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PROF. DR. VICTORIA KOLB-BACHOFEN

THE INDUCIBLE NITRIC OXIDE SYNTHASE EXERTS ANTIOXIDANT EFFECTS IN ENDOTHELIAL CELLS VIA A ZINC-DEPENDENT SIGNALING PATHWAY

[Die induzierbare Stickstoffmonoxid-Synthase
induziert endotheliale Resistenz gegen oxidativen Stress
über einen zinkabhängigen Signalweg]

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Miriam Margherita Cortese
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Co-Referent: Univ.-Prof. Dr. Peter Proksch
Co-Referent: Univ.-Prof. Dr. Ulrich Fösterman

Tag der mündlicher Prüfung 26.01.2007

This work is dedicated
to Daniel and Yasemin

GUTTA CAVAT LAPIDEM

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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Düsseldorf, den 28.08.2006

Miriam Margherita Cortese

ZUSAMMENFASSUNG

Entzündung, oxidativer Stress und Lipid-Stoffwechselstörungen gelten als Hauptursachen für die endotheliale Dysfunktion und für die Initiation der Arteriosklerose. Während eines entzündlichen Stimulus können Endothelzellen den indizierbaren Isotyp der NO-Synthasen (iNOS) exprimieren und so über einen längeren Zeitraum relativ hohe Konzentrationen an Stickstoffmonoxid (NO) produzieren („high-output" Synthese). In der vorliegenden Arbeit wurde eine 24-stündige Inkubation von Endothelzellkulturen mit H_2O_2 als Modell für den oxidativen Stress gewählt. In diesem Modell konnte bestätigt werden, dass der Peroxid-induzierte Zelltod vollständig aufgehoben wurde in Anwesenheit eines exogen zugegebenen NO-Donormoleküls oder alternativ durch die endogene „high-output" NO-Synthese über die iNOS. Dieser NO-vermittelte Schutz wird durch Blockade der *de novo* Proteinsynthese völlig aufgehoben. Somit wird die protektive Wirkung von NO über eine Transkriptionsregulation vermittelt.

Hier konnte gezeigt werden, dass das GSH-System eine zentrale Rolle im NO-vermittelten Schutz darstellt. Die Inhibition der GSH-Peroxidase, aber nicht der Katalase, hebt die Schutzwirkung von NO auf. Es zeigte sich, dass NO die Expression der Glutamat-Cystein Ligase (GCL) induziert, die den geschwindigkeitslimitierenden Schritt der GSH-Synthese katalysiert. Die erhöhte Expression der GCL resultiert in einem signifikant erhöhten intrazellulären GSH-Spiegel. Eine Inhibition der GCL hebt den NO-vermittelten Schutz auf. Die zellulären GSH-Spiegel korrelieren signifikant und direkt mit dem Überleben der Zellen nach der Peroxyd-Behandlung. Daraus lässt sich schließen, dass die antioxidative Wirkung von NO hauptsächlich auf der Erhöhung der Transkription der GCL und damit der GSH-Konzentration in der Zelle beruht.

Es ist bekannt, dass die „high-output" NO-Synthese die Labilisierung von Zink aus Zink-Schwefel-Clustern über S-Nitrosierung bewirkt. Da Zink ein bekannter transkriptioneller Regulator mit einer antioxidativen Wirkung ist, wurde die Hypothese aufgestellt, dass eine NO-induzierte Zink-Umverteilung den molekularen Mechanismus darstellt, der zum Zellschutz vor oxidativem Stress führt. Dies würde einen neuen Signaltransduktionsweg darstellen. Es konnte gezeigt werden, dass durch Zinksupplementation gleiche Effekte wie mit NO erzielt werden, die sich aber in ihrer Kinetik unterscheiden, nämlich eine Erhöhung der GCL-Expression, der zellulären GSH-Spiegel und des Zellschutzes. Zusätzlich wurde in dieser Arbeit herausgefunden, dass Zink-Defizienz, erreicht durch einen Zink-spezifischen Chelator (TPEN), zur Aufhebung der NO-induzierten Effekte – also der Erhöhung der GCL -Expression, der Erhöhung der GSH-Spiegel und dem Schutz vor Peroxyd – führt. Daraus lässt sich schließen, dass die iNOS-vermittelte NO Synthese Endothelzellen hauptsächlich über eine zinkabhängige Verstärkung der *Gclc*-Gentranskription schützt.

In dieser Arbeit wurde auch nach der Beteiligung von spezifischen Transkriptionsfaktoren, insbesondere als Zielmoleküle für die NO-induzierte Zink-Labilisierung, gesucht. Mit den Methoden der RNA-Interferenz und der konfokalen Lichtmikroskopie konnte gezeigt werden, dass NO die Expression des *Gclc*-Gens über eine zinkabhängige Aktivierung des Transkriptionsfaktors Nrf2 induziert. Des Weiteren konnte auch festgestellt werden, dass NO die Expression von Zielgenen für MTF-1

zinkabhängig induziert. Dies zeigt, dass NO sowohl Nrf2 als auch MTF-1 über eine Zinklabilisierung aktiviert. Zusätzlich wurden auch Hinweise auf eine Regulation der NFκB-Aktivierung gefunden. Es ist bekannt, dass NFkB die iNOS-Expression reguliert und dass die NFκB-Aktivität durch Zink inhibiert wird. Hier konnte gezeigt werden, dass eine zusätzliche Gabe von Zink unter proinflammatorischen Bedingungen die iNOS-Promotoraktivität, -Expression und NO-Synthese reduziert. Dies deutet auf eine zinkvermittelte Inhibition der NFκB-Aktivität und damit auf einen „feedback" Regulationszyklus hin, der die endotheliale Aktivierung limitiert.

Zusammenfassend konnte gezeigt werden, dass die Induktion einer lang andauernden endogenen, antioxidativen Schutzreaktion, über die Erhöhung der intrazellulären GSH-Spiegel, einen wesentlichen Weg darstellt, über den NO oxidative Schäden vermindert und vor endothelialem Zelltod schützt. Weiterhin demonstrieren die hier erzielten Ergebnisse, dass intrazellulär labilisiertes Zink als Botenstoff in einer neuen NO-vermittelten Signalkaskade wesentlich ist. Die Aufdeckung von Interaktionen zwischen den zentralen Faktoren der Entzündungsreaktion, insbesondere des Zusammenspiels von iNOS-vermittelter NO-Synthese und Zink, sollen helfen die Mechanismen zu verstehen, die zu endothelialer Dysfunktion und zu chronisch-entzündlichen Prozessen führen.

SUMMARY

Inflammation, oxidative stress, and dyslipidemia have been indicated as main causes of endothelial dysfunction and thus of the onset of the atherosclerotic process. Under pro-inflammatory conditions, endothelial cells express the inducible form of nitric oxide synthase (iNOS), which produces high output synthesis of nitric oxide (NO). Using a 24-hour treatment with H_2O_2 as a model for oxidative stress, the present study confirms that peroxide-induced endothelial cell death can be completely inhibited by exogenously applied NO or iNOS-derived NO. The presence of a protein synthesis inhibitor blocks the NO-mediated protection, indicating that NO exerts its protective activity via transcriptional regulation.

This study shows that the glutathione (GSH) system plays a central role in NO-mediated antioxidant activity. In fact, the inhibition of GSH peroxidase, but not of catalase, fully blocks the NO-mediated protective activity. NO induces the expression of glutamate-cysteine ligase (GCL), the enzyme catalysing the rate limiting step of the *de novo* synthesis of GSH. This increase in GCL enzyme levels produces an increase of total GSH. The inhibition of GCL fully abrogates the NO-mediated cytoprotection. Moreover, the GSH levels of the cells significantly and directly correlate with the rate of cell survival after the peroxide treatment. Therefore, the antioxidant activity of NO depends mainly on the transcriptional increase of GCL and on the increase of the GSH levels in the cells.

It has been previously shown that high-output NO-synthesis induces zinc release from zinc-sulfur clusters via S-nitrosation. Since a signalling role of zinc has been demonstrated, the NO-mediated zinc redistribution might be a novel mechanism of signal transduction, leading to cellular protection against oxidative stress. The present study shows that in endothelial cells, zinc supplementation produces effects similar to NO. For instance, an increase in GCL expression, in total GSH cellular content, and in cell viability is observed. In addition, zinc deficiency, achieved by adding the cell-permeable zinc chelator TPEN, fully inhibits the NO-mediated protection against oxidative stress. Therefore the data presented here show that iNOS-derived NO protects endothelial cells against oxidative stress mainly via activating the transcription of *Gclc* in a zinc-dependent fashion.

In addition, the involvement of specific transcription factors in NO-mediated protection, and particularly as targets of NO-mediated zinc redistribution, was also investigated here. Findings obtained by RNA interference and confocal microscopy demonstrate that NO induces the expression of *Gclc* by activating the transcription factor Nrf2. Moreover, the present study also shows that NO induces the expression of MTF-1 target genes in a zinc-dependent fashion, proving that both Nrf2 and MTF-1 are targets of NO-mediated zinc redistribution. An additional target of the NO-mediated zinc redistribution might be the transcription factor NFκB, as proposed here. It is well known that zinc inhibits the binding and activation of NFκB. In this study, it has been shown that under pro-inflammatory conditions zinc supplementation reduces the iNOS promoter activity, iNOS expression, and NO production. Since NFκB is the main regulator of

iNOS expression, the zinc-mediated inhibition of NFκB might be a sort of feedback mechanism that limits endothelial activation.

To conclude, the present study shows that the induction of long-term endogenous antioxidant defense mechanisms, and particularly the GSH system, may represent the major pathway by which NO limits oxidative injury and prevents endothelial cell death. Moreover, the data presented here clearly show that intracellular labile zinc is involved in the antioxidant activity of NO, pointing to a role of zinc as second-messenger in a novel NO-induced signaling pathway. Uncovering the interactions between the central players of inflammatory pathways, such as iNOS-derived NO and zinc contributes to understanding the mechanisms leading to endothelial dysfunction and chronic inflammatory conditions.

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ABBREVIATIONS

18S rRNA ribosomal RNA 18S
A549/8 iNOS reporter cell line stably transfected with a 16kB segment of the human iNOS promoter
AC adenylate cyclase
AP-1 activator protein-1 (transcription factor)
ARE antioxidant responsive element
ATA 3-amino-1,2,4-triazole (catalase inhibitor)
ATP adenosine-triphosphate
BCA Bicinchoninic Acid
BH_4 tetrahydrobiopterine
BSO buthionine sulfoximine (glutathione-cysteine ligase inhibitor)
CAT catalase
cGMP cyclic guanosine-monophosphate
COX cyclooxygenase
DAB 3-3'-diaminobenzidine
DABCO 1,4 diazabicyclo-[2.2.2]octane
dATP 2′-Deoxyadenisine 5′-triphosphate
dCTP 2′-Deoxycytosine 5′-triphosphate
DETA Diethylentriamine
DETA/NO Diethylentriamine/nitric oxide adduct or (Z)-1-[N-(2-aminoethyl)-N-(2-aminoethyl)-amino]diazen-1-ium-1,2-diolate
DETC diethyldithiocarbamate
dGTP 2′-Deoxyguanosine 5′-triphosphate
dH_2O distilled water
ddH_2O double distilled water
dUTP 2′-Deoxyuridine 5′-triphosphate
EC endothelial cell
ECGS endothelial cell growth supplements
EDRF endothelium derived relaxing factor
EDTA Ethylenediaminetetraacetic acid
eNOS endothelial nitric oxide synthase
ET endothelin
FAD flavin adenine dinucleotide
FCS fetal calf serum
Fe-S clusters iron-sulfur clusters
FMN flavin mononucleotide
GABA gamma aminobutirric acid receptor
GAPDH glyceryl-aldehyde-6-P-dehydrogenase
GC guanylate cyclase
GCL glutathione-cysteine ligase
GPx glutathione peroxidase
GR glucocorticoid receptor
GSH gamma glutamyl-cinseinyl-glycine or glutathione (reduced)
GSNO S-nitrosoglutathione
GSSG gluthathione disulfide
GTP guanosine-triphosphate
h hours
HBSS Hank´s Balanced Salt Solution
HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HO-1 heme oxigenase-1
HSP70 heat shock protein 70kD
Hyperfilm ECL electron chemiluminescence
ICAM intracellular adhesion molecule
IFN-γ interferon-gamma
IκB inhibitor of kappa B
IL-1β interleukin-1-beta
iNOS inducible nitric oxide synthase
IRE interferon responsive element
IRF interferon regulatory factor
Keap-1 Kelch-like ECH-associated protein
L-NIO L-N5-(1-iminoethyl)-ornithine-dihydrochloride
LPS lipopolysaccharide
MAPK protein kinase
MRE metal responsive element

MS 2-mercaptosuccinate (glutathione peroxidase inhibitor)
MT metallothionein (protein)
Mt1a metallothionein-1a (gene)
MTF-1 metal-regulatory transcription factor-1 or metal responsive element transcription factor-1
NADPH Nicotinamide adenine dinucleotide phosphate, reduced
NFkB nuclear factor-kappa B
NMDA n-Methyl-D-Aspartat (Glutamat, -R: Rezeptor):
nNOS neuronal nitric oxide synthase
NO or $NO^{\cdot}$ nitric oxide (radical)
NO^{+} nitrosonium cation
NOS nitric oxide synthase
NOx nitrogen oxides
Nrf2 NF-E2-related factor 2
$O_2^{\cdot -}$ superoxide anion radical
$ONOO^{-}$ peroxynitrite anion
PBS phosphate buffered saline
PDE nucleotide phosphodiesterase
PK protein kinase
PKC protein kinase C
ponceau S 3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]phenylazo)-2,7-naphthalenedisulfonic acid sodium salt
PTP protein tyrosine phosphatase
PVDF Polyvinylidenfluorid
QRT-PCR quantitative (or real-time) reverse transcription-polymerase chain reaction
RE responsive element
ROS reactive oxygen species
RPMI 1640 Roswell park memorial institute (commercial culture medium)
RPMI 1640 complete RPMI 1640 + additives without FCS
RT room temperature
RT-PCR reverse transcription-polymerase chain reaction
sGC soluble guanylate cyclase
siGAPDH silencing RNA directed against the *Gapdh* mRNA
siMTF-1 silencing RNA directed against the *Mtf-1* mRNA
siNrf2 silencing RNA directed against the *Nrf2* mRNA
siRNA silencing RNA
SNOC S-nitrosocysteine
SOD superoxide dismutase
tBHQ tertbutylhydroquinone
TBS tris buffered solution
TNF-α tumor necrosis factor-alpha
TPEN N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine
Tris 2-Amino-2-(hydroxymethyl)-1,3-propanediol or tris(hydroxymethyl)aminoethane
Tris-HCl Tris-hydrochloride
T-TBS 0,05% Tween-tris buffered solution
VCAM vascular cell adhesion molecule
VEGF vascular endothelium growth factor
vWF von Willebrand factor
WHO world health organisation
ZnT-1 zinc transporter-1 or soluble carrier 30kD (Slc30)

NOTE: gene symbols are indicated in italics and protein symbols are represented in standard fonts, as indicated in the "Guidelines for Human Gene Nomenclature"(Wain *et al.*, 2002)

INTRODUCTION

THE ENDOTHELIUM

Due to its particular anatomic location, the endothelial wall controls many biological events occurring either on the endoluminal or the interstitial side of the vasculature. The endothelium has long been considered a "layer of nucleated cellophane," endowed with passive properties, the most important being its ability to act as a non-thrombogenic substrate for blood (Cines *et al.,* 1998). As such, endothelial cells were thought to be essentially a target for injurious agents. This view has changed radically. It is now evident that haemostasis and haematopoiesis, inflammatory reactions, antigen presentation, immunity, and lipoprotein metabolism involve close interactions between immuno-competent cells and vascular endothelium (Mantovani *et al.,* 1997; Rotella *et al.,* 1996) .

Inflammation and endothelial cells activation

The first step in the development of an inflammatory process induced by external agents such as bacterial lipopolysaccaride (LPS) or by tissue injury (e.g., determined by hypoxia) consists in the local production of pro-inflammatory mediators, such as pro-inflammatory cytokines (Osborn, 1990). This process is accompanied by the activation of the endothelial cell, which results in the expression of its pro-inflammatory properties.

Stimuli producing endothelial cell activation such as cytokines, LPS, or hypoxia/reoxygenation, activate specific membrane receptors. The signal is then transduced through different protein kinase-dependent pathways, mainly leading to the activation of the transcription factor NF-κB (Karin, 1992; Magnuson *et al.,* 1989). Under steady-state conditions, NF-κB is bound to IκB, the cytosolic inhibitory protein that keeps NF-κB inactive (Collins *et al.,* 1995). When activated, the NF-κB-IκB complex is phosphorylated, and becomes dissociated. Once dissociated, IκB is degraded rapidly, while NF-κB accumulates in the nucleus, where it promotes the transcription of its target genes and determining endothelial cell activation. In addition, NF-κB induces the

transcription of the gene for IκB, increasing the *de novo* synthesis of IκB and activating a feedback inhibition loop (Collins *et al.,* 1995).

Activated endothelial cells produce inflammatory cytokines, chemokines, coagulation factors, and vasoactive agents, as well as surface adhesion molecules, which promotes leukocyte adhesion (Meager, 1999). Of interest, under pro-inflammatory conditions endothelial cells express the inducible form of nitric oxide synthase (iNOS), which produces high-output synthesis of nitric oxide (NO) (Suschek *et al.,* 1994; Suschek *et al.,* 1993).

Endothelial cells pathology and atherosclerosis: the role of endothelial dysfunction, inflammation and oxidative stress

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. This deposition leads to the proliferation of vascular cells within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. In the last 150 years there have been numerous efforts to explain the complex events associated with the pathogenesis of this disease. In this endeavor, three distinct hypotheses have emerged (Stocker *et al.,* 2004).

First, the "response-to-injury" hypothesis focuses on endothelial injury as the inciting event in atherosclerosis, permitting the deposition of lipids within the endothelial wall and promoting inflammation. Second, the "response-to-retention" hypothesis focuses more on the demonstrated correlation between hyperlipidemia and onset of the disease, asserting that the interaction between lipoproteins and the extracellular matrix is the critical event in early atherosclerosis. Third, the "oxidative modification" hypothesis focuses on the involvement of oxidative stress in the pathogenesis of atherosclerosis, indicating the oxidation of lipoproteins as the initial event causing the recruitment of inflammatory cells and the development of vascular diseases. Although each hypothesis points to its own critical initiating events, they present many common features, i.e., the central role played by the endothelium, by the lipoproteins, and by inflammation (Stocker *et al.,* 2004). The three initially well-separated hypotheses are now evolving into a more complex theory.

First, the prominent role played by inflammation in the pathogenesis of atherosclerosis and its complications has been recognized. In fact, early atherogenesis is

characterized by leukocyte recruitment and expression of pro-inflammatory cytokines (Libby, 2002). Moreover, in the late stages of atherosclerosis, inflammatory pathways promote the occurrence of complications, such as thrombosis, which are responsible for myocardial infarctions and most strokes (Libby, 2002).

In addition, a new role has been attributed to endothelial damage in the development of vascular diseases. For instance, it has been observed that an injured endothelium is also dysfunctional. It is characterized by an impairment of endothelial-dependent vasorelaxation, mainly caused by a loss of NO bioactivity. Other characteristics of endothelial dysfunction are: the alteration of the anticoagulant and anti-inflammatory properties of the endothelium, the impaired regulation of vascular growth, and the dysregulation of vascular remodeling (Cai *et al.,* 2000). A dysfunctional endothelium not only permits the development of atherosclerosis, as proposed in the response-to-injury theory, but indeed facilitates and boosts the propagation of the atherosclerotic lesions. Moreover, the occurrence of endothelial dysfunction has been strictly correlated to the presence of chronic inflammation and oxidative stress. For instance, the decline in NO bioavailability observed in a dysfunctional endothelium seems to be determined by an accelerated degradation of NO by radical oxygen species (ROS) during oxidative stress, as well as decreased activity or expression of eNOS (Cai *et al.,* 2000).

On the other hand, oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the oxidants (Sies, 1991), is now considered to be a consequence of, as well as a boost to, the inflammatory process (Stocker *et al.,* 2004). Within the vessel wall, oxidants can originate from extracellular or intracellular sources and be produced in enzymatic or non-enzymatic reaction pathways (Stocker *et al.,* 2004). An extracellular source of radicals for endothelial cells is the blood that contains various kinds of cell soluble components, vasoactive substances, as well as reactive oxygen species (Cai *et al.,* 2000). In addition, also non-enzymatic oxidant species (e.g., transition metals or heme, derived from hemolysis) have been implicated in the catalysis of oxidative reactions within the vascular wall (Stocker *et al.,* 2004). Instead, intracellular sources of oxidants are mitochondrial respiration and enzymes catalyzing oxidative reactions such as: NAPDH oxidase, xantine oxidase, myeloperoxidase, and lipooxigenase

(Stocker *et al.,* 2004; Wassmann *et al.,* 2004). In addition, under pro-inflammatory conditions, mediators such as pro-inflammatory cytokines, angiotensin II, Thrombin, VEGF, and shear stress induce the expression of oxidative enzymes, promoting the production of ROS and, consequently, the occurrence of oxidative stress (Wassmann *et al.,* 2004).

Strategies of defense against oxidative stress: the role of GSH and of the adaptive response

Among the eukaryotic cells, vascular cells are particularly well equipped with defense mechanisms for the detoxification of oxidants and xenobiotics (Sies, 1993; Wassmann *et al.,* 2004). Defense strategies against oxidative stress damage include three levels of protection: prevention (i.e., protection against the formation of radical species, for example by chelation of transition metals), deactivation (i.e., interception of damaging radical species by endogenously produced antioxidant species or by antioxidant enzymes), and repair (i.e., restitution or replenishment of the damaged functionality) A further level of protection exists in eukaryotic cells. For instance, after the damage has occurred, an adaptive response is activated in order to prevent further damage. The adaptive response consists of the transcriptional activation of protective proteins able to induce long-term protection against oxidative damage (Sies, 1993).

The integrity and the functionality of the endothelium are, therefore, maintained by the coordinate activation of different levels of protective mechanisms. If the defense system fails, or an imbalance between antioxidant mechanism and oxidants occurs, as for example under pathological conditions such as chronic inflammation, hypoxia, or ischemia-reperfusion, endothelial cells die or become dysfunctional (Wassmann *et al.,* 2004).

Among the different levels of defense mechanisms, the ability of eukaryotic cells and, particularly, of vascular cells to deactivate the ROS produced and to mount an adaptive response to prevent future damage is the most powerful.The deactivation of ROS in mammalian cells and, in particular, in endothelial cells is achieved by endogenous production of antioxidant molecules able to scavenge radicals, such as α-tocopherol (vitamin E), ascorbate (vitamin C), β-carotene, lycopene, and glutathione (γ-Glutamyl-cyteinyl-glycine; GSH) (Wu *et al.,* 2004), as well as through the activity of

antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Sies, 1993; Wassmann *et al.,* 2004).

Among the antioxidant species produced in mammalian cells, GSH is the most abundant (Sies, 1999) and exerts antioxidant activity by directly scavenging free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2), or by participation in enzymatic reactions as a cofactor (Sies, 1999). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase (Fig. 1). The main GSH-dependent antioxidant enzyme is glutathione peroxidase (a selenium-containing enzyme), which catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides (Fig. 1). A similar function is also carried out by catalase, a GSH independent enzyme (Fig. 1).

The *de novo* synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, glutamate-cysteine ligase (GCL) (also called γ-glutamylcysteine synthetase), which catalyses the rate limiting step (Jones, 2002), and GSH synthetase (Lu, 2000). The enzyme GCL consists of a catalytically active heavy subunit (*Gclc*; 73 kDa) and a light regulatory (GCLr; 31 kDa) subunit (Lu, 2000).

The concentration of total cellular GSH (reduced + oxidated) (Sies, 1999) is determined by: the rate of *de novo* synthesis; consumption and export in the form of GSH (from hepatocytes, in the form of GSSG), which contributes to the net loss of intracellular GSH; as well as in the formation of thioethers (S-conjugates), which are usually committed to detoxication and elimination. Other important products derived from GSH include thiolesters, S-nitrosoglutathione (GSNO), and the glutathionyl radical (Sies, 1999; Wu *et al.,* 2004).

In order to prevent further damage, an adaptive response is activated in mammalian cells, which consists of the transcriptional activation of protective proteins able to exert long-term protection by preventing, deactivating, and repairing oxidative damage (Sies, 1993). Such protective proteins are the described antioxidant enzymes (e.g., SOD, catalase, and GSH peroxidase), phase II detoxification enzymes (e.g., GSH-S-transferase, NADPH:quinine oxidoreductase, and enzymes of the GSH redox cycle), and stress inducible cytoprotective genes (e.g., the chaperon HSP70 and the antioxidant enzyme heme oxigenase-1) (Sies, 1993). Moreover, under pro-inflammatory conditions,

activated endothelial cells express iNOS. The resulting high-output synthesis of NO has been shown to exert antioxidant and protective activity in the vessel wall, which is discussed later (Suschek *et al.,* 2003).

