein was cannulated for drug administration and changes in BP were detected using a pressure transducer and PowerLab data acquisition system (ADInstruments, Spechbach, Germany). Hypotensive responses to acetylcholine (0.02–2 μM/kg BW) were recorded as a transient fall in MAP and calculated as % fall in MAP with respect to baseline MAP before each response. In a separate set of mice, hypertensive responses to a bolus application of NOS-inhibitor L-NAME (32 mg/kg BW) were evaluated.

2.4. Vasorelaxation studies

Isolated aortic arteries were cannulated and pressurized as described previously [6,19,20]. Function of the endothelium was examined in aortic rings of C57BL/6, eNOS-KO and C101A/eNOS-KO by cumulative addition of acetylcholine (0.01–10 μmol/L) after submaximal precontraction with 0.2 μmol/L phenylephrine [20]. Thereafter, vasorelaxation to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 1–10 μmol/L) or diethylamine/nitric oxide (DEA/NO 1–10 μmol/L) was measured. In some experiments the NOS inhibitor L-NAME (1 mM) or the colloidal $Fe(DETC)_2$ spin trap (0.5 mM) were administrated 30 min before application of acetylcholine.

2.5. Real-time PCR

RNA of frozen tissues was extracted by RNeasy® Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) using the TissueRuptor® (Qiagen, Hilden, Germany). Synthesis of cDNA was performed with 500 ng RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantification of bovine eNOS mRNA was carried out with bovine-specific primer (Bt03217671_m1, TaqMan®, Life Technologies GmbH). No cross-reactivity for this bovine primer with Mus musculus was detected in wild type probes. Experiments were performed using Abi Prism 7900HT Sequence Detector System (Applied Biosystems, Weiterstadt, Germany). Samples were analyzed in triplicate and normalized to the housekeeping gene RPL0 (Mm01974474_gH (TaqMan®, Life Technologies GmbH) as described [21].

2.6. SDS–PAGE and immunoblotting

Western blots for eNOS, nNOS, ecSOD, sGCβ1, sGCα1, nitrotyrosine residues, and actin were performed in different mouse tissues using standard techniques as described previously [6,17,19,20,22].

2.7. Immunohistochemistry

Endothelium specific overexpression was analyzed by eNOS/CD31-double staining as described previously [17].

2.8. Statistics

All data are expressed as mean ± s.e.m. of n individual samples. Statistical comparisons between groups were performed by t-tests, Newman–Keuls multiple comparisons post-hoc test following one-way ANOVA (more than two groups) or two-way ANOVA (concentration–response curves). $P < 0.05$ was considered statistically significant.

3. Results

3.1. eNOS expression in eNOS-Tg/KO mice

Real-time PCR using bovine-specific primers confirmed eNOS mRNA expression in aorta and brain stem of eNOS-Tg/KO, while no signal for bovine eNOS mRNA was detectable in C57BL/6 (Fig. S2a). Western blot demonstrated eNOS protein expression in a variety of organs of eNOS-Tg/KO as depicted in Fig. 1A. However, in each organ of eNOS-Tg/KO expression of eNOS was significantly lower than in C57BL/6 (Fig 1A, Fig. S2b–f). There was no Tie-2-driven eNOS protein expression in circulating or bone marrow mononuclear cells or in whole bone marrow extracts (data not shown) and only an infinitesimal eNOS mRNA content not exceeding 1% of that detected in aortic tissue was found in blood and bone marrow mononuclear cells (Fig. S3). These findings are consistent with previous data [17,22,23]. In addition, we comparatively analyzed the expression of several other proteins which were shown to be associated with vascular bioavailability or function of NO such as the vascular NO receptor soluble guanylyl cyclase (sGC) β1- and α1-subunits, extracellular superoxide dismutase (ecSOD) protecting NO from interstitial

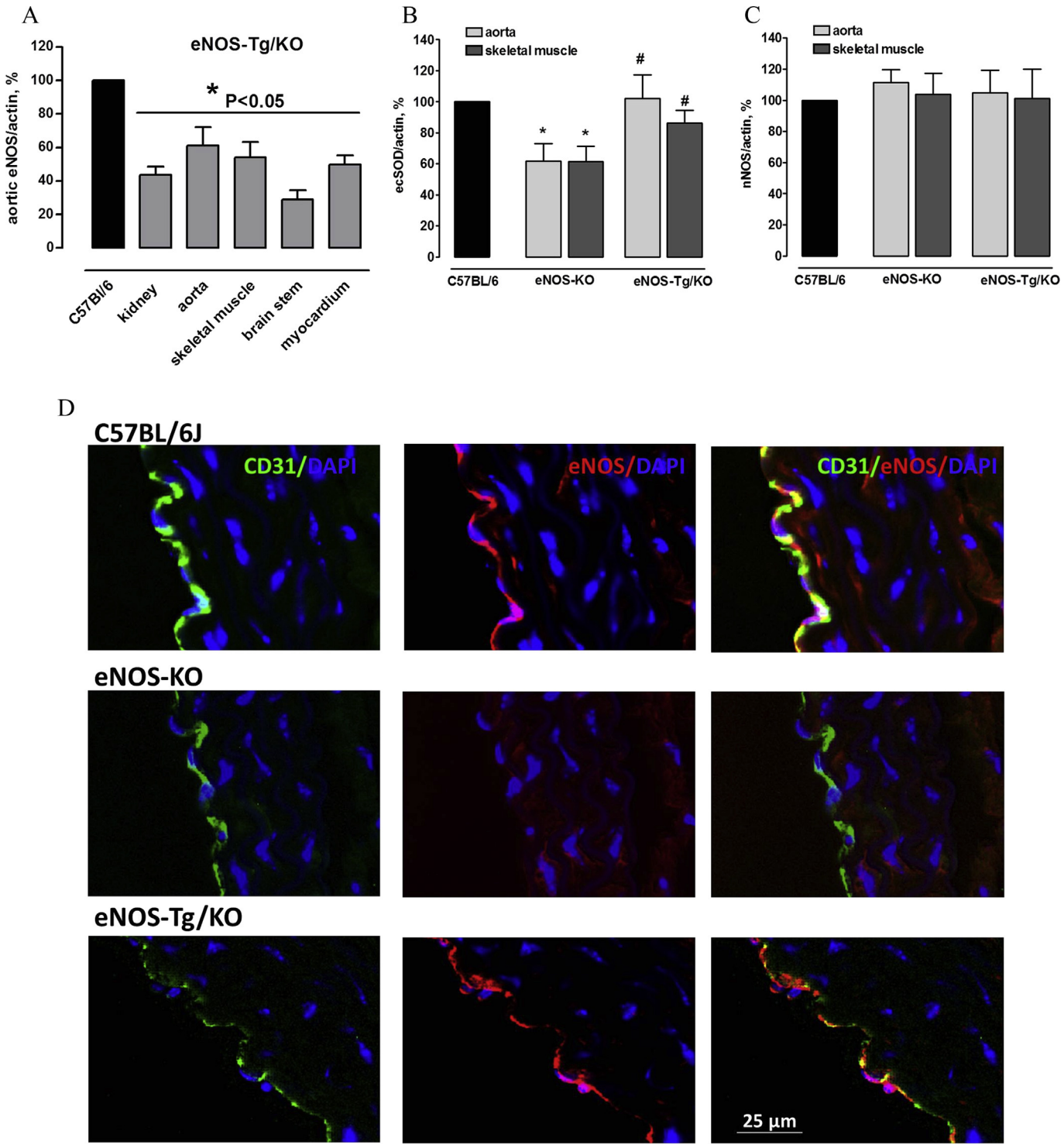


Fig. 1. Characterization of eNOS-Tg/KO mice. (A) Protein expression of eNOS in aorta (n = 11), skeletal muscle (n = 10), kidney, brain stem and myocardium (n = 4, each) of eNOS-Tg/KO (*P < 0.05 vs. C57BL/6). The control values referring to eNOS protein expression in every tissue of C57BL/6 was set to 100% and is reflected by just 1 bar. (B) Aortic and skeletal muscle ecSOD expression (*P < 0.05 vs. C57BL/6, #P < 0.05 vs. eNOS-KO n = 4–7, One-way ANOVA). (C) nNOS protein in aorta (n = 5) and skeletal muscle (n = 7) of eNOS-Tg/KO, eNOS-KO and C57BL/6 mice (P > 0.05, One-way ANOVA). (D) Images showing immunofluorescent staining for eNOS (red) and CD31 (pseudocolored in green) in the aorta of C57BL/6, eNOS-KO and C101A/eNOS-KO mice. Nuclei are stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

oxidation [24], neuronal NOS (nNOS) and protein nitrotyrosine residues. Expression of ecSOD was significantly higher in aorta and skeletal muscle of eNOS-Tg/KO then in eNOS-KO and similar to that of C57BL/6 (Fig. 1B). There were no other expression changes in these tissues comparing C57BL/6, eNOS-Tg/KO and eNOS-KO (Fig. 1C; Fig. S4). The localization of eNOS-Tg was restricted to endothelium as evidenced by the overlay with an endothelial-specific marker CD31 (Fig. 1D).

3.2. Conductance vessel reactivity of eNOS-Tg/KO

Aortic relaxation in response to acetylcholine was identical in C57BL/6 and eNOS-Tg/KO, while a slight vasoconstriction occurred in eNOS-KO (Fig. 2A). Preincubation of aortic rings with the NOS-inhibitor L-NAME abolished acetylcholine-induced vasodilation in eNOS-Tg/KO and C57BL/6 and induced a slight vasoconstriction as observed in eNOS-KO (Fig. 2B). Likewise, the cell membrane permeable NO-scavenger $Fe(DETC)_2$ completely inhibited acetylcholine-induced aortic dilation of eNOS-Tg/KO and C57BL/6 suggesting that it was mediated by NOS-derived NO (Fig. 2C). Furthermore, the strongly increased sensitivity of aortic tissue of eNOS-KO or C57BL/6 treated with L-NAME to alpha-adrenergic stimulation by phenylephrine [6] was completely reversed in eNOS-Tg/KO which showed a concentration-dependent vasoconstriction similar to C57BL/6 (Fig. 2D). We also observed that the hypersensitivity of aortic rings of eNOS-KO to NO-donors [6] was completely abolished in eNOS-Tg/KO (Fig. 2E and F). Taken together,

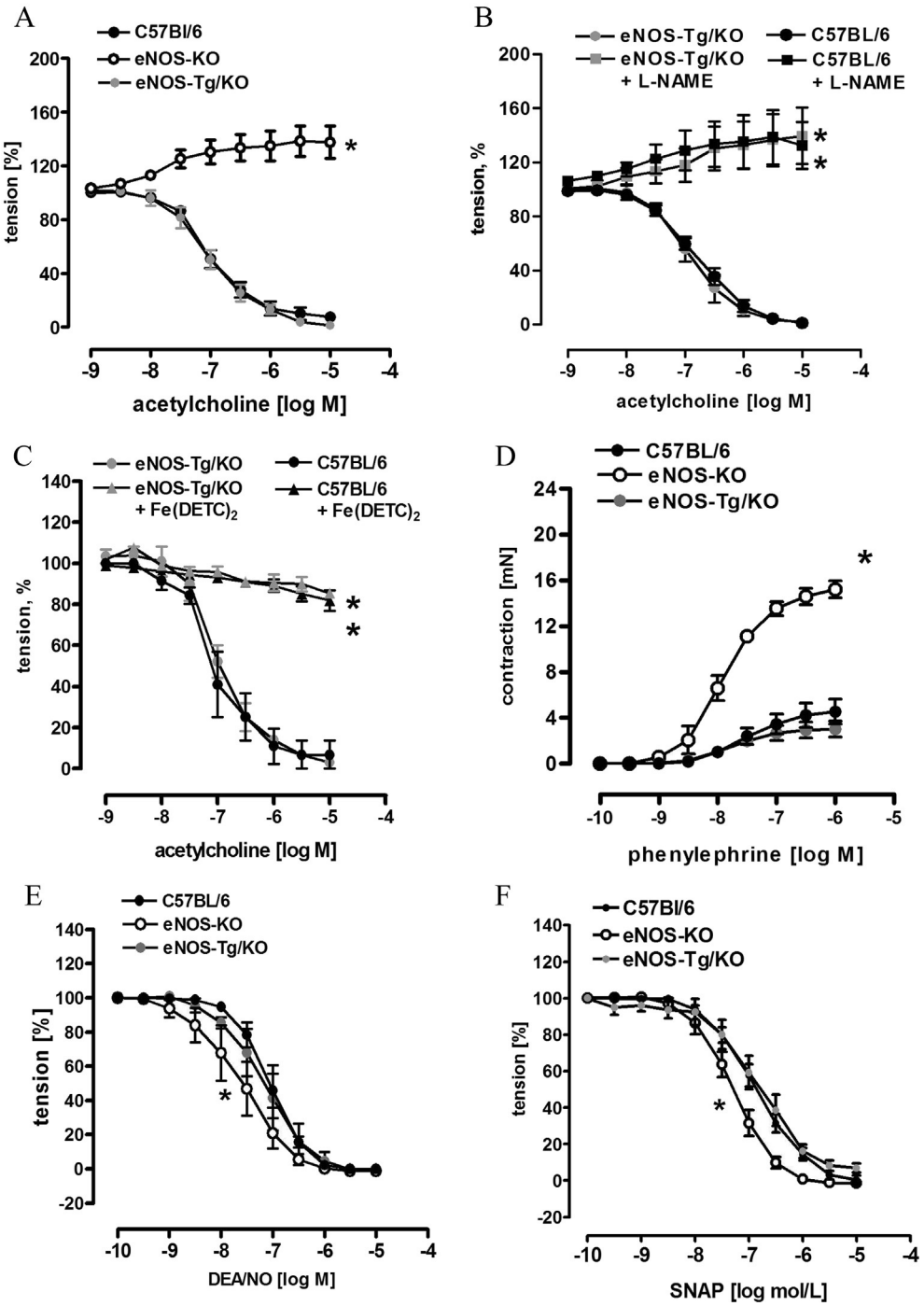


Fig. 2. Aortic reactivity of eNOS-Tg/KO. (A) Aorta of eNOS-Tg/KO (n = 8) showed a complete restoration of endothelium-dependent relaxation to acetylcholine (P = 0.311 vs C57BL/6, n = 11). (B) Effects of the NOS-inhibitor L-NAME (1 mM), and (C) the NO-scavenger $Fe(DETC)_2$ (0.5 mM) on acetylcholine-induced relaxation of aortic rings of eNOS-Tg/KO and C57BL/6 (*P < 0.05 vs. C57BL/6, Two-way ANOVA, n = 4). Aortic reactivity to (D) phenylephrine (*P < 0.0001, n = 5–7, Two-way ANOVA), (E) NO-donors diethylamine/NO (*P < 0.0001, n = 4–5, Two-way ANOVA) and (F) S-nitroso-N-acetyl-D,L-penicillamine (*P < 0.0001, n = 11, Two-way ANOVA) vs. C57BL/6 in all investigated strains.

these data suggest a normal function of eNOS in conductance vessels of eNOS-Tg/KO.

3.3. Resistance vessel reactivity of eNOS-Tg/KO

In all mice i.v. acetylcholine caused a dose-dependent and substantial reduction of MAP but the sensitivity to acetylcholine was significantly different between the strains (Fig. 3A). The fall of MAP occurring during infusion of 0.0002–0.02 μg/kg BW of acetylcholine is likely eNOS-dependent since it is absent in eNOS-KO. Higher concentrations of acetylcholine induced BP reduction in eNOS-KO as well. Data analysis by Two-way ANOVA revealed a significant decrease in sensitivity to acetylcholine-induced hypotension in eNOS-KO ($P = 0.0003$). We observed a hypotensive response to acetylcholine in eNOS-Tg/KO that was almost similar to C57BL/6 ($P = 0.376$) but significantly more sensitive than that of eNOS-KO ($P = 0.012$, Fig. 3A). In accordance, i.v. injection of the NOS-inhibitor L-NAME caused a rapid increase of sBP, dBP and MAP in C57BL/6 and eNOS-Tg/KO but not in eNOS-KO. Original tracings and mean values are shown in Fig. 3B–E. These data suggest that reintroduced eNOS in resistance arteries of eNOS-Tg/KO was as functional as in aortic rings.

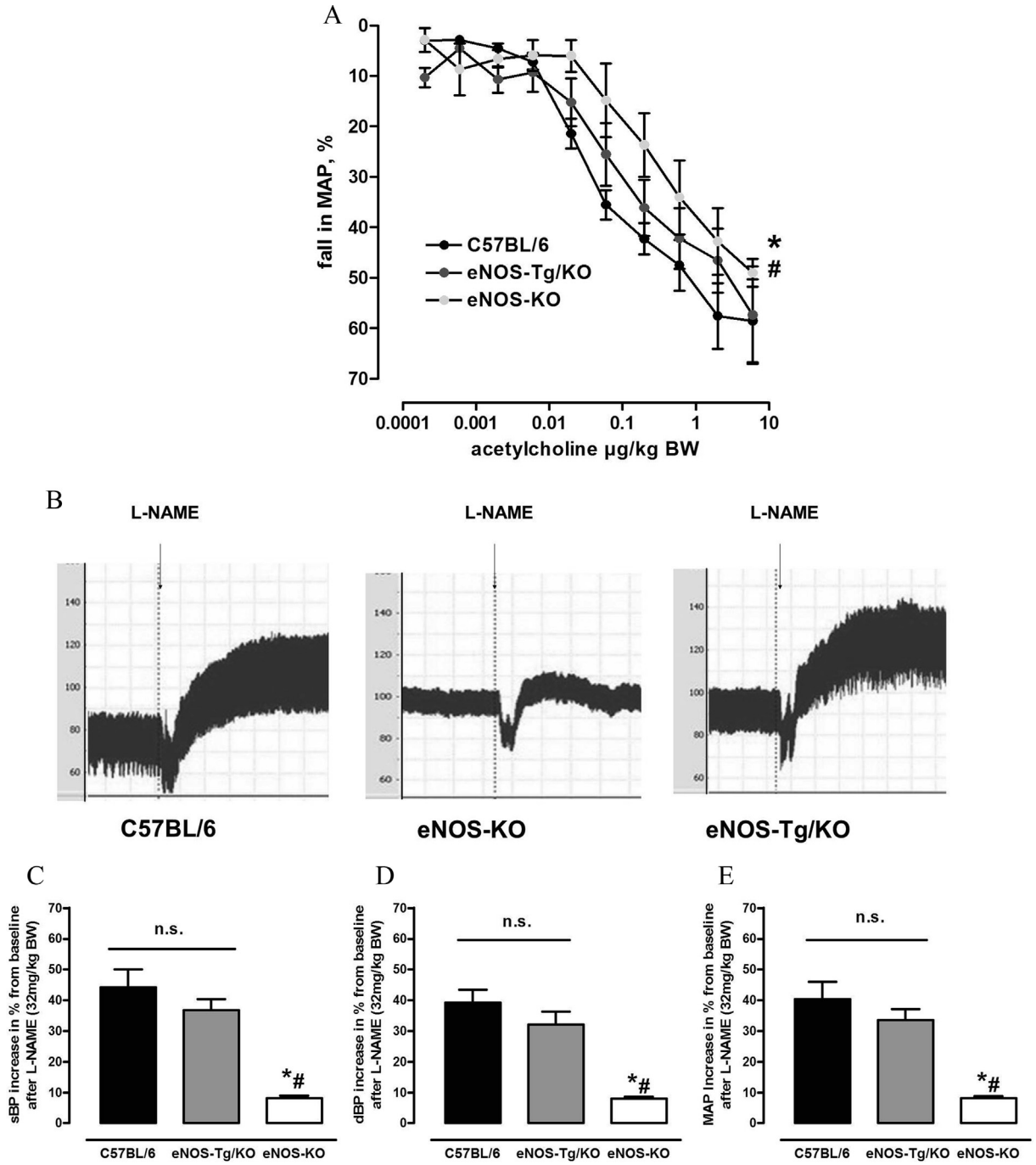


Fig. 3. Resistance vessel reactivity of eNOS-Tg/KO. (A) Acetylcholine-induced fall in mean arterial pressure (MAP) measured invasively in C57BL/6, eNOS-KO and eNOS-Tg/KO (*P < 0.05 vs. C57Bl/6, #P < 0.05 eNOS-Tg/KO vs. eNOS-KO, n = 3–4, Two-way ANOVA). (B) Representative original recordings of L-NAME-induced increase of MAP in C57BL/6, eNOS-KO, eNOS-Tg/KO and mean summary of L-NAME-induced changes of (C) systolic (sBP), (D) diastolic (dBP) and (E) MAP (*P < 0.001 vs. C57BL/6, n = 4, #P < 0.001 vs. eNOS-Tg/KO, n = 6, One-way ANOVA).

3.4. Hypertension and bradycardia in eNOS-Tg/KO

Our findings demonstrate that bovine eNOS of eNOS-Tg/KO is functional in both conductance and resistance arteries. Thus, we expected that hypertension occurring in eNOS-KO would not be a phenotype of eNOS-Tg/KO but this was not the case. Using a well-established tail-cuff system [19,25], we observed hypertension in eNOS-Tg/KO that was indistinguishable from eNOS-KO (Fig. 4A). In addition, bradycardia occurring in eNOS-KO was evident in eNOS-Tg/KO as well (Fig. 4B). The finding of sustained hypertension in eNOS-Tg/KO was confirmed by invasive BP measurement with a pressure transducer placed into the right carotid artery (Fig. 4C). Long-term oral treatment with L-NA caused an increase of BP only in C57BL/6 so that there was no BP difference between the 3 strains anymore (Fig. S5a). Likewise, the significant reduction of heart rate induced by L-NA was similar in eNOS-KO and eNOS-Tg/KO but more pronounced in C57BL/6 (Fig. S5b). These data suggest that endothelial-specific reintroduction of functionally active eNOS is not sufficient to rescue hypertension and bradycardia of eNOS-KO.

4. Discussion

Accumulating evidence, including observations that mice lacking the eNOS gene are hypertensive, has demonstrated that eNOS-derived NO plays an important role in the regulation of BP. Although it is generally assumed that hypertension in eNOS-KO is caused by the lack of endothelium-dependent NO induced vasodilation, there is in fact no evidence from knockout models clearly linking eNOS activity in endothelial cells to the regulation of BP. To address this question we have reintroduced eNOS into eNOS-KO in an endothelium-specific manner. Surprisingly, we found not even a partial reduction of BP in eNOS-KO. Therefore, our data suggest that the activity of eNOS expressed in non-vascular tissue might play a role in physiologic BP regulation.

Endothelial-specific reintroduction of eNOS in eNOS-KO resulted in a lower eNOS protein expression in different tissues of eNOS-Tg/KO. Therefore, we investigated vascular eNOS activity by acetylcholine-induced endothelium-dependent vasodilation in conductance and resistance vessels, i.e. in aortic rings and in mice in-vivo. We observed no aortic response to acetylcholine in eNOS-KO [1,6] but a complete and indistinguishable dilation in C57BL/6 and eNOS-Tg/KO. Thus, aortic eNOS in eNOS-Tg/KO appears to be functionally active and the lower level of expression didn't impair the magnitude and sensitivity of endothelium-dependent aortic relaxation. Previous investigations have shown that the sensitivity of the NO-receptor sGC likely adapts to variations of eNOS expression and tonic vascular NO generation inasmuch as NO can induce nitrosylation of sGC which inhibits its activity. This was demonstrated in smooth muscle and endothelial cells upon treatment with the NO-donors or vascular endothelial growth factor [26] and in aortic and lung tissues of eNOS-Tg or of C57BL/6 treated with L-NA [17]. Such sGC S-nitrosylation might also underlie the hypersensitivity of aortic rings of eNOS-KO to organic nitrates such as glycerol trinitrate [6], and the spontaneous NO-donor spermine-

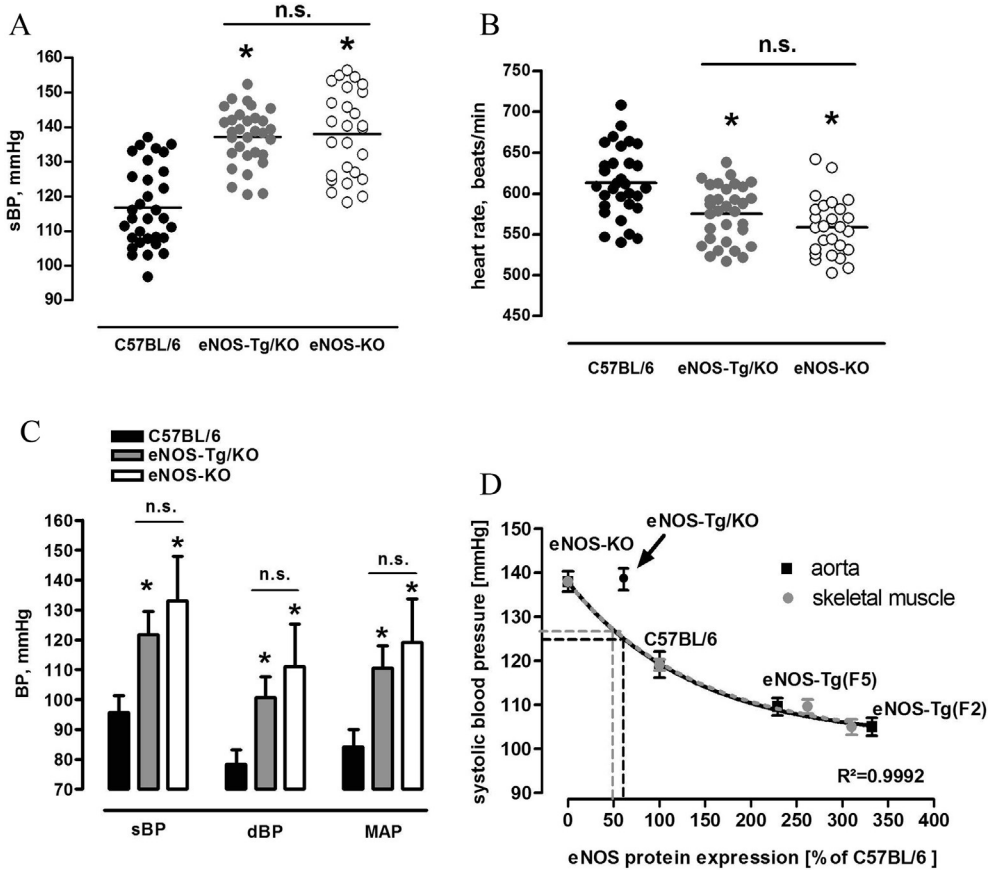


Fig. 4. Sustained hypertension in eNOS-Tg/KO. (A) sBP and (B) heart rate measured by tail-cuff method in eNOS-Tg/KO (n = 31), eNOS-KO (n = 26) and C57BL/6 (n = 32, $P < 0.001$ vs.C57BL/6, One-way ANOVA) (C) sBP, dBP and MAP measured invasively in all strains (*$P < 0.05$, n = 4–7, One-way ANOVA). (D) Non-linear correlation between aortic and skeletal muscle eNOS protein expression and sBP measured by tail cuff method in eNOS-KO, C57BL/6 and two different colonies of eNOS-Tg (F2 and F5). Endothelial-specific targeting of eNOS in eNOS-KO to 61.1% of the aortic level of C57BL/6 was expected to lower sBP in eNOS-Tg/KO to appr. 124.5 mmHg (black dotted lines and an arrow). 54.2% of the skeletal muscle eNOS expression (as compared to C57BL/6) was expected to reduce sBP to 126 mmHg (grey dotted line).

NONOate [27]. Thus, correction of the NO-hypersensitivity occurring in eNOS-KO can be viewed as further evidence for normal aortic eNOS function in eNOS-Tg/KO. In accordance, the increased sensitivity of aortic tissue of eNOS-KO to alpha-adrenergic stimulation by phenylephrine as described previously [6] was completely reversed in eNOS-Tg/KO which showed a concentration-dependent vasoconstriction similar to C57BL/6. Taken together, these data suggest a normal function of eNOS in conductance vessels of eNOS-Tg/KO.

To investigate endothelium-dependent vasodilation of resistance arteries in-vivo we invasively monitored BP of mice subjected to increasing doses of i.v. acetylcholine as performed previously in rabbits [5] and rats [18]. Acetylcholine produced a dose-dependent and strong fall of BP in all strains including eNOS-KO albeit with a lower sensitivity to acetylcholine. Therefore, other endothelium-dependent vasodilators appear to compensate for the lack of eNOS in resistance arteries. For example, the dilatory response of coronary arteries to acetylcholine is preserved by compensatory generation of e.g. cyclooxygenase products [11,12]. Furthermore, preservation of endothelium-dependent vasodilation has been demonstrated in femoral and mesenteric vessels [13], cerebral arteries [14,16] and small skeletal muscle arterioles [15] of eNOS-KO mice. While these data indicate compensation for the lack of endothelial generation of NO in eNOS-KO, our data suggests that endothelial-specific reintroduction of eNOS to eNOS-KO partially reverses such compensation since there was no statistically significant difference in sensitivity to acetylcholine between eNOS-Tg/KO and C57BL/6. Furthermore, the strong and comparable increase of systolic, diastolic and mean arterial pressure to i.v. L-NAME in C57BL/6 and eNOS-Tg/KO suggests the reversion of compensatory mechanisms and indicates a functional eNOS enzyme in resistance arteries of eNOS-Tg/KO as observed in aortic rings.

In view of the hypotensive effect of endothelial-specific overexpression of eNOS [17,19,28] we correlated skeletal muscle eNOS expression with sBP including data on colony 5 of eNOS-Tg [17] which was used to generate eNOS-Tg/KO (Fig. 4D). We found a highly significant one phase exponential decay (R^2 =0.992) described by the equation $Y = SPAN \cdot e^{(-K \cdot X)} + Plateau$ (Y = sBP; SPAN = 35.56; X = Expression; K = 0.00720; Plateau = 102.3) and similar values were found for the correlation of aortic eNOS expression and sBP (R^2 =0.9992; SPAN = 35.92; K = 0.00727; Plateau = 102.0). Using these equations the expected sBP calculated for eNOS-Tg/KO was 126.0 and 124.5 mmHg, respectively. Hence, the predicted reduction of ≥12 mmHg of sBP in eNOS-Tg/KO did not occur. Moreover, we observed not even a part of this expected reduction of sBP regardless of the method of measurement suggesting that endothelial-specific and thus vascular expression of eNOS alone appears not sufficient to reduce BP in eNOS-KO.

It has been shown that both expression and activity of vascular extracellular SOD (ecSOD) are dependent on bioavailability of vascular endogenous NO and are largely reduced in eNOS-KO [24,29]. Likewise, upregulation of eNOS by exercise triggers induction of ecSOD expression [24]. In striking contrast to lower expression levels of ecSOD in eNOS-KO, the expression of ecSOD in eNOS-Tg/KO was elevated to the levels observed in aorta and in skeletal muscle of C57BL/6. We observed also no difference in the expression of other important proteins which may modify and/or compensate for the activity of vascular NO. For example, there was no change in the expression of the NO receptor sGCβ1- and α1-subunits which confirms previous results on sGC expression and activity in eNOS-KO [6] as well as eNOS-Tg [17]. Despite the small amount of nNOS present in blood vessel nerves, it has been reported that low levels of nNOS-derived NO could compensate for the lack of eNOS when eNOS activity is compromised. For instance, nNOS-cGMP-dependent pathways dilated pial arterioles [14] and nNOS–derived NO contributed to the flow-induced responses in coronary arteries of eNOS-KO [30]. However, there was no compensatory upregulation of nNOS protein in aortic and skeletal muscles of eNOS-Tg/KO as compared to C57BL/6 suggesting that such effects unlikely contributed in eNOS-Tg/KO to the effects of acetylcholine and L-NAME in aortic rings and in vivo. Finally, we investigated whether eNOS-Tg/KO show an increase of protein tyrosine nitration which likely reduces vascular NO-bioavailability [31] and found no differences between the strains. Thus, examination of the expression levels of important proteins associated with vascular NO-activity showed no alterations which would call into question a normal vascular NO activity.

Taken together our data demonstrate a previously unrecognized obligatory role of extra-endothelial eNOS in the physiologic regulation of BP. They open the perspective that non-endothelial eNOS impairment may contribute to the pathogenesis of hypertension which is complex, multifactorial and incompletely understood [32]. Several extra-endothelial locations of eNOS might be of particular importance [33–35]. For example, there is considerable evidence that NO in the central nervous system affects sympathetic nerve activity and modulates BP and heart rate [36,37]. Our novel mouse model might also be a useful tool to study whether current antihypertensive treatments are similarly effective if non-endothelial eNOS is missing.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

This study was supported by research Grant of DFG (SU 783/1-1 to T.S.) and Forschungs-kommission of the Heinrich-Heine-University Düsseldorf (Project 9772 446 to T.S.).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.152.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.152.

References

[1] P.L. Huang, Z.H. Huang, H. Mashimo, K.D. Bloch, M.A. Moskowitz, J.A. Bevan, M.C. Fishman, Hypertension in mice lacking the gene for endothelial nitric oxide synthase, Nature 377 (1995) 239–242.
[2] E.G. Shesely, N. Maeda, H.S. Kim, K.M. Desai, J.H. Krege, V.E. Laubach, P.A. Sherman, W.C. Sessa, O. Smithies, Elevated blood pressures in mice lacking endothelial nitric oxide synthase, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13176–13181.
[3] A.R. Gregg, A. Schauer, O. Shi, Z. Liu, C.G. Lee, W.E. O'Brien, Limb reduction defects in endothelial nitric oxide synthase-deficient mice, Am. J. Physiol. 275 (1998) H2319–H2324.
[4] H.M. Stauss, A. Gödecke, R. Mrowka, J. Schrader, P.B. Persson, Enhanced blood pressure variability in eNOS knockout mice, Hypertension 33 (1999) 1359–1363.
[5] D.D. Rees, R.M.J. Palmer, S. Moncada, Role of endothelium derived nitric oxide in the regulation of blood pressure, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 646–652.
[6] G. Kojda, J.B. Laursen, S. Ramasamy, J.D. Kent, S. Kurz, J. Burchfield, E.G. Shesely, D.G. Harrison, Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control, Cardiovasc. Res. 42 (1999) 206–213.
[7] P. Vallance, J. Collier, S. Moncada, Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man, Lancet 334 (1989) 997–1000.

[8] L.A. Barouch, R.W. Harrison, M.W. Skaf, G.O. Rosas, T.P. Cappola, Z.A. Kobeissi, I.A. Hobai, C.A. Lemmon, A.L. Burnett, B. O'Rourke, E.R. Rodriguez, P.L. Huang, J.A.C. Lima, D.E. Berkowitz, J.M. Hare, Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms, Nature 416 (2002) 337–340.
[9] M. Tsutsui, H. Shimokawa, Y. Otsuji, N. Yanagihara, Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases, Pharmacol. Ther. 128 (2010) 499–508.
[10] R.S. Scotland, M. Morales-Ruiz, Y. Chen, J. Yu, R.D. Rudic, D. Fulton, J.P. Gratton, W.C. Sessa, Functional reconstitution of endothelial nitric oxide synthase reveals the importance of serine 1179 in endothelium-dependent vasomotion, Circ. Res. 90 (2002) 904–910.
[11] A. Gödecke, U. Decking, Z. Ding, J. Hirchenhain, H.J. Bidmon, S. Godecke, J. Schrader, Coronary hemodynamics in endothelial NO synthase knockout mice, Circ. Res. 82 (1998) 186–194.
[12] K.G. Lamping, D.W. Nuno, E.G. Shesely, N. Maeda, F.M. Faraci, Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice, Am.J.Physiol Heart Circ.Physiol 279 (2000) H1906–H1912.
[13] G.J. Waldron, H. Ding, F. Lovren, P. Kubes, C.R. Triggle, Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase, Br. J. Pharmacol. 128 (1999) 653–658.
[14] W. Meng, C. Ayata, C. Waeber, P.L. Huang, M.A. Moskowitz, Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice, Am.J.Physiol. Heart Circ.Physiol 274 (1998) H411–H415.
[15] D. Sun, A. Huang, C.J. Smith, C.J. Stackpole, J.A. Connetta, E.G. Shesely, A. Koller, G. Kaley, Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice, Circ. Res. 85 (1999) 288–293.
[16] A. Drouin, N. Thorin-Trescases, E. Hamel, J.R. Falck, E. Thorin, Endothelial nitric oxide synthase activation leads to dilatory H2O2 production in mouse cerebral arteries, Cardiovasc. Res. 73 (2007) 73–81.
[17] M. Oppermann, T. Suvorava, T. Freudenberger, V.T. Dao, J.W. Fischer, M. Weber, G. Kojda, Regulation of vascular guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification, Basic Res. Cardiol. 106 (2011) 539–549.
[18] K.M. Desai, V. Gopalakrishnan, L.M. Hiebert, J.R. McNeill, T.W. Wilson, EDHF-mediated rapid restoration of hypotensive response to acetylcholine after chronic, but not acute, nitric oxide synthase inhibition in rats, Eur. J. Pharmacol. 546 (2006) 120–126.
[19] T. Suvorava, N. Lauer, S. Kumpf, R. Jacob, W. Meyer, G. Kojda, Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo, Circulation 112 (2005) 2487–2495.
[20] T. Suvorava, N. Lauer, G. Kojda, Physical inactivity causes endothelial dysfunction in healthy young mice, J. Am.Coll.Cardiol 44 (2004) 1320–1327.
[21] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.
[22] T. Suvorava, S. Kumpf, B.H. Rauch, V.T. Dao, V. Adams, G. Kojda, Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity, Free Radic.Res. 44 (2010) 199–207.
[23] G. Douglas, J.K. Bendall, M.J. Crabtree, A.L. Tatham, E.E. Carter, A.B. Hale, K.M. Channon, Endothelial-specific Nox2 overexpression increases vascular superoxide and macrophage recruitment in ApoE(-)/(-) mice, Cardiovasc. Res. 94 (2012) 20–29.
[24] T. Fukai, M.R. Siegfried, M. Ushio-Fukai, Y. Cheng, G. Kojda, D.G. Harrison, Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training, J. Clin. Invest. 105 (2000) 1631–1639.
[25] J.H. Krege, J.B. Hodgin, J.R. Hagaman, O. Smithies, A noninvasive computerized tail cuff system for measuring blood pressure in mice, Hypertension 25 (1995) 1111–1115.
[26] N. Sayed, P. Baskaran, X. Ma, A.F. van den, A. Beuve, Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 12312–12317.
[27] M.B. Hussain, A.J. Hobbs, R.J. MacAllister, Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta, Br. J. Pharmacol. 128 (1999) 1082–1088.
[28] Y. Ohashi, S. Kawashima, K. Hirata, T. Yamashita, T. Ishida, N. Inoue, T. Sakoda, H. Kurihara, Y. Yazaki, M. Yokoyama, Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase [see comments], J. Clin. Invest. 102 (1998) 2061–2071.
[29] M. Oppermann, V. Balz, V. Adams, V.T. Dao, M. Bas, T. Suvorava, G. Kojda, Pharmacologic induction of vascular extracellular superoxide dismutase expression in-vivo, J.Cell Mol.Med 13 (7) (2009) 1271–1278.
[30] A. Huang, D. Sun, E.G. Shesely, E.M. Levee, A. Koller, G. Kaley, Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice, Am.J.Physiol Heart Circ. Physiol. 282 (2002) H429–H436.
[31] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol. 271 (1996) C1424–C1437.
[32] D.G. Harrison, The mosaic theory revisited: common molecular mechanisms coordinating diverse organ and cellular events in hypertension, J. Am.Soc.Hypertens 7 (2013) 68–74.
[33] L.H. Lin, O. Taktakishvili, W.T. Talman, Identification and localization of cell types that express endothelial and neuronal nitric oxide synthase in the rat nucleus tractus solitarii, Brain Res. 1171 (2007) 42–51.
[34] P.A. Ortiz, J.L. Garvin, Cardiovascular and renal control in NOS-deficient mouse models, Am.J.Physiol Regul.Integr. Comp. Physiol. 284 (2003) R628–R638.
[35] P. Kleinbongard, R. Schulz, T. Rassaf, T. Lauer, A. Dejam, T. Jax, I. Kumara, P. Gharini, S. Kabanova, B. Ozuyaman, H.G. Schnurch, A. Godecke, A.A. Weber, M. Robenek, H. Robenek, W. Bloch, P. Rosen, M. Kelm, Red blood cells express a functional endothelial nitric oxide synthase, Blood 107 (2006) 2943–2951.
[36] J. Zanzinger, J. Czachurski, H. Seller, Inhibition of sympathetic vasoconstriction is a major principle of vasodilation by nitric oxide in vivo, Circ. Res. 75 (1994) 1073–1077.
[37] P.G. Guyenet, The sympathetic control of blood pressure, Nat. Rev. Neurosci. 7 (2006) 335–346.

ANTIOXIDANTS & REDOX SIGNALING
Volume 23, Number 9, 2015

DOI: 10.1089/ars.2014.6059

Discoveries

ORIGINAL RESEARCH COMMUNICATION

Impact of eNOS-Dependent Oxidative Stress on Endothelial Function and Neointima Formation

Tatsiana Suvorava,[1] Nadine Nagy,[1] Stephanie Pick,[1] Oliver Lieven,[2] Ulrich Rüther,[2] Vu Thao-Vi Dao,[1] Jens W. Fischer,[1] Martina Weber,[3] and Georg Kojda[1]

Abstract

Aims: Vascular oxidative stress generated by endothelial NO synthase (eNOS) was observed in experimental and clinical cardiovascular disease, but its relative importance for vascular pathologies is unclear. We investigated the impact of eNOS-dependent vascular oxidative stress on endothelial function and on neointimal hyperplasia. ***Results:*** A dimer-destabilized mutant of bovine eNOS where cysteine 101 was replaced by alanine was cloned and introduced into an eNOS-deficient mouse strain (eNOS-KO) in an endothelial-specific manner. Destabilization of mutant eNOS in cells and eNOS-KO was confirmed by the reduced dimer/monomer ratio. Purified mutant eNOS and transfected cells generated less citrulline and NO, respectively, while superoxide generation was enhanced. In eNOS-KO, introduction of mutant eNOS caused a 2.3–3.7-fold increase in superoxide and peroxynitrite formation in the aorta and myocardium. This was completely blunted by an NOS inhibitor. Nevertheless, expression of mutant eNOS in eNOS-KO completely restored maximal aortic endothelium-dependent relaxation to acetylcholine. Neointimal hyperplasia induced by carotid binding was much larger in eNOS-KO than in mutant eNOS-KO and C57BL/6, while the latter strains showed comparable hyperplasia. Likewise, vascular remodeling was blunted in eNOS-KO only. ***Innovation:*** Our results provide the first *in vivo* evidence that eNOS-dependent oxidative stress is unlikely to be an initial cause of impaired endothelium-dependent vasodilation and/or a pathologic factor promoting intimal hyperplasia. These findings highlight the importance of other sources of vascular oxidative stress in cardiovascular disease. ***Conclusion:*** eNOS-dependent oxidative stress is unlikely to induce functional vascular damage as long as concomitant generation of NO is preserved. This underlines the importance of current and new therapeutic strategies in improving endothelial NO generation. *Antioxid. Redox Signal.* 23, 711–723.

Introduction

NO SYNTHESIS BY NOS enzymes is known to strictly require several cofactors, such as flavin adenine dinucleotide, flavin mononucleotide, and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4) (10). According to the crystal structure of the heme domain (oxygenase domain) of bovine endothelial NO synthase (eNOS) (44), binding and correct orientation of BH_4 is critically dependent on the three-dimensional structure of the homodimeric protein. Dimerization itself depends on a zinc ion tetrahedrally coordinated to pairs of cysteine residues, that is, Cys96 and Cys101 of bovine eNOS in each monomer. Thus, genetic disruption of the Zn ion complex would be expected to decrease dimer stability, to impair correct BH_4 orientation and NO synthesis, and to increase eNOS-dependent superoxide and peroxynitrite formation. For example, replacement of cysteine 99 to alanine in human eNOS resulted in a reduction of BH_4 affinity, enzyme stability, citrulline formation, and an irreversible loss of heme (5).

BH_4 plays a key role for normal NOS function inasmuch as it inhibits the generation of superoxide instead of NO by autooxidation of the intermediate oxyferrous complex (14). Endothelial BH_4 deficiency has been shown to be associated with eNOS-dependent vascular oxidative stress in several

[1]Institute for Pharmacology and Clinical Pharmacology and [2]Institute for Animal Developmental and Molecular Biology, Heinrich-Heine-University, Düsseldorf, Germany.
[3]Division of Cardiology, Emory University School of Medicine, Atlanta, Georgia.

Innovation

The results of this study provide the first *in vivo* evidence that oxidative stress generated by an impairment of endothelial NO synthase (eNOS) activity is unlikely to be an initial cause of impaired endothelium-dependent vasodilation and/or a pathologic factor promoting intimal hyperplasia. Therefore, other important enzymatic sources of vascular oxidative stress such as the many known oxidases appear to be more important. Our findings might be explained by concomitant generation of NO by eNOS, which is a fundamental difference from oxidases. They are in line with beneficial effects of therapeutic strategies improving eNOS expression and/or activity and substantiate the importance of vasoprotection conferred by endothelial NO.

models of cardiovascular disease, such as hypertension, atherosclerosis, and diabetic endothelial dysfunction (10, 13, 17, 61). Moreover, supplementation of BH_4 was reported to improve endothelial function in human studies (38), although clinical trials with BH_4 analogs revealed contradictory results (6, 13).

Many oxidative enzymes have been implicated in vascular pathologies and in a variety of cardiovascular diseases, including those mentioned above and stroke (18, 22, 29, 35, 43). This makes it difficult to define the relative role for eNOS-dependent formation of superoxide and peroxynitrite in cardiovascular pathologies using animal models. The aim of this study was to investigate the impact of eNOS-dependent vascular oxidative stress on endothelial function and on neointimal hyperplasia. To accomplish this, a mutant of bovine eNOS where cysteine 101 was replaced by alanine was cloned and introduced into eNOS-KO in an endothelial-specific manner.

Results

C101A mutation of eNOS increases oxidative stress

Despite having similar protein levels of the respective wild-type bovine eNOS (WT-eNOS) and C101A-eNOS following transfection to human embryonic kidney (HEK) cells (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars), the basal WT-eNOS activity in cell homogenates was 6-fold higher than C101A-eNOS activity (WT: 601 ± 233 and C101A-eNOS: 98 ± 5 pmol citrulline/mg/min, Fig. 1A). With increasing concentrations of BH_4, the specific eNOS activities increased, but the maximal specific C101A-eNOS activity in the cell homogenates (554 ± 173 pmol citrulline/mg/min) was significantly lower than the maximal WT-eNOS activity (927 ± 311 pmol citrulline/mg/min, $p = 0.0055$, $n = 3$). Similar results were obtained using purified enzyme preparations (Fig. 1B). Furthermore, the mutation destabilized the enzyme as evidenced by increased monomer formation corresponding to a decreased dimer/monomer ratio during separation on a low-temperature sodium dodecyl sulfate (SDS) gel (Fig. 1C). Using electron spin resonance, it was demonstrated that basal and stimulated C101A-eNOS produced less NO (Supplementary Fig. S1B, C), but more superoxide (Supplementary Fig. S1D, E), than WT-eNOS. The latter increase was completely inhibited by NG-nitro-L-arginine methyl ester (L-NAME), identifying C101A-eNOS as the underlying source (Supplementary Fig. S1F). C101A-eNOS HEK cells showed a significantly higher amount of amino acid carbonyl groups, indicating increased oxidative protein modification, while in cells transfected with the pcDNA3 vector, carbonyl formation was identical to that observed in WT-eNOS-transfected HEK cells (Fig. 1D, E).

C101A-eNOS activity induces vascular oxidative stress in vivo

Mice expressing C101A-eNOS (C101A-Tg) were generated by cloning C101A-eNOS in a construct that allows endothelium-specific expression (Fig. 2A). Western blot analysis demonstrated increased eNOS protein signals in the aorta ($151\% \pm 16\%$) and the left myocardium ($135\% \pm 16\%$) of C101A-Tg (Fig. 2B and Supplementary Fig. S2A, B). Aortic endothelium-dependent relaxation of C101A-Tg and their transgene-negative littermates was indistinguishable (Fig. 2C), despite a significant increase of tyrosine nitration in C101A-Tg (Fig. 2D), which confirms the data obtained in HEK cells. Double transgenic mice expressing endothelial C101A-eNOS only (C101A/eNOS-KO) were obtained by crossing C101A-Tg with eNOS-deficient mice (eNOS-KO, backcrossed to C57BL/6) (51). The genotype of C101A/eNOS-KO was confirmed by sequencing the C101A mutation, by polymerase chain reaction (PCR) using mutation-specific primers, by primers binding in the neomycin cassette, and by primers detecting the Tie-2-C101A-eNOS construct (Supplementary Fig. S2C–E). On visual inspection, C101A/eNOS-KO do not appear different from C57BL/6. However, compared with C57BL/6, their litter size is lower and they show increased blood pressure, decreased heart rate, and myocardial hypertrophy just like eNOS-KO (Supplementary Fig. S2F–H). Furthermore, 3 weeks of treatment with the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NA), evoked an increase of blood pressure in C57BL/6 to the level observed in eNOS-KO, while L-NA had no effect on blood pressure in eNOS-KO and C101A/eNOS-KO (Supplementary Fig. S2F). The localization of C101A-eNOS *in vivo* was restricted to endothelial cells as evidenced by fluorescence microscopy (Supplementary Fig. S3). Compared with C57BL/6, C101A/eNOS-KO expressed lower levels of eNOS in the aorta, the lung, the myocardium, and the skeletal muscle (Fig. 2E and Supplementary Fig. S4A–D) and increased eNOS phosphorylation on Ser 1176 (Ser 1179 in bovine eNOS) (Supplementary Fig. S4E). Similar to HEK cells, we observed increased eNOS monomer formation during separation of lung homogenates on a native SDS gel (Fig. 2F).

Generation of superoxide was assessed by following the cumulative photon emission from thoracic aortic segments (Fig. 3A) and the left ventricular myocardium (Fig. 3B) incubated with 5 μM lucigenin at 37°C and found a 2.5- to 3-fold stronger formation of superoxide in tissues of C101A/eNOS-KO compared with C57BL/6 and eNOS-KO, while there was no difference between the latter two strains (Fig. 3A, B). Furthermore, this increase was dependent on C101A-eNOS as demonstrated by complete inhibition following *in vitro* incubation with L-NAME in the aortic (Fig. 3C) and myocardial tissues (Fig. 3D). In striking contrast, L-NAME tended to increase aortic and myocardial superoxide formation in C57BL/6, while having no effect in eNOS-KO (Fig. 3C, D). To investigate peroxynitrite formation, we used Western blot

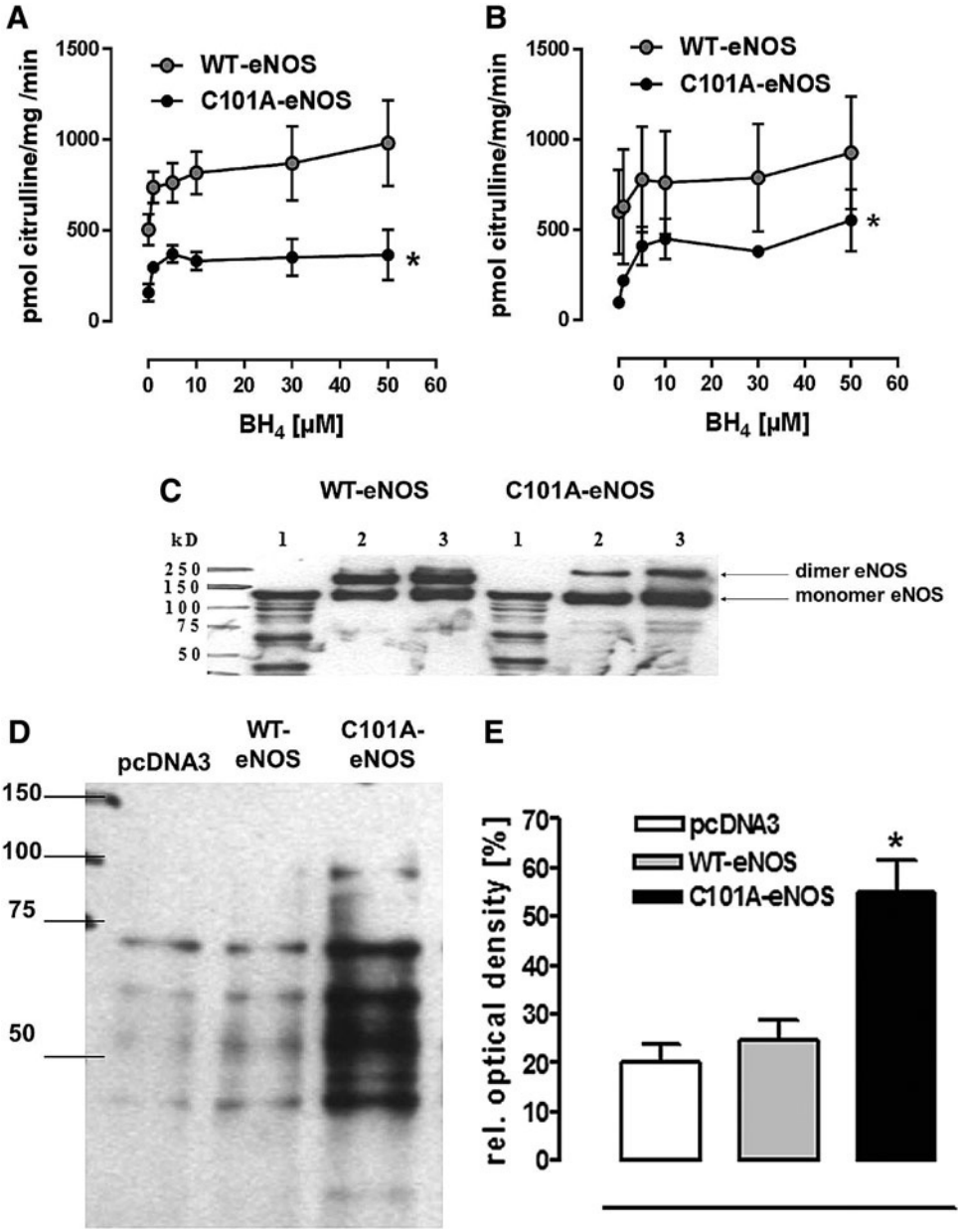


FIG. 1. Characterization of C101A-eNOS *in vitro*. **(A)** Specific eNOS activity in purified WT- and C101A-eNOS preparations and **(B)** cell homogenates from stably transfected WT- and C101A-eNOS cells in dependence on BH_4 concentrations. Both enzymes were activated by BH_4, but the basal and the maximal specific eNOS activity in the C101A-eNOS cells was significantly lower than the activity of the WT cells (*$p = 0.006$, $n = 3$). **(C)** Western blot of a low-temperature SDS-gel representing a small amount of C101A-eNOS dimer in the purified mutant enzyme. Lane 1: denatured protein as monomer control, lane 2: 25 μg WT/MT-eNOS, lane 3: 50 μg WT/MT-eNOS. **(D)** Representative Western blot of pcDNA3-, WT-, and C101A-eNOS-transfected cells for detection of carbonyl groups as a marker for cellular protein oxidation. Twenty micrograms of total protein was loaded, and the band below 75 kDa was used for densitometric analysis. **(E)** Relative optical density (%) from six Oxyblots. The amount of carbonyl groups in the C101A-eNOS cells was significantly higher than in WT or pcDNA3- HEK 293 cells (*$p = 0.002$, $n = 6$). BH_4, (6R-)5,6,7,8-tetrahydro-L-biopterin; eNOS, endothelial NO synthase; HEK 293, human embryonic kidney 293.

detection of tyrosine nitration in aortic and myocardial homogenates. In the aorta, myocardium, and skeletal muscle of C101A/eNOS-KO, we observed a significantly higher nitrotyrosine level compared with C57BL/6 and eNOS-KO (Supplementary Fig. S4F–H). The increase of nitrotyrosine levels was completely inhibited by oral treatment with L-NA, demonstrating the dependence on C101A-eNOS (Fig. 3E, F).

Similar to increase of protein carbonylation in C101A-eNOS HEK cells, a significantly higher amount of amino acid carbonyl groups was detected in aortic and myocardial tissues of C101A/eNOS-KO compared with wild type and eNOS-KO (Supplementary Fig. S5).

C101A-eNOS activity restores endothelial function in eNOS-KO

Endothelial function was studied using acetylcholine-induced endothelium-dependent dilation of aortic rings, as described previously (11). We found a concentration-dependent reduction of vascular tone in C101A/eNOS-KO, while there was just a slight vasoconstriction in eNOS-KO (Fig. 4A). These data suggest that the expression level of eNOS in the aortic endothelium may fall by as much as 60% without impairing maximal endothelium-dependent vasodilation. Furthermore, this vasorelaxation occurred despite a strong increase of vascular oxidative stress as shown in Figure 3A, C, and E. There was, however, a significant rightward shift of the concentration–response curves to acetylcholine in C101A/eNOS-KO *versus* C57BL/6 (Fig. 4A). The corresponding pD_2 values were 6.85 ± 0.08 ($n = 14$) and 6.58 ± 0.1 ($n = 11$, $p = 0.0358$), respectively. To study whether the rightward shift of the acetylcholine concentration–response curve is induced by increased superoxide generation, aortic rings of C57BL/6 and C101A/eNOS-KO were preincubated with 100 U/ml of pegylated superoxide dismutase (SOD), but the pD_2 values for

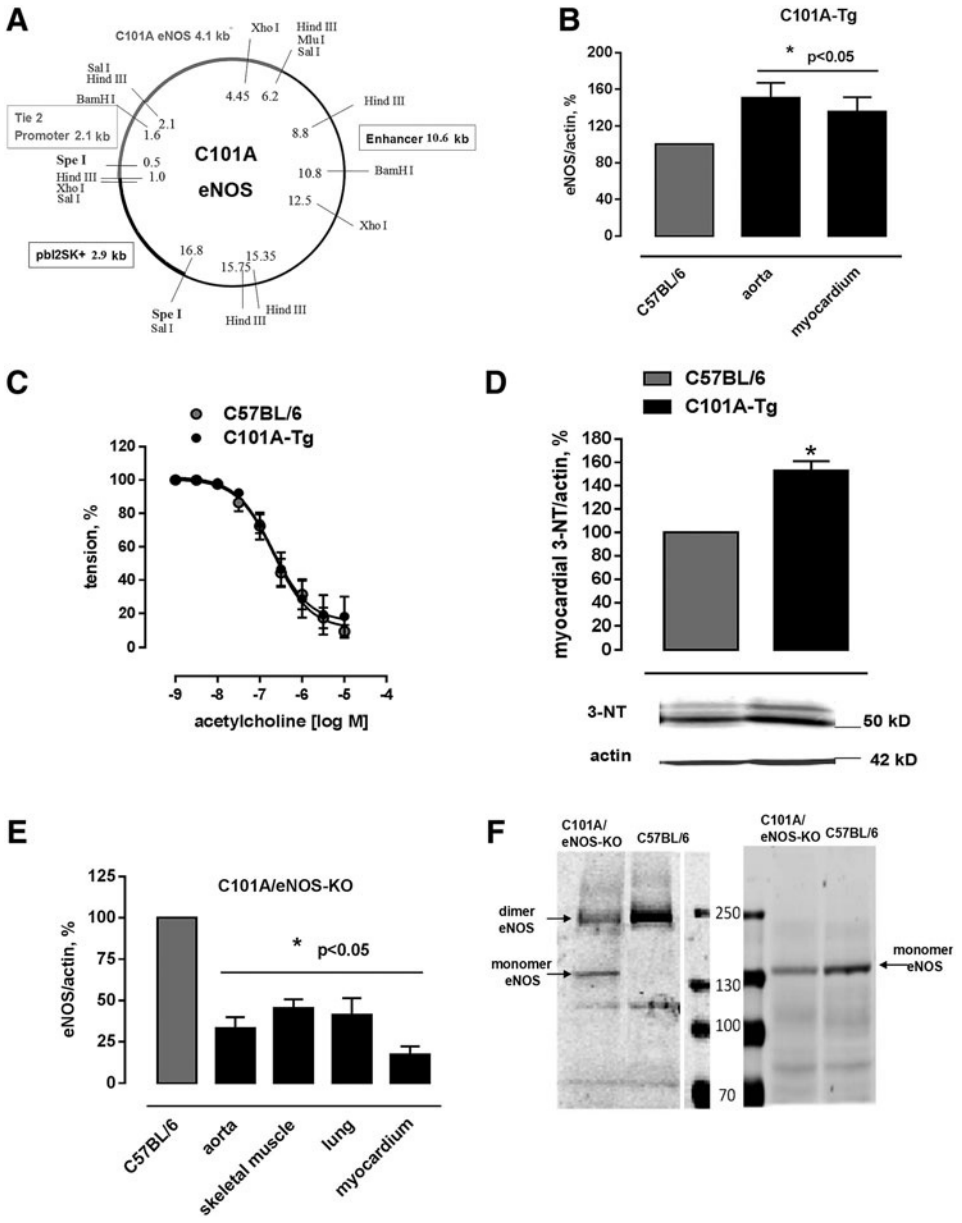


FIG. 2. Generation and basal characterization of C101A-Tg and C101A/eNOS-KO. (A) Construct used for insertion into fertilized eggs of C57BL/6 to generate C101A-eNOS-expressing mice (C101A-Tg) consisting of a 2.1 kb Tie-2 promoter, 4.1 kb bovine C101A-eNOS DNA, and a 10.6 kb Tie-2 enhancer. SpeI restriction sites were used for linearization of the plasmid before insertion. **(B)** Aortic and myocardial eNOS protein expression standardized to actin in C101A-Tg (mean ± SEM $*p < 0.05$ *vs.* controls, $n = 5$–7). **(C)** No effect of C101A-eNOS overexpression on aortic endothelium-dependent relaxation induced by cumulative addition of acetylcholine (ACh, $p = 0.5548$, $n = 6$, two-way ANOVA). **(D)** Significant increase in nitrotyrosine residues as a marker for peroxynitrite formation (mean ± SEM *upper panel*, $*p < 0.05$, $n = 6$) and representative Western blot of protein nitrotyrosine residues (*middle panel*) standardized to actin (*lower panel*) in myocardial tissue of C101A-Tg *versus* C57BL/6. **(E)** Protein expression of eNOS in the aorta, skeletal muscle, lung, and myocardium of C101A/eNOS-Tg ($*p < 0.05$ *vs.* C57BL/6, $n = 6$–12). The control values referring to eNOS protein expression in every tissue of C57BL/6 were set to 100% and are reflected by just one bar. **(F)** A representative experiment showing an increase of eNOS monomer level in native and denatured lung homogenates of C101A/eNOS-KO *versus* C57BL/6 (100 μg of total protein per lane). ANOVA, analysis of variance.