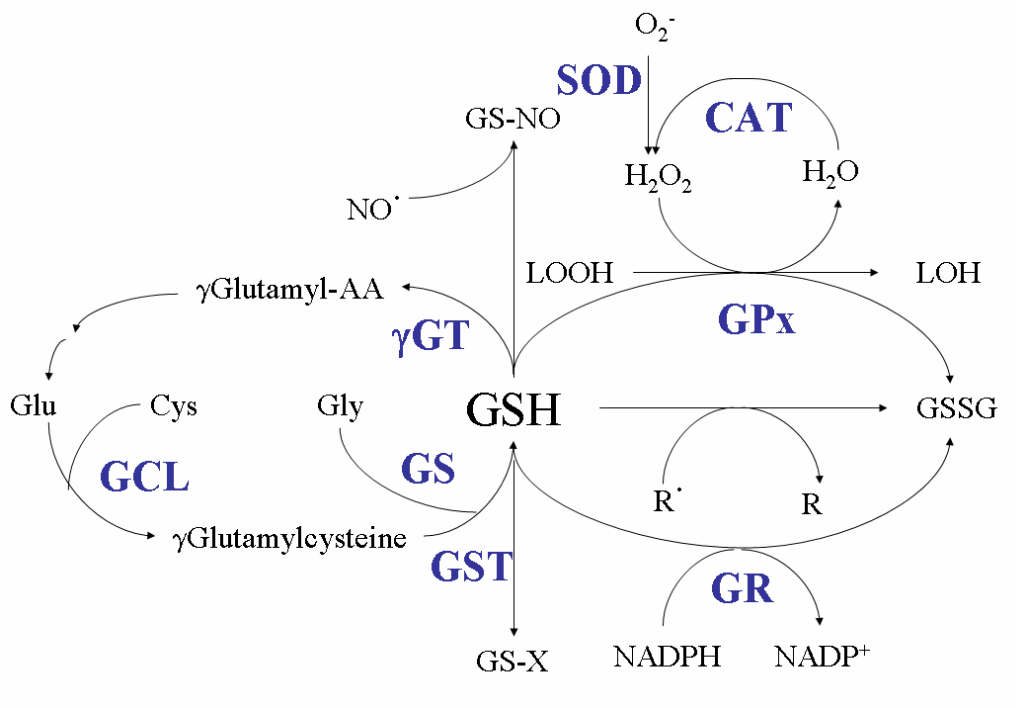


FIGURE 1. The antioxidant defence mechanisms of endothelial cells and the metabolism of GSH.

The de novo synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by: glutamate-cysteine ligase (GCL), which catalyses the rate limiting step; and by GSH synthetase (GS). GSH exerts its antioxidant activity by directly scavenging radical species (R), and is a co-factor in reactions catalysed by antioxidant enzymes such as glutathione peroxidase (GPx), which reduce H_2O_2 with consequent oxidation of GSH to GSSG. The GSSG is reduced by the NADPH-dependent enzyme glutathione reductase (GR). Alternatively, the reduction of H_2O_2 to H_2O is catalysed by the antioxidant enzyme catalase (CAT). The enzyme superoxide dismutase (SOD) catalyses the reduction of superoxide to peroxide. GSH can be conjugated to electrophiles, physiological metabolites, and xenobiotics (X) to form mercapturates in reactions initiated by the enzyme glutathione-S-transferase (GST), a phase II detoxification enzyme. Alternatively, GSH conjugates with NO to form an S-nitrosoglutathione adduct (GS-NO). The catabolism of GSH is catalysed by the enzyme γ-glutamyl transferase (γ-GT). LOOH, lipid hydroperoxide. (Adapted from Wu, et al. 2004)

With transcriptional activation as the basic mechanism of the adaptive response, the transcription factors involved in the activation of protective genes become the key players in this response (Otterbein *et al.,* 2002). The redox-sensitive transcription factors, NFκB and activator protein (AP)-1, are two of the most prominent regulators of cellular responses to oxidative stress, but also participate in the regulation of a plethora of

biologic processes, including inflammation (Otterbein *et al.,* 2002). AP-1 belongs to the family of the bZIP transcription factors characterized by a region containing heptad repeats of leucine. A subclass of the AP-1 class of transcription factors family is the "cap'n'collar" CNC-bZIP, presenting high homology with a transcription factor isolated from *Drosophyla Melanogaster* (McGinnis *et al.,* 1998; Moi *et al.,* 1994).

The oxidative stress-sensitive transcription factor Nrf2 is the most prominent member of this subclass in mammals, and is constitutively expressed in a variety of tissues (Moi *et al.,* 1994; Otterbein *et al.,* 2002). Several studies suggest that Nrf2 regulates the transcription of phase II detoxifying enzymes by recognizing the antioxidant response element (ARE) present in the promoter of those genes (Chan *et al.*, 1999; Chan *et al.*, 1996; Jaiswal, 2004; Otterbein *et al.*, 2002; Wasserman *et al.*, 1997).

The activation mechanism of Nrf2 is currently under discussion. Under normal conditions, Nrf2 exists in an inactive state in the cytoplasm because it has bound to the inhibitor Keap1 (Kelch-like ECH-associated protein). After exposure to electrophiles, heavy metals, or ROS, Nrf2 is translocated into the nucleus where it binds to the antioxidant responsive elements (ARE) and activates the transcription of its target genes (Itoh *et al.*, 2004; Itoh *et al.*, 2003; Nguyen *et al.*, 2005; Wakabayashi *et al.*, 2004).

NITRIC OXIDE

The first biological activity attributed to NO was the ability to induce relaxation of aortic rings, which has been named the endothelium-derived relaxing factor (EDRF) (Furchgott *et al.,* 1980). NO not only plays a central role in the patho-physiology of vascular cells (Vallance *et al.,* 1989), but also in neurotransmission (Bredt *et al.,* 1994), both in the central and peripheral nervous system, as well as in inflammatory processes (Bogdan, 2001a; Bogdan, 2001b; Kröncke *et al.*, 2001; Suschek *et al.*, 2004).

Nitric oxide synthases

The enzymes class of nitric oxide synthases (NOS) is composed of three isoenzymes and catalyses the five-electron oxidation of the guanidino nitrogen of L-arginine, which produces the gaseous free radical nitric oxide and citrulline in equimolar concentrations. Essential cofactors for this reaction are flavin mononucleotide (FMN),

flavin adenine dinucleotide (FAD), protoporphyrin IX heme, and tetrahydrobiopterin (BH_4). The proposed reaction mechanism involves an electron transfer from the flavin to the heme group, where the oxidation of guanidino nitrogen of L-arginine takes place. Tetrahydrobiopterin appears to be important in maintaining the enzyme in its active dimeric form (Kröncke *et al.,* 1998; Nathan, 1992).

Of the three isozymes of NOS, two are constitutively expressed primarily in vascular endothelia cells (eNOS), neuronal cells, and skeletal muscle (nNOS), and produce low amounts of NO in a pulsatile and Ca^{2+}-regulated fashion. A third type, the inducible NOS (iNOS), is induced after inflammatory stimuli (such as cytokines or gram negative bacteria), and produces high-output synthesis of NO in a Ca^{2+}-independent fashion. Each of the three isoforms is transcribed from a different gene (Zhang *et al.,* 2000).

The constitutively expressed enzymes eNOS and nNOS are activated after stimulation of specific receptors by various agonists (e.g., acetycholine, bradykinin, serotonin, adenosine, ADP:ATP, histamine, and thrombin), with the following increase of intracellular free Ca^{2+}. The binding of Ca^{2+} to the Ca^{2+}/Calmodulin subunit of the enzymes activates the enzyme to produce NO. Subsequentely, the binding of NO to the Fe^{2+}-heme of soluble guanylate cyclase (sGC) activates this enzyme to produce cyclic guanosine-3-5P-monophosphate (cGMP) from guanosine-5P-triphosphate (GTP). The signal is then transduced to downstream elements of the signaling cascade, i.e. cGMP-dependent protein kinases, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase (Denninger *et al.,* 1999). The effects produced by eNOS-derived NO include smooth muscle relaxation, blood pressure regulation, and platelet disaggregation.In the central nervous system, nNOS-derived NO is involved in the processes of long-term potentiation and depression (Buechler *et al.,* 1994; Jaffrey *et al.,* 1995; Warner *et al.,* 1994) In the autonomic nervous system in non-adrenergic, non-cholinergic nerves, NO functions as an inhibitory neurotransmitter, producing relaxation in the gastrointestinal tract (Bult *et al.,* 1990; Desai *et al.,* 1991) and in the corpus cavernosum, thereby mediating penile erection (Burnett *et al.,* 1992).

On the other hand, iNOS activation is mainly transcriptionally regulated and the molecular mechanism of iNOS gene induction presents species-dependent variations. It

appears that the higher the species, the more complex and longer the iNOS gene promoter, suggesting an evolutionary change in the mechanisms of iNOS induction. The transcriptional binding sites mostly responsible for cytokine-, or LPS-mediated iNOS induction, are the NF-κB binding sites (Lowenstein *et al.,* 1993; Xie *et al.,* 1994; Xie *et al.,* 1993). However, the importance of other transcriptional binding sites in mediating iNOS induction has also been reported. For example, interferon-γ (IFN-γ) exerts a synergistic effect on the LPS-mediated induction of iNOS, because of the presence of a interferon-responsive, factor-responsive element (IRF-E) in position -923 to -913 bp (Martin *et al.,* 1994; Spink *et al.,* 1997).

Impact of nitric oxide on cellular functions

NO and its oxidised redox forms like N_2O_3,NO_2 or NOO^- show different chemical reactivity toward various target molecules, explaining the breadth of its effects (Kröncke et al., 1997; Kröncke et al., 1998; Kröncke et al., 1995). The most important targets of NO are iron-containing prosthetic groups of enzymes and the thiol group of cysteines.

NO directly binds to the Fe^{2+}-heme prosthetic group, not only producing the activation of sGC as previously described, but also inhibiting the activity of other heme enzymes such as cytochrome oxidase, catalase, and cytochrome P-450 (Kerwin *et al.,* 1995). In addition, NO also binds to iron-sulfur (Fe-S) clusters, which are responsible for the activity of many enzymes (Beckman *et al.,* 1996; Hausladen *et al.,* 1994). Most Fe-S clusters (e.g., the electron-transferring Fe-S centers in the mitochondrial electron transfer chain) are deeply buried within proteins and, therefore, are relatively inaccessible to NO. However, at high NO concentrations, reactions with Fe_4S_4 clusters in proteins inaccessible to solvent were shown to occur (Forster *et al.,* 1999).

The S-nitrosation of cysteine residues achieved by NO in the presence of oxygen seems to play a central role in NO signaling. First, the formation of S-nitrosothiols, by NO-mediated S-nitrosation of small peptides, could be a mechanism for extending the half life of NO and prolonging its effects. In fact S-nitrosothiols, such as S-nitrosocystein, S-nitrosohomocystein, S-nitrosoglutathione (GSNO), and S-nitrosoalbumin have a longer half life than NO itself and can release NO by transnitrosation or oxidative reactions, acting as NO-reservoirs (Stamler, 1994). Second, by S-nitrosation of critic cysteines, NO can modify the activity of targeted proteins and exert a direct signaling function. For

example, the nitrosation of Cys 163 inhibits the activity of caspase-3 (Jia *et al.,* 1996; Rossig *et al.,* 1999; Stamler, 1994; Stamler *et al.,* 1992). Other demonstrated targets of NO-mediated S-nitrosation are signaling proteins such as p21ras (Lander *et al.,* 1995) and c-Jun N-terminal kinase 2 (So *et al.,* 1998), or transcription factors such as NF-kB (Matthews *et al.,* 1996) and AP-1 (Tabuchi *et al.,* 1994).

In addition, it has been shown that the S-nitrosation of Zn-S clusters, which mainly serve as structural elements within proteins (e.g., zinc fingers), destroys these clusters and produces the release of protein-bound Zn^{2+} within cells. (Berendji *et al.,* 1997b; Tartler *et al.,* 2000). Initially, it has been proposed that the S-nitrosation of zinc finger-dependent transcription factors and the ejection of Zn^{2+} thereby lead to the inhibition of DNA in their binding activity (Kröncke *et al.,* 1994).

On the other hand, it has been observed that zinc itself serves signaling tasks in mammalian cells (Beyersmann *et al.,* 2001) and that the zinc, which has been released by oxidant species from zinc clusters, can be transferred to target enzymes leading to their regulation (Jacob *et al.,* 1998). In addition, highly conserved zinc-sensor molecules have been isolated from mammalian cells (Andrews, 2001). Therefore, it has been proposed that the NO-mediated zinc release from zinc clusters could be a novel signal transduction mechanism, producing NO-induced, zinc-dependent, downstream effects (Kröncke, 2001a; Spahl *et al.*, 2003; St Croix *et al.*, 2002).

Given the multiplicity of the targets and the breadth of the effects exerted by NO, a simple classification of the NO-mediated signaling pathways in cGMP-dependent or –cGMP-independent has been proposed. Among the cGMP-dependent effects are the regulation of blood pressure and flow by eNOS-derived NO and neurotransmission by nNOS-derived NO. Instead, cGMP-independent responses are mainly induced by S-nitrosation of cysteine residues and play a central role in cellular signal transduction. S-nitrosation can modify the activity of targeted proteins or lead to the formation of S-nitrosothiols, which could prolong or transduce the signal.

The protective actions of high-output nitric oxide synthesis

Because of its chemical nature, iNOS-generated NO has long been considered a cytotoxic molecule (Kröncke *et al.,* 1997). However, a paper by Mannick et al. in 1994 forced a paradigm shift (Mannick *et al.,* 1994). These authors showed that endogenous

iNOS expression, or exposure to low doses of NO donors, inhibited apoptosis in human B lymphocytes. Following this report, similar findings on NO-mediated inhibition of apoptosis induced by different stimuli, including oxidative stress, were described in several *in vitro* cell culture systems (Beauvais *et al.,* 1995; Genaro *et al.,* 1995; Hebestreit *et al.,* 1998; Kim *et al.,* 1997b; Saavedra *et al.,* 1997), including endothelial cells (Dimmeler *et al.,* 1997) and hepatocytes (Kim *et al.,* 1997a; Kim *et al.,* 1997b; Saavedra *et al.,* 1997). *In vivo* experiments confirmed these observations (Saavedra *et al.,* 1997).

Multiple mechanisms for the inhibition of oxidative stress-induced apoptosis by NO may co-exist in a single cell type. NO may exert its protective actions through the reaction with alkoxyl and peroxyl radicals, thus inhibiting lipid peroxidation (Rubbo *et al.,* 1994); it may suppress the superoxide/hydrogen peroxide-mediated cytotoxic effect by acting as a scavenger of ROS (Brune *et al.*, 1997; Brüne *et al.*, 1999). In addition, NO induces long term protection against oxidative stress via inducing the expression of protective genes.

The transcriptional activity of NO is well known. Recent microarray-based investigations show that iNOS-derived NO alters gene expression of many different genes (Ehrt *et al.,* 2001; Zamora *et al.,* 2002). Among the many genes that have been shown to be transcriptionally regulated by NO are also protective genes such as anti-apoptotic genes, (e.g., Bcl-2) or antioxidant genes, (e.g., HO-1 and VEGF) (Suschek *et al.,* 2001; Suschek *et al.,* 2003; Suschek *et al.,* 2004). Therefore, NO is able to induce an adaptive response in the cells, which then results in protection against noxious stimuli, such as pro-apoptotic stimuli and oxidants.

Before the cytoprotective activity of high-output synthesis of NO had been fully recognized, it was observed that different cell types exhibit huge differences towards the sensitivity to NO-mediated cytotoxicity. In the search for an explanation for this observation, some researchers focused on the analysis of the interrelation between NO and the GSH redox cycle, which plays a major role in the antioxidant defense of the cells. Interestingly, it has been shown that either no effects, or an increase of GSH, was measured, which was explained as an adaptive response by the cells to the GSH depletion by NO and a consequent replenishment in response to the NO-mediated cytotoxicity (Berendji *et al.,* 1999; Luperchio *et al.,* 1996). More recently Moellering et al. reported

that, in bovine aortic endothelial cells, a treatment with exogenously-applied NO increased the total GSH levels of the cells, as well as the protein level of glutamate-cysteine ligase, catalyzing the rate limiting step of *de novo* synthesis of GSH (Moellering *et al.,* 1999; Moellering *et al.,* 1998). Because of the antioxidant roles exerted by GSH in mammalian cells, the increase in GSH by NO is a potential mechanism for enhancing the antioxidant defenses of the cell.

ZINC

Zinc is a micronutrient essential for growth and development of all organisms on earth (Vallee *et al.,* 1993). Over 300 enzymes require zinc for their function, in which it exerts a structural or catalytic role (Vallee *et al.,* 1993). Zinc accomplishes its biochemical functions primarily when bound to enzymes and other proteins and under physiological conditions; it does not undergo reduction or oxidation (Vallee *et al.,* 1993). It has a variable coordination sphere and the stereochemical adaptability to assume multiples coordination geometries, thus becoming a versatile interactant for different donor groups such as nitrogen-, oxygen-, and sulphur-containing ligands (Auld, 2001). The tridentate coordination with aspartate, cysteine, glutamate, or hystidine residues (the most common being hystidine) is characteristic for active sites of enzymes containing zinc with catalytic activity because it provides coordination sites open to water and/or substrate (Auld, 2001; Vallee *et al.,* 1990). The tetradentate coordination with four cysteines and/or hystidines is defined as a zinc-thiolate cluster with high stability constants, allowing local conformations and structures to be conserved (Auld, 2001; Vallee *et al.,* 1990).

Zinc biology and signalling functions

Unlike other transition metals, including those of the IIB series, zinc is virtually non–toxic (Vallee *et al.,* 1993). The homeostatic mechanisms that regulate its entry into, distribution in, and excretion from cells are so efficient that no disorders are known to be associated with its excessive accumulation, in contrast to iron, copper, mercury, and other metals (Vallee *et al.,* 1993). On the other hand, zinc is an essential nutrient and organisms must maintain adequate intracellular zinc concentrations. To accomplish this, a complex

system of integral membrane transport proteins that move the zinc through the cell membrane exists in eukaryotic cells (Gaither *et al.,* 2001). Two families of zinc transporters have been identified in eukaryotes. The Zrt-, Irt-like proteins (ZIP) family plays a prominent role in zinc uptake, transporting zinc from outside the cell or from intracellular compartment into the cytoplasm (Cousins *et al.*, 2000; Gaither *et al.*, 2001; Liuzzi *et al.*, 2004; McMahon *et al.*, 1998).

The Cation Diffusion Facilitator (CDF) family of transporters move zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell or into the lumen of an organelle (Cousins *et al.*, 2000; Gaither *et al.*, 2001; Liuzzi *et al.*, 2004; McMahon *et al.*, 1998). A prominent member of this last family is ZnT-1, expressed on the cell membrane (Palmiter *et al.,* 1995).

Virtually all zinc found in cells is bound to macromolecules in the form of zinc proteins/enzymes or nucleotides, RNA and DNA. The corresponding apo-proteins, apo-enzymes, or nucleic acids are normally not identified in biological systems (Outten *et al.,* 2001; Vallee *et al.,* 1993). A pool of labile-bound zinc, which can be rapidly mobilised by oxidative stimuli such as H_2O_2 and NO, is stored in organelles called zincosomes (Berendji *et al.*, 1997a; Jacob *et al.*, 1998; Kröncke *et al.*, 2002; Maret *et al.*, 1998; Spahl *et al.*, 2003; St Croix *et al.*, 2004).

There is also evidence of a direct signalling function of zinc (Beyersmann *et al.,* 2001). Zinc has been involved in: extracellular signal recognition, regulating the GABA and NMDA receptors in mammalian brain cells (Canzoniero *et al.,* 1997; Cuajungco *et al.,* 1997); in second messenger metabolism; in protein phosphorylation and dephosphorylation; and in the regulation of the activity of transcription factors, as depicted in Fig 5. Zinc can indirectly regulate the activity of transcription factors by modulating their phosphorilation state; for example, by regulating the activity of cyclic nucleotide phosphodiesterase (PDE), protein kinases (PKs), such as PKC or MAPK, or protein tyrosine phosphatase (PTP).

Alternately, zinc can directly modulate the activity of transcription factors. In fact, highly-conserved proteins, able to sense small variations of labile zinc, have been isolated in organisms ranging from bacteria to mammals (Beyersmann *et al.,* 2001; Maret *et al.,*

1998). The best characterised zinc-sensitive transcription factor is the metal-responsive transcription factor (MTF)-1, which regulates the expression of genes responsible for zinc homeostasis, such as the zinc-binding protein metallothionein-1 (*Mt1)* (Heuchel *et al.,* 1994) and the zinc exporter *Znt1* (Langmade *et al.,* 2000). MTF-1 contains six zinc finger structures, of which the first binds zinc with low affinity. After activation by zinc binding, MTF-1 translocates from the cytoplasm into the nucleus (Smirnova *et al.,* 2000), where it binds to the metal-responsive elements present on the promoter of its target genes (Andrews, 2001).

In addition, other transcription factors that are subject to modulation by zinc have been identified. For example, zinc inhibits the binding of steroids to the murine glucocorticoid receptor (Telford *et al.,* 1997). The inhibition of NFkB transcriptional activity, which occurs via inhibition of the NFkB activation in the cytoplasm (Jeon *et al.,* 2000), as well as binding to the DNA (Kim *et al.*, 1999a), appears to be responsible for the anti-inflammatory properties exerted by zinc (Connell *et al.*, 1997; Hennig *et al.*, 1999). In addition, other genes show zinc responsiveness (Lichtlen *et al.*, 2001), such as molecular chaperons, heat shock proteins, and the antioxidant protein HO-1, but the mechanisms and the transcription factors involved have not been fully elucidated.

It has been found that the NO-mediated zinc release from zinc clusters was reversible (Kröncke *et al.*, 2000) and could represent a mechanism of transcriptional regulation (Kröncke *et al.*, 2002). Moreover, it has been observed that the zinc released from the clusters by oxidants could be transferred to target enzymes, regulating their activity (Jacob *et al.*, 1998; Kröncke, 2003; Kröncke, 2001b). Therefore, considering the previous finding and the demonstrated signaling activity of zinc, the hypothesis of a "second-messenger" like role of zinc in NO-induced signaling pathways has been elaborated.

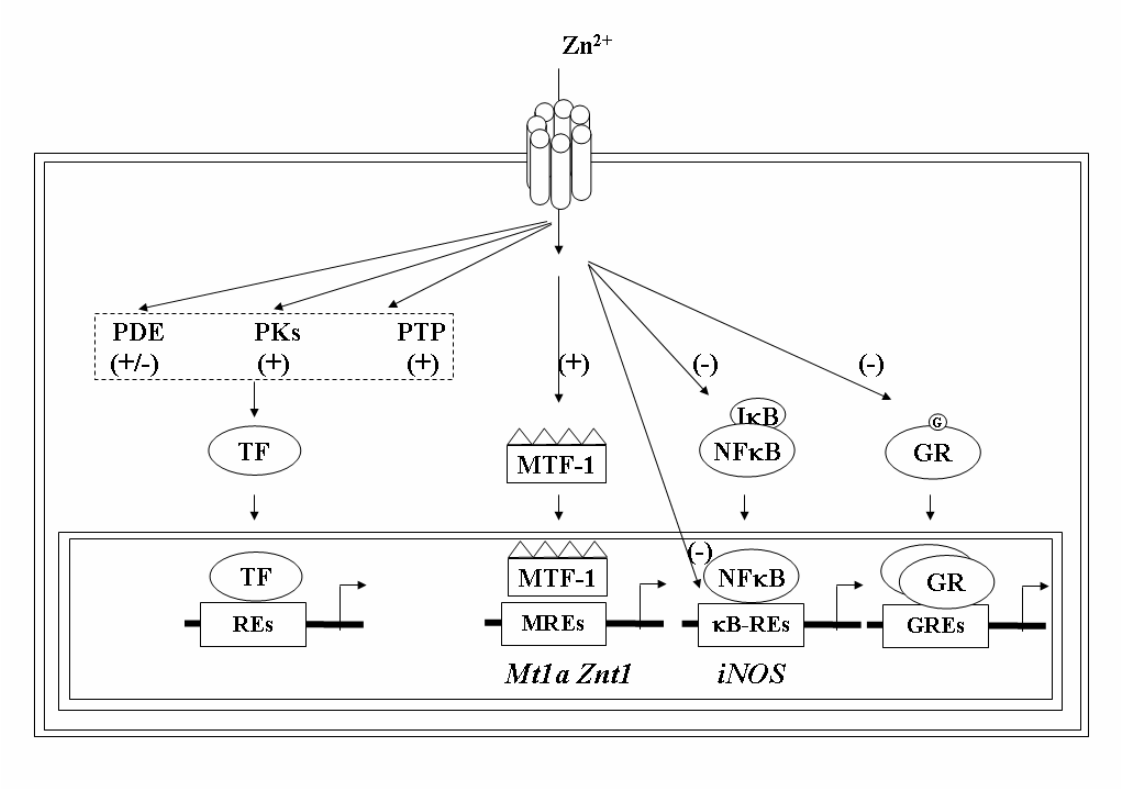


FIGURE 2. Zinc-mediated signalling functions.

Zinc can modulate the phosphoyilation state of transcription factors by modulating the activity of: cyclic nucleotide phosphodiesterase (PDE); protein kinases (PKs), such as PKC or MAPK; or protein tyrosine phosphatase (PTP). Zinc can also directly modulate the activity of transcription factors. The best characterised is MTF-1, which binds to metal responsive elements and modulates the expression of genes involved in zinc homeostasis, such as MT1a and Znt1. Zinc also inhibits the activation and/or the binding to DNA of NFκB, as well as the binding of glucocorticoids (G) to their receptor(GR).
GRE glucocorticoids responsive element, (+) activation, (-) inhibition. Adapted from Beyermann and Haase 2001.