C57BL/6 (7.01 ± 0.126) and C101A/eNOS-KO (6.573 ± 0.07) remained significantly different ($*p < 0.05$, $n = 4$, Fig. 4B). Likewise, a direct comparison revealed that 100 U/ml of pegylated SOD did not change acetylcholine-dependent vasodilation in C57BL/6 or C101A/eNOS-KO (Supplementary Fig. S6A, B). Rubbing off the endothelial layer completely inhibited acetylcholine-induced vasodilation in C101A/eNOS-KO (data not shown).

Several approaches were accomplished to investigate the molecular mechanism of aortic vasodilation to acetylcholine in C101A/eNOS-KO. First, it was observed that L-NAME reversed endothelium-dependent vasodilation to

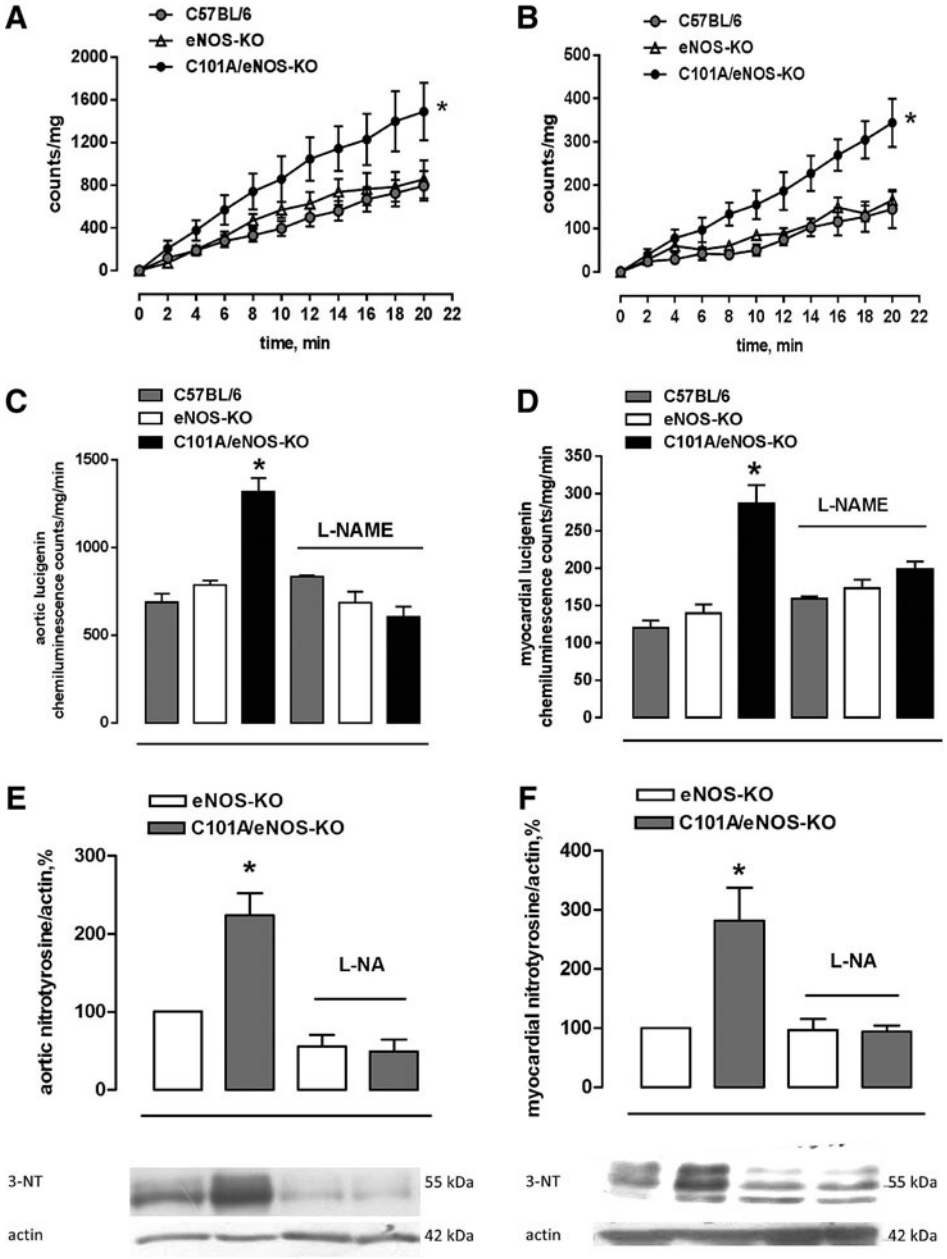


FIG. 3. Role of C101A-eNOS as a source of increased vascular oxidative stress in C101A/eNOS-KO. Increased **(A)** aortic and **(B)** myocardial basal superoxide production in C101A/eNOS-KO, eNOS-KO, and C57BL/6. The cumulative counts/mg of dried tissue measured during incubation with 5 μM lucigenin are given ($*p < 0.05$, two-way ANOVA, $n = 6–13$). **(C)** Effect of NOS inhibition (L-NAME, 1 mM) on aortic and **(D)** myocardial superoxide production assessed by lucigenin chemiluminescence in C101A/eNOS-KO, eNOS-KO, and C57BL/6. Results are expressed as counts/tissue dry weight/min (mean ± SEM) averaged over the last 8 min of measurement ($*p < 0.05$ *vs.* all other conditions, one-way ANOVA, $n = 5–8$). **(E)** Aortic and **(F)** myocardial nitrotyrosine levels before and after oral treatment with the NO synthase inhibitor, L-NA, in eNOS-KO and C101A/eNOS-KO. Results are given as mean ± SEM percentage relative to eNOS-KO (*upper panel*, $*p < 0.05$ *vs.* eNOS-KO, one-way ANOVA, $n = 5–11$). The *middle panel* shows a representative Western blot for protein nitrotyrosine residues, the *lower panel* shows β-actin expression as loading control. L-NA, N^{ω}-nitro-L-arginine; L-NAME, NG-nitro-L-arginine methyl ester.

vasoconstriction in C101A/eNOS-KO and C57BL/6 and that vasoconstriction did not differ between the strains (Fig. 4C). Thus, endothelium-dependent vasodilation to acetylcholine in C101A/eNOS-KO is dependent on the activity of C101A-eNOS. Second, incubation of aortic rings with 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) to inhibit soluble guanylyl cyclase (sGC) completely abolished the vasodilator response to acetylcholine with no difference between both strains (Fig. 4D). These data demonstrate the crucial involvement of the NO/cGMP pathway. Third, these data were confirmed by subjecting aortic rings to the spin trap $Fe(DETC)_2$. As shown in Figure 4E, scavenging of NO completely inhibited acetylcholine-induced vasodilation in both strains. Finally, it was investigated whether hydrogen peroxide might be involved in endothelium-dependent vasodilation. However, neither conventional nor pegylated catalase (Supplementary Fig. S6C, D) had any influence on the vasodilation. Investigation of the sensitivity of the NO/cGMP pathway confirmed the previously reported sensitization in eNOS-KO (25) and demonstrated that a similar hypersensitivity to NO donors, such as S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (Fig. 4F) and diethylamine/nitric oxide (DEA/NO) (Supplementary Fig. S6E), occurred in C101A/eNOS-KO. There was no change of the expression of sGC subunit $\beta 1$ (Supplementary Fig. S6F). In addition, aortic and skeletal muscle expression of nNOS was not changed in C101A/eNOS-KO (Supplementary Fig. S7A–C). Taken together, these data demonstrate that C101A-eNOS-dependent vascular oxidative stress had no influence on complete vasodilation to acetylcholine in aortic rings of C101A/eNOS-KO. The relaxation was entirely endothelium dependent and mediated by the same pathway as in C57BL/6, that is, generation of NO by eNOS and activation of the NO/cGMP pathway.

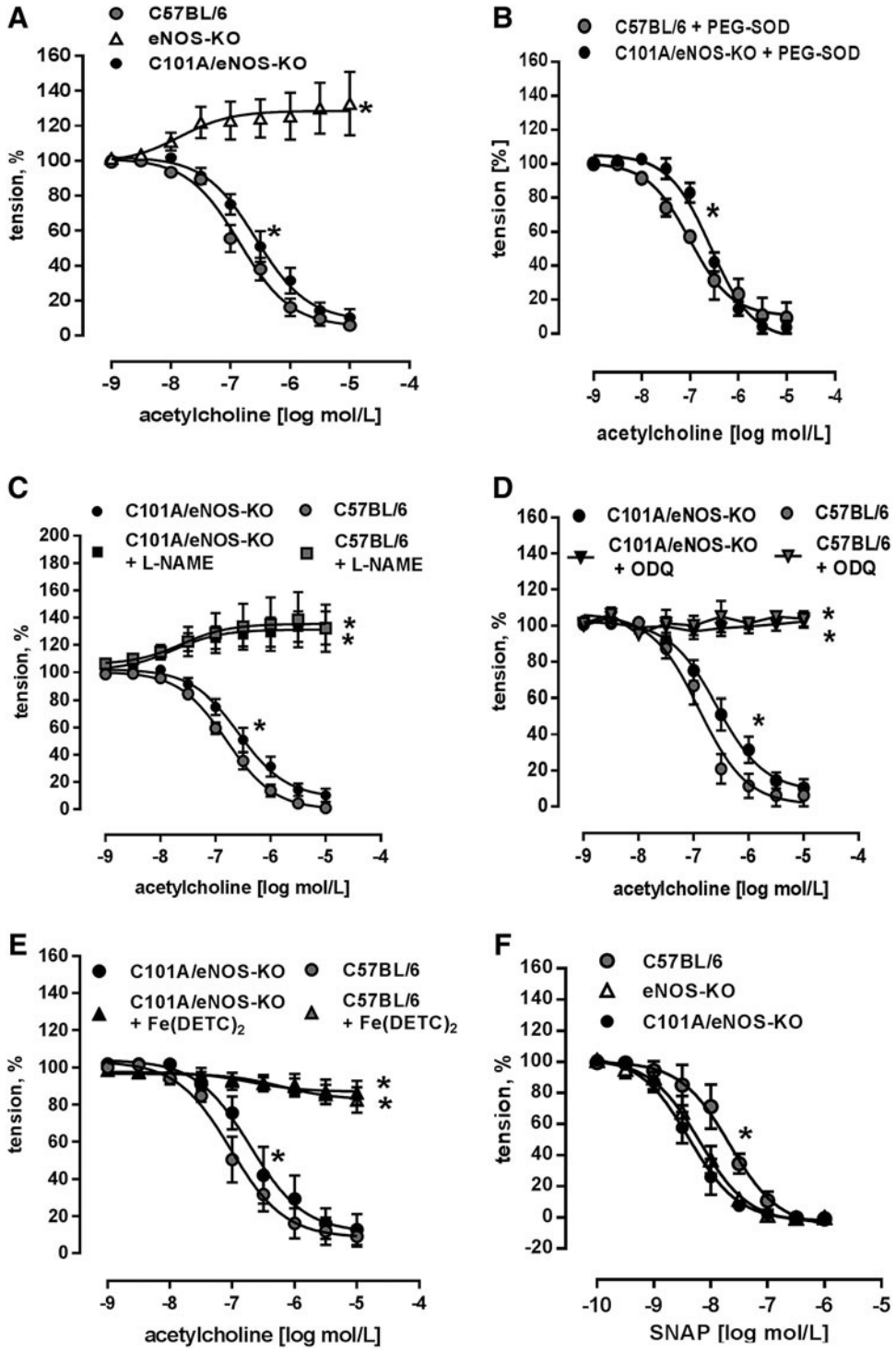


FIG. 4. Preserved endothelial function in C101A/eNOS-KO. **(A)** Relaxation response of aortic rings of C101A/eNOS-KO, eNOS-KO, and C57BL/6 induced by cumulative addition of acetylcholine (*$p < 0.05$ *vs.* C57BL/6, two-way ANOVA, $n = 6$–14). **(B)** Aortic acetylcholine-induced relaxation in C101A/eNOS-KO in the presence of 100 U/ml of pegylated SOD (*$p < 0.05$, $n = 4$). **(C)** Effect of the NOS inhibitor, L-NAME, **(D)** the sGC inhibitor, ODQ, and **(E)** the NO scavenger, $Fe(DETC)_2$, on relaxation response to acetylcholine in aortic rings of C101A/eNOS-KO and C57BL/6 (*$p < 0.05$ *vs.* C57BL/6, two-way ANOVA, $n = 4$–9). **(F)** Aortic relaxation response to cumulative addition of NO donor, SNAP, in C101A/eNOS-KO, eNOS-KO, and C57BL/6 (*$p < 0.05$ for pD_2 and two-way ANOVA, $n = 5$–6). ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; SOD, superoxide dismutase.

C101A-eNOS activity inhibits increased neointima formation in eNOS-KO

In an attempt to investigate whether increased vascular oxidative stress present in C101A/eNOS-KO may play a role in the development of vascular pathologies such as neointimal hyperplasia, we subjected C57BL/6, eNOS-KO, and C101A/eNOS-KO to ligation for 4 weeks of the left common carotid artery proximal to the bifurcation. In C57BL/6, this procedure resulted in a strong formation of neointima, which declined with increasing distance from the ligation as reported previously (Fig. 5A–C) (16). Likewise, the lumen area was largely decreased, while the media were enlarged (Supplementary Fig. S8A, B). This pronounced development of neointimal hyperplasia resulted in an increased intima/media ratio, which declined with increasing distance from the ligation (Fig. 5D, E). In eNOS-KO, neointimal hyperplasia was much more pronounced as evidenced by a significantly

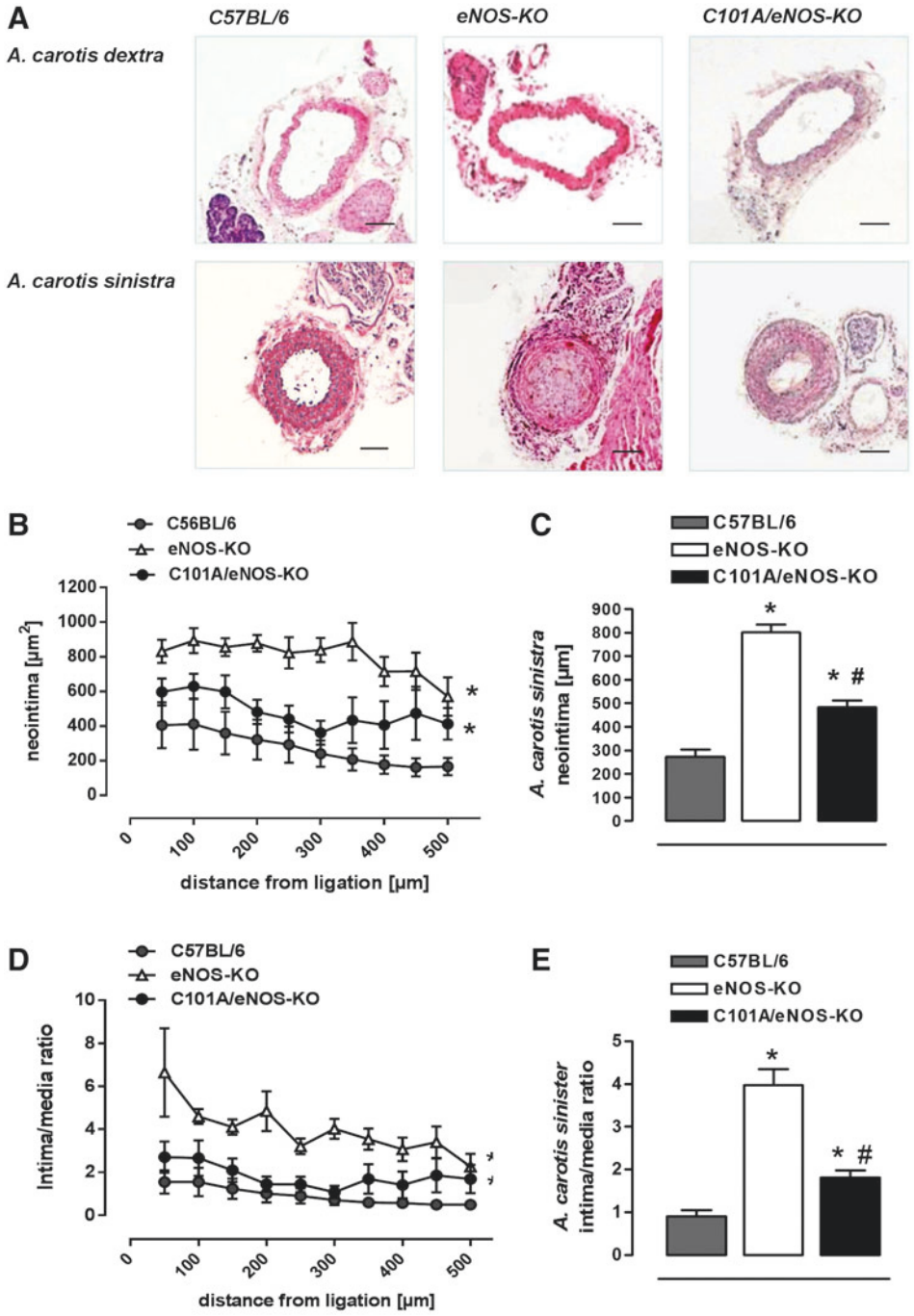


FIG. 5. Negligible effects of C101A-eNOS on neointimal hyperplasia. **(A)** Representative sections stained by hematoxylin and eosin demonstrate pronounced neointimal hyperplasia, media thickening, and luminal narrowing in the ligated *A. carotis sinistra* of all studied genotypes (450 μm from the ligation). Scale bar indicates 100 μm. **(B)** Neointima formation is shown for different distances from the ligation site in C57BL/6, eNOS-KO, and C101A/eNOS-KO. Values are mean ± SEM, $^{*}p < 0.05$ *vs.* C57BL/6, two-way ANOVA, $n = 4$–6). **(C)** Statistical evaluation of the neointimal area ($^{*}p < 0.05$ *vs.* C57BL/6, $^{\#}p < 0.05$ *vs.* eNOS-KO, $n = 4$–6). **(D)** Intima/media ratio for different distances from ligation in C57BL/6, eNOS-KO, and C101A/eNOS-KO ($^{*}p < 0.05$ *vs.* C57BL/6, two-way ANOVA, $n = 4$–6) and **(E)** statistical analysis after summarizing the ratios for each animal at each distance point ($^{*}p < 0.05$ *vs.* C57BL/6, $^{\#}p < 0.05$ *vs.* eNOS-KO, one-way ANOVA, $n = 4$–6). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

increased neointima area (Fig. 5A–C) and intima/media ratio (Fig. 5D, E). In striking contrast, neointimal hyperplasia in ligated carotid arteries of C101A/eNOS-KO resembled that observed in C57BL/6. The small increase of neointima and intima/media ratio *versus* C57BL/6 did not reach statistical significance (Fig. 5C, E). In addition, neointimal hyperplasia in C101A/eNOS-KO significantly differed from that in eNOS-KO. Thus, despite strongly increased vascular oxidative stress, mild hypertension, and a low level of C101A-eNOS expression, neointimal hyperplasia was not accelerated in C101A/eNOS-KO. These data suggest that the lack of NO rather than increased levels of eNOS-derived vascular oxidative stress accelerates neointimal hyperplasia in response to ligation.

C101A-eNOS activity partially restores vascular remodeling in eNOS-KO

To estimate remodeling following the ligation procedure, the left common carotid vessel area was measured 500–1000 μm distal to the ligation and the averaged values were compared with those obtained from the unligated right common carotid artery. As shown in Figure 6A, there was still considerable neointimal hyperplasia in this region of the

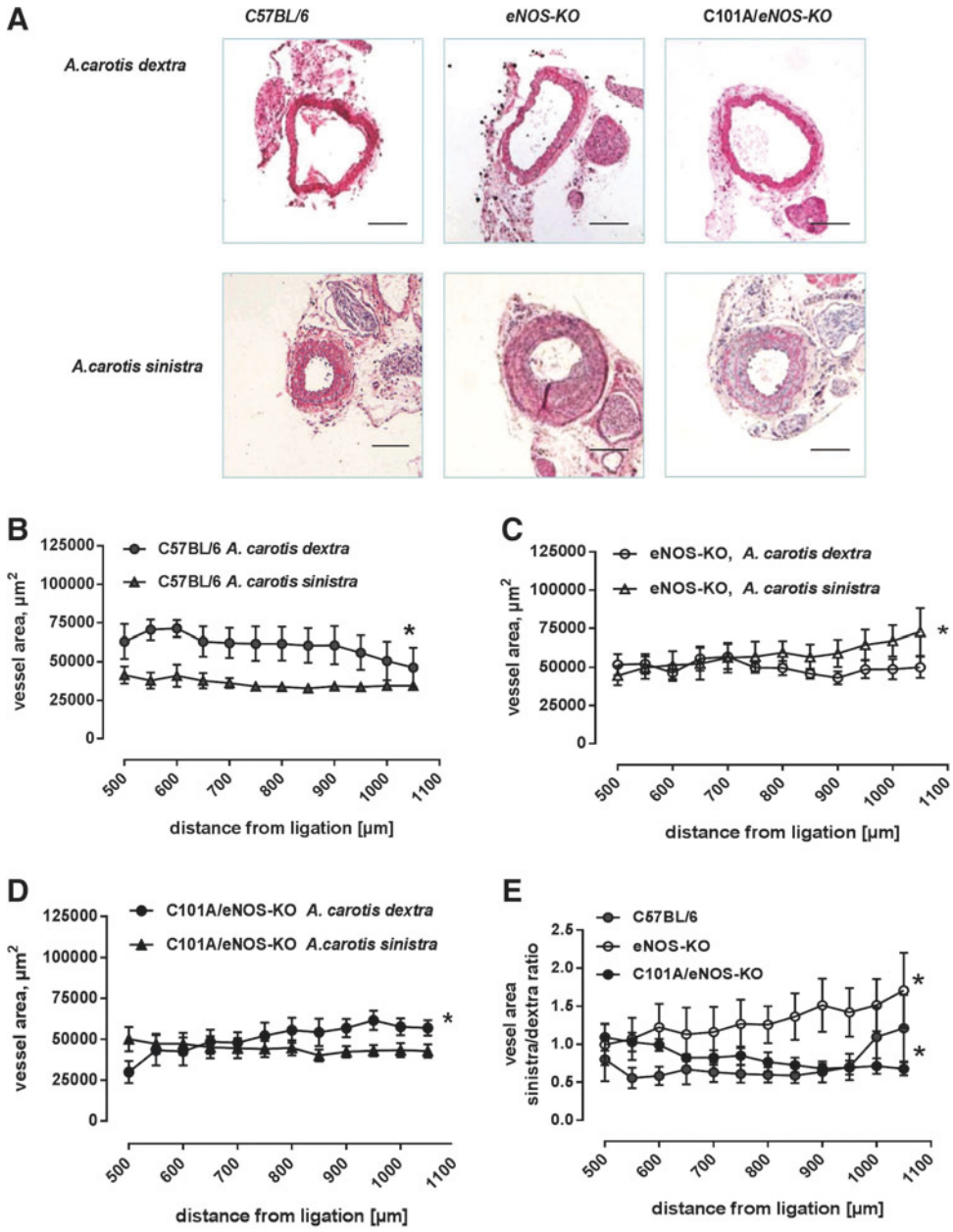


FIG. 6. Effects of C101A-eNOS on vascular remodeling. (A) Representative photomicrographs of hematoxylin and eosin-stained transverse sections of the right (unligated side, *A. carotis dextra*) and left (ligated side, *A. carotis sinistra*) common carotid arteries (850 μm from the ligation). Scale bar indicates 200 μm. **(B)** Effects of *A. carotis sinistra* ligation on the vessel area in C57BL/6, **(C)** eNOS-KO, and **(D)** C101A/eNOS-KO (*$p < 0.05$ *vs. A. carotis dextra*, two-way ANOVA, $n = 4–5$). **(E)** Vessel area ratio of left common carotid artery from the ligated animal over the intact right common carotid artery from the same mouse (*$p < 0.05$ *vs.* C57BL/6, two-way ANOVA, $n = 4–5$). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

ligated artery. In C57BL/6, there was a statistically significant difference between the vessel area of the left and right common carotid artery, suggesting the occurrence of vascular remodeling induced by the reduction of flow in the ligated and/or an increase of flow in the unligated vessels (Fig. 6B). In striking contrast, in eNOS-KO, the vessel area distal to the ligation was significantly increased over the vessel area of the unligated right common carotid artery (Fig. 6C), suggesting that vascular remodeling was inversed. In C101A/eNOS-KO, this abnormal vascular response was partially restored, that is, the vessel area of the ligated carotid artery was smaller than that of the unligated right common carotid artery, although the difference was not as pronounced as in C57BL/6 (Fig. 6D). In accordance, the ratio of the vessel area of the ligated *versus* the unligated carotid artery, which was calculated in each separate animal and then averaged for each distance point, clearly demonstrated a mouse strain-dependent significant increase over the investigated distance from the ligation in eNOS-KO, while C57BL/6 and C101A/eNOS-KO showed a decrease (Fig. 6E).

Discussion

This study aimed to investigate the impact of eNOS-dependent vascular oxidative stress on endothelial function and on neointimal hyperplasia. A mutant destabilized eNOS, which was introduced in eNOS-KO in an endothelial-specific manner, (i) largely increased vascular generation of superoxide and protein nitrotyrosine formation, but (ii) had little impact on aortic endothelium-dependent vasodilation by acetylcholine, and (iii) corrected the accelerated neointimal hyperplasia and the abnormal vascular remodeling observed in eNOS-KO. These data suggest that vascular oxidative stress generated by an impairment of eNOS activity has a

limited effect on endothelial function of conduit arteries, presumably because of concomitant generation of NO by eNOS.

As predicted by the crystal structure of the oxygenase domain of eNOS, genetic disruption of its Zn ion complex disturbs dimer formation and thereby binding and correct orientation of BH_4 (44). BH_4 deficiency promotes the transfer of an electron to molecular oxygen and thereby the generation of the superoxide (14). Thus, a replacement of cysteine 101 by alanine destabilizes eNOS, resulting in reduced NO formation and increased generation of superoxide in transfected HEK cells and in different tissues of C101A-Tg and C101A/eNOS-KO. Our data on decreased catalytic activity of C101A-eNOS are consistent with previous reports describing eNOS mutations at one or both Zn-binding cysteines (5, 9). Of note, mutation of eNOS by replacing both Zn-binding cysteines with serine did not abolish formation of L-citrulline from L-arginine (9), suggesting partial preservation of NO generation, despite decreased dimer stability as reported in this study with C101A-eNOS. The destabilization of C101A-eNOS was also evident by the decrease of the dimer/monomer ratio following separation on native SDS gels, and similar findings have been reported previously (45). Moreover, increased superoxide generation induced by C101A-eNOS in transfected HEK cells and in aortic and myocardial tissues of C101A/eNOS-KO was abolished by L-NAME, identifying C101A-eNOS as the underlying source both in cells and in transgenic mice.

Previous studies with purified eNOS have demonstrated that BH_4 deficiency strongly increases the eNOS-derived superoxide electron spin resonance (ESR) signal and that BH_4 supplementation inhibits this oxidase activity in a concentration-dependent manner (57, 58). In striking contrast, citrulline formation by purified C101A-eNOS never reached the maximal rate that was achieved with WT-eNOS, not even at BH_4 concentrations of 50 μM in the reaction tube, demonstrating that the ability of C101A-eNOS to generate citrulline and hence NO cannot be improved by excess BH_4. Taken together, these results are in line with previous investigations on the importance of BH_4 (5, 14, 17, 44) and strongly suggest that an impairment of binding and correct orientation of BH_4 underlies the increase of superoxide production observed in cells and tissues harboring C101A-eNOS.

A surprising finding of this study is that activation of C101A-eNOS by acetylcholine in aortic rings resulted in complete endothelium-dependent vasodilation in C101A-Tg and C101A/eNOS-KO, despite reduced NO formation and increased vascular oxidative stress induced by C101A-eNOS. The relaxation by acetylcholine in aortic rings of C101A/eNOS-KO was found to be mediated by NO and developed at tissue levels of superoxide and/or peroxynitrite, resembling those occurring in various animal models with clearly detectable impairment of eNOS function and/or endothelial-dependent vasodilation (2, 7, 15, 21, 32, 33, 61). However, in each of these studies, concomitant conditions, such as atherosclerosis, severe hypertension, diabetes, or disruption of certain genes such as interleukin 10 or Bmal1, were present, which might have contributed to endothelial dysfunction beside oxidative and/or nitrosative stress. Nevertheless, there was a significant rightward shift of the acetylcholine concentration–response curve, indicating a reduced efficacy of eNOS activation in aortic rings of C101A/eNOS-KO. A contribution of increased generation of superoxide is most likely not involved since pegylated SOD had no impact on acetylcholine-induced aortic dilation. It is also possible that the reduction of eNOS expression and its ability to generate NO in C101A/eNOS-KO compared with C57BL/6 is the underlying cause. According to the data obtained from the carotid-binding experiments, this small shift appears to be rather unimportant for NO bioavailability *in vivo*. Taken together, our data suggest that oxidative stress generated by eNOS appears to have little impact on endothelium-dependent vasodilation in conduit arteries.

It might be argued that the lack of effect of C101A-eNOS on mild hypertension of eNOS-KO demonstrates an effect of oxidative stress generated by an impairment of eNOS activity on resistance vessels and blood pressure. One would expect a blood pressure-lowering effect of endothelial expression of eNOS as reported previously (40, 41, 54); however, data of a further study have shown that even endothelial-specific expression of normal eNOS does not reduce the hypertension of eNOS-KO (55). Thus, the persistence of hypertension in C101A-eNOS-KO is probably not provoked by C101A-eNOS-dependent oxidative stress and is unlikely to represent a specific phenotype caused by pronounced vascular oxidative stress in this animal model. On the other hand, our data certainly do not exclude that oxidative stress due to an impairment of eNOS activity may have an impact on the regulation of blood pressure, for example, in more severe hypertensive cardiovascular disease states (34).

Smooth muscle proliferation is an obvious event during the development of atherosclerosis and restenosis (36, 39, 46). It is generally believed that oxidative stress plays a crucial role in this process (35, 48) and that this pathologic role of oxidative stress is closely linked to a downregulation of vascular NO bioavailability (56). Furthermore, inhibition of oxidative stress is considered to be a key component of the overall vasoprotective effect of endothelial eNOS activity (12). In this study, neointima formation following carotid ligation (26) was used to estimate the vascular bioavailability and vasoprotective activity of endothelial NO *in vivo*. In this model, neointima formation occurs in the presence of intact endothelial lining and is directly related to the alteration of the blood flow pattern, that is, turbulent rather than laminar flow, as well as to the infiltration of monocytes (20). Consequently, neointima formation is largest close to ligation and becomes smaller with increasing distance (26) and a similar pattern was observed in C57BL/6, C101A/eNOS-KO, and eNOS-KO. However, neointima formation was significantly more pronounced in eNOS-KO than in C57BL/6, confirming that vascular eNOS elicits protective effects in the setting of carotid ligation (59). Neointima formation in C101A/eNOS-KO tended to increase in comparison with C57BL/6, but the difference did not reach statistical significance, while the difference compared with eNOS-KO was obvious. Furthermore, this difference was evident, despite increased oxidative stress in C101A/eNOS-KO, which does not occur in eNOS-KO, suggesting that C101A/eNOS-KO generate bioavailable NO sufficiently to inhibit smooth muscle proliferation as observed in C57BL/6. Finally, these data obtained from the carotid ligation experiments indicate that increased neointima formation in eNOS-KO appears to be caused by the lack of endothelial NO formation and not by the presence of mild hypertension.

Another important role of NO is to mediate vascular remodeling in response to flow (28, 47). Although carotid ligation is not a good model to investigate flow-dependent vascular remodeling due to the neointima formation, the ratio of ligated to unligated vessel area, which indicates vascular remodeling, was almost similar in C57BL/6 and C101A/eNOS-KO. In these strains, the ratio decreased with increasing distance from the ligation and reached values well below one, while in eNOS-KO this was not the case. These data again suggest a limited impairment of NO bioavailability in C101A/eNOS-KO.

The alteration of activity of C101A-eNOS might resemble the features of uncoupled eNOS, a term which has been used to describe early findings with purified NOS protein showing increased production of superoxide and hydrogen peroxide in the absence of important cofactors such as BH_4 (19). In contrast, mutant C101A-eNOS produces less NO and more superoxide in response to dimer destabilization and subsequent impairment of binding and correct orientation of BH_4. Hence, this approach shows a direct relationship between a reduced stability of homodimers and eNOS function. In contrast, there is no evidence that the presence of monomers and a reduced dimer/monomer ratio are directly related to uncoupling of the enzyme since only the dimeric form has been shown to be active and generate NO and superoxide (46). Moreover, we believe that an advantage of this approach is the possibility to study the importance of eNOS dimer destabilization and impaired BH_4 binding for vascular physiology and pathophysiology in otherwise healthy animals. This largely reduces unpredictable confounding factors, such as complex disease processes occurring in hypertension, atherosclerosis, and/or diabetes, which are associated with an impairment of eNOS cofactor availability or proven increases of other components such as the eNOS inhibitor, ADMA (3). Nevertheless, concomitant generation of NO and superoxide by C101A-eNOS probably closely resembles the situation in the endothelium of diseased vessels when uncoupled eNOS molecules and coupled eNOS molecules can exist in the same cell at the same time (35).

Taken together, the results of this study suggest that vascular oxidative stress generated by an impairment of eNOS activity might not be as important as estimated previously and rather highlight the contribution of oxidative stress to vascular pathologies caused by other enzymatic sources (18, 22, 29, 36, 43, 52). A reasonable explanation for the limited importance of eNOS-dependent oxidative stress for functional vascular damage might be the concomitant generation of NO by eNOS, which is a fundamental difference from oxidases. Hence, our findings substantiate the importance of vasoprotection conferred by endothelial NO (12), which appears to be able to cope with even larger increases of vascular oxidative stress. Similar findings have been reported for the vasoprotective activity of eNOS in murine atherosclerosis (42). Although it is difficult to extrapolate our data to clinical conditions as the transgenic approach used does not directly match clinically occurring disease pathologies, our data support previous observations on interventions associated with increased eNOS protein expression and improvement of endothelial NO production, such as regular exercise training (24), pharmacotherapy with statins (31), and treatment with inhibitors of the renin–angiotensin system (12) have provided superior cardiovascular prevention than targeting vascular oxidative stress (4, 18a, 37, 50, 60).

Materials and Methods

Stable transfection of HEK 293 cells

Using primer overlap extension PCR, we genetically destabilized bovine eNOS by replacement of Cys 101 to Ala (C101A). The mutation was confirmed by sequencing. The cDNAs for wild-type bovine eNOS (WT) and the mutant (MT) C101A eNOS were cloned into the pcDNA3 vector (Invitrogen Corporation, Carlsbad, CA) and stably transfected in HEK 293 cells.

ESR measurements

ESR measurements were performed as described previously (8, 27). Cellular basal and Ca^{2+} ionophore A23187-stimulated NO production was measured using 0.2 *M* of the spin trap iron/diethyldithiocarbamic acid, $Fe(DETC)_2$. CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine) at 100 μM was used for superoxide detection in the absence and presence of the NOS inhibitor, L-NAME (1 *M*).

eNOS activity

The specific activity of WT- and C101A-eNOS in cell homogenates from stably transfected HEK cells and in isolated enzyme preparations was determined by conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline as described previously (49) in the presence of increasing concentrations of BH_4 (0–50 μM).

Detection of oxidative protein modification

Protein modifications caused by additional carbonyl groups were detected in C101A-eNOS and WT-eNOS-transfected HEK cells and aortic and myocardial tissue of C57BL/6, eNOS-KO, and C101A/eNOS-KO using the Oxyblot™ system (Intergen, Burlington, MA).

Transgenic C101A-eNOS mice

We generated a DNA construct, in which C101A-eNOS cDNA (4.1 kb) was inserted between the murine Tie-2 promoter (2.1 kb) cDNA and a 10 kb Tie-2 intron fragment, designated as Tie-2 enhancer. This construct was used to target C101A-eNOS gene expression to the vasculature, as described previously (30). Double transgenic mice expressing endothelial C101A-eNOS only (C101A/eNOS-KO) were obtained by crossing C101A-Tg with eNOS-deficient mice (eNOS-KO, backcrossed > 20 times to C57BL/6J) (51). Additional groups of C57BL/6J, eNOS-KO, and C101A/eNOS-KO received L-NA (100 mg L-NA/kg BW/day; Sigma, Munich, Germany) for 3 weeks before the experiments. Permission for the animal studies was provided by the regional government of Germany (AZ 50.05-230-3-94/00, AZ 50.05-230-18/06, AZ 8.87-51.04.20.09.383), and the experiments were performed according to the guidelines for the use of experimental animals, as given by the German ''Tierschutzgesetz'' and the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health.

Vasorelaxation studies

Function of the endothelium was examined in aortic rings of C57BL/6, eNOS-KO, and C101A/eNOS-KO by cumulative addition of acetylcholine (0.01 to 10 μM) after submaximal

precontraction with 0.2 μM phenylephrine, as described previously (53). Thereafter, vasorelaxation to the NO donor, SNAP (1 nM to 10 μM) or DEA/NO (1 nM to 10 μM), was studied by cumulative application. In some experiments, the NOS inhibitor, L-NAME (1 mM), or the sGC inhibitor, ODQ (10 μM), was administrated 30 min before application of acetylcholine. To determine the contribution of hydrogen peroxide to aortic relaxation in C101A/eNOS-KO, acetylcholine concentration–response curves were recorded in the absence and presence of either conventional (1200 U/ml) or pegylated catalase (1400 U/ml). To investigate a possible interaction between endothelium-dependent relaxation in C101A/eNOS-KO and increased aortic superoxide formation, we studied the acetylcholine concentration–response curve in aortic segments of wild type and C101A/eNOS-KO preincubated for 30 min with pegylated SOD (100 U/ml). In another experimental set, aortic rings of C101A/eNOS-KO and C57BL/6 were incubated with the colloidal $Fe(DETC)_2$ spin trap solution before application of the increasing acetylcholine concentrations. The spin trap was freshly prepared by mixing equal amounts of deoxygenated 1.6 mM $FeSO_4$ and 3.2 mM diethyldithiocarbamate (DETC) solutions (Noxigen, Elzach, Germany). In all experiments, only one acetylcholine concentration–response curve per aortic ring was recorded and analyzed.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Western blots for eNOS, nNOS, immunoprecipitated eNOS phosphorylated at Ser1176 (Ser 1179 in bovine eNOS), sGC-β1, nitrotyrosine residues, von Willebrand factor, and actin were performed in different mouse tissues using standard techniques, as described previously (25, 41) (please also refer to Supplementary Materials and Methods section).

Immunohistochemistry

Colocalization of eNOS and endothelium-specific marker, CD31, in aortas of C57BL/6, eNOS-KO, and C101A/eNOS-KO was performed as described previously (41) (please also refer to Supplementary Materials and Methods section).

Lucigenin superoxide detection

Lucigenin-enhanced chemiluminescence detection of superoxide in the intact aorta and left ventricular myocardium of C101A/eNOS-KO, eNOS-KO, and C57BL/6 was measured as described previously (23).

Measurement of systolic blood pressure

Systolic blood pressure and heart rate were measured in awake, 3–4-month-old male C57BL/6, eNOS-KO, and C101A/eNOS-KO using an automated tail-cuff system (Visitech Systems, Apex, NC), as described previously (25). In some experiments, establishment of basal blood pressure was followed by L-NA treatment with the drinking water, and blood pressure recordings were continued for another 21 days.

Neointimal hyperplasia

Ten-week-old C57BL/6, eNOS-KO, and C101A/eNOS-KO ($n = 9$–10) were anesthetized and the left common carotid artery was dissected and ligated near the carotid bifurcation, as described previously (26). All animals were sacrificed 28 days after ligation. After excision of the left and right common carotid arteries, the vessels were fixed in 4% p-formaldehyde for 6 h, dehydrated, and embedded in paraffin. Serial 5-μm sections were cut, and sections of every 50 μm were used for morphometric analysis using hematoxylin–eosin staining (vessels with thrombus formation were excluded). Morphometric analysis was carried out using image analysis software (Leica Microsystems, Wetzlar, Germany). The circumference of the lumen, elastic lamina interna, and elastic lamina externa were determined by tracing along the different vessel layers.

Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC Software, version 4.0) and are expressed as mean ± SEM of n individual samples. Statistical comparisons between groups were performed by t-tests, the Newman-Keuls multiple comparisons *post-hoc* test following one-way analysis of variance (ANOVA, for more than two groups) or two-way ANOVA (concentration–response curves). $p < 0.05$ was considered statistically significant.

Materials and reagents

All chemicals were purchased from Sigma or Merck (Darmstadt, Germany), except otherwise stated.

A more detailed description of the Materials and Methods section is provided in the online Supplementary Materials and Methods section.

Acknowledgments

This study was supported by the Forschungskommission of the Heinrich Heine University Düsseldorf (Project 9772 272 to G.K. and 9772 446 to T.S.) and by the DFG research grant, SU 783/1-1, to T.S.

Author Disclosure Statement

No competing financial interests exist.

References

1. This reference has been deleted.
2. Anea CB, Cheng B, Sharma S, Kumar S, Caldwell RW, Yao L, Ali MI, Merloiu AM, Stepp DW, Black SM, Fulton DJ, and Rudic RD. Increased superoxide and endothelial NO synthase uncoupling in blood vessels of Bmal1-knockout mice. *Circ Res* 111: 1157–1165, 2012.
3. Boger RH. Asymmetric dimethylarginine (ADMA): a novel risk marker in cardiovascular medicine and beyond. *Ann Med* 38: 126–136, 2006.
4. Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, and Rasmussen K. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med* 354: 1578–1588, 2006.
5. Chen PF, Tsai AL, and Wu KK. Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem Biophys Res Commun* 215: 1119–1129, 1995.
6. Cunnington C, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM, Antoniades C, Margaritis M, Lee R,

Cerrato R, Crabtree MJ, Francis JM, Sayeed R, Ratnatunga C, Pillai R, Choudhury RP, Neubauer S, and Channon KM. Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease. *Circulation* 125: 1356–1366, 2012.
7. Didion SP, Kinzenbaw DA, Schrader LI, Chu Y, and Faraci FM. Endogenous interleukin-10 inhibits angiotensin II-induced vascular dysfunction. *Hypertension* 54: 619–624, 2009.
8. Dikalov S and Fink B. ESR techniques for the detection of nitric oxide *in vivo* and in tissues. *Methods Enzymol* 396: 597–610, 2005.
9. Erwin PA, Lin AJ, Golan DE, and Michel T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 280: 19888–19894, 2005.
10. Forstermann U and Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 33: 829–837d, 2012.
11. Furchgott RF and Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373–376, 1980.
12. Gewaltig MT and Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc Res* 55: 250–260, 2002.
13. Gielis JF, Lin JY, Wingler K, Van Schil PE, Schmidt HH, and Moens AL. Pathogenetic role of eNOS uncoupling in cardiopulmonary disorders. *Free Radic Biol Med* 50: 765–776, 2011.
14. Gorren AC and Mayer B. Nitric-oxide synthase: a cytochrome P450 family foster child. *Biochim Biophys Acta* 1770: 432–445, 2007.
15. Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, and Malinski T. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension* 26: 854–857, 1995.
16. Harmon KJ, Couper LL, and Lindner V. Strain-dependent vascular remodeling phenotypes in inbred mice. *Am J Pathol* 156: 1741–1748, 2000.
17. Harrison DG, Chen W, Dikalov S, and Li L. Regulation of endothelial cell tetrahydrobiopterin pathophysiological and therapeutic implications. *Adv Pharmacol* 60: 107–132, 2010.
18. Harrison DG and Gongora MC. Oxidative stress and hypertension. *Med Clin North Am* 93: 621–635, 2009.
18a. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 360: 23–33, 2002.
19. Heinzel B, John M, Klatt P, Böhme E, and Mayer B. Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem J* 281: 627–630, 1992.
20. Hui DY. Intimal hyperplasia in murine models. *Curr Drug Targets* 9: 251–260, 2008.
21. Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, and Brandes RP. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* 109: 1795–1801, 2004.
22. Kang DH and Kang SW. Targeting cellular antioxidant enzymes for treating atherosclerotic vascular disease. *Biomol Ther (Seoul)* 21: 89–96, 2013.
23. Kojda G, Hacker A, and Noack E. Effects of non-intermittent treatment of rabbits with pentaerythritol tetranitrate on vascular reactivity and vascular superoxide production. *Eur J Pharmacol* 355: 23–31, 1998.
24. Kojda G and Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res* 67: 187–197, 2005.
25. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, and Harrison DG. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res* 42: 206–213, 1999.
26. Kumar A and Lindner V. Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol* 17: 2238–2244, 1997.
27. Kuzkaya N, Weissmann N, Harrison DG, and Dikalov S. Interactions of peroxynitrite with uric acid in the presence of ascorbate and thiols: implications for uncoupling endothelial nitric oxide synthase. *Biochem Pharmacol* 70: 343–354, 2005.
28. Langille BL and O'Donnell F. Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. *Science* 231: 405–407, 1986.
29. Lassegue B, San MA, and Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* 110: 1364–1390, 2012.
30. Lauer N, Suvorava T, Rüther U, Jacob R, Meyer A, Harrison DG, and Kojda G. Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase. *Cardiovasc Res* 65: 254–262, 2005.
31. Laufs U, La Fata V, Plutzky J, and Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 97: 1129–1135, 1998.
32. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, and Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* 95: 588–593, 1997.
33. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, and Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 103: 1282–1288, 2001.
34. Lee MY and Griendling KK. Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal* 10: 1045–1059, 2008.
35. Li H, Horke S, and Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis* 237: 208–219, 2014.
36. Libby P, Ridker PM, and Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 473: 317–325, 2011.
37. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, and Genest J, Jr. Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 354: 1567–1577, 2006.
38. Maier W, Cosentino F, Lutolf RB, Fleisch M, Seiler C, Hess OM, Meier B, and Luscher TF. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *J Cardiovasc Pharmacol* 35: 173–178, 2000.
39. Napoli C, De Nigris F, Williams-Ignarro S, Pignalosa O, Sica V, and Ignarro LJ. Nitric oxide and atherosclerosis: an update. *Nitric Oxide* 15: 265–279, 2006.
40. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, and Yokoyama M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest* 102: 2061–2071, 1998.
41. Oppermann M, Suvorava T, Freudenberger T, Dao VT, Fischer JW, Weber M, and Kojda G. Regulation of vascular

guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification. *Basic Res Cardiol* 106: 539–549, 2011.
42. Ponnuswamy P, Schrottle A, Ostermeier E, Gruner S, Huang PL, Ertl G, Hoffmann U, Nieswandt B, and Kuhlencordt PJ. eNOS protects from atherosclerosis despite relevant superoxide production by the enzyme in apoE mice. *PLoS One* 7: e30193, 2012.
43. Radermacher KA, Wingler K, Langhauser F, Altenhofer S, Kleikers P, Hermans JJ, Hrabe de AM, Kleinschnitz C, and Schmidt HH. Neuroprotection after stroke by targeting NOX4 as a source of oxidative stress. *Antioxid Redox Signal* 18: 1418–1427, 2013.
44. Raman CS, Li H, Martasek P, Kral V, Masters BS, and Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 95: 939–950, 1998.
45. Ravi K, Brennan LA, Levic S, Ross PA, and Black SM. S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci U S A* 101: 2619–2624, 2004.
46. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 340: 115–126, 1999.
47. Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, and Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 101: 731–736, 1998.
48. Satoh K, Nigro P, and Berk BC. Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A. *Antioxid Redox Signal* 12: 675–682, 2010.
49. Schmidt K, Andrew P, Schrammel A, Groschner K, Schmitz V, Kojda G, and Mayer B. Comparison of neuronal and endothelial isoforms of nitric oxide synthase in stably transfected HEK 293 cells. *Am J Physiol Heart Circ Physiol* 281: H2053–H2061, 2001.
50. Sesso HD, Buring JE, Christen WG, Kurth T, Belanger C, MacFadyen J, Bubes V, Manson JE, Glynn RJ, and Gaziano JM. Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA* 300: 2123–2133, 2008.
51. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, and Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 93: 13176–13181, 1996.
52. Sugamura K and Keaney JF, Jr. Reactive oxygen species in cardiovascular disease. *Free Radic Biol Med* 51: 978–992, 2011.
53. Suvorava T, Lauer N, and Kojda G. Physical inactivity causes endothelial dysfunction in healthy young mice. *J Am Coll Cardiol* 44: 1320–1327, 2004.
54. Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, and Kojda G. Endogenous vascular hydrogen peroxide regulates arteriolar tension *in vivo*. *Circulation* 112: 2487–2495, 2005.
55. Suvorava T, Stegbauer J, Thieme M, Pick S, Friedrich S, Rump LC, Hohlfeld T, and Kojda G. Sustained hypertension despite endothelial-specific eNOS rescue in eNOS-deficient mice. *Biochem Biophys Res Commun* 458: 576–583, 2015.
56. Tabima DM, Frizzell S, and Gladwin MT. Reactive oxygen and nitrogen species in pulmonary hypertension. *Free Radic Biol Med* 52: 1970–1986, 2012.
57. Vásquez-Vivar J, Kalyanaraman B, Martásek P, Hogg N, Masters BSS, Karoui H, Tordo P, and Pritchard KA, Jr. Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc Natl Acad Sci U S A* 95: 9220–9225, 1998.
58. Xia Y, Tsai AL, Berka V, and Zweier JL. Superoxide generation from endothelial nitric-oxide synthase—a Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 273: 25804–25808, 1998.
59. Yogo K, Shimokawa H, Funakoshi H, Kandabashi T, Miyata K, Okamoto S, Egashira K, Huang P, Akaike T, and Takeshita A. Different vasculoprotective roles of NO synthase isoforms in vascular lesion formation in mice. *Arterioscler Thromb Vasc Biol* 20: E96–E100, 2000.
60. Yusuf S, Dagenais G, Pogue J, Bosch J, and Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 342: 154–160, 2000.
61. Zou MH, Shi C, and Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 109: 817–826, 2002.

Address correspondence to:
Dr. Georg Kojda
Institute for Pharmacology and Clinical Pharmacology
Heinrich-Heine-University
Universitätsstr. 1
Düsseldorf 40225
Germany

E-mail: kojda@uni-duesseldorf.de

Date of first submission to ARS Central, July 28, 2014; date of final revised submission, March 3, 2015; date of acceptance, March 11, 2015.

Abbreviations Used

ANOVA = analysis of variance
BH_4 = (6R-)5,6,7,8-tetrahydro-L-biopterin
C101A-eNOS = bovine eNOS destabilized by replacement of Cys 101 to Ala
C101A/eNOS-KO = double transgenic mice expressing destabilized C101A-eNOS exclusively in the endothelium
C101A-Tg = mice with endothelium-specific overexpression of destabilized C101A-eNOS
DEA/NO = diethylamine/nitric oxide
DETC = diethyldithiocarbamate
eNOS = endothelial NO synthase
eNOS-KO = eNOS-deficient mice
ESR = electron spin resonance
HEK 293 = human embryonic kidney 293
L-NA = N^{ω}-nitro-L-arginine
L-NAME = NG-nitro-L-arginine methyl ester
ODQ = 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sGC = soluble guanylyl cyclase
SNAP = S-nitroso-N-acetyl-D,L-penicillamine
SOD = superoxide dismutase
WT-eNOS = wild-type bovine eNOS

Basic Res Cardiol
DOI 10.1007/s00395-011-0160-5

ORIGINAL CONTRIBUTION

Regulation of vascular guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification

Marc Oppermann · Tatsiana Suvorava · Till Freudenberger · Vu Thao-Vi Dao · Jens W. Fischer · Martina Weber · Georg Kojda

Received: 3 September 2010 / Revised: 14 January 2011 / Accepted: 27 January 2011

Abstract In isolated cells, soluble guanylyl cyclase (sGC) activity is regulated by exogenous nitric oxide (NO) via downregulation of expression and posttranslational *S*-nitrosylation. The aim of this study was to investigate whether such regulatory mechanism impact on endothelium-dependent vasodilation in a newly developed mouse strain carrying an endothelial-specific overexpression of eNOS ($eNOS^{++}$). When compared with transgene negative controls ($eNOS^{n}$), $eNOS^{++}$-mice showed a 3.3-fold higher endothelial-specific aortic eNOS expression, increased vascular cGMP and VASP phosphorylation, a L-nitroarginine (L-NA)-inhibitable decrease in systolic blood pressure, but normal levels of peroxynitrite and nitrotyrosine formation, endothelium-dependent aortic vasodilation and vasodilation to NO donors. Western blot analysis for sGC showed similar protein levels of sGC-α1 and sGC-β1 subunits in $eNOS^{n}$ and $eNOS^{++}$. In striking contrast, the activity of isolated sGC was strongly decreased in lungs of $eNOS^{++}$. Semiquantitative evaluation of sGC-β1-*S*-nitrosylation demonstrated that this loss of sGC activity is associated with increased nitrosylation of the enzyme in $eNOS^{++}$, a difference that disappeared after L-NA-treatment. Our data suggest the existence of a physiologic NO-dependent posttranslational regulation of vascular sGC in mammals involving *S*-nitrosylation as a key mechanism. Because this mechanism can compensate for reduction in vascular NO bioavailability, it may mask the development of endothelial dysfunction.

Keywords Nitric oxide · Endothelial nitric oxide synthase · Soluble guanylyl cyclase · *S*-Nitrosylation · Endothelial dysfunction

M. Oppermann and T. Suvorava contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00395-011-0160-5) contains supplementary material, which is available to authorized users.

M. Oppermann · T. Suvorava · T. Freudenberger · V. T. Dao · J. W. Fischer · G. Kojda (✉)
Institute for Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Moorenstr. 5, 40225 Düsseldorf, Germany
e-mail: kojda@uni-duesseldorf.de

M. Weber
Division of Cardiology, Emory University School of Medicine, Atlanta, GA, USA

Nitric oxide (NO) is involved in physiological processes, such as smooth muscle relaxation, neurotransmission, platelet aggregation, host defense mechanisms and apoptosis and has antioxidative effects [11, 28]. The effects of NO greatly contribute to the physiologic vascular functions and likely protect the vascular wall from vasotoxic compounds, such as reactive oxygen species [11, 19]. In the vasculature, the majority of NO effects are mediated by activation of soluble guanylyl cyclase (sGC), generation of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG) and phosphorylation of various cellular proteins regulating calcium homeostasis [15]. The sGC enzyme is composed of two subunits, α and β, and a prosthetic heme group. The majority of vascular sGC is formed by the subunits α1 (sGC-α1) and β1 (sGC-β1) [5, 35]. Studies with NO donors in cultured rat aortic smooth muscle cells have provided evidence for a NO-dependent downregulation of sGC protein expression suggesting a negative feedback loop, where NO acts as a signaling molecule regulating sGC expression [9]. In another approach, rat pulmonary artery smooth muscle cells

Published online: 06 February 2011

responded to treatment with lipopolysaccharide, which is known to induce the expression of inducible nitric oxide synthase with a downregulation of sGC mRNA levels [39]. Likewise, transfection of HEK cells with an endothelial nitric oxide synthase (eNOS) plasmid and incubation with NO donors reduced sGC expression [37, 42].

In contrast, a strain of mice strongly overexpressing vascular eNOS driven by the prepro-endothelin promoter showed partial resistance to endothelium-dependent and NO-induced vasodilatation, but no decrease in sGC expression [43]. Instead, the authors described a 50% reduction of basal unstimulated sGC activity and a 20% reduction of PKG expression. Other groups reported that changes in PKG activity do not occur in eNOS knockout mice, but showed that there is no change of sGC protein expression in this animal model [4, 12, 14]. Inhibition of NOS by chronic treatment of mice with the NOS-inhibitor L-NAME had no effect on vascular sGC expression as well, but potentiated the aortic cGMP response to the NO-donor sodium nitroprusside [30]. In a recent investigation with increasing doses of different organic nitrates, we failed to detect a NO-dependent feedback loop controlling sGC expression in vivo [32].

Considering these contrasting data, we aimed to investigate the regulation of sGC activity by endothelial NO in vivo. To accomplish this, we generated a new transgenic mouse strain characterized by moderate endothelial-specific overexpression of eNOS and studied the expression and activity of vascular sGC under various conditions. Our data suggest the existence of a physiologic NO-dependent posttranslational regulation of vascular sGC involving *S*-nitrosylation as a key mechanism.

Methods

Materials and reagents

All chemicals were purchased from Sigma (Munich, Germany) or Merck (Darmstadt, Germany), except otherwise stated in this section or in the Supplemental Materials and Methods.

Transgenic eNOS^{++} mice

We generated a DNA construct, in which bovine eNOS cDNA (4.1 kb) was inserted between the murine Tie-2 promoter (2.1 kb) cDNA and a 10 kb Tie-2 intron fragment, designated as Tie-2-enhancer and this construct was used to target eNOS gene expression to the vasculature as described previously [25]. For detailed methods, please refer to Supplemental Materials and Methods.

Founder mice showing high eNOS expression as compared to controls were crossed ten times to C57BL/6 mice to generate a C57BL/6 background. Three different colonies were established and the strain with the highest overexpression was used in all further experiments (eNOS^{++}). Male mice were used at 12–16 weeks of age. Transgene negative littermates (eNOS^{n}) served as controls. In some experiments, C57Bl/6 mice served as transgenic-negative controls. Additional groups of mice received N$^{\omega}$-nitro-L-arginine (L-NA, Sigma, Germany) (100 mg L-NA/kg BW/day) for 3 weeks before the experiments. Permission for the animal studies was provided by the regional Government of Germany (AZ 23.05-230-3-77/99, AZ 23.05-230-3-52/99, AZ 50.05-230-3-65/99, AZ 50.05-230-3-94/00 AZ 50.05-230-18/06), and the experiments were performed according to the guidelines for the use of experimental animals, as given by the German "Tierschutzgesetz" and the "Guide for the Care and Use of Laboratory Animals" of the US National Institutes of Health.

Electron spin resonance measurements

Electron spin resonance measurements were performed together with Noxygen GmbH, Elzach, Germany, as described previously [7, 24]. Spintrap was $Fe^{2+}(DETC)_2$ (iron/diethyldithiocarbamic acid) at 0.2 mmol/L for NO and CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine) at 100 µmol/L for superoxide. Urate (100 µmol/L) was used to quench peroxynitrite signals detected by CPH.

Measurement of blood pressure

Systolic blood pressure and heart rate were measured in awake 3 to 4-month-old male eNOS^{++} ($n = 4$) and eNOS^{n} ($n = 4$) mice using an automated tailcuff system (Visitech Systems, Apex, NC, USA) as described previously [22]. After 7 days of starting the measurement mice received L-NA with the drinking water and blood pressure recordings were continued for 36 days.

Vasorelaxation studies

Mice were killed by inhalation of carbon dioxide, and tissues were used for organ bath studies (aorta) or immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting. Function of the endothelium was examined by cumulative addition of acetylcholine (ACh, 0.01–10 µmol/L) after submaximal precontraction with 0.2 µmol/L phenylephrine as described previously [40]. Thereafter, vasorelaxation to the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 1 nmol/L to 10 µmol/L), or diethylamine/nitric oxide

(DEA/NO) was studied by cumulative application after precontraction with phenylephrine (0.2 μmol/L) in both endothelium intact and endothelium denuded thoracic rings.

Immunohistochemistry and Western blot

Immunohistochemistry was performed in mouse carotid arteries to characterize localization of overexpressed eNOS. Western blots were performed in mouse tissues for eNOS, both sGC subunits, vasodilator-stimulated phosphoprotein (VASP) phosphorylation, and protein nitrotyrosines (please refer to Supplemental Materials and Methods).

Determination of sGC activity

Specific activity of sGC was measured in aliquots of the 100,000×*g* supernatants of mouse lungs by formation of [α-^{32}P]-cGMP from [α-^{32}P]-GTP (guanosine triphosphate) after stimulation with increasing doses of SNAP as described previously [21, 38]. Tissue cGMP levels were measured by enzyme immunoassay (Cayman, Ann Arbor, MI, US) in mouse lung according to the manufacturer's protocol.

Determination of *S*-nitrosylation of sGC-β1

S-Nitrosylation of β1-subunit of sGC was evaluated semiquantitavely in lung cytosols of transgenic and L-NA treated mice as described previously (please refer to Supplemental Materials and Methods) [16, 17].

Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC software, version 3.0) and are expressed as mean ± SEM of n individual samples. Statistical comparisons between groups were performed by *t* tests, Newman–Keuls multiple comparisons post hoc test following one-way analysis of variance (ANOVA, for more than two groups) or two-way ANOVA (concentration–response curves). $P < 0.05$ was considered statistically significant.

Results

Characterisation of eNOS^{++} mice

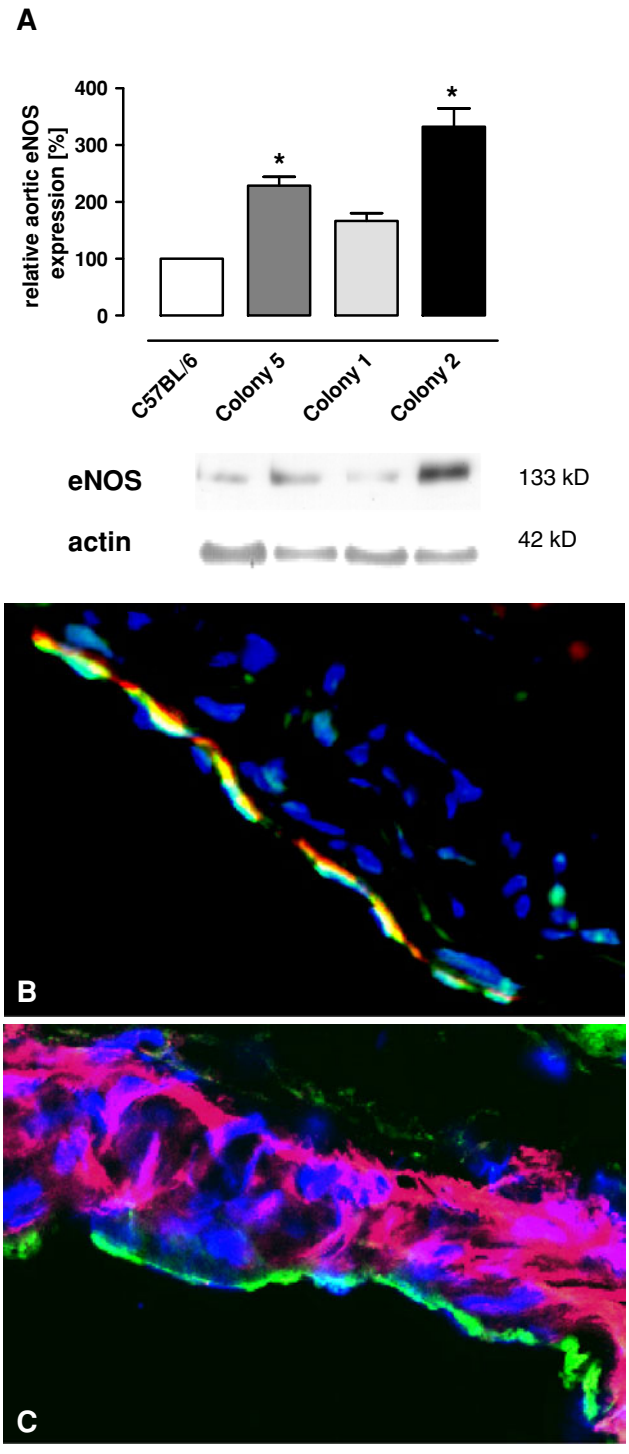
To investigate the regulation of sGC by NO in vivo, we established a new mouse model carrying a moderate endothelial-specific overexpression of eNOS. As shown in Fig. 1a, the level of overexpression as standardized to actin varied among different colonies of eNOS^{++} mice. The expression values were 166 ± 13.7% colony 1 ($P > 0.05$ vs. C57BL/6, $n = 5$, ANOVA, post hoc test), 332 ± 32.1% for colony 2 ($P < 0.001$, $n = 7$) and 229 ± 15.5% for colony 5 ($P < 0.01$, $n = 6$). Colony 2 was used for further experiments. In this colony, the eNOS Western blot signal was additionally standardized to von Willebrand factor which yielded a similar level of overexpression (please refer to Fig. 4, Supplementary Material).

Immunohistochemical detection of eNOS in vascular tissue of eNOS^{++}-mice confirmed the exclusive expression in the endothelium. The overlay in Fig. 1b reveals a yellow coloured area which indicates a co-localization of eNOS with the endothelial marker CD31. The green-stained eNOS is not expressed in tissues other than endothelium (Fig. 1c).

To determine whether the overexpression of eNOS was associated with increased eNOS activity in vivo we measured blood pressure in eNOS^{++} and eNOS^{n}. While the systolic blood pressure in eNOS^{n} was 118.1 ± 1.4 mmHg ($n = 4$), it was strongly reduced to 109.6 ± 2.0 mmHg in eNOS^{++}mice ($n = 4$, $P = 0.0126$, *t* test, Fig. 2a). The treatment with the NOS-inhibitor L-NA increased blood pressure in both eNOS^{n} and eNOS^{++} mice. The difference in blood pressure between the two groups rapidly decreased during the treatment and was finally abolished. On day 30 of L-NA treatment, eNOS^{n} mice had a blood pressure of 128.9 ± 6.8 mmHg and eNOS^{++} a blood pressure of 135.2 ± 3.9 mmHg ($n = 4$, $P = 0.1921$, *t* test, Fig. 2a). These data suggest that the decrease in blood pressure measured in eNOS^{++} mice is largely due to eNOS overexpression in these transgenic animals.

In eNOS^{++} mice, lung cGMP levels were increased to 33.7 ± 3.0 pmol/g ($n = 6$), when compared with eNOS^{n} (19.4 ± 3.5 pmol/g, $n = 5$, $P = 0.0126$, *t* test, Fig. 2b). Furthermore, relative serine-239 VASP phosphorylation was increased in eNOS^{++} to 165 ± 31.5% ($n = 4$, $P < 0.05$ ANOVA post hoc test, Fig. 2c). L-NA treated eNOS^{n} and eNOS^{++} mice showed a significantly decreased VASP phosphorylation of 31.6 ± 7.7 and 40.3 ± 23.0%, respectively ($n = 4$, $P < 0.05$ vs. untreated eNOS^{n}). Moreover, L-NA-treatment abolished the difference between the two groups (Fig. 2c, $P > 0.05$, ANOVA post hoc test).

Direct comparative assessment of NO levels in the aorta was accomplished by electron-spin resonance using $Fe^{2+}(DETC)_2$ spintrap. We found that the amount of NO more than doubled in eNOS^{++} as compared to eNOS^{n}, but this difference did not reach statistical significance (Table 1). Control experiments with the CPH spintrap demonstrated that in myocardial tissue neither superoxide, nor peroxynitrite concentration differed between the two



◀ **Fig. 1** eNOS overexpression in transgenic mice. **a** Protein expression of eNOS in transgenic eNOS^{++} mice (aorta, $*P < 0.05$ vs. controls) given as mean ± SEM (*upper panel*) and representative Western blot signals (*lower panel*). Colony 2 showed the highest overexpression and was used for experiments. In this colony, the eNOS Western blot signal was additionally standardized to von Willebrand factor which yielded a similar level of overexpression (please refer to Fig. 4 of the online Supplemental Materials). **b** Confocal image of a carotid artery of an eNOS^{++} mouse stained for eNOS (*green*) and CD31 (*red*) and **c** stained for eNOS (*green*) and α-SM-actin (*magenta*). Nuclei are stained with DAPI (*blue*). Original magnification in **b** ×100, original magnification in **c** ×400

strains. Similar results were obtained in aortic and lung tissues.

ESR findings on peroxynitrite were confirmed with Western blots of heart tissue using an antibody against nitrotyrosine residues, a surrogate marker for tissue peroxinitrite levels. This antibody showed three bands at around 50 kDa which were evaluated densitometrically. There was no significant difference between eNOS^{n} (100%) and eNOS^{++} mice (121.2 ± 20.6%, $n = 6$, $P = 0.3498$, t test, Fig. 2d).

Expression and activity of sGC in eNOS^{++} mice

The expression levels of sGC-α1 and sGC-β1 in lung cytosolic fractions of eNOS^{++} were identical to those measured in eNOS^{n}, i.e. in eNOS^{++} the relative expression of sGC-α1 was 103.4 ± 10.3% ($n = 11$) and that of sGC-β1 was 109.7 ± 28.6% ($n = 9$, $P = 0.2700$ for both, ANOVA, Fig. 3a).

The sGC activity (in pmol cGMP/mg protein/min) of mouse lung cytosols showed a strong decrease in maximal activity in eNOS^{++} ($P = 0.0007$, two-way ANOVA, Fig. 3b). In contrast, the sensitivity of sGC to stimulation by the NO-donor SNAP was unchanged as indicated by similar pD_2 values in eNOS^{n} (3.144 ± 0.241) and eNOS^{++} (3.223 ± 0.488, $P = 0.9170$, t test). The treatment of both mouse strains with L-NA revealed that inhibition of eNOS completely abolished the difference between eNOS^{n} and eNOS^{++} suggesting that sGC activity in vivo is indeed dependent on the bioavailability of endothelial NO. Moreover, L-NA treatment increased sGC activity in both strains (Fig. 3c) demonstrating that endothelial NO regulates sGC activity in normal C57Bl/6 mice as well, i.e. this regulation likely occurs on a physiologic level. These data suggest that endogenous NO formation does not decrease sGC expression, but greatly reduces its response to NO. Thus, in vivo regulation of sGC by NO appears to involve posttranslational protein modifications rather than variation of expression.

S-Nitrosylation of sGC-β1

To evaluate a posttranslational modification of sGC which is associated with a decreased activity, we investigated the magnitude of *S*-nitrosylation of sGC-β1. In lung tissue of eNOS^{++} increased *S*-nitrosylation of sGC-β1 was evident (147.7 ± 12.0%, $n = 6$, $P = 0.0106$, t test, Fig. 4a) when compared with eNOS^{n} mice (set to 100%). Additional experiments showed that treatment of both groups with L-NA for 3 weeks decreased this difference to a relative

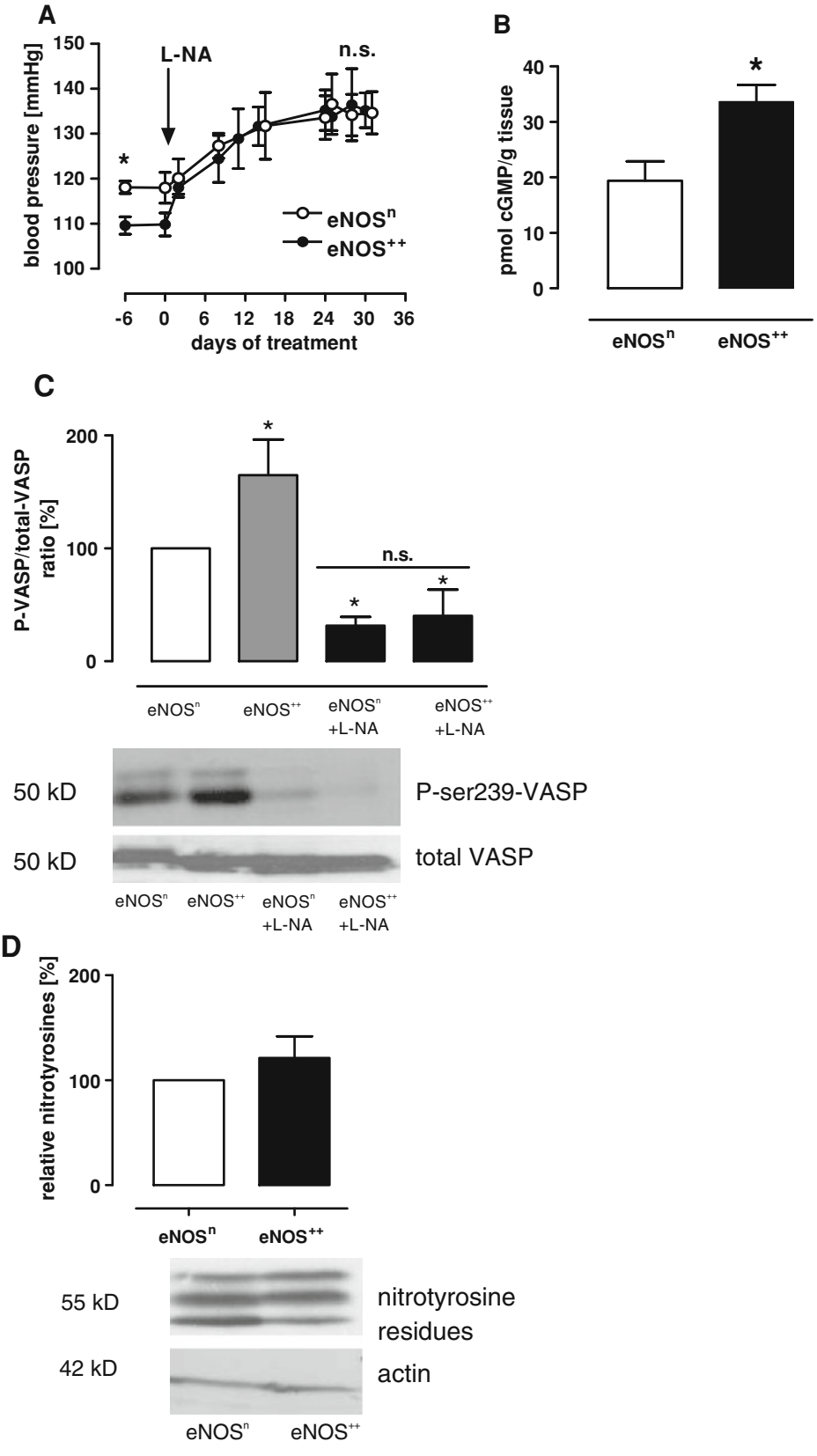


Fig. 2 Characterization of eNOS-overexpressing mice ($eNOS^{++}$). **a** Systolic blood pressure in awake $eNOS^{++}$ and their transgene negative littermates ($eNOS^{n}$) before and during treatment with the NO-synthase inhibitor L-NA ($n = 4$, $P < 0.05$). **b** cGMP levels in lung tissue of $eNOS^{n}$ and $eNOS^{++}$ ($^{*}P < 0.05$). **c** Western blot analysis of Ser^{239}-VASP-phosphorylation. A significantly increased phosphorylation in $eNOS^{++}$-mice ($^{*}P < 0.05$ vs. $eNOS^{n}$) disappeared after treatment with L-NA (n.s.). **d** Western blots for nitrotyrosine residues in heart homogenates of transgenic and control mice showed no difference between the groups ($P > 0.05$, as for full scale blots, please refer to Fig. 2 of the online Supplemental Materials)

Table 1 ROS production in (pmol/mg protein/min) measured by ESR with $Fe^{2+}(DETC)_2$ (nitric oxide), CPH (superoxide), CPH $^{+}$ urate (peroxynitrite), $n = 4$–6

ROS	$eNOS^{n}$	$eNOS^{++}$	*P* value
Aorta			
Nitric oxide	19.1 ± 8.7	49.4 ± 19.7	0.1898
Superoxide	22.7 ± 9.3	20.3 ± 5.7	0.8371
Peroxynitrite	52.6 ± 16.1	22.8 ± 7.0	0.1185
Lung			
Superoxide	21.9 ± 5.6	19.9 ± 5.8	0.8137
Peroxynitrite	27.5 ± 5.6	28.3 ± 4.2	0.9127
Heart			
Superoxide	79.3 ± 40.4	53.0 ± 22.2	0.5884
Peroxynitrite	114.0 ± 47.9	39.8 ± 18.9	0.2627

sGC-β1 *S*-nitrosylation of 128.6 ± 32.2% in $eNOS^{++}$ ($n = 4$, $P = 0.4400$, *t* test). Furthermore, control experiments in L-NA-treated and untreated C57Bl/6 mice showed that this treatment decreases *S*-nitrosylation of sGC-β1 as well (Fig. 4b). Taken together, these data suggest that sGC is *S*-nitrosylated in a physiologic manner and that this posttranslational modification is associated with decreased sGC activity (Fig. 3b, c).

Functional activity of the vascular NO/cGMP pathway

Despite the reduction in sGC activity combined with increased *S*-nitrosylation in $eNOS^{++}$, endothelium-dependent vasodilation induced by increasing concentrations of acetylcholine in aortic rings of $eNOS^{++}$ mice was unchanged as compared to $eNOS^{n}$ (Fig. 5a). Furthermore, the overexpression of eNOS did not lead to a significant change of the relaxation response to the NO-donor SNAP as indicated by identical pD_2 values in $eNOS^{++}$ (6.66 ± 0.12) and $eNOS^{n}$ (6.76 ± 0.07, $n = 5$, $P = 0.5202$, Fig. 5b). Denudation of the endothelium led to a significant leftward shift of the SNAP response in both $eNOS^{n}$ and $eNOS^{++}$ mice (Fig. 5c, d). The corresponding pD_2 value for denuded $eNOS^{n}$ aorta was increased to 7.12 ± 0.08 ($n = 5$, $P = 0.0097$ vs. intact $eNOS^{n}$ aorta, *t* test, Fig. 5c), and that for $eNOS^{++}$ was increased to 7.23 ± 0.08 ($n = 5$, $P = 0.0026$ vs. intact $eNOS^{++}$ aorta, *t* test, Fig. 5d). However, there was no difference to SNAP responses between the strains after denudation ($P = 0.1667$ for pD_2 values of $eNOS^{n}$ vs. $eNOS^{++}$, *t* test). Similarly, pD_2 values obtained with the NO donor DEA/NO were the same in $eNOS^{n}$ ($pD_2 = 7.475 \pm 0.029$, $n = 5$) and $eNOS^{++}$ (7.432 ± 0.043, $n = 6$, $P = 0.4480$). In addition, endothelial denudation leads to a significant leftward shift in both the groups (Fig. 5e, f). Likewise, there was no significant difference between pD_2 values in endothelium denuded rings of both strains (7.944 ± 0.038, $n = 5$ for $eNOS^{n}$, 7.920 ± 0.061, $n = 6$ for $eNOS^{++}$, $P = 0.7571$).

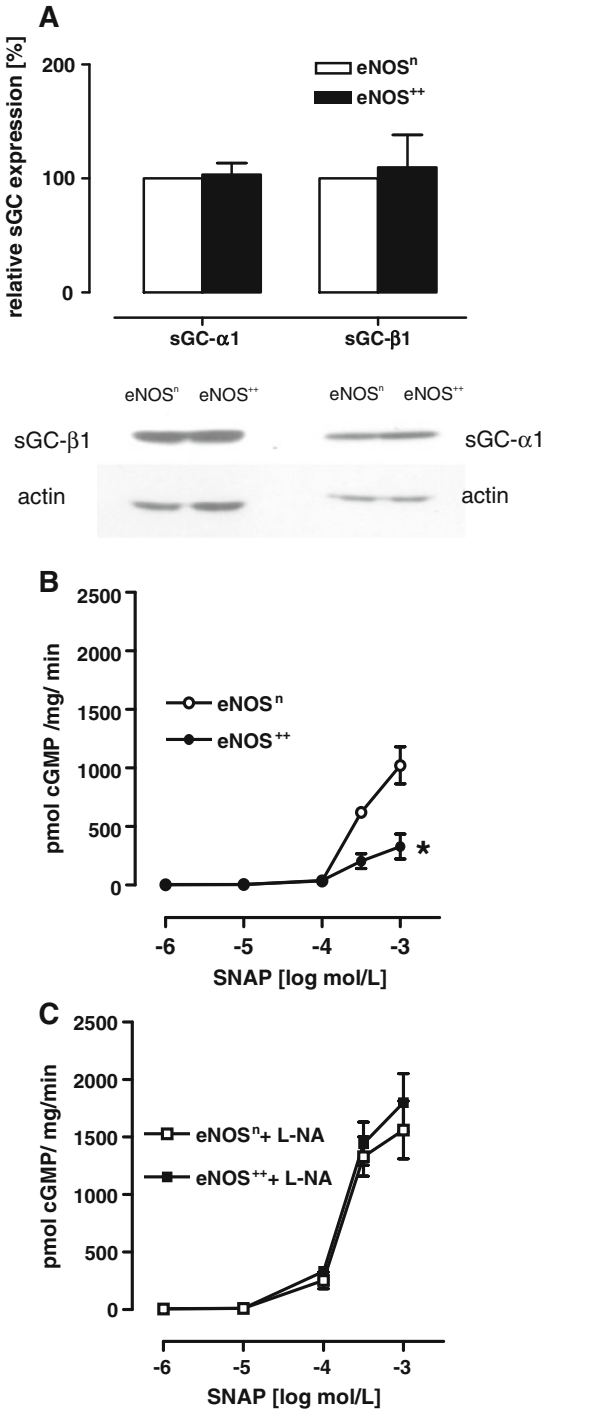


Fig. 3 Effect of eNOS overexpression in mice ($eNOS^{++}$) on sGC. **a** Protein expression of sGC-α1 and sGC-β1 (*upper panel*) standardized to actin (*lower panel*) in $eNOS^{++}$e when compared with controls ($eNOS^{n}$). **b** Decreased activity of sGC protein in $eNOS^{++}$ when compared with $eNOS^{n}$ (*$P = 0.0027$ vs. $eNOS^{n}$). **c** The activity of sGC protein in $eNOS^{++}$ when compared with $eNOS^{n}$ after 3 weeks of treatment with the NOS-inhibitor L-NA

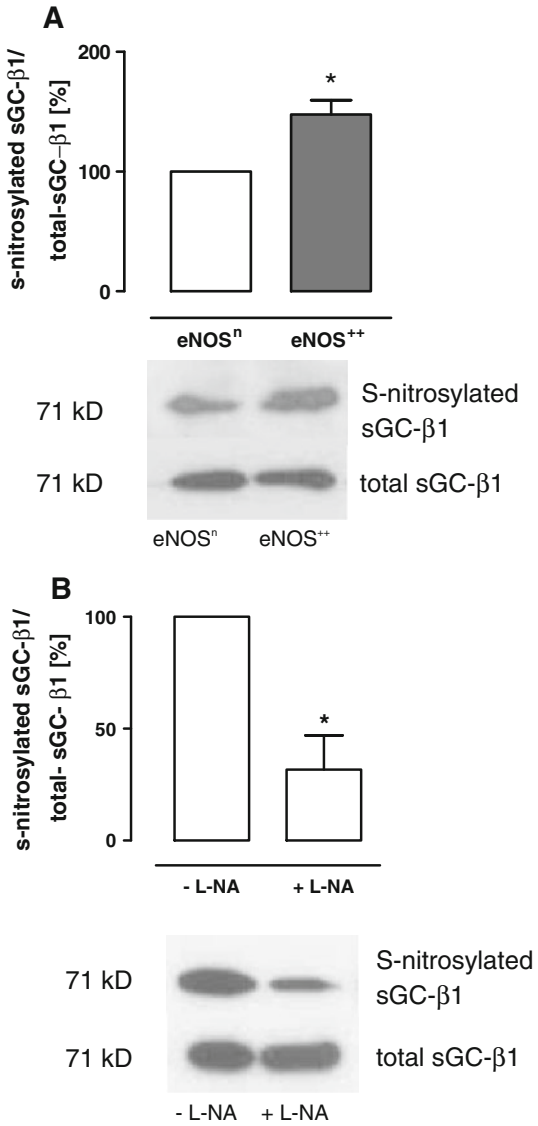


Fig. 4 **a** *S*-Nitrosylation of sGC-β1 in lung cytosols of transgenic eNOS^{++} mice as compared to transgene-negative littermates given as mean ± SEM (*upper panel*) and representative Western blot signals (*lower panel* $n = 6$, *: $P < 0.05$, *t* test). **b** *S*-Nitrosylation of sGC-β1 in lung cytosols of normal C57Bl/6 mice with (+L-NA) and without treatment with L-NA (-L-NA) given as mean ± SEM (*upper panel*) and representative Western blot signals (*lower panel*, $n = 5$, $^*P = 0.0111$, *t* test). As for full-scale Western blots, please refer to Fig. 3 of the Online Supplemental Materials

Discussion

The aim of this study was to determine the influence of endogenous NO on the expression and function of vascular sGC in vivo. Our major new finding is that endothelial NO bioavailability triggers *S*-nitrosylation of its key receptor sGC and thereby negatively regulates sGC activity in a reversible manner in vivo. Despite significant changes in aortic NO bioavailability, the functional efficacy of the vascular NO/cGMP pathway appeared to be maintained over a considerable range of vascular NO levels. Our data suggest that *S*-nitrosylation by endothelial NO regulates vascular sGC activity in vivo. This posttranslational regulation can compensate for a loss of vascular NO bioavailability and may mask the development of endothelial dysfunction.

We generated a transgenic animal model to investigate long-term effects of moderately increased vascular NO bioavailability on vascular sGC expression. This new transgenic mouse strain showed a 3.3-fold increase in vascular eNOS expression. The overexpressed eNOS protein was functionally active as indicated by a significant reduction in blood pressure which was completely inhibited by treatment with the NOS-inhibitor L-NA. Further analysis confirmed that the Tie-2 promoter provided an endothelial-specific transgene expression. Moreover, we found no evidence for a resistance to vasodilator effects of endothelial NO and NO donors. Likewise, there was no evidence of nitrosative stress as vascular levels of superoxide and peroxynitrite, as well as the abundance of protein nitrotyrosine residues were identical to the transgene negative littermates. Thus, this new transgenic strain shows several important differences to the previously published mouse strain [31]. These mice showed a pronounced resistance to the vasodilator effects of endothelial NO and NO donors [43] suggesting a rather pathological increase of vascular NO bioavailability presumably associated with a considerable increase of vascular nitrosative stress, e.g. resulting in protein tyrosine nitration. In accordance, a later study showed accelerated atherosclerosis in a double transgenic strain generated by crossing the eNOS strain with apoE knockouts demonstrating the pathophysiologic importance of nitrosative stress [33].

In this study, we found evidence for the existence of a regulatory mechanism in eNOS^{++} which limits the efficacy of vascular cGMP generation upon activation with NO. Although intact rings of thoracic aorta of eNOS^{++} showed no impairment of the vasodilator efficacy of the NO donors SNAP and DEA/NO, we found a strong potentiation after endothelial denudation. Evaluation of sGC activity in lung cytosolic fractions showed an approximately 50% reduction in the maximal activity of sGC in eNOS^{++} as compared to eNOS^{n}. To further substantiate a causal role for eNOS overexpression, we treated eNOS^{++} and eNOS^{n} with L-NA for 3 weeks and found that this completely abolished the desensitization of sGC in eNOS^{++}. In addition, L-NA-treatment potentiated sGC activity in eNOS^{n} as well suggesting that endothelial-specific overexpression of eNOS potentiated a desensitization mechanism of sGC which is also present in normal mice. Of note, these experiments also demonstrate that the physiologically occurring sGC desensitization is reversible. In striking contrast, the expression of both subunits of sGC

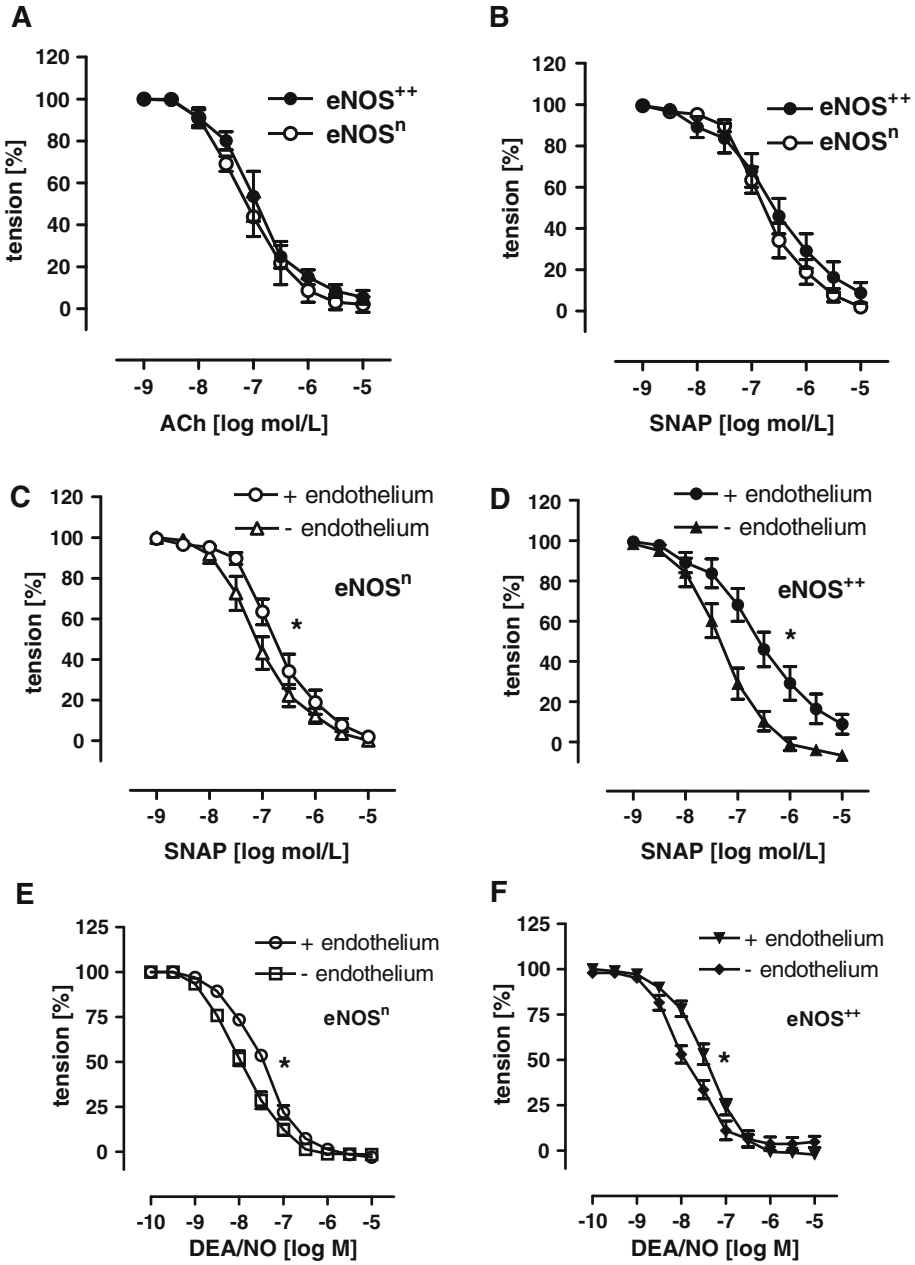


Fig. 5 Relaxation response of aortic ring segments of NOS^{++} and $eNOS^{n}$ induced by **a** cumulative addition of acetylcholine (ACh, $n = 5$, $P = 0.0991$, two-way ANOVA) and **b** SNAP ($n = 5$, $P = 0.1859$, two-way ANOVA). **c**, **d** Relaxation response of endothelium intact and denuded aortic ring segments of NOS^{++} and $eNOS^{n}$ to cumulative addition of SNAP (*$P<0.05$ for pD_2 and two-way ANOVA). **e**, **f** Relaxation response of endothelium intact and denuded aortic ring segments of NOS^{++} and $eNOS^{n}$ to cumulative addition of DEA/NO (*$P < 0.05$ for pD_2 and two-way ANOVA)

was not different between $eNOS^{++}$ and $eNOS^{n}$ which demonstrates that the downregulation of sGC activity in our experiments was completely dependent on a post-translational modification of the enzyme.

Several mechanisms of desensitization of sGC are described. A rapid desensitization has been reported in intact astrocytes and platelets [3], but this desensitization was lost when the cells were lysed. A similar form of desensitization might be induced by normal continuous endothelial NO generation, but this form is associated with a reduction in NO-dependent vasodilation [29]. Recently, a new mechanism of sGC desensitization was described which occurred following the stimulation of smooth muscle cells with nitrosocysteine [36], a compound which releases NO, stimulates sGC and induces vasodilation [2, 34]. It was found that *S*-nitrosylation of cysteine-234 of the sGC-α1 subunit and cysteine-122 of sGC-β1 strongly correlated with decreased sGC activity. *S*-Nitrosylation of sGC was also observed after treatment of endothelial cells with vascular endothelial growth factor suggesting physiologic relevance in endothelial cells. The authors suggested that a change in the conformation of the heme-binding histidine-105 may impair binding of NO and subsequent activation of the enzyme. Furthermore, a recent study by another group showed that a stoichiometric nitrosation in fact results in complete deactivation of the purified enzyme

[26]. Thus, we investigated whether *S*-nitrosylation might account for the observed reversible downregulation of sGC activity in $eNOS^{++}$.

Investigation of *S*-nitrosylation of sGC was performed using the biotin-switch assay in two different experimental approaches. The comparison between $eNOS^{n}$ and $eNOS^{++}$ demonstrated a statistically significant upregulation of sGC *S*-nitrosylation in $eNOS^{++}$. In addition, this difference disappeared after the treatment of mice with the NOS inhibitor L-NA. Of note, the treatment of normal C57Bl/6 mice with L-NA profoundly reduced sGC nitrosylation as well. Although our data are limited due to the semiquantitative approach, they demonstrate a correlation between sGC *S*-nitrosylation and sGC activity in all experimental procedures performed in this investigation and strongly suggest that *S*-nitrosylation of sGC is a physiologic post-translational modification which regulates sGC activity in an NO-dependent manner. In striking contrast, downregulation of sGC expression, which can be demonstrated by treating isolated cells or arteries with high concentrations of NO [9], is most likely unimportant in vivo.

Recently, Zhao et al. [44] reported another inactivation mechanism of the NO-cGMP pathway in caveolin-1 deficient mice which operates at increased levels of endothelial NO only and induces pulmonary hypertension in 9-month old mice. In this study, increased nitration of pulmonary protein kinase G was identified as an important underlying mechanism. It is not known whether *S*-nitrosylation of sGC might contribute to increased pulmonary vascular resistance in caveolin-1-deficient mice. In addition, an increase in blood pressure has not been found consistently in caveolin-1-deficient mice suggesting that nitration of PKG in resistance arteries compromising the NO-cGMP pathway does not occur [6, 8].

Denudation of aortic rings of $eNOS^{++}$ mice slightly increased the maximal relaxation to SNAP, but not to DEA/NO. This finding might be related to a small difference between both NO donors [23]. SNAP is a nitrosothiol that does not release NO in solutions free of even trace amounts of heavy metals, such as copper or iron, while in vivo (or in isolated organs) this membrane permeable nitrosothiol is undergoing *trans*-nitrosylation with endogenous thiols, such as reduced glutathione to form nitrosoglutathione which spontaneous releases NO at physiologic pH [1, 27]. In contrast, DEA/NO spontaneously degrades in any solution at physiologic pH [18]. Thus, the ability of SNAP to release NO is dependent on the availability of free tissue thiols. For this reason, the release of NO in buffer solutions, as well as activation of isolated sGC is approximately 10 times lower with SNAP than with DEA/NO, while both drugs are equipotent vasodilators [23]. We repeated the experiments with DEA/NO to evaluate whether the slight increase in the maximal relaxation to SNAP following endothelial denudation in $eNOS^{++}$ can be confirmed using DEA/NO, but that was not the case. Thus, we suggest that the slightly different relaxation response to SNAP of denuded aortic rings between $eNOS^{n}$ and $eNOS^{++}$ is not due to a difference of the response to NO itself.

Our data suggest that sGC *S*-nitrosylation is a physiologic response to changes of vascular NO bioavailability in both directions. Thus, *S*-nitrosylation by endothelial NO of sGC likely compensates for limited changes of vascular NO bioavailability. For example, acetylcholine-dependent vasodilation was identical in $eNOS^{++}$ and $eNOS^{n}$ although one would have expected that a moderate increase in endothelial eNOS expression would potentiate this response. In accordance, this compensatory mechanism might contribute to explain why exercise training, another approach to increase endothelial eNOS expression and vascular NO bioavailability, is not consistently associated with improved endothelium-dependent vasodilatation in healthy mammals [20]. On the other hand, a reduction in vascular NO bioavailability as accomplished by L-NA treatment potentiates vascular sGC activity. Finally, our results may also be important for the recently reported effects of NO on ischemia–reperfusion, e.g. by adrenomedullin [13], eNOS gene transfer [41] and the eNOS enhancer AVE9488 [10]. Therefore, the reduction in NO bioavailability which is associated with the development of endothelial dysfunction, e.g. in atherosclerosis, might be masked and, therefore, not be detected until the NO levels fall beyond the potentiator effect on sGC activity induced by reversal of sGC *S*-nitrosylation.

Acknowledgments This study was supported by the Forschungskommission of the Heinrich-Heine-Universität Düsseldorf (Project 9772 109 to G.K., and project 9772 345 to T.S.).

Conflict of interest None declared.

References

1. Barnett DJ, McAninly J, Williams DLH (1994) Transnitrosation between nitrosothiols and thiols. J Chem Soc Perkin Trans 21131–21133. doi:10.1039/P29940001131
2. Batenburg WW, De Vries R, Saxena PR, Danser AH (2004) L-*S*-Nitrosothiols: endothelium-derived hyperpolarizing factors in porcine coronary arteries? J Hypertens 22:1927–1936
3. Bellamy TC, Wood J, Goodwin DA, Garthwaite J (2000) Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses. Proc Natl Acad Sci USA 97:2928–2933
4. Brandes RP, Kim DY, Schmitz-Winnenthal FH, Amidi M, Gödecke A, Mülsch A, Busse R (2000) Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice—role of soluble guanylyl cyclase. Hypertension 35:231–236

5. Buechler WA, Nakane M, Murad F (1991) Expression of soluble guanylate cyclase activity requires both enzyme subunits. Biochem Biophys Res Commun 174:351–357. doi:10.1016/0006-291X(91)90527-E
6. Desjardins F, Lobysheva I, Pelat M, Gallez B, Feron O, Dessy C, Balligand JL (2008) Control of blood pressure variability in caveolin-1-deficient mice: role of nitric oxide identified in vivo through spectral analysis. Cardiovasc Res 79:527–536. doi:10.1093/cvr/cvn080
7. Dikalov S, Fink B (2005) ESR techniques for the detection of nitric oxide in vivo and in tissues. Methods Enzymol 396:597–610. doi:10.1016/S0076-6879(05)96052-7
8. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV (2001) Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science 293:2449–2452. doi:10.1126/science.1062688
9. Filippov G, Bloch DB, Bloch KD (1997) Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. J.Clin.Invest. 100:942–948. doi:10.1172/JCI119610
10. Frantz S, Adamek A, Fraccarollo D, Tillmanns J, Widder JD, Dienesch C, Schafer A, Podolskaya A, Held M, Ruetten H, Ertl G, Bauersachs J (2009) The eNOS enhancer AVE 9488: a novel cardioprotectant against ischemia reperfusion injury. Basic Res Cardiol 104:773–779. doi:10.1007/s00395-009-0041-3
11. Gewaltig MT, Kojda G (2002) Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc Res 55:250–260. doi:10.1016/S0008-6363(02)00327-9
12. Godecke A, Schrader J (2000) Adaptive mechanisms of the cardiovascular system in transgenic mice—lessons from eNOS and myoglobin knockout mice. Basic Res Cardiol 95:492–498
13. Hamid SA, Totzeck M, Drexhage C, Thompson I, Fowkes RC, Rassaf T, Baxter GF (2010) Nitric oxide/cGMP signalling mediates the cardioprotective action of adrenomedullin in reperfused myocardium. Basic Res Cardiol 105:257–266. doi:10.1007/s00395-009-0058-7
14. Hussain MB, Hobbs AJ, MacAllister RJ (1999) Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta. Br J Pharmacol 128:1082–1088. doi:10.1038/sj.bjp.0702874
15. Ignarro LJ, Cirino G, Casini A, Napoli C (1999) Nitric oxide as a signaling molecule in the vascular system: an overview. J Cardiovasc Pharmacol 34:879–886
16. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH (2001) Protein *S*-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol 3:193–197. doi:10.1038/35055104
17. Jaffrey SR, Snyder SH (2001) The biotin switch method for the detection of *S*-nitrosylated proteins. Sci STKE 2001:L1. doi:10.1126/stke.2001.86.pl1
18. Keefer LK, Nims RW, Davies KM, Wink DA (1996) "NONOates" (1-substituted diazen-1-ium-1, 2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. Methods Enzymol 268:281–293. doi:10.1016/S0076-6879(96)68030-6
19. Kelm M, Rath J (2001) Endothelial dysfunction in human coronary circulation: relevance of the L-arginine-NO pathway. Basic Res Cardiol 96:107–127. doi:10.1007/s003950170061
20. Kojda G, Hambrecht R (2005) Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? Cardiovasc Res 67:187–197. doi:10.1016/j.cardiores.2005.04.032
21. Kojda G, Kottenberg K, Hacker A, Noack E (1998) Alterations of the vascular and the myocardial guanylate cyclase/cGMP-system induced by long-term hypertension in rats. Pharm Acta Helv 73:27–35. doi:10.1016/S0031-6865(97)00044-7
22. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, Harrison DG (1999) Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. Cardiovasc Res 42:206–213. doi:10.1016/S0008-6363(98)00315-0
23. Kojda G, Patzner M, Hacker A, Noack E (1998) Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate. A novel mechanism to explain preferential venodilation of organic nitrates. Mol Pharmacol 53:547–554
24. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S (2005) Interactions of peroxynitrite with uric acid in the presence of ascorbate and thiols: implications for uncoupling endothelial nitric oxide synthase. Biochem Pharmacol 70:343–354. doi:10.1016/j.bcp.2005.05.009
25. Lauer N, Suvorava T, Rüther U, Jacob R, Meyer A, Harrison DG, Kojda G (2005) Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase. Cardiovasc Res 65(1):254–262. doi:10.1016/j.cardiores.2004.09.010
26. Mayer B, Kleschyov AL, Stessel H, Russwurm M, Munzel T, Koesling D, Schmidt K (2009) Inactivation of soluble guanylate cyclase by stoichiometric *S*-nitrosation. Mol Pharmacol 75:886–891. doi:10.1124/mol.108.052142
27. Meyer DJ, Kramer H, Özer N, Coles B, Ketterer B (1994) Kinetics and equilibria of *S*-nitrosothiol-thiol exchange between glutathione, cysteine, penicillamines and serum albumin. FEBS Lett 345:177–180. doi:10.1016/0014-5793(94)00429-3
28. Moncada S, Higgs A (1993) Mechanisms of disease: the L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012
29. Moncada S, Rees DD, Schulz R, Palmer RMJ (1991) Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. Proc Natl Acad Sci USA 88:2166–2170
30. Mullershausen F, Russwurm M, Koesling D, Friebe A (2003) The enhanced NO-induced cGMP response induced by long-term L-NAME treatment is not due to enhanced expression of NO-sensitive guanylyl cyclase. Vascul Pharmacol 40:161–165. doi:10.1016/S1537-1891(03)00049-1
31. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, Yokoyama M (1998) Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase (see comments). J Clin Invest 102:2061–2071. doi:10.1172/JCI4394
32. Oppermann M, Dao VT, Suvorava T, Bas M, Kojda G (2008) Effect of oral organic nitrates on expression and activity of vascular soluble guanylyl cyclase. Br J Pharmacol 155:335–342. doi:10.1038/bjp.2008.269
33. Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, Hirata K, Yasui H, Sakurai H, Yoshida Y, Masada M, Yokoyama M (2002) Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. J Clin Invest 110:331–340. doi:10.1172/JCI15215
34. Riego JA, Broniowska KA, Kettenhofen NJ, Hogg N (2009) Activation and inhibition of soluble guanylyl cyclase by *S*-nitrosocysteine: involvement of amino acid transport system L. Free Radic Biol Med 47:269–274. doi:10.1016/j.freeradbiomed.2009.04.027
35. Russwurm M, Koesling D (2004) NO activation of guanylyl cyclase. EMBO J 23:4443–4450. doi:10.1038/sj.emboj.7600422
36. Sayed N, Baskaran P, Ma X, van den Beuve A AF (2007) Desensitization of soluble guanylyl cyclase, the NO receptor, by *S*-nitrosylation. Proc Natl Acad Sci USA 104:12312–12317. doi:10.1073/pnas.0703944104
37. Schmidt K, Andrew P, Schrammel A, Groschner K, Schmitz V, Kojda G, Mayer B (2001) Comparison of neuronal and

endothelial isoforms of nitric oxide synthase in stably transfected HEK 293 cells. Am J Physiol Heart Circ Physiol 281:H2053–H2061
38. Schultz G, Böhme E (1984) Guanylate Cyclase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinheim, FRG, pp 379–389
39. Scott WS, Nakayama DK (1998) *Escherichia coli* lipopolysaccharide downregulates soluble guanylate cyclase in pulmonary artery smooth muscle. J Surg Res 80:309–314. doi:10.1006/jsre.1998.5442
40. Suvorava T, Lauer N, Kojda G (2004) Physical inactivity causes endothelial dysfunction in healthy young mice. J Am Coll Cardiol 44:1320–1327. doi:10.1016/j.jacc.2004.06.030
41. Szelid Z, Pokreisz P, Liu X, Vermeersch P, Marsboom G, Gillijns H, Pellens M, Verbeken E, Van de WF, Collen D, Janssens SP (2010) Cardioselective nitric oxide synthase 3 gene transfer protects against myocardial reperfusion injury. Basic Res Cardiol 105:169–179. doi:10.1007/s00395-009-0077-4
42. Weber M, Lauer N, Mulsch A, Kojda G (2001) The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. Free Radic Biol Med 31:1360–1367. doi:10.1016/S0891-5849(01)00706-7
43. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Rikitake Y, Inoue N, Hirata K, Akita H, Yokoyama M (2000) Mechanisms of reduced nitric oxide/cGMP-mediated vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. Hypertension 36:97–102
44. Zhao YY, Zhao YD, Mirza MK, Huang JH, Potula HH, Vogel SM, Brovkovych V, Yuan JX, Wharton J, Malik AB (2009) Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration. J Clin Invest 119:2009–2018. doi:10.1172/JCI33338

Free Radical Research, February 2010; 44(2): 199–207

informa
healthcare

Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity

TATSIANA SUVORAVA[1], STEPHANIE KUMPF[1], BERNHARD H. RAUCH[1], VU THAO-VI DAO[1], VOLKER ADAMS[2], & GEORG KOJDA[1]

[1]*Institute of Pharmacology and Clinical Pharmacology, Heinrich Heine University, Duesseldorf, Germany, and* [2]*Heart Center Leipzig, Clinic for Internal Medicine/Cardiology Leipzig, Germany*

(Received: 15 June 2009; revised: 23 September 2009)

Abstract
The number of circulating stem cells with endothelial progenitor capacity (EPCs) inversely correlates with the number of cardiovascular risk factors. In this study we sought to investigate the effects of vascular H_2O_2 on circulating EPC levels. In C57BL/6 mice 3 weeks of freely moving or forced physical activity or voluntary exercise failed to increase circulating EPCs defined as double positive for Flk-1 and CD34, CD133 or Sca-1. Likewise, neither insertion of additional genes encoding for catalase (cat^{++}) or eNOS nor eNOS knock-out changed EPCs in resting mice. In striking contrast, inhibition of catalase by aminotriazole strongly reduced circulating EPCs in sedentary cat^{++} and their transgen-negative littermates (cat^{n}), while forced or voluntary exercise training of cat^{++} mice significantly increased the number of circulating EPCs. The latter effect was completely inhibitable by aminotriazole. These data suggest that endogenous vascular H_2O_2 likely contributes to the impairment of important stem cell-induced vascular repair mechanisms in cardiovascular disease.

Keywords: *Hydrogen peroxide, endothelial progenitor cells, exercise, reactive oxygen species.*

Introduction

Recent evidence suggests that regeneration of damaged vascular endothelium involves the participation of stem cells mobilized from the bone marrow. Asahara et al. [1] demonstrated that circulating $CD34^{+}$-angioblasts within human peripheral blood are able to differentiate *in vitro* to endothelial phenotype. These 'endothelial progenitor cells' contribute to important vascular repair mechanisms such as re-endothelialization and can improve organ blood flow by homing into ischemic regions. Here, EPCs contribute to the formation of entirely new vessels and/or release angiogenic factors such as vascular endothelial growth factor (VEGF) in a paracrine manner [2–4]. These potentially beneficial effects are impaired when the number and/or functional activities of EPCs are reduced.

Clinical trials revealed that the number of circulating EPCs and EPC homing is decreased in subjects with cardiovascular risk factors, such as hypercholesterolaemia, diabetes and smoking [5,6]. All these pathologies are associated with increased vascular oxidative stress and it has been suggested that a lower number of circulating EPCs indicates the progression of cardiovascular damage [7]. Proliferative EPCs showed decreased clonogenic capacity after treatment with oxidants such as H_2O_2 [8] and an increase of reactive oxygen species such as H_2O_2 likely impairs vessel growth [9]. Therefore, reactive oxygen species may directly influence stem cell-induced vascular repair mechanisms by reducing the number of circulating EPCs.

Exercise has been reported to increase the number of circulating EPCs in mice [10]. Furthermore, exercise is an effective anti-oxidative intervention which can prevent acute cardiovascular events and reduce cardiovascular mortality [11]. We hypothesized that the strong vascular oxidant H_2O_2 interferes with

Correspondence: Tatsiana Suvorava, PhD, Institute of Pharmacology and Clinical Pharmacology, University Hospital Duesseldorf, Moorenstr. 5 40225 Duesseldorf, Germany. Tel: +49-211-81-12511. Fax: +49-211-81-14781. Email: tasuv@gmx.de

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715760903402898

exercise-induced increases of circulating EPCs. In the present study we investigated the effect of H_2O_2 on the number of circulating EPCs in sedentary mice and in mice subjected to exercise. To accomplish this, we used normal C57Bl/6 mice and mice of a transgenic strain carrying an endothelial-specific overexpression of catalase [12,13].

Material and methods

Experimental animals

We generated a transgenic construct, in which human catalase was inserted between murine Tie-2 promotor (2.1 kb) and a 10 kb Tie-2-enhancer and this construct was used to target catalase gene expression to the vasculature as described previously [13]. Founder mice were crossed 15 times to C57BL/6 mice to generate a C57BL/6 background. Transgene negative littermates (cat^n) served as controls. In some experiments mice deficient for eNOS gene ($eNOS^{-/-}$) and new generated mouse strain with a endothelial-specific over-expression of bovine eNOS driven by the murine Tie-2 promotor were used ($eNOS^{++}$) [13,14]. In addition, C57Bl/6 mice were used to study the effect of different exercise protocols on circulating levels of EPCs.

Sedentary, moving and exercised mice

All mice were bred at the university's animal facilities in an SPF-area. Mice were randomly assigned to one of four groups: (1) sedentary mice living alone for 5 weeks in small cages with a floor space of 0.3 m^2 (Figure 1A), (2) moving mice housed in groups of four-to-five males in cages with a floor space of 0.9 m^2 (moving mice, Figure 1A), (3) forced exercised group (15 days lasting exercise programme on a treadmill, Figure 1B) or (4) voluntary exercised mice (Figure 1C). Mice living in groups were fighting, running and climbing for the most part of their active daily cycle, while singularized mice were predominantly resting during their active daily cycle and showed a low physical activity as evidenced by measuring the heart weight/body weight ratio and skeletal citrate synthase activity [15]. In some experiments soleus weight/body weight and soleus weight/tibia length ratios were calculated as additional exercise performance measures. Citrate synthase activity was measured in soleus muscle as described previously [15].

Exercise protocols

For voluntary training, the mice were housed individually in cages supplied with running wheels (0.25 m in diameter, Tecniplast, Germany) and equipped with counters to record the daily running distance. The mean running distance for C57Bl/6 was 4.65 ± 0.3 km/24 h and did not differ significantly between C57Bl/6, cat^n (4.725 ± 0.15, $n=6$) and cat^{++} (5.11 ± 0.33, $n=8$). For comparison, treadmill training was performed using a previously established protocol [16]. After i.p. injection of heparin (Liquemin, Roche Pharma AG, Switzerland) mice were euthanized by CO_2 inhalation. Blood samples were collected from the heart for EPCs measurement. The aortas and soleus muscles were immediately frozen in liquid nitrogen and kept at –80°C for further analysis. Permission for this study was provided by the regional government and the experiments were performed according to the guidelines for the use of experimental animals as given by 'Deutsches Tierschutzgesetz' and to the 'Guide for the care and use of laboratory animals' of the US National Institutes of Health.

Fluorescence-activated cell sorter analysis (FACS)

Circulating EPCs in mouse peripheral blood were counted using FACS Calibur (Beckton Dickinson, USA). Aliquots of 100 µl of mouse peripheral blood were incubated with an antibody combination of anti CD3-APC, anti Flk-1-PE and anti CD34-FITC or anti CD3-APC, anti Flk1-PE and anti Sca-1-PE or anti CD3-APC, anti Flk-1-PE (all BD Pharmingen, USA) and anti CD133-FITC (eBioscience, Natutec, Germany). After an incubation period of 15 min at room temperature the erythrocytes were lysed using FACS lysing solution (BD Biosciences, San Jose, CA) and after several washing steps the remaining cells were fixed for FACS analysis. Isotype specific antibodies served as controls in every experiment (BD Pharmingen, eBioscience). To quantify the amount of EPCs in the mouse blood, only cells negative for the marker CD3 and double positive for CD34 and Flk-1 ($CD34^+/Flk\text{-}1^+$) or negative for CD3 and double positive for Sca-1 and Flk-1 ($Sca\text{-}1^+/Flk\text{-}1^+$) or negative for CD3 and double positive for CD133 and Flk-1 ($CD133^+/Flk\text{-}1^+$) were counted as EPCs. Another set of experiments was done for confirmation of our results with another FACS analyser (BD Coulter) using the same antibodies combinations except for CD3-APC which was replaced by CD3-PerCP (BD Bioscience, USA).

RT-PCR

Expression of transgene-specific mRNA in hearts of cat^{++}, cat^n and C57Bl/6 was analysed by RT-PCR using three different pairs specific to human catalase cDNA. The sense primers were TTCTGTTGAAGATGCGGCG (S1), TTTAACGCCATTGCCACA (S2), antisense-primers TGTTGTTCCGGAGCACCA (AS1), TCCGCACTTCTCCAGAAT (AS2), 1st primers pair S1/AS1, 2nd pair S2/AS1, 3rd pair S1/AS2.

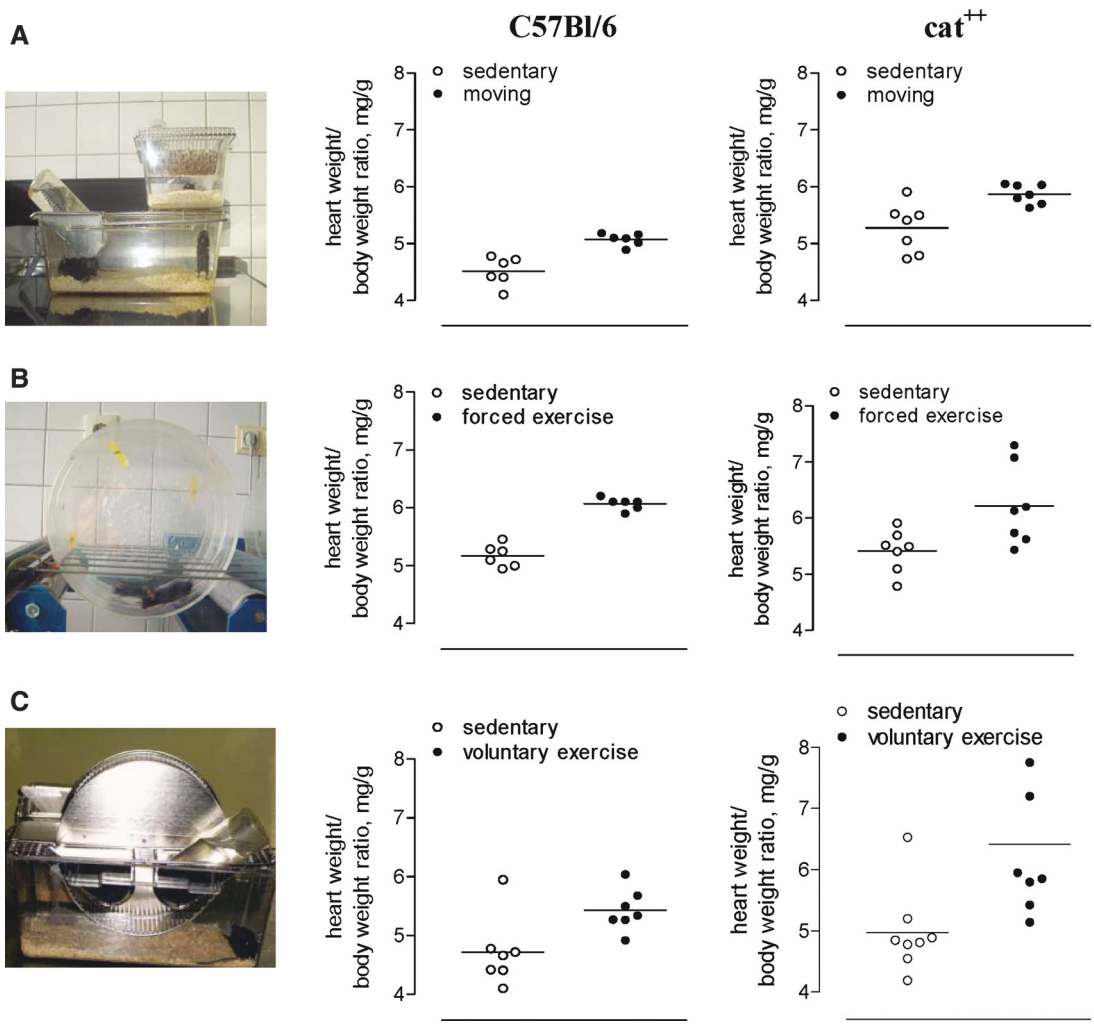


Figure 1. Details of the cages used to house and to exercise mice and heart weight/body weight ratio (mg/g) in sedentary and freely moving (A), forced exercised (B) and voluntary exercised (C) C57Bl/6 and cat^{++} mice. All exercise protocols significantly increased heart weight/body weight ratio in C57Bl/6 and cat^{++} mice almost identically when compared to sedentary singularized controls (n=6–7, p>0.05).

Western blotting

Mice thoracic aortas and bone marrow extracts were flash frozen in liquid nitrogen, homogenized, solubilized in lysis buffer [17] and centrifuged for 10 min at 100 x g. Supernatants were stored at –80°C until used for western blots. Western blot analysis was performed as described previously [16] using a monoclonal antibody directed against eNOS (Transduction Laboratories, Lexington, Kentucky) and a polyclonal antibody for catalase (Calbiochem, Darmstadt, Germany). Blots were developed using enhanced chemiluminescence (Roche, Mannheim, Germany) and exposed to x-ray film. The autoradiographs were analysed by densitometry (Geldoc, Bio-Rad, Muenchen, Germany). Total protein levels were determined by the Bradford method [18].

Measurement of blood pressure and heart rate

Systolic blood pressure (sBP) and heart rate were measured in awake male cat^{++} (n=8), cat^{n} (n=8) and C57Bl/6 (n=6) at 3–4 months of age using an automated tailcuff system (Visitech Systems, Apex, NC) as described previously [19]. In some experiments cat^{++} and cat^{n} mice (n=8, each) were treated with the catalase inhibitor aminotriazole (670 mg/kg/day, dissolved in drinking water) for 14 days. In another subset of experiments cat^{++} (n=6) and cat^{n} (n=5) and eNOS^{n} and eNOS^{++} (n=6 each) were treated for 28 days with N$^{\omega}$-nitro-L-arginine (L-NA, 100 mg/kg BW/day) and sBP and heart rate were recorded before, during and after the treatment period.

Substances and solutions

All chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany) in analytical grade.

Statistics

All data were analysed by standard computer programs (GraphPad Prism PC Software, Version 3.0,

Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (SEM). Significant differences were evaluated using either Newman-Keuls Multiple Comparison Test following OneWay-ANOVA or students *t*-test. A *p*-value below 0.05 was considered as significant.

Results

Characterization of transgenic mice

Briefly, cat^{++} showed human catalase mRNA expression as evidenced by transgene-specific PCR (Figure 2A) and increased catalase protein expression in both conductance (Figure 2B) and myocardial resistance vessels and associated with an increase of catalase mRNA, protein and activity [13]. Furthermore, there was a marked reduction of endothelial steady-state concentration of endogenous H_2O_2 measured by dichlorofluoresceine fluorescence in cat^{++} mice [12,13]. Since Tie-2 may also be expressed in neutrophils [20] we have measured catalase protein expression in the bone marrow extracts of cat^{n} and cat^{++} and have found no difference between the strains (Figure 2B). Furthermore, no difference between cat^{n} and cat^{++} was found by measurements of dihydroethidine fluorescence in leucocytes using FACS analysis suggesting absence of catalase over-expression in non-vascular cells [12]. Taken together, these data strongly suggest endothelial-specific over-expression of catalase and reduction of endogenous vascular H_2O_2 in our transgenic cat^{++} mouse model.