Zinc, endothelium and oxidative stress

It is estimated that zinc deficiency affects one third of the World's population and is the major factor contributing to 1.4% of all death worldwide (WHO, 2002). Although acute deficiency in developed countries is rare, marginal deficiency is thought to be relatively common (Hambidge, 2000). The pathological outcome of zinc deficiency is mainly observed in highly proliferating systems like the immune system, the reproductive system, the skin, and the blood. Considerable attention has been recently focused on the antioxidant and anti-apoptotic activity of zinc *in vitro* or *in vivo* (Alam *et al.,* 1999). Zinc supplementation decreases the sensitivity to oxidative stress and pro-apoptotic stimuli in

many different cell types (Fukamachi *et al.,* 1998; Meerarani *et al.,* 2000; Szuster-Ciesielska *et al.,* 2000; Tang *et al.,* 2001), as well as in animal models (Bediz *et al.,* 2006; Sahin *et al.,* 2005; Zhou *et al.,* 2005). Conversely, zinc deprivation increases oxidative stress and induces apoptotic cell death (Ho *et al.*, 2002; Oteiza *et al.*, 2000; Oteiza *et al.*, 1995). Whether the antioxidant and protective effects exerted by zinc in eukariotic cells are well established, the molecular mechanisms responsible for these effects are currently under active investigation. It has been proposed that zinc exerts an anti-oxidative function by increasing the stability of bio-membranes (Verstraeten *et al.,* 2004), by protecting SH groups from oxidation, and by inducing the expression of protective genes (Klotz *et al.,* 2003; Powell, 2000; Rink *et al.,* 2001).

Because of its role of antioxidant, anti-apoptotic molecule, and membrane stabiliser, the possibility that zinc protects the vascular endothelium against oxidative stress by maintaining the integrity of endothelial cells has been raised (Hennig *et al.,* 1996). In particular, it has been proposed that an inadequate zinc concentration in the plasma or vascular tissues may be involved in either initiation of cell injury, potentiation of oxidative stress and inflammatory response, or lack of protection against apoptosis (Beattie *et al.,* 2004), which are all events leading to endothelial dysfunction and vascular diseases. The antiatherogenic properties have been demonstrated on the basis of the effects exerted by zinc supplementation on cultured endothelial cells or smooth muscle cells. It has been shown that zinc supplementation protects endothelial cells against lipid-induced endothelial cell injury, as well as attenuates cytokines-induced apoptosis, as reviewed by Henning *et al.,* 1996. In addition, the protective activity exerted by zinc supplementation or the permissive activity of zinc deficiency against the insurgence of atherosclerosis has also been tested in animal models. It has been shown that zinc deficiency increases plasma lipids, atherosclerotic markers, and inflammation in low density lipoprotein-receptor deficient mice (Reiterer *et al.,* 2005). In addition zinc deficiency decreases the plasma levels and hepatic mRNA abundance of the atheroprotective apolipoprotein apoA1 in rats and hamsters (Wu *et al.,* 1998). Zinc supplementation also inhibited aortic atherogenesis in cholesterol-fed rabbits, determining an increased expression of superoxide dismutase, a decreased lipid peroxidation, and a decreased lesion area (Alissa *et al.,* 2004)

In clinical and epidemiological studies, however, zinc deficiency has not been firmly associated with an increased incidence of atherosclerosis, probably because of the lack of a good indicator of the zinc status (Wood, 2000) In fact, studies of experimental depletion/repletion of zinc in humans have shown that plasma zinc levels are not a good indicator of the zinc status of the individual. In addition, human subjects are resistant to zinc depletion obtained by low-zinc diet or subministration of zinc chelators, such as orto-phenanthroline or phytate (Wood, 2000) On the other hand, the beneficial effects of zinc supplementation have been observed. For example, in a recent clinical study that analysed the impact of alcohol consumption and nutritional status on the insurgence of vascular diseases in post menopausal women, zinc supplementation showed an inverse association with cardiovascular disease mortality in women consuming more than 10g alcohol per day (Lee *et al.,* 2005b)

In conclusion, a growing set of evidence indicates that zinc could exert anti-atherogenic effects because of its anti-oxidative, anti-apoptotic, and anti-inflammatory properties. Zinc deficiency has been discussed as a possible risk factor of atherosclerosis. In order to define the role of nutritional zinc in endothelial cell integrity, more research has to be done, either to investigate the molecular mechanism underlying the effects observed in cell culture, or to determine a good indicator for the zinc status in humans, thus permitting epidemiological as well as clinical studies.

AIM OF THE STUDY

Dyslipidemia, inflammation and the resulting oxidative stress have been proposed as main causes of endothelial dysfunction and of the onset of the atherosclerotic process. Under pro-inflammatory conditions, endothelial cells express iNOS, which produces high-output NO synthesis. Whether iNOS-derived NO exerts a deleterious or a protective role in endothelial cells is currently under discussion. Particularly, as regards the induction of endothelial dysfunction and the development of the atherosclerotic plaque, the activation of iNOS may exert complex effects.

Using a treatment with H_2O_2 as a model for oxidative stress, it has been previously shown that ROS-induced endothelial cell death can be completely inhibited by high-output NO synthesis, through the inhibition of lipid peroxidation and of cytochrome c leakage, and through the increase in bcl-2-gene expression.The present study is focused on the transcriptional activity of high-output synthesis of NO in endothelial cells and particularly on the molecular mechanisms responsible for the NO-mediated regulation of gene expression.

Our group and others have shown that high-output synthesis of NO produces zinc redistribution in endothelial cells and other cell types.this effects is induced via NO-mediated S-nitrosation of the cysteines belonging to zinc- sulphur clusters. Since it has been observed that in the presence of oxidants zinc can be transferred to target molecules regulating their activity, it has been proposed that the zinc released exerts a signalling role.

Moreover, a growing amount of evidence, mainly obtained through experiments of zinc supplementation and/or depletion, recognizes zinc as a signalling molecule in eukaryotic cells with an established transcriptional activity. In fact, highly specific zinc sensor transcription factors, able to sense small variations of zinc concentration, have been isolated in organisms ranging from bacteria to mammals.

The aim of this study is to analyse the long-term protective activity of iNOS derived NO in endothelial cells and the involvement of zinc in the NO-mediated effects. The hypothesis is that NO-mediated zinc redistribution could be a novel mechanism of

signal transduction leading to cellular protection against oxidative stress. This study analyses the antioxidants effects exerted by high-output synthesis of NO and by zinc supplementation in endothelial cells, as well as the deleterious effects of zinc depletion. In particular this study focuses on the involvement of transcriptional regulation in the observed effects, analysing the participation of transcription factors such as Nrf2, MTF-1 and NFκB.

Since chronic inflammation and oxidative stress play a major role in the onset of endothelial dysfunction and vascular diseases, identifying the central players regulating inflammatory pathways, such as iNOS-derived NO and zinc, potentially contribute to a better understanding of inflammatory diseases such as atherosclerosis.

MATERIALS AND METHODS

MATERIALS

Chemicals

The manufacturer names and the products are given in alphabetic order.
Coenzyme-A and D-luciferine were from **Applichem** (Darmstadt, Germany). Chelex 100 and non fat dry milk were from **BioRad** (Munich, Germany). Biotin-dUTP, dATP, dCTP, dGTP, Ethylenediaminetetraacetic acid (EDTA), Geneticin G418, Kornberg polymerase from **Boehringer Mannheim** (Mannheim, Germany). If not otherwise specified, chemicals for the preparation of buffers were all from **Merk** (Darmstad, Germany). RNase-inhibitor was from **Promega** (Mannheim, Germany). Complete mini-protease inhibitors cocktails and Reporter Gene Assay Lysis Buffer was from **Roche diagnostics GmbH** (Mannheim, Germany). Sodium dodecyl sulfate (SDS), Tris, and Tris-HCl were purchased from **Roth** (Carl Roth GmbH+Co KG, Karlsruhe, Germany). 2'-[etoxyphenyl]-5-[4-metyl-1-piperazinyl]-2',5'-bi-1H-benzimidodezol (Hoechst 33342), 3,3'-diaminobenzidine (DAB), Hematoxylin were purchased from **Serva** (Heidelberg, Germany). 3-amino-1,2,4-triazole (ATA), 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (cyclohexymide, =94% TLC from *Streptomyces griseus*), adenosine 5'-triphosphate disodium salt (Na_2ATP) grade II or molecular biology grade (for the Luciferase assay), bovine serum albumin (BSA) molecular biology grade, Catalase from bovine liver (lyophilized powder, =10,000 units/mg protein), Collagenase (from *Clostridium. histolyticum*), desoxycholic acid sodium salt 97%, diethyldithiocarbamate (DETC), diethylenetriamine (DETA), DL-dithiothreitol (DTT), endothelial cell growth supplement (ECGS), Glutathione reductase (from *Saccaromyces cerevisiae*), L-buthionine-[S,R]-sulfoximine (BSO), L-glutathione oxidized disodium salt (Na_2GSSG), L-glutathione reduced (GSH, min 99%), mercaptosuccinic acid (MS, 97%, Aldrich), N-[Tris(hydroxymethyl)methyl]glycine (Tricine), Neutral Red (3% solution), Nonidet P40 sobstitute (Fluka), ß-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (Na_4NADPH), *tert*-Butylhydroquinone (purum, =98.0% HPLC, Fluka), Tween 20 (Fluka), type I collagen, were purchased from **Sigma-Aldrich** (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). 2-mercaptoethanol and L-N5-(1-iminoethyl)-ornithine-dihydrochloride (L-NIO) were from **Strathmann Biotec** (Hannover, Germany).

Cytokines

The recombinant cytokines human interleukin 1β (IL-1β), rat interferon γ (IFN-γ) and human tumor necrosis factor α (TNF-α) were from Strathmann Biotec (Hannover, Germany).

Antibodies

All the antibodies in use for this study, the manufacturer and the respective dilutions are indicated in Tab. 1.

Table 1: Antibodies used in this study

Name (Producer)	**Description**	**Dil.**	**App.**	**incubation**
Human von Willebrand factor (vWF) (Sigma-Aldrich chemie GmbH, Steinheim, Germany)	Rabbit polyclonal antibody	1 : 50	IHC	45 min / RT
Bovine eNOS (Sigma-Aldrich)	Mouse monoclonal antibody from mouse ascites fluid (IgA)	1 : 50	IHC	45 min / RT
iNOS/NOS type II (BD Biosciences, Heidelberg, Germany)	Mouse monoclonal antibody	1 : 2500	WB	1 h / RT
Human Gamma glutamyl cyteine syntetase (GCS) (Dunn labortechnik GmbH, Asbach, Germany)	Rabbit polyclonal antibody	1 : 400	WB	2 h / RT
Human Beta-actin (BD Biosciences)	Mouse monoclonal antibody	1 : 5000	WB	1 h / RT
Nrf2 (H-300) (Santa Cruz Biotechnology, Heidelberg, Germany)	Rabbit polyclonal antibody	1 : 100	IF	1 h / RT
Universal DAKO Cytomation LSAB+System HRP (DAKO Cytomation Carpineria, CA, USA)	Biotinylated anti-rabbit, anti-mouse, anti-human immunoglobulins + Biotinylated link universal streptavidin HRP-	See manifacturer instructions	IHC	45 min / RT
HRP anti-mouse antiserum (BD Biosciences)	Goat polyclonal antibody	1 : 2500	WB	1 h / RT
HRP anti-rabbit antiserum (BD Biosciences)	Goat polyclonal antibody	1 : 5000	WB	1 h / RT
AlexaFluor 594 anti-rabbit antiserum (Invitrogen, Paisley, UK)	Goat polyclonal antibody	1: 250	IF	1 h / RT

Media and additives for cell culture

Cell culture dishes and sterile materials for cell culture were purchased from Greiner Labortechnik (Frickenhausen, Germany). Medium RPMI 1640, high quality fetal calf serum (FCS) endotoxin free, Sodium pyruvate solution (100X), MEM non essential amino acids, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 1M, Penicillin-Streptomycin Trypsin EDTA and Trypsin were purchased by PAA Laboratories (Pasching, Austria), L-alanyl-glutamine 100X (GlutaMax) was from Gibco (Gibco Laboratories, Eggenstein, Germany)
The complete medium for cell culture (later referred as 10% FCS-RPMI 1640 complete) was prepared by adding 5 ml of sodium pyruvate solution (100X), 5 ml of MEM non essential amino acids, 5 ml of HEPES 1M, 5 ml Penicillin-Streptomycin, 5 ml of glutaMAX, 50 ml of endotoxyn free FCS to 500 ml of RPMI 1640.

Nitric oxide donors

The NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-aminoethyl)-amino]-diazen-1-ium-1,2-diolate (DETA/NO) was kindly provided by Prof. Dr. K.-D. Kröncke (Institute for Biochemistry and Molecular Biology I, Heinrich-Heine-University, Düsseldorf) and synthesized as described (Hrabie *et al.*, 1993). The NO donor DETA/NO at 37°C in cell culture medium exhibits a half life of approximately 8 hours. Stock solutions of 50 mM were prepared immediately prior to the respective experiment and diluted to the end concentrations indicated with 10% FCS RPMI complete culture medium.

The stock solution of the NO donor S-nitrosocysteine (SNOC; 100 mM) was synthesized at 4°C immediately before use by mixing 192 µl of a CysHCl solution (7.04 mg CysHCl in 192 µl ddH_2O) and 192 µl of $NaNO_2$ solution (2.76 mg $NaNO_2$ in 192µl ddH_2O) with 8 µl HCl 1M and incubating on ice for 2 min. Then, 7 µl of NaOH were added to equilibrate the solution to neutral pH. The solution was the diluted with 10% FCS RPMI complete culture medium, and the The NO donor SNOC at 37°C in cell culture medium exhibits a half life of approximately 25 minutes. As control, the respective mother compounds were added where indicated.

CELL CULTURE AND EXPERIMENTAL METHODS

Animals

Male Wistar rats (about 30 days old and 150 g of body weight) were obtained from the university breeding facility. All animals received a standard diet and tap water *ad libitum*.

Isolation and culture of rat aorta endothelial cells

Isolation. The rat was scarified by CCl_4 asphyxia and abdominal aorta was explanted following standard surgical procedures. Then, aorta was washed in sterile PBS and cut transversally to obtain aortic rings. Rat aorta endothelial cells (EC) were isolated by outgrowth from aortic rings on a collagen type I matrix, as described (McGuire *et al.*, 1987; Suschek *et al.*, 1993). Briefly, aortic segments were placed on top of a collagen gel

(1.8 mg collagen/ml) in 24-well tissue culture plates and incubated in 20 % FCS RPMI 1640 complete, containing 100 µg endothelial cell growth supplement (ECGS)/ml in a humidified incubator at 37°C in a 95 % air/5 % CO_2 atmosphere for 4 to 6 days, depending on the degree of cellular outgrowth. Three days after, outgrowth of endothelial cells can be observed in the vicinity of the aortic ring (Fig. 6). The aortic ring has been then removed to avoid contamination with smooth muscle cells and fibroblasts, which have a slower growth rate. One or two days later, depending on the density of the culture, cells were detached with 0.25 % collagenase in Hank´s Balanced Salt Solution (HBSS) and after 5 min centrifugation at 500 g (1000 rpm) in Allegra 6KR Centrifuge (Beckman Coulter GmbH - Diagnostics, Krefeld, Germany), cells were transferred onto plastic culture dishes in 10 % FCS RPMI 1640 complete.
In a semi-confluent culture endothelial cells show a tri-polygonal morphology and form circular or net shaped colonies. After reaching confluence they present the typical cobblestone morphology.

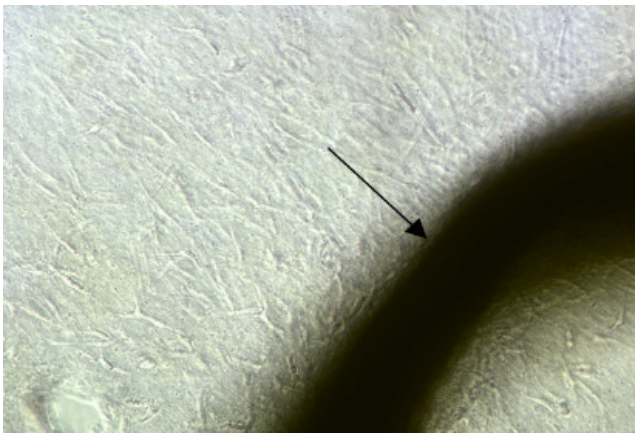


FIGURE 3. Isolation of rat aortic endothelial cells.

Aortic rings are implanted on a collagen matrix. After three days an outgrowth of endothelial cells can be observed in the vicinity of the explants (Photo by M. Lenzen).

Cell culture. Cells were subcultured for up to 10 passages. Removal from culture dishes for each passage was performed by treatment with 0.05 % trypsin/0.02 % EDTA in isotonic 0.9% NaCl for 2 min. Reaction was stopped by adding 10 % FCS RPMI 1640 complete. Cells were centrifuged at 500g (1000 rpm) in Allegra 6KR Centrifuge (Beckman Coulter GmbH - Diagnostics). Cells were then resuspended in 10 % FCS RPMI 1640. Cells were counted in Neubauer Chamber (0.0025 mm^2 x 0.1 mm depth; Brand, Wertheim, Germany) using Zeiss Axiovert 200 (Carl Zeiss MicroImaging GmbH, Cologne, Germany) and then seeded into plastic culture dishes. The number of cells seeded and the type of dish used is in the description of each experimental procedure.

Immunocytological characterization of cultured cells

Rat endothelial cells (EC) were characterized by using cross-reacting rabbit-anti-human-von Willebrand Factor (vWF) antiserum, the mouse monoclonal antibody anti-bovine eNOS. For signal detection the commercial kit Universal DAKO Cytomation Labeled streptavidin biotin (LSAB)+ kit, peroxidase and the liquid 3,3' aminobenzidine (DAB) chromogen (DAKO Cytomation, Carpinteria, CA, USA) were used, flowing the manufacturer instructions. As a control, cells were stained with the universal negative control mouse IgG1 or rabbit (DAKO Cytomation), as also indicated in the

manufacturer's instructions. Briefly, 10^4 cells were cultured in sterile glass Chamber-Tek slides for 24 hours. Cells were dried and fixed with acetone at -20°C for 10 minutes. To inhibit the endogenous peroxidase activity specimen were treated with 0,3% H_2O_2 for 5 min. Specimens were washed with TBS, (10 mM Tris, 100 mM NaCl) then incubated with a cross-reacting rabbit-anti-human-vWF antiserum (1:50) or with a mouse monoclonal anti-eNOS antibody (1:50) diluted in 1% BSA in TBS at room temperature for 45 minutes. Then specimens were washed extensively with TBS and incubated in with biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulin (DAKO Cytomation LSAB+kit) for 15 minutes at room temperature. After extensive washing TBS specimens are incubated with peroxidase-labeled streptavidin for 15 min. After washing, specimens were then incubated with the substrate-chromogen solution containing DAB. Counterstaining of the nuclei was performed by incubation with Haematoxylin for 30 sec. After washing in distilled H_2O, specimens were dehydrated incubating them in increasing concentration of ethanol (3 min each) and ending with xylene (5 min). Mounting was performed with Roti®-Histokitt (Roth, Karlsruhe, Deutschland) Specimen were analysed in Zeiss Axioplan (Carl Zeiss MicroImaging GmbH). Micrographs were taken using RT color photocamera (Diagnostic instruments Inc, Visiotron system GmbH, Puchheim, Germany)

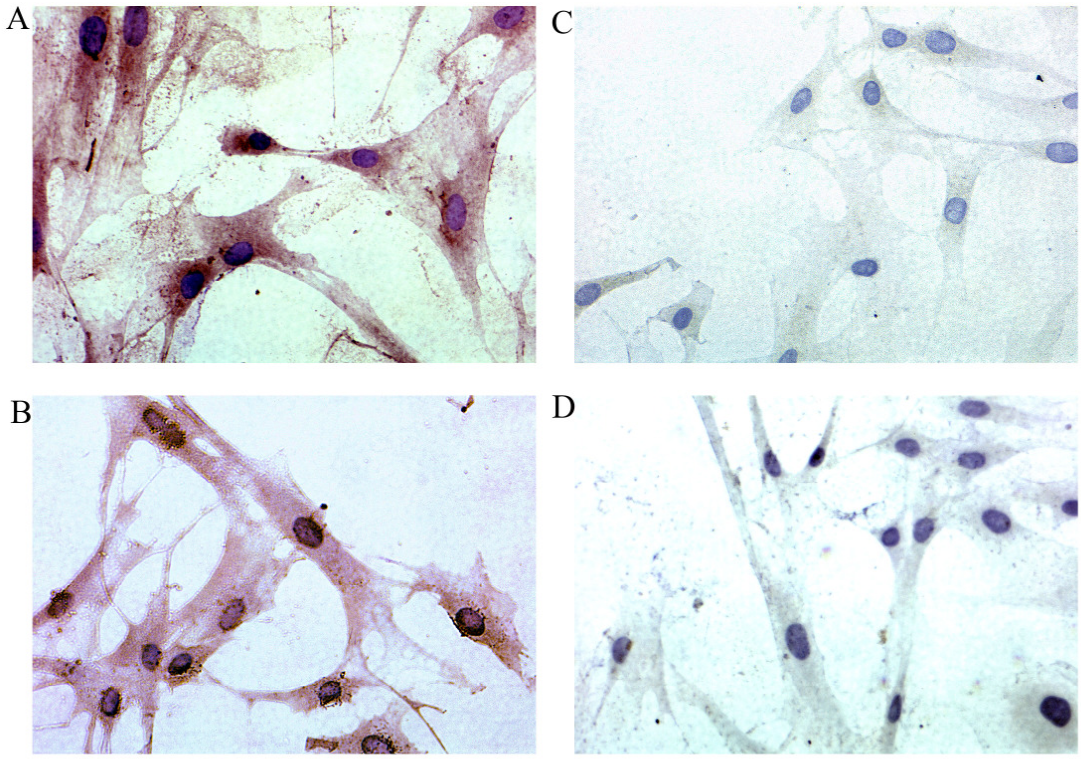


FIGURE 4. Immunecytological characterisation of endothelial cells.

*Freshly isolated endothelial cells from the Wistar rat aorta grown on Chamber-Tek slides for 24 hours were stained with antibody rabbit anti-human vWF antiserum and mouse monoclonal anti-eNOS, then conterstained with hematoxylin.. **A)** Anti-vW antibody. **B)** Universal negative control rabbit. **C)** Anti-eNOS antibody **D)** universal negative control mouse. Magnification: 40x*

Culture of A549/8 iNOS cells

The human alveolar epithelium-like A549/8 cells stably transfected with a 16kB fragment of the human iNOS promoter in front of a luciferase reporter gene and a neomycin resistance gene was a kind gift of Prof. Dr. H. Kleinert (Institut für Pharmakologie, Johannes Gutenberg-Universität Mainz) (de Vera *et al.*, 1996; Kleinert *et al.*, 1996). Cells were grown in 10% FCS RPMI complete supplemented with 1mg/ml Geneticin G418 and detached incubating them for 3 min with 0.05% trypsin-EDTA, using the procedure described for rat aortic EC. If cells were prepared used for experimental purpose, they were cultured in the absence of Geneticin G418.

Determination of cell viability by trypan blue exclusion assay

The viability of EC and of A549/8 iNOS cells was routinely controlled using the trypan blue exclusion assay; all the cells which exclude the dye are viable (Phillips *et al.*, 1957). Briefly, cells were stained for 5 min at RT with trypan blue solution (0.4% in 0.9% NaCl) and then counted in Neubauer Chamber (Brand) using Zeiss Axiovert 200 (Carl Zeiss MicroImaging GmbH).

Determination of cell viability by neutral red staining

Cells were incubated for 90 minutes with Neutral Red solution (1:100 dilution of a 3% solution, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), washed twice with PBS, dried completely and then lysed with 0.05M HCl in isopropanol. Then, 100 µl aliquot of cell lysate measured in double at 530 nm in microtiter plate reader (FLUOstar OPTIMA by BMG Labtech, Offenburg, Germany). Only live cells can be stained with neutral red and the extinctions are linearly correlated to the number of live cells (Finter, 1969).The number of live cells is expressed as percentage, calculated against the untreated control.

Detection of nuclear chromatin condensation and nuclear fragmentation by Hoechst staining

EC were cultured for 24 hours in 10% FCS RPMI 1640 complete in 12-well tissue culture plates ($5x10^5$ cell/well), in the absence or presence of the respective additives at the concentrations indicated. Then cells were washed with PBS and stained with Hoechst dye H33342 (5 µg/ml in PBS) for 5 minutes. Nuclei were visualized using Zeiss Axioplan fluorescence microscope (excitation 355 nm, emission: 465 nm; Carl Zeiss MicroImaging GmbH). Micrographs were taken using RT color photocamera (Diagnostic instruments Inc, Visiotron system GmbH).