Reduction of blood pressure is one obvious phenotype of cat^{++} [12]. In the animals used for this study, we found a similar decrease of sBP from 116±2.6 mmHg (cat^{n}) to 101.4±2.5 mmHg in cat^{++} (n=8, p=0.0007). This effect was completely inhibited by treatment of mice with aminotriazole (117.4±4.0 mmHg, n=8, p=0.7653). The difference in sBP between cat^{n} and cat^{++} remained after inhibition of eNOS with oral L-NA treatment for 3 weeks (Figure 2C) suggesting that eNOS is not involved in sBP reduction in cat^{++}. Both findings confirm previous observations in these mice [12,13]. The $eNOS^{++}$ mice used in this study showed a 3.32±0.32-fold stronger aortic eNOS expression (n=7, p<0.001) as compared to $eNOS^{n}$. This was associated with a decrease of sBP from 118.1±2.4 mmHg in $eNOS^{n}$ to 105.6±3.3 mmHg in $eNOS^{++}$ (n=6, p=0.0151). In contrast to cat^{++}, this decrease of blood pressure was completely dependent on eNOS activity as evidenced by oral treatment with L-NA which increased sBP to a similar degree in $eNOS^{n}$ (134.5±3.1 mmHg, n=6) and $eNOS^{++}$ (132.9±2.1 mmHg, n=6, p=0.343).

The effect of training induced by different exercise protocols

To evaluate the efficacy of training we measured heart weight/body weight ratio. As shown in Figure 1, all training protocols significantly increased heart weight/body weight ratio in C57Bl/6 mice to a comparable extent suggesting a comparable cardiovascular adaptation to the different exercise protocols. Similar effects were observed in cat^{++} (Figure 1) demonstrating that the response to the each exercise protocol was not different between transgenic mice and C57Bl/6. Skeletal muscle citrate synthase activity was used as a metabolic marker for oxidative capacity to check for the changes in skeletal muscle energetics. While citrate synthase activity was increased in moving mice (55.5±4.3 mU/mg protein, n=5) and after forced physical activity (141.3±15.4 mU/mg protein, n=7) as compared to sedentary controls (40.3±2.8 mU/mg protein, n=7, p<0.05), we were unable to detect such a difference in voluntary running mice (55.2±5 mU/mg protein, n=7) vs sedentary controls (57.8±1.4 mU/mg protein, n=8, p=0.6038). However, voluntary training significantly increased soleus weight relative to body weight (0.356±0.03 vs 0.280±0.005 mg/g, n=4–5, p=0.027) and relative to tibia length (5.40±0.34 vs 4.17±0.20 mg/cm, n=4–5, p=0.0126).

The effect of training on the number of EPCs in C57Bl/6 mice

In a first set of experiments we used the stem cell marker CD34 and the endothelial cell marker Flk-1 to detect the effect of different training protocols on circulating EPCs. As shown in Figure 3, there were no changes of circulating EPCs induced by exercise. Likewise, no alterations in circulating CD34/Flk-1 double positive cells were detected after forced exercise (101.6±22.2 vs 90.4±17.1, n=8 each, p>0.05) and after voluntary running (81.8±9.9 vs 68.9±8.0, n=8 each, p>0.05) when another FACS analyser (BD Coulter) and the same antibodies combinations except for CD3-APC which was replaced by CD3-PerCP were used to count for EPCs.

To scrutinize this result, we investigated the effects of the same exercise protocol (voluntary running) on EPCs by using the three different stem cell markers CD34, Sca-1 and CD133 in combination with Flk-1. Again, there were no changes in EPC counts (Figure 4). Similarly, measurements using another FACS analyser (FACS Calibur) confirmed no changes in EPC numbers (369±109 vs 425±147 Sca1/Flk-1 double positive cells, n=5–6, p>0.05 or CD34/Flk-1 double positive cells (Figure 3C)). Likewise, we observed that the number of EPCs identified with Sca-1/Flk-1 was unchanged in freely moving compared to sedentary mice (577±128 vs 425±147 per 500 000 events, n=5, p=0.4588). The same was true when we used CD133/Flk-1 to identify EPCs in mice which underwent forced physical activity compared to sedentary controls (39±11 vs 36±17 per 500 000 events, n=6, p=0.8988).

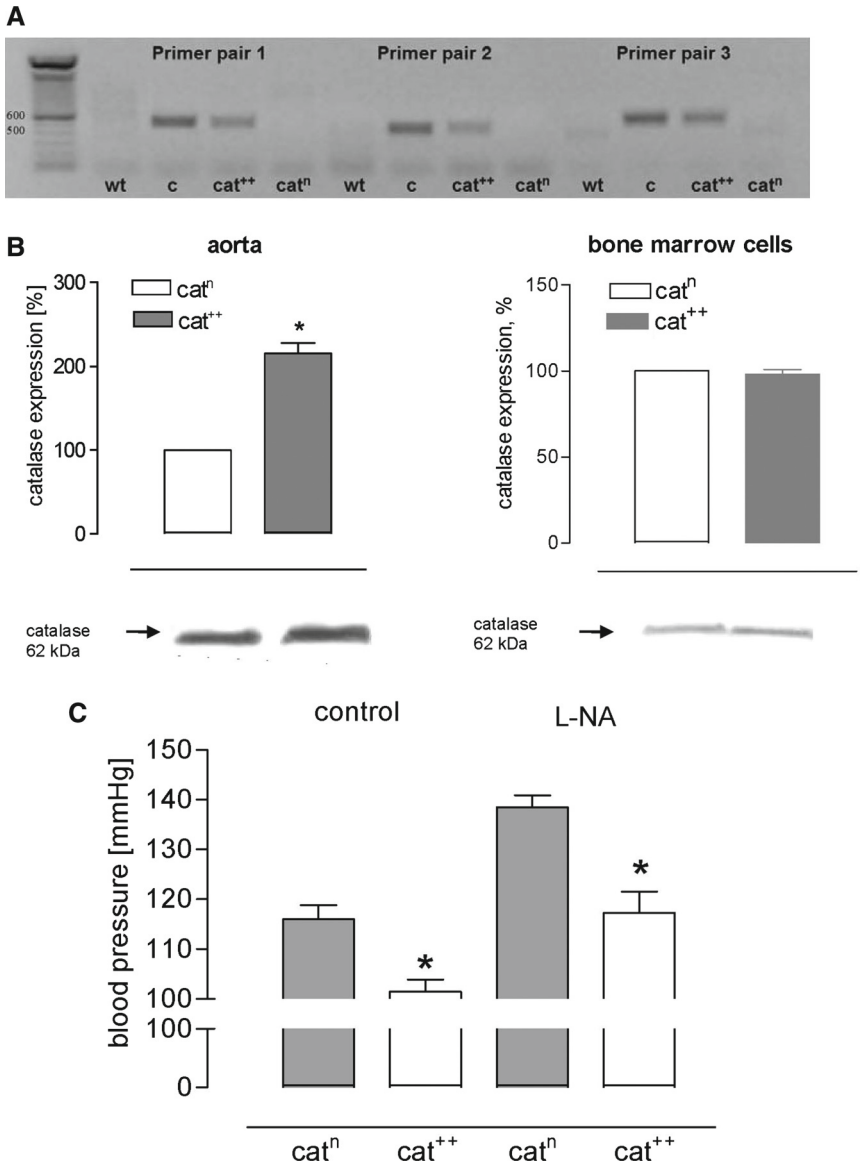


Figure 2. Evidence for functionally active over-expression of human catalase in transgenic mice. (A) Transcription of human catalase in heart of cat^{++} as evident by transgene-specific RT-PCR. The expected size bands of 510, 449 and 557 bp were detected in transgene-positive mice (cat^{++}) using three different pairs of primers specific for human catalase cDNA, while there were no signals in transgene-negative littermates (cat^{n}). Total mRNA from heart of C57Bl/6 mice served as negative control (wt), mRNA isolated from human umbilical vein served as positive control (c). (B) Catalase protein expression measured by western blotting in the aortic homogenates and in the bone marrow cells of cat^{++} and cat^{n}. (C) Effect of NO-synthase inhibitor L-NA (100 mg/kg BW/day) on systolic blood pressure in cat^{++} and cat^{n}. Measurements were obtained in resting awake animals using a tail-cuff method (* n=5-6, $p<0.001$, one way ANOVA).

The effect of eNOS and catalase on the number of EPCs in transgenic mice

All experiments in transgenic mice were done using CD34/Flk-1 to identify EPCs. To investigate an effect of H_2O_2 on the number of circulating EPCs we compared cat^{++}-mice with cat^{n}-mice but found no effect of the transgene (Figure 5A). In contrast, treatment of these mice with aminotriazole resulted in a large decrease of circulating EPCs suggesting that increased H_2O_2 is the underlying mediator of this effect (Figure 5A). It has been published that cat^{++} have hypotension and that inhibition of catalase in these mice by aminotriazole increases sBP back to normal values [12]. Thus, blood pressure might be a confounding factor in our study. To further investigate a possible role of blood pressure we used two other strains of transgenic mice, $eNOS^{-/-}$ having hypertension [19] and $eNOS^{++}$ having hypotension [12]. As shown in Figures 5B and C the numbers of EPCs were unchanged in these animal models suggesting

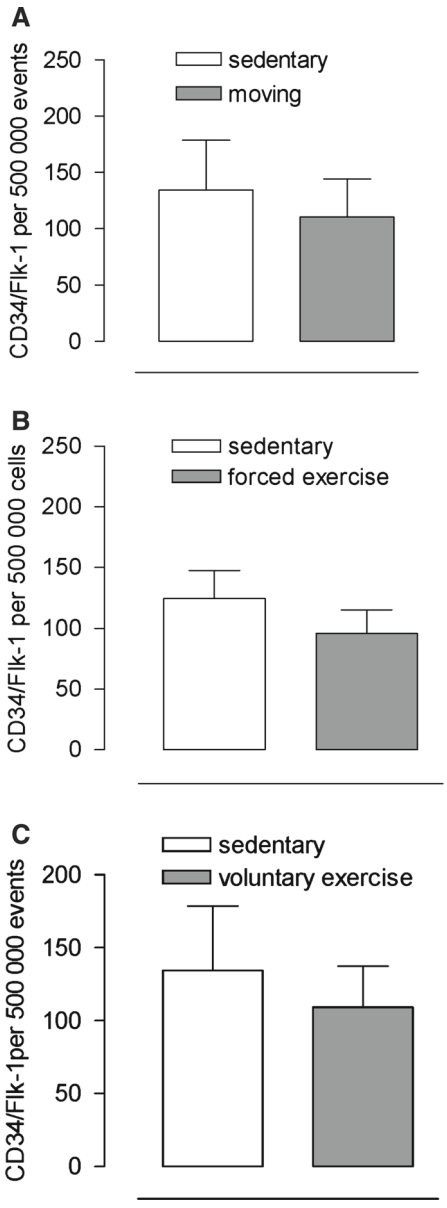


Figure 3. No difference in circulating EPCs measured as CD34/Flk-1 positive cells using FACS Calibur (Beckton Dickinson) in peripheral blood of freely moving (A), forced (B) and voluntary (C) exercised C57Bl/6 mice (n=5–9, p>0.05).

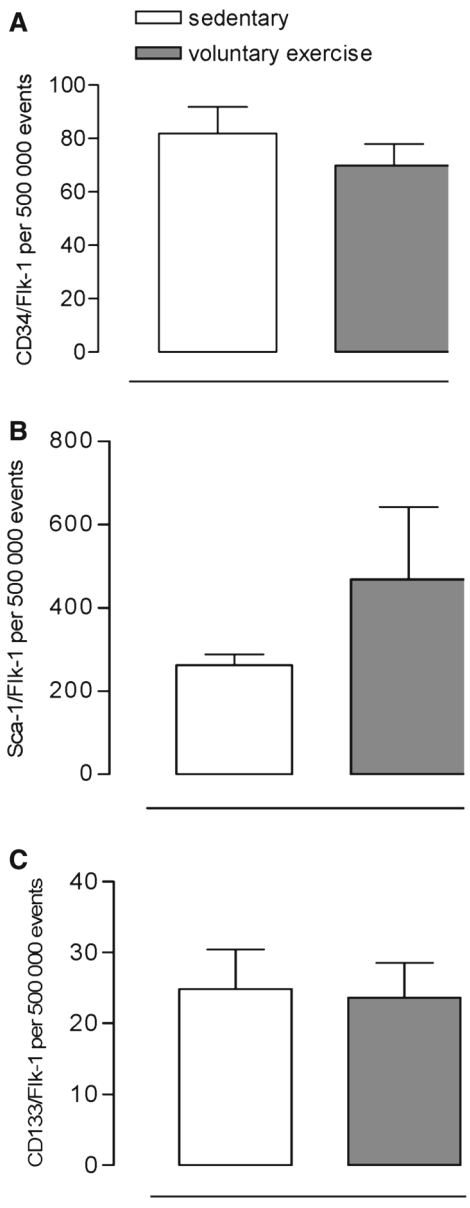


Figure 4. Voluntary exercise training had no effect on circulating EPCs in C57Bl/6 mice as measured by FACS analyser (BD Coulter) using different stem cell markers. Circulating EPCs were defined (A) as CD34/Flk-1 (n=8, p=0.365); (B) as Sca-1/Flk-1 (n=7–8, p=0.292) and (C) as CD133/Flk-1 (n=6–7, p=0.876) double positive cells.

that neither eNOS activity nor blood pressure appears to be important for the number of circulating EPCs in resting healthy mice.

The effect of the catalase transgene on circulating EPCs after exercise training

A comparison of the response of cat^{++} to two different training protocols showed a strong and significant increase of circulating EPCs induced by forced physical activity (Figure 6A) and voluntary exercise (Figure 6B). These data suggest that H_2O_2 inhibits exercise-induced augmentation of circulating EPCs in cat^{n} and C57Bl/6 mice (Figure 3). In accordance with this suggestion we found that aminotriazole completely inhibited the augmenting effect of exercise on circulating EPCs in cat^{++} (Figure 6C).

Discussion

The aim of this study was to investigate the effects of vascular H_2O_2 on the number of circulating EPCs and on changes thereof in response to a 3–4 weeks moderate exercise training period. Our major new finding is that H_2O_2 as an important component of total vascular oxidative stress inhibits exercise-induced

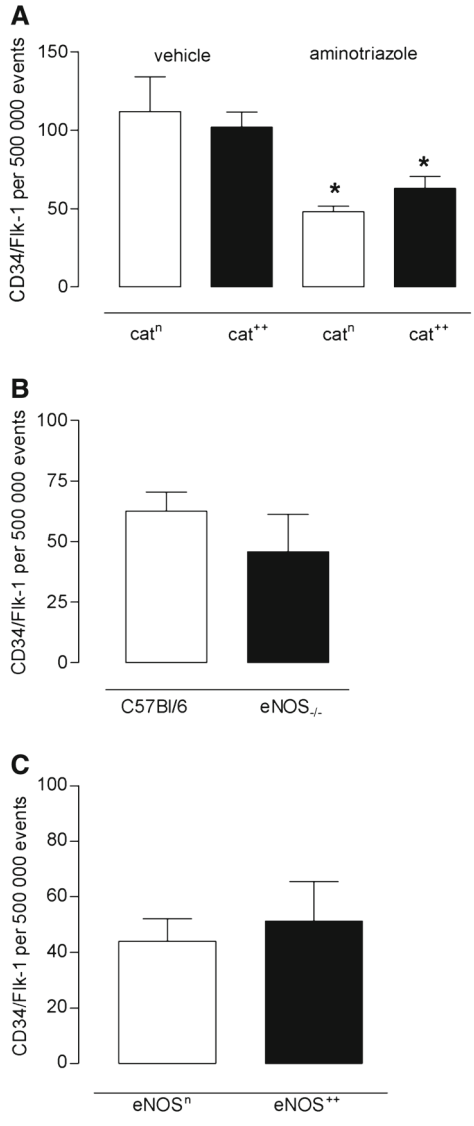


Figure 5. Circulating EPCs in peripheral blood of different transgenic mouse strains. (A) There was no difference between basal EPC levels in cat^n and cat^{++} mice. Oral treatment with catalase inhibitor aminotriazole significantly decreased EPCs in peripheral blood of cat^n and cat^{++} (* n=5–8, p<0.05). (B) Circulating EPCs in eNOS knockout mice ($eNOS^{-/-}$) and (C) mice with endothelium-specific over-expression of eNOS ($eNOS^{++}$). (C) Neither eNOS knockout (n=6, p=0.6625) nor insertion of eNOS gene (n=7, p=0.358) changed EPC counts in resting mice.

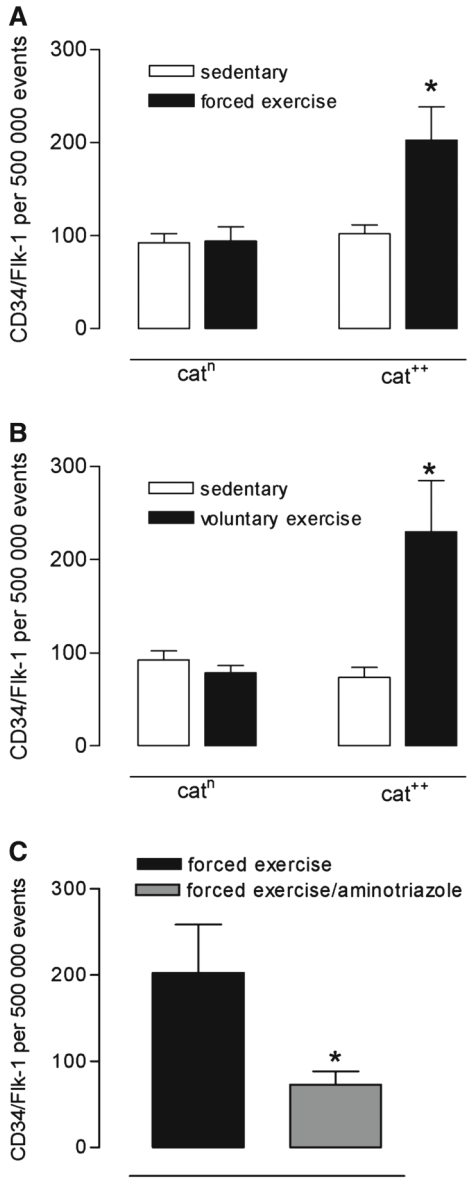


Figure 6. Moderate forced (A) and voluntary (B) exercise training strongly increased circulating EPCs in cat^{++} mice and this was completely abolished by treatment with catalase inhibitor aminotriazole (C) (n=5–8, p<0.05).

upregulation of circulating hematopoietic stem cells with endothelial progenitor capacity.

Vascular H_2O_2 is known to be an important part of vascular oxidative stress and exerts several deleterious effects in the cardiovascular system such as increased smooth muscle proliferation, stimulation of inflammation, promotion of the atherosclerotic process, generation of tissue-toxic hydroxyl radicals and alterations of vascular tone [21]. Here we describe a new and potentially hazardous activity of vascular H_2O_2, i.e. a reduction of the number of circulating EPCs. Several lines of evidence support this suggestion. Aminotriazole, which inhibits catalase activity and accumulates tissue H_2O_2, strongly decreased circulating EPCs in resting animals. Likewise, endothelial-specific over-expression of catalase and subsequent reduction of vascular H_2O_2 [12,13] resulted in an increased number of circulating EPCs in response to different exercise protocols. Finally, the exercise-induced increase of EPCs was completely abolished in cat^{++} treated with aminotriazole. These data suggest that H_2O_2 is involved in the physiologic non-ischemic regulation of the number of circulating EPCs both at rest and by exercise.

According to previous investigations, the number of EPCs was increased by non-ischemic exercise in mice [10] but not in patients [22,23]. To further investigate the

role of non-ischemic exercise on the number of circulating EPCs, we used several different exercise protocols. The different protocols appear to be equally effective as indicated by a comparable increase of heart weight/body weight ratio. However, the distance voluntarily running mice ran each night was almost 10 times greater than the daily distance made by mice subjected to forced physical activity. Furthermore, freely moving mice and those who underwent daily forced physical activity appear to be much more exposed to stress factors such as the aggressive nature of the C57Bl/6 strain in freely moving mice living in groups and the experimental procedure of forced running mice in rotating wheels. While such maximal strains are absent in singularized voluntarily running mice, this type of exercise training rather matches endurance training with a lower but more constant training intensity. In addition, voluntary running matches the preferred running time and speed for each individual mouse. In general, mice appear to voluntarily run in short bouts of 2–3 min at a mean speed of 2–3 km/h for a total of 100–120 bouts per night [24]. While individual bouts may be of high intensity, they are of short duration and do not invoke comparable adaptation in mitochondrial enzyme activity in slow-twitch soleus muscle. The absence of increase in citrate synthase activity has been reported previously [25,26]. Although we didn't analyse voluntary running patterns in detail, many aspects such as total running distance, changes of heart weight/body weight, soleus weight/ body weight and soleus weight/tibia length ratios and daily running distance demonstrate that mice run sufficiently in our voluntary model of exercise.

Identification of EPCs by FACS analysis relies on the combined expression of proteins indicative for stem cells such as CD34, CD133 and Sca-1 and for endothelial cells such as the VEGFR2 (Flk-1). Cell culture experiments have shown that human EPCs are not a homogenous population of stem cells. For example, early and late outgrowth EPCs can be differentiated [27]. Furthermore, Sca-1 positive non-bone marrow-derived progenitor cells are able to differentiate into both, endothelial and smooth muscle cells [28]. It was also noted that Sca-1 is expressed on mature endothelial cells and this might hold true for CD34 as well [5]. Accordingly, CD133/Flk-1 double staining is currently considered to be the most reliable identification for EPCs [29,30]. However, in mice the number of these cells is extremely low and this makes a quantitative evaluation very difficult. Therefore, we have compared the data obtained with all three stem cell markers and found similar results. The only trend for an increase of circulating EPCs we have detected was when Sca-1/Flk-1 double positive cells were counted following voluntary running and a similar observation has been made previously [10]. Thus, it appears indeed important not to rely on measurements with just one stem cell marker [30].

Blood pressure might [31] or might not [7] impact on the number of circulating EPCs in humans. In mice, we observed an exercise-induced increase of EPCs in cat^{++} only, a strain showing both decreased steady state levels of vascular H_2O_2 and reduced blood pressure as compared to cat^{n} and C57Bl/6. Thus, blood pressure might have been a confounding factor in our study. However, resting cat^{++} and cat^{n} treated with aminotriazole showed a comparable decrease of EPCs, although aminotriazole treatment increased blood pressure in cat^{++} but not in cat^{n}. In order to further clarify whether blood pressure changed the effect of exercise on the number of EPCs in our study, we evaluated other transgenic mouse strains. We generated mice using the same promotor and enhancer element as in cat^{++} but inserted bovine eNOS ($eNOS^{++}$). Furthermore, eNOS knock-out mice ($eNOS^{-/-}$) were investigated.

While $eNOS^{-/-}$ have hypertension, $eNOS^{++}$ have hypotension which was completely dependent on treatment with the NOS-inhibitor L-NA, suggesting NO-dependent reduction of blood pressure. All of these different animal strains didn't differ in terms of basal (resting) number of circulating EPCs. These data strongly suggest that changes of blood pressure unlikely change the number of circulating EPCs in mice. A similar situation appears to hold true for the effects of exercise. Aminotriazole completely inhibited exercise-induced up-regulation of circulating EPCs in cat^{++}-mice and increased blood pressure at the same time. However, treatment of cat^{n} with animotriazole didn't change blood pressure and EPC-response to exercise, suggesting that changes of blood pressure had no effect on exercise-induced increase of circulating EPCs in our strains of mice.

Maintenance and mobilization of EPCs in the bone marrow is determined by the local microenvironment, which consists of fibroblasts and endothelial cells. Recently we have found significantly increased level of vascular MMP-9, a key matrix metalloproteinase involved in mobilization of EPCs in cat^{++} mice (our unpublished data). H_2O_2 is able to accumulate extracellularly in the tissue and survive long enough to induce paracrine functions, even in more distant cells. We speculate that vascular H_2O_2 can diffuse from the vascular niche to the stem cell niche of the bone marrow and have an inhibitory effect on the local MMP-9. This effect is abolished in mice with endothelial-specific over-expression of catalase. Further studies are necessary to clarify molecular mechanisms of H_2O_2-dependent bone marrow MMP-9 regulation.

In summary, we reported a new and potentially hazardous activity of vascular endogenous H_2O_2, i.e. a reduction of the number of circulating EPCs. These data suggest that H_2O_2, a known component of vascular oxidative stress, likely contributes to the impairment of important stem cell-induced vascular repair mechanisms in cardiovascular disease.

Acknowledgements

This study was supported by the Forschungskommission of the Heinrich-Heine-University Duesseldorf (project 9772272, to G.K. and project 9772 345 to T.S.).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.

[2] Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest. 2005;115:572–583.

[3] Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation. 2001;104:1046–1052.

[4] Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. Blood. 2006;107:3546–3554.

[5] Zampetaki A, Kirton JP, Xu Q. Vascular repair by endothelial progenitor cells. Cardiovasc Res. 2008;78:413–421.

[6] Urbich C, Dernbach E, Rossig L, Zeiher AM, Dimmeler S. High glucose reduces cathepsin L activity and impairs invasion of circulating progenitor cells. J Mol Cell Cardiol. 2008; 45:429–436.

[7] Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med. 2005;353:999–1007.

[8] Ingram DA, Krier TR, Mead LE, McGuire C, Prater DN, Bhavsar J, Saadatzadeh MR, Bijangi-Vishehsaraei K, Li F, Yoder MC, Haneline LS. Clonogenic endothelial progenitor cells are sensitive to oxidative stress. Stem Cells. 2007;25:297–304.

[9] Roy S, Khanna S, Sen CK. Redox regulation of the VEGF signaling path and tissue vascularization: Hydrogen peroxide, the common link between physical exercise and cutaneous wound healing. Free Radic Biol Med. 2008;44:180–192.

[10] Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G. Physical training increases endothelial progenitor cells, inhibits neointimaformation, and enhances angiogenesis. Circulation. 2004;109:220–226.

[11] Kojda G, Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? Cardiovasc Res. 2005;67:187–197.

[12] Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G. Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo. Circulation. 2005;112:2487–2495.

[13] Lauer N, Suvorava T, Rüther U, Jacob R, Meyer A, Harrison DG, Kojda G. Critical Involvement of Hydrogen Peroxide In Exercise-Induced Upregulation Of Endothelial NO-Synthase. Cardiovasc Res. 2005;65(1):254–262.

[14] Suvorava T, Kojda G. Reactive oxygen species as cardiovascular mediators: lessons from endothelial-specific protein overexpression mouse models. Biochim Biophys Acta. 2009; 1787:802–810.

[15] Suvorava T, Lauer N, Kojda G. Physical inactivity causes endothelial dysfunction in healthy young mice. J Am Coll Cardiol. 2004;44:1320–1327.

[16] Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. Circulation. 2001;103:2839–2844.

[17] Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. Circ Res. 1998;83:334–341.

[18] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72: 248–254.

[19] Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, Harrison DG. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. Cardiovasc Res. 1999;42:206–213.

[20] Lemieux C, Maliba R, Favier J, Theoret JF, Merhi Y, Sirois MG. Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. Blood. 2005;105:1523–1530.

[21] Ardanaz N, Pagano PJ. Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. Exp Biol Med (Maywood). 2006;231:237–251.

[22] Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, Tarnok A, Gielen S, Emmrich F, Schuler G, Hambrecht R. Increase of circulating endothelial progenitor cells in patientswith coronary artery disease after exercise-induced ischemia. Arterioscler Thromb Vasc Biol. 2004;24:684–690.

[23] Sandri M, Adams V, Gielen S, Linke A, Lenk K, Krankel N, Lenz D, Erbs S, Scheinert D, Mohr FW, Schuler G, Hambrecht R. Effects of exercise and ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes: results of 3 randomized studies. Circulation. 2005;111:3391–3399.

[24] De Bono JP, Adlam D, Paterson DJ, Channon KM. Novel quantitative phenotypes of exercise training in mouse models. Am J Physiol Regul Integr Comp Physiol. 2006;290: R926–R934.

[25] Momken I, Lechene P, Ventura-Clapier R, Veksler V. Voluntary physical activity alterations in endothelial nitric oxide synthase knockout mice. Am J Physiol Heart Circ Physiol. 2004; 287:H914–H920.

[26] Noble EG, Moraska A, Mazzeo RS, Roth DA, Olsson MC, Moore RL, Fleshner M. Differential expression of stress proteins in rat myocardium after free wheel or treadmill run training. J Appl Physiol. 1999;86:1696–1701.

[27] Elsheikh E, Uzunel M, He Z, Holgersson J, Nowak G, Sumitran-Holgersson S. Only a specific subset of human peripheral-blood monocytes has endothelial-like functional capacity. Blood. 2005;106:2347–2355.

[28] Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest. 2004;113:1258–1265.

[29] Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res. 2004; 95:343–353.

[30] Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C, Avogaro A. Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau. Atherosclerosis. 2008; 197:496–503.

[31] Xiao Q, Kiechl S, Patel S, Oberhollenzer F, Weger S, Mayr A, Metzler B, Reindl M, Hu Y, Willeit J, Xu Q. Endothelial progenitor cells, cardiovascular risk factors, cytokine levels and atherosclerosis—results from a large population-based study. PLoS One. 2007;2:e975.

This paper was first published online on Early Online on 19 November 2009.

Journal of Cellular and Molecular Medicine
A JOURNAL OF TRANSLATIONAL MEDICINE

J. Cell. Mol. Med. Vol 15, No 11, 2011 pp. 2326-2334

Catalase activity prevents exercise-induced up-regulation of vasoprotective proteins in venous tissue

Vu Thao-Vi Dao [a], Melanie Floeren [a], Stephanie Kumpf [a], Charlotte Both [a], Bärbel Peter [b], Vera Balz [c], Tatsiana Suvorava [a], Georg Kojda [a, *]

[a] *Institute for Pharmacology and Clinical Pharmacology, University Hospital, Heinrich-Heine-University, Duesseldorf, Germany*
[b] *Animal facilities, University Hospital, Heinrich-Heine-University, Duesseldorf, Germany*
[c] *Institute for Transplantationdiagnostic and Cell Therapeutics, University Hospital, Heinrich-Heine-University, Duesseldorf, Germany*

Received: June 15, 2010; Accepted: November 15, 2010

Abstract

Physical activity induces favourable changes of arterial gene expression and protein activity, although little is known about its effect in venous tissue. Although our understanding of the initiating molecular signals is still incomplete, increased expression of endothelial nitric oxide synthase (eNOS) is considered a key event. This study sought to investigate the effects of two different training protocols on the expression of eNOS and extracellular superoxide dismutase (ecSOD) in venous and lung tissue and to evaluate the underlying molecular mechanisms. C57Bl/6 mice underwent voluntary exercise or forced physical activity. Changes of vascular mRNA and protein levels and activity of eNOS, ecSOD and catalase were determined in aorta, heart, lung and vena cava. Both training protocols similarly increased relative heart weight and resulted in up-regulation of aortic and myocardial eNOS. In striking contrast, eNOS expression in vena cava and lung remained unchanged. Likewise, exercise up-regulated ecSOD in the aorta and in left ventricular tissue but remained unchanged in lung tissue. Catalase expression in lung tissue and vena cava of exercised mice exceeded that in aorta by 6.9- and 10-fold, respectively, suggesting a lack of stimulatory effects of hydrogen peroxide. In accordance, treatment of mice with the catalase inhibitor aminotriazole for 6 weeks resulted in significant up-regulation of eNOS and ecSOD in vena cava. These data suggest that physiological venous catalase activity prevents exercise-induced up-regulation of eNOS and ecSOD. Furthermore, therapeutic inhibition of vascular catalase might improve pulmonary rehabilitation.

Keywords: physical activity • catalase • endothelial nitric oxide synthase • pulmonary rehabilitation

Introduction

Physical activity has been shown to reduce cardiovascular morbidity and mortality and is an important non-pharmacological strategy to prevent and treat cardiovascular diseases [1, 2]. Different exercise protocols induce favourable changes of various cardiovascular functions such as reduction in heart rate and blood pressure, increase of maximal myocardial oxygen uptake, various metabolic modifications and physiologic adaptations such as angiogenesis and arteriogenesis involving skeletal muscle and cardiac muscle [3–6]. One of the key events in terms of vascular adaptations to exercise training appears to be an up-regulation of endothelial nitric oxide synthase (eNOS). This vascular adaptation was initially described in 1994 [7] and has been confirmed many times in many species including human beings [1]. Likewise, exercise was shown to increase vascular nitric oxide bioavailability [8–10]. Furthermore, we could demonstrate that induction of eNOS by exercise triggers induction of extracellular SOD expression [11]. The dependence of ecSOD up-regulation by vascular nitric oxide was recently confirmed using a transgenic animal and treatment with exogenous nitric oxide [12].

The mechanism of exercise-induced up-regulation of eNOS is multifactorial [1]. Experimental evidence suggest that activation of cSrc [13] and vascular hydrogen peroxide [14] are critically involved. Both conditions are closely related to increased shear stress suggesting that activation of shear stress regulatory *cis*-elements in the promoter of the eNOS gene play an important

*Correspondence to: Georg KOJDA,
Institut fuer Pharmakologie und klinische Pharmakologie,
Heinrich-Heine-Universitaet, Moorenstr. 5,
40225 Duesseldorf, Germany.
Tel.: +49-211-81-12518
Fax: +49-211-81-14781
E-mail: kojda@uni-duesseldorf.de

doi: 10.1111/j.1582-4934.2010.01227.x

role [15]. However, our understanding of the signals initiating up-regulation of arterial eNOS is still incomplete.

All these published data rely on studies in arteries and richly arterialized tissue such as the left ventricular myocardium. In striking contrast, little is known about the effect of exercise in veins and/or low-pressure tissues such as the lung. In an attempt to understand the importance of exercise on venous vascular biology and the underlying mechanisms, we investigated whether exercise increases the expression of eNOS and ecSOD in venous tissue such as vena cava and lung. We used two strikingly different training protocols to further answer the question whether the type of exercise training, *e.g.* short-term high-intensity (forced physical activity) or long-term low-intensity (voluntary running), is important in this context.

Materials and methods

Permission for this study was provided and the experiments were performed according to the guidelines for the use of experimental animals as given by the Deutsches Tierschutzgesetz.

Animals

A total of 60 male C57BL/6 mice, 2–3 months of age were used. All mice were bred at the university's animal facilities in a specified pathogen-free area and were singularized for 8 weeks prior exercise training. Sedentary mice and forced exercise mice lived individually in small cages (0.036 m^2) for the whole experimental period of 12 weeks, voluntary running mice were transferred after 8 weeks of singularization in 0.036 m^2 cages to larger cages and remained there for 4 weeks. These cages offered a floor space of 0.051 m^2 and were supplied with running wheels as described below (Fig. 1A). Groups of sedentary mice, forced exercise mice and voluntary running mice ($n = 8$ each) were used for the initial investigation. Additional 20 male C57BL/6 mice underwent voluntary running to obtain further data in vena cava and 16 male C57BL/6 mice were treated with the catalase inhibitor aminotriazole (Sigma, Munich, Germany) for 6 weeks and 12 underwent voluntary running during the last 4 weeks. All mice were killed and the tissues were snap-frozen in liquid nitrogen.

Exercise training

After a 5 day adaptation training program forced exercise mice were exercised 5 days a week for 30 min. at 0.25 m/sec. for 3 weeks in a self-build exercise treadmill especially designed for mice (Fig. 1B). During exercise, control sedentary mice were placed in small cages at the sides of the training apparatus to expose them to the same stress, noise and vibration. Voluntary running mice underwent training for 3 weeks in running wheels (Fig. 1B) especially designed to record the daily running distance for each mouse (Techniplast, Hohenpeissenberg, Germany). The adaptation to this exercise protocol lasted 1 week (Fig. 1C). Control mice were housed in cages of the same size but without a running wheel. Within 16 to 20 hrs after the last training mice were killed by inhalation of carbon dioxide and the heart weight, body weight, soleus weight and tibia length were determined (Tables 1 and 2).

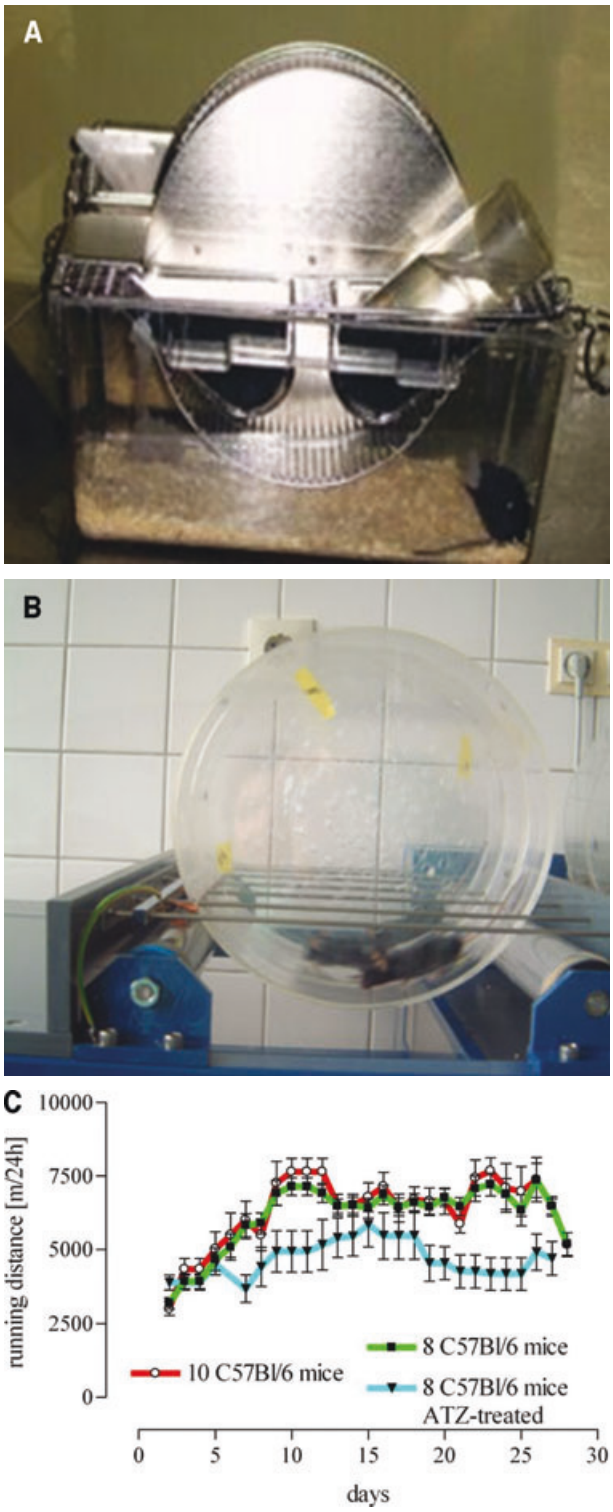


Fig. 1 (**A**) Image of the forced exercise machine and (**B**) a representative cage equipped with running wheels as described previously and (**C**) activity of voluntary running mice expressed as daily running distance (m/24 hrs).

Table 1 Training efficacy as measured results from voluntary running mice compared to sedentary controls

Measure	Sedentary	Voluntary running	*P*-value
HW (g)	0.1391 ± 0.0029	0.1536 ± 0.0024	0.0013
BW (g)	31.50 ± 0.9443	28.61 ± 0.5552	0.0168
SW (g)	0.009567 ± 0.00039	0.01082 ± 0.00029	0.0365
TL (cm)	1.903 ± 0.03142	1.871 ± 0.03736	n.s.
HW/TL (mg/cm)	75.14 ± 1.96	83.60 ± 1.57	0.004
SW/BW (mg/g)	0.2982 ± 0.008	0.3756 ± 0.0094	0.0001
SW/TL (mg/cm)	5.157 ± 0.2	5.871 ± 0.16	0.0132

Values are means ± SE. BW, body weight; HW, heart weight; TL, tibia length; SW, soleus weight.

Table 2 Measures of training efficacy in aminotriazole treated voluntary running mice C57Bl/6 mice compared to sedentary controls

Measure	Sedentary	Voluntary running	*P*-value
HW (g)	0.09180 ± 0.003443	0.1044 ± 0.003434	0.0210
BW (g)	26.57 ± 0.5615	25.76 ± 0.6854	n.s.
SW (g)	0.00926 ± 0.000305	0.01187 ± 0.000327	<0.0001
TL (cm)	1.751 ± 0.02597	1.696 ± 0.02243	n.s.
HW/BW (mg/g)	3.456 ± 0.1129	4.058 ± 0.09696	0.0012
HW/TL (mg/cm)	52.55 ± 2.254	61.60 ± 1.874	0.0080
SW/BW (mg/g)	0.3484 ± 0.009816	0.4618 ± 0.01126	<0.0001
SW/TL (mg/cm)	5.283 ± 0.1470	7.000 ± 0.1598	<0.0001

Values are means ± SE. BW, body weight; HW, heart weight; TL, tibia length; SW, soleus weight.

Measurements of citrate synthase activity

Citrate synthase activity was measured in soleus muscle by the method of Kusnetzov *et al.* [16] as described previously [17]. The samples were measured at 412 nm at 30°C for 5 min. Determination of total protein in the homogenates was done following Bradford method [18] and citrate synthase activity was calculated in U/μg.

Determination of eNOS mRNA expression

Total RNA isolation and real-time measurements were performed as described previously [12]. Briefly, real-time PCR was performed with the TaqMan Gene Expression Assay (Applied Biosystems, Weiterstadt, Germany) for the eNOS gene and the endogenous control hypoxanthine guanine phosphoribosyl transferase 1.

The relative eNOS expression was determined using the $\Delta\Delta C_t$ method. All real-time PCR experiments were carried out on an ABI PRISM 7500 Real PCR System (Applied Biosystems).

Immunoblotting

Western blot analysis was performed as described previously [17], using commercially available monoclonal antibody directed against human eNOS (BD, Heidelberg, Germany), polyclonal antibody against rabbit Ser^{1177} (Santa Cruz, Heidelberg, Germany), polyclonal antibody against rabbit catalase (Calbiochem, Darmstadt, Germany) and goat ecSOD (R&D Systems, Wiesbaden-Nordenstadt, Germany). Blots were subsequently challenged with a horseradish peroxidase-conjugated antibody antimouse IgG (Biorad, Munich, Germany), anti-rabbit antibody (Calbiochem) or donkey anti-goat (Santa Cruz). Western blot signals of eNOS phosphorylation Ser^{1177} were related to total eNOS protein detected by co-incubation as described above. All blots were standardized to polyclonal anti-actin (Sigma). Blots were developed using ECL (Roche, Mannheim, Germany) and exposed to X-ray film and analysed by densitometry (Gel Doc 1000; Bio-Rad, Munich, Germany). Comparative quantitative evaluation was performed with signals appearing on the same blot only.

Measurements of catalase activity

Catalase activity was measured by the method of Cohen *et al.* [19] as described previously [14]. The measurements were performed with 100 × *g* homogenates of the aorta and the lung isolated from untrained C57Bl/6 mice. The absorbance of residual $KMnO_4$ obtained after $KMnO_4$ reduction was measured spectrophotometrically at 480 nm wavelength.

Statistics

All data were analysed by standard computer program (GraphPad Prism PC software, version 3.03) and are expressed as mean ± S.E.M of *n* individual samples. Statistical comparisons between groups were performed by either Student's t-tests or Newman–Keuls multiple comparisons test following one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Efficacy of physical activity

All forced exercised mice (5.149 ± 0.049 mg/g) and all voluntary running mice (5.250 ± 0.128 mg/g) showed a higher heart weight/body weight ratio than sedentary controls (4.706 ± 0.08 mg/g, P = 0.0007, Fig. 2A). There was a significant increase in citrate synthase activity in soleus muscle of forced exercise mice as compared to sedentary control (Fig. 2B). In contrast, citrate synthase activity did not change in voluntary running mice (Fig. 2B), although these mice ran a mean distance of 5.8 km/24 hrs (Fig. 1C). However, other measures for exercise efficacy such as the ratios heart weight/body weight, heart weight/tibia length, soleus weight/body weight and soleus weight/tibia length were increased in this group (Table 1) and in aminotriazole-treated voluntary running mice (Table 2).

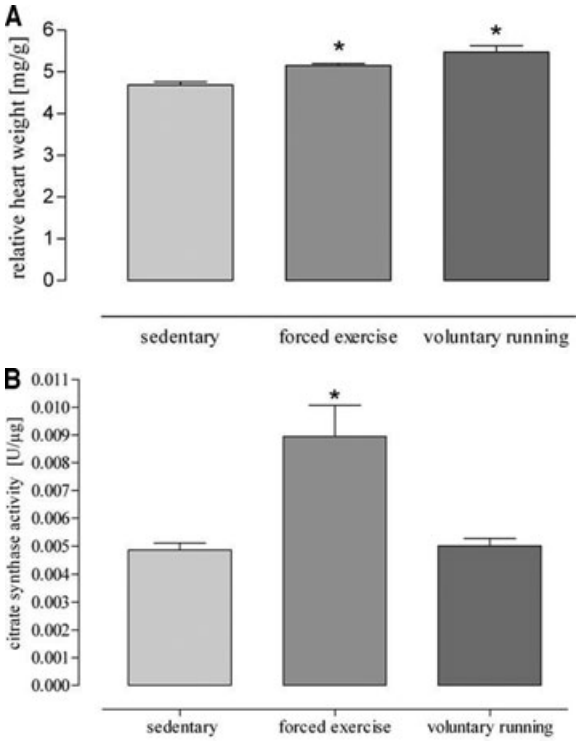


Fig. 2 (**A**) Changes of heart weight body weight ratio induced by both exercise protocols. Forced physical activity and voluntary running induced a significant higher heart weight body weight ratio as compared to sedentary controls ($^{*}P = 0.0007$ *versus* sedentary controls). (**B**) Citrate synthase activity of soleus muscle of sedentary and exercised mice ($^{*}P < 0.0001$ *versus* sedentary controls).

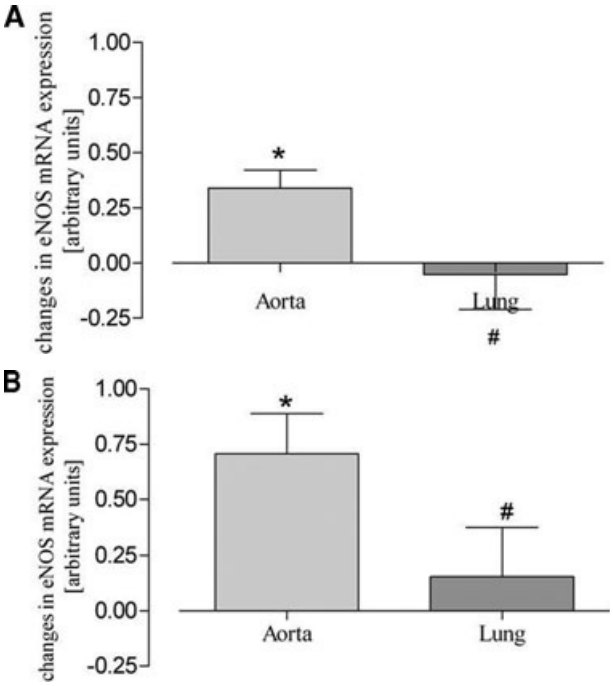


Fig. 3 Real-time mRNA measurement of aortic and lung tissues. Aortic and lung eNOS mRNA expression after (**A**) forced physical activity and (**B**) voluntary running compared to sedentary controls (set to zero) ($^{*}P < 0.05$ *versus* controls, $^{\#}P < 0.05$ *versus* aorta).

Effect of physical activity on eNOS and ecSOD expression in mice

Both training protocols showed a significantly higher expression level of eNOS mRNA compared to both aortic tissue of sedentary mice and lung tissue of exercised mice (Fig. 3). In striking contrast, such changes did not occur in lung tissues (Fig. 3). Likewise, physical activity according to both training protocols significantly increased eNOS protein expression in aorta and heart but not in lung tissue and/or vena cava (Fig. 4). Finally, eNOS phosphorylation at Ser^{1177}, which is an estimate for shear stress, was increased by both training protocols in the aorta, in lung tissue and in vena cava (Fig. 5). These data demonstrate that exercise activates eNOS-Ser^{1177} phosphorylation in venous tissue but initiation of the expression of eNOS does not occur.

To further assess the functional activity of exercise-induced eNOS expression, eNOS-dependent induction of ecSOD was measured [11]. While a higher eNOS expression level was associated with increased ecSOD expression in aortic and heart tissue, there was no change detectable in lung tissue. This was true for both training protocols (Fig. 6). These data suggest that exercise, independently of its type, selectively increases eNOS and subsequently ecSOD expression in arterial but not in venous tissue.

Effect of exercise on catalase expression

According to the results of our previous investigations [14, 20] we determined catalase protein expression levels in vena cava and lung in comparison to aorta and myocardium. In sedentary mice, we found a 4.5-fold higher catalase expression in vena cava compared to aorta and after voluntary running this difference increased to 10.5-fold (Fig. 7). Likewise, in sedentary mice we found a 2.9-fold higher catalase expression in lung tissue compared to aorta and after voluntary running this difference increased to 6.9-fold (Fig. 7). In accordance, catalase activity was higher in lung tissue than in aorta (Fig. 7D). These data suggest that high catalase activity in vena cava and lung tissue compared to aorta reduces the steady state level of venous hydrogen peroxide which in turn may impair exercise-induced up-regulation of eNOS expression.

Effect of aminotriazole treatment on exercise-induced eNOS expression

To challenge the hypothesis mentioned above, catalase was blocked by treating the mice with aminotriazole. During this treatment mice

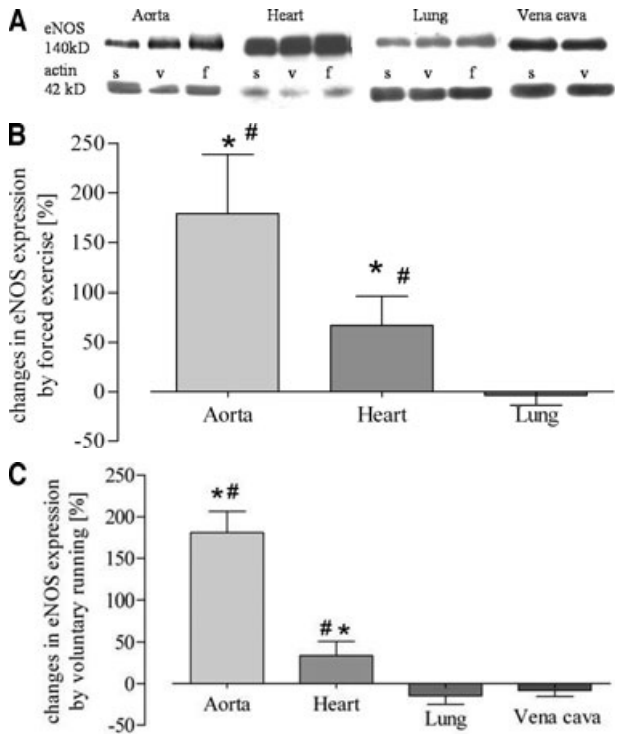


Fig. 4 Quantitative evaluation of eNOS protein expression in the aorta, heart, lung and vena cava. (**A**) Representative Western blot of aorta, heart, lung and vein homogenates from sedentary controls (s), forced exercise (f) and voluntary running mice (v); (**B**) quantitative evaluation of Western blot signals following forced exercise ($^{*}P = 0.0012$ *versus* control) and (**C**) voluntary running ($^{*}P < 0.0001$ *versus* control). Sedentary controls (bars not shown) were set to zero ($^{\#}P < 0.05$ *versus* lung and vein).

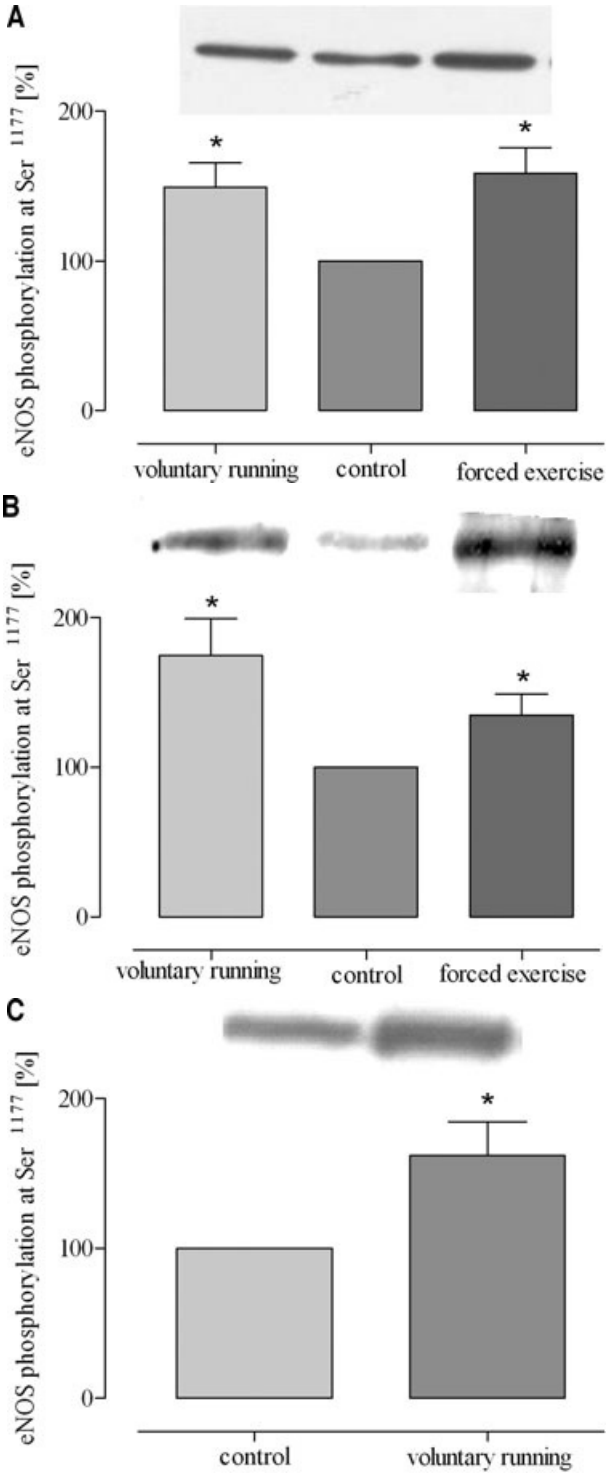


Fig. 5 Phosphorylation of eNOS (eNOS-Ser^{1177}) in response to exercise in (**A**) aorta ($^{*}P = 0.0326$ *versus* control), (**B**) lung tissues ($^{*}P = 0.0113$ *versus* control) and (**C**) vena cava ($^{*}P < 0.05$ *versus* control). Blot signals for controls were set to 100%.

were subjected to voluntary running. Inhibition of catalase blunted the arterio-venous difference and resulted in a significant exercise-induced up-regulation of eNOS in vena cava and lung which equalized the effect in the aorta (Fig. 8B). Furthermore, up-regulation of eNOS in vena cava and lung tissue was associated with augmented ecSOD expression (Fig. 8C) indicating increased NO-bioavailability. These data suggest that physiological catalase activity in vena cava may prevent exercise-induced up-regulation of eNOS.

Discussion

We intended to investigate the effects of two different training protocols on the expression of eNOS and ecSOD in venous and lung tissue and to evaluate the underlying molecular mechanisms. Our major new finding is that – in striking difference to arterial tissue – exercise had no effect on the expression of eNOS and ecSOD in venous tissue such as vena cava and lung. Furthermore, treatment with the catalase inhibitor aminotriazole resulted in up-regulation

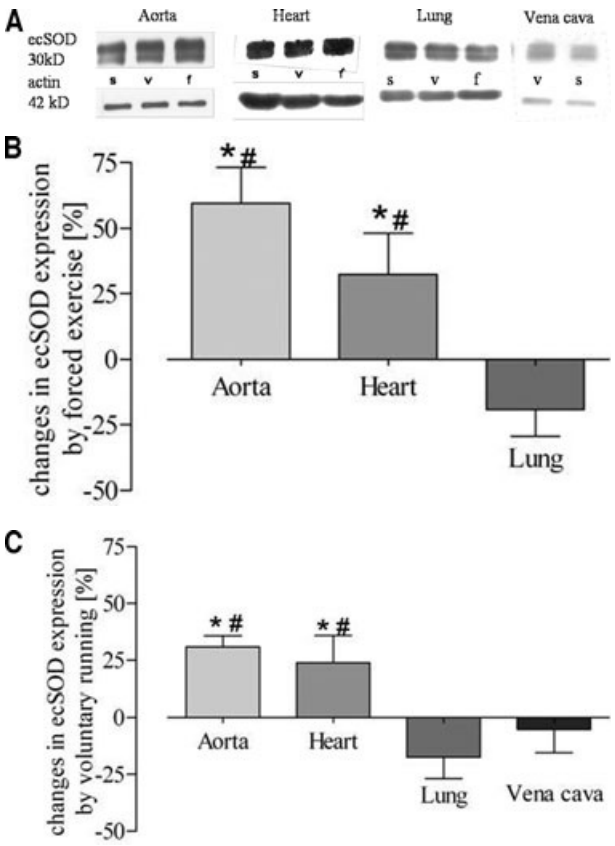


Fig. 6 Quantitative evaluation of ecSOD protein expression in the aorta, heart, lung and vena cava. (**A**) Representative Western blot of aorta, heart, lung and vein homogenates from sedentary controls (s), forced exercise (f) and voluntary running mice (v); (**B**) quantitative evaluation of Western blot signals following forced exercise ($^{*}P = 0.0001$ *versus* control) and (**C**) voluntary running ($^{*}P < 0.0001$ *versus* control). Sedentary controls (bars not shown) were set to zero ($^{\#}P < 0.05$ *versus* lung and vein).

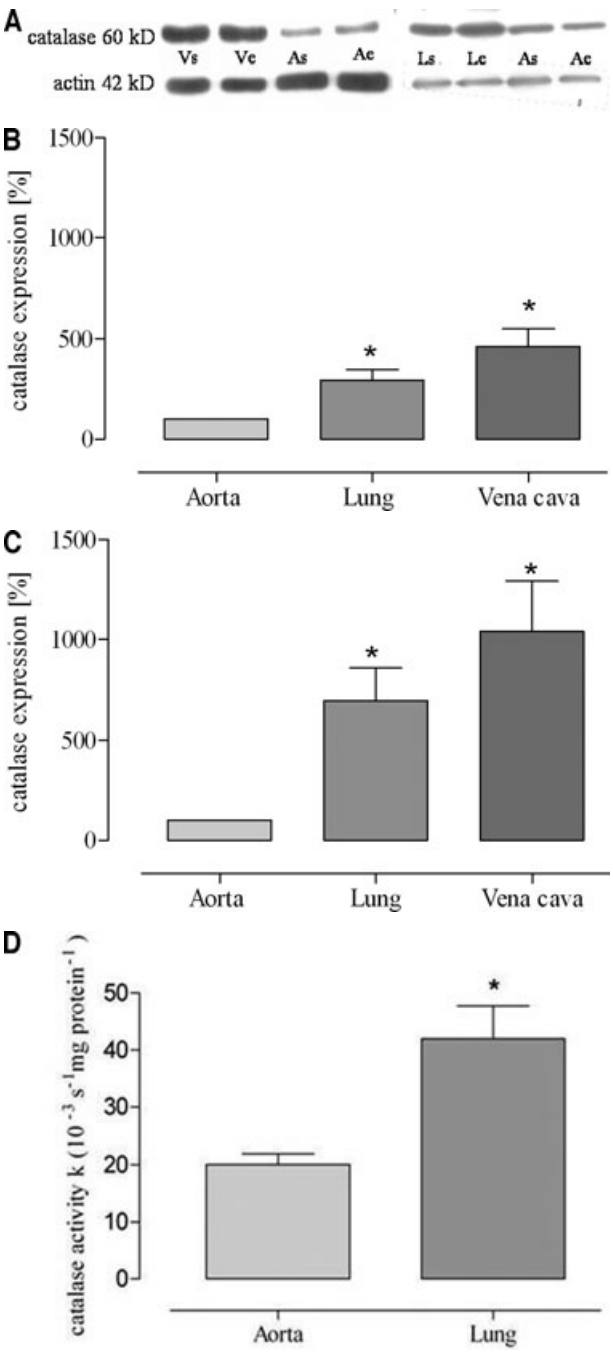


Fig. 7 Expression of catalase protein as (**A**) representative Western blots of vena cava (V), lung (L) and aorta in sedentary (s) and voluntary running mice (e) and as quantitative evaluation in (**B**) sedentary mice ($^{*}P = 0.0066$ *versus* aorta) and (**C**) voluntary running mice ($^{*}P = 0.0093$ *versus* aorta). Catalase activity is shown in aorta and lung homogenates of sedentary mice ($^{*}P = 0.0026$ *versus* aorta) (**D**). Catalase expression in aortic tissues of voluntary running mice compared to sedentary mice was not significantly changed and amounted to 119% ± 24.81 (P = 0.4788).

of eNOS in vena cava tissue. These data suggest that physiological venous catalase activity prevents exercise-induced up-regulation of eNOS and ecSOD.