Detection of DNA strand breaks by *in situ* nick translation

DNA strand breaks of cells grown on 8-well Chamber-Tek slides were visualized by the in situ nick-translation method (Fehsel *et al.*, 1991). After treating the cells for 24 hours with H_2O_2 or medium, cells were dried and fixed with acetone at -20ºC. Then, endogenous peroxidase activity was blocked with 0.3 % H_20_2 in methanol for 30 min. The reaction buffer contained dGTP, dATP, dCTP (3 µM each), 50 mM Tris-HCL, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT). The enzyme Kornberg polymerase (5 U/100 µl of reaction buffer) and 3 µM biotin-dUTP were added to the reaction buffer and the reaction was performed at for 20 min at RT. Slides were washed in PBS and then

incubated with biotin-labeled UTP. After 4 washes with PBS, specimens were incubated with peroxidase-labeled avidin. After 4 washes with PBS, the peroxidase activity was detected by a colorimetric reaction using DAB as substrate (DAKO Cytomation). Specimens were visualized using Zeiss Axioplan microscope (Carl Zeiss MicroImaging GmbH). Micrographs were taken using RT color photocamera (Diagnostic instruments Inc, Visiotron system GmbH).

Induction of endogenous high-output nitric oxide synthesis

Endogenous NO production under pro-inflammatory conditions was achieved by cytokine challenge with a mix of IL-1β (200 U/mL) and IFN-γ (100 U/mL) in the absence or in the presence of 500 U/ml TNF-α, which leads to nearly maximal iNOS enzyme activity. Inhibition of NOS activity was performed by adding L-NIO (0.5 mM).

Zinc supplementation, zinc depletion and measurement of zinc content by atomic adsorption spectrometry

Measurement of zinc content by atomic adsorption spectrometry. Aliquots of RPMI 1640, FCS (3 different batches), complete culture medium 10% FCS RPMI 1640 complete (freshly prepared or after EC culture) and PBS were analyzed to determine the zinc content using a flame atomic adsorption spectrophotometer (PE1100B, Perkin-Elmer LAS GmbH, Rodgau - Jügesheim, Germany). Measurements were performed by Ms Stemmans in collaboration with Dr. M. Falck (Zentralinstitut für Klinische Chemie und Laboratoriumsdiagnostik, Heinrich-Heine-University).

Zinc supplementation. A stock solution of 5 mM $ZnSO_4$, dissolved in physiologic solution (0.9 % NaCl) and stored at 4˚C, was diluted immediately before each experiment in culture medium to reach the concentrations indicated. The toxicity after 24 hours treatment was determined by neutral red staining.

Zinc depletion. To deplete EC of "labile" zinc, the membrane-permeable zinc-chelator N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; 3.3 µM) was added to the culture medium (containing 2.3±0.3 µM Zn^{2+}) in the absence or in the presence of DETA/NO or 6h after the cytokine-mix addition. As a control, an equimolar concentration of $ZnSO_4$ (3.3 µM) was added, as indicated. The toxicity after 24 hours treatment was determined by neutral red staining.
Alternatively, zinc-free medium was prepared by mixing FCS (containing 24,98±2.00 µM Zn^{2+}) with the ionic exchange resin Chelex 100 following the manufacturer instructions. Briefly, Chelex 100 (5 g/ 100 ml of high quality FCS) was added to the FCS and stirred for 1h at room temperature. The resin was then removed by filtration and the zinc-free FCS was then used for the preparation of the complete culture medium, as previously indicated. The toxicity was determined by neutral red staining.

Peroxide-induced cell death

Measurements were performed with EC from passages 2 through 8. EC were cultured for 24 hours in RPMI 1640/10% FCS in 12-well tissue culture plates ($5x10^5$ cell/well), in the absence or presence of the respective additives at the concentrations indicated. After

extensive wash oxidative stress-derived endothelial cell death was induced by a 24-hour incubation with H_2O_2 at the concentrations indicated and cell viability was assessed using neutral red. The effectiveness of the H_2O_2 treatment was controlled by adding the enzyme catalase (4000 U/ml) during the treatment. The cell viability is expressed as percentage of live cells, obtained calculating the ratio between the number of live cells after the treatment with H_2O_2 and the respective control cells. Control cells were grown in the same plate as the H_2O_2 treated cells and received the same pre-treatment as the H_2O_2-treated cells, but were not treated with H_2O_2. As a further control, in each plate a well containing untreated cells was always included.
Where indicated, the inhibitor of catalase, 3-amino-1,2,3-triazole (ATA, 0.5 mM), the inhibitor of glutathione peroxidase, 2-mercaptosuccinate (MS, 1 mM), dissolved in medium containing 20 mM HEPES, or the inhibitor of glutamate-cysteine ligase, L-buthionine sulfoximine (BSO, 0.05 mM) were added together with H_2O_2 (Chaudiere *et al.*, 1984; Feinstein *et al.*, 1964; Griffith *et al.*, 1979).

CONFOCAL MICROSCOPY

Zinquin staining

Endothelial cells ($5x10^4$ cells/well) cultured on 22 mm ∅-coverslips in 6-well plate for 24 hours were washed with PBS and incubated for 15 min with Zinquin (25 µM / PBS). Coverslips were then transferred in the observation plate, containing 1 ml PBS and incubated at 37˚C. The NO-donor SNOC (5 mM) fresh prepared or $ZnSO_4$ (100 µM or 200 µM, as indicated) were added were indicated. Fluorescence was monitored by confocal microscopy, by taking a picture every 30 sec. The micrographs here presented represent the results after 15 min incubation with the stimuli and were made using a Zeiss LSM 510 confocal laser scanning microscope and a Zeiss Plan Neofluar 40x/1.3 oil DIC objective. To excite Zinquin, the 364 nm line of an UV enterprise laser was used. Zinquin emission was recorded with a 375 nm beam splitter and a 385 nm long pass filter. Micrographs were taken with the expert technical help of Wiebke Wetzel (Institute of Biochemistry and Molecular Biology II, Heinrich-Heine-University)

Determination of Nrf2 translocation by confocal microscopy

Endothelial cells ($1x10^4$cells) were seeded on 22mm∅-coverslips and cultured for 24 hours in 1.5 ml complete medium in 6-well plate. Then, fresh medium alone or containing the indicate additives was added and cells were incubated for
1 hour. Then, cells were washed twice with warm (37˚C) PBS and then fixed in 4% formaldehyde in PBS for 20 min. Specimens were then washed 3 times with T-PBS (0.1 % Tween in PBS) for 5 min each, then blocked overnight in 5% BSA-T-PBS (blocking buffer). After 1 wash in T-PBS, specimens were incubated for 1 hour with rabbit anti-Nrf2 antiserum (1:100) diluted in blocking buffer. Specimen were then washed 5 times with T-PBS for 5 min each and then incubated with the secondary antibody, respectively goat anti-rabbit antibody conjugated with Alexafluor 535 (1:250) diluted in blocking buffer. After 1 wash in T-PBS, specimens were incubated with Hoechst dye (5 µg/ml) in T-PBS of for 5 min. After 3 additional washes in PBS, specimens were mounted using

2.5% (w/v) 1,4 diazabicyclo-[2.2.2]octane (DABCO, Sigma-Aldrich) dissolved in Mowiol (Sigma-Aldrich) and after 24 hours analysed under the Zeiss LSM 510 confocal laserscanning microscope and a Zeiss Plan Neofluar 40x/1.3 oil DIC objective. To excite Hoechst the 364 nm line of an UV enterprise laser was used. Hoechst emission was recorded with a BP 385-485 nm filter. The Alexa 594 was excited with a HeNe 543 nm laser using a UV/488/543/633 nm beam splitter. Fluorescence was recorded with a 560-615 nm band pass filter Micrographs were taken with the expert technical help of Wiebke Wetzel (Institute of Biochemistry and Molecular Biology II, Heinrich-Heine-University).

BIOCHEMICAL METHODS

Nitrite determination by Griess assay

After 24 hours of incubation with pro-inflammatory cytokines (as indicated in the figure legend) in the absence or in the presence of L-NIO (an inhibitor of NOS activity), the nitrite concentration in culture supernatants was determined using the diazotization reaction (Griess assay), as modified by Wood et al (Wood *et al.*, 1990) and $NaNO_2$ as standard. Briefly, the nitrite content of 100 µM aliquots of supernatants were measured in double in a microtiter plate by adding 50 µl of Griess solution I (1% sulfanilamide in 2.5% H_2PO_4). After 15 min of incubation, 50 µl of Griess solution II (1% N-1-Naphthylendiamin-dihydrochlorid in 2.5% H_2PO_4) were added. Then, 20 µl of methanol were added to each well, to eliminate any bubble. The formation of the pink color was measured at 540 nm in a microtiterplate reader FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany). Standard were prepared diluting a stock solution of 100 mM $NaNO_2$ in H_2O in 10% FCS RPMI 1640 complete, in a way that the standards were prepared exactly in the same way as the probes. The nitrite concentrations were normalized against the number of live cells measured by neutral red staining.

Total glutathione determination

Total cellular glutathione (reduced + oxidated) was determined enzymatically according to (Abdelmohsen *et al.*, 2003). Briefly, cells grown on 7 cm^2 dishes were scraped in 150 µl of ice-cold HCl (0.01 M) and sonicated on ice. Aliquots of the crude lysate were kept for protein determination according to bicinchoninic acid (BCA) assay (Pierce, Perbio Science Deutschland GmbH, Bonn, Germany) and following the manufacturer's instructions. Samples were diluted 1:5 in Tris 1 M pH 8. Standard were prepared diluting a stock solution of 2% BSA in H_2O in a buffer obtained diluting 1:5 the lysis buffer HCl 0.05 M in Tris 1 M pH 8, in a way that the standards were prepared exactly in the same way as the probes. Proteins in crude lysates were precipitated with 5% (w/v; final concentration) 5-sulfosalicylic acid by centrifugation at 10000 g for 20 min at 4ºC in Universal 30RF (Hettich Zentrifugen, Mülheim a.d. Ruhr, Germany). The total GSH concentration was determined kinetically measuring the formation of 5-thio-2-nitrobenzoic acid from 5,5'-dithionitrobenzoic acid in the presence of NADPH and glutathione reductase at 405 nm in a microtiter plate reader (FLUOstar OPTIMA, BMG Labtech.) The reaction mixture was prepared at 4°C in a minicentrifuge tube by addition of 40 µl of sample, 20 µl of DTNB stock solution (10 mM DTNB in 0.1M $NaHCO_3$) to

340µl of reaction buffer (1.5 mM NADPH, 0.1 mM EDTA, 0.2 M Tris pH 8). After vortexing, 100 µl of reaction mixture was added to the wells of a microtiterplate and 10 µl of enzyme-containing solution (2.99 U/ml in ddH_2O) were added. The reaction was followed by measuring the sample at 405 nm every 5 minutes for 30 minutes. Standard were prepared diluting in 0.01 HCl a fresh prepared stock solution of GSSG (10 mM Na_2GSSG in 0.01 M HCl). The same amount of 5-sulfosalicylic acid used to precipitate the proteins in crude lysates was also added to the standards, in a way that the standards were prepared exactly in the same way as the probes. The slopes of the curves (obtained by plotting the average absorbance values as a function of time) were plotted against the standard GSSG concentrations to obtain a standard curve. The GSH amount was calculated by multiplying for 2 the GSSG amount calculated. The amount of total glutathione was then normalized to protein contents and expressed as mmol GSH / mg protein.

Protein determination

Cell lysis. Endothelial cells cultured for 24 hours in a 6-well plate were washed 2 times with warm PBS, then 100 µl of RIPA lysis buffer (1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS in PBS) completed with a commercial cocktail of protease inhibitors, prepared following the manufacturer's instructions (Complete-mini protease inhibitors, Roche Diagnostic GmbH) was added to each well. Cells were scraped and the lysates were transferred in a pre-chilled microcentrifuge tube, sonicated on ice for 10 sec and centrifuged at 10000 g for 10 min at 4ºC in Universal 30RF (Hettich Zentrifugen). The lysates were aliquoted and stored at -20 ºC. Total protein concentration was determined by the Lowry assay using a commercial kit (DC Protein Assay, Bio-Rad), and following the manufacturer's instructions. Samples were diluted 1:4 in PBS. Standards were prepared diluting a stock solution of 2% BSA in H_2O in PBS, in a way that the standards were prepared exactly in the same way as the probes.

Western blot. The western blot mini chamber XCell SureLock and all materials were purchased from Invitrogen (Paisley, UK) and the manufacturer instructions were followed. Briefly, 30 µg of total cell lysate were mixed to NuPAGE LDS sample buffer and NuPAGE reducing Agent and heated for 10 min at 72 ºC. Samples were loaded in 7% NuPAGE Novex Tris/Acetate pre-cast gel and the runned for 1 hour at 150 V in Tris–Acetate SDS running buffer. The proteins were transferred on PVDF membrane Hybond P (Amersham Biosciences, Freiburg, Germany) for 1 hour at 30V constant in 10% Methanol 1x NuPAGE transfer buffer.
The transfer efficiency was controlled by staining the membrane with Ponceau S (0.2% in 3% TCA, Serva Electrophoresis GmbH, Heidelberg, Germany). After blocking overnight with 5% non fat dry milk (Bio-Rad, Munich, Germany) in T-TBS (0,1% Tween in TBS; TBS: 10 mM Tris, 100 mM NaCl), the membrane was incubated for 1 hour with 2.5 µg/ml of the polyclonal rabbit anti GCL (Dunn Labortechnik Gmbh, Asbach, Germany), washed for 1 hour in T-TBS (0.1% Tween in TBS) and then incubated with horseradish peroxidase conjugated goat anti-rabbit antibody (BD Biosciences, Heidelberg, Germany) diluted 1:5000 in blocking buffer. Alternatively, as a loading control, the membrane was incubated with mouse anti-human β-actin diluted 1:5000 and then with Horseradish peroxidase conjugated goat anti-mouse antibody (BD Biosciences).

The bands were visualized by autoradiography on Hyperfilm ECL (Amersham Biosciences) using SuperSignal West Pico Chemioluminescent Substrate (Pierce).

MOLECULAR BIOLOGY METHODS

Characterization of gene expression

Cell lysis. Cell lysis and homogenization was performed in 1% 2-mercaptoethanol in RLT lysis buffer using QIAschredder spin columns (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total cellular RNA was isolated by RNeasy Mini Kit and RNase-free DNase Set (Qiagen) following the manufacturer's protocols.

Real time RT-PCR. The RNA (0.5-1 µg) was reversed transcribed for 1 hours at 37°C in a GeneAmp® PCR System 9700 (Applied Biosystem, Foster City, CA, USA), using Omniscript RT Kit and primed both with oligo(dT) and with random octamers (purchased from Operon, Technologies, Huntsville, USA). The reaction was stopped by denaturing the enzyme at 95°C for 5 min.The cDNA (5 ng) or control RNA in triplicate was used as template for quantitative polymerase chain reaction (Q-PCR), performed using TaqMan® Universal PCR Master Mix in ABI PRISM 7900 (Applied Biosystem). Primers and probes were designed by and purchased from Applied Biosystem: *Mt1a* Rn00821759-g1), *Znt1* (Rn00575737-m1), *Gclc* (Rn00563101-m1), *Ho-1* (Rn00561387) 18srRNA (Eukaryotic 18S rRNA endogenous control VIC/TAMRA Probe, primer limited).
Alternatively, the template was amplified using SyBrgreen Master Mix (Qiagen). The primers were designed using Primer Express software 2.0 (Applied Biosystem), purchased from Operon Technologies and the sequences are described in Tab. 2. Primer efficiency and specificity was verified respectively by amplifying standard dilutions of a probe obtained by pooling all the samples and by melting curve analysis. Results were analyzed following established procedures (Muller *et al.*, 2002; Pfaffl *et al.*, 2002). Briefly, for each experiments triplicates of Ct values were sorted if the standard error was greater that 0.2 (SE) were discarded. The Ct values were averaged and calculation of folds induction was performed using the formula # 1 using $E_{target} = 2$ and $E_{18srRNA} = 1.8$. The standard error of fold induction was calculated using formula # 2.

 # 1

$$\frac{E_{target}^{(Ct_{ctrl} - Ct_{sample})}}{E_{18srRNA}^{(Ct_{ctrl} - Ct_{sample})}}$$

 # 2

$$SE = \frac{E_{target}^{(Ct_{ctrl} - Ct_{sample})}}{E_{18srRNA}^{(Ct_{ctrl} - Ct_{sample})}} \sqrt{(\ln(E_{target}) \cdot SE_{target})^2 (\ln(E_{18srRNA}) \cdot SE_{18srRNA})^2}$$

Gene silencing experiments

The siRNA sequences targeting *Nrf2* mRNA and *Mtf-1*mRNA were designed and synthesized by Qiagen, on the basis of the target sequences indicated in Tab 2. The negative control, a sequence not recognizing any eukaryotic mRNA, was purchased from Qiagen. The best silencing efficiency was obtained by incubation of $1.5x10^5$ cells per well of a 6-well plate with complexes formed of 5 nM siRNA and 9µl of HiPerfect transfection reagent (Qiagen) dissolved in 100 µl medium without additives. The positive control recognizing GAPDH was purchased from Ambion (Huntingdon, UK). SiRNA/HiPerfect transfection reagent complexes were prepared in medium without additives following the manufacturer instruction. For transfection, cells were seeded in 0.9 ml complete medium and then 100µl of complexes were added drop-wise onto the cells. Then, 24 hours after transfection, 1 ml of fresh complete medium was added. Then, 48-hours after transfection, medium was changed and stimuli such as DETA/NO 1 mM or $ZnSO_4$ 100 µM were added to the cells and incubated for 24 hours. For the analysis of silencing efficiency the expression of *Nrf2*, *Mtf-1*, *Gapdh* were determined by real time RT-PCR using 18srRNA as housekeeping gene (Tab. 2) and SyBrgreen (Qiagen), as described in the real time PCR session.

Determination of iNOS Promoter Activity by Luciferase Assay

A549/8 iNOS cells were treated for 6 hours as indicated and then lysed with Reporter Gene Assay Lysis Buffer (Roche Diagnistic GmbH). The cell lysate was transferred to a microcentrifuge tube and centrifuged at maximal speed for 2 min at 4 °C in Universal 30RF (Hettich Zentrifugen). The supernatant was transferred to a clean microfuge tube, quick frozen in liquid N_2, and stored at -80 °C until Luciferase assays was performed.
Luciferase assay was carried out in Luciferase assay buffer (30 mM tricine, 0.1 mM EDTA, 15 mM $MgSO_4$, 10 mM DTT) supplemented with 0.27 mM coenzyme-A (Applichem, Darmstadt, Germany) and 0.53 mM ATP and with 0.5 mM of the substrate D-luciferine (Applichem). 20 µl of cell lysate was assayed in double for luminescence using a microtiterplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). The light units (LU) of the luciferase assay were normalized by protein content and were calculated as relative iNOS-promoter activity given in percent as compared to the controls (=100 %).

STATISTICAL ANALYSIS

Data are presented as mean±SEM and were analysed using ANOVA followed by an appropriate post hoc multiple comparison test (Tukey or Student's T test).

Table 2: Real time PCR primers and siRNA sequences

Name	GeneBank Accession Number	Primer sequence	siRNA target sequence	siRNA duplex (sense, antisense)
MTF-1	XM_342919	5'-GAAAGTGGAGCAGGTGTACTTTACC-3' 5'-AGGAACACTGAGGCCAATCTG-3'	TCGGAATGTCCTGAAACGAAA	GGAAUGUCCUGAAACGAAAdTdT UUUCGUUUCAGGACAUUCCdGdA
Nrf2	NM_031789	5'-CAGTGACTCGGAAATGGAAGAG-3' 5'-AATGTGTTGGCTGTGCTTTAGG-3'	CCGGCATTTCACTGAACACAA	GGCAUUUCACUGAACACAA dTdT UUGUGUUCAGUGAAAUGCCdGdG
GAPDH	NM_017008	5'-ATG ACT CTA CCC ACG GCA AG-3' 5'-CTG GAA GAT GGT GAT GGG TT-3'	Commercial siRNA (sequence N/A)	
Negative Control	-	-	AATTCTCCGAACGTGTCACGT	UUCUCCGAACGUGUCACGUdT ACGUGACACGUUCGGAGAAdTdT
18s rRNA	X01117	5'-GCC GCT AGA GGT GAA ATT CTT G-3' 5'-CAT TCT TGG CAA ATG CTT TCG-3'	-	-

RESULTS

NITRIC OXIDE AND ZINC EXERT ANTIOXIDANT EFFECTS IN ENDOTHELIAL CELLS

Endothelial cells are cultured in the presence of H_2O_2 as a model of oxidative stress

Endothelial cells were treated with increasing concentrations of H_2O_2 for 24 hours, as a model of oxidative stress-mediated damage. The treatment resulted in concentration-dependent cell death with a half-maximal lethal dose (LD_{50}) of 1 mM H_2O_2 (Fig. 5). As a control the enzyme catalase, catalyzing the reduction of H_2O_2 to H_2O was added. In the presence of catalase the H_2O_2 treatment did not exert any toxicity (Fig.5).

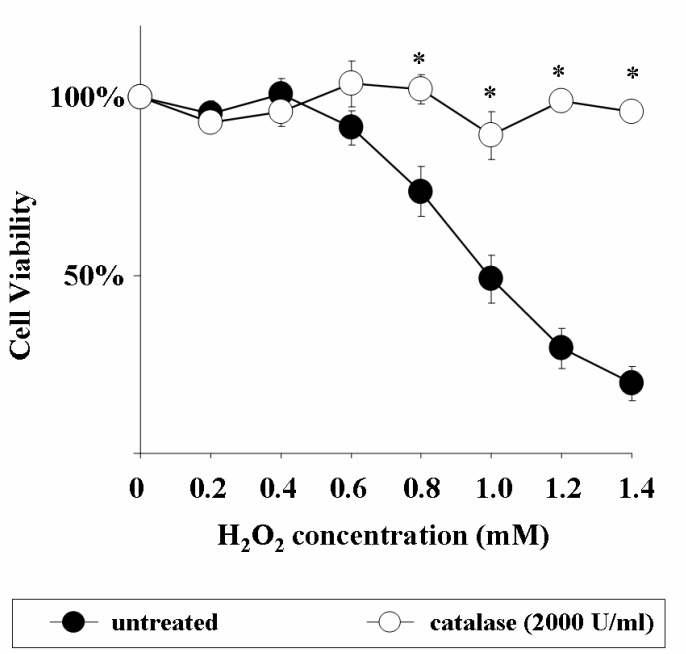


FIGURE 5. Endothelial cells are cultured in the presence of H_2O_2 as a model of oxidative stress

*Cell viability after 24 hours of incubation with the indicated concentrations of H_2O_2 was determined by neutral red staining (*p<0,05 n=3-6).The LD_{50} of the H_2O_2-treatment is 1 mM. As a control or in the enzyme catalase (2000 U/ml) was added during the H_2O_2 treatment.*

Cell death occurs via apoptosis, as revealed by staining H_2O_2-treated cells with Hoechst dye (Fig. 6B) or by performing *in situ* nick-translation (Fig. 6F).

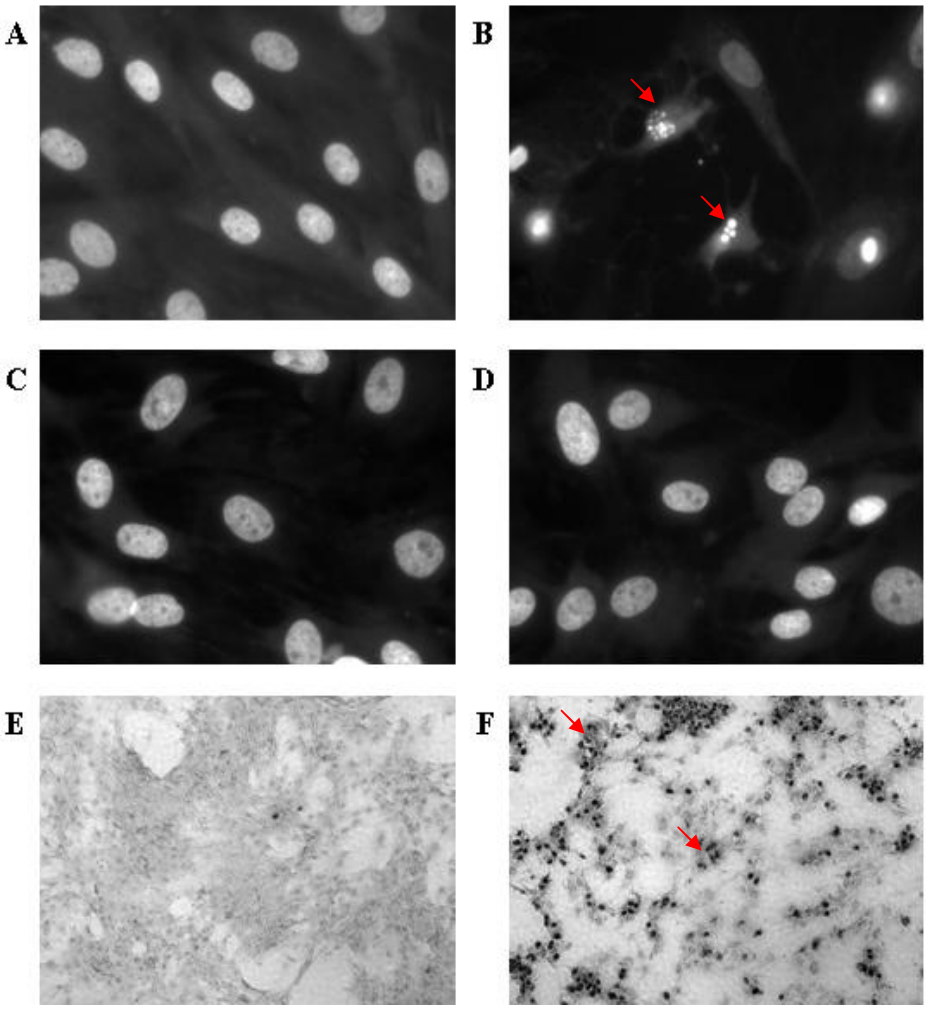


FIGURE 6. The treatment with H_2O_2 induces apoptotic cell death

A, B, C, D) Hoechst staining. *EC were treated with 1 mM H_2O_2 for 24 hours and then stained with Hoechst dye. After incubation with H_2O_2, pyknotic and shrunken cells with condensed or fragmented chromatin/nuclei are detected by Hoest staining and evidenced by the arrows (B). If cells were pre treated with 1 mM DETA/NO (C) or with 100 µM $ZnSO_4$ (D), no nuclear fragmentation is detected. (A)Untreated cells. Magnification: 650x.*

E,F) In situ nick translation. *EC seeded on Chamber-Tek slides were treated with medium only (E) or with medium containing 1 mM H_2O_2 for 24 hours (F). Then, in situ nick translation was performed. DNA strand brakes are detected in H_2O_2-treated cells through a colorimetric reaction and appear like black spots (see arrows). Magnification:40x*

Activation of iNOS in rat aortic endothelial cells

Endothelial cells were incubated with different combinations of pro-inflammatory cytokines (200 U/ml hIL-1β, 500 U/ml, hTNF-α and 100 U/ml rIFN-γ) for 24 hours. The nitrite accumulation in culture supernatant was measured as a marker of endothelial activation, of iNOS expression and of high-output NO production (Hrabie *et al.*, 2000). As also previously shown (Suschek *et al.*, 1994; Suschek *et al.*, 1993), nitrite accumulates in the culture media after addition of hIL-1β (Fig.7A, lane 3).

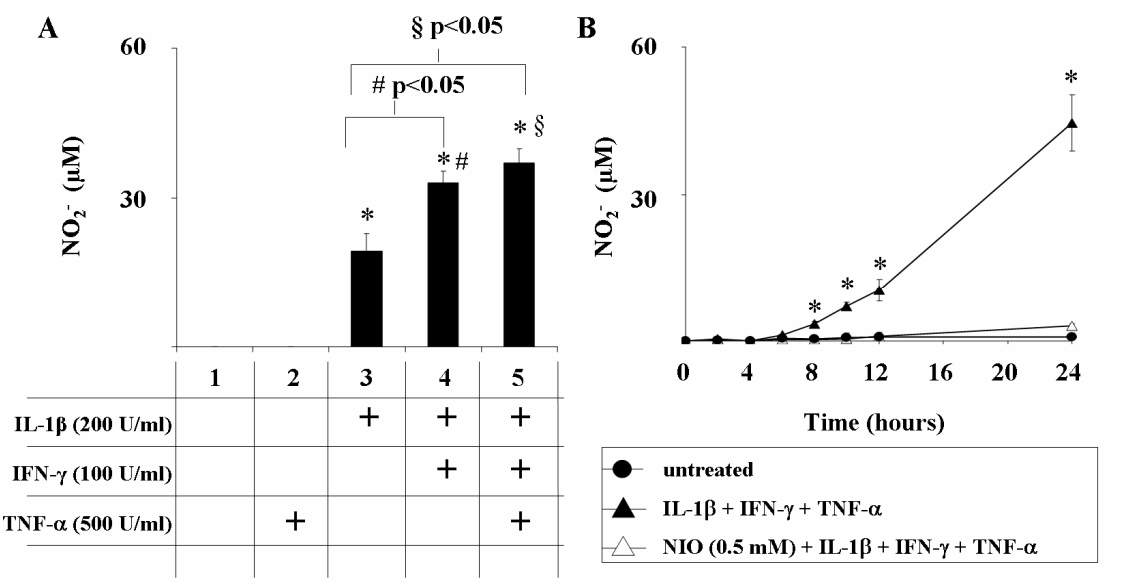


FIGURE 7. Activation of iNOS in rat aortic endothelial cells.

A) *EC were incubated with different combinations of pro-inflammatory cytokines (200 U/ml hIL-1β, 500 U/ml, hTNF-α and 100 U/ml rIFN-γ for 24 hours, as indicated. Nitrite accumulation in culture supernatant was measured by the Griess reaction as a marker of endothelial activation. Concentration was normalised against the number of live cell measured by neutral red staining.* ***B)*** *EC were incubated with pro-inflammatory cytokines (200 U/ml hIL-1β and 100 U/ml rIFN-γ). At the times indicated, a 100 μl aliquot of the supernatant was taken and 100 μl of fresh medium was added. The nitrite concentration was measured by the Griess reaction and normalised for the number of live cells, measured by neutral red stainig at the end of the experiment. Values represent the mean±SEM. (n=6; *p<0.05)*

The accumulation of nitrite in the supernatants indicates that the treatment with pro-inflammatory cytokines produces EC activation. The nitrite accumulation is already measurable after 8 hours of incubation.

The presence of rIFN-γ and or hTNF-α exerts a synergic effect on hIL-1β-induced increase in nitrite accumulation (Fig.7A, lane 4, 5). The kinetics of iNOS activation was also studied by collecting aliquots of the medium at different time-points during the 24-hour treatment with cytokines (Fig. 7B). An increase of nitrite concentration in the

surnatants was observed starting from the aliquot collected after 8 hours of incubation (Fig. 7B).

Nitric oxide protects endothelial cells against oxidative stress

To verify that NO protects endothelial cells against H_2O_2-induced cell death, endothelial cell cultures were pre-treated for 24 hours with the NO donor DETA/NO (Fig.8A) or with pro-inflammatory cytokines (Fig.8B) or as a control with medium only, and then were treated with H_2O_2.

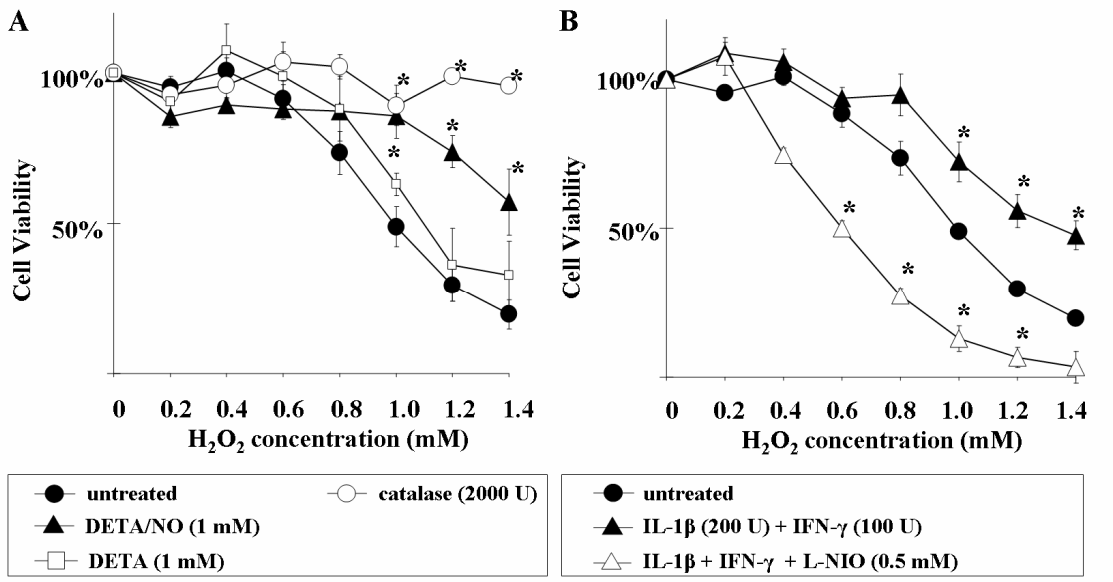


FIGURE 8. Nitric oxide protects endothelial cells against oxidative stress.

*EC were cultured for 24 hours in the absence or in the presence of the indicated additives and after extensive washing treated for 24 hours with 1 mM H_2O_2. Cell viability was determined by neutral red staining. Values represent the mean±SEM. (n=3-6; *p<0.05).*
Cells pre-treated with DETA/NO or with pro-inflammatory cytokines are protected against peroxide-induced toxicity.

Cells pre-treated with DETA/NO (1 mM) or with pro-inflammatory cytokines were fully protected against the H_2O_2-induced toxicity (Fig. 8 A,B). Nuclei of cells pre-treated with DETA/NO and subsequently treated with H_2O_2 did not show any morphological alteration (Fig. 6C). Control treatment with the mother substance DETA (1 mM) did not show any protective effect (Fig. 8A). The inhibition of cytokines-induced NO synthesis by addition of the NOS-specific inhibitor L-NIO (0.5 mM) completely reversed the protective effects, thus underlining that the cytokine-induced protection fully depends on endogenous NO-synthesis (Fig. 8B).

These results show that the pre-incubation of endothelial cells with NO or pro-inflammatory cytokines exerts protective activity against oxidative stress.

Effects of zinc supplementation and depletion on endothelial cells

To study the effects of zinc on endothelial cells, the culture medium was supplemented with $ZnSO_4$ dissolved in physiologic solution (0.9% NaCl). Endothelial cells show high tolerance to a 24-hour treatment with zinc; in fact the LD_{50} resulted 0.55 mM (Fig.9A).

To verify the effects of zinc depletion, cells were treated for 24 hours with a non toxic concentration of the membrane-permeable zinc chelator N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; K_d for Zn^{2+}-TPEN is 6.3×10^{-16} M at pH 7.6) (Cousins *et al.*, 2003; Kimura *et al.*, 2003). The concentration in use (3.3 µM) completely removed zinc from the culture medium, which contains 2,3±0,3 µM zinc, as determined by atomic adsorption spectrometry, but did not show any toxicity after 24-hour incubation, as determined by neutral red staining (Fig. 9B). Alternatively, zinc depletion was performed by culturing the cells for three days in zinc-free-medium.

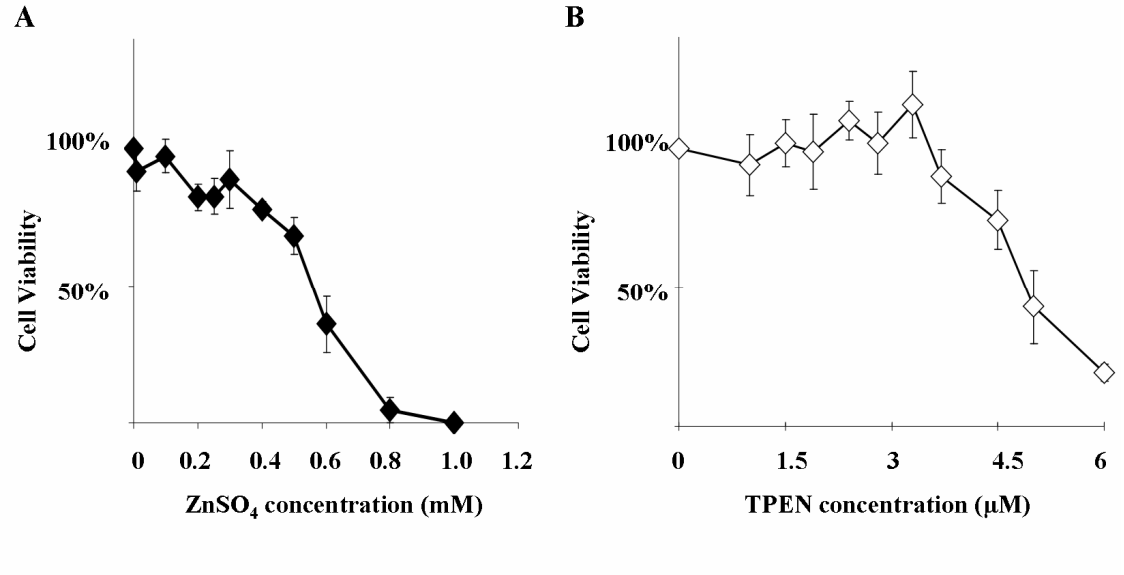


FIGURE 9. The effects of zinc supplementation and zinc depletion

A) *EC were cultured for 24 hours in the presence of $ZnSO_4$ at the concentrations indicated. Cell viability was detected by neutral red staining (*p<0,05 n=3-6). The non toxic concentration of 100 µM was chosen.* ***B)*** *EC were cultured for 24 hours in the presence of TPEN at the concentrations indicated. Cell viability was detected by neutral red staining (*p<0,05 n=3-6) The non toxic concentration of 3.3 µM was chosen.*

In this study the non toxic concentration of 100 µM $ZnSO_4$, 200 µM $ZnSO_4$, and 3.3 µM TPEN were chosen.

Zinc protects endothelial cells against oxidative stress.

The antioxidant effects exerted by zinc on endothelial cells were verified by analysing the effects of zinc supplementation or depletion on peroxide-induced toxicity.

Zinc supplementation was performed by addition of $ZnSO_4$ at the concentrations indicated. Cells pre-treated with 100 µM or 200 µM $ZnSO_4$ were significantly resistant to peroxide-induced toxicity (in untreated cells the LD_{50} = 1 mM H_2O_2; in cell pre-treated with 100 µM or 200 µM $ZnSO_4$ the LD_{50}> 1.5 mM H_2O_2, Fig. 10).

Zinc depletion was performed by addition of 3.3 µM TPEN. Cell pre-treated with TPEN showed an increased susceptibility to peroxide-induced toxicity (LD_{50} = 0.4 mM, Fig. 10). Alternatively, zinc depletion was performed by culturing the cells for three days in zinc-free medium. If cells were cultured in zinc-free medium and then treated with H_2O_2 for 24 hours, an increase of peroxide-induced toxicity versus the control is observed (LD_{50} = 0.6 mM, Fig. 10).

Therefore zinc supplementation protects endothelial cells against peroxide-induced toxicity, whereas zinc deprivation increases the rate of cell death.

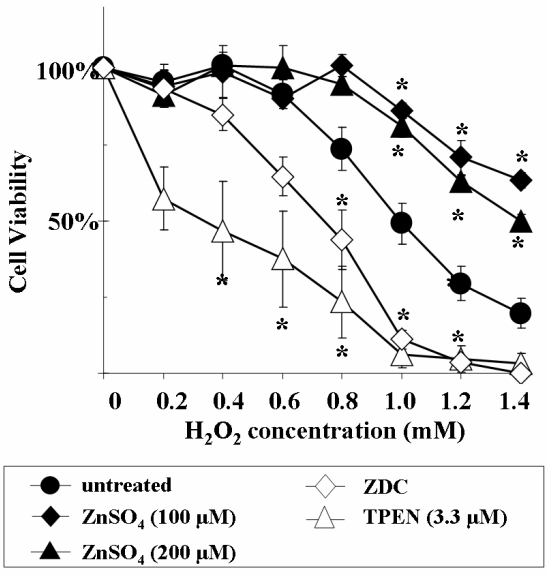


FIGURE 10. Zinc protects endothelial cells against oxidative stress

*EC were cultured for 24 hours in the absence or in the presence of the indicated additives and after extensive washing treated for 24 hours with 1 mM H_2O_2. Cell viability was determined by neutral red staining. Values represent the mean±SEM. (n=3-6; *p<0.05).*
ZDC = zinc-depleted cells, cell cultured in zinc free medium
Cell pre-incubated with zinc are fully protected against H_2O_2-mediated toxicity. Zinc-depleted cells are more susceptible to peroxide-induced toxicity

Nitric oxide induces zinc redistribution from zinc clusters.

The NO-mediated intracellular zinc redistribution was studied by staining the endothelial cells with Zinquin, a Zn^{2+}-specific fluorophore that forms fluorescent zinquin-Zn^{2+} complexes in either 1:1 or 2:1 ratios (K_d of 370 ± 60 nM and 850 ± 160 nM, respectively)(Kimura *et al.*, 2003). Results are shown in Fig. 11.

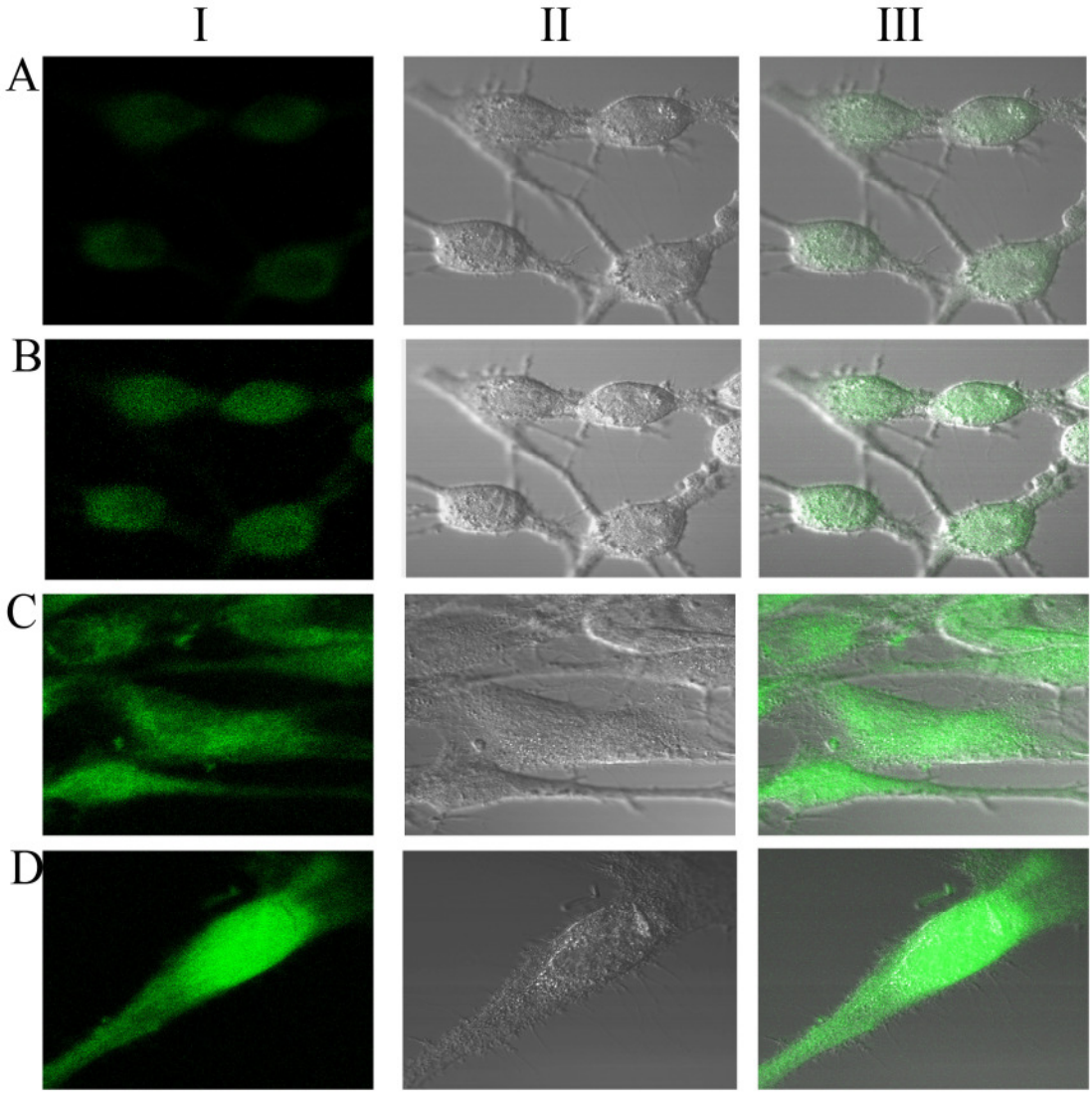


FIGURE 11. NO increases labile zinc in endothelial cells.

EC were cultured for 24 hours, after washing the cells were incubated with Zinquin for 15 min in PBS. Then, cell were washed again and incubated with ***A)*** *medium* ***B)*** *5 mM SNOC* ***C)*** *100 µM $ZnSO_4$* ***D)*** *200 µM $ZnSO_4$. After 15 min cells were analysed by LSM laser scanning microscopy. After addition of the NO donor SNOC, as shown in panel B an increase of fluorescence vs. the control (panel A) is observed in endothelial cells. If medium is supplemented with 100 µM $ZnSO_4$ (panel C) or 200 µM $ZnSO_4$ (panel D), an increase of fluorescent is also observed.*
After the NO-treatment an increase of zinc-specific fluorescence is measured, indicating that NO induces zinc redistribution in endothelial cells

An increase of fluorescence was observed after the addition of a NO donor such as SNOC (t1/2= ~25 min), showing that NO increases labile zinc in endothelial cells (Fig.11 B). As a control, the increase of intracellular labile zinc concentration after zinc addition in the medium is also showed, (Fig. 11C, D).

These results show that NO increases the intracellular labile zinc concentration in endothelial cells, thus they prove that NO produces zinc redistribution from zinc clusters within the cell.

Nitric oxide protects endothelial cells in a zinc-dependent fashion

Since NO induces zinc redistribution and both NO and zinc protect endothelial cells against peroxide-induced toxicity, an interrelation between the NO and zinc-mediated protective pathways might exist. Thus, the next step was to determine if zinc played a role in the NO-induced protection against H_2O_2-induced toxicity.

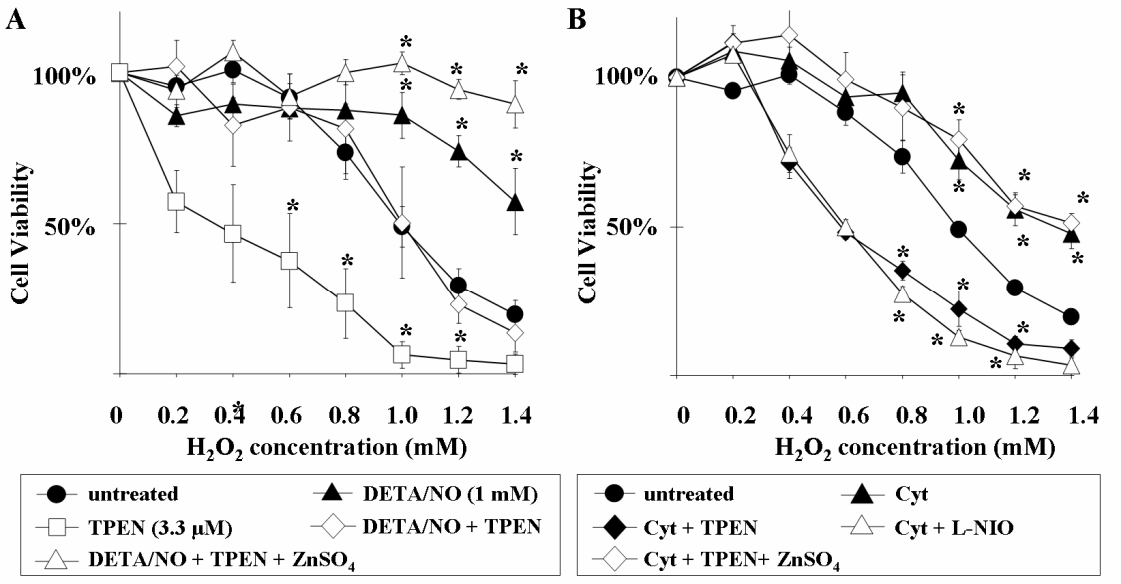


FIGURE 12. NO protects endothelial cells in a zinc-dependent fashion.

A) *EC were cultured for 24 hours in the absence or in the presence of the indicated additives and after extensive washing treated for 24 hours with 1 mM H_2O_2. Cell viability was determined by neutral red staining. Values represent the mean±SEM. (n=3-6; *p<0.05).* ***B)*** *EC were pre-activated for 6h with the cytokine mix indicated in the absence or in the presence of the NOS inhibitor L-NIO. Then the specific zinc chelator TPEN (3.3µM) in the absence or in the presence of equimolar concentration of zinc was added. 24 hours after the addition of cytokines, the cells were washed and then treated for 24 hours with 1 mM H_2O_2. Cell viability was determined by neutral red staining. Values represent the mean±SEM. (n=3-6; *p<0.05).*
The presence of TPEN blocks the protective activity of DETA/ NO and endogenous NO-synthesis, indicating that the NO-mediated protection is zinc dependent.

Endothelial cell cultures were incubated with DETA/NO in the presence of the membrane-permeable zinc chelator TPEN. As already shown in Fig.8, cells pre-incubated with the NO donor DETA/NO (Fig. 8A and Fig.12A) or with pro-inflammatory cytokines (Fig. 8B and Fig.12B) are protected against peroxide-induced toxicity. Pre-incubation of the cells with DETA/NO or cytokines in the presence of TPEN failed to protect endothelial cells against H_2O_2 (Fig. 12A, B). The titration of TPEN with 3.3 µM $ZnSO_4$ restored the NO-mediated protection (Fig. 12A, B), showing the specificity of the TPEN-induced effects.

This data show that zinc depletion inhibits the NO-mediated protective effects, indicating that zinc participates in the antioxidant activity of NO.

Nitric oxide and zinc protect endothelial cells via gene expression

Next the mechanism of NO- and zinc-induced protection was investigated. The addition of the protein-synthesis inhibitor cycloheximide during the pre-treatment with DETA/NO or $ZnSO_4$ completely abolished both the NO-mediated and the zinc mediated protection, indicating that the protective effects of both molecules depend on the activation of gene expression (Fig. 13).