Previous investigations including those from our lab have shown that physical activity increases the expression of arterial eNOS in animals and human beings [1]. These investigations have been performed almost exclusively in large conduit arteries such as aorta or coronary arteries and some data suggest that this effect does not occur in a similar manner throughout the arterial tree [21]. In striking contrast, little is known about the expression and activity of eNOS in low-pressure vasculature such as vena cava or the lung circulation. In one investigation there was a small increase of eNOS after 1 week of exercise in pulmonary arteries of miniature pigs [22] and another investigation showed similar effects after an acute bout of exercise in rats [23]. Thus, the lack of eNOS up-regulation in the venous circulation following exercise is a new observation.

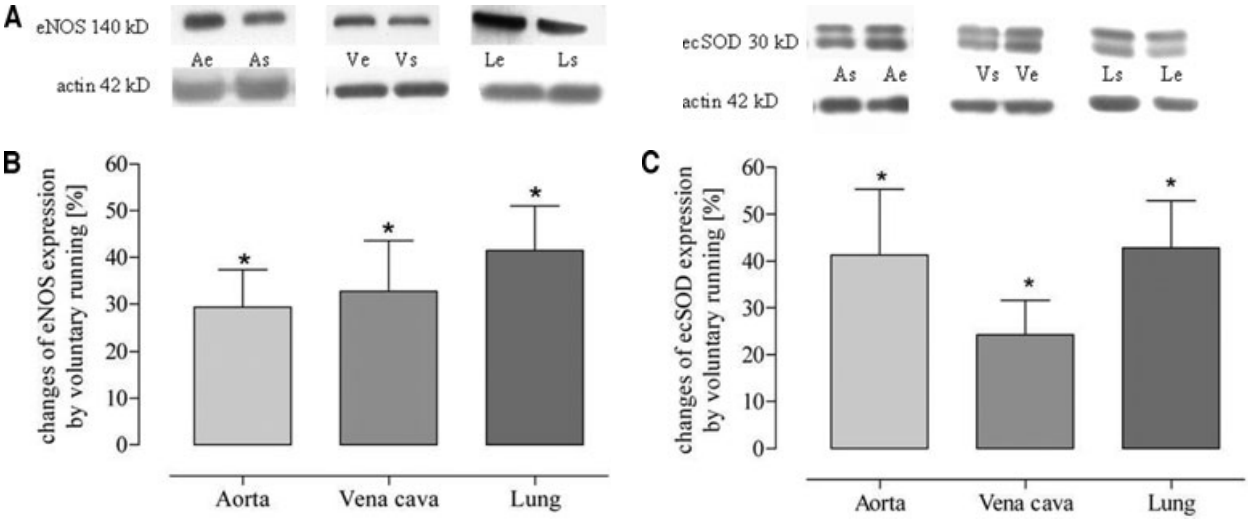


Fig. 8 Effect of the catalase inhibitor aminotriazole on exercise-induced up-regulation of eNOS and ecSOD protein expression in voluntary running mice (**A**) as representative Western blots in isolated aorta (A), lung (L) and vena cava (V); (**B** and **C**) as quantitative evaluation ($^*P < 0.0001$ and $^*P = 0.0021$, respectively) compared to sedentary controls (set to zero).

It has been shown that exercise-induced up-regulation of eNOS is completely inhibited in mice with an endothelium-specific overexpression of catalase [14] suggesting that hydrogen peroxide appears to be an important mediator in this process. Several sources of hydrogen peroxide during exercise may be considered. First, exercise increases shear–stress which has been shown to activate endothelial NADPH oxidase and subsequent superoxide generation [24]. In accordance, Ashton *et al.* demonstrated that exercise induced an increase of a carbon-centred radical in plasma of human beings [25]. Secondly, exercise increases ATP production in skeletal and myocardial muscle during which the coenzyme Q radical can transfer its unpaired electron to molecular oxygen, thereby generating superoxide [26]. In contrast to superoxide itself, hydrogen peroxide generated from non-endothelial superoxide by SODs may reach endothelial cells by diffusion, since the molecule is uncharged. Of note, shear stress is a potent stimulus for the expression of Cu/ZnSOD [27] and exercise has been shown to up-regulate ecSOD in mice [11]. Thus, steady-state levels of hydrogen peroxide likely contribute to the regulation of eNOS expression induced by exercise.

Given this, we have comparatively measured catalase expression and found much more catalase protein in venous tissues such as vena cava and lung. This difference was more pronounced after training, because the expression of catalase increased in the lung, while catalase expression in the aorta and in the heart does not respond to exercise training with a change of catalase expression [14]. In addition, *in vivo* treatment of normal mice with the catalase inhibitor aminotriazole resulted in up-regulation of eNOS in vena cava and this finding strongly supports a crucial role of catalase in preventing exercise-induced up-regulation of eNOS in venous tissue. Aminotriazole has been shown to irreversibly inhibit human erythrocyte catalase activity in the presence of a constant source of hydrogen peroxide [28]. The daily dose of 666 mg/kg body weight which was used in this study is likely effective *in vivo* in mice. A previous study showed that this dose completely corrected hypotension in transgenic mice characterized by a 2.5-fold endothelium-specific overexpression of catalase [29].

Physical forces such as shear are important mediators of exercise-induced regulation of eNOS expression [30]. However, blood flow velocity, which strongly contributes to the magnitude of shear stress on endothelial cells *in vivo*, largely decreases from the aorta to the arterioles and is lowest in veins. Despite this, we could demonstrate that Ser^{1177} phosphorylation of eNOS in vena cava and lung strongly increases in response to exercise suggesting that increased shear is induced by exercise in these tissues. Nevertheless, this increase of the shear signal did not induce eNOS expression in venous tissue.

The up-regulation of eNOS mRNA and protein expression in arterial tissue *in vivo* is associated with increased eNOS activity. This has been demonstrated in animals and human beings, *e.g.* by increased generation of vascular cGMP [8] and by increased coronary flow [9]. In the present investigation we assessed eNOS activity by monitoring the expression of ecSOD as previously described [11]. While exercise-induced up-regulation of ecSOD expression was detected in arterial tissues, there was no ecSOD up-regulation in the lung. This effect is most likely not caused by an insensitivity of venous smooth muscle cells to up-regulate ecSOD in response to exercise, since up-regulation of lung ecSOD was observed in non-exercised trangenic mice carrying an endothelial-specific overexpression of eNOS [12]. Our new data suggest that exercise-induced eNOS expression following inhibition of catalase activity initiates ecSOD expression in venous tissue.

The lack of exercise-induced up-regulation of eNOS and ecSOD in the lung was observed with two different training protocols. Forced physical activity is a short-term high-intensity intervention which might be more efficient than endurance training as indicated by activation of skeletal muscle adaptations [31, 32]. Likewise, we repeatedly detected profound changes in the skeletal muscle such as an increase of citrate synthase activity [17]. In contrast, such changes were absent following voluntary running which is a long-term low intensity training protocol consisting of short bouts of 2–3 min. for a total of 100–120 bouts per night [33]. The absence of increased citrate synthase activity following voluntary training of mice has been observed by other investigators as well [34] and many aspects such as the total running distance and changes of heart weight/body weight ratio, soleus weight/body weight ratio and soleus weight/tibia length ratio closely matches previously reported results and prove the efficacy of this training protocol [33–35]. Despite these considerations, there was no effect of the training protocol on the observed changes of arterial and venous expression of eNOS and ecSOD.

Our data might be helpful to explain why pulmonary rehabilitation programs do not improve lung mechanics and gas exchange, while exercise improves arterial functions such as endothelium-dependent vasodilation and organ perfusion [9, 36]. In view of the significance of lung perfusion for blood arterialization, exercise-induced up-regulation of eNOS in the pulmonary circulation could theoretically improve pulmonary gas exchange. Furthermore, up-regulation of eNOS might reduce the transmural pressure of pulmonary blood vessels by shear-induced endothelium-dependent vasodilation and this might have a protective effect on the blood gas barrier [37]. According to our data, it might be possible to achieve exercise-induced up-regulation of eNOS in pulmonary vessels by therapeutic inhibition of vascular catalase. This would be accomplished by the expense of hypocatalasemia, though. Using such a pharmacological approach, *i.e.* a pill that works with exercise only [38], might be associated with some side effects which might accelerate cardiovascular disease by increasing vascular oxidative stress and accelerating atherosclerosis. Data from patients with acatalesemia suggest that a complete loss of catalase activity might be associated with a higher incidence of diabetes mellitus, high levels of blood lipids and hyperhomocysteinemia [39]. Furthermore, oral gangrene and ulceration (Takahara disease) might occur. However, in the rare cases of acatalesemia reported so far, only about half of the affected individuals have mild symptoms and the comparison of patients and their non-affected relatives showed no change in life-span, despite the increased risk of diabetes mellitus and arteriosclerosis [39].

Acknowledgement

This study was supported by the Forschungskommission of the Heinrich-Heine-Universitaet Duesseldorf (Projects 9772408 and 9772345).

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Kojda G, Hambrecht R**. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res*. 2005; 67: 187–97.
2. **Gielen S, Schuler G, Hambrecht R**. Exercise training in coronary artery disease and coronary vasomotion. *Circulation*. 2001; 103: E1–6.
3. **Prior BM, Yang HT, Terjung RL**. What makes vessels grow with exercise training? *J Appl Physiol*. 2004; 97: 1119–28.
4. **Brown MD, Hudlicka O**. Modulation of physiological angiogenesis in skeletal muscle by mechanical forces: involvement of VEGF and metalloproteinases. *Angiogenesis*. 2003; 6: 1–14.
5. **Green DJ, Maiorana A, O'Driscoll G, *et al***. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol*. 2004; 561: 1–25.
6. **Bowles DK, Wamhoff BR**. Coronary smooth muscle adaptation to exercise: does it play a role in cardioprotection? *Acta Physiol Scand*. 2003; 178: 117–21.
7. **Sessa WC, Pritchard K, Seyedi N, *et al***. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res*. 1994; 74: 349–53.
8. **Kojda G, Cheng YC, Burchfield J, *et al***. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation*. 2001; 103: 2839–44.
9. **Hambrecht R, Wolf A, Gielen S, *et al***. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *N Engl J Med*. 2000; 342: 454–60.
10. **Hambrecht R, Adams V, Erbs S, *et al***. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation*. 2003; 107: 3152–8.
11. **Fukai T, Siegfried MR, Ushio-Fukai M, *et al***. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest*. 2000; 105: 1631–9.
12. **Oppermann M, Balz V, Adams V, *et al***. Pharmacologic induction of vascular extracellular superoxide dismutase expression *in-vivo*. *J Cell Mol Med*. 2009; 13: 1271–8.
13. **Davis ME, Cai H, McCann L, *et al***. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. *Am J Physiol Heart Circ Physiol*. 2003; 284: H1449–53.
14. **Lauer N, Suvorava T, Rüther U, *et al***. Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase. *Cardiovasc Res*. 2005; 65: 254–62.

15. **Fleming I, Busse R**. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol*. 2003; 284: R1–12.
16. **Kusnetzov A, Lassnig B, Gnaiger E**. Laboratory protocol – citrate synthase, mitochondrial marker enzyme. *Mitochondrial Physiology Network*. 2003; 8.14: 1–7, www.oroboros.at.
17. **Suvorava T, Lauer N, Kojda G**. Physical inactivity causes endothelial dysfunction in healthy young mice. *J Am Coll Cardiol*. 2004; 44: 1320–7.
18. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72: 248–54.
19. **Cohen G, Dembiec D, Marcus J**. Measurement of catalase activity in tissue extracts. *Anal Biochem*. 1970; 34: 30–8.
20. **Suvorava T, Kumpf S, Rauch BH, *et al*.** Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity. *Free Radic Res*. 2010; 44: 199–207.
21. **Laughlin MH, Rubin LJ, Rush JW, *et al*.** Short-term training enhances endothelium-dependent dilation of coronary arteries, not arterioles. *J Appl Physiol*. 2003; 94: 234–44.
22. **Johnson LR, Rush JW, Turk JR, *et al*.** Short-term exercise training increases ACh-induced relaxation and eNOS protein in porcine pulmonary arteries. *J Appl Physiol*. 2001; 90: 1102–10.
23. **Miyauchi T, Maeda S, Iemitsu M, *et al*.** Exercise causes a tissue-specific change of NO production in the kidney and lung. *J Appl Physiol*. 2003; 94: 60–8.
24. **Laurindo FR, Pedro Mde A, Barbeiro HV, *et al*.** Vascular free radical release: *ex vivo* and *in vivo* evidence for a flow-dependent endothelial mechanism. *Circ Res*. 1994; 74: 700–9.
25. **Ashton T, Rowlands CC, Jones E, *et al*.** Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol*. 1998; 77: 498–502.
26. **Finkel T, Holbrook NJ**. Oxidants, oxidative stress and the biology of ageing. *Nature*. 2000; 408: 239–47.
27. **Inoue N, Ramasamy S, Fukai T, *et al*.** Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *Circ Res*. 1996; 79: 32–7.
28. **Ou P, Wolff SP**. Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide *in vitro*. *Biochem Pharmacol*. 1993; 46: 1139–44.
29. **Suvorava T, Lauer N, Kumpf S, *et al*.** Endogenous vascular hydrogen peroxide regulates arteriolar tension *in vivo*. *Circulation*. 2005; 112: 2487–95.
30. **Uematsu M, Ohara Y, Navas JP, *et al*.** Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol Cell Physiol*. 1995; 269: C1371–8.
31. **Gibala MJ, Little JP, van Essen M, *et al*.** Short-term sprint interval *versus* traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol*. 2006; 575: 901–11.
32. **Gibala MJ**. Molecular responses to high-intensity interval exercise. *Appl Physiol Nutr Metab*. 2009; 34: 428–32.
33. **De Bono JP, Adlam D, Paterson DJ, *et al*.** Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290: R926–34.
34. **Noble EG, Moraska A, Mazzeo RS, *et al*.** Differential expression of stress proteins in rat myocardium after free wheel or treadmill run training. *J Appl Physiol*. 1999; 86: 1696–701.
35. **Laufs U, Werner N, Link A, *et al*.** Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation*. 2004; 109: 220–6.
36. **Casaburi R, ZuWallack R**. Pulmonary rehabilitation for management of chronic obstructive pulmonary disease. *N Engl J Med*. 2009; 360: 1329–35.
37. **West JB**. Comparative physiology of the pulmonary blood-gas barrier: the unique avian solution. *Am J Physiol Regul Integr Comp Physiol*. 2009; 297: R1625–34.
38. **Goodyear LJ**. The exercise pill–too good to be true? *N Engl J Med*. 2008; 359: 1842–4.
39. **Goth L, Rass P, Pay A**. Catalase enzyme mutations and their association with diseases. *Mol Diagn*. 2004; 8: 141–9.

J. Cell. Mol. Med. Vol 13, No 7, 2009 pp. 1271-1278

Pharmacological induction of vascular extracellular superoxide dismutase expression *in vivo*

Marc Oppermann [a, #], Vera Balz [b, #], Volker Adams [c], Vu Thao-Vi Dao [a], Murat Bas [d], Tatsiana Suvorava [a], Georg Kojda [a, *]

[a] *Institute for Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Duesseldorf, Germany*
[b] *Otorhinolaryngology Clinic, Heinrich-Heine-University, Duesseldorf, Germany*
[c] *Heart Center Leipzig, Clinic for Internal Medicine/Cardiology Leipzig, Germany*
[d] *Hals-Nasen-und Ohrenklinik rechts der Isar, Technische Universitaet Munich, Germany*

Received: September 12, 2008; Accepted: December 11, 2008

Abstract

Pentaerythritol tetranitrate (PETN) treatment reduces progression of atherosclerosis and endothelial dysfunction and decreases oxidation of low-density lipoprotein (LDL) in rabbits. These effects are associated with decreased vascular superoxide production, but the underlying molecular mechanisms remain unknown. Previous studies demonstrated that endogenous nitric oxide could regulate the expression of extracellular superoxide dismutase (ecSOD) in conductance vessels *in vivo*. We investigated the effect of PETN and overexpression of endothelial nitric oxide synthase ($eNOS^{++}$) on the expression and activity of ecSOD. C57BL/6 mice were randomized to receive placebo or increasing doses of PETN for 4 weeks and $eNOS^{++}$ mice with a several fold higher endothelial-specific eNOS expression were generated. The expression of ecSOD was determined in the lung and aortic tissue by real-time PCR and Western blot. The ecSOD activity was measured using inhibition of cytochrome C reduction. There was no effect of PETN treatment or eNOS overexpression on ecSOD mRNA in the lung tissue, whereas ecSOD protein expression increased from 2.5-fold to 3.6-fold ($P < 0.05$) by 6 mg PETN/kg body weight (BW)/day and 60 mg PETN/kg BW/day, respectively. A similar increase was found in aortic homogenates. $eNOS^{++}$ lung cytosols showed an increase of ecSOD protein level of 142 $\pm$ 10.5% as compared with transgene-negative littermates ($P < 0.05$), which was abolished by N^{ω}-nitro-L-arginine treatment. In each animal group, the increase of ecSOD expression was paralleled by an increase of ecSOD activity. Increased expression and activity of microvascular ecSOD are likely induced by increased bioavailability of vascular nitric oxide. Up-regulation of vascular ecSOD may contribute to the reported antioxidative and anti-atherosclerotic effects of PETN.

Keywords: endothelial nitric oxide synthase • extracellular superoxide dismutase • nitric oxide • pentaerythritol tetranitrate

Introduction

Nitric oxide exhibits a variety of anti-atherogenic effects such as vasodilation, anti-aggregation, anti-apoptosis, anti-adhesion, anti-proliferation and antioxidation [1]. In common conditions such as hypertension, coronary artery disease and type 2 diabetes, the bioavailability of nitric oxide is reduced, as demonstrated by blunted endothelium-dependent vasodilation [2]. Such endothelial dysfunction is likely a consequence of increased vascular oxidative stress, a condition characterized by a misbalance of endogenous production of vascular reactive oxygen species (ROS) and the vascular antioxidative capacity. Although a variety of mediators have been described to contribute to vascular oxidative stress, both the generation and the detoxification of superoxide most likely play a major role in this process.

Superoxide is generated as a metabolic by-product, for example, in the mitochondria, by cytochrome P450 (CYP) oxygenases, by xanthine oxidase, by nitric oxide synthases (NOS) and particularly by NAD(P)H-oxidases [3]. It is also produced as a signalling molecule for certain cellular processes such as hypertrophy, smooth muscle cell growth, Ras signalling and $NF\kappa B$ activation [4]. There are highly specific mechanisms to rapidly detoxify superoxide and thereby prevent unwanted oxidative processes in

[#]Both authors contributed equally to this work.
*Correspondence to: Georg KOJDA, Pharm.D., Ph.D.,
Institut fuer Pharmakologie und Klinische Pharmakologie,
Heinrich-Heine-Universitaet, Moorenstr. 5,
40225 Duesseldorf, Germany.
Tel.: +49-211-81-12518
Fax.: +49-211-81-14781
E-mail: kojda@uni-duesseldorf.de

doi:10.1111/j.1582-4934.2008.00627.x

cells and tissues. Of these, the superoxide dismutases I (CuZnSOD), II (MnSOD) and III (extracellular SOD [ecSOD]) are of utmost importance. Although CuZnSOD and MnSOD provide intracellular and intramitochondrial protection against superoxide, respectively, ecSOD does so in the interstitium. It is expressed in vascular smooth muscle cells and is subsequently secreted to the interstitium [5]. It binds to polyanionic sites such as heparin sulphates at the outer membrane of endothelial cells and likely protects endothelial nitric oxide while it traverses to the smooth muscle cell layer of the vascular wall [6]. The highest ecSOD concentrations are found between the endothelial cell layer and the smooth muscle, and 99% of ecSOD is tissue-bound.

Previous investigations have shown that endogenous nitric oxide is an essential stimulus of ecSOD expression in large conductance vessels such as the aorta, and that an increase of endothelial (e)NOS expression by exercise results in a consecutive overexpression of ecSOD [7]. Hence, driving the expression of ecSOD appears to be an important mechanism underlying the antioxidative effects of nitric oxide. We have also found in several studies that the nitric oxide donors such as pentaerythritol tetranitrate (PETN) [8, 9] and isosorbide mononitrate [10] exert substantial anti-atherosclerotic effects in rabbits, as demonstrated by a reduction in aortic plaque load, an improvement of endothelial function and a reduction of vascular superoxide production. One experimental study reported that PETN increases vascular haemoxygenase expression by approximately 1.5-fold, and it is likely that such a mechanism may play a role in the anti-atherosclerotic effects of PETN [11].

The aim of this study was to investigate whether PETN can increase ecSOD expression and activity in the microvasculature and whether such effects are induced by endogenous nitric oxide, as generated by endothelial-specific overexpression of eNOS as well.

Materials and methods

C57BL/6 mice

A total of 39 C57BL/6 male mice were randomly assigned to three groups and were treated with placebo (PETN-0) and 6 mg (PETN-6) or 60 (PETN-60) mg oral PETN/kg BW/day for 4 weeks. PETN was provided by Actavis, Langenfeld, Germany. Plasma concentrations of pentaerythritol dinitrate (PEDN) and pentaerythritol mononitrate (PEMN) were determined by gas chromatography/mass spectrometry (GC/MS; HP6890; Hewlett-Packard, Boelingen, Germany) after liquid–liquid extraction with ethyl acetate, as described previously [10].

Transgenic eNOS^{++} mice

Bovine eNOS complementary DNA (cDNA; 4.1 kb) was inserted between the murine Tie-2 promoter (2.1 kb) cDNA and a 10-kb Tie-2 intron fragment, designated as Tie-2-enhancer, and this construct was used to target eNOS gene expression to the vasculature, as described previously [12, 13]. Founder mice showing high eNOS expression (eNOS^{++}) compared with controls were crossed eight times to C57BL/6 mice to generate a C57BL/6 background. The mice were used at 12–16 weeks of age. Transgene-negative littermates (eNOS^{n}) served as controls. Permission for the animal studies was provided by the regional government of Germany, and the experiments were performed according to the guidelines for the use of experimental animals, as given by the German "Tierschutzgesetz" and the "Guide for the Care and Use of Laboratory Animals" of the U.S. National Institutes of Health.

Measurement of blood pressure

Systolic blood pressure and heart rate were measured in awake male eNOS^{++} ($n = 6$) and eNOS^{n} ($n = 6$) mice at 3–4 months of age using an automated tailcuff system (Visitech Systems, Apex, NC, USA), as described previously [14]. On day 6, the mice received N$^{\omega}$-nitro-L-arginine (L-NA, Sigma, Munich, Germany) with the drinking water (100 mg L-NA/kg BW/day), and blood pressure measurement was continued for a maximum of 30 days. Blood pressure of PETN-treated mice was measured from the day before treatment until 6 days after treatment.

Determination of ecSOD mRNA concentration

Total RNA was isolated from snap-frozen lung tissue with the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNAse digestion. Complementary DNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. A primer/probe set directed to amplify an 82-bp fragment overlapping the exon2/exon3 boundary was designed with the online software provided by Qiagen (http://www.qiagen.com) and it revealed the following primers: sense: 5′-CTGACAGGTGCAGAGAA-3′, antisense: 5′-ACATGGTGACAGAGCCACA-3′, probe: 5′-CTACGGCTTGC-TACTGG-3′. For normalization purposes, coamplification of hypoxanthine guanine phosphoribosyl transferase (HPRT) cDNA was performed. The probes for ecSOD and HPRT were labelled with 6-carboxy-fluorescein and Yakima Yellow, respectively. The relative ecSOD expression was determined using the $\Delta\Delta C_t$ method. A second set of normalization experiments was based on 18S rRNA or smooth muscle actin (SMA) using 18S rRNA: 5′-ATACAGGACTCTTTCGAGGCCC-3′ and 5′-CGGGACACTC AGCTAAGAG-CAT-3′ annealing at 61°C; SMA-U: 5′-AGAGCAAGAGAGGGATCCTGA-3′ and SMA-L: 5′-GTCGTCCCAGTTGGTGATGAT-3′ annealing at 57°C. All real-time PCR experiments were carried out on an ABI PRISM 7900HT Sequence Detector System (Applied Biosystems, Weiterstadt, Germany).

Western blot

Preparation of cytosols from mouse lungs was performed as described previously [15]. The tissues were flash frozen in liquid nitrogen and stored until use at –80°C. The supernatants of mouse lung and aortic homogenates (10 μg of total protein per lane) as well as blood plasma (10 and 20 μg) were fractionated on denaturing 12% polyacrylamide gels, blotted on PVDF membranes (Protrans; Schleicher & Schuell, Berlin, Germany) and stained with a monoclonal antibody (R&D Systems, Wiesbaden, Germany) directed against ecSOD. Actin staining served as a loading control (polyclonal anti-actin antibody; Sigma, Munich, Germany), and autoradiographs were analysed by densitometry. For confirmation

purpose, Western blot analyses were repeated with polyclonal antibodies (Biomol GmbH, Stressgen, Hamburg, Germany; and R&D Systems, Wiesbaden, Germany). Western blots for eNOS in mouse crude aortic homogenates were done with a monoclonal eNOS antibody (Transduction Lab, BD Biosciences, Heidelberg, Germany).

Measurement of ecSOD activity

The lungs were homogenized in 50 mM potassium phosphate (pH 7.4) containing 0.3 mol/l potassium bromide, as described previously [16]. Chromatography of homogenates on concanavalin A sepharose (GE Healthcare, Uppsala, Sweden) has been used to separate ecSOD from other SOD isoenzymes [16]. The SOD activity was determined spectrophotometrically by monitoring the inhibition of the rate of xanthine oxidase-mediated reduction of cytochrome C, as described previously [17]. The effectiveness of ecSOD separation was confirmed by Coomassie native gel staining and Western blot analysis. The lungs from 6 individual animals were pooled and two separate pools were analysed.

Statistics

All data were analysed by a standard computer program (GraphPad Prism PC software, version 3.03, Graphpad Software, La Jolla, CA, USA) and are expressed as mean ± S.E.M. of *n* individual samples. Statistical comparisons between the groups were performed by either the t-tests or the Newman–Keuls multiple comparisons test following one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

PETN metabolites and blood pressure data

The plasma concentrations of PETN metabolites detectable by GC/MS, PEDN and PEMN, rose with the daily oral dose of PETN. PEMN increased from 167.0 ± 104.8 ng/ml (PETN-6, $n = 3$) to 242.0 ± 48.4 ng/ml (PETN-60, $n = 4$, $P = 0.5059$), and PEDN from 17.3 ± 3.3 ng/ml (PETN-6, $n = 3$) to 120.8 ± 32.7 ng/ml (PETN-60, $n = 4$, $P = 0.0444$), indicating successful PETN resorption. Six days of treatment with PETN had no effect on blood pressure in awake mice. In PETN-6 mice ($n = 4$), the initial blood pressure of 121.8 ± 1.4 mmHg remained unchanged after treatment (119.1 ± 7.7 mmHg, $P = 0.7414$). Similar results were obtained before (125.0 ± 4.6 mmHg) and after (114.1 ± 8.3 mmHg, $P = 0.2915$) treatment in PETN-60 mice ($n = 4$; Fig. 1A). These data suggest that PETN, even at doses exceeding therapeutical doses by 20-fold, had no effect on resting blood pressure in mice.

Blood pressure and eNOS expression in eNOS-overexpressing mice

Systolic blood pressure in $eNOS^{++}$ mice was reduced (Fig. 1B; $P = 0.0126$, $n = 4$). Treatment with the eNOS-inhibitor L-NA increased blood pressure in both $eNOS^{n}$ and $eNOS^{++}$ mice, and the difference between the two groups disappeared after 25 days of L-NA treatment ($P = 0.4407$, $n = 4$). Western blot analysis showed increased expression of aortic eNOS protein in $eNOS^{++}$ mice of 2.8 ± 0.5-fold ($P = 0.0369$, $n = 4$; Fig. 1C).

ecSOD mRNA levels

The mRNA expression of lung ecSOD relative to HPRT did not show significant changes in the PETN-treated groups compared with the PETN-0-treated groups (each $n = 6$, $P = 0.7558$, ANOVA; Fig. 2A). In $eNOS^{++}$ mice, ecSOD mRNA expression (0.522 ± 0.080) was not significant different from $eNOS^{n}$ mice (0.637 ± 0.156, $n = 6$, $P = 0.5238$; Fig. 2B). Likewise, $eNOS^{++}$ aortic ecSOD mRNA levels were unchanged. Similar results on aortic ecSOD mRNA were obtained after treatment with L-NA and when SMA and 18S rRNA were used as standards (Fig. 2C).

ecSOD protein expression

PETN increased ecSOD protein expression in the lungs to 251 ± 91.0% ($n = 5$) in the PETN-6 group and to 362 ± 84.9% ($n = 6$) in the PETN-60 group ($n = 6$, $P = 0.0479$ *versus* PETN-0; Fig 3A). A comparable result was obtained with a polyclonal ecSOD antibody (data not shown). A *post-hoc* analysis revealed statistical significance for PETN-60 *versus* PETN-0 but not for PETN-6 *versus* PETN-0 (Fig. 3A). Similar data were obtained in the aortas of the PETN-treated mice (Fig. 3B). The expression in PETN-6 mice was 152 ± 18.9% ($n = 6$) and that in PETN-60 was 207 ± 34.7% ($n = 5$) as compared with PETN-0 mice ($P = 0.0116$, for ANOVA; * = $P < 0.01$ for PETN-0 *versus* PETN-60, *post-hoc* analysis).

The lung tissue of $eNOS^{++}$ mice showed increased ecSOD protein as well (142 ± 10.5%, $n = 5$, $P < 0.05$ *versus* $eNOS^{n}$). Treatment with L-NA strongly reduced ecSOD expression ($P = 0.0003$, ANOVA; Fig. 3C) to 59.8 ± 18.6% in $eNOS^{n}$ mice ($n = 6$, $P < 0.001$ *versus* untreated) and to 44.4 ± 12.0% in $eNOS^{++}$ mice ($n = 6$, $P < 0.001$ *versus* untreated). Furthermore, the difference in ecSOD protein expression disappeared (Fig. 3C; $P > 0.05$ for $eNOS^{++}$/L-NA *versus* $eNOS^{n}$/L-NA). Additionally, $eNOS^{++}$ mice showed increased ecSOD blood plasma levels of 122 ± 5.6% ($n = 3$, $P = 0.0021$) compared with $eNOS^{n}$ mice (Fig. 3D).

ecSOD activity

Changes in ecSOD activity paralleled changes in ecSOD expression ($n = 7$, $P < 0.0001$, ANOVA; Fig 4A). In the lungs of the PETN-treated mice, the ecSOD activity was 24.2 ± 2.23 U/mg (PETN-6, $n = 7$, $P < 0.05$, *post-hoc* analysis) and 37.3 ± 2.77 U/mg (PETN-60, $n = 6$, $P < 0.01$, *post-hoc* analysis) compared with the ecSOD activity of 16.1 ± 1.95 U/mg in PETN-0 mice. Likewise, the ecSOD activity was significantly increased in $eNOS^{++}$ mice (63.5 ± 4.24 U/mg, $n = 6$) compared with $eNOS^{n}$ mice (51.3 ± 2.95 U/mg, $n = 6$, $P = 0.0401$; Fig. 4B).

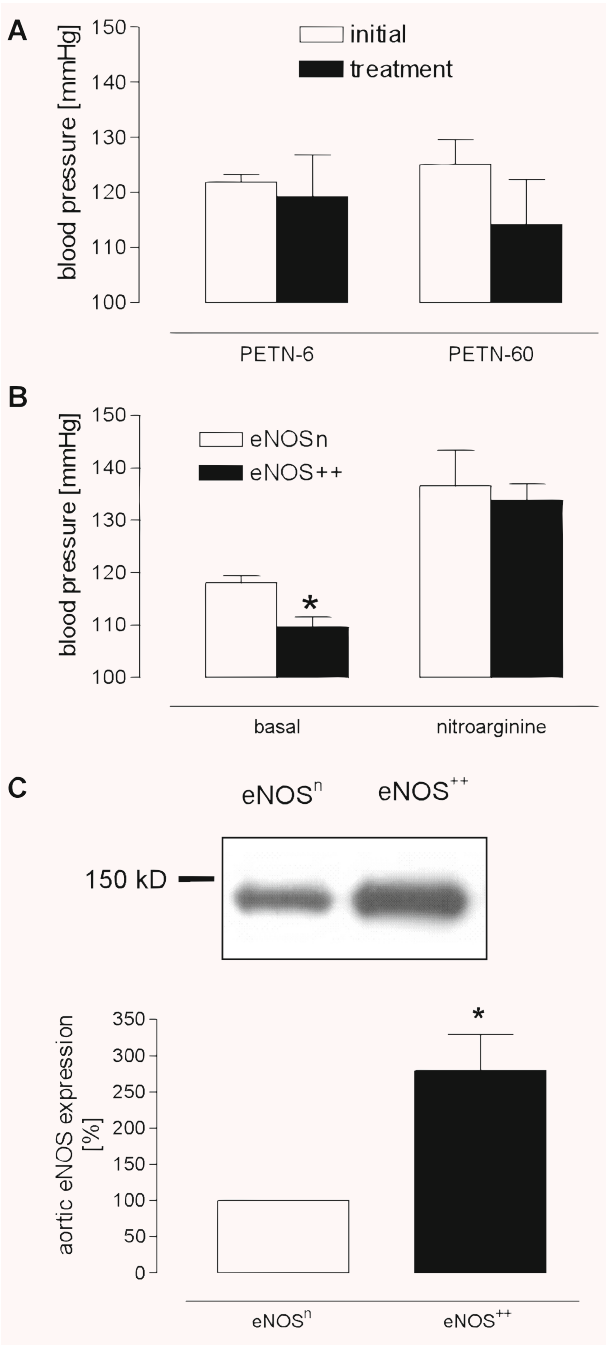


Fig. 1 eNOS and blood pressure in eNOS-overexpressing mice. (**A**) Blood pressure in C57Bl/6 mice before (initial) and after treatment with PETN (not significant). (**B**) Untreated eNOS^{++} mice have a significant lower blood pressure than eNOS^{n} (* = $P < 0.05$). After 25 days of treatment with the eNOS inhibitor L-NA, this difference disappeared ($P > 0.05$). (**C**) Upper panel: Western blot for eNOS in aortic homogenates of eNOS^{++}; lower panel ($n = 4$): a mean of 2.8 ± 0.5-fold greater eNOS expression than in eNOS^{n} ($n = 4$, * = $P < 0.05$).

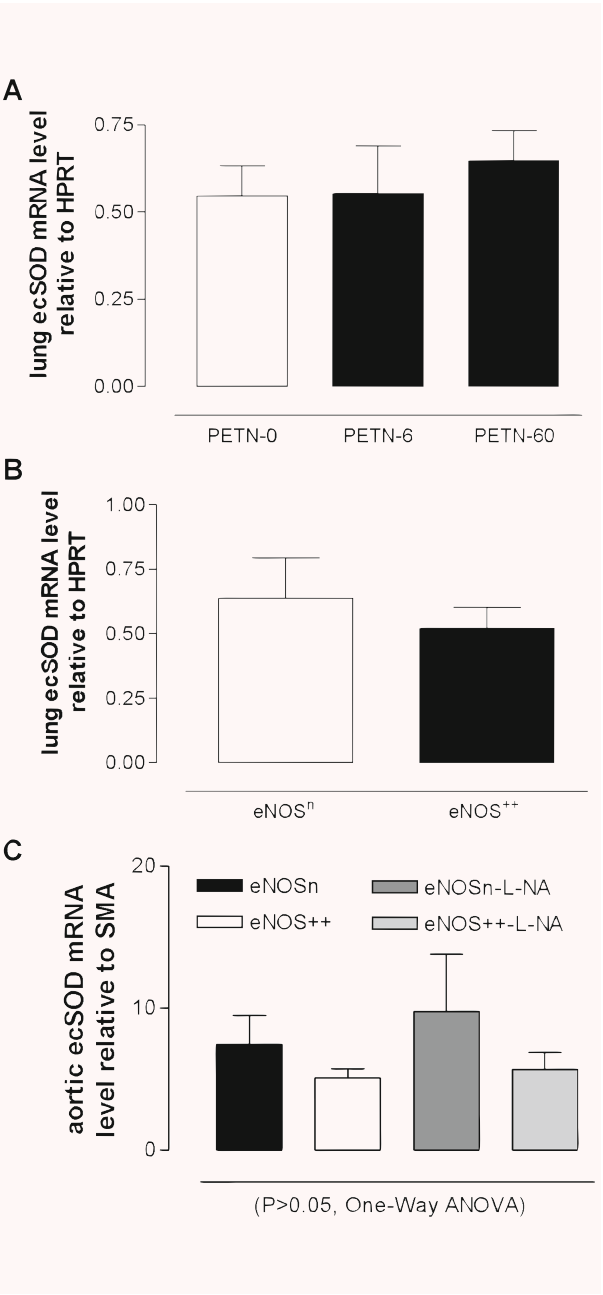


Fig. 2 ecSOD mRNA levels. (**A**) Treatment of C57Bl/6 mice with 6 mg/kg/day (PETN-6) or 60 mg/kg/day (PETN-60) PETN induced no changes in lung ecSOD mRNA expression relative to HPRT ($P > 0.05$ *versus* PETN-0). (**B**) eNOS^{++} mice induced no changes in lung ecSOD mRNA expression relative to HPRT ($P > 0.05$ *versus* eNOS^{n}). (**C**) eNOS^{++} mice induced no changes in aortic ecSOD mRNA expression relative to SMA ($P > 0.05$ *versus* eNOS^{n}), irrespective of oral treatment with L-NA.

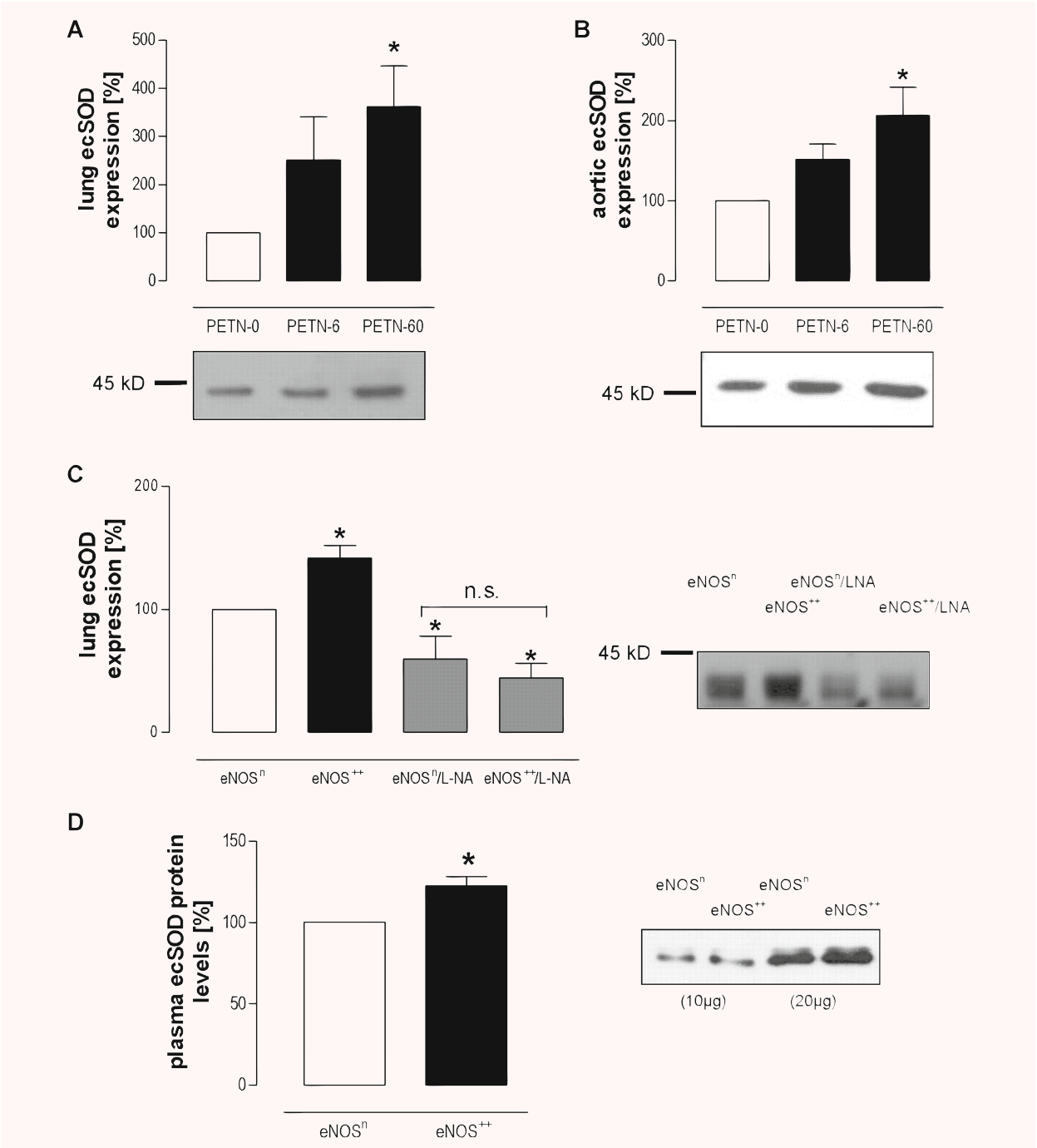


Fig. 3 ecSOD protein expression. (**A**) Increased ecSOD protein expression in lung cytosolic fractions of C57Bl/6 mice treated with 0, 6 or 60 mg/kg/day PETN (PETN-0, PETN-6 or PETN-60, respectively, * = $P < 0.05$ for PETN-60 *versus* PETN-0). (**B**) Increased ecSOD protein expression in aortic homogenates of PETN-treated mice (* = $P < 0.01$ *versus* PETN-0). (**C**) Increased ecSOD protein expression in lung cytosols of $eNOS^{++}$ *versus* $eNOS^{n}$ mice ($P < 0.05$). Treatment with L-NA significantly lowered ecSOD expression in both groups ($eNOS^{n}$/L-NA and $eNOS^{++}$/L-NA; each * = $P < 0.05$ *versus* $eNOS^{n}$) and blunted the difference between both groups (N.S. = $P > 0.05$). (**D**) ecSOD protein in blood plasma of $eNOS^{++}$ mice was significantly increased compared with transgene-negative littermates ($eNOS^{n}$, * = $P < 0.01$).

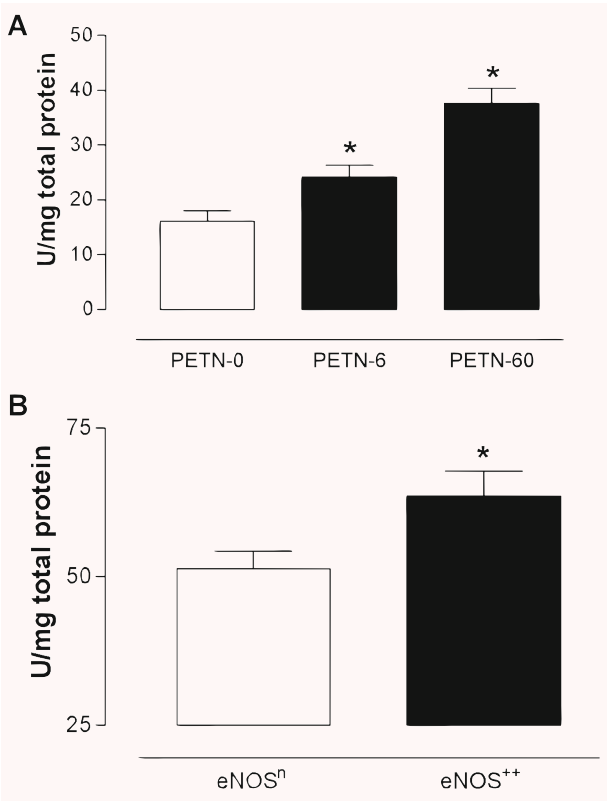


Fig. 4 ecSOD activity. (**A**) The activity of extracellular superoxide dismutase (ecSOD) in lung tissue of eNOS^{++} mice was significantly increased compared with eNOS^{n} mice (* = $P < 0.05$). (**B**) Treatment of C57Bl/6 mice with 6 or 60 mg/kg/day pentaerythritol tetranitrate (PETN-6 or PETN-60, respectively) similarly resulted in increased activities compared with control mice (PETN-0). * = $P < 0.05$ *versus* PETN-0.

Discussion

The aim of this study was to investigate whether vascular expression of ecSOD is subject to genetic and pharmacological regulation. Our findings are that the organic nitrate PETN increases the expression and activity of microvascular and macrovascular ecSOD in mice *in vivo*, and that a similar increase was observed in untreated transgenic mice with an endothelium-specific overexpression of bovine eNOS. These data extend previous observations and suggest that increased bioavailability of vascular nitric oxide regulates ecSOD expression, and that this effect might contribute to the antioxidative and anti-atherosclerotic effects of PETN observed previously.

This is the first study that demonstrates that PETN can induce the expression of ecSOD. PETN is an organic nitrate that is known to exert its anti-anginal effect by nitric oxide-dependent activation of soluble guanylyl cyclase (sGC), subsequent cyclic guanosine monophosphate (cGMP)-induced activation of protein kinase G and vasodilation of conductive arteries [18]. There is some debate whether nitric oxide is indeed the pharmacologically active principle of organic nitrates [19], although spin-trap-based nitric oxide analyses in rabbits have demonstrated vascular nitric oxide formation from glyceryl trinitrate in both venous and arterial vessels [20]. Detailed investigations identified a novel reductase activity of mitochondrial aldehyde dehydrogenase as the most important pathway for glyceryl trinitrate bioactivation, whereas nitrite but no nitric oxide was detectable as an intermediate of this reaction [21]. It has been shown that PETN and its trinitrate metabolite PETriN is effectively metabolized by aldhyde dehydrogenase (ALDH)-2 [22], whereas PEDN and PEMN are likely bioactivated by other pathways, for example, in a cytochrome P 450-dependent manner [23, 24]. Thus, there is neither evidence nor reasons to conclude that PETN increases vascular superoxide generation *in vivo*.

There is evidence that PEMN and PEDN generate nitric oxide. PETN and all metabolites are able to release nitric oxide in a cysteine-dependent manner. Likewise, the activation of sGC occurs in a cystein-dependent fashion. Furthermore, there are excellent correlations between cystein-dependent nitric oxide release and *in vitro* vasodilation expressed as pD2 values [18]. Neither PETN nor PETriN appears in blood plasma, suggesting that PETN and PETriN undergo extensive hepatic metabolism following intestinal absorption, the so-called first-pass effect [25]. Furthermore, intragastral PETN reduced the mean aortic pressure in anaesthetized rabbits by 20%, and the same quantitative effect was measured by intravenous PEDN, suggesting that PEDN is the major vasoactive metabolite [26]. Furthermore, the plasma half-lives of PEDN and PEMN are closely correlated with the duration of the anti-anginal action of PETN [25]. These data strongly suggest that vascular nitric oxide formation from PEDN and PEMN is a prerequisite for the therapeutical efficacy of PETN.

According to the metabolism pathways discussed previously, it seems likely that the up-regulation of ecSOD protein expression was mediated not directly by PETN but rather by nitric oxide released from its metabolites PEDN and PEMN. To further substantiate this, we used our transgenic mouse model with an endothelial overexpression of eNOS. In these mice, overexpression of eNOS was functionally active, as demonstrated by a significant reduction of blood pressure, which was completely blunted by oral treatment with the NOS inhibitor L-NA. We found a significant up-regulation of ecSOD protein expression and activity in cytosols of the lungs and aorta, suggesting that nitric oxide is the active mediator. To further challenge this hypothesis, we treated both eNOS^{n} and eNOS^{++} mice with the NOS inhibitor L-NA and observed a strong down-regulation of ecSOD protein expression. This result provides additional evidence for a regulatory role of vascular nitric oxide for ecSOD protein expression. Likewise, this result demonstrates that the increase of ecSOD in eNOS^{++} mice is not an unspecific consequence of genetic manipulation. Finally, the reduction of blood pressure itself unlikely influenced the expression of ecSOD. This up-regulation was evident either at reduced blood pressure in eNOS^{++} mice or at normal blood pressure following PETN treatment.

Our earlier observations with spontaneous nitric oxide donors in smooth muscle cells and aortic organoid cultures and with

exercise in mice *in vivo* [7] are consistent with the suggestion that nitric oxide is an important mediator driving ecSOD expression. In that same study, it was shown that the effect of nitric oxide on ecSOD expression seems to be cGMP-dependent, as it was prevented by the selective sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and was mimicked by a cGMP analogue. So far, it is not known whether other nitric oxide/superoxide interactions such as suppression of thioredoxin-interacting protein [27] or alterations in superoxide release by neutrophils [28] may be involved in or modify ecSOD up-regulation.

In human vascular smooth muscle cells, nitric oxide was shown to increase the transcription but not the half-life of ecSOD mRNA, and the effect on transcription was critically dependent on the activity of p38 mitogen activated protein (MAP) kinase. In contrast to these findings in cultured human cells, we were not able to detect a change in mRNA levels in murine lung and aortic tissue after PETN treatment and in $eNOS^{++}$ mice as well. One alternative pathway could be that ecSOD is produced in other organs or tissues and reaches the lungs by travelling through the circulation. In this case, one would expect an increase of circulating ecSOD protein, which was indeed measurable. However, it remains unclear whether the small increase of circulating ecSOD is sufficient to account for the increase of ecSOD found in the aorta and lungs. Alternatively, a yet unresolved, solely post-translational *in vivo* regulation of ecSOD protein expression might occur. Peng *et al*. recently showed a nitric oxide-induced inhibition of the ubiquitin–proteasome system in murine primary cortical neurons [29]. Given this, one could speculate that the increase of ecSOD protein expression and activity *in vivo* could be a yet undiscovered effect of nitric oxide that needs to be substantiated further. However, other studies on this matter have suggested that nitric oxide rather increases proteasomal degradation of proteins by nitric oxide-dependent protein modification such as S-nitrosylation [30]. Taken together, established mechanisms of ecSOD regulation like the influence of proteolytic removal of the heparin-binding domain [31] and inflammatory cytokines [32] might extend to a yet unknown nitric oxide-dependent mechanism that deserves further interest and investigation.

Up-regulation of ecSOD likely appears to be a mechanism that contributes to the antioxidative and anti-atherosclerotic effects of PETN observed previously. PETN was shown to reduce the development and progression of experimental atherosclerosis [8], and this effect was evident even in established atherosclerosis [9]. Further activities of PETN such as inhibition of endothelial dysfunction and LDL oxidation are consistent with an inhibition of superoxide formation as well [9], although it is not sure that the observed effect is active in a similar manner in pathological settings. Additionally, PETN does not induce the development of *in vivo* nitrate tolerance [18], which strikingly contrasts the effect of glyceryl trinitrate, which is a well-known inducer of nitrate tolerance associated with increased vascular oxidative stress [33]. The lack of nitrate tolerance induction by PETN was shown in experimental [26, 34] and clinical studies [35]. Hence, the increased expression of ecSOD might contribute to the lack of nitrate tolerance induction by PETN as well.

By induction of ecSOD expression, PETN likely increases the bioavailability of endogenous nitric oxide, as superoxide and nitric oxide rapidly form peroxynitrite, a strong oxidant contributing to vascular oxidative stress and a source for further reactive radicals. These related products involve peroxynitrous acid or decomposition products of the carbon dioxide adduct of peroxynitrite such as the nitrogen dioxide radical [36–38]. However, superoxide is rapidly detoxified to hydrogen peroxide, which has been shown to induce both the expression and the activity of eNOS [39]. Thus, a higher activity of ecSOD will not only reduce extracellular superoxide but also protect readily formed nitric oxide. By increasing hydrogen peroxide, it most likely increases endothelial nitric oxide synthesis as well. This effect of hydrogen peroxide has been shown to contribute to the up-regulation of vascular nitric oxide synthesis induced by exercise training [12].

Acknowledgements

We like to thank Dr. Dirk Stalleicken (Actavis Deutschland GmbH & Co. KG, Langenfeld, Germany) for providing PETN. This study was supported by the Forschungskommission of the Heinrich-Heine-Universitaet Duesseldorf (Project 9772 109 to GK and project 9772 345 to TS). GK has received an educational grant from Actavis GmbH, Germany.

References

1. **Gewaltig MT, Kojda G**. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc Res*. 2002; 55: 250–60.
2. **Kojda G, Harrison DG**. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res*. 1999; 43: 562–71.
3. **Griendling KK, Sorescu D, Ushio-Fukai M**. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000; 86: 494–501.
4. **Griendling KK, Harrison DG**. Out, damned dot: studies of the NADPH oxidase in atherosclerosis. *J Clin Invest*. 2001; 108: 1423–4.
5. **Stralin P, Karlsson K, Johansson BO, Marklund SL**. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler Thromb Vasc Biol*. 1995; 15: 2032–6.
6. **Jung O, Marklund SL, Geiger H, *et al.*** Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: *in vivo* and *ex vivo* evidence from ecSOD-deficient mice. *Circ Res*. 2003; 93: 622–9.
7. **Fukai T, Siegfried MR, Ushio-Fukai M, *et al.*** Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest*. 2000; 105: 1631–9.
8. **Kojda G, Stein D, Kottenberg E, *et al.*** *In vivo* effects of pentaerythrityl-tetranitrate and isosorbide- 5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. *J Cardiovasc Pharmacol*. 1995; 25: 763–73.

9. **Hacker A, Müller S, Meyer W, Kojda G**. The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis. *Br J Pharmacol*. 2001; 132: 1707–14.
10. **Muller S, Konig I, Meyer W, Kojda G**. Inhibition of vascular oxidative stress in hypercholesterolemia by eccentric isosorbide mononitrate. *J Am Coll Cardiol*. 2004; 44: 624–31.
11. **Wenzel P, Oelze M, Coldewey M, *et al.*** Heme oxygenase-1: a novel key player in the development of tolerance in response to organic nitrates. *Arterioscler Thromb Vasc Biol*. 2007; 27: 1729–35.
12. **Lauer N, Suvorava T, Rüther U, *et al.*** Critical Involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase. *Cardiovasc Res*. 2005; 65: 254–62.
13. **Suvorava T, Lauer N, Kumpf S, *et al.*** Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo. *Circulation*. 2005; 112: 2487–95.
14. **Kojda G, Laursen JB, Ramasamy S, *et al.*** Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res*. 1999; 42: 206–13.
15. **Koesling D**. Modulators of soluble guanylyl cyclase. *Naunyn Schmiedebergs Arch Pharmacol*. 1998; 358: 123–6.
16. **Marklund SL**. Extracellular superoxide dismutase. *Methods Enzymol*. 2002; 349: 74–80.
17. **Crapo JD, McCord JM, Fridovich I**. Preparation and assay of superoxide dismutases. *Methods Enzymol*. 1978; 53: 382–93.
18. **Kojda G, Hacker A, Noack E**. Effects of non-intermittent treatment of rabbits with pentaerythritol tetranitrate on vascular reactivity and vascular superoxide production. *Eur J Pharmacol*. 1998; 355: 23–31.
19. **Kleschyov AL, Oelze M, Daiber A, *et al.*** Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circ Res*. 2003; 93: e104–12.
20. **Mülsch A, Mordvintcev P, Bassenge E, *et al.*** *In vivo* spin trapping of glyceryl trinitrate-derived nitric oxide in rabbit blood vessels and organs. *Circulation*. 1995; 92: 1876–82.
21. **Chen Z, Zhang J, Stamler JS**. Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci USA*. 2002; 99: 8306–11.
22. **Wenzel P, Hink U, Oelze M, *et al.*** Number of nitrate groups determines reactivity and potency of organic nitrates: a proof of concept study in ALDH-$2^{-/-}$ mice. *Br J Pharmacol*. 2007; 150: 526–33.
23. **Minamiyama Y, Takemura S, Akiyama T, *et al.*** Isoforms of cytochrome P450 on organic nitrate-derived nitric oxide release in human heart vessels. *FEBS Lett*. 1999; 452: 165–9.
24. **McGuire JJ, Anderson DJ, McDonald BJ, *et al.*** Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate. *Biochem Pharmacol*. 1998; 56: 881–93.
25. **Weber W, Michaelis K, Luckow V, *et al.*** Pharmacokinetics and bioavailability of pentaerythrityl tetranitrate and two of its metabolites. *Arzneim Forsch*. 1995; 45: 781–4.
26. **Mullenheim J, Muller S, Laber U, *et al.*** The effect of high-dose pentaerythritol tetranitrate on the development of nitrate tolerance in rabbits. *Naunyn Schmiedebergs Arch Pharmacol*. 2001; 364: 269–75.
27. **Schulze PC, Liu H, Choe E, *et al.*** Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity. *Arterioscler Thromb Vasc Biol*. 2006; 26: 2666–72.
28. **Siminiak T, Abramowska A, Czechowska K, *et al.*** Intravenous isosorbide dinitrate inhibits neutrophil aggregation and plasma-mediated stimulation of superoxide anion production. *Int J Cardiol*. 1994; 45: 171–5.
29. **Peng ZF, Chen MJ, Yap YW, *et al.*** Proteasome inhibition: an early or late event in nitric oxide-induced neuronal death? *Nitric Oxide*. 2008; 18: 136–45.
30. **Lee CM, Kim BY, Li L, Morgan ET**. Nitric oxide-dependent proteasomal degradation of cytochrome P450 2B proteins. *J Biol Chem*. 2008; 283: 889–98.
31. **Enghild JJ, Thogersen IB, Oury TD, *et al.*** The heparin-binding domain of extracellular superoxide dismutase is proteolytically processed intracellularly during biosynthesis. *J Biol Chem*. 1999; 274: 14818–22.
32. **Stralin P, Marklund SL**. Multiple cytokines regulate the expression of extracellular superoxide dismutase in human vascular smooth muscle cells. *Atherosclerosis*. 2000; 151: 433–41.
33. **Münzel T, Sayegh H, Freeman BA, *et al.*** Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J Clin Invest*. 1995; 95: 187–94.
34. **Fink B, Bassenge E**. Unexpected, tolerance-devoid vasomotor and platelet actions of pentaerythrityl tetranitrate. *J Cardiovasc Pharmacol*. 1997; 30: 831–6.
35. **Jurt U, Gori T, Ravandi A, *et al.*** Differential effects of pentaerythritol tetranitrate and nitroglycerin on the development of tolerance and evidence of lipid peroxidation: a human in vivo study. *J Am Coll Cardiol*. 2001; 38: 854–9.
36. **Bonini MG, Radi R, Ferrer-Sueta G, *et al.*** Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J Biol Chem*. 1999; 274: 10802–6.
37. **Lymar SV, Hurst JK**. Rapid reaction between peroxonitrite ion and carbon dioxide: implications for biological activity. *J Am Chem Soc*. 1995; 117: 8867–8.
38. **Pfeiffer S, Mayer B, Hemmens B**. Nitric oxide: chemical puzzles posed by a biological messenger. *Angew Chem Int Ed*. 1999; 38: 1714–31.
39. **Drummond GR, Cai H, Davis ME, *et al.*** Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000; 86: 347–54.

British Journal of Pharmacology (2008) 155, 335–342

0007–1188/08 $32.00
www.brjpharmacol.org

RESEARCH PAPER

Effect of oral organic nitrates on expression and activity of vascular soluble guanylyl cyclase

M Oppermann[1], VT-V Dao[1], T Suvorava[1], M Bas[2] and G Kojda[1]

[1]*Institute for Pharmacology and Clinical Pharmacology, Heinrich-Heine-Universität, Düsseldorf, Germany and* [2]*Hals-, Nasen- und Ohrenklinik rechts der Isar, Technische Universität München, München, Germany*

Background and purpose: The regulation of vascular soluble guanylyl cyclase (sGC) expression by nitric oxide (NO) is still under discussion. *In vitro*, NO has been shown to downregulate the expression of sGC but it is unclear if this mechanism is operative *in vivo* and occurs during nitrate treatment.
Experimental approach: We investigated whether high dose isosorbide mononitrate (ISMN) or pentaerythrityl tetranitrate (PETN) treatment changes vascular sGC expression and activity *in vivo*. New Zealand White rabbits received a standard diet, 2 or 200 mg ISMN kg^{-1} d^{-1} for 16 weeks, and C57BL/6 mice received a standard diet, 6, 60 or 300 mg PETN kg^{-1} d^{-1} for four weeks. Absorption was checked by measuring the plasma levels of the drug/metabolite.
Key results: Western blots of rabbit aortic rings showed similar protein levels of sGC α1- ($P=0.2790$) and β1-subunits ($P=0.6900$) in all groups. Likewise, ANOVA showed that there was no difference in the expression of sGC in lungs of PETN-treated mice ($P=0.0961$ for α1 and $P=0.3709$ for β1). The activities of isolated sGC in response to SNAP (1 μM–1 mM) were identical in aortae of ISMN-treated rabbits ($P=0.0775$) and lungs of PETN-treated mice ($P=0.6348$). The aortic relaxation response to SNAP slightly decreased at high ISMN but not at high PETN.
Conclusions and implications: These data refute the hypothesis that therapeutic treatment with long acting NO donors has a significant impact on the regulation of vascular sGC expression and activity *in vivo*.
British Journal of Pharmacology (2008) **155,** 335–342; doi:10.1038/bjp.2008.269; published online 30 June 2008

Keywords: organic nitrates; nitric oxide; soluble guanylyl cyclase; endothelium; nitric oxide synthase; chronic treatment

Abbreviations: eNOS, endothelial nitric oxide synthase; ISMN, isosorbide mononitrate; NO, nitric oxide; PETN, pentaerythrityl tetranitrate; sGC, soluble guanylyl cyclase; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; VASP, vasodilator-stimulated phosphoprotein

Introduction

Organic nitrates are commonly used drugs for prevention and acute treatment of coronary artery disease symptoms. They are activated to nitric oxide (NO), which is involved in physiological processes such as smooth muscle relaxation, neurotransmission, platelet aggregation, host defence mechanisms and apoptosis and has antioxidative effects (Moncada and Higgs, 1993). The effects of NO greatly contribute to the physiological vascular functions and probably protect the vascular wall from vasotoxic compounds such as reactive oxygen species (Gewaltig and Kojda, 2002). In the vasculature, the majority of the effects of NO are mediated by the activation of soluble guanylyl cyclase (sGC), generation of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG) and phosphorylation of various cellular proteins regulating calcium haemostasis (Ignarro *et al*., 1999). The sGC enzyme is composed of two subunits, α and β, and a prosthetic haem group. The vast majority of vascular sGC is formed by the subunits α1 and β1 (Buechler *et al*., 1991; Russwurm and Koesling, 2004).

A disturbance of the NO–cGMP pathway induced by changes of the expression of sGC has been previously observed. The expression of vascular sGC is downregulated in spontaneously hypertensive rats (Bauersachs *et al*., 1998; Ruetten *et al*., 1999), in smooth muscle cells of Fischer 344 rats (Chen *et al*., 2000) and in aged Wistar Kyoto rats (Kloss *et al*., 2000; Friebe and Koesling, 2003). Other studies have shown a downregulation of sGC mRNA and protein expression by endotoxin, cAMP, cytokines and oestradiol (Shimouchi *et al*., 1993; Papapetropoulos *et al*., 1996; Kojda *et al*., 1998a; Ruetten *et al*., 1999; Krumenacker *et al*., 2001; Takata *et al*., 2001; Friebe and Koesling, 2003). In contrast, hypercholesterolaemia increases the expression of rabbit aortic vascular sGC, particularly in atherosclerotic plaques

Correspondence: Professor G Kojda, Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, Düsseldorf 40225, Germany.
E-mail: kojda@uni-duesseldorf.de
Received 18 March 2008; revised 29 May 2008; accepted 9 June 2008; published online 30 June 2008

(Laber *et al*., 2002), and a qualitatively similar but smaller effect has been observed in experimental chronic myocardial infarction and in nitrate tolerance (Bauersachs *et al*., 1998; Mulsch *et al*., 2001). In late-stage atherosclerosis, this may be different, in particular in the neointima (Melichar *et al*., 2004).

The mechanisms underlying these changes of sGC expression are currently unknown. Studies in cultured smooth muscle cells have suggested a crucial role of vascular NO generation, which may downregulate sGC expression in a negative feedback manner (Filippov *et al*., 1997; Weber *et al*., 2001). Likewise, transfection of HEK 293 cells with an endothelial nitric oxide synthase (eNOS)-containing plasmid and incubation of rat aortic rings with the NO-donor *N*-[4 [1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (SPER/NO) reduced mRNA expression of the β1 subunit (Schmidt *et al*., 2001; Weber *et al*., 2001).

To investigate whether alterations of sGC protein expression and activity may be involved in oral therapy with long-acting NO donors such as isosorbide mononitrate (ISMN) and pentaerythrityl tetranitrate (PETN), we sought to determine the effect of nitrate treatment on vascular smooth muscle sGC *in vivo*. To accomplish this, we measured the vascular sGC protein expression and activity as well as NO effects in aortic segments after treatment with different doses of ISMN and in mice treated with different doses of PETN.

Methods

Test systems used

A total of 30 New Zealand white rabbits (10–12 weeks old) with a mean body weight of 2105 ± 47 g were housed individually as previously described (Kojda *et al*., 1995). The rabbits were randomly assigned to three groups of 10 animals and were fed a standard diet (ISMN-0) and a diet supplemented with ISMN to achieve a daily dose of ISMN of 2 mg kg^{-1} (ISMN-2) or 200 mg kg^{-1} (ISMN-200) for 16 weeks. The dose of ISMN was given in two identical portions in the morning at 0800 hours and in the early afternoon at 1500 hours. Body weight was determined weekly and the animals were supervised by a veterinerian. Previous studies have shown that 200 mg kg^{-1} per day of ISMN induces a nitrate tolerance as indicated selectively by reduced vasodilator activity of ISMN, both in normal and in hypercholesterolaemic rabbits (Muller *et al*., 2003, 2004).

C57BL/6 mice (male, 5-month old) were divided into four groups and were then randomly allocated to receive placebo ($n = 8$) or PETN treatment ($n = 12$ per group) for 4 weeks. The groups were treated with placebo (PETN-0) or 6 (PETN-6), 60 (PETN-60) or 300 mg kg^{-1} per day (PETN-300) of PETN, according to an average body weight of 25 g and an assumed daily amount of 5 g of consumed food. According to the manufacturer's recommendations, PETN was given continuously.

Permission for the animal studies was provided by the regional government of Germany (AZ 23.05-230-3-77/99, AZ 23.05-230-3-52/99, AZ 50.05-230-3-65/99, AZ 50.05-230-3-94/00 AZ 50.05-230-18/06). The experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' and the '*Guide for the Care and Use of Laboratory Animals*' of the US National Institutes of Health.

Measurements of plasma levels of drug/metabolite

Plasma concentrations of ISMN, pentaerythrityl dinitrate and mononitrate were determined by ACC GmbH (Leidensbach, Germany) with gas chromatography/mass spectrometry (GC/MS, HP6890, Hewlett-Packard, Germany) after liquid–liquid extraction with ethyl acetate as previously described (Muller *et al*., 2004).

Measurement of blood pressure

Systolic blood pressure and heart rate were measured in awake male C57BL/6 ($n = 4$ of each PETN treatment group) at 3–4 months of age using an automated tailcuff system as previously described (Kojda *et al*., 1999). On day 7, the PETN diet was started and blood pressure measurement was continued for a maximum of 7 days.

Vasorelaxation studies, sGC activity and western blots

Preparation of rabbit thoracic ring segments, equilibration and relaxation to the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) as well as preparation of cytosols from rabbit aorta and mouse lung were performed as previously described (Kojda *et al*., 1995; Laber *et al*., 2002). Specific activity of sGC was measured as described by Kojda *et al*. (1998a) and Schultz and Böhme (1984). Western blots for sGC α1 and β1 subunits were performed in rabbit aorta and mouse lung using specific antibodies. In addition, blots for vasodilator-stimulated phosphoprotein (VASP) were performed in lung and heart homogenates of PETN-treated mice. Please refer to Supplementary information for detailed protocols.

Data analyses and statistical procedures

All data were analysed by a standard computer programme (GraphPad Prism PC software, version 3.03) and are expressed as mean ± s.e.m. of n individual samples. Statistical comparisons between groups were performed by Newman–Keuls multiple comparisons test following analysis of variance (ANOVA) for pD_2 values and protein expression or two-way ANOVA for concentration–response curves. $P < 0.05$ was considered statistically significant.

Drugs, chemical reagents and other materials

[α-^{32}P]-GTP was obtained from PerkinElmer (Rodgau, Germany). Antibodies against sGC α1 subunit (catalogue no. G4280) and actin (no. A2066) were from Sigma (Munich, Germany); against sGC β1 subunit (no. 160897) from Cayman (Biozol, Eching, Germany); against rabbit immunoglobulin G (IgG) (no. 401315) from Calbiochem (Darmstadt, Germany); against phosphorylated and total VASP (no. 804–240 and no. 210–880) from Alexis Biochemicals (Lörrach, Germany) and against mouse IgG (no. 170–6516) from Bio-Rad (Munich, Germany). Polyvinylidene fluoride membranes were from

Millipore (Schwalbach, Germany). SNAP was synthesized in our laboratory as previously described (Kojda *et al*., 1996). ISMN was provided by Schwarz Pharma (Monheim, Germany); PETN by Actavis (Langenfeld, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma in analytical grade. The stock solutions of acetylcholine (10 mM) and phenylephrine (10 mM) and ISMN (100 mM) were prepared in distilled water. Solutions of SNAP (200 mM) were prepared in dimethylsulphoxide. All stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice and protected from daylight until use. The blood pressure measurement system was from Visitech Systems (Apex, NC, USA).

The molecular target nomenclature in this paper conforms with the BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Results

ISMN-treated rabbits

Treatment with ISMN resulted in plasma ISMN concentrations of 6.2 ± 1.9 ng mL^{-1} ($n = 10$) in ISMN-2 and 1.77 ± 0.469 μg mL^{-1} ($n = 9$) in ISMN-200. Densitometric analyses of the α1 subunit revealed that its expression in the ISMN-2 and ISMN-200 groups (each $n = 10$) was not different from that in the ISMN-0 group (Figure 1a). Expression of β1 in the ISMN-2 and ISMN-200 groups also showed no difference from that in the ISMN-0 group (Figure 1b). In addition, we measured sGC protein expression using actin protein as a standard. These data showed protein levels of $77.4 \pm 21.0\%$ ($n = 10$) in ISMN-2 and $97.5 \pm 18.9\%$ ($n = 8$) in ISMN-200 for α1 ($P = 0.1598$, ANOVA) and $105.1 \pm 17.9\%$ (ISMN-2) or $136.7 \pm 26.9\%$ (ISMN-200) for β1 ($P = 0.3072$, each $n = 10$, ANOVA). Thus, the method of standardization had no influence on the main result.

Measurement of sGC activity in response to increasing concentrations of SNAP (1 μM–1 mM) in aortic cytosols showed comparable pD_2 values (half-maximal effective concentrations in −logM) in ISMN-2 (3.41 ± 0.24, $n = 6$), ISMN-200 (3.05 ± 0.39, $n = 7$) and ISMN-0 (3.39 ± 0.26, $n = 6$, $P = 0.6524$, ANOVA). Likewise, the dose–response curves showed no significant differences between all three groups, as determined by two-way ANOVA (Figure 1c).

The dose–response curves for SNAP-induced vasorelaxation in rabbit aortic rings were slightly shifted to the right in the ISMN-2 and ISMN-200 groups. However, the shift was very small and the maximal response to SNAP was not changed. The corresponding pD_2 values were significantly less in the treated groups compared to the untreated group (Figure 2a). Measurements after removal of the endothelium did not significantly change this SNAP response pattern or pD_2 values for ISMN-0 (Figure 2b), ISMN-2 (Figure 2c) and ISMN-200 (Figure 2d). Again, significant differences occurred between treatment groups and ISMN-0 ($P = 0.0206$, ANOVA) but not between ISMN-2 and ISMN-200.

PETN-treated mice

The plasma concentrations of the PETN dinitrate (PEDN) and mononitrate (PEMN) metabolites, which were detectable by

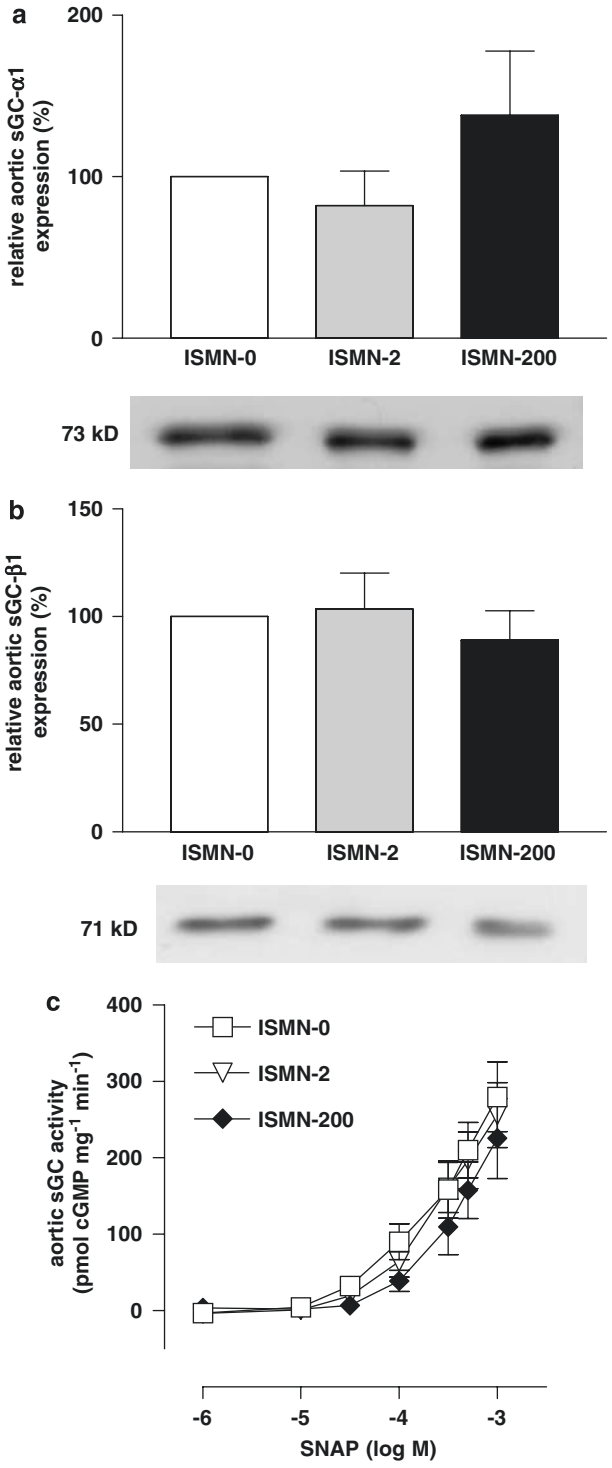


Figure 1 Effect of isosorbide mononitrate (ISMN) treatment on soluble guanylyl cyclase (sGC) in rabbits. Protein expression of (**a**) sGC α1 subunit ($P = 0.2790$) and (**b**) sGC β1 subunit in aortae of ISMN-treated rabbits measured by western blot densitometry. Neither the low-dose (ISMN-2) nor the high-dose (ISMN-200, each $n = 10$, $P = 0.6900$, analysis of variance (ANOVA)) group showed a significant change in protein expression. (**c**) Activity of sGC protein measured in pmol cGMP per mg protein per min in ISMN-treated rabbits. There was no significant difference among all three groups ($n = 6$–7, $P = 0.0775$, ANOVA). Results shown represent mean ± s.e.m.

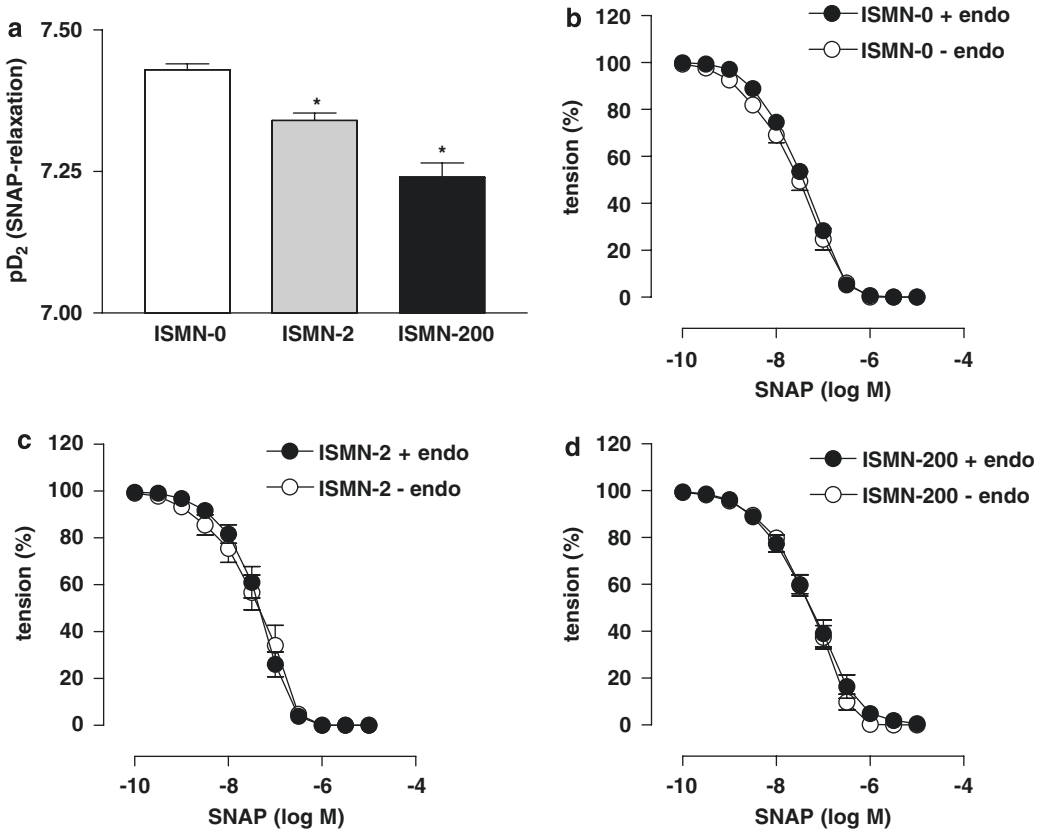


Figure 2 Vasorelaxation experiments with rabbit aorta. (**a**) The nitric oxide (NO)-sensitivity of aortic rings progressively declined with increasing isosorbide mononitrate (ISMN) dosage ($n=9$–10, $P=0.0161$, analysis of variance (ANOVA)). (**b**–**d**) Removal of the endothelium (–endo) in all three groups did not change the responses to *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) in any of the groups ($P=0.1999$ for ISMN-0, $P=0.3706$ for ISMN-2 and $P=1.000$ for ISMN-200, $n=9$–10, *t*-tests). All values represent mean ± s.e.m.

GC/MS, increased with the daily oral dose of PETN. The concentrations of both metabolites increased in a directly proportional way (Figure 3).

Western blot analyses of the sGC α1 subunit standardized by total protein revealed that its expression in the lungs of PETN-treated mice did not differ significantly from that in the untreated (PETN-0) group (Figure 4a). Likewise, expression of the β1 subunit showed no significant difference between the groups (Figure 4b). Additional western blots for β1 in aortic tissues showed an aortic expression of 109.8 ± 25.8% in PETN-6 and 144.9 ± 25.3% in PETN-60 group ($n=5$, $P=0.3135$, ANOVA).

Phosphorylation of VASP at serine 239 relating to total VASP showed no difference induced by PETN treatment, either. In lung tissue, phosphorylation was 103 ± 47.4% (PETN-6, $n=6$) and 121 ± 34.0% (PETN-60, $n=5$, $P=0.8996$, ANOVA) compared to PETN-0 ($n=6$), in hearts 112.3 ± 32.0% (PETN-6, $n=7$) and 99.7 ± 42.8% (PETN-60, $n=7$, $P=0.9460$, ANOVA).

The maximal activities of sGC in response to 1 mM SNAP in lung cytosols (in pmol cGMP per mg protein per min) did not differ significantly between the groups (Figure 4c). Likewise, the pD_2 values (in −logM) for SNAP in PETN-6 (3.84 ± 0.07), PETN-60 (3.89 ± 0.15) and PETN-300 (3.73 ± 0.14) were not significantly different from PETN-0 (3.86 ± 0.31, each $n=7$, $P=0.9412$, ANOVA), as well as the dose–response curves ($P=0.6348$, two-way ANOVA).

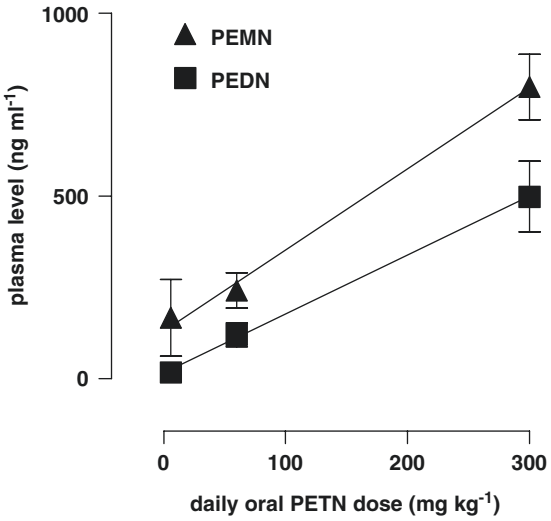


Figure 3 Plasma levels of the pentaerythrityl tetranitrate (PETN) metabolites PETN dinitrate (PEDN) and mononitrate (PEMN) detected in PETN-treated mice. The concentrations of both metabolites increased significantly with increased PETN dose in a directly proportional manner; r^2 is 0.5606 for PEDN (significant deviation from 0, $P=0.0013$) and 0.7374 for PEMN ($P<0.0001$, $n=7$). All values represent mean ± s.e.m.

The NO-dependent vasorelaxation was determined in organ bath experiments using the NO-donor SNAP in concentrations from 1 nM up to 10 mM. The dose–response

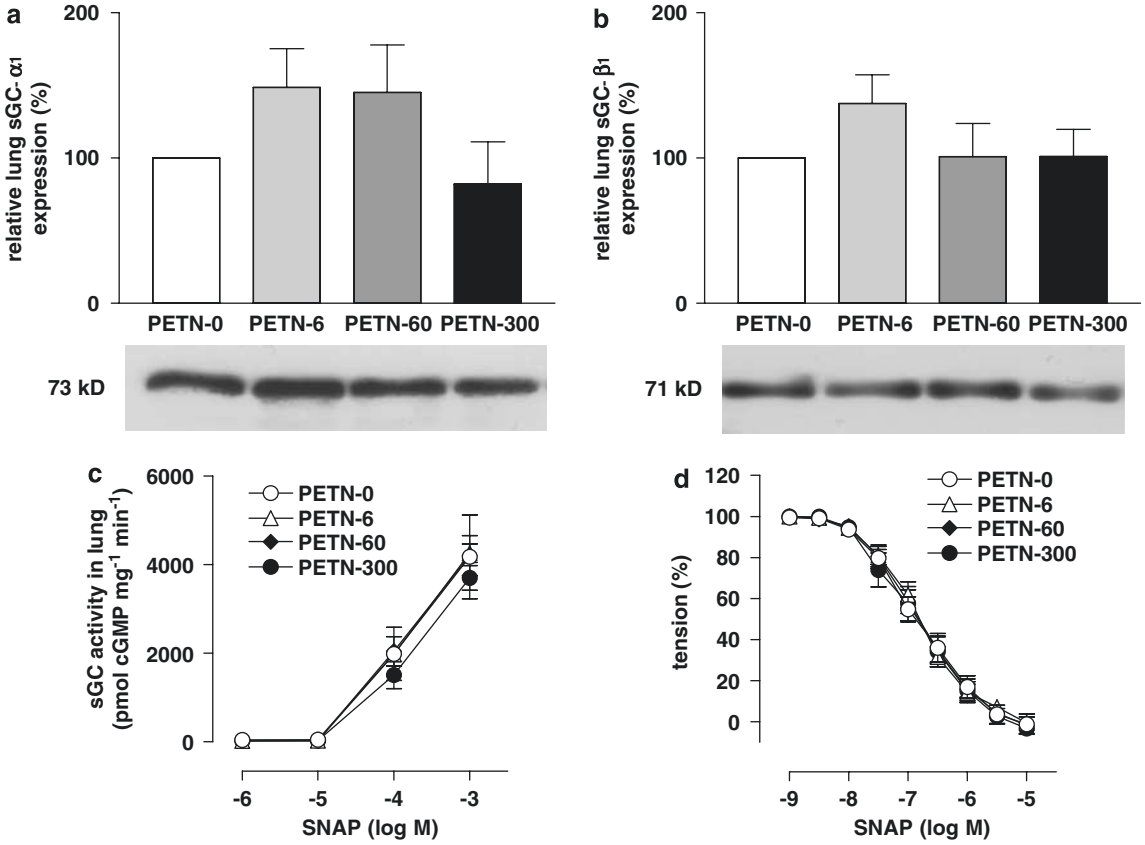


Figure 4 Effects of pentaerythrityl tetranitrate (PETN) treatment on soluble guanylyl cyclase (sGC) in mice. Protein expression of (**a**) sGC α1 subunit ($P=0.0961$, $n=8$) and (**b**) sGC β1 subunit ($P=0.3709$, $n=8$, analysis of variance (ANOVA)) in lungs of PETN-treated mice measured by western blot densitometry. None of the groups treated with different doses of PETN showed a significant change in protein expression. (**c**) Activity of sGC protein measured in pmol cGMP per mg protein per min in PETN-treated mice. There was no significant difference among all four groups ($P=0.6348$, $n=7$, two-way ANOVA). (**d**) NO-dependent vasorelaxation in aortic rings of PETN-treated mice induced by the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Again, there was no significant difference between the groups ($P=0.8865$, two-way ANOVA). All values represent mean ± s.e.m.

patterns did not differ between the groups (Figure 4d), and the half-maximal concentrations of SNAP in this experiment (−logM) were similar in all four groups (PETN-0: 6.85 ± 0.08, PETN-6: 6.82 ± 0.06, PETN-60: 6.81 ± 0.07 and PETN-300: 6.84 ± 0.09, $P=0.7217$, ANOVA).

PETN had no effect on systolic blood pressure, either on the first day (acute effect) or on days 5–7 of treatment (chronic effect) in all three groups as compared to pretreatment values (Figure 5a). Measurements of heart rate in the three PETN-treated groups also showed no significant differences between the groups and from the pretreatment values (Figure 5b).

Discussion and conclusions

The aim of this study was to determine the influence of pharmacological nitrate treatment on the expression and function of vascular sGC *in vivo*. Our main finding is that none of the conditions changed the protein expression of sGC α1 and β1 subunits and sGC activity. In addition, the functional efficacy of the vascular NO–cGMP pathway was maintained in all experimental models used. These data suggest that oral therapy with long-acting nitrates does not impair the vascular NO–cGMP pathway.

Studies in cultured rat aortic smooth muscle cells have provided evidence for an NO-dependent downregulation of sGC protein expression, suggesting a negative feedback loop where NO acts as a signalling molecule regulating sGC expression (Filippov *et al*., 1997). In another study, rat pulmonary artery smooth muscle cells responded to treatment with lipopolysaccharide, which is known to induce the expression of inducible NOS, with a downregulation of sGC mRNA levels (Scott and Nakayama, 1998). In eNOS-transfected cells showing a strong western blot signal for eNOS, we found an approximately fourfold downregulation of sGC mRNA expression and a reduction of sGC activity (Schmidt *et al*., 2001). Likewise, incubation of rat aortic rings with the NO-donor SPER/NO at NO generation levels of approximately $2\,\mu mol\,L^{-1}\,min^{-1}$ resulted in both, a reduction of activity and of protein expression (Weber *et al*., 2001). Therefore, it appears that our *in vivo* findings contradict the results obtained in cultured cells (Filippov *et al*., 1997; Scott and Nakayama, 1998) and isolated rat aortic rings (Weber *et al*., 2001).

In contrast, our data are consistent with reports that failed to show the existence of an NO-dependent feedback loop

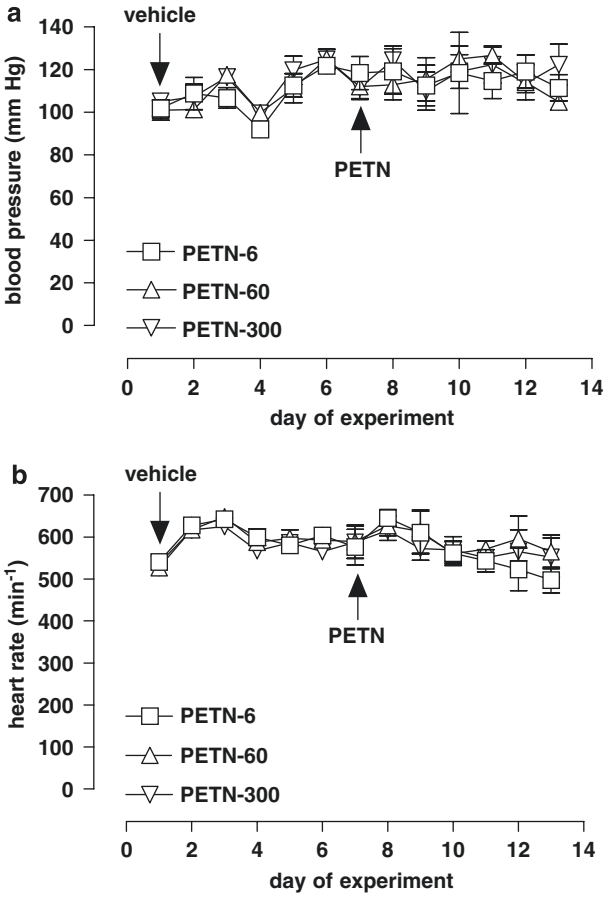


Figure 5 Effects of pentaerythrityl tetranitrate (PETN) treatment on blood pressure and heart rate. (**a**) Blood pressure measurements before and during PETN treatment of C57BL/6 mice. On day 7, vehicle food was replaced by diets enriched with 6, 60 or 300 mg kg^{-1} per day of PETN. None of these doses showed a significant acute or chronic effect on the blood pressure compared to pretreatment measurements ($P=0.5314$, $n=4$, two-way analysis of variance (ANOVA)). (**b**) Heart rate of PETN-treated mice before and during treatment. The different PETN doses had no significant effect on the heart rate compared to pretreatment measurements ($P=0.4416$, $n=4$, two-way ANOVA). All values represent mean ± s.e.m.

controlling sGC expression *in vivo*. Mice overexpressing eNOS in the vasculature show a resistance to endothelium-dependent and NO-induced vasodilatation but not a decrease of sGC expression (Yamashita *et al*., 2000). Instead, the authors describe a 50% reduction of basal unstimulated sGC activity and a 20% reduction of PKG expression. However, other groups have found that changes of PKG activity do not occur in eNOS knockout mice and that there is no change of sGC protein expression in this animal model (Hussain *et al*., 1999; Brandes *et al*., 2000). Inhibition of NOS by chronic treatment of mice with the NOS inhibitor L-NAME has been shown to have no effect on vascular sGC expression but potentiate the aortic cGMP response to the NO-donor sodium nitroprusside (Mullershausen *et al*., 2003).

There is some debate as to whether NO is indeed the pharmacologically active principle of organic nitrates (Kleschyov *et al*., 2003), although spin trap-based NO analyses in rabbits have demonstrated vascular NO formation from glyceryl trinitrate in both venous and arterial vessels (Mülsch *et al*., 1995). Detailed investigations identified a novel reductase activity of mitochondrial aldehyde dehydrogenase as the most important pathway for glyceryl trinitrate bioactivation, whereas nitrite but no NO was detectable as an intermediate of this reaction (Chen *et al*., 2002). It has been shown that PETN and its trinitrate metabolite PETriN is effectively metabolized by ALDH-2 (Wenzel *et al*., 2007), whereas the PEDN and PEMN metabolites as well as ISMN are probably bioactivated by other pathways, for example, in a cytochrome P 450-dependent manner to generate NO or at least an sGC stimulating intermediate compound (McGuire *et al*., 1998; Minamiyama *et al*., 1999). These data suggest that vascular generation of such a compound or NO from PEDN, PEMN and ISMN is a prerequisite for the therapeutic efficacy of PETN and ISMN.

The apparent contradiction between the *in vitro* and *in vivo* results (Francois and Kojda, 2004) might be caused by various mechanisms, such as the difference between bioactive concentrations of vascular NO, the time of exposure and possibly associated compensatory changes of the NO–cGMP pathway. In this study, increasing doses of the NO-donor ISMN in rabbits causing a 280-fold increase of maximal ISMN plasma concentrations in ISMN-200 compared to ISMN-2 and a moderate nitrate tolerance (Muller *et al*., 2003) had no effect on sGC activity and protein expression, whereas the NO sensitivity of aortic rings progressively declined with increasing ISMN dosage. Although, the overall effect on SNAP-induced vasodilatation was very small. These data suggest that the bioactive concentration of vascular NO even in the ISMN-200 group did not reach a concentration threshold necessary to induce inhibition of sGC expression.

To confirm these results and to avoid species- or treatment-specific artefacts, we repeated our experiments using a different species (mice) and various doses of a different nitrate (PETN). Although plasma concentrations of PEDN and PEMN were detectable in a dose-dependent manner, PETN treatment did not have an effect on sGC activity, sGC expression, NO-dependent vasorelaxation and blood pressure. However, our VASP measurements in the lung and in the heart showed no difference as well, although the plasma concentrations of PEDN and PEMN at 60 mg kg^{-1} per day of PETN largely exceed any therapeutic plasma concentration. So far, there is no other report available showing blood pressure reduction in mice receiving long-term treatment with PETN and just one paper describes a blood pressure drop 3 h after oral bolus application of 20 mg kg^{-1} of ISMN, a dose exceeding the human dose by approximately 80-fold (Momi *et al*., 2007), whereas another newly developed nitrate had no effect. Of the several possible explanations for the lack of blood pressure reduction in response to PETN, physiological adaptation to any initial arterial vasodilator effect appears to be the most likely. Furthermore, PETN has been shown to dilate venous vessels predominantly (Mullenheim *et al*., 2001). The supine position of the animals may blunt the blood pressure response as well.

Nevertheless, our data suggest that the bioactive concentration of vascular NO even in the PETN-300 and ISMN-200

groups did not reach a concentration threshold necessary to induce a negative feedback signalling on sGC expression that has been described to occur *in vitro*. One explanation for this phenomenon might be that vascular metabolism of organic nitrates to NO is decreased by NO itself (Kojda *et al*., 1998b). Using ^{14}C-glyceryl trinitrate, we have previously provided data to support this hypothesis.

In another study, chronic glyceryl trinitrate treatment induced severe nitrate tolerance and elicited a small upregulation of sGC that could be related to a negative feedback signalling between NO and sGC expression (Mulsch *et al*., 2001), but the results of this study and those previously obtained *in vivo* (Laber *et al*., 2002) and *in vitro* (Weber *et al*., 2001) rather suggest that this effect was mediated by the increase of vascular superoxide production associated with nitrate tolerance. Taken together, these results suggest that treatment with NO donors does not increase vascular bioavailable NO enough to induce a negative feedback signalling on sGC expression.

Previous *in vitro* observations in cultured smooth muscle cells expressing either sGC or PKG showed that transfection of PKG-deficient smooth muscle cells with the catalytic domain of PKG-I reduced protein expression of the β1 subunit, whereas transfection of sGC-deficient smooth muscle cells with both α1 and β1 subunits reduced protein expression of PKG after a 48 h treatment with the NO-donor 2,2′-(hydroxynitrosohydrazono)-bis-ethanimine (DETA/NO) (Browner *et al*., 2004). The effect of NO on the expression of sGC appears to involve a post-transcriptional regulation (Filippov *et al*., 1997), which might be caused by decreased binding of the sGC-mRNA-stabilizing family protein HuR (Kloss *et al*., 2003). In view of our *in vivo* results we suggest such inhibition by NO of sGC expression occurs at concentrations of NO exceeding normal *in vivo* concentrations and which are not even achievable with extremely high nitrate doses.

Acknowledgements

We thank Dr Dirk Stalleicken (Actavis Deutschland GmbH and Co. KG, Langenfeld, Germany) for providing PETN and Dr Gudrun Walosek (Schwarz Pharma AG, Monheim, Germany) for providing ISMN.

This study was supported by the Forschungskommission of the Heinrich-Heine-Universität Düsseldorf (projects 9772 109 and 9772 272 to GK and project 9772 345 to TS).

Conflict of interest

GK received an unrestricted small educational grant from Actavis GmbH, Germany.

References

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC). *Br J Pharmacol*, 3rd edn. **153** (Suppl 2): S1–S209.

Bauersachs J, Bouloumié A, Mülsch A, Wiener G, Fleming I, Busse R (1998). Vasodilator dysfunction in aged spontaneously hypertensive rats: changes in NO sythase III and soluble guanylyl cyclase expression, and in superoxide anion production. *Cardiovasc Res* **37**: 772–779.

Brandes RP, Kim DY, Schmitz-Winnenthal FH, Amidi M, Gödecke A, Mülsch A *et al*. (2000). Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice—role of soluble guanylyl cyclase. *Hypertension* **35**: 231–236.

Browner NC, Dey NB, Bloch KD, Lincoln TM (2004). Regulation of cGMP-dependent protein kinase expression by soluble guanylyl cyclase in vascular smooth muscle cells. *J Biol Chem* **279**: 46631–46636.

Buechler WA, Nakane M, Murad F (1991). Expression of soluble guanylate cyclase activity requires both enzyme subunits. *Biochem Biophys Res Commun* **174**: 351–357.

Chen LH, Daum G, Fischer JW, Hawkins S, Bochaton-Piallat ML, Gabbiani G *et al*. (2000). Loss of expression of the β subunit of soluble guanylyl cyclase prevents nitric oxide-mediated inhibition of DNA synthesis in smooth muscle cells of old rats. *Circ Res* **86**: 520–525.

Chen Z, Zhang J, Stamler JS (2002). Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci USA* **99**: 8306–8311.

Filippov G, Bloch DB, Bloch KD (1997). Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *J Clin Invest* **100**: 942–948.

Francois M, Kojda G (2004). Effect of hypercholesterolemia and of oxidative stress on the nitric oxide–cGMP pathway. *Neurochem Int* **45**: 955–961.

Friebe A, Koesling D (2003). Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* **93**: 96–105.

Gewaltig MT, Kojda G (2002). Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc Res* **55**: 250–260.

Hussain MB, Hobbs AJ, MacAllister RJ (1999). Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta. *Br J Pharmacol* **128**: 1082–1088.

Ignarro LJ, Cirino G, Casini A, Napoli C (1999). Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol* **34**: 879–886.

Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E *et al*. (2003). Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circ Res* **93**: e104–e112.

Kloss S, Bouloumie A, Mulsch A (2000). Aging and chronic hypertension decrease expression of rat aortic soluble guanylyl cyclase. *Hypertension* **35**: 43–47.

Kloss S, Furneaux H, Mulsch A (2003). Post-transcriptional regulation of soluble guanylyl cyclase expression in rat aorta. *J Biol Chem* **278**: 2377–2383.

Kojda G, Kottenberg K, Hacker A, Noack E (1998a). Alterations of the vascular and the myocardial guanylate cyclase/cGMP-system induced by long-term hypertension in rats. *Pharm Acta Helv* **73**: 27–35.

Kojda G, Kottenberg K, Nix P, Schlüter KD, Piper HM, Noack E (1996). Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ Res* **78**: 91–101.

Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J *et al*. (1999). Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res* **42**: 206–213.

Kojda G, Patzner M, Hacker A, Noack E (1998b). Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate. A novel mechanism to explain preferential venodilation of organic nitrates. *Mol Pharmacol* **53**: 547–554.

Kojda G, Stein D, Kottenberg E, Schnaith EM, Noack E (1995). *In vivo* effects of pentaerythrityl-tetranitrate and isosorbide-5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. *J Cardiovasc Pharmacol* **25**: 763–773.

Krumenacker JS, Hyder SM, Murad F (2001). Estradiol rapidly inhibits soluble guanylyl cyclase expression in rat uterus. *Proc Natl Acad Sci USA* **98**: 717–722.

Laber U, Kober T, Schmitz V, Schrammel A, Meyer W, Mayer B *et al*. (2002). Effect of hypercholesterolemia on expression and function of vascular soluble guanylyl cyclase. *Circulation* **105**: 855–860.

McGuire JJ, Anderson DJ, McDonald BJ, Narayanasami R, Bennett BM (1998). Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate. *Biochem Pharmacol* **56**: 881–893.

Melichar VO, Behr-Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A *et al*. (2004). Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. *Proc Natl Acad Sci USA* **101**: 16671–16676.

Minamiyama Y, Takemura S, Akiyama T, Imaoka S, Inoue M, Funae Y *et al*. (1999). Isoforms of cytochrome P450 on organic nitrate-derived nitric oxide release in human heart vessels. *FEBS Lett* **452**: 165–169.

Momi S, Impagnatiello F, Guzzetta M, Caracchini R, Guglielmini G, Olivieri R *et al*. (2007). NCX 6560, a nitric oxide-releasing derivative of atorvastatin, inhibits cholesterol biosynthesis and shows anti-inflammatory and anti-thrombotic properties. *Eur J Pharmacol* **570**: 115–124.

Moncada S, Higgs A (1993). Mechanisms of disease: the L-arginine-nitric oxide pathway. *N Engl J Med* **329**: 2002–2012.

Mullenheim J, Muller S, Laber U, Thamer V, Meyer W, Bassenge E *et al*. (2001). The effect of high-dose pentaerythritol tetranitrate on the development of nitrate tolerance in rabbits. *Naunyn Schmiedebergs Arch Pharmacol* **364**: 269–275.

Muller S, Konig I, Meyer W, Kojda G (2004). Inhibition of vascular oxidative stress in hypercholesterolemia by eccentric isosorbide mononitrate. *J Am Coll Cardiol* **44**: 624–631.

Muller S, Laber U, Mullenheim J, Meyer W, Kojda G (2003). Preserved endothelial function after long-term eccentric isosorbide mononitrate despite moderate nitrate tolerance. *J Am Coll Cardiol* **41**: 1994–2000.

Mullershausen F, Russwurm M, Koesling D, Friebe A (2003). The enhanced NO-induced cGMP response induced by long-term L-NAME treatment is not due to enhanced expression of NO-sensitive guanylyl cyclase. *Vascul Pharmacol* **40**: 161–165.

Mülsch A, Mordvintcev P, Bassenge E, Jung F, Clement B, Busse R (1995). *In vivo* spin trapping of glyceryl trinitrate-derived nitric oxide in rabbit blood vessels and organs. *Circulation* **92**: 1876–1882.

Mulsch A, Oelze M, Kloss S, Mollnau H, Topfer A, Smolenski A *et al*. (2001). Effects of *in vivo* nitroglycerin treatment on activity and expression of the guanylyl cyclase and cGMP-dependent protein kinase and their downstream target vasodilator-stimulated phosphoprotein in aorta. *Circulation* **103**: 2188–2194.

Papapetropoulos A, Abou-Mohamed G, Marczin N, Murad F, Caldwell RW, Catravas JD (1996). Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1β. *Br J Pharmacol* **118**: 1359–1366.

Ruetten H, Zabel U, Linz W, Schmidt HHHW (1999). Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats. *Circ Res* **85**: 534–541.

Russwurm M, Koesling D (2004). NO activation of guanylyl cyclase. *EMBO J* **23**: 4443–4450.

Schmidt K, Andrew P, Schrammel A, Groschner K, Schmitz V, Kojda G *et al*. (2001). Comparison of neuronal and endothelial isoforms of nitric oxide synthase in stably transfected HEK 293 cells. *Am J Physiol Heart Circ Physiol* **281**: H2053–H2061.

Schultz G, Böhme E (1984). Guanylate Cyclase. In: Bergmeyer HU (ed). *Methods of Enzymatic Analysis*. Verlag Chemie: Weinheim, FRG, pp 379–389.

Scott WS, Nakayama DK (1998). *Escherichia coli* lipopolysaccharide downregulates soluble guanylate cyclase in pulmonary artery smooth muscle. *J Surg Res* **80**: 309–314.

Shimouchi A, Janssens SP, Bloch DB, Zapol WM, Bloch KD (1993). cAMP regulates soluble guanylate cyclase β1-subunit gene expression in RFL-6 rat fetal lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* **265**: L456–L461.

Takata M, Filippov G, Liu H, Ichinose F, Janssens S, Bloch DB *et al*. (2001). Cytokines decrease sGC in pulmonary artery smooth muscle cells via NO-dependent and NO-independent mechanisms. *Am J Physiol Lung Cell Mol Physiol* **280**: L272–L278.

Weber M, Lauer N, Mulsch A, Kojda G (2001). The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. *Free Radic Biol Med* **31**: 1360–1367.

Wenzel P, Hink U, Oelze M, Seeling A, Isse T, Bruns K *et al*. (2007). Number of nitrate groups determines reactivity and potency of organic nitrates: a proof of concept study in ALDH-2−/− mice. *Br J Pharmacol* **150**: 526–533.

Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Rikitake Y, Inoue N *et al*. (2000). Mechanisms of reduced nitric oxide/cGMP-mediated vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *Hypertension* **36**: 97–102.

Supplementary Information accompanies the paper on British Journal of Pharmacology website (http://www.nature.com/bjp)

Endogenous Vascular Hydrogen Peroxide Regulates Arteriolar Tension In Vivo

Tatsiana Suvorava, MS*; Nadine Lauer, PharmD, PhD*; Stephanie Kumpf, BSc; Ralf Jacob, PhD; Wilfried Meyer, PhD; Georg Kojda, PharmD, PhD

Background—Although many studies suggested direct vasomotor effects of hydrogen peroxide (H_2O_2) in vitro, little is known about the vasomotor effects of H_2O_2 in vivo.

Methods and Results—We have generated mice overexpressing human catalase driven by the Tie-2 promoter to specifically target this transgene to the vascular tissue. Vessels of these mice (cat^{++}) expressed significantly higher levels of catalase mRNA, protein, and activity. The overexpression was selective for vascular tissue, as evidenced by immunohistochemistry in specimens of aorta, heart, lung, and kidney. Quantification of reactive oxygen species by fluorescence signals in cat^{++} versus catalase-negative (cat^{n}) mice showed a strong decrease in aortic endothelium and left ventricular myocardium but not in leukocytes. Awake male cat^{++} at 3 to 4 months of age had a significantly lower systolic blood pressure (sBP, 102.7±2.2 mm Hg, n=10) compared with their transgene-negative littermates (cat^{n}, 115.6±2.5 mm Hg, P=0.0211) and C57BL/6 mice (118.4±3.06 mm Hg, n=6). Treatment with the catalase inhibitor aminotriazole increased sBP of cat^{++} to 117.3±4.3 mm Hg (P=0.0345), while having no effect in cat^{n} (118.4±2.4 mm Hg, n=4, P>0.05). In contrast, treatment with the NO-synthase inhibitor nitro-L-arginine methyl ester (100 mg · kg BW^{-1} · d^{-1}) increased sBP in cat^{++} and C57Bl/6 to a similar extent. Likewise, phosphorylation of vasodilator-stimulated phosphoprotein in skeletal muscle, left ventricular myocardium, and lung was identical in cat^{++} and cat^{n}. Endothelium- and NO-dependent aortic vasodilations were unchanged in cat^{++}. Aortic KCl contractions were significantly lower in cat^{++} and exogenous H_2O_2 (10 μmol/L)–induced vasoconstriction.

Conclusions—These data suggest that endogenous H_2O_2 may act as a vasoconstrictor in resistance vessels and contribute to the regulation of blood pressure. (***Circulation*. 2005;112:2487-2495.**)

Key Words: hydrogen peroxide ■ blood pressure ■ catalase, vascular ■ aminotriazole ■ contractility

Hydrogen peroxide is a nonradical reactive oxygen species that is produced in many different cell types in the human body, including vascular endothelial and smooth muscle cells.[1] A large number of reports during the past 20 years have suggested that hydrogen peroxide may be an important mediator in the vasculature, eg, a regulator of vasomotor tone.[2–4] However, in vitro studies have yielded conflicting results on the effect of hydrogen peroxide on vasomotor tone,[5,6] suggesting that the vasomotor response to hydrogen peroxide depends on experimental conditions, such as the type of vessel studied, the species studied, and the concentration range used. For example, endothelial hydrogen peroxide has been shown to relax mouse and human mesenteric arteries[7,8] but not human radial arteries.[9]

The steady-state concentration of hydrogen peroxide in human plasma, blood cells, and vascular cells is unknown but is most likely in the lower micromolar range or less.[1] In contrast, the vast majority of studies investigated the vasomotor effects of exogenous hydrogen peroxide at much larger concentrations (up to 10 mmol/L), and both vasodilator and vasoconstrictor effects were reported.[3–5,10] Vasodilator effects of hydrogen peroxide may be mediated by activation of K^+ channels,[7] activation of endothelial NO production,[11,12] decreased myosin phosphorylation,[13] and inhibition of myosin ATPase,[14] whereas vasoconstriction is attributed to an increase of intracellular Ca^{2+},[15] to the generation of arachidonic acid metabolites with vasoconstrictor activity,[16] and to direct Ca^{2+}-independent tonic effects on the smooth muscle contractile apparatus.[17,18]

Recent studies in mouse and rat mesenteric arteries and mouse aorta showed vasoconstrictor effects at up to 10 μmol/L of hydrogen peroxide, whereas higher concentrations induced sustained and almost irreversible vasodilation.[16,19]

Clinical Perspective p 2495

Received February 15, 2005; revision received July 25, 2005; accepted July 27, 2005.

From the Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Düsseldorf (T.S., N.L., S.K., G.K.); the Institut für Zytobiologie und Zytopathologie, Philipps-Universität, Marburg (R.J.); and the Anatomisches Institut, Tierärztliche Hochschule, Hannover (W.M.), Germany.

*The first 2 authors contributed equally to this work.

Correspondence to Georg Kojda, PharmD, PhD, Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany. E-mail kojda@uni-duesseldorf.de

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.543157**

These data suggest a biphasic action of hydrogen peroxide on the vasomotor tone of conductance and small mesenteric arteries, in which lower micromolar concentrations induce predominantly vasoconstrictor effects. Although there are no data on the effects of hydrogen peroxide on the vascular tone of small skeletal muscle resistance arteries, it seems possible that the vasomotor effects of endogenously produced hydrogen peroxide contribute to the regulation of blood pressure. In hypertension, the plasma concentration of hydrogen peroxide is increased and is positively correlated to plasma renin activity and systolic blood pressure (sBP) but negatively correlated to cardiac contractility and renal function.[20] To investigate the effects of endogenous vascular hydrogen peroxide on small skeletal resistance vessels in vivo, we studied a transgenic mouse with a vascular-specific overexpression of human catalase.

Methods

Generation of Transgenic Mice

We generated a transgenic construct in which human catalase (hCat) was inserted between murine Tie-2 Promotor (2.1 kb) and a 10-kb Tie-2 intron fragment, designated as Tie-2 enhancer, and this construct was used to target catalase gene expression to the vasculature as described previously.[21] Founder mice showing approximately 100-fold higher catalase expression were crossed 10 times to C57BL/6 mice to generate pure C57BL/6 background (cat^{++}). Mice were used at 12 to 16 weeks of age. Transgene-negative littermates (cat^{n}) served as controls. In addition, C57BL6 mice were used as nontransgenic controls. In some experiments, mice carrying a vascular-specific overexpression of wild-type endothelial nitric oxide synthase (eNOS) driven by the murine Tie-2 promotor were used. Vascular overexpression of eNOS ($eNOS^{++}$) was evident by a 1.66±0.13-fold eNOS protein content in the aorta (n=5, $P<0.05$) compared with transgene negative littermates ($eNOS^{n}$).

Ethical Statement

Permission for this study was provided by the regional government (AZ 23.05-230-3-94/00 and 23.05-230-3-65/99), and the experiments were performed according to the guidelines for the use of experimental animals as given by the Deutsches Tierschutzgesetz and to the *Guide for the Care and Use of Laboratory Animals* of the US National Institutes of Health.

Preparation of 100*g* Supernatants

To prepare protein homogenates, mouse thoracic aortas were flash-frozen in liquid nitrogen, homogenized, solubilized in lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L DTT, 1 μmol/L proteinase inhibitors), and centrifuged 10 minutes at 100*g*. Supernatants were stored at −70°C until used for Western blots.

Western Blotting

Western blot analysis was performed in a blinded manner, as described previously,[22] using commercially available monoclonal antibodies directed against eNOS (Transduction Laboratories), rabbit antiserum M4 against vasodilator-stimulated protein (VASP, Alexis), monoclonal anti-phospho-Ser239 antibody 16C2 (Vasopharm), and a polyclonal antibody for catalase (Calbiochem) as well. Blots were developed by use of enhanced chemiluminescence (Roche) and exposed to x-ray film. The autoradiographs were analyzed by densitometry (Geldoc, Bio-Rad). Total protein levels were determined by the Bradford method.[23]

Immunohistochemistry

Different organs of the mice (heart, aorta, lung, kidney) were removed, immediately fixed in Bouin's fluid, and embedded in paraffin wax. Sections 8 μm thick were stained for catalase (antibody diluted 1:100, anti-human, from rabbit; Calbiochem). After incubation for 60 minutes at room temperature, the reaction was detected by an ABC system (Vector Elite Kit) using biotinylated second antibodies and PO-conjugated streptavidin. Before the reaction, all sections were digested for 30 minutes with 0.1% trypsin.

Smooth Muscle Layer Thickness

Aortic rings (1 cm long) of cat^{++} and cat^{n} were fixed in buffered 4% formalin and embedded ethanol-free in the water-soluble plastic resin Technovit 7100 (Heraeus-Kulzer) to prevent shrinkage and lipid extraction. Sections 4 μm thick were stained with 1% toluidine blue, and intima-media thickness was measured by use of the image analyzing system CUE-3 (Olympus Ltd, version 4.5, 1993). The measurements were routinely performed in a double-blind experimental approach by a technical assistant who had no knowledge of the specific scientific background of the study.

Detection of Reactive Oxygen Species

The hydrogen peroxide level was monitored with 5- and 6-carboxy-2′7′-dichlorodihydrofluorescein diacetate bis(acetomethyl)ester (DCDHF-DA, Molecular Probes), which can be oxidized to the fluorescent compound 2′7′-dichlorofluorescein.[24] The intimal layer of longitudinally cut mouse aortic rings was incubated with 5 μmol/L DCDHF-DA for 30 minutes at 37°C and rinsed 3 times with PBS, and fluorescence was visualized on a Leica TCS SP2 confocal microscope (excitation, 488 nm; emission, 525 nm). Slices of left ventricle of cat^{++} and cat^{n} mice were pretreated for 1 hour at 37°C with vehicle or with the catalase inhibitor aminotriazole (1 mmol/L), followed by incubation with 5 μmol/L DCDHF-DA. and quantification of fluorescence intensity. In addition, the basal level of oxidative stress was measured in leukocytes by means of dihydroethidine fluorescence intensity as analyzed by fluorescence-activated cell sorter (FACS).[25]

Measurement of Blood Pressure and Heart Rate

SBP and heart rate were measured in awake male cat^{++} (n=8), cat^{n} (n=8), and C57Bl/6 mice (n=6) at 3 to 4 months of age using an automated tail-cuff system (Visitech Systems) as described previously.[26] In some experiments, cat^{++} and cat^{n} mice (n=4 each) were treated with aminotriazole (666 $mg \cdot kg^{-1} \cdot d^{-1}$, dissolved in drinking water) for 14 days. In another subset of experiments, cat^{++} (n=6) and C57Bl/6 (n=5) mice were treated for 28 days with N^{ω}-nitro-L-arginine (100 $mg \cdot kg\ BW^{-1} \cdot d^{-1}$), and blood pressure and heart rate were recorded before, during, and after the treatment period.

Organ Bath Experiments

Preparation of thoracic ring segments was performed in HEPES-containing Krebs-Henseleit buffer, and the organ bath experiments were performed in the same buffer lacking HEPES. After a 60-minute equilibration period, aortic rings were repeatedly subjected to 80 mmol/L KCl. The vasoconstriction that developed during the last of 3 KCl applications was taken as the maximal receptor-independent vasoconstriction. The function of the endothelium was examined by cumulative addition of acetylcholine (1 nmol/L–10 μmol/L) after submaximal precontraction with phenylephrine. In cat^{++} (n=4) and cat^{n} (n=4) mice, endothelium-dependent vasodilation to the NO donor DEA-NO (0.1 nmol/L–10 μmol/L) was followed by a cumulative application of phenylephrine (1 nmol/L–10 μmol/L). Thereafter, the aortic rings were incubated for 30 minutes with aminotriazole (1 mmol/L) or vehicle, and a concentration-response curve for phenylephrine (1 nmol/L–10 μmol/L) was performed. Reaction to increasing concentrations of hydrogen peroxide (10 nmol/L–10 mmol/L) was examined in endothelium-intact or denuded aortic segments of cat^{++} and cat^{n}/C57BL/6 mice after precontraction with phenylephrine (0.1 μmol/L).

Substances and Solutions

All chemicals were obtained from Merck or from Sigma in analytical grade. Stock solutions were prepared daily, diluted with Krebs-buffer as required, kept on ice, and protected from daylight until use. All

concentrations indicated in the text and figures are expressed as final bath concentrations.

Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0, ANOVA) and are expressed as mean values and SEM. Significant differences were evaluated by use of either the Newman-Keuls multiple comparison test after 1-way ANOVA, 2-way ANOVA, or Student *t* test. A probability value of less than 0.05 was considered significant.

Results

Characterization of Catalase Overexpression

Injection of the catalase-Tie-2-promotor construct resulted in a marked overexpression of catalase, as evidenced by Southern blot in the myocardium (not shown), competitive reverse transcription–polymerase chain reaction (31.7±4.2 6 pg mRNA/μg total RNA in cat^{++} versus 4.5±0.6 pg mRNA/μg total RNA in cat^{n}, n=3, $P<0.01$), Western blot (2.2±0.3-fold, $P<0.05$), and activity (43.2±11.5×10^{-3} s^{-1}/mg protein of cat^{++} versus 14.2±2.0×10^{-3} s^{-1}/mg protein in cat^{n}, n=4, $P<0.05$) in the aorta, the lung, and the heart. Immunohistochemistry performed in cross sections of the aorta, the heart, the lung, and the kidney of cat^{++} showed that the overexpression occurred specifically in blood vessels (Figure 1). Examination of the steady-state content of hydrogen peroxide in endothelial cells by confocal microscopy using longitudinally cut aortic segments pretreated with DCDHF-DA revealed a significant reduction of the fluorescence signal in cat^{++} (1973±244) compared with cat^{n} (3808±664, n=8, $P=0.0021$). Likewise, DCDHF-DA–induced fluorescence was much smaller in slices of left ventricular myocardium of cat^{++} compared with cat^{n}, whereas this difference disappeared in the presence of aminotriazole, which increased the fluorescence signal to the same degree in mice of both strains (Figure 2). In striking contrast, oxidative stress in leukocytes as measured by dihydroethidine fluorescence using FACS analysis was not different in cat^{n} (43±6 U, n=10) and cat^{++} (44±8 U, n=9) but increased strikingly in cat^{++} treated with aminotriazole (107±5, n=5). These data indicate that cat^{++} mice specifically overexpress catalytically active catalase protein in vascular cells.

Smooth Muscle Layer Thickness

To evaluate a possible effect of catalase overexpression on the media thickness of the vasculature, we measured the smooth muscle layer thickness of different arteries. There was no difference between cat^{n} and cat^{++} (n=3, each) in the aorta (45.18±2.37 versus 45.25±1.06 μm, $P=0.978$) and in arterioles of the heart (5.0±0.23 versus 5.12±0.08 μm, $P=0.642$) and the lung (9.33±0.44 versus 8.91±0.24 μm, $P=0.455$). These data suggest that catalase overexpression has no effect on vascular media thickness.

Blood Pressure and Heart Rate

sBP was significantly lower in cat^{++} mice (102.7±2.2 mm Hg, n=10) compared with their transgene-negative littermates (cat^{n}, 115.6±2.5 mm Hg, n=8) or C57BL/6 mice (118.4±3.06 mm Hg, n=6, Figure 3A). Catalase overexpression had no effect on heart rate (data not shown). The decrease of sBP in cat^{++} was identical to that in eNOS^{++} mice (105.3±2.6 mm Hg, n=6, Figure 3B). These data indicate that overexpression of catalase causes the same degree of sBP reduction as overexpression of eNOS.

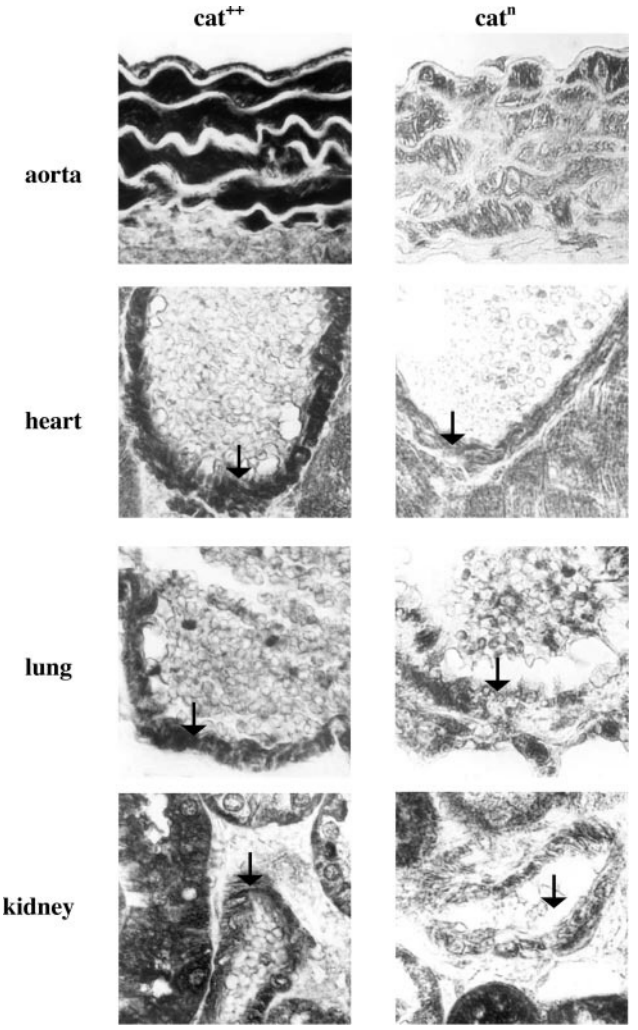


Figure 1. Demonstration of vascular-specific overexpression of human catalase in aorta, heart, lung, and kidney of cat^{++} and cat^{n} (magnification as indicated). Arrows indicate a vascular wall.