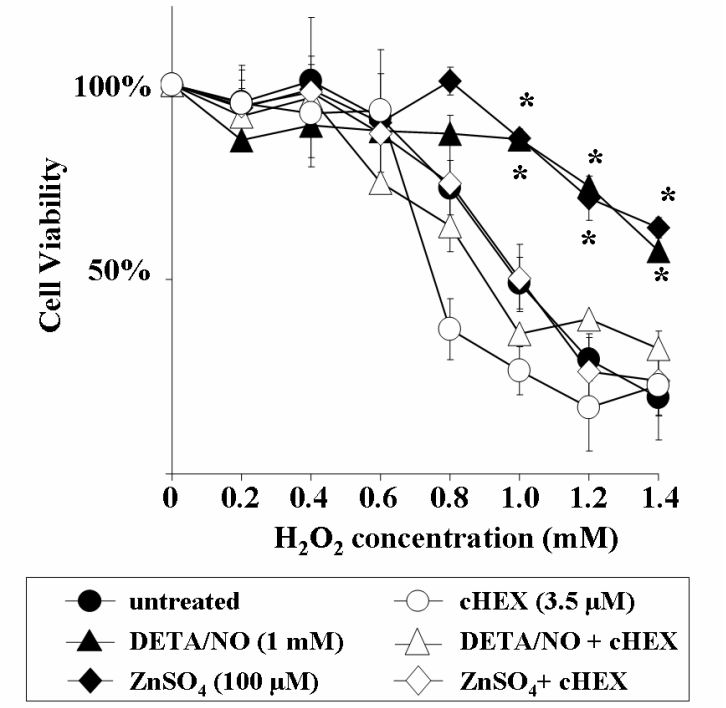


FIGURE 13. Cyclohexymide inhibits both the NO- and the zinc-mediated protection against H_2O_2.

*EC were cultured for 24 hours with DETA/NO (1 mM) or with $ZnSO_4$ (100 µM) in the absence or in the presence of the protein synthesis inhibitor cyclohexymide (cHEX) and after extensive washing treated for 24 hours with 1 mM H_2O_2. Cell viability was determined by neutral red staining. Values represent the mean±SEM. (n=3-6; *p<0.05). The inhibition of protein synthesis blocks the protective activity of both NO and zinc, indicating that the protective effects of both molecules depend on the activation of gene expression.*

Glutathione peroxidase plays a central role in nitric oxide-mediated antioxidant activity

The role exerted by the enzymes catalase and glutathione peroxidase in the NO- and zinc-mediated cyto-protective activity was also examined here. Both enzymes catalyze the reduction of H_2O_2 within eukaryotic cells

The treatment with H_2O_2 was performed in the presence of non toxic concentrations of the catalase inhibitor 3'-aminotriazole (ATA, 0.5 mM) or the glutathione peroxidase inhibitor β-mercaptosuccinate (MS, 1 mM).

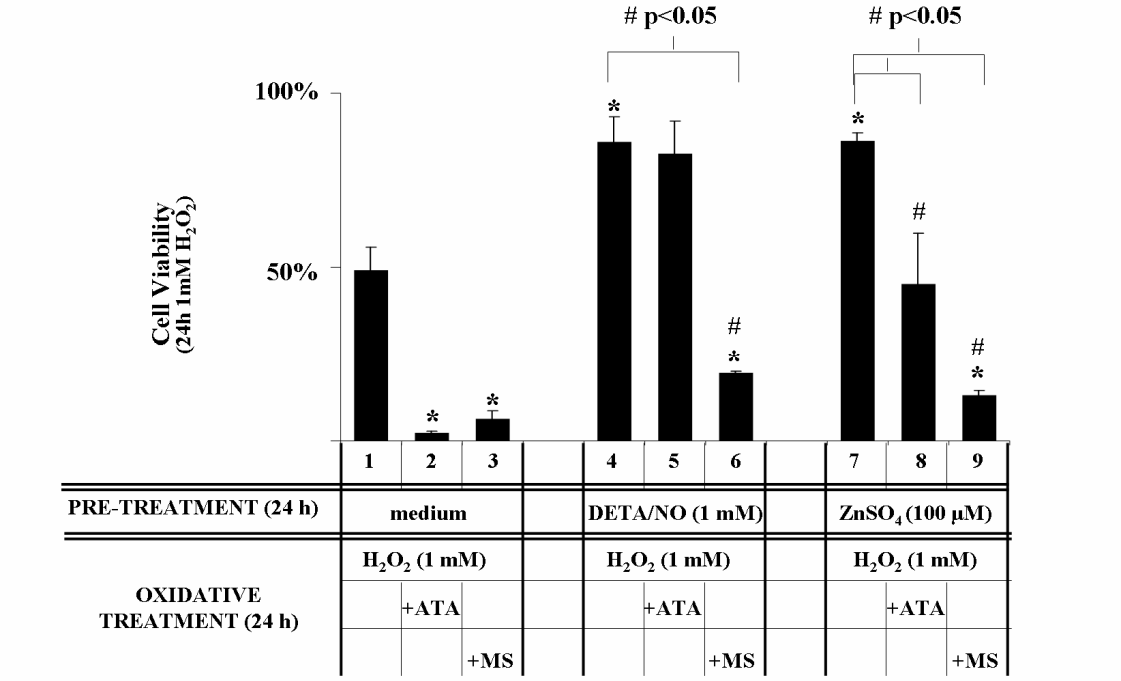


FIGURE 14. The GSH redox cycle plays a central role in the NO-mediated as well as in the zinc-mediated protection against H_2O_2.

*EC were cultured for 24 hours in complete medium (lane 1,2,3) or in the presence of 1 mM DETA/NO (lane 4,5,6) or 100 µM $ZnSO_4$ (lane 7,8,9) and after extensive washing treated for 24 hours with 1 mM H_2O_2 alone (lane 1,4,7) or in the presence of the inhibitor of catalase 3-amino-1,2,4-triazole (ATA, 0.5 mM; lane 2,5,8), or the inhibitor of glutathione peroxidase β–mercaptosuccinate (MS, 1 mM; lane 3,6,9). Cell viability was determined by neutral red staining. Values represent the mean±SEM (n=6; * p<0.05 compared to the control (Tukey test); # p<0.05 comparison (T-test) between the series indicated).*
The inhibition of glutathione peroxidase completely blocks both the NO- and zinc-mediated cyto-protective activity indicating that the GSH redox cycle plays a central role in the effects induced by both molecules.

The presence of both inhibitors significantly increased the toxicity of the H_2O_2 treatment (Fig. 14), showing the effectiveness of the inhibition. If endothelial cells were pre-

incubated with DETA/NO, only the inhibitor of glutathione peroxidase (MS) was able to abolish the NO-mediated protection, whereas the inhibitor of catalase did not exert any effect (Fig. 14). In cells pre-treated with $ZnSO_4$ the addition of both the inhibitor of catalase (ATA) and the inhibitor of glutathione peroxidase (MS) significantly impeded the zinc-mediated protective activity; but MS was the most effective (Fig. 14).

Thus, the inhibition of glutathione peroxidase completely blocks both the NO- and zinc-mediated cytoprotective activities, indicating that the GSH redox cycle plays a central role in the protection induced by both molecules

Exogenously applied nitric oxide increases the *de novo* synthesis of GSH.

Since GSH is an essential cofactor of glutathione peroxidase, the activity of this enzyme strictly depends on the cellular GSH levels, which are determined by the rate of GSH *de novo* synthesis and consumption. The enzyme glutamate-cysteine ligase (GCL) catalyses the rate limiting step of GSH *de novo* synthesis and consists of two

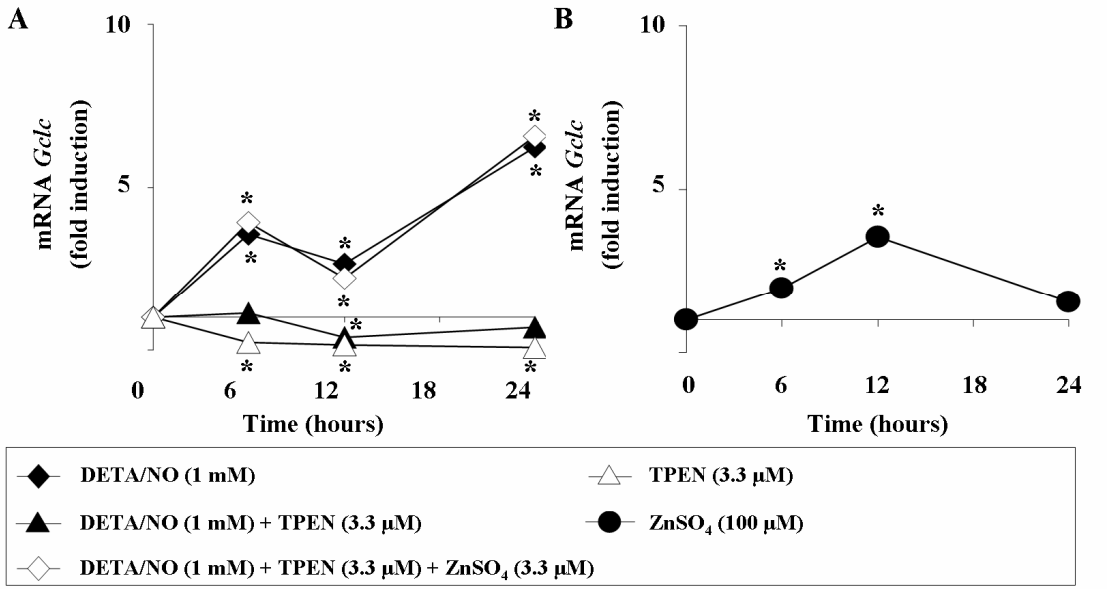
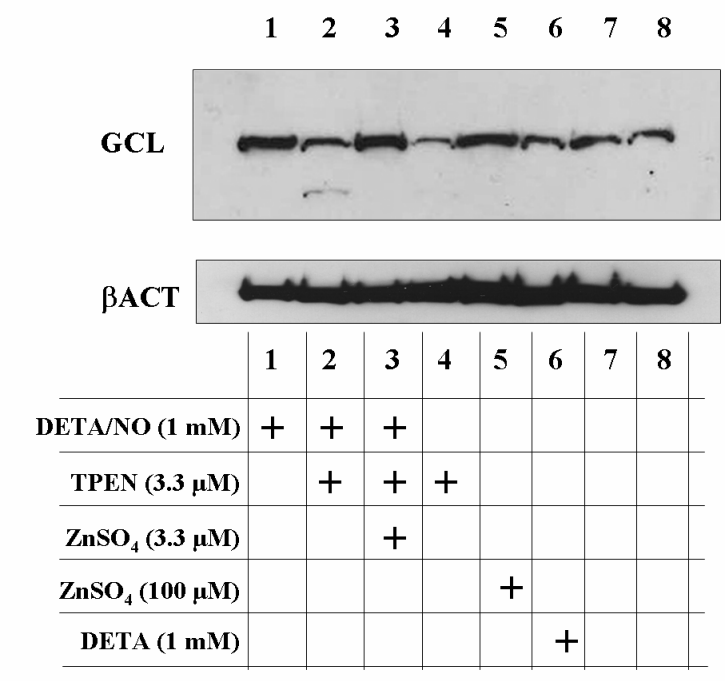


FIGURE 15. The NO-mediated induction of Gclc is zinc dependent.

*EC were cultured for 24 hours in the absence or in the presence of the additives indicated. At the time-points indicated total mRNA was extracted and the expression of Gclc was studied by real time RT-PCR using the TaqMan technology. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. Statistical analysis was performed by randomization test (n=3; *p<0.05).*
Both NO and zinc induces the expression of Gclc; the addition of TPEN blocks the NO-mediated response, indicating that NO induces Gclc in a zinc-dependent fashion.

transcriptionally regulated subunits: a catalytically active subunit (GCLc) and a regulatory subunit (GCLr).

The mRNA levels of *Gclc*[1] were determined by qRT-PCR to examine whether the expression of GCL is modified after challenge with NO, with zinc, or during zinc depletion. A 24-hour incubation with DETA/NO increases endothelial expression of *Gclc* with a maximal expressional peak at 24 hours (Fig. 15A *Gclc* mRNA; Fig. 16, lane 1 GCL protein). In addition, zinc depletion by TPEN induces a strong down-regulation of *Gclc* mRNA (Fig. 15A), as well as protein expression (Fig. 16, lane 4). Interestingly, the addition of TPEN during the treatment with DETA/NO fully blocked the NO-induced up-regulation of *Gclc* mRNA (Fig. 15A) as well as GCL protein (Fig.16, lane 2).



	1	2	3	4	5	6	7	8
DETA/NO (1 mM)	+	+	+					
TPEN (3.3 μM)		+	+	+				
$ZnSO_4$ (3.3 μM)			+					
$ZnSO_4$ (100 μM)					+			
DETA (1 mM)						+		

FIGURE 16. The treatment with DETA/NO and $ZnSO_4$ increases the expression of GCL in a zinc-dependent fashion.

EC were incubated for 24 hours with or without the indicated additives. Then 30 μg of total protein extract was separated and analyzed by western blot using anti GCL antibody and anți β–actin antibody as a loading control.

[1] Gene symbols are indicated in italics and protein symbols are represented in standard fonts, as indicated in the "Guidelines for Human Gene Nomenclature" (Wain *et al*., 2002).

If TPEN was counterbalanced by the addition of an equimolar concentration of $ZnSO_4$ (3.3 μM), the NO-induced response was fully restored (Fig.15A; Fig. 16, lane 3). On the contrary, zinc supplementation increases the expression of *Gclc* (Fig. 15B) and the production of GCL (Fig. 16, lane 5). The induction kinetics of *Gclc* mRNA after NO and zinc supplementation are clearly different, NO showing an earlier and longer lasting response.

These data show that NO induces the expression of GCL in a zinc dependent fashion.

Nitric oxide and zinc increase total cellular GSH levels

Next, the effect of exogenously applied NO and "labile" zinc depletion on the activity of GCL was examined, by measuring total cellular GSH (oxidized + reduced) levels. A 24-hour incubation with DETA/NO increases the total cellular GSH levels by 2-folds (vs. untreated cells) and the mother substance DETA did not exert any effect (Fig. 17, lane 2,3). Zinc depletion, obtained by the addition of TPEN alone, resulted in a significant decrease of total GSH levels (Fig. 17, lane 6). The addition of TPEN in the presence of DETA/NO completely abolished the NO-induced increase of GSH (Fig. 17, lane 4). If TPEN was counterbalanced by equimolar concentrations of $ZnSO_4$, the GSH increase was restored (Fig. 17, lane 5). This indicates that NO increases the GSH levels of the cells in a zinc-dependent fashion.

On the other hand, the addition of $ZnSO_4$ increases the GSH levels of the cells (Fig. 17, lane 8).

To verify if NO and zinc increase the total GSH levels of the cells via inducing the expression of GCL, the activity of GCL was inhibited by adding a non-toxic concentration of the specific inhibitor buthionine sulfoximine (BSO, 50 μM). The inhibition of GCL by BSO produces a strong decrease of the GSH levels, even in the presence of NO and zinc, confirming the central role played by GCL in controlling the GSH levels of the cells under study.

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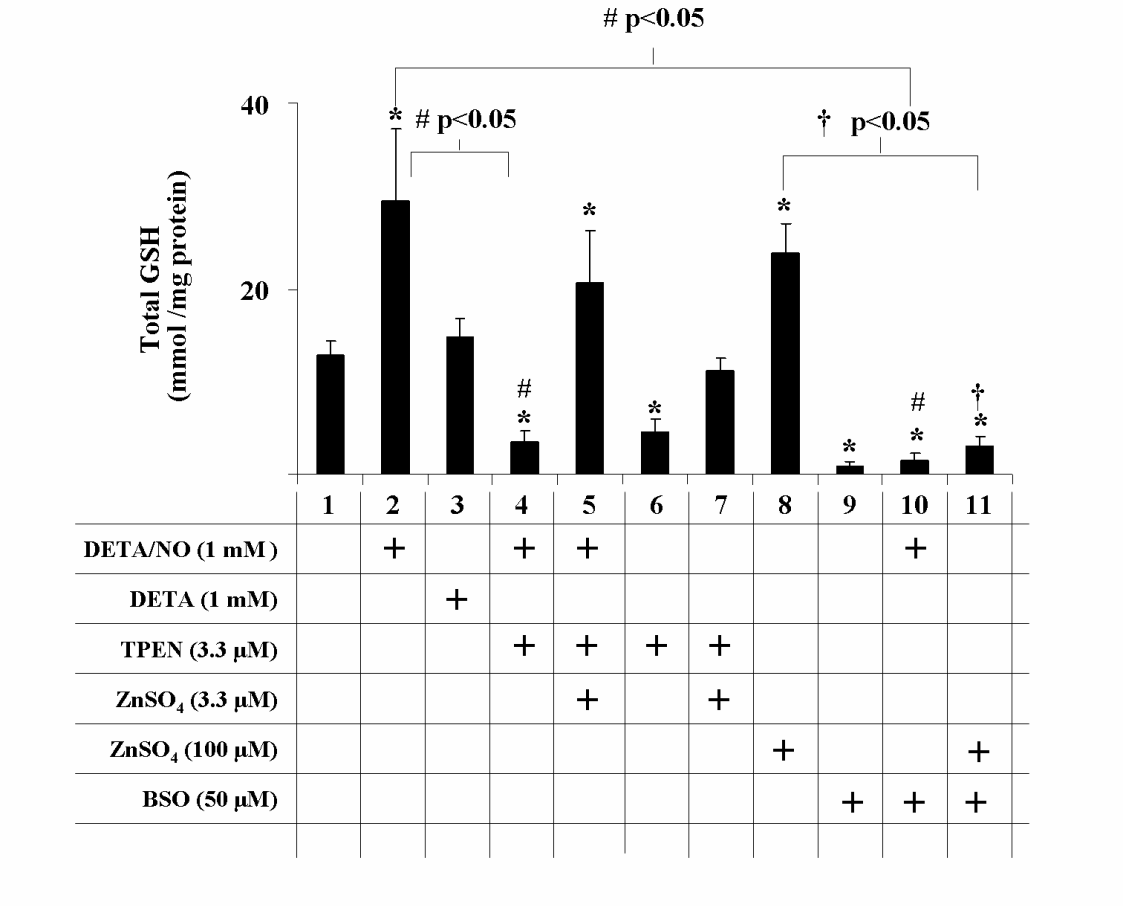

FIGURE 17. The treatment with DETA/NO and $ZnSO_4$ increases total cellular GSH levels; the NO-mediated increase of total GSH is zinc dependent.

*EC were cultured for 24 hours in the absence (lane 1) or in the presence of the indicated additives. Total GSH was assayed in cell lysates by the GSH reductase recycling method and expressed as mmol GSH per mg protein. Values represent the mean±SEM (n=5; * p<0.05* p<0.05 compared to the control by using the Tukey test-; #,† p<0.05 comparison between the series indicated performed using T-test).*
Both NO and zinc increases the total GSH levels of the cells. The presence of TPEN blocks the NO-mediated increase of GSH levels, indicating that the NO-mediated increase of GSH levels is fully zinc dependent. If the de novo synthesis of GSH is blocked by addition of BSO, a decrease of GSH levels is observed, indicating that the de novo synthesis of GSH plays a central role in the control of the total cellular GSH levels.

Since GSH exerts antioxidant activity, the observed increase in GSH levels might be correlated to the protective effects exerted by both NO and zinc against the peroxide-induced toxicity. To verify if a statistically relevant correlation between the GSH levels and cell viability after treatment with peroxide existed, a Pearson linearity test was performed.

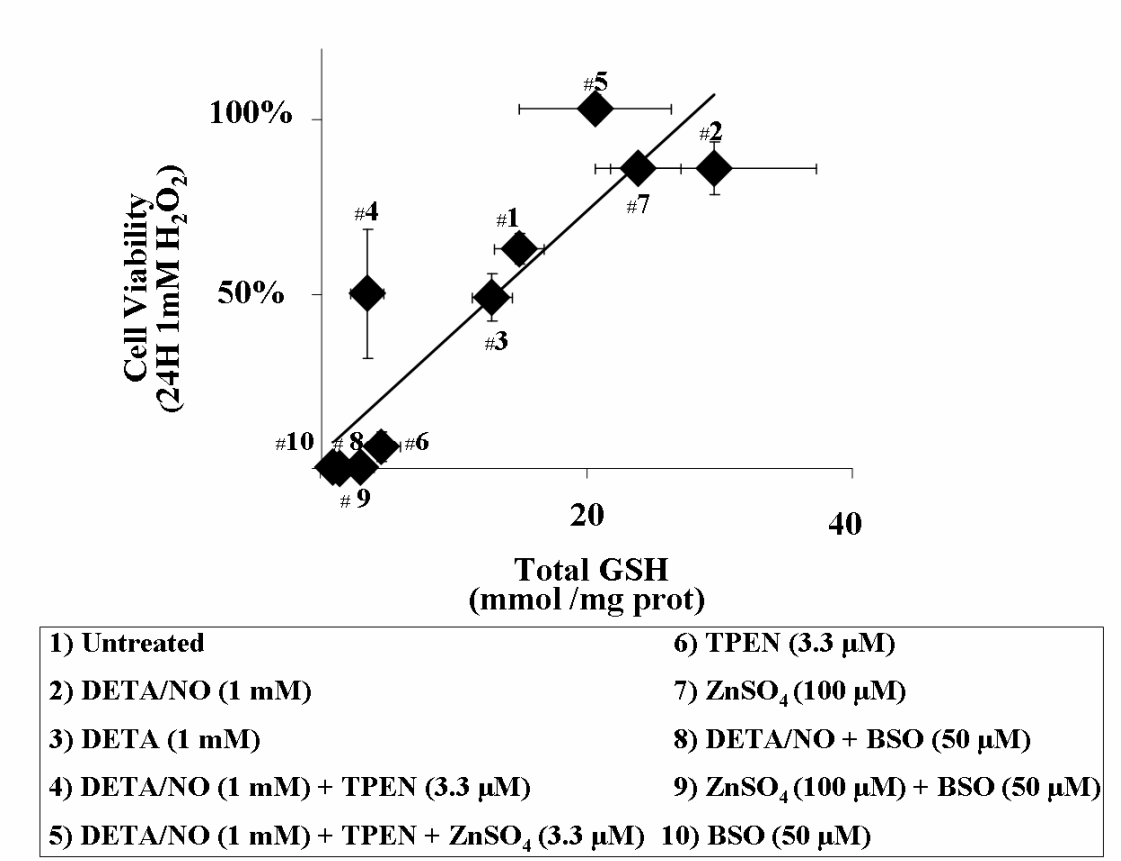

FIGURE 18. The sensitivity of endothelial cells to oxidative stress directly correlates with their content of GSH.

The number of live cells measured after the treatment with 1 mM H_2O_2, (showed in Fig 8A, Fig. 10 , Fig.12A and Fig. 19) directly correlates with the amount of total GSH levels measured before the peroxide treatment and showed in Fig. 17. Pierson correlation coefficient r= 0.879 (p<.0.05; n = 4-6).

The number of live cells after the treatment with 1 mM H_2O_2, shown in Fig 8A, Fig. 10, Fig.12A and Fig. 19, strongly correlates with the amount of total cellular GSH levels measured before the H_2O_2 addition and shown in Fig. 17. (Pierson correlation coefficient

r = 0.8709 p<0.05, Fig. 18). The cell viability after the peroxide treatment relative to samples pre-treated with DETA/NO+BSO or $ZnSO_4$+BSO are shown in Fig. 19.

The existence of a direct correlation between the GSH levels and the survival of the cells after the treatment with H_2O_2 indicates that the sensitivity of the endothelial cells to peroxide-induced toxicity strictly depends on their total GSH content.

The GSH levels of the cells strictly depends on the expressional activation of GCL

To further confirm that NO and zinc protect the cells against H_2O_2 by increasing the expression of *Gclc* and the production of GSH, the effects exerted by the inhibition of GCL on cell viability has been examined here.

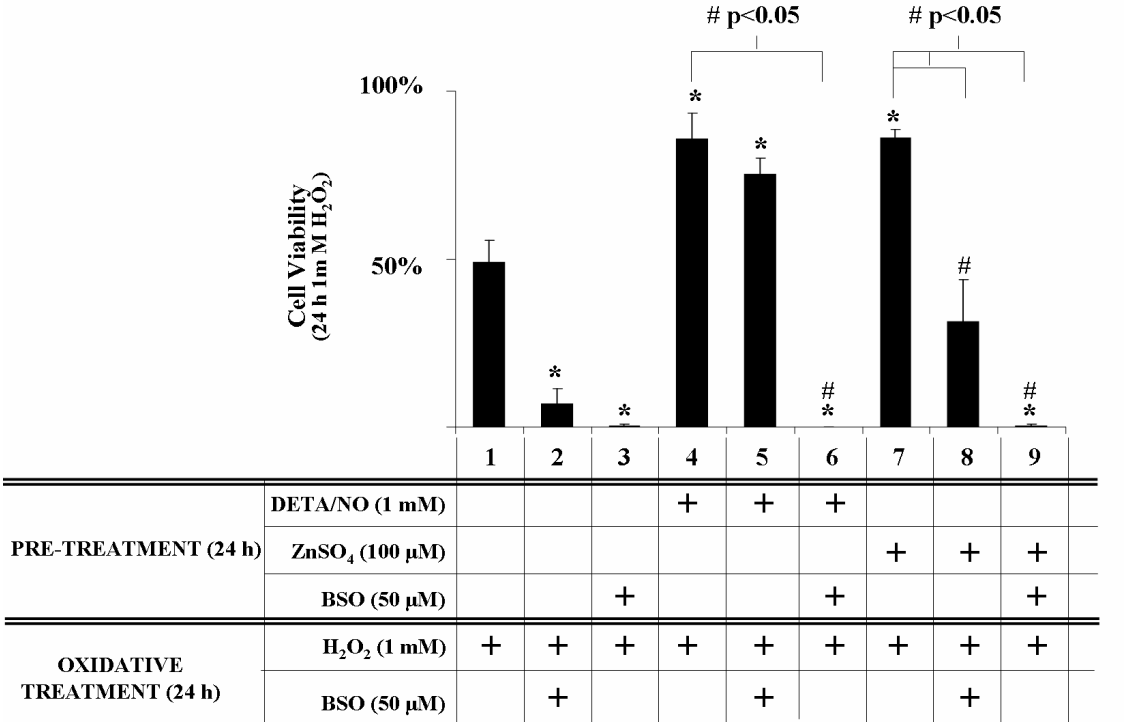

FIGURE 19. If the GCL activity is inhibited by BSO, both DETA/NO and $ZnSO_4$ are not effective in protecting the cells against H_2O_2.