Aminotriazole Treatment

Treatment of cat^{++} (n=4) and cat^{n} (n=4) with the catalase inhibitor aminotriazole significantly raised sBP in cat^{++} mice, whereas it had little effect on the sBP of cat^{n} (Figure 3C). There was no change of heart rate caused by inhibition of catalase (data not shown). The recovery of sBP by aminotriazole treatment suggests that the reduction of blood pressure in cat^{++} mice is caused by overexpression of catalase and a subsequent decrease of the vascular hydrogen peroxide steady-state concentration.

Aortic eNOS Expression

To evaluate a possible effect of catalase overexpression on vascular eNOS expression, we compared aortic eNOS protein levels in cat^{n} and cat^{++}. As shown in Figure 4, overexpression

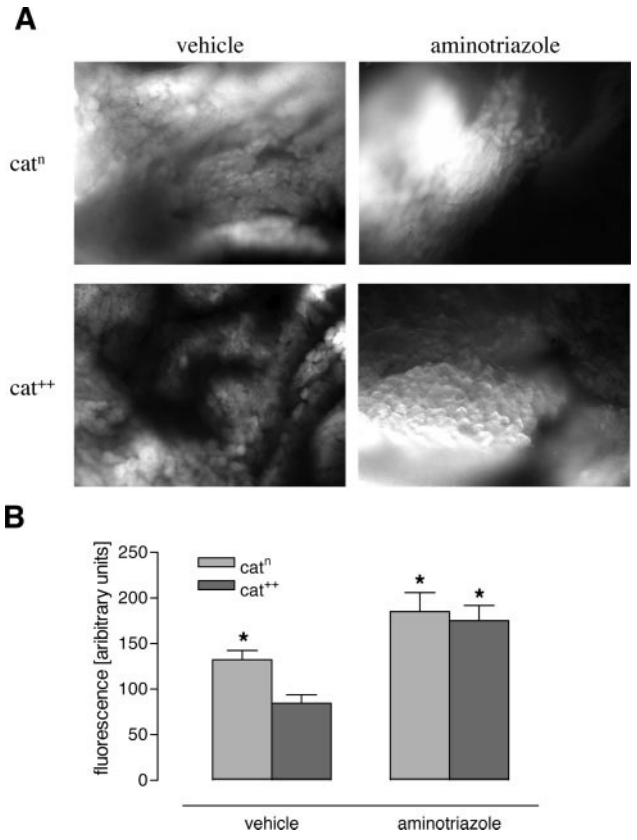


Figure 2. Effect of catalase inhibitor aminotriazole on endogenous hydrogen peroxide content in the slices of left ventricle of cat^{++} and cat^{n}. A, Endothelial hydrogen peroxide levels after treatment with vehicle or catalase inhibitor aminotriazole (1 mmol/L) as measured by use of dichlorofluorescein fluorescence. B represents the mean dichlorofluorescein fluorescence intensity in arbitrary units (*$P<0.05$ vs cat^{++}, n=4, 1-way ANOVA).

of catalase had no effect on aortic eNOS protein content. In contrast, Western blot analysis in $eNOS^{++}$ mice demonstrated a significantly elevated aortic eNOS protein level of 166±13.7% (n=5, $P<0.05$, Figure 4). These findings indicate that the reduction of sBP in cat^{++} mice is not caused by an increase of aortic eNOS protein expression.

NO Bioavailability

To evaluate whether overexpression of catalase changes the bioavailability of endogenous NO, C57BL/6 and cat^{++} mice were treated with the NO synthase inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) for 6 weeks. As expected, L-NAME elevated blood pressure significantly in C57BL/6 mice, from 118±3 to 146±5.6 mm Hg (n=6, $P<0.001$, Figure 5A). This 23.3±4.8% increase of sBP observed in C57BL/6 mice was almost identical to the 20.3±2.8% increase of sBP in cat^{++} (Figure 5B), in which an elevation from 103±2.3 to 125±4.5 mm Hg occurred (n=6, $P<0.001$). In both mouse strains, termination of L-NAME treatment induced a complete restoration of sBP to pretreatment values. We have also measured VASP phosphorylation to further substantiate that the NO/cGMP system is not involved. There was no change in the total VASP protein content in cat^{++} in the lung (120±12%, n=4), the skeletal muscle (92±10%, n=4), and the heart muscle (109±19%, n=3), tissues known to contain mostly resistance vessels. Likewise, phosphorylated VASP protein was also identical in cat^{n} and cat^{++}, as indicated by the relative Western blot signal intensity in cat^{++} in the lung (117±36%, n=8), the skeletal muscle (126±20%, n=4), and the heart (95±29%, n=4).

Studies of Isolated Aortic Segments

Aortic responses to increasing concentrations of acetylcholine (Figure 6A) were similar in cat^{++} and cat^{n}, confirming that the bioavailability of endothelial NO is not altered by a reduction of vascular hydrogen peroxide levels. Likewise, the efficiency of the NO/cGMP pathway remained in cat^{++}, as evidenced by aortic responses to the spontaneous NO donor DEA-NO (Figure 6B). Thus, vascular overexpression of catalase had no effect on endothelium-dependent and -independent NO-mediated relaxation of conductance vessels.

Subjection of aortic rings to a single dose of 80 mmol/L KCl induced a strong vasoconstriction in cat^{n} (9.65±0.34 mN, n=4). Aortic rings of cat^{++} showed a significantly lower maximal response to 80 mmol/L KCl of 8.05±0.46 mN (n=4, $P=0.0322$), although there was no change of the media thickness in this vessel type (see above). These data suggest that overexpression of catalase decreases the contractile response of vascular smooth muscle cells caused by depolarization. In contrast, concentration-dependent vasoconstriction to phenylephrine was similar in cat^{n} and cat^{++} (Figure 7). Neither the half-maximal effective concentration given as pD_2 values in −log mol/L for phenylephrine in cat^{++} (6.46±0.2, n=4) and cat^{n} (6.45±0.2, n=4) nor maximal vasoconstrictions to phenylephrine in cat^{++} (5.49±0.54 mN, n=4) and cat^{n} (5.48±0.54 mN, n=4) were different. However, although inhibition of catalase with aminotriazole had only little effect on phenylephrine constrictions in cat^{++}, it significantly increased phenylephrine constrictions in cat^{n} ($P=0.023$, n=4, Figure 7), suggesting a contribution of endogenous hydrogen peroxide to adrenergic vasoconstriction in conductance vessels.

To investigate vasoconstrictor effects of exogenous hydrogen peroxide, denuded aortic rings of cat^{n} and cat^{++} were subjected to increasing concentrations of hydrogen peroxide. Small vasoconstrictor effects were observed up to 50 μmol/L hydrogen peroxide in cat^{n} only, whereas higher concentrations induced strong and irreversible vasodilator effects in cat^{n} and cat^{++} (Figure 8). After washout of vasodilator concentrations of hydrogen peroxide, aortic rings did not respond to KCl or phenylephrine any longer (data not shown).

Discussion

The aim of this study was to determine the effect of endogenous vascular hydrogen peroxide on the vasomotor tone of resistance vessels in vivo. Our new finding is that a reduction of steady-state concentrations of vascular hydrogen peroxide induced by a vascular specific overexpression of human catalase resulted in a marked reduction of sBP in mice. This hypotension was completely reversed by treatment with the catalase inhibitor aminotriazole but was independent of vascular eNOS protein content, eNOS activity, and the

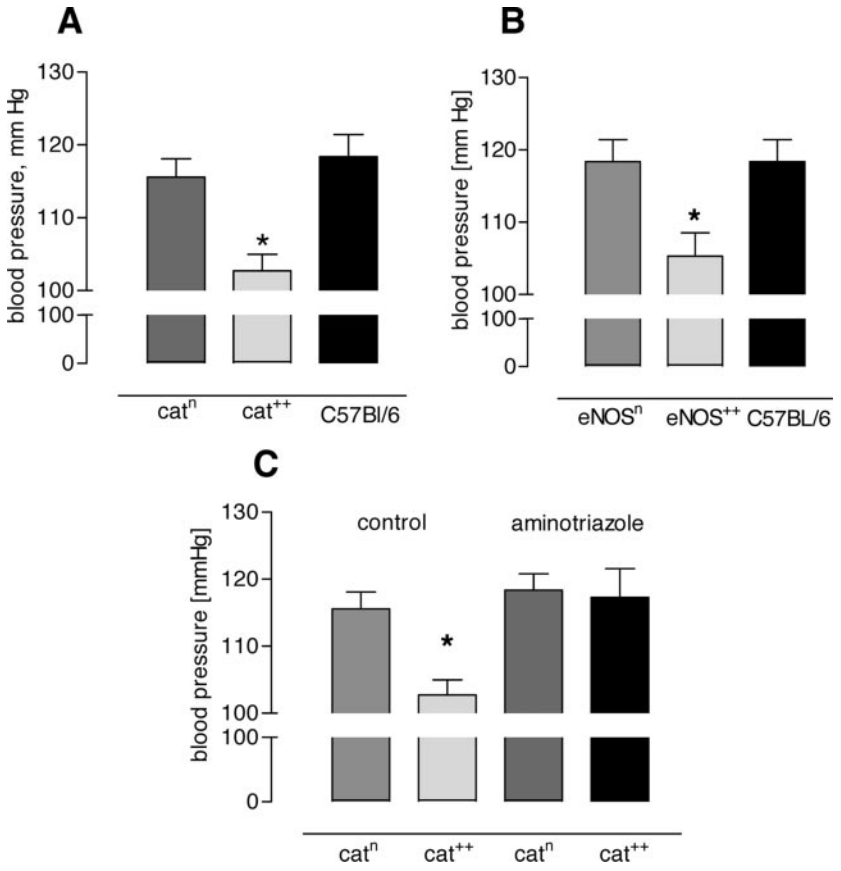


Figure 3. Blood pressure in C57Bl/6 mice, cat^{++}, and cat^{n} (A) and in C57Bl/6, $eNOS^{++}$, and $eNOS^{n}$ (B). Measurements were obtained in resting awake animals using a tail-cuff method (*$P<0.01$, n=6 to 10, 1-way ANOVA). C, Restoration of sBP by treatment with catalase inhibitor aminotriazole. Treatment with aminotriazole (666 mg · kg^{-1} · d^{-1} for 2 weeks) normalized sBP in cat^{++} but had no influence on blood pressure in cat^{n} (*$P<0.01$, n=4 to 8, for all comparisons with control/cat^{++}).

efficiency of the NO/cGMP pathway as evidenced by Western blot analysis, chronic treatment with the NOS inhibitor L-NAME, and organ bath experiments, respectively. These data suggest that endogenous hydrogen peroxide is a vasoconstrictor in resistance vessels in vivo. We assume that the vasoconstrictor effects of hydrogen peroxide in resistance vessels might contribute to the development of essential hypertension.

Our data obtained in transgenic mice overexpressing catalase strongly support the concept that reactive oxygen species, such as hydrogen peroxide, generated in the vascular wall can modulate arterial tone and are involved in the pathogenesis of such vascular diseases as hypertension.[27,28] As predicted,[29] catalase overexpression driven by the Tie-2 promotor construct was vascular-specific in our mouse model. This was evident in the aorta, the lung, the heart, and the kidney. Vascular specificity was further investigated by measuring dihydroethidine fluorescence in leukocytes by FACS analysis, and no difference was found between cat^{n} and cat^{++}, suggesting an absence of catalase overexpression in nonvascular cells, as found in a variety of organs. Our cat^{++} mouse is the first transgenic animal model in which a mammalian gene was overexpressed, driven by the Tie-2 promotor. In the original publication describing the discovery of the Tie-2 promotor, Schläger et al[29] suggested an endothelium-specific expression induced by this promotor. However, this was not directly proved by histology of cross sections of vascularized tissues, and our results rather suggest a vascular-specific instead of an endothelium-specific expression pattern induced by the Tie-2 promotor.

In our animal model, catalase overexpression was associated with a substantial reduction of blood pressure in resting awake mice, and this hypotension was completely reversible by the catalase inhibitor aminotriazole. Recently, Yang et al[30] reported that catalase-overexpressing mice generated by means of an 80-kb P1 clone containing the entire human catalase gene show no change of hydrogen peroxide release from the aorta and have normal blood pressure. Thus, specific targeting of catalase overexpression to the vasculature appears to be important to unmask a vasotonic effect of endogenous hydrogen peroxide.

It has been shown that vascular hydrogen peroxide has many associations with the vascular NO/cGMP pathway.[3,4] For example, hydrogen peroxide may either enhance endogenous NO generation by increasing the activity and expression of eNOS[11,12,31] or impair endothelial production of NO in response to such mediators as the calcium ionophore A23187, bradykinin, and ADP.[32] Hydrogen peroxide might also play a role in flow-mediated vasodilation, and catalase most likely consumes NO, which partially inhibits the reaction of the enzyme with hydrogen peroxide.[33,34] We investigated whether hypotension in cat^{++} is associated with changes of the vascular eNOS content or the bioavailability of endogenous NO. Aortic eNOS protein was not changed, suggesting that the reduction of vascular hydrogen peroxide in cat^{++} had no impact on the regulation of endothelial eNOS expression.

The lack of influence on eNOS expression of a reduced vascular hydrogen peroxide content probably results from the complex regulation of vascular eNOS expression, leaving a

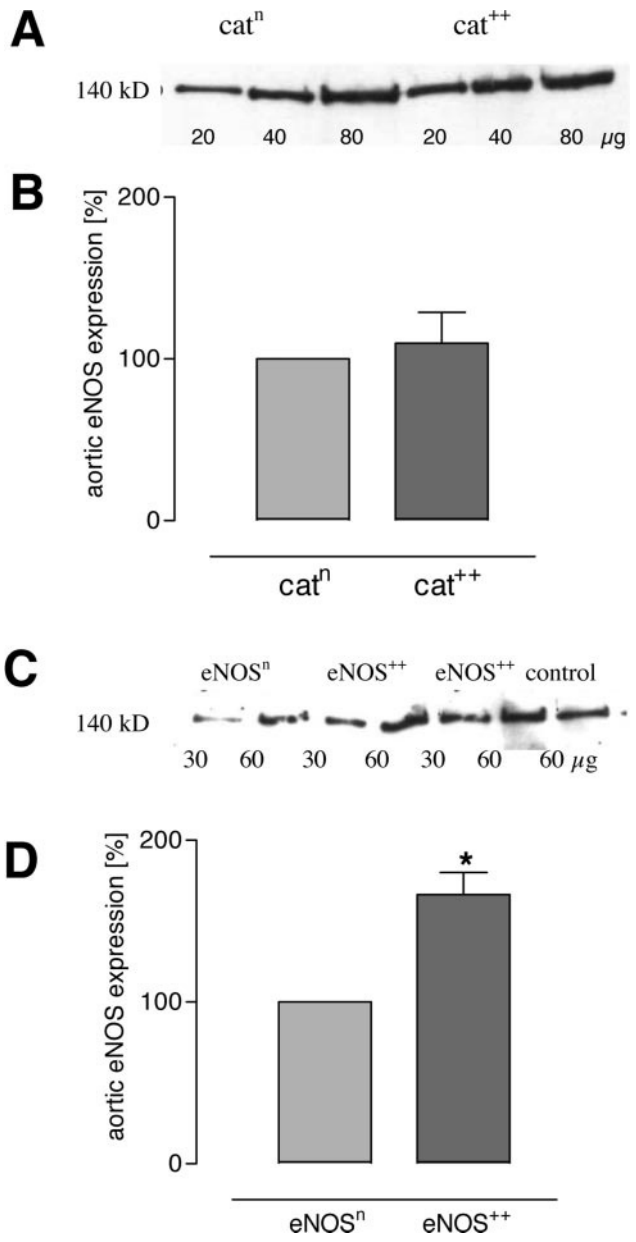


Figure 4. Quantitative evaluation of aortic eNOS expression in cat^n and cat^{++} and in $eNOS^n$ and $eNOS^{++}$. A and C, Representative blots for eNOS in cat^n and cat^{++} (A) and in $eNOS^n$ and $eNOS^{++}$ (C). The plots in B and D represent the mean values of eNOS protein expression in the aorta of cat^n and cat^{++} (B) and $eNOS^n$ and $eNOS^{++}$ (D) after densitometric analysis (n=5, each).

lot of compensatory mechanisms.[35] We were also unable to detect differences of the bioavailability of vascular NO in resistance and conductance vessels. Oral treatment with the NOS inhibitor L-NAME induced an identical rise of sBP in cat^{++} and cat^n, and the approximately 15-mm Hg difference in sBP remained. Likewise, aortic endothelium-dependent vasodilation in cat^{++} was normal, and measurement of serine-239–VASP phosphorylation[36] showed a similar degree of activation of the NO/cGMP pathway in tissues known to contain mostly resistance vessels. These data do not support the hypothesis that endogenous hydrogen peroxide impairs endogenous NO production and bioavailability in BP-regulating skeletal resistance vessels.

Previous data obtained in endothelium-denuded mouse and rat mesenteric arteries have suggested that concentrations of exogenous hydrogen peroxide (0.1 to 10 µmol/L), which probably resemble in vivo conditions, induce either no response or a vasoconstriction.[16,19] Such vasoconstrictor effects of hydrogen peroxide might be mediated by an increase of intracellular Ca^{2+},[15] the generation of arachidonic acid metabolites with vasoconstrictor activity,[16] and a direct Ca^{2+}-independent tonic effect on the smooth muscle contractile apparatus.[17,18] These in vitro vasocontractile activities of exogenous hydrogen peroxide might also occur in vivo and might explain hypotension in cat^{++}, which is dependent on catalase activity, as evidenced by the complete reversal after aminotriazole treatment. Inhibition of catalase activity by aminotriazole also unmasked a potentiation of adrenergic vasoconstriction by phenylephrine in aortic rings of cat^n that was attenuated in cat^{++}, as expected. In addition, concentrations of hydrogen peroxide that most likely resemble in vivo conditions induced vasoconstrictor effects in aortic rings submaximally precontracted with phenylephrine. We suggest that the vasoconstrictor and/or calcium-sensitizing effect of hydrogen peroxide in vivo is already exploited at normal physiological concentrations that occur in the vascular wall. A further increase by treatment with aminotriazole does increase blood pressure slightly but not significantly. Likewise, aminotriazole did increase DCDHF-DA fluorescence of ventricular slices of cat^n slightly but not significantly, while having a much stronger effect in cat^{++}.

Vasoconstriction to phenylephrine is mediated by $\alpha_{1A/C}$ receptors on vascular smooth muscle cells.[37] Activation of these G-protein–coupled receptors results in stimulation of different intracellular enzymes that are involved in the signal transduction. The second messengers 1,4,5-inositol triphosphate (IP_3) and diacyl glycerol are generated by phospholipase C. Whereas IP_3 releases calcium from the sarcoplasmic reticulum and thereby directly initiates vasoconstriction, diacyl glycerol activates protein kinase C and supports the intracellular calcium release.[38] Hydrogen peroxide is known to interact with some proteins of this signal transduction pathway, such as phospholipase C, protein kinase C, and phosphoinositide 3-kinase, which might explain the molecular mechanisms underlying hydrogen peroxide–induced increases of vascular tone.[39] It might be argued that aortic rings are not a suitable model to investigate the effects of hydrogen peroxide on resistance vessels. However, our data are consistent with the observed change of sBP, with the effects of aminotriazole in vivo and in vitro, and with a previous report showing that increases of blood pressure in response to vasoconstrictor agents such as norepinephrine and angiotensin II were less pronounced in mice with an unspecific overexpression of catalase.[30] Thus, the involvement of endogenous hydrogen peroxide in adrenergic constriction of resistance vessels in vivo most likely contributes to hypotension in cat^{++}.

We found that aortic rings of cat^{++} had a significantly lower maximal vasoconstrictor response to KCl. KCl induces a depolarization of the smooth muscle cell membrane and a subsequent influx of calcium through L-type calcium channels.[40,41] It has been shown that the increase of the intracel-

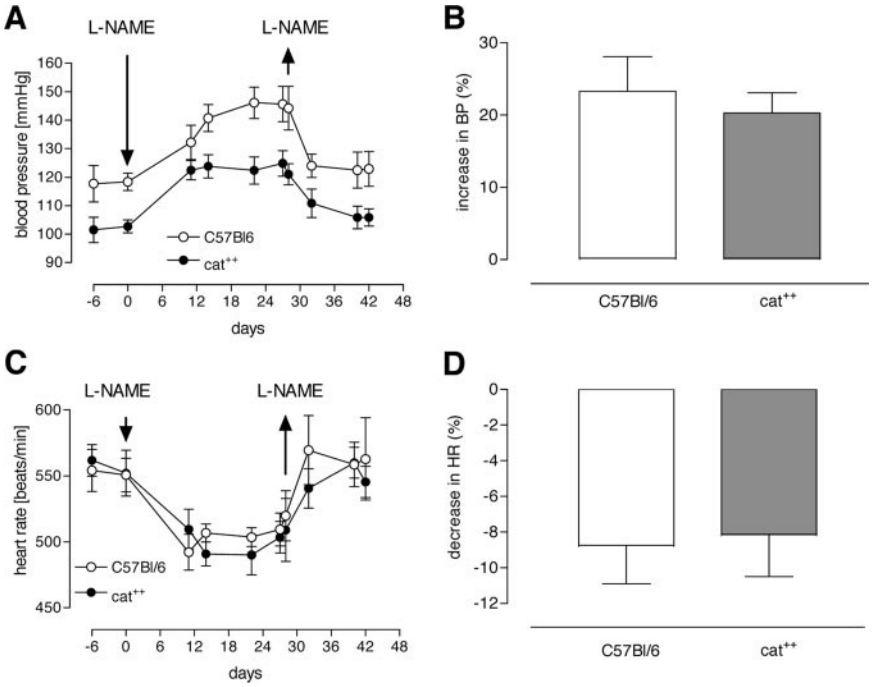


Figure 5. Time-dependent changes of sBP (A) and heart rate (C) in cat^{++} (n=6) and C57Bl/6 (n=5) mice during (4 weeks) and after (2 weeks) L-NAME (100 mg · kg BW^{-1} · d^{-1}) treatment. There was no effect of L-NAME application on the difference (in % from control level) in sBP (B) and heart rate (D) between C57Bl/6 and cat^{++}. Application (+) and removal (−) of the NOS inhibitor L-NAME indicated by arrows.

lular calcium concentration induced by KCl is not accompanied by a calcium release from the sarcoplasmic reticulum.[42] This selective dependency of KCl-induced vasoconstrictions on the influx of extracellular calcium is widely used to initially evaluate the response of isolated vascular preparations to increased cytosolic calcium concentrations in smooth muscle cells.[40] Hence, our data suggest that the vascular calcium contraction coupling might be less efficient in cat^{++}. In mouse-tail arterioles, elevation in transmural pressure causes generation of reactive oxygen species, of which particularly hydrogen peroxide initiates myogenic constriction.[18]

It is interesting to speculate on a causal role of hydrogen peroxide in the development of essential hypertension. Experimental as well as clinical hypertension is associated with oxidative stress.[27,43] Furthermore, previous studies indicate that in hypertension, the plasma concentration of hydrogen peroxide is increased and is positively correlated to plasma renin activity and sBP but negatively correlated to cardiac contractility and renal function.[20] Still, it is not known whether vascular oxidative stress is a result of the disease or may be one underlying cause. Treatment options that have been shown to reduce vascular oxidative stress also reduce blood pressure.[27,43] The direct demonstration of hypotension in mice carrying a vascular specific overexpression of catalase strongly argues for both direct vasoconstrictor effects of endogenous hydrogen peroxide on resistance vessels and a possible contribution of these effects to the development of hypertension.

One might consider limitations of our study on the basis of the fact that neither aminotriazole nor DCDHF-DA and dihydroethidine are entirely specific compounds. Unfortunately, there are no alternative chemical tools showing better specificity.[44] Although DCDHF-DA detects primarily hydrogen peroxide, it is also oxidized by superoxide, although to a much lesser extent. Likewise, dihydroethidine detects primarily superoxide but is also known to be oxidized by hydrogen peroxide. The DCDHF-DA signal was much lower in the

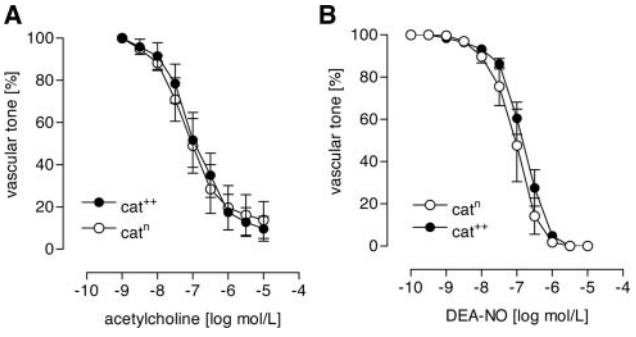


Figure 6. Endothelium-dependent relaxation to acetylcholine (A) and NO-dependent vasodilation to NO donor DEA-NO (B) in aortic rings of cat^{++} and cat^{n} mice. Overexpression of catalase had no influence on these vascular responses to acetylcholine (P=0.75, n=4) and DEA-NO (P=0.82, respectively, n=4).

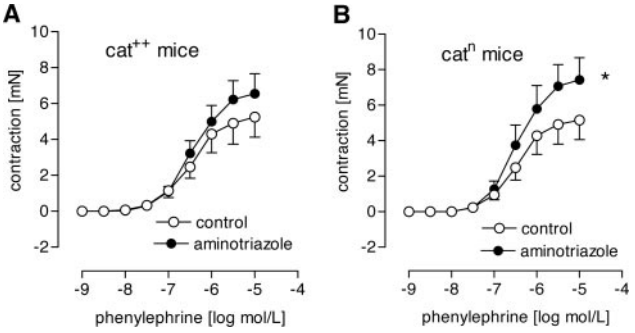


Figure 7. Concentration-dependent constrictor response to increasing concentrations of phenylephrine in aortic rings of cat^{++} (A) and cat^{n} (B) after preincubation with vehicle (control) or aminotriazole (1 mmol/L, *P<0.05, n=4, ANOVA).

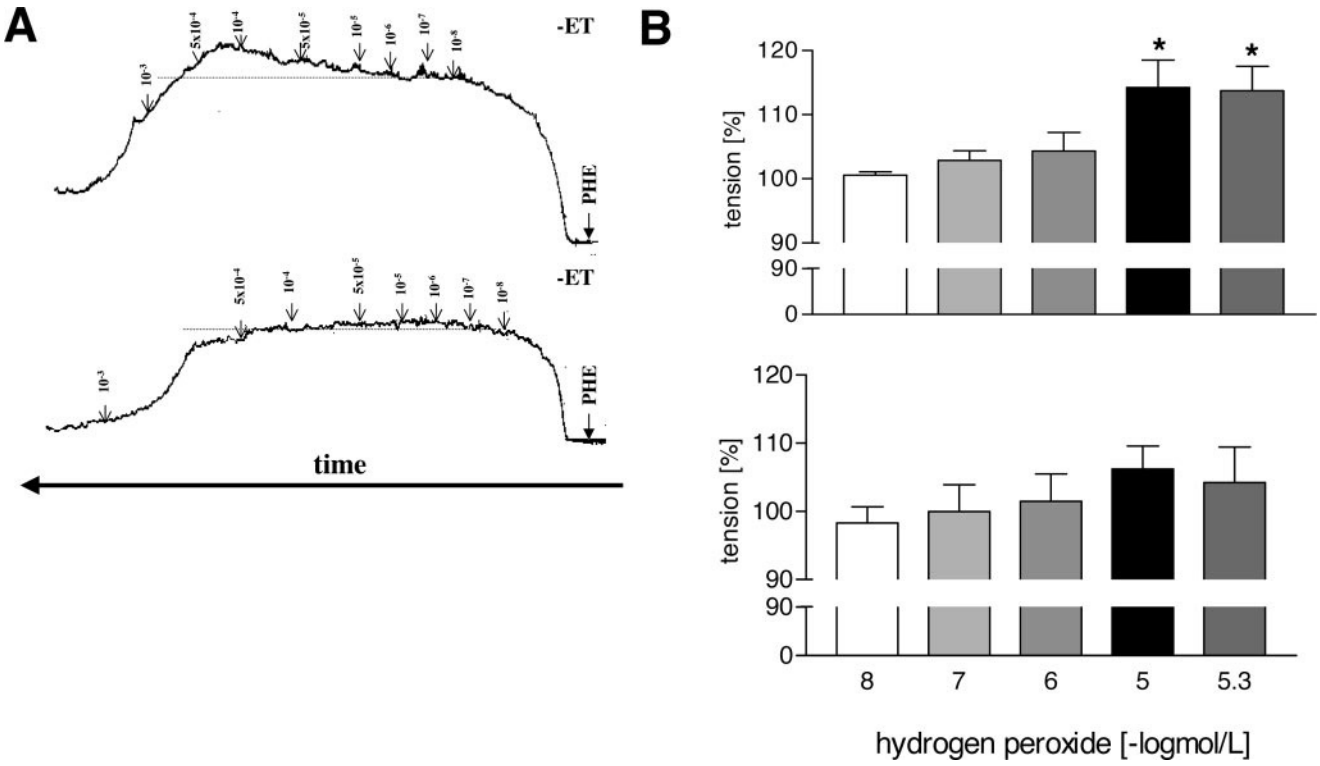


Figure 8. Vasoconstrictor response to increasing concentrations of hydrogen peroxide. A, Original recordings in endothelium-denuded (−ET) aortic rings of cat^{n} (top) and cat^{++} (bottom). B, Summary of 9 individual experiments in cat^{n} (top) and cat^{++} (bottom, $^{*}P<0.05$ vs 0.01 μmol/L hydrogen peroxide, ANOVA).

aortic endothelium and the left ventricular myocardium in cat^{++} compared with cat^{n}, and this was associated with (1) a 6-fold increase of catalase mRNA expression, (2) a 2-fold increase of catalase protein expression, (3) a 3-fold increase of catalase activity in these tissues[21], and (4) a 2-fold increase of the DCDHF-DA signal after treatment with aminotriazole, leaving little room to explain the oxidation of the fluorescent dyes by superoxide. In vitro data suggest that aminotriazole might inhibit not only catalase but also glutathione peroxidase, but the latter does not seem to occur in cardiovascular tissue of mammals in vivo.[45] These data are consistent with our observation the aminotriazole strongly increased the blood pressure of cat^{++} but had only a small effect in cat^{n}. However, we cannot completely rule out the possibility that aminotriazole slightly inhibited glutathione peroxidase in cardiovascular tissue of cat^{++} and cat^{n}.

Acknowledgments

This study was supported by the Forschungskommission of the Heinrich-Heine-Universität Düsseldorf (Project 9772 109) and research scholarships of the Deutscher Akademischer Austauschdienst and the Federation of European Biochemical Societies (to T. Suvorava).

References

1. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. *FEBS Lett*. 2000;486:10–13.
2. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.
3. Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol*. 2000;20:1430–1442.
4. Ellis A, Triggle CR. Endothelium-derived reactive oxygen species: their relationship to endothelium-dependent hyperpolarization and vascular tone. *Can J Physiol Pharmacol*. 2003;81:1013–1028.
5. Jones RD, Morice AH. Hydrogen peroxide: an intracellular signal in the pulmonary circulation: involvement in hypoxic pulmonary vasoconstriction. *Pharmacol Ther*. 2000;88:153–161.
6. Griffith TM. Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? *Br J Pharmacol*. 2004; 141:881–903.
7. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;106: 1521–1530.
8. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, Mukai Y, Hirakawa Y, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun*. 2002;290:909–913.
9. Hamilton CA, McPhaden AR, Berg G, Pathi V, Dominiczak AF. Is hydrogen peroxide an EDHF in human radial arteries? *Am J Physiol*. 2001;280:H2451–H2455.
10. Wolin MS, Gupte SA, Oeckler RA. Superoxide in the vascular system. *J Vasc Res*. 2002;39:191–207.
11. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000;86:347–354.
12. Cai H, Li Z, Davis ME, Kanner W, Harrison DG, Dudley SC Jr. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Mol Pharmacol*. 2003;63:325–331.
13. Lorenz RR, Warner DO, Jones KA. Hydrogen peroxide decreases Ca^{2+} sensitivity in airway smooth muscle by inhibiting rMLC phosphorylation. *Am J Physiol*. 1999;277:L816–L822.
14. Perkins WJ, Lorenz RR, Bogoger M, Warner DO, Cremo CR, Jones KA. A novel mechanism by which hydrogen peroxide decreases calcium sensitivity in airway smooth muscle. *Am J Physiol*. 2003;284: L324–L332.

15. Yang ZW, Zheng T, Wang J, Zhang A, Altura BT, Altura BM. Hydrogen peroxide induces contraction and raises $[Ca^{2+}]_i$ in canine cerebral arterial smooth muscle: participation of cellular signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol*. 1999;360:646–653.
16. Gao YJ, Hirota S, Zhang DW, Janssen LJ, Lee RM. Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. *Br J Pharmacol*. 2003;138:1085–1092.
17. Pelaez NJ, Braun TR, Paul RJ, Meiss RA, Packer CS. H_2O_2 mediates Ca^{2+}- and MLC_{20} phosphorylation-independent contraction in intact and permeabilized vascular muscle. *Am J Physiol*. 2000;279:H1185–H1193.
18. Nowicki PT, Flavahan S, Hassanain H, Mitra S, Holland S, Goldschmidt-Clermont PJ, Flavahan NA. Redox signaling of the arteriolar myogenic response. *Circ Res*. 2001;89:114–116.
19. Ellis A, Pannirselvam M, Anderson TJ, Triggle CR. Catalase has negligible inhibitory effects on endothelium-dependent relaxations in mouse isolated aorta and small mesenteric artery. *Br J Pharmacol*. 2003;140:1193–1200.
20. Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. *Hypertension*. 2000;36:878–884.
21. Lauer N, Suvorava T, Rüther U, Jacob R, Meyer A, Harrison DG, Kojda G. Critical involvement of hydrogen peroxide in exercise-induced upregulation of endothelial NO-synthase. *Cardiovasc Res*. 2005;65:254–262.
22. Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation*. 2001;103:2839–2844.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
24. Schwarz MA, Lazo JS, Yalowich JC, Reynolds I, Kagan VE, Tyurin V, Kim YM, Watkins SC, Pitt BR. Cytoplasmic metallothionein overexpression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity. *J Biol Chem*. 1994;269:15238–15243.
25. Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol*. 1994;55:253–258.
26. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, Harrison DG. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res*. 1999;42:206–213.
27. Kojda G, Harrison DG. Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res*. 1999;43:562–571.
28. Touyz RM. Reactive oxygen species in vascular biology: role in arterial hypertension. *Expert Rev Cardiovasc Ther*. 2003;1:91–106.
29. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci U S A*. 1997;94:3058–3063.
30. Yang H, Shi M, VanRemmen H, Chen X, Vijg J, Richardson A, Guo Z. Reduction of pressor response to vasoconstrictor agents by overexpression of catalase in mice. *Am J Hypertens*. 2003;16:1–5.
31. Thomas SR, Chen K, Keaney JF Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem*. 2002;277:6017–6024.
32. Jaimes EA, Sweeney C, Raij L. Effects of the reactive oxygen species hydrogen peroxide and hypochlorite on endothelial nitric oxide production. *Hypertension*. 2001;38:877–883.
33. Liu Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC, Gutterman DD. Mitochondrial sources of H_2O_2 generation play a key role in flow-mediated dilation in human coronary resistance arteries. *Circ Res*. 2003;93:573–580.
34. Brunelli L, Yermilov V, Beckman J. Modulation of catalase peroxidatic and catalatic activity by nitric oxide. *Free Radic Biol Med*. 2001;30:709–714.
35. Li H, Wallerath T, Forstermann U. Physiological mechanisms regulating the expression of endothelial-type NO synthase. *Nitric Oxide*. 2002;7:132–147.
36. Smolenski A, Burkhardt AM, Eigenthaler M, Butt E, Gambaryan S, Lohmann SM, Walter U. Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn Schmiedebergs Arch Pharmacol*. 1998;358:134–139.
37. Ratz PH. Regulation of ERK phosphorylation in differentiated arterial muscle of rabbits. *Am J Physiol*. 2001;281:H114–H123.
38. Graham RM, Perez DM, Hwa J, Piascik MT. α_1-Adrenergic receptor subtypes: molecular structure, function, and signaling. *Circ Res*. 1996;78:737–749.
39. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol*. 2000;20:2175–2183.
40. Bolton TB. Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol Rev*. 1979;59:606–718.
41. Neild TO, Kotecha N. Relation between membrane potential and contractile force in smooth muscle of the rat tail artery during stimulation by norepinephrine, 5-hydroxytryptamine, and potassium. *Circ Res*. 1987;60:791–795.
42. Daniel EE. Cellular calcium mobilization. *J Cardiovasc Pharmacol*. 1984;6(suppl 4):S622–S629.
43. Landmesser U, Harrison DG. Oxidative stress and vascular damage in hypertension. *Coron Artery Dis*. 2001;12:455–461.
44. Tarpey MM, Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res*. 2001;89:224–236.
45. Antonenkov VD, Panchenko LF. Effect of chronic ethanol treatment under partial catalase inhibition on the activity of enzymes related to peroxide metabolism in rat liver and heart. *Int J Biochem*. 1988;20:823–828.

CLINICAL PERSPECTIVE

The term vascular oxidative stress denotes an increase of a great variety of reactive oxygen species generated in the vascular wall. Although vascular oxidative stress is considered an important pathogenic factor in cardiovascular diseases, there is some evidence for physiological or even beneficial effects. A large number of reports during the past 20 years have suggested that the oxidant hydrogen peroxide may act as a regulator of vasomotor tone, but these in vitro studies have yielded conflicting results. The aim of our in vivo study was to evaluate possible physiological effects of the "hair bleach" hydrogen peroxide in the vasculature. By generation of a transgenic mouse with a vascular-specific overexpression of catalase, we established an animal model with a strongly reduced steady-state concentration of vascular hydrogen peroxide. In these animals, we found a profound hypotension that was completely reversed by treatment with the catalase inhibitor aminotriazole. These observations suggest a tonic effect of hydrogen peroxide on vascular smooth muscle and might be interpreted as a dual vascular role of hydrogen peroxide, again confirming Paracelsus' law: "The dose determines a poison." We cannot tell whether this newly discovered physiological effect of hydrogen peroxide occurs in humans, but this appears to be not entirely unlikely. We hope that our data might encourage clinical researchers to evaluate whether this tonic effect of hydrogen peroxide might contribute to the development of essential hypertension as previously suggested by the results of small clinical trials.

ELSEVIER

Cardiovascular Research 65 (2005) 254–262

Cardiovascular Research

www.elsevier.com/locate/cardiores

Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase

Nadine Lauer[a], Tatsiana Suvorava[a], Ulrich Rüther[b], Ralf Jacob[c], Wilfried Meyer[d], David G. Harrison[e], Georg Kojda[a,*]

[a]*Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Düsseldorf, Germany*
[b]*Institut für Entwicklungsbiologie der Tiere, Heinrich-Heine-Universität, Düsseldorf, Germany*
[c]*Institut für Zellbiologie und Zellpathologie, Philipps-Universität, Marburg Germany*
[d]*Anatomisches Institut, Tierärztliche Hochschule, Hanover, Germany*
[e]*Cardiology Division, Emory University, Atlanta, GA, USA*

Received 27 February 2004; received in revised form 13 September 2004; accepted 13 September 2004
Available online 7 October 2004
Time for primary review 19 days

Abstract

Objective: Recent studies from our groups have indicated that endothelial nitric oxide synthase (eNOS) expression is increased in cell culture by both shear stress and by hydrogen peroxide (H_2O_2). In vivo, exercise training, known to increase both endothelial shear stress and oxidative stress, also increases eNOS expression. It is unclear if H_2O_2 contributes to an increase in eNOS expression in response to exercise training.
Methods: To address this question, we generated mice overexpressing human catalase (hCat) driven by the murine Tie-2 promoter to specifically target this transgene to the endothelium (cat^{++}).
Results: Vessels of cat^{++} expressed significantly higher levels of catalase mRNA and catalase protein and activity but normal levels of eNOS. Exercise alone had no effect on catalase expression in C57BL/6. Wild-type littermates of cat^{++} showed an increase in eNOS expression with 3 weeks of exercise (2.53±0.42-fold) comparable to C57BL/6 (2.93±0.45-fold). In striking contrast, 3 weeks of exercise had no effect on aortic (1.33±0.32-fold) and myocardial (1.1±0.2-fold) eNOS expression in catalase transgenic mice.
Conclusions: These data suggest that endogenous H_2O_2 plays a key role in the endothelial adaptation to exercise training by stimulating an up-regulation of eNOS.

Keywords: Oxidative stress; Hydrogen peroxide; Exercise; Endothelial nitric oxide synthase; Catalase; Vascular endothelial function

1. Introduction

Regular physical activity is an important factor in the prevention of cardiovascular diseases [1]. The effects of exercise on organ function are associated with an approximately 30% reduction of mortality in patients with coronary artery disease or heart failure [2]. Even daily walking of 2 miles reduces the mortality of nonsmoking retired men [3]. Exercise induces a reduction of heart rate and blood pressure, increases maximal myocardial oxygen uptake and leads to several physiological adaptations involving skeletal muscle, cardiac muscle, circulating blood volume and a variety of metabolic modifications [4–7]. In contrast, little is known about the mechanisms leading to the positive effects of exercise in the vasculature.

Several studies suggest an important role of endogenous production of nitric oxide (NO) in the beneficial effects of exercise [8]. In-vivo studies in animals and humans have shown that exercise results in an increased vascular

* Corresponding author. Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany. Tel.: +49 211 81 12518; fax: +49 211 81 14781.
E-mail address: kojda@uni-duesseldorf.de (G. Kojda).

0008-6363/$ - see front matter © 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2004.09.010

expression of the endothelial cell eNOS [9–12]. This potentially important adaptation to exercise training seems to require two intact endothelial nitric oxide synthase (eNOS) genes [13] and might contribute to the improvement of endothelial function in coronary conductance and resistance vessels of exercised coronary artery disease patients [14]. On the other hand, exercise is associated with increased vascular oxidative stress which is considered to be a pathogenic factor in cardiovascular disease [15].

It is not yet known how the induction of vascular eNOS by exercise is mediated. Increased shear stress potently stimulates an increase in eNOS expression. In addition, previous studies from our laboratory have shown that hydrogen peroxide (H_2O_2) increases the expression and activity of eNOS in endothelial cells [16–18]. The increase of eNOS activity in response to H_2O_2 comes along with a change of eNOS phosphorylation and is thought to be an acute cellular adaptation to an increase in oxidant stress [19,20]. This is relevant to exercise, which is associated with an increase in oxidative stress within the skeletal muscle and an increase in circulating levels of H_2O_2 and markers of lipid oxidation. We have also demonstrated that exercise increases expression of vascular extracellular superoxide dismutase (ecSOD) which could enhance the formation of H_2O_2 [21]. Given these considerations, we hypothesized that endogenously produced H_2O_2 would contribute to the up-regulation of eNOS expression caused by exercise training. Unfortunately, there is no reliable method to directly measure vascular H_2O_2 levels in vivo [22]. Thus, we generated transgenic mice with vascular-specific overexpression of human catalase (hCat) and compared the effect of exercise training on eNOS expression in these and wild-type animals.

2. Methods

2.1. Generation of transgenic mice

To target catalase gene expression to the endothelium, we generated a transgenic construct, in which human catalase (hCat) was inserted between murine Tie-2 promotor (2.1 kb) and a 10-kb Tie-2 intron fragment, designated as Tie-2 enhancer (Fig. 1A).

2.1.1. Plasmid construction

By PCR, a Sse83871 restriction site was added to the 5′ site and a MluI site was added to the 3′ site of hCat cDNA. The following primers were used: sense (5′ GAGAGACCTGCAGGAGCAAACCGCACGCTATG); antisense: (5″GAGAGAACGGCTCCAGTGATGAGCGGGTTAC). The resulting 1.7-kb Sse8387I-hCat-MluI fragment was confirmed by restriction analysis and was sequenced by cycle sequencing with dye terminators. Sse8387I-hCat-MluI fragment was isolated and inserted downstream of the Tie-2-promotor (a generous gift from T. Schläger, M.-Planck Institut, Bad Nauheim) and upstream of the Tie-2-enhancer. Plasmid (PCK), confirmed by restriction analysis, comprised pBluescriptII SK(+) backbone (Stratagene, La Jolla, CA), 2.1-kb Tie-2-promotor, 1.7-kb hCat-

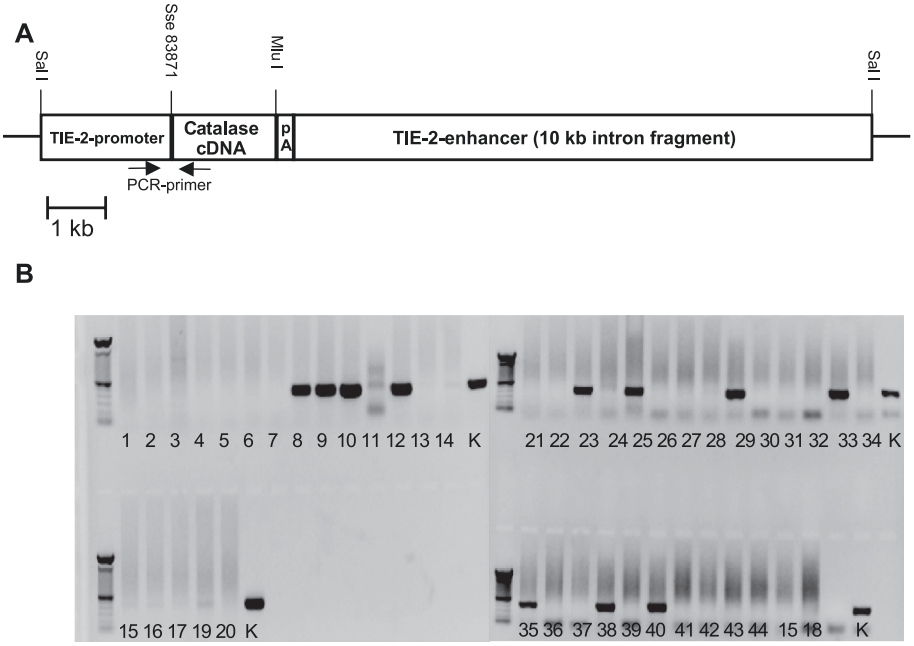


Fig. 1. (A) Construct inserted into fertilized eggs of C57BL/6 mice to generate catalase overexpressing mice consisting of a 2.1-kb Tie-2-promotor, 1.7-kb hCat-cDNA, 250 bp SV40 poly A signal and 10-kb Tie-2 enhancer. Sal I restriction sites used for linearization of the plasmid and Sse83871/MluI sites added to catalase cDNA for ligation are indicated. PCR primers used for genotyping PCR are indicated by arrows. (B) PCR identifying 11 of 44 founder animal harbouring the transgene. A 470 bp transgene specific fragment is amplified in transgenic animals. Plasmid-DNA (100 ng) of the construct served as control (K).

cDNA, 250-bp SV40 poly A signal and 10-kb Tie-2 enhancer (see Fig. 1).

2.1.2. Microinjection

The Tie-2-promotor-hCat-Tie-2 enhancer construct was excised from the vector, purified and microinjected in fertilized F_2-eggs of F_1 (C57BL/6×C3H/He) mice. Microinjected eggs were transferred into the oviducts of pseudopregnant mice and allowed to develop to term.

2.1.3. PCR

Eleven of 44 founder mice were identified harboring the transgene by PCR of genomic DNA isolated from tail biopsies at 4–5 weeks of age. Tail DNA was extracted after proteinase K (0.5 mg/ml) digestion by subsequent isopropanol precipitation and purification. Subsequent genotyping PCRs were performed using transgene specific primers, i.e, sense primer at the 3′ end of Tie-2 promotor (5′ -GGGAAGTCGCAAAGTTGTGAGTT) and antisense primer at the 5′ end of hCat cDNA (5′ -CCGATTCTC-CAGCAACAG) identifying a 470 bp transgene specific fragment (Fig. 1B).

By southern blot, identifying transgenic DNA (not shown), and by competitive PCR, using an internal standard fragment of 364 bp, the transgene copy numbers of different founder lines were estimated. One line showed approximately 100-fold higher catalase expression compared to other lines. Animals of this line were crossed 10 times to C57/BL6 mice to generate pure C57BL/6 background (cat^{++}). Mice were used at 12 to 16 weeks of age. Transgene negative littermates (cat^{n}) served as controls. In addition, C57BL6 mice were used as nontransgenic controls to evaluate changes of catalase expression and of eNOS expression induced by exercise.

2.1.4. Ethical statement

Permission for this study was provided by the regional government (AZ 23.05-230-3-94/00 and 23.05-230-3-65/99), and the experiments were performed according to the guidelines for the use of experimental animals as given by "Deutsches Tierschutzgesetz" and to the "*Guide for the care and use of laboratory animals*" of the US National Institutes of Health.

2.1.5. Competitive RT–PCR

Catalase mRNA was measured in hearts of cat^{++}–mice in comparison to cat^{n}–mice by competitive RT–PCR specific for human, transgenic mRNA as well as murine, native mRNA. An internal deleted standard of 444 bp was constructed by linker-primer–PCR, cloned into pCRII-TOPO Cloning Vector, (Invitrogene, Karlsruhe) and confirmed by restriction analysis. The standard was in vitro transcribed into cRNA, (RNA Transcription Kit, Stratagene, Amsterdam) and quantified spectrophotometrically. Total RNA was isolated from mice tissues with "Rneasy Mini Kit", (Qiagen, Hilden, Germany). In competitive RT–PCR experiments, 500 ng of total RNA was incubated in separate reactions with defined amounts of an internal deleted catalase cRNA standard, reverse transcribed into cDNA (Superscript™ first-strand system for RT–PCR, Invitrogene) and amplified by PCR; sense-primer: 5′-GGTTTCTTTCTTGTTCAGTG, antisense-primer: 5′ -CGGTAGGGACAGTTCACA. A 554-bp fragment results for the native RNA, a 443 bp-fragment for the standard RNA. Fragments were separated through agarose gel electrophoresis, and the optical density of each fragment was measured (Gel Doc 1000, Bio-Rad, Münich, Germany). The respective RNA amounts were calculated using double logarithmic plots of quotients of standard to native PCR fragment density and determining the equivalence point.

2.2. Exercise protocol

Mice were exercised following a previously established protocol [23]. Briefly, mice ran in a newly established self-build exercise wheel treadmill especially designed for mice. Five animals were studied simultaneously. Mice were initially trained three times for 10 min every other day for 10 days. The maximal velocity of the treadmill was 0.25 m/s. After this training, mice were exercised for 3 weeks at 5 days a week for 30 min at 0.25 m/s. Thus, 8 days of training corresponds to a total time of 10 days (2 days without training) and 15 days of training to a total time of 3 weeks (4 days without training). The training was executed during the active cycle of the animals. Nonexercised controls were exposed to the same noise and the vibration of the environment. All mice completed the exercise protocol without signs of exhaustion. There was no obvious difference in exercise performance between the different strains of mice. Previous experiments revealed that 3 weeks of exercise greatly increased the activity of citrate synthase in soleus muscle from 40.3±2.8 mU/mg protein to 141.0±18.0 mU/mg protein (n=7) [24]. Within 16 to 20 h after termination of the last training, mice were sacrificed by inhalation of carbon dioxide, and their aortas and hearts were immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting.

2.3. Preparation of 100×g supernatants

To prepare protein homogenates, mice tissues (thoracic aorta, left ventricle) were flash frozen in liquid nitrogen, homogenized, solubilized in lysis buffer (50 mM Tris–HCL, 1 mM DTT, 1 μM proteinase inhibitors) and centrifuged 10 minutes at 100×g. Supernatants were stored at −70 °C until used for either Western blots or determination of catalase activity.

2.4. Western blotting

Western blotting was performed as described previously [25]. Briefly, increasing amounts of total protein per lane were loaded and 0.5 μg of mouse catalase (Sigma) was used as standard. Blots were incubated with a polyclonal antibody to catalase (Calbiochem, Darmstadt, Germany). Western blotting for eNOS was performed using a monoclonal antibody directed against human eNOS (Transduction Laboratories). Blots were subsequently challenged with a horseradish peroxidase-conjugated antibody (antimouse IgG, Biorad, Munich, Germany, antirabbit IgG, Calbiochem). Blots were developed using ECL (Roche, Mannheim, Germany) and exposed to X-ray film. The autoradiographs were analyzed by densitometry (Geldoc, Bio-Rad). The transfer efficiency was detected by staining the gel with Coomassie after the blotting procedure. In none of the gels, we found visible blue bands. Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%.

2.5. Determination of catalase activity

Catalase activity was measured by $KMnO_4$-reduction assay, as described by Cohen et.al. [26]. 100×*g* supernatants were incubated with 1.2 μmol of H_2O_2 for 3 min. After the reaction has been stopped with 240 μmol of H_2SO_4, 2.8 μmol of $KMnO_4$ was added. Residual $KMnO_4$ was immediately measured spectrophotometrically at 480-nm wavelength.

2.6. Immunohistochemistry

Different organs of the mice were removed, immediately fixed in Bouin's fluid and embedded in paraffin wax. Eight-micrometer sections were stained for catalase (antibody dilute 1:100, antihuman, from rabbit; Calbiochem). Following incubation for 60 min at room temperature, the reaction was detected by an ABC system (Vector Elite Kit) using biotinylated second antibodies and PO-conjugated streptavidin. Before the reaction, all sections were digested for 30 min with 0.1% trypsin.

The H_2O_2 level was monitored with 5- and 6-carboxy-2′ 7′ -dichlorodihydrofluoresceine diacetate bis(acetomethyl) ester (DCDHF-DA, Molecular Probes), which can be oxidized to the fluorescent compound 2′ 7′ -dichlorofluoresceine [27]. Mouse aortic slices were incubated with 5 μM of DHCDF-DA for 30 min at 37 °C, rinsed three times with phosphate-buffered saline and visualized on a Leica TCS SP2 confocal microscope (excitation 488 nm; emission 525 nm).

2.7. Substances and solutions

All chemicals were obtained from Merck, Darmstadt, Germany, or from Sigma, Deisenhofen, Germany, in analytical grade. Stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

2.8. Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0, Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (S.E.M.). Significant differences were evaluated using either Newman–Keuls multiple comparison test following one-way ANOVA (expression of murine catalase after 10 and 15 days of exercise) or student's *t*-test. A *P* value below 0.05 was considered as significant.

3. Results

3.1. Characterization of catalase overexpression

Injection of the catalase-Tie-2-promotor construct resulted in a marked overexpression of catalase as evidenced by Southern blot (not shown), competitive RT–PCR (Fig. 2), Western blot and activity (Fig. 3). Immunohistochemistry performed in cross-sections of the aorta and the heart of cat^{++} showed that the overexpression occurred specifically in

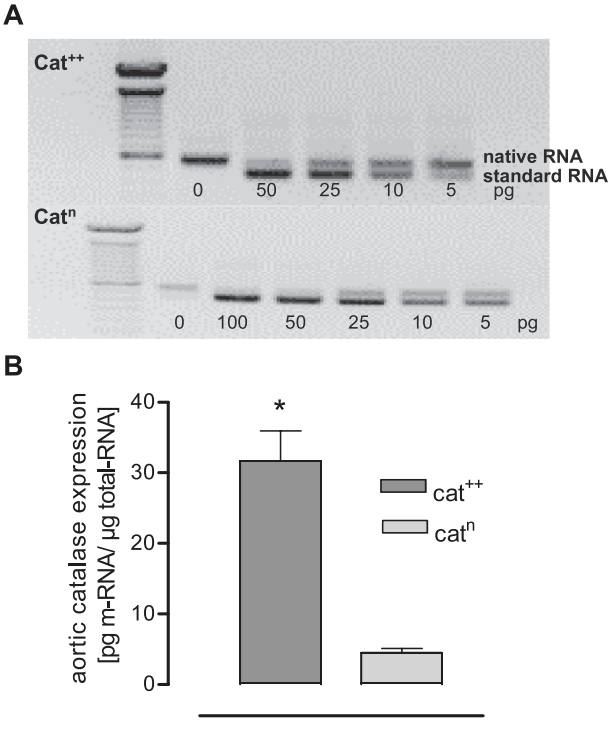


Fig. 2. Quantitative evaluation of catalase mRNA expression in the mouse aorta. The upper panel (A) shows a representative gel comparing mRNA expression in the aorta of cat^{++} and of cat^{n}. The plot in the lower panel (B) shows the mean values of mRNA expression after densitometric analysis based on the ratios of the optical density values for native and standard RNA-bands (n=5). These ratios were calculated for each standard concentration and plotted against the amount of standard mRNA (*P<0.01).

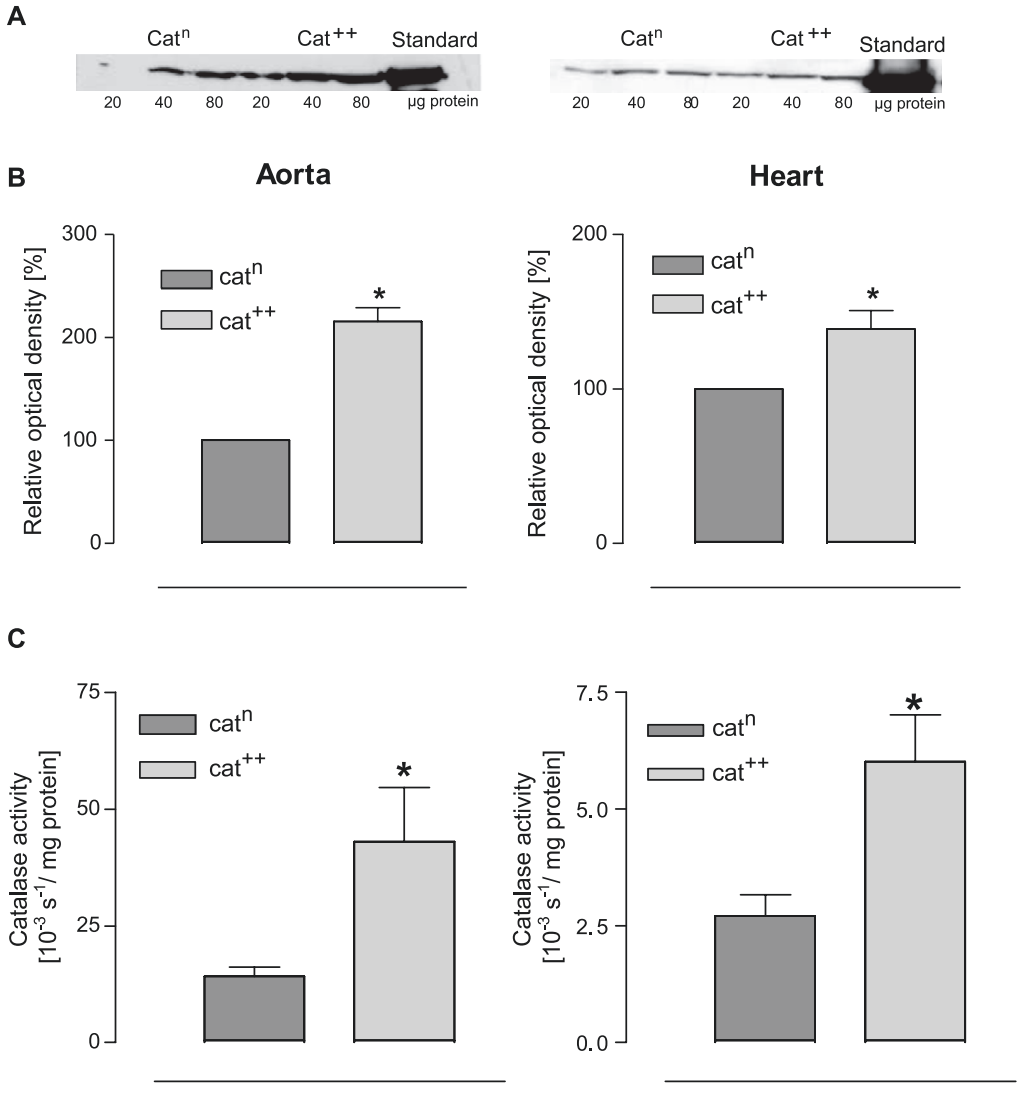


Fig. 3. Quantitative evaluation of catalase protein expression and activity in mouse aorta and heart. The upper panel (A) shows representative gels comparing protein expression in the aorta (upper left panel) and the left ventricular myocardium (upper right panel) of cat^{++} and cat^{n}. The plots in panel (B) show the mean values of catalase protein expression in the aorta (middle left panel) and the left ventricular myocardium (middle right panel) after densitometric analysis (n=5, each). Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%. Panel (C) shows mean values of catalase activity in the aorta (lower left panel) and the left ventricular myocardium (lower right panel; *P<0.05).

blood vessels (Fig. 4). This was also evident in the lung, liver and kidney (not shown) and was associated with a marked reduction of the H_2O_2 content in the aortic endothelial layer as measured using dichlorofluoresceine-fluorescence (in arbitrary units, n=8) from 3808±664 in cat^{n} to 1973±244 in cat^{++} (example given in Fig. 4, top panel). These data indicate that these mice specifically overexpressed catalase in vascular cells.

3.2. Effect of catalase overexpression on basal eNOS expression

To evaluate a possible effect of catalase overexpression on the expression of eNOS, we compared aortic eNOS protein expression in cat^{n} and cat^{++}. Overexpression of catalase had no effect on basal protein expression of aortic eNOS (110±19% in cat^{++} vs. cat^{n}, n=5). These data suggest that there is no change of basal eNOS expression in cat^{++}.

3.3. Effect of exercise on basal catalase expression

To evaluate a possible effect of exercise on the expression of aortic and myocardial catalase, we exercised C57BL/6 mice for both 10 days (n=3) and 3 weeks (n=6). Exercise had a small but nonsignificant effect on the expression of catalase in C57BL/6 mice both in the aorta and in left ventricular myocardium (Fig. 5). Thus, exercise

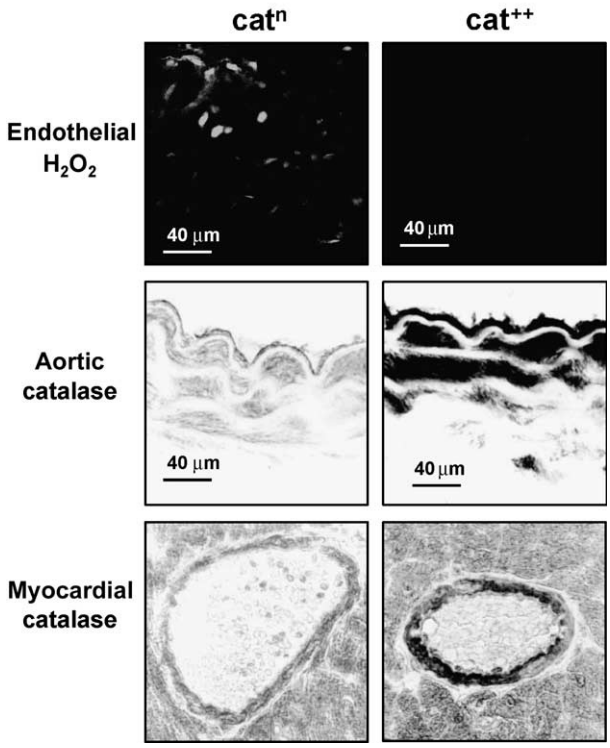


Fig. 4. Demonstration of endothelial H_2O_2 content (bright spots, top panels, direct view on top of the endothelial layer of longitudinally cut ring preparations); of overexpression of human catalase in the aorta (cross-sections, middle panels) and in left ventricular myocardium arterioles (cross-section, lower panels, identical magnification as indicated) in mice with a vascular specific overexpression of catalase (cat^{++}) and their transgene negative littermates (cat^{n}). Determination of H_2O_2 was accomplished by measurement of dichlorofluoreceine-fluorescence, while catalase protein was visualized using a standard catalase antibody (for details please refer to Methods).

itself does not change catalase protein content in the cardiovascular system.

3.4. Efficiency of exercise and its effect on eNOS expression in C57BL/6, cat^{n} and cat^{++}

Three weeks of exercise significantly increased the heart weight/body weight ratio (in mg/g) of C57BL/6 mice from 5.48±0.22 (control, n=11) to 6.32±0.24 (exercise, n=12, P=0.0171), indicating efficient training. This was associated with a significant increase of eNOS expression in the aorta of 2.93±45.2-fold (P<0.01) and the left heart ventricle of 1.88±33.1-fold (P<0.05). A similar result was obtained in cat^{n} (n=4, Fig. 6), where 3 weeks of exercise increased eNOS expression by 2.52±0.464-fold in the aorta and by 1.9±0.37-fold in the left myocardial ventricle (Fig. 6). In striking contrast, there was no effect of exercise on eNOS expression in the aorta and the heart of cat^{++} (Fig. 6), although the heart weight/body weight ratio (in mg/g) increased from 5.15±0.16 to 6.04±0.22 (n=8, P=0.0056). These data suggest that endogenous H_2O_2 plays a key role in exercise-induced up-regulation of cardiovascular eNOS.

3.5. Effect of aminotriazole exercise-induced eNOS expression in cat^{++}

Preliminary experiments indicated that 5 weeks of treatment with aminotriazole at a dosage of ≈666 mg/kg body weight tended to reduce the increase of aortic eNOS expression in response to 3 weeks of exercise (P=0.3023, data not shown). In striking contrast, aminotriazole treatment resulted in a significant increase of eNOS expression in response to 3 weeks of exercise in cat^{++} mice (Fig. 7B, P<0.01) as compared to vehicle-treated cat^{++} mice (Fig. 7A, P>0.05). These data show that the activity of catalase in cat^{++} mice is critically involved in the inhibition of exercise-induced vasular eNOS expression.

4. Discussion

In this study, we generated a new transgenic mouse line with vascular-specific overexpression of human catalase to investigate the importance of vascular H_2O_2 for the increase of vascular eNOS expression induced by exercise. In striking contrast to the wild-type littermates, there was no increase of eNOS expression when cat^{++} mice were subjected to exercise. Based on these data, we suggest that

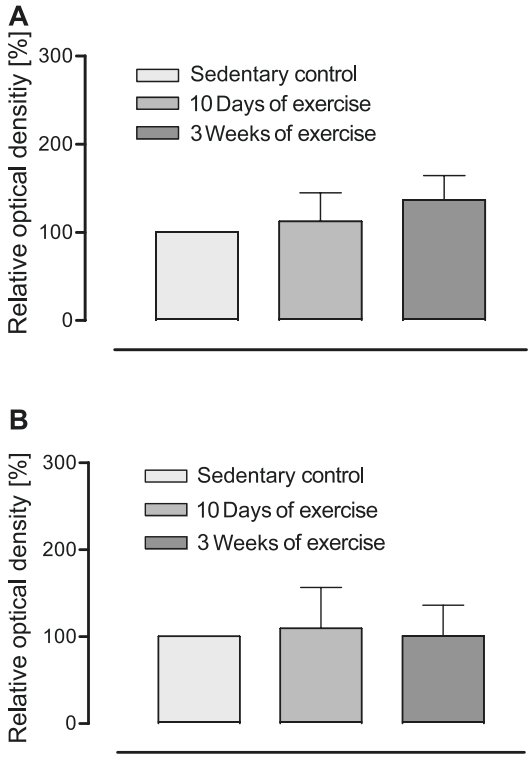


Fig. 5. Lack of effect of exercise on the expression of catalase protein in the aorta (A) and the left ventricular myocardium (B) of healthy 3- to 4-month old C57BL6 mice. Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%.

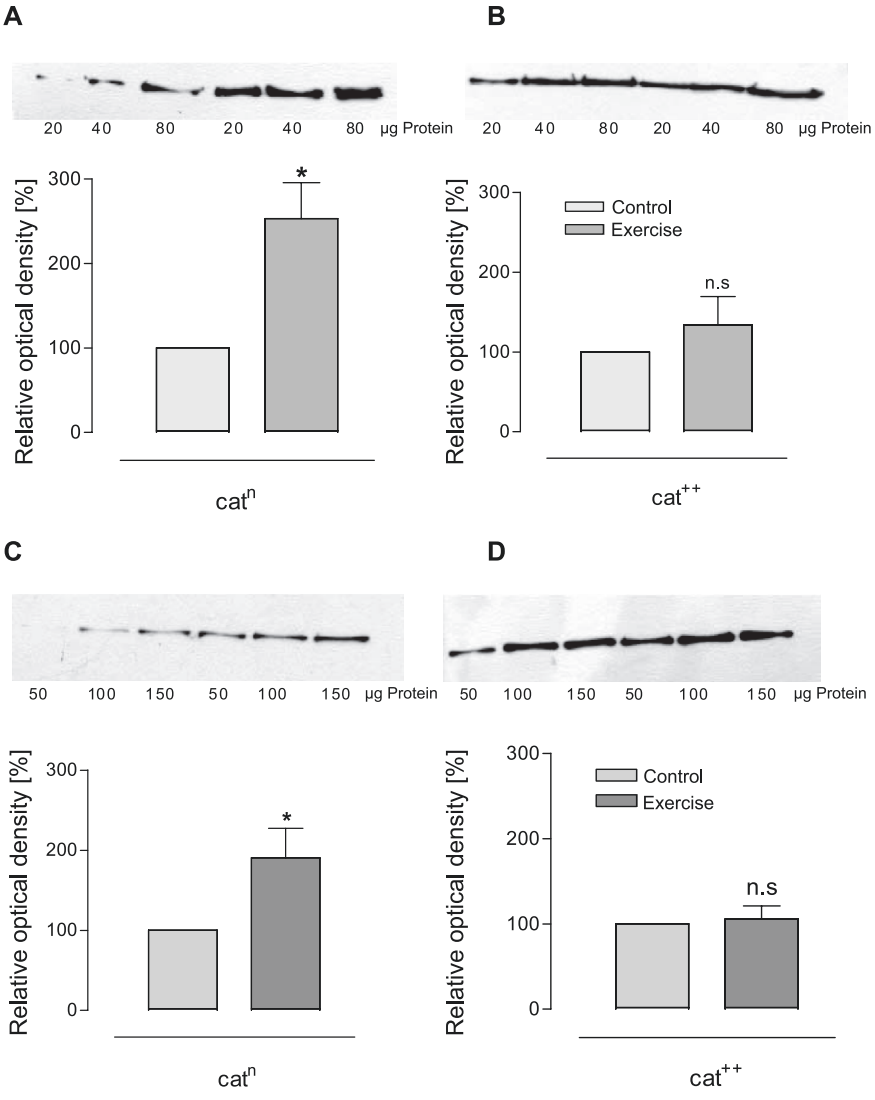


Fig. 6. Effect of 3 weeks of exercise on eNOS protein expression in the aorta of cat^{n} (A) and cat^{++} (B) and the left ventricular myocardium of cat^{n} (C) and cat^{++} (D) as quantified by Western blot (for details see Methods). Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%.

endogenously produced H_2O_2 contributes to the beneficial effects of exercise on endothelial function.

Our data strongly support the concept that H_2O_2 is critically involved in up-regulation of eNOS by exercise, as catalase prevented this response. The vascular specific overexpression of catalase was evident in both conductance and myocardial resistance vessels and associated with an increase of catalase mRNA, protein and activity, as well as a marked reduction of the endothelial steady-state concentration of reactive oxygen species including H_2O_2. Although vascular specific overexpression of catalase had no effect on basal expression of eNOS, it almost completely inhibited the increase of eNOS expression induced by exercise in the aorta and in left ventricular arterioles. Moreover, treatment of mice with the catalase inhibitor aminotriazole restored the up-regulation of aortic eNOS in response of cat^{++} to exercise, strongly supporting the concept that H_2O_2 is involved in this physiologic vascular adaptation to exercise training. Unfortunately, there is no reliable method to directly measure vascular H_2O_2 levels during exercise [22]. In addition, H_2O_2 is so fleeting that even rapid removal of vessels after an acute bout of exercise will unlikely reveal a change in its tissue level. This might be considered a limitation of our study; however, taken together, the effects of catalase overexpression and its reversal strongly support a role of H_2O_2 in regulation of eNOS expression during exercise.

Our current studies do not identify a specific site or source of H_2O_2. High levels of shear stress, as encountered during exercise as a result of increased cardiac output, have been shown to stimulate vascular superoxide production, which would predispose to increased levels of H_2O_2 via dismutation [28]. Importantly, it is possible that H_2O_2 is produced either in adjacent tissues or in blood borne cells during bouts of exercise. In keeping with this concept, Ashton et al. [29] have

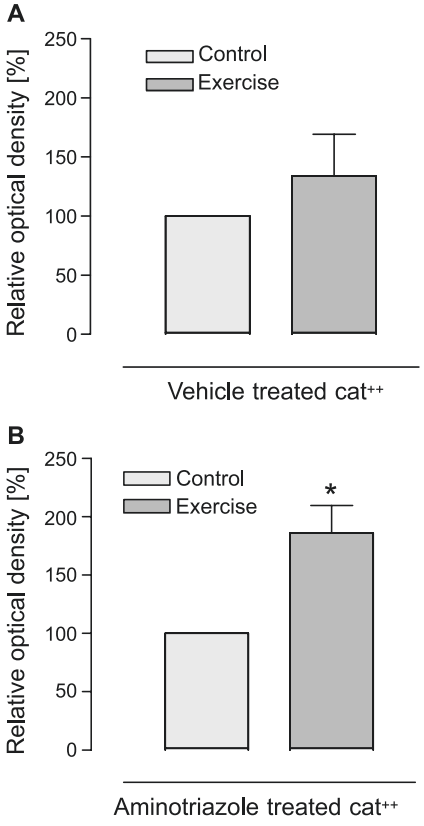


Fig. 7. Effect of 3 weeks of exercise on the expression of eNOS protein in the aorta of cat^{++} mice treated with either vehicle (A) or the catalase inhibitor aminotriazole (10 mg/ml of drinking water, corresponds to ≈666 mg/kg body weight, [B]) for 5 weeks. Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%.

shown that exercise acutely increases what appears to be a carbon-centered radical in the plasma of humans, using electron spin resonance spectroscopy. A variety of studies have indicated that exercise is associated with increased ATP synthesis in skeletal and myocardial muscle [30], and we have found a more than threefold increase of citrate synthase activity [24]. During ATP synthesis, the coenzyme Q radical can transfer its unpaired electron to molecular oxygen, which increases superoxide and subsequently the generation of H_2O_2 [31]. In contrast to superoxide, H_2O_2 is not charged and therefore able to freely diffuse within tissue. Thus, mitochondrial H_2O_2 likely contributes to increased vascular oxidative stress during exercise, particularly in myocardial and skeletal muscle arterioles where the diffusion distance is comparatively small. Furthermore, exercise increases heart rate, and this will almost certainly enhance the mechanical forces of blood flow, such as shear stress, pressure and cyclic strain on the vascular wall [1]. In conductance arteries, shear stress has been shown to increase endothelial superoxide generation [28]. Further studies have identified the endothelial NADPH oxidase as a major source of vascular superoxide induced by laminar and oscillatory shear [32].

Previous studies in our laboratories have shown that exercise not only induces the expression of eNOS but also that of ecSOD [13,21]. In addition, shear stress is a potent stimulus for expression of the endothelial cell Cu/Zn SOD [33]. The SODs not only reduce ambient levels of superoxide but, in doing so, generate H_2O_2. Thus, superoxide generated during exercise training may be more readily dismutated to H_2O_2. The two major enzymes which detoxify H_2O_2 are catalase and glutathione peroxidase. Of these, glutathione peroxidase is inhibited by NO in a concentration-dependent manner [34]. It is also known that exercise increases endothelial NO generation, suggesting a significant inhibition of glutathione peroxidase. Therefore, catalase may be more important for detoxification of exercise-induced generation of H_2O_2 because exercise also increases endothelial NO generation [13,21].

Regulation of eNOS expression is highly complex. A variety of factors, such as shear stress, lysophosphatidylcholine, cGMP analogs, lipoproteins, inhibitors of protein kinase C and different cytokines, are known to alter eNOS expression [35,36]. In addition, previous studies in endothelial cells have shown that H_2O_2 increases eNOS expression as well [16]. The mechanism underlying this up-regulation includes a calcium-dependent increase of calmodulin kinase II phosphorylation leading to activation of janus kinase II [17]. The latter tyrosine kinase phosphorylates other protein kinases, such as Ras, which directly activates transcription factors. Shear stress is also considered an important stimulus for eNOS expression. Recent studies have shown that the underlying signaling mechanism is completely different from that of H_2O_2, involving activation of the tyrosine kinase cSrc, which in turn initiates both eNOS transcription and eNOS mRNA stabilization via divergent pathways [18]. In recent studies, we have also found that mice with reduced cSrc are unable to increase eNOS expression in response to exercise training [37]. Taken together with our current data, it seems that both vascular H_2O_2 and cSrc are critical in allowing endothelial cells to increase eNOS expression during exercise training. The manner in which these signals interact in vivo remain poorly defined.

Our findings may have implications for the use of antioxidants and drugs that suppress the production of reactive oxygen species in vivo. While reactive oxygen species produced in large quantities clearly mediate cellular damage, it is clear that low levels of reactive oxygen species like H_2O_2 may have important signaling properties. Complete suppression of cellular production of reactive oxygen species would therefore likely be undesirable, as it might lead to loss of these signaling events and, in the case illustrated in this study, an inability to increase eNOS in response to exercise training.

Acknowledgements

This study was supported by the Forschungskommission of the Heinrich-Heine-Universität, Projekt 9772 179 (to GK) and by a research scholarship of the DAAD (to TS).

References

[1] Gielen S, Schuler G, Hambrecht R. Exercise training in coronary artery disease and coronary vasomotion. Circulation 2001;103:E1.

[2] O'Connor GT, Buring JE, Yusuf S, Goldhaber SZ, Olmstead EM, Paffenbarger RSJ, et al. An overview of randomized trials of rehabilitation with exercise after myocardial infarction. Circulation 1989;80:234–44.

[3] Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, et al. Effects of walking on mortality among nonsmoking retired men [see comments]. N Engl J Med 1998;338:94–9.

[4] Laslett LJ, Paumer L, Amsterdam EA. Increase in myocardial oxygen consumption indexes by exercise training at onset of ischemia in patients with coronary artery disease. Circulation 1985;71:958–62.

[5] Rogers MA, Yamamoto C, Hagberg JM, Holloszy JO, Ehsani AA. The effect of 7 years of intense exercise training on patients with coronary artery disease. J Am Coll Cardiol 1987;10:321–6.

[6] Hagberg JM. Physiologic adaptations to prolonged high-intensity exercise training in patients with coronary artery disease. Med Sci Sports Exerc 1991;23:661–7.

[7] Niebauer J, Hambrecht R, Velich T, Hauer K, Marburger C, Kälberer B, et al. Attenuated progression of coronary artery disease after 6 years of multifactorial risk intervention—role of physical exercise. Circulation 1997;96:2534–41.

[8] Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, Hintze TH. Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. Med Sci Sports Exerc 1995; 27:1125–34.

[9] Sessa WC, Pritchard Jr KA, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349–53.

[10] Delp MD, Laughlin MH. Time course of enhanced endothelium-mediated dilation in aorta of trained rats. Med Sci Sports Exerc 1997;29:1454–61.

[11] Woodman CR, Muller JM, Laughlin MH, Price EM. Induction of nitric oxide synthase mRNA in coronary resistance arteries isolated from exercise-trained pigs. Am J Physiol Heart Circ Physiol 1997; 273:H2575–9.

[12] Maroun MJ, Mehta S, Turcotte R, Cosio MG, Hussain SN. Effects of physical conditioning on endogenous nitric oxide output during exercise. J Appl Physiol 1995;79:1219–25.

[13] Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. Circulation 2001;103:2839–44.

[14] Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease [see comments]. N Engl J Med 2000;342:454–60.

[15] Kojda G, Harrison DG. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999;43:562–71.

[16] Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 2000;86: 347–54.

[17] Cai H, Davis ME, Drummond GR, Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/ calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. Arterioscler Thromb Vasc Biol 2001;21:1571–6.

[18] Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. Circ Res 2001;89:1073–80.

[19] Thomas SR, Chen K, Keaney Jr JF. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem 2002;277:6017–24.

[20] Cai H, Li Z, Davis ME, Kanner W, Harrison DG, Dudley Jr SC. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. Mol Pharmacol 2003;63:325–31.

[21] Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. J Clin Invest 2000;105:1631–9.

[22] Tarpey MM, Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. Circ Res 2001;89:224–36.

[23] Kojda G, Cheng Y, Burchfield J, Harrison DG. Dysfunctional regulation of eNOS expression in response to exercise in mice lacking one eNOS gene. Circulation 2001;103:2839–44.

[24] Suvorava T, Lauer N, Kojda G. Physical inactivity causes endothelial dysfunction in healthy young mice. J Am Coll Cardiol 2004;44:1320–7.

[25] Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, et al. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. Cardiovasc Res 1999;42:206–13.

[26] Cohen G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Anal Biochem 1970;34:30–8.

[27] Schwarz MA, Lazo JS, Yalowich JC, Reynolds I, Kagan VE, Tyurin V, et al. Cytoplasmic metallothionein overexpression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity. J Biol Chem 1994; 269:15238–43.

[28] Laurindo FRM, De Almeida Pedro M, Barbeiro HV, Pileggi F, Carvalho MHC, Augusto O, et al. Vascular free radical release: ex vivo and in vivo evidence for a flow-dependent endothelial mechanism. Circ Res 1994;74:700–9.

[29] Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B, et al. Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. Eur J Appl Physiol Occup Physiol 1998;77:498–502.

[30] Ji LL. Antioxidants and oxidative stress in exercise. Proc Soc Exp Biol Med 1999;222:283–92.

[31] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239–47.

[32] De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander KK, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. Circ Res 1998;82:1094–101.

[33] Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. Circ Res 1996;79:32–7.

[34] Asahi M, Fujii J, Suzuki K, Seo HG, Kuzuya T, Hori M, et al. Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. J Biol Chem 1995;270:21035–9.

[35] Li H, Wallerath T, Munzel T, Forstermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. Nitric Oxide 2002;7:149–64.

[36] Li H, Wallerath T, Forstermann U. Physiological mechanisms regulating the expression of endothelial-type NO synthase. Nitric Oxide 2002;7:132–47.

[37] Davis ME, Cai H, McCann L, Fukai T, Harrison DG. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. Am J Physiol Heart Circ Physiol 2003;284: H1449–53.

Journal of the American College of Cardiology
© 2004 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 44, No. 6, 2004
ISSN 0735-1097/04/$30.00
doi:10.1016/j.jacc.2004.06.030

Physical Inactivity Causes Endothelial Dysfunction in Healthy Young Mice

Tatsiana Suvorava, PhD, Nadine Lauer, PharmD, PhD, Georg Kojda, PharmD, PhD
Duesseldorf, Germany

OBJECTIVES We sought to determine if physical inactivity affects endothelial function in young healthy individuals.

BACKGROUND Recent studies have linked exercise training to increased bioavailability of vascular nitric oxide (NO) and to improved endothelial function in patients with cardiovascular disorders. The effects of physical inactivity on normal vascular endothelial function are not known.

METHODS Healthy young male C57Bl/6 mice living in groups of five in large cages, where they were running, climbing, and fighting during their active cycle, were randomly assigned to stay there or to live alone in small cages where they were predominantly resting. After five and nine weeks citrate synthase activity (a measure of mitochondrial respiratory chain activity), heart weight/body weight ratio, vascular reactivity, and protein expression of endothelial nitric oxide synthase (eNOS) were assessed.

RESULTS Singularized mice showed a reduction of citrate synthase activity ($p < 0.05$), of endothelium-dependent vasorelaxation (to 65 ± 5% of control levels; $p < 0.001$), and of eNOS protein expression (to 53 ± 8% of control levels; $p < 0.01$). In striking contrast, vascular responses to potassium chloride, phenylephrine, and the NO-donor racemic S-nitroso-N-acetylpenicillamine were unchanged. The alterations of vascular eNOS-activity were completely reversible when singularized mice underwent exercise. In mice living in groups, exercise showed only a small effect on aortic eNOS expression.

CONCLUSIONS In young healthy individuals physical inactivity induces endothelial dysfunction, which is completely reversible by a short period of moderate exercise training. We suggest that physical inactivity, the so-called sedentary lifestyle, increases cardiovascular risk in young healthy individuals by inducing endothelial dysfunction. (J Am Coll Cardiol 2004;44:1320–7)

Physical inactivity, the so-called sedentary lifestyle, increases cardiovascular morbidity and mortality, as indicated by studies comparing individuals with different levels of daily exercise (1,2). Regarding the underlying molecular mechanism, exercise training is associated with significant physiologic adaptations involving skeletal muscle, cardiac muscle, circulating blood volume, and a variety of metabolic modifications (3). Recently, it has become apparent that exercise induces an increased expression of vascular endothelial nitric oxide synthase (eNOS) as well (4,5). This adaptation most likely improves the bioavailability of endogenous nitric oxide (NO) in mice and humans (5–7). At the same time, exercise induces the expression of extracellular superoxide dismutase (8), which may contribute to the improvement of NO bioavailability.

Bioavailability of endogenous NO may determine exercise capacity by its vasodilatory action. In addition, it has been established that NO not only produces vasodilation but also inhibits platelet aggregation and has antioxidant, antiproliferative, and antiapototic properties (9). These effects suggest that increased NO production induced by exercise may also slow the progression of vascular disease (3). Pharmacologic inhibition of eNOS or eNOS gene disruption has been shown to accelerate the atherosclerotic process (10,11), whereas treatment with NO donors such as organic nitrates can reduce lesion formation, vascular superoxide production, oxidation of low-density lipoprotein, and endothelial dysfunction in cholesterol-fed rabbits (12,13).

It is unknown if physical inactivity affects endothelial function in healthy young individuals. Most epidemiologic and prospective clinical trials investigated the effect of exercise on disease progression in patients with coronary artery disease and heart failure, where endothelial function is already impaired (14). In contrast, the Multiple Risk Factor Interventiona Trial (MRFIT) study surveyed 12,138 men without cardiovascular disease for 16 years and found a significantly reduced incidence of cardiovascular deaths in men with normal daily physical activity compared to men with a sedentary lifestyle (1). We speculated that an impairment of endothelial function may be a factor associated with a sedentary lifestyle in healthy individuals and investigated if physical inactivity affects endothelial function in young normal mice.

METHODS

Moving and sedentary mice. A total of 81 male C57Bl/6 mice, four to six months of age, were used for the main study. The study consisted of three arms: 1) a pilot study to assess potential differences in training effects on heart weight/body weight ratio; 2) an exercise restriction arm to

From the Institut fuer Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universitaet, Duesseldorf, Germany. This study was supported by the Forschungskommission of the Heinrich-Heine-Universität Düsseldorf (Project 9772 109). The first two authors contributed equally to this work.

Manuscript received March 31, 2004; revised manuscript received May 25, 2004, accepted June 7, 2004.

Abbreviations and Acronyms

ACh	= acetylcholine
ANOVA	= analysis of variance
eNOS	= endothelial nitric oxide synthase
KCl	= potassium chloride
NO	= nitric oxide
pD_2	= half maximal effective concentration in −log mol/l
PE	= phenylephrine
SNAP	= racemic S-nitroso-N-acetylpenicillamine

assess the effects of a sedentary lifestyle on endothelial function and citrate synthase activity; and 3) a training intervention arm to assess the reversibility of changes induced by activity restriction. A total of 19 mice were used in preliminary studies to determine changes of heart weight/body weight ratio. Fourteen of these mice were singularized for five weeks and seven of them underwent three weeks of exercise. All mice were bred at the university's animal facilities in a specified pathogen-free area. After weaning, mice from different litters were housed together in groups of five males in cages with a floor space of 900 cm^2 (moving mice) (Fig. 1) at a temperature of 18°C to 20°C, a humidity of 50% to 60%, and a day-night rhythm of 12 h. Mice received food and water (pH 3) ad libitum. Fifty-two of 81 mice were randomly assigned to live alone in small cages (sedentary mice) with a floor space of 300 cm^2 (Fig. 1) for five weeks (n = 31) and nine weeks (n = 21). Some mice of the five-week singularized group (n = 10) and the nine-week singularized group (n = 7) and of the moving group (n = 5) underwent a controlled eight to 15 days' lasting exercise program. All other conditions were identical. Mice living in groups were fighting, running, and climbing for the most part of their active daily cycle, whereas singularized mice were predominantly resting during their active daily cycle and showed a low physical activity, as evidenced by measuring the heart weight/body weight ratio and skeletal and cardiac citrate synthase activity.

Permission for this study was provided by the regional government and the experiments were performed according to the guidelines for the use of experimental animals as given by "Deutsches Tierschutzgesetz" and the "Guide for the Care and Use of Laboratory Animals" of the U.S. National Institutes of Health.

Figure 1. Details of the cages used to house mice in groups **(bottom cage)** and singularized mice **(top cage)**.

Exercise protocol. Mice were exercised following a previously established protocol (5). Briefly, mice ran in a newly established self-build exercise treadmill especially designed for mice. Five animals were studied simultaneously. Mice were initially trained three times for 10 min every other day for 10 days. The maximal velocity of the treadmill was 0.25 m/s. After this training, mice were exercised for three weeks at five days a week for 30 min at 0.25 m/s. Thus, eight days of training corresponded to a total time of 10 days (2 days without training) and 15 days of training to a total time of three weeks. The training was executed during the active cycle of the animals. Non-exercised controls were exposed to the same noise and the vibration of the environment. All mice completed the exercise protocol without signs of exhaustion. There was no obvious difference in exercise performance between sedentary and moving mice. Within 16 to 20 h after termination of the last training, mice were sacrificed by inhalation of carbon dioxide and their aortas and hearts were immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting.

Measurement of citrate synthase activity. Citrate synthase activity was measured according to Kusnetzov et al. (15). Briefly, soleus muscle and left ventricular cardiac muscle were harvested and snap-frozen in liquid nitrogen. Samples were homogenized in triton X-100 (0.1 % V/V) containing tris buffer (0.1 M, pH 8.1) and then repeatedly thawed and refrozen to further disrupt mitochondria. Five μl of the homogenate was used to start the reaction (generation of the mercaptide) in a volume of 1 ml aqueous reaction volume containing 5,5-dithiobis(2-nitrobezoate) (DTNB, 0.1 mM), acetyl-CoA (0.3 mM), oxaloacetate (0.5 mM) and triton X-100 (0.25 % V/V). Changes in absorbance at 412 nm were measured at 30°C for 5 min. Total protein levels in the homogenate was determined by the Bradford method (16) and citrate synthase activity is given in mU/mg total protein.

Vasorelaxation studies. Preparation of thoracic ring segments was performed in HEPES-containing Krebs-Henseleit buffer, whereas the organ bath experiments were done in the same buffer lacking HEPES. After a 60-min equilibration period, aortic rings were repeatedly subjected to 80 mmol/l potassium chloride (KCl). The vasoconstriction that developed during the last of three KCl applications was taken as the maximal receptor-independent vasoconstriction. Function of endothelium was examined by cumulative addition of acetylcholine (ACh) (10^{-9} to 10^{-5} mol/l) following submaximal precontraction with phenylephrine (PE). In moving mice and those subjected to five weeks of singularization, endothelium-dependent vasodilation was followed by a cumulative application of PE (10^{-9}–10^{-5} mol/l). Thereafter, the aortic rings

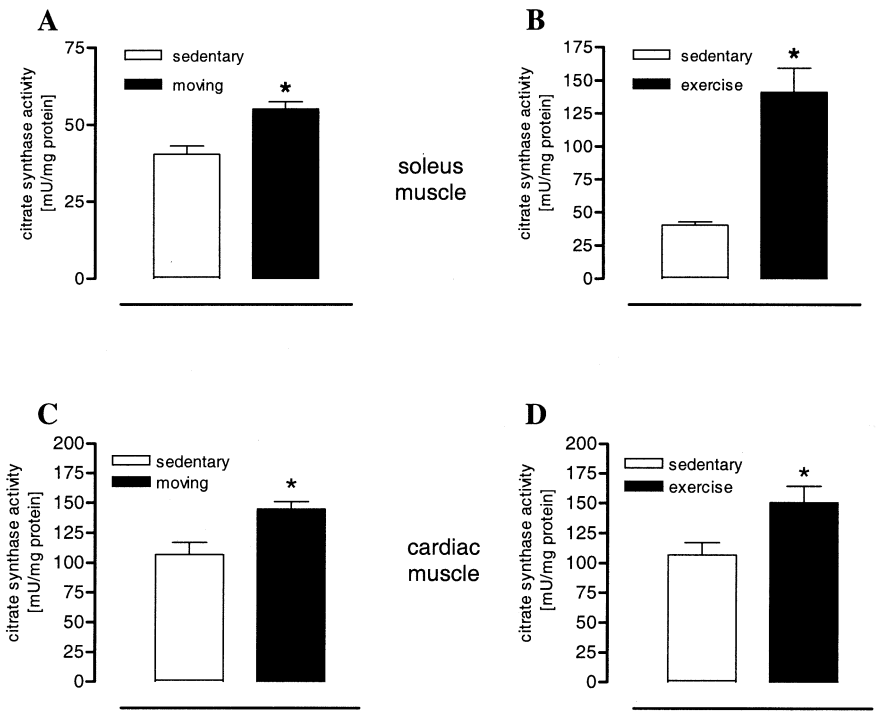


Figure 2. Activity of citrate synthase in homogenates of soleus **(A, B)** and cardiac left ventricular muscle **(C, D)** of singularized mice (sedentary, n = 7, all panels), mice living in groups (moving, n = 5) **(A, C)** and singularized mice that underwent three weeks of endurance training (exercise, n = 7) **(B, D)** (*$p < 0.05$, unpaired two-tailed Student *t* test).

were precontracted with 1-μmol/l PE and a concentration-response curve for racemic S-nitroso-N-acetylpenicillamine (SNAP) (10^{-9}–10^{-5} mol/l) was performed.

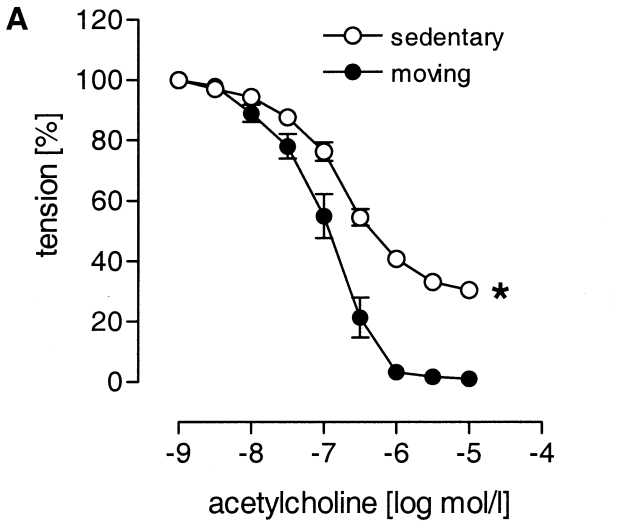
Determination of aortic eNOS expression. Western blot analysis was done in a blinded fashion. Frozen aortas from moving, sedentary (five weeks), and exercised mice were immersed in iced tris-buffer (5 mmol/l, pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aprotinin, phenylmethylsulfonyl fluoride, and antipain (10 μg/ml). The tissues were homogenized for 30 s using a polytron homogenizer. The homogenates were then centrifuged for 10 min at 100 × g to remove particulate matter and unbroken cells. Total protein levels were determined by the Bradford method (16). Western blot analysis was performed as described previously (5) using a commercially available monoclonal antibody (Transduction Laboratories, Lexington, Kentucky) and the enhanced chemiluminescence detection system (Amersham).

Substances and solutions. Racemic S-nitroso-N-acetylpenicillamine was synthesized in our laboratory as described previously (17). All other chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany) in analytical grade. The stock solutions of ACh (10 mmol/l), PE (10 mmol/l), and KCl (4 mol/l) were prepared in distilled water. Solutions of SNAP (200 mmol/l) were prepared in dimethylsulfoxide. All stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice, and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

Statistics. All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0; analysis of variance [ANOVA]) and are expressed as mean values and standard error of the mean. Significant differences were evaluated using either unpaired two-tailed Student *t* test, ordinary two-way-ANOVA (without post-tests) and one-way-ANOVA with subsequent Newman-Keuls multiple comparison test. A p value below 0.05 was considered significant.

RESULTS

Quantitation of physical activity. There was a significant increase of soleus muscle citrate synthase activity in moving and exercised mice as compared with sedentary mice (Figs. 2A and 2B), and this increase was much more pronounced after exercise. Likewise, citrate synthase activity was enhanced in cardiac muscle of moving and exercised mice. A comparison between the two muscle types reveals some differences. As expected, the citrate synthase activity was greater in cardiac muscle of sedentary mice as compared with soleus muscle, suggesting a higher mitochondrial activity in the former tissue. Furthermore, the increase of citrate synthase activity in cardiac muscle was identical in moving and exercised mice (Figs. 2C and 2D), suggesting an already high activity as previously reported (18). To further quantify the level of physical activity, we determined the body weight/heart weight ratio in sedentary mice with (n = 7) and without (n = 7) three weeks of exercise and in moving mice (n = 5). We found that the heart weight/body



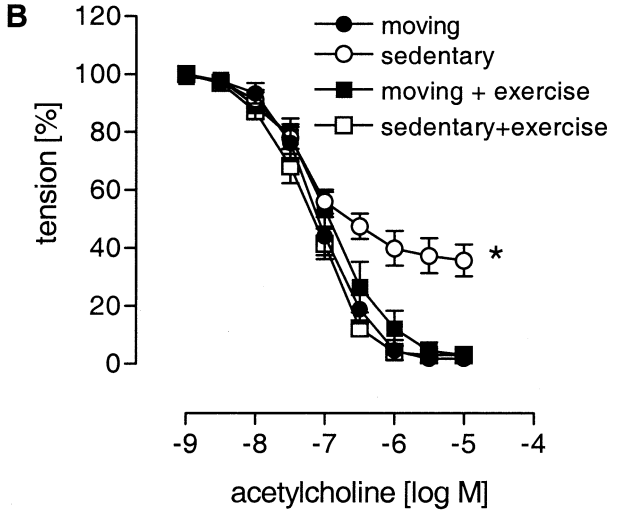


Figure 3. Endothelium-dependent vasodilation in aortic rings of sedentary mice after **(A)** five weeks of singularization (n = 6) compared with mice living in groups of five animals per cage (moving mice, n = 6) and **(B)** after nine weeks of singularization (n = 7) compared with moving mice (n = 5) and with sedentary (n = 7) and moving mice (n = 5) that underwent the exercise program. Physical inactivity induced by singularization resulted in a significant impairment of endothelium-dependent vasorelaxation (* = $p < 0.001$, analysis of variance). Three weeks of exercise completely reversed this impairment, whereas exercise training had no effect on endothelium-dependent vasodilation in moving mice.

weight ratio (in mg/g) of sedentary mice (5.2 ± 0.09) was increased in moving mice (5.5 ± 0.1; $p < 0.05$) and after three weeks of exercise (6.04 ± 0.04; $p < 0.01$).

Endothelium-dependent vasodilation. Endothelial function of moving and sedentary mice was assessed by examination of endothelium-dependent vasodilation to ACh. The concentration-response curves for ACh demonstrate that five (Fig. 3A) and nine weeks (Fig. 3B) of forced physical inactivity as initiated by singularization in small cages results in endothelial dysfunction. After five weeks of singularization we found a reduction of the maximal vasodilation to 10

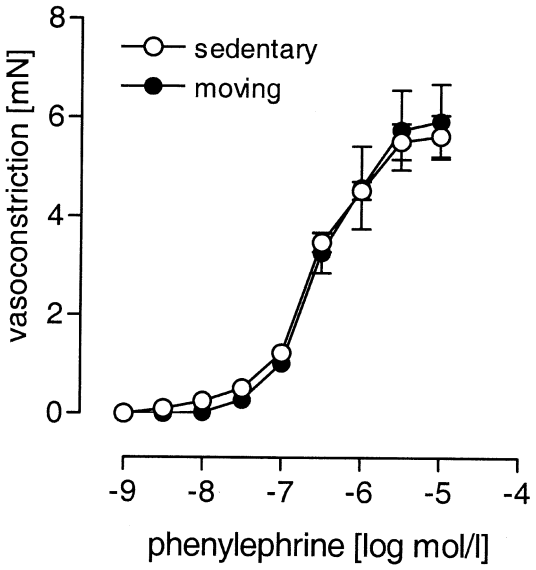


Figure 4. Vasoconstrictor response to increasing concentrations of phenylephrine in aortic rings of singularized sedentary mice (n = 6) and moving mice (n = 6). There was no difference between the groups ($p = 0.6262$, analysis of variance).

μmol/l ACh from 99.4 ± 0.3% in moving mice to 69.4 ± 1.9% in sedentary mice ($p < 0.0001$). After nine weeks of singularization this vasodilator response was reduced from 91.7 ± 2.7% in moving mice to 64.3 ± 5.5% in sedentary mice ($p = 0.0008$). These data suggest that five weeks of physical inactivity is sufficient to induce a degree of endothelial dysfunction in mice that is not further aggravated by prolonging the time period of physical inactivity. Sedentary mice that underwent the exercise program regained their normal ACh response, whereas exercise had no effect on endothelium-dependent vasorelaxation in moving mice (Fig. 3B).

Vasocontractile responses. Vasocontractile responses were not different in moving and sedentary mice. Subjection of aortic rings to a single dose of 80 mmol/l KCl resulted in an identical rise of maximal vascular tension in sedentary (6.4 ± 0.4, n = 6) and moving mice (6.6 ± 0.4, n = 6, $p = 0.728$). Likewise, neither the half maximal effective concentration in −log mol/l (pD_2) values for PE in moving (6.479 ± 0.21) and sedentary mice (6.569 ± 0.12, $p = 0.7157$) nor the maximally inducible vasoconstrictions different ($p = 0.7919$) (Fig. 4).

NO-dependent vasodilation. Contrary to endothelium-dependent vasodilation, the NO-dependent vasodilation induced by the NO donor SNAP showed identical pD_2 values in moving (7.393 ± 0.071) and sedentary mice (7.302 ± 0.03422, $p = 0.2180$) (Fig. 5). These data suggest that an impairment of the NO/cGMP-pathway is most likely not involved in endothelial dysfunction induced by a sedentary lifestyle in mice.

Aortic eNOS protein expression. Assessment of eNOS protein expression showed a significant reduction in sedentary mice that was evident in each animal examined (relative

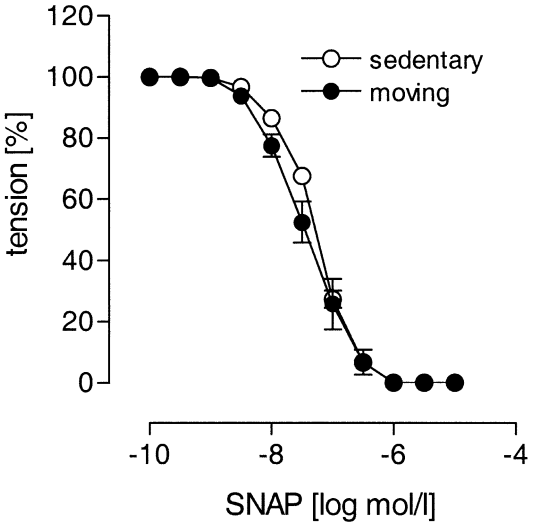


Figure 5. Vasodilator response to increasing concentrations of the nitric oxide donor racemic S-nitroso-N-acetylpenicillamine in aortic rings of singularized sedentary mice (n = 6) and of moving mice (n = 6). There was no difference between the groups (p = 0.4059, analysis of variance).

densities ranging from 0.31 to 0.64; $p < 0.01$) (Fig. 6A). These data suggest that the overall halved expression of eNOS contributes to the impairment of endothelium-dependent vasodilation induced by physical inactivity.

Effect of exercise on eNOS expression in sedentary and moving mice. Sedentary mice were exercised by a standard protocol for eight days and 15 days. Overall, there was a time-dependent several-fold increase of aortic eNOS-protein expression (p = 0.0147, ANOVA) (Fig. 7A). Subsequent Newman-Keuls multiple comparison tests of the data depicted in Figure 7A revealed a significant difference only for the comparison sedentary versus 15 days of exercise. Although a similar trend was observed in left ventricular myocardium, the increase of eNOS protein expression was not significant in this tissue (p = 0.1637, ANOVA) (Fig. 7B). In striking contrast, aortic eNOS protein expression did not change after 15 days of exercise in moving mice ($p > 0.05$) (Fig. 6B).

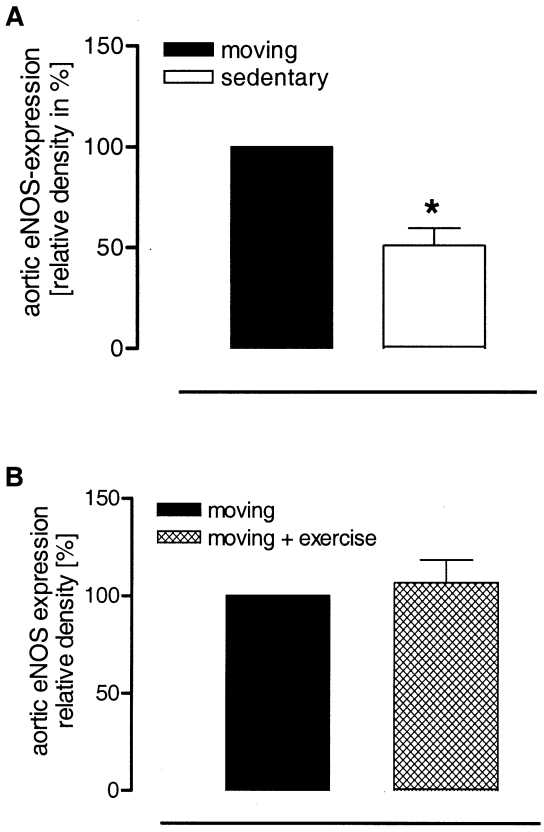


Figure 6. (A) Protein expression of eNOS in aortic rings of singularized sedentary mice (n = 5) compared with moving mice (n = 5) and **(B)** of moving mice (n = 4) compared with moving mice that underwent an exercise program (n = 4). Singularization resulted in a significant reduction of eNOS protein expression (**A,** $p < 0.01$), whereas exercise had no effect on eNOS protein expression in moving mice (**B,** $p > 0.05$, unpaired two-tailed Student *t* test).

DISCUSSION

The aim of this study was to determine the influence of physical inactivity on vascular endothelial function. We demonstrate for the first time that a sedentary lifestyle, mimicked by forced physical inactivity in young healthy mice, can induce a specific impairment of endothelium-dependent vasodilation, whereas vasocontractile activity and NO-donor-induced vasorelaxation were not changed. Further experiments showed that a reduction of vascular eNOS protein expression most likely contributes to this endothelial dysfunction. When sedentary mice underwent exercise, the expression of eNOS increased by severalfold, whereas exercise had only a small effect on aortic eNOS-expression in moving mice. Thus, our data suggest that physical inactivity can reduce vascular expression of eNOS and thereby elicit endothelial dysfunction in the absence of other cardiovascular risk factors.

Endothelial dysfunction is a well-known pathologic condition that is associated with a variety of cardiovascular diseases such as coronary artery disease, hypertension, heart failure, and diabetes. The mechanism of endothelial dysfunction is multifactorial and most likely depends on the underlying pathologic process. It is generally accepted that vascular inflammation, vascular oxidative stress, and aging are important factors in cardiovascular diseases (19–21). Here we describe for the first time the development of endothelial dysfunction in healthy young mice with no signs of vascular inflammation or oxidative stress that had been subjected to forced physical inactivity. At the same time, the protein expression of endothelial nitric oxide synthase was reduced by one-half, whereas activation of the NO/cGMP pathway by an NO donor was equally effective in sedentary and moving mice. Therefore, downregulation of eNOS expression appears to be a key mechanism underlying the impairment of endothelial function in young healthy sedentary mice. However, in cardiovascular patients other mechanisms such as increased eNOS phosphorylation have been shown to follow vigorous exercise (7). Other mecha-

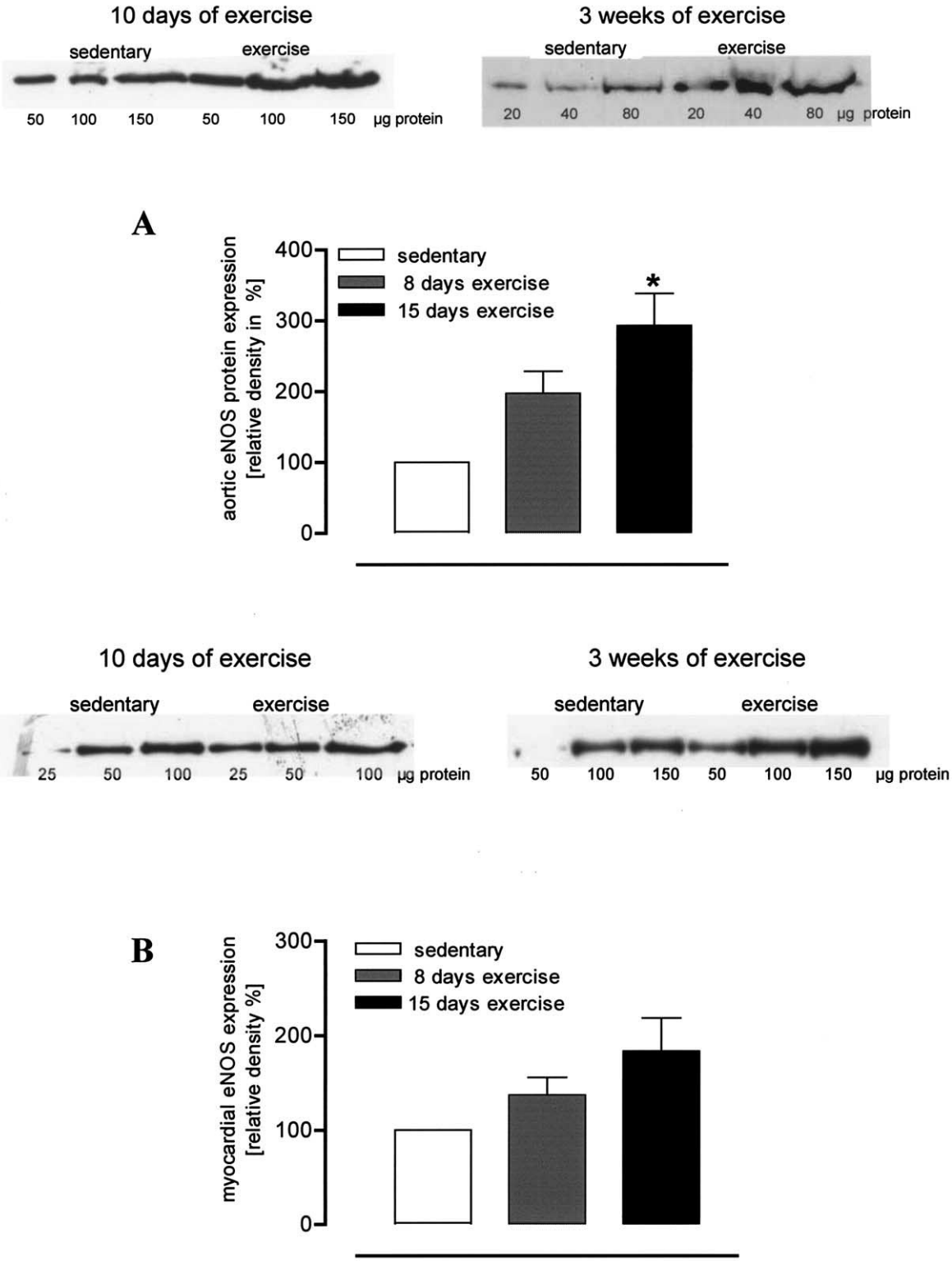


Figure 7. Effect of 8 days (n = 5) and 15 days of exercise (n = 5) on eNOS-protein expression in **(A)** aortic rings and **(B)** left ventricular myocardium of singularized sedentary mice (n = 5 for each exercise period). Although exercise induced a significant and time-dependent upregulation of eNOS protein expression in aortic rings ($p = 0.0147$, analysis of variance), there was only a trend in left ventricular myocardium ($p = 0.1637$). Subsequent Newman-Keuls multiple comparison test of the data depicted in **A** revealed a significant difference only for the comparison sedentary versus 15 days' exercise as indicated (*, $p < 0.05$, analysis of variance).

nisms decreasing the bioavailability of endothelial NO in sedentary mice, such as increased oxidation by superoxide radicals owing to a reduced expression of extracellular superoxide dismutase, might also play a role (8).

Many different conditions, endogenous mediators, and drugs have been shown to modulate the expression of eNOS (22). Among these, mechanisms modifying eNOS expression in response to exercise might be particularly important, because it seems conceivable that a reversion of these mechanisms occurs in sedentary mice. In accordance, we demonstrate that exercise reversed endothelial dysfunction induced by physical inactivity. Recent reports suggest that there are at least two different mechanisms underlying the upregulation of eNOS expression by exercise. First, exercise increases heart rate, which in turn increases blood flow and most likely vascular shear stress. Besides its role as the most important physiologic activator of endothelial NO production, shear stress has been shown to increase vascular eNOS expression (23,24). Further studies suggested that this process is dependent on c-Src and involves an increase of transcription and of messenger ribonucleic acid stability as well (25). Thus, a decreased intensity of physiologic shear stress, as expected in sedentary mice, might diminish vascular eNOS expression by reducing the shear stress-dependent activity of c-Src in endothelial cells.

Second, exercise increases not only oxygen consumption but also the generation of reactive oxygen species such as superoxide and hydrogen peroxide (26). It is well known that superoxide generation is increased in a nonenzymatic fashion during adenosine triphosphate synthesis by an electron transfer from coenzyme Q to molecular oxygen (27). Furthermore, and likely more important, shear stress has been shown to increase the vascular generation of reactive oxygen species by an endothelium-dependent mechanism (28). A later analysis of this phenomenon showed an activation of endothelial nicotinamide adenine dinucleotide phosphate, reduced form oxidase as a possible underlying cause (29). Although superoxide barely traverses cell membranes and is rapidly converted by superoxide dismutases, the resulting product hydrogen peroxide can diffuse through the vascular wall and is much more stable (20). Previous data have shown that hydrogen peroxide can increase the expression and activity of eNOS by phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II/janus kinase 2 (30,31). By generating transgenic mice with an endothelial-specific overexpression of catalase, we have recently provided evidence that hydrogen peroxide contributes to exercise-induced upregulation of eNOS. In these transgenic mice, three weeks of exercise performed according to the protocol used in the present study had no effect on vascular eNOS expression (32).

Western blotting for eNOS was done in a blinded fashion, but the organ bath studies were not. This might be considered a limitation of our study. However, in striking contrast to the observed endothelial dysfunction, the vascular responses to the vasoconstrictors KCl and PE and to the vasodilatory activity of the NO donor SNAP were normal. These data demonstrate that five weeks of forced physical inactivity has no impact on receptor-independent and alpha-adrenergic vasoconstriction. In addition, there seems to be no functionally important impairment of the vascular NO/cGMP pathway. In contrast, vascular oxidative stress and inflammatory stimuli such as cytokines, which typically induce endothelial dysfunction, are known to increase PE responses, presumably by activation of protein kinase C (33,34). Furthermore, oxidative stress and atherosclerosis have been shown to strongly impair the NO/cGMP pathway by inhibition of the catalytic activity of soluble guanylyl cyclase (35). In view of these studies, it is unlikely that vascular inflammation and/or oxidative stress have caused endothelial dysfunction induced by forced physical inactivity in healthy young mice.

Regarding the wildlife habits, singularization of mice is an artificial situation. This holds also true for mice being caged in groups. Interestingly, we found identical endothelium-dependent vasodilation in exercised and moving mice, although there was a large and significant difference concerning citrate synthase activity, which is known to correlate with physical activity (18). This suggests that low-intensity physical activity may be sufficient to maintain normal endothelial function in young healthy individuals. Furthermore, vigorous exercise has no further effect on endothelium-dependent vasodilation and eNOS expression in normally active mice. Thus, it seems likely that the differences in physical activity in our experimental setup mimic to a certain extent the situation in humans. For example, it was shown that there is an identical rate of cardiovascular events between women who simply walked for exercise and those who underwent vigorous exercise (2).

Recent data suggest that endothelial dysfunction is an independent predictor of cardiovascular event rates (36–38). Thus, endothelial dysfunction induced by a sedentary lifestyle might be an important pathophysiologic event. Our data suggest that this unfavorable change of vascular function can be prevented or remarkably delayed in young individuals by either a daily short-lasting high-intensity training period or continuous low-intensity physical activity. Based on our observations, we propose that regular physical activity may exert beneficial effects in two different ways. In patients with cardiovascular disease, exercise reduces the degree of endothelial dysfunction, whereas in young healthy individuals normal physical activity and/or moderate exercise might delay the development of cardiovascular disorders by maintaining normal endothelial function.

Reprint requests and correspondence: Dr. Georg Kojda, Institut fuer Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universitaet, Moorenstr. 5, 40225 Duesseldorf, Germany. E-mail: kojda@uni-duesseldorf.de.

REFERENCES

1. Leon AS, Myers MJ, Connett J. Leisure time physical activity and the 16-year risks of mortality from coronary heart disease and all-causes in the Multiple Risk Factor Intervention Trial (MRFIT). Int J Sports Med 1997;18 Suppl 3:S208–15.
2. Manson JE, Greenland P, LaCroix AZ, et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women. N Engl J Med 2002;347:716–25.
3. O'Connor GT, Buring JE, Yusuf S, et al. An overview of randomized trials of rehabilitation with exercise after myocardial infarction. Circulation 1989;80:234–44.
4. Sessa WC, Pritchard KA Jr., Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349–53.
5. Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. Circulation 2001;103:2839–44.
6. Hambrecht R, Wolf A, Gielen S, et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease. N Engl J Med 2000;342:454–60.
7. Hambrecht R, Adams V, Erbs S, et al. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. Circulation 2003;107:3152–8.
8. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. J Clin Invest 2000;105:1631–9.
9. Gewaltig MT, Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc Res 2002;55:250–60.
10. Naruse K, Shimizu K, Muramatsu M, et al. Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta: PGH_2 does not contribute to impaired endothelium-dependent relaxation. Arterioscler Thromb 1994;14: 746–52.
11. Moroi M, Zhang L, Yasuda T, et al. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. J Clin Invest 1998;101:1225–32.
12. Kojda G, Stein D, Kottenberg E, Schnaith EM, Noack E. In vivo effects of pentaerythrityl-tetranitrate and isosorbide-5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. J Cardiovasc Pharmacol 1995;25:763–73.
13. Hacker A, Müller S, Meyer W, Kojda G. The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis. Br J Pharmacol 2001;132:1707–14.
14. Gielen S, Schuler G, Hambrecht R. Exercise training in coronary artery disease and coronary vasomotion. Circulation 2001;103:E1–6.
15. Kusnetzov A, Lassnig B, Gnaiger E. Laboratory protocol—citrate synthase, mitochondrial marker enzyme. Mitochondr Physiol Network 2003;8,14:1–7.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
17. Kojda G, Kottenberg K, Nix P, Schlüter KD, Piper HM, Noack E. Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. Circ Res 1996;78:91–101.
18. Siu PM, Donley DA, Bryner RW, Alway SE. Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. J Appl Physiol 2003;94:555–60.
19. Brasier AR, Recinos A III, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. Arterioscler Thromb Vasc Biol 2002; 22:1257–66.
20. Kojda G, Harrison DG. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999;43:562–71.
21. Tschudi MR, Barton M, Bersinger NA, et al. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. J Clin Invest 1996;98:899–905.
22. Li H, Wallerath T, Munzel T, Forstermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. Nitric Oxide 2002;7:149–64.
23. Uematsu M, Ohara Y, Navas JP, et al. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. Am J Physiol Cell Physiol 1995;269:C1371–8.
24. Busse R, Fleming I. Pulsatile stretch and shear stress: physical stimuli determining the production of endothelium-derived relaxing factors. J Vasc Res 1998;35:73–84.
25. Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. Circ Res 2001;89:1073–80.
26. Ji LL. Antioxidants and oxidative stress in exercise. Proc Soc Exp Biol Med 1999;222:283–92.
27. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239–47.
28. Laurindo FRM, De Almeida Pedro M, Barbeiro HV, et al. Vascular free radical release: ex vivo and in vivo evidence for a flow-dependent endothelial mechanism. Circ Res 1994;74:700–9.
29. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. Circ Res 1998;82:1094–101.
30. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 2000;86: 347–54.
31. Cai H, Davis ME, Drummond GR, Harrison DG. Induction of endothelial NO synthase by hydrogen peroxide via a Ca^{2+}/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. Arterioscler Thromb Vasc Biol 2001;21:1571–6.
32. Lauer N, Harrison DG, Kojda G. Hydrogen peroxide supports upregulation of eNOS induced by exercise (abstr). Free Rad Biol Med 2002;33 Suppl 2:S367.
33. Münzel T, Harrison DG. Evidence for a role of oxygen-derived free radicals and protein kinase C in nitrate tolerance. J Mol Med 1997;75:891–900.
34. Berk BC, Abe JI, Min W, Surapisitchat J, Yan C. Endothelial atheroprotective and anti-inflammatory mechanisms. Ann N Y Acad Sci 2001;947:93–109; discussion 109–11:93–109.
35. Laber U, Kober T, Schmitz V, et al. Effect of hypercholesterolemia on expression and function of vascular soluble guanylyl cyclase. Circulation 2002;105:855–60.
36. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000;101:1899–906.
37. Al Suwaidi J, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Jr., Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000;101:948–54.
38. Perticone F, Ceravolo R, Pujia A, et al. Prognostic significance of endothelial dysfunction in hypertensive patients. Circulation 2001; 104:191–6.