*EC were pre-treated for 24 hours with medium (lane 1,2,3), with DETA/NO (1 mM; lane 4,5,6), with $ZnSO_4$ (100 µM; lane 7,8,9) alone or in the presence of buthionine sulfoximine (BSO 50 µM;lane 2,5,8), a specific inhibitor of GCL. After extensive wash, EC were treated for 24 hours with 1 mM H_2O_2 in the absence (lane 1,2,4,5,7,8) or in the presence of BSO (lane 3,6,9). Cell viability was determined by neutral red staining. Values represent the mean±SEM (n=6; *p<0.05 compared to the control (Tukey test); # p<0.05 comparison (T-test) between the series indicated).*
The inhibition of GCL during the treatment with DETA/NO or with zinc completely blocks the protective activity of both molecules, confirming the central role played by GCL in the NO- and zinc-mediated protection

Endothelial cells were pre-treated for 24 hours with BSO alone or in the presence of DETA/NO or $ZnSO_4$ and after extensive wash treated with H_2O_2. Alternatively, BSO was added during the treatment with H_2O_2 (Fig. 19). If cells were pre-incubated (for 24 hours) with BSO (Fig. 19, lane 3) or with DETA/NO+BSO (Fig. 19, lane 6) or with $ZnSO_4$+BSO (Fig. 19, lane 9) and then treated with H_2O_2 (for 24 hours), the toxicity of peroxide reaches the maximum with an LD_{50} <0.2 mM (in Fig. 19 the data relative to 1 mM H_2O_2 only are shown).

If cells were pretreated with medium (Fig. 19, lane 2) or with $ZnSO_4$ (Fig. 19, lane 8) and then treated with H_2O_2 in the presence of BSO, cell viability was strongly affected. Instead in DETA/NO pretreated cells, the addition of BSO during the treatment with H_2O_2 (Fig. 19, lane 5) did not exert any effect.

Thus, the inhibition of the GCL activity during the treatment with DETA/NO or with $ZnSO_4$ completely blocks the NO- and zinc-mediated protective effects, confirming the central role played by GCL in the NO- and zinc-mediated protection. Instead, if the inhibition of GCL is performed during the H_2O_2 treatment, the NO-mediated protection is not affected.

Endogenous nitric oxide synthesis regulates cellular GSH levels in a zinc-dependent fashion.

Next it has been investigated whether NO produced endogenously by iNOS exerted the same effects observed after incubation with the NO donor DETA/NO.

Cytokine-mediated iNOS expression and the resulting endogenous high-output NO-synthesis significantly increases the *Gclc* mRNA to comparable levels than found after exogenous NO addition (Fig. 20A, lane 2). The addition of the NOS inhibitor L-NIO (Fig. 20A, lane 3) but also of TPEN (Fig. 20A, lane 4) fully abrogated the *Gclc* induction, demonstrating that the *Gclc* increase depends on both, on endogenous NO-synthesis and on the presence of “labile” zinc.

The cellular GSH levels in cytokine-activated cells have been also measured here (Fig. 20B). Both the inhibition of NO formation (Fig. 20B, lane 3) and zinc deprivation (Fig. 20B, lane 4) significantly decrease cellular GSH levels, showing that under pro-inflammatory conditions the GSH levels of the cells depend on endogenous NO synthesis and on the presence of zinc.

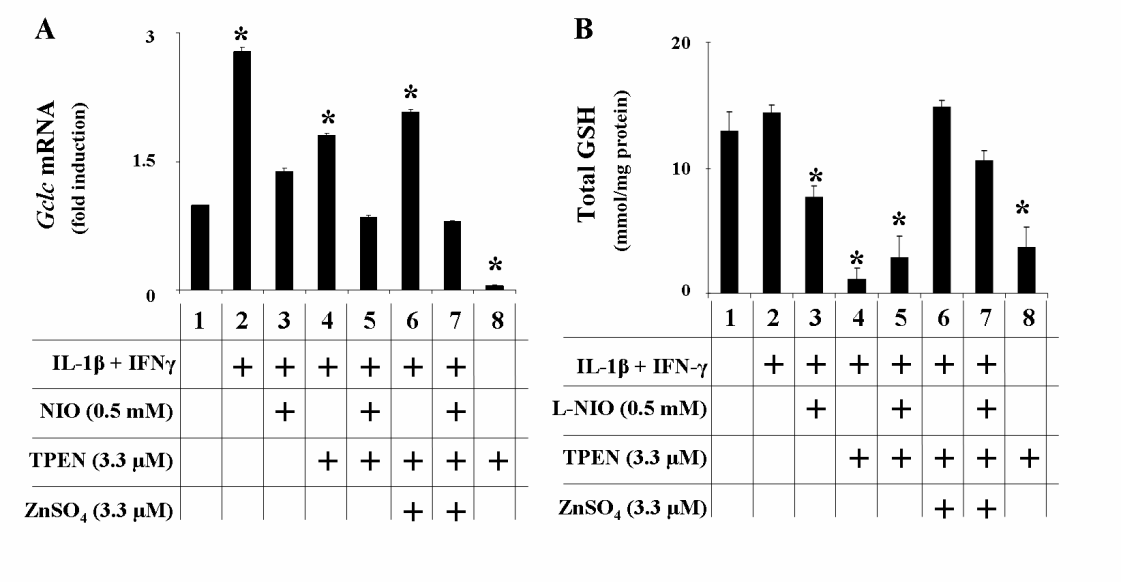

FIGURE 20. Endogenous NO synthesis regulates Gclc and total cellular GSH levels in a zinc-dependent fashion.

EC were pre-activated for 6 hours with the cytokine mix indicated, in the absence or in the presence of the NOS inhibitor L-NIO. Then the specific zinc chelator TPEN (3.3 μM) was added in the absence or in the presence of equimolar concentration of zinc. ***A)*** *24 hours after activation, total mRNA was extracted and the expression of Gclc was studied by real time RT-PCR using the TaqMan technology. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. (n=3; * p<0.05).* ***B)*** *24 hours after activation, total GSH was assayed in cell lysates by the GSH reductase recycling method and expressed as mmol GSH per mg protein. Values represent the mean±SEM (n=5; * p<0.05).*
Endogenous NO synthesis induces the expression of Gclc in a zinc-dependent fashion. Under pro-inflammatory conditions the GSH levels of the cells depend on endogenous NO synthesis and on the presence of zinc.

NITRIC OXIDE REGULATES THE GSH SYSTEM VIA NRF2

The results presented in this study clearly indicate that NO-mediated and zinc dependent induction of GCL regulates the total GSH levels of endothelial cells and consequently their sensitivity towards peroxide-induced toxicity. The mechanism controlling transcriptional regulation of GCL has been studied mainly in mice models and human cell culture-based experiments. According to these studies the expression of GCL and of the other members of the class of phase II detoxifying enzymes is regulated at the transcriptional level by the antioxidant transcription factor Nrf2, which binds to AREs present in the promoters of the respective genes (Jaiswal, 2004; Moi *et al.*, 1994; Nguyen *et al.*, 2005; Wakabayashi *et al.*, 2004). Little information about the promoter of rat *Gclc* is present in the literature (Yang *et al.*, 2005; Yang *et al.*, 2001). The expression of *Gclc* is Nrf1- and Nrf2-dependent, but both transcription factors appear to participate in the regulation of *Gclc* in an indirect fashion (Yang *et al.*, 2005).

Nitric oxide and zinc induces the translocation of Nrf2 into the nucleus

In this model, the Nrf2 activation by NO or zinc was verified by using confocal microscopy. In rat aortic endothelial cells Nrf2 immune-reactivity was detected in the cytoplasm only and not in the nucleus, indicating that Nrf2 is dominantly located in the cytoplasm during the steady state (Fig. 21A, I, II, III). Instead, endothelial cells treated for 1 h with the NO donor DETA/NO (Fig. 21B, I, II, II), or with $ZnSO_4$ (Fig. 21C, I, II, III), as well as with the established Nrf2 activator tert-butylhydroquinone (tBHQ; Fig. 21D, I, II, III) presented cytoplasmic as well as prominent nuclear Nrf2 staining. This shows that both high-output synthesis of NO and zinc supplementation induce Nrf2 translocation from the cytoplasm into the nucleus.

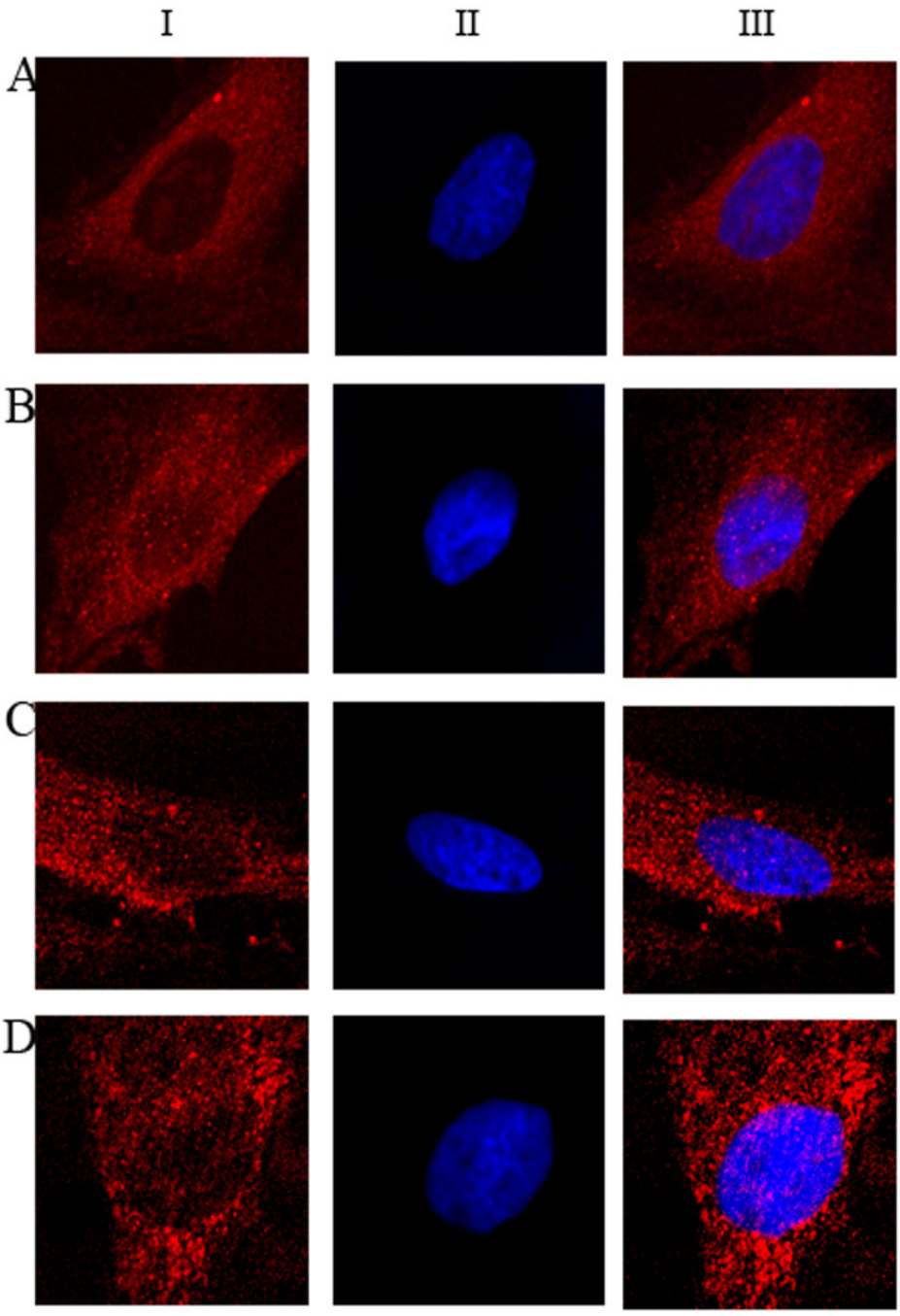

FIGURE 21. NO and zinc induce the nuclear translocation of Nrf2.

EC were seeded on 22 mm ∅ cover glasses and grow for 24 hours. Cells were then cultured for 1 hour with A) medium ***B)*** *DETA/NO (1 mM)* ***C)*** *$ZnSO_4$ (100 µM)* ***D)*** *tert-butylhydroquinone (tBHQ 100 µM), as positive control. Then, cells were stained with an antibody anti-Nrf2 (panel I) and nuclei were stained with Hoechst dye (panel II), overlap (panel III). In untreated cells the Nrf2 immune-reactivity was detected in the cytoplasm only. Instead in cells treated with DETA/NO (panel B) or zinc (panel C) the Nrf2 signal was detected also in the nucleus, indicating that NO and zinc induce the nuclear translocation of Nrf2*

The antioxidant transcription factor Nrf2 is involved in the nitric oxide-mediated gene regulation of *Gclc*.

To further investigate if Nrf2 activation by NO and zinc plays a role in *Gclc* transcriptional regulation the expression Nrf2 was silenced by using RNA interference.

Little information is present in the literature regarding the regulation of the rat Gclc. Since the zinc sensor MTF-1 is involved in the regulation of mouse *Gclc,* but it not of human *Gclc*, the involvement of MTF-1 in the regulation of rat *Gclc* has been also taken in consideration. Endothelial cells were transfected in parallel with a silencing RNA (siRNA) sequence directed against *Nrf2* mRNA (siNrf2) and a sequence directed against *Mtf-1*mRNA (siMTF-1)

To evaluate whether RNA interference mediated by siNrf2 and siMTF-1 was effective, the levels of the targeted mRNAs in each sample has been determined by qRT-PCR. A strong reduction of both *Mtf-1* and *Nrf2* mRNA was measured (Fig.25 A, B).

To control the transfection and the silencing efficiency, cells were transfected in parallel with a commercially available siRNA directed against *Gapdh*. The mRNA levels of *Gapdh* were measured in each experiment. A strong reduction for *Gapdh* mRNA was always observed (Fig. 22 C). In addition, as a control for silencing specificity and for eventual off-target effects, cells were also transfected with a commercially available non-silencing siRNA (NS siRNA), which does not recognize any eukaryotic sequence. The expression of *Mtf-1*, *Nrf2* and *Gapdh* are not modified by transfection of NSsiRNA, therefore no off target effects of RNA interference were detected (Fig. 22A, B, C).

The expression of *Gclc* was assayed by real time PCR. After Nrf2 silencing, a significant down-regulation of *Gclc* was observed at each time point analyzed (Fig. 23A, B, C; lane CTRL). Instead, silencing of MTF-1 did not exert any effect on the basal expressional level of *Gclc* (Fig. 23 A, B, C; lane CTRL). Of interest, if Nrf2 was silenced, neither NO after 6,12 or 24 hours of incubation (Fig. 23A,B,C lane DETA/NO) nor cytokines after 24 hours of incubation (Fig. 23C lane Cyt) were effective anymore in inducing the expression of *Gclc* in endothelial cells (Fig. 23A,B,C; lane CTRL). The silencing of MTF-1 instead did not exert any effect on the induction of *Gclc* by NO or cytokines (Fig.26A, B, C).

Therefore, in rat aortic endothelial cells, Nrf2 regulates both the basal, as well as the NO-and zinc-mediated induction of Gclc. Instead, MTF-1 does not play any role.

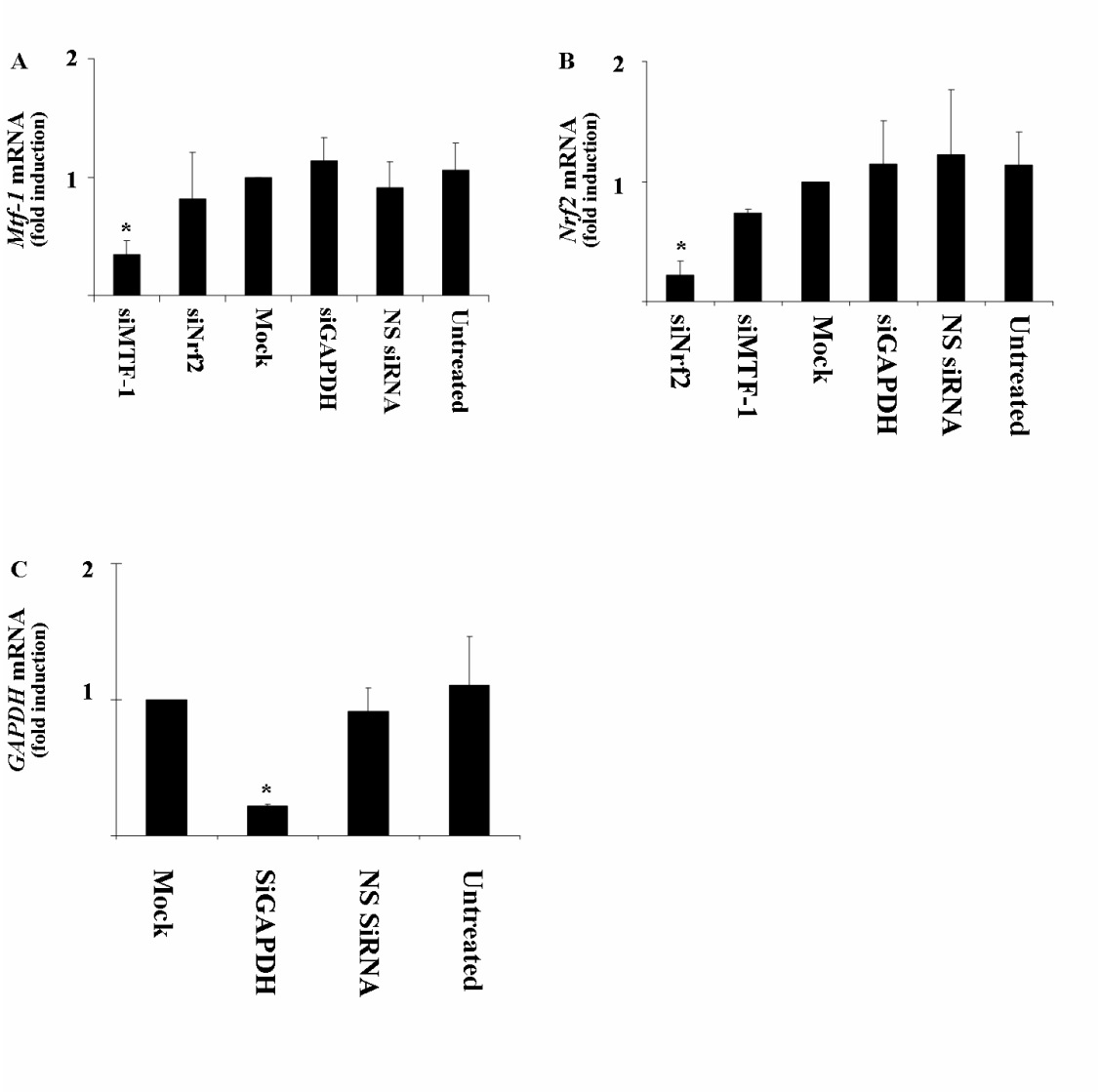

FIGURE 22. Evaluation of the silencing efficiency.

EC were seeded and immediately transfected with 5 nM of SiNrf2 or SiMtf1 and 9 µl of transfection reagent in 1 ml of complete medium. As a control, cells were transfected with 5 nM of SiGapdh or a non silencing (NS) siRNA or with transfection reagent only (Mock) or with medium only (untreated). After 24 hours, 1ml of fresh medium was added. Then, 48 hours after the transfection, the medium was changed. Then, 72h after the transfection, cells were lysed and total mRNA was extracted.
The mRNA levels of ***A)*** *Mtf-1* ***B)*** *Nrf2 and* ***C)*** *Gapdh were evaluated by real time RT-PCR using SyBrgreen. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA (n=3,* p<0.05 vs. Mock).*

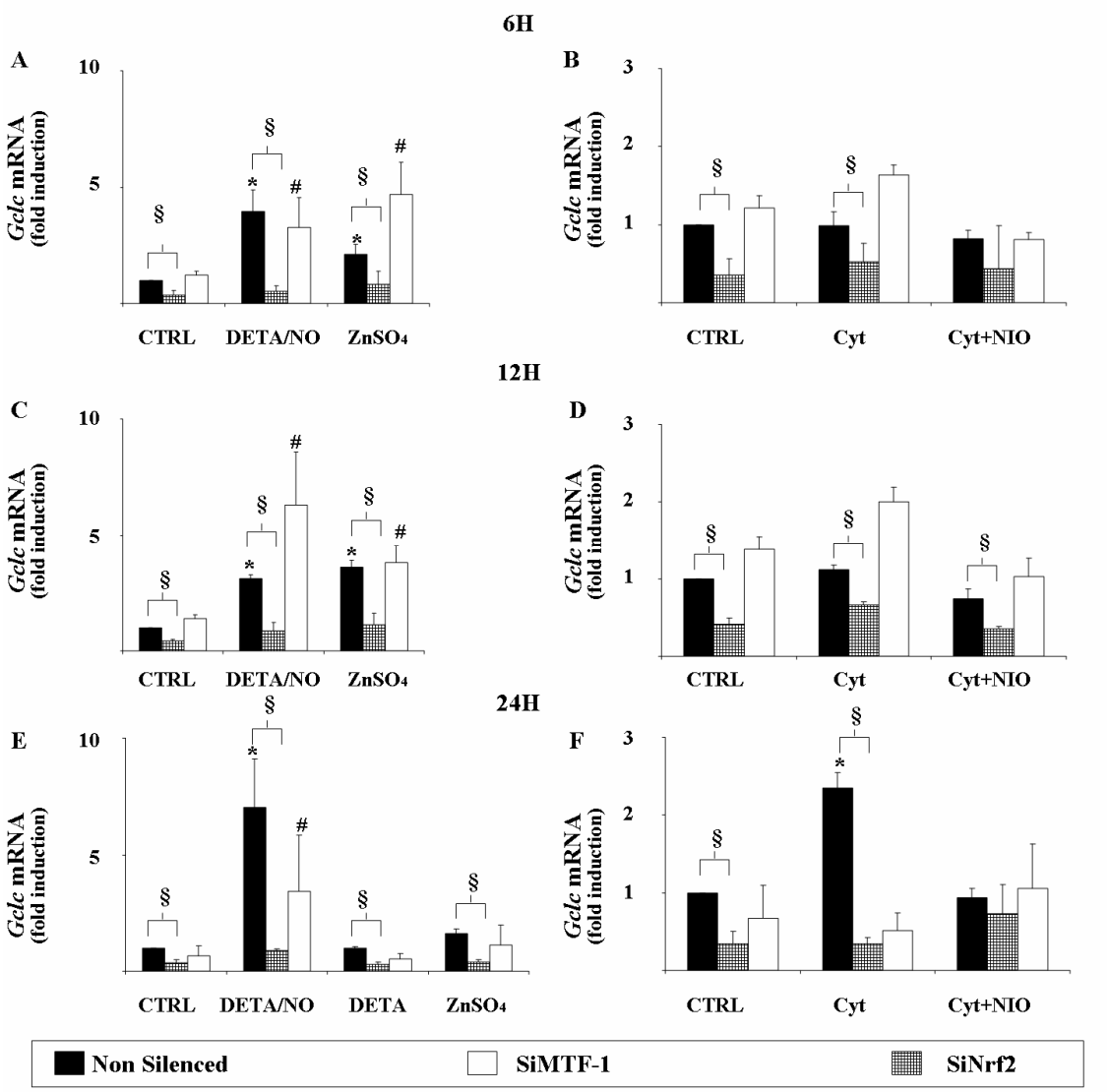

FIGURE 23. The expression of Gclc is affected by Nrf2 silencing, but not by MTF-1 silencing.

*EC were seeded and immediately transfected with 5 nM of SiNrf2 or SiMtf-1 and 9 µl of transfection reagent in 1 ml of complete medium, as indicated. After 24 hours, 1ml of fresh medium was added. 48 hours after transfection, medium was changed and stimuli were added, as indicated. Then, 72h after transfection cells were lysed and total mRNA was extracted. The expression of Gclc was studied by real time RT-PCR using the TaqMan tecnology. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. Values represent the mean±SEM (n=3; * series "Non Silenced", significantly different vs. the untreated control p<0.05; # series "siMTF-1", significantly different vs. the control p<0.05; § p<0.05 comparison between the series indicated performed using T-test).*
Nrf2 regulates both the basal, as well as the NO-and zinc-mediated induction of Gclc. Instead, MTF-1 does not play any role.

Evaluation of the expression of the established Nrf2 target gene *Ho-1*

The antioxidant gene *Ho-1* is an established Nrf2 target, also in rat models. Therefore, the expression of *Ho-1* has been measured as a further control.

DETA/NO and $ZnSO_4$ induced the expression of *Ho-1* already after 6 hours of incubation (Fig. 24 A). If endothelial cells were treated with pro-inflammatory cytokines, an increase of *Ho-1* mRNA is measured after 24 hours (Fig. 24C), as expected considering that the endogenous NO synthesis is detectable only 8 hours after cytokine challenge (Fig. 8B). The cytokine-induced up-regulation of *Ho-1* is fully NO-dependent, as the inhibition of NO synthesis by addition of L-NIO blocked the increase of the *Ho-1* mRNA (Fig. 24C). If Nrf2 was silenced, DETA/NO as well as cytokines treatment do not induce *Ho-1* transcription, indicating that NO-mediated up-regulation of *Ho-1* strictly depends on Nrf2 (Fig. 24 A,B,C). Surprisingly the zinc-mediated activation of *Ho-1* was not affected by Nrf2 silencing (Fig. 24A, B, and C).

Thus, in the cells under study both exogenously applied NO and endogenously produced NO induce the expression of the antioxidant enzyme Gclc and *Ho-1* through the activation of Nrf2, confirming the central role played by Nrf2 in the antioxidant defense of endothelial cells. .

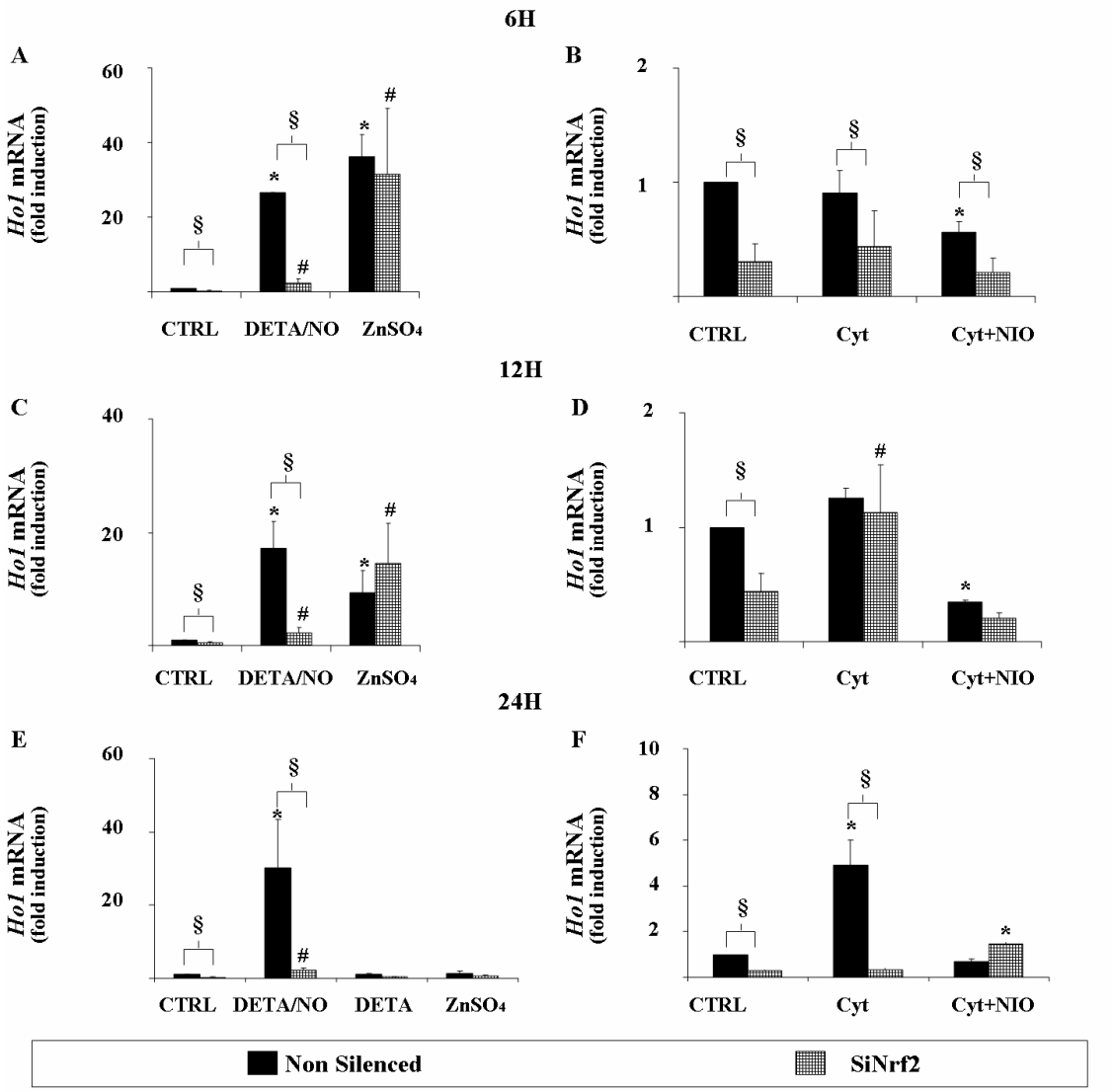

FIGURE 24. Nrf2 regulates the expression of Ho-1

*EC were transfected with 5 nM of SiNrf2, as indicated in Fig. 23. Then, 72h after transfection, the expression of Ho1 was studied by real time RT-PCR. using the TaqMan technology. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. Values represent the mean±SEM (n=3; * series "Non Silenced", significantly different vs. the untreated control p<0.05; # series "siNrf2", significantly different vs. the control p<0.05; § p<0.05 comparison between the series indicated performed using T-test).*
Both exogenously applied NO and endogenously produced NO induce the expression of Ho-1 via the activation of Nrf2

NITRIC OXIDE INDUCES MTF-1-DEPENDENT TRANSCRIPTIONAL REGULATION

The transcription factor MTF-1 is a highly conserved zinc sensor, able to sense small variations of intracellular zinc concentration. It is activated in response to heavy metals and oxidative stress. The involvement of MTF-1 in the NO-mediated transcriptional regulation was verified by analyzing the expression of established MTF-1 target genes, such as *Mt1a* and *Znt1*, after NO-challenge. Moreover, the effects of both the silencing of MTF-1 by RNA interference and zinc depletion on the expression of MTF-1 target genes have been also analyzed here.

Nitric oxide induces the expression of *Mt1a* via MTF-1 activation.

As expected, zinc-treated cells present a high increase in *Mt1a* expression. After MTF-1 silencing the basal expression, as well the zinc-mediated up-regulation of *Mt1a* is strongly decreased.

Metallothioneins play a central role in the cellular defense against oxidative stress. It has been shown that the promoter of *Mt1a* contains active AREs (Andrews, 2000; Dalton *et al.*, 1994). Therefore, to verify if Nrf2 participates in the NO-mediated regulation of metallothionein, the *Mt1a* expression was analyzed also after silencing of *Nrf2* (Fig. 25). The silencing of Nrf2 do not affect the basal mRNA levels of *Mt1a* (Fig. 25B, D, F; lane CTRL), but surprisingly blocks the zinc-mediated up-regulation (Fig. 25A, C, E; lane $ZnSO_4$), indicating that Nrf2 participate in the zinc-mediated transcriptional regulation of *Mt1a*.

Of interest, exogenously applied NO induces the expression of Mt1a already after 6 hours of incubation (Fig. 25B; lane DETA /NO). If MTF-1 is silenced, the DETA/NO mediated induction of *Mt1a* observed after 6 hours of incubation is inhibited (Fig. 25B, lane DETA/NO). Instead, no effects are observed for the expressional peaks observed after 12 hours or 24 hours of incubation (Fig. 25D,F; lane DETA/NO), indicating that only the peak at 6 hours is MTF-1-dependent.

The silencing of Nrf2 did not produce any significant effect on NO-induced *Mt1a* expression (Fig. 25B, D, F; lane DETA/NO).

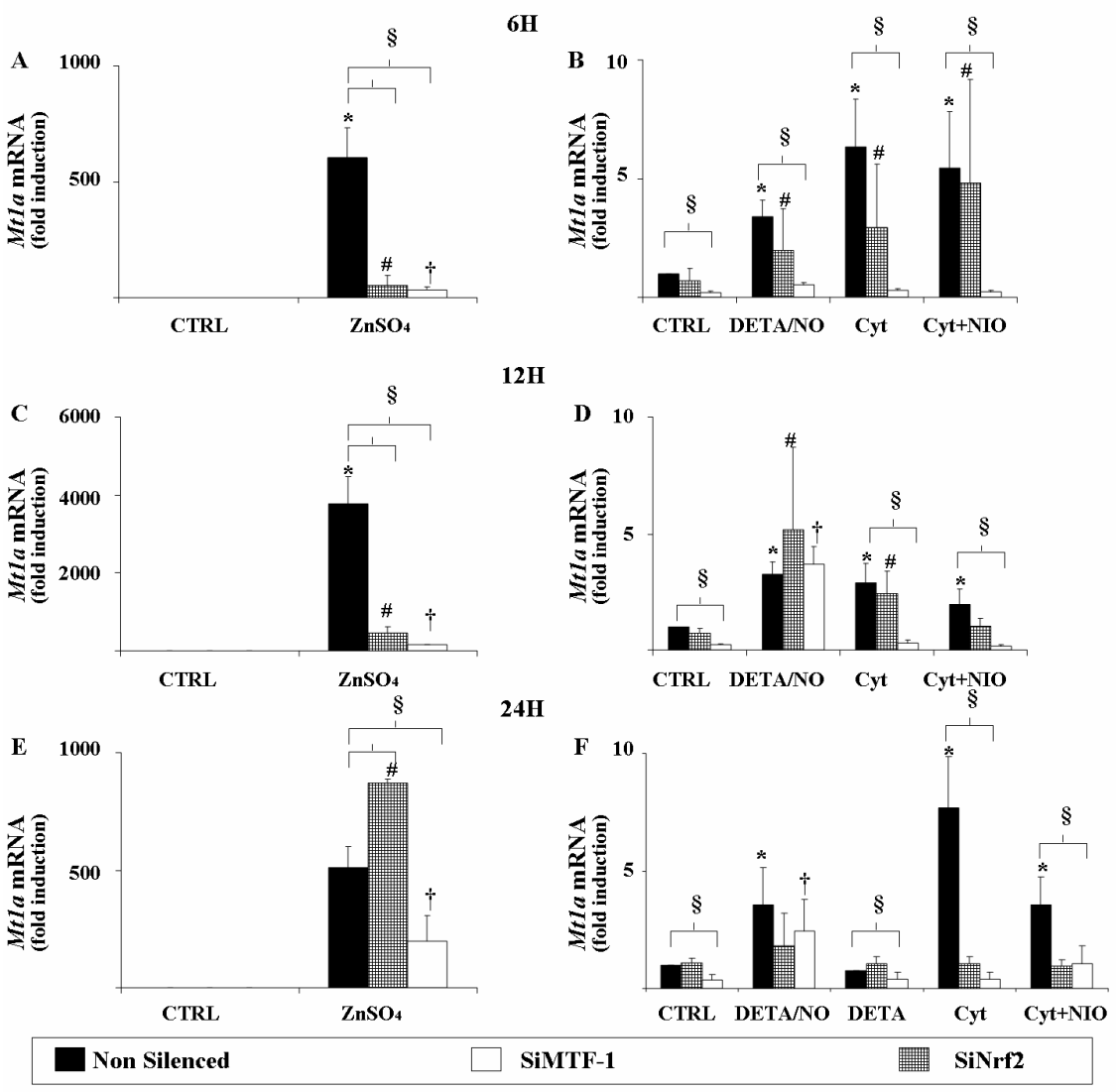

FIGURE 25. Both MTF-1 and Nrf2 participate in the gene expression regulation of Mt1a.

*EC were transfected with 5 nM of SiNrf2 or SiMtf1, as indicated in Fig 23. Then, 72h after transfection, the expression of Mt1a was studied by real time RT-PCR using the TaqMan tecnology. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. Values represent the mean±SEM (n=3; * series "Non Silenced", significantly different vs. the untreated control p<0.05; # series "siMTF-1", significantly different vs. the control p<0.05; † series "siNrf2", significantly different vs. the control; § p<0.05 comparison between the series indicated performed using T-test).*
The MTF-1 silencing reveals that the basal mRNA levels (CTRL), the zinc-mediated increase in mRNA levels ($ZnSO_4$), and the NO-mediated increase in mRNA levels (DETA/NO-6h and Cyt-24h) are fully MTF-1-dependent. The Nrf2 silencing reveals that Nrf2 participates in the zinc-mediated as well as in the cytokine-mediated transcriptional regulation of Mt1a. Thus, both MTF-1 and Nrf2 participate in the gene expression regulation of Mt1a

The treatment with pro-inflammatory cytokines produces a transcriptional activation already after 6 hours of incubation (Fig. 25B, lane Cyt), which is not determined by

endogenous NO-production, as the addition of L-NIO does not produce any effect (Fig. 25B, lane Cyt+L-NIO). Interestingly, the cytokine-mediated *Mt1a* induction after 6 hours and 12 hours of incubation is MTF-1-dependent and Nrf2-independent (Fig. 25B, D; lane Cyt). Instead, the cytokines induced up-regulation of *Mt1a* after 24 hours of incubation is NO-dependent, as the addition of L-NIO completely block the response (Fig. 25F, lanes Cyt and Cyt + L-NIO). After silencing of neither MTF-1 nor Nrf2, the 24-hour treatment with cytokines is effective in inducing *Mt1a* transcriptional up-regulation (Fig. 25F, lanes Cyt and Cyt + L-NIO), indicating that both MTF-1 and Nrf2 are involved in the regulation of *Mt1a* under pro-inflammatory conditions.

The MTF-1 silencing reduces the basal mRNA levels as well as the zinc-mediated expressional levels, confirming that the expression of Mt1a is regulated mainly by MTF-1. Of interest, both exogenously applied NO and endogenously produced NO induce the *Mt1a* expression in a MTF-1-dependent fashion, indicating that NO activates the expression of *Mt1a* via MTF-1. In addition, the Nrf2 silencing reveals that Nrf2 participates in the zinc-mediated, as well as in the cytokine-mediated transcriptional regulation of *Mt1a*. Thus, both MTF-1 and Nrf2 are involved in the regulation of *Mt1a*, particularly under pro-inflammatory conditions.

Nitric oxide increases the expression of *Znt1* via MTF-1 activation

The expression of the MTF-1 target gene *Znt1* has been also analyzed. The treatment with zinc, and more importantly the treatment with both the NO-donor DETA/NO and with pro-inflammatory cytokines induce the expression of *Znt1* (Fig. 28C, lanes DETA/NO and Cyt). After MTF-1 silencing, the basal expression of *Znt1*, as well the zinc-mediated up-regulation is strongly decreased (Fig. 25 C, lanes CTRL and Fig. 25 A, B, C; lane $ZnSO_4$). Interestingly, the NO-induced expression of *Znt1* is fully inhibited by the MTF-1 silencing, indicating that NO increases the expression of *Znt1* via MTF-1 activation (Fig. 28C, lane DETA/NO).

Control incubation with DETA did not exert any significant effects (Fig. 28C, lane DETA).

Thus, NO induces the expression of both *Mt1a* and *Znt1* via the activation of MTF-1, demonstrating that MTF-1 is a NO-target

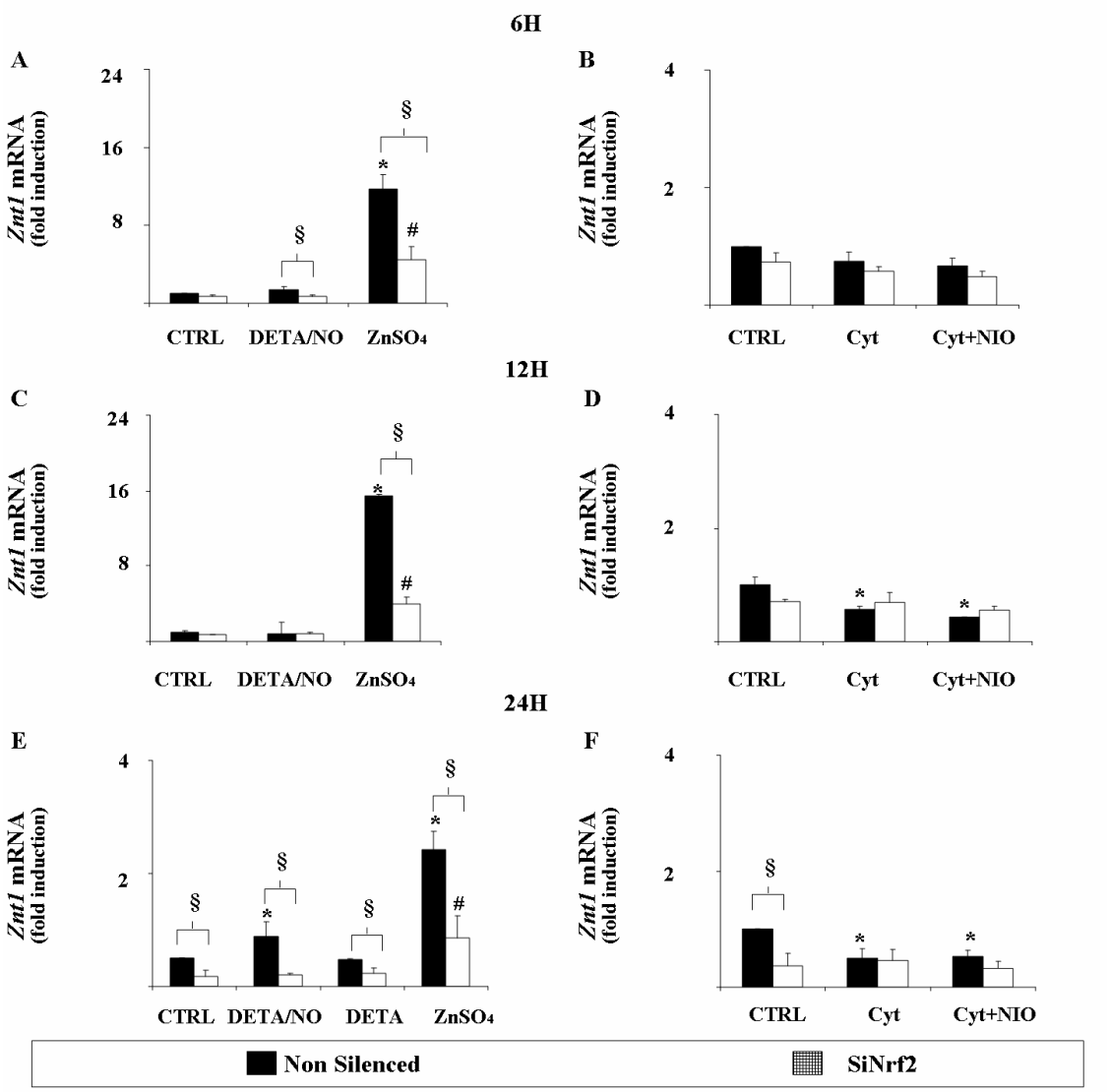

FIGURE 26. MTF-1 regulates the NO-mediated expression of Znt1

*EC were transfected with 5 nM of SiMTF-1, as indicated in Fig. 23. Then, 72h after transfection, the expression of Znt1 was studied by real time RT-PCR. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. Values represent the mean±SEM (n=3; * series "Non Silenced", significantly different vs. the untreated control p<0.05; # series "siMTF-1", significantly different vs. the control p<0.05; § p<0.05 comparison between the series indicated performed using T-test).*
After 24 hours of incubation, the silencing of MTF-1 reduces the basal mRNA levels (CTRL) and blocks the zinc-mediated $ZnSO_4$ 6 h, 12 h, 24 h) as well as the NO-mediated (DETA/NO 24 h) transcriptional increases of Znt1, demonstrating that MTF-1 regulates the basal expression and particularly the NO-mediated induction of Znt1.

Nitric oxide induces the expression MTF-1 target genes in a zinc-dependent fashion

The data presented show that NO-induces the expression of Znt1 and Mt1a via MTF-1. Next, it has been analyzed whether the NO-dependent regulation of MTF-1 target genes is affected by depletion of "labile" zinc, obtained by incubating the cells with the membrane-permeable zinc chelator TPEN (3.3 μM). Interestingly, TPEN treatment led to a significant down-regulation of the MTF-1 target *Znt1*, but had little impact on *Mt1a* expression (Fig. 27A, C).

The NO-mediated up-regulation of *Znt1* was completely abolished in the presence of TPEN. If TPEN was counterbalanced by adding an equimolar concentration of $ZnSO_4$ (3.3 μM), the NO-induced response was fully restored (Fig. 27 C).

On the contrary, zinc depletion by 3.3 μM TPEN did not abrogate the NO-mediated induction of *Mt1a* transcription and counterbalancing TPEN with zinc did not significantly alter this response (Fig. 28A). The addition of 5 μM TPEN strongly reduced the basal *Mt1a* levels and blocked the NO-induced up-regulation of *Mt1a* (Fig. 27B).

The data presented show that NO-induces the expression of *Znt1* and *Mt1a* via MTF-1 and that NO induces the expression of MTF-1 target genes in a zinc-dependent fashion. Thus, NO might regulate the MTF-1 activation via zinc labialization.

In addition, the effects of zinc depletion on the expression of the Nrf2 target gene *Ho-1* have been also analyzed here. The treatment with TPEN induces an increase of the *Ho-1* expression (Fig. 27D). Of interest, the presence of TPEN limits NO-mediated activation of HO-1 at 24 hours (Fig. 27D), indicating that the NO-mediated expression of *Ho-1* is partially zinc-dependent.

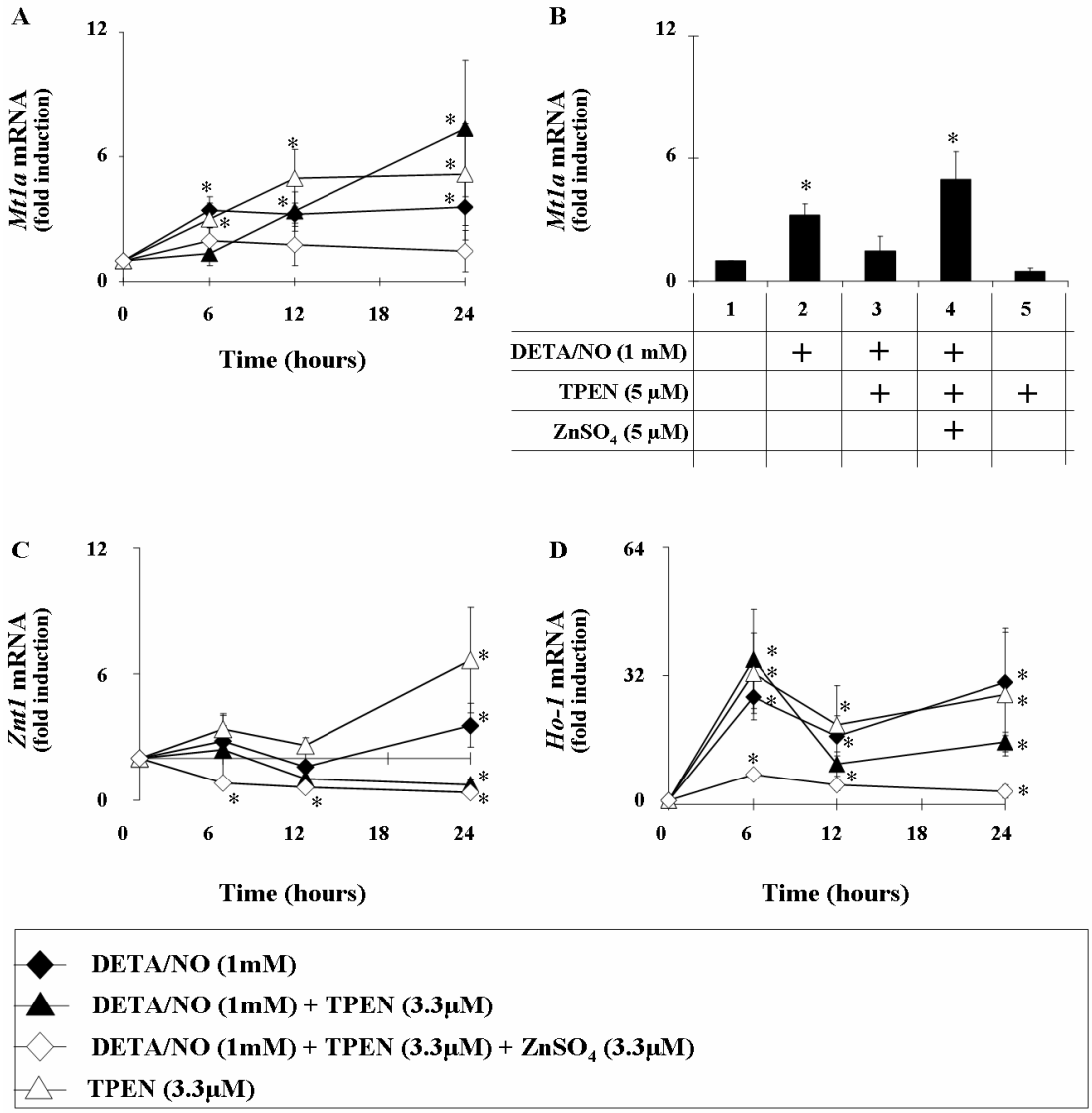

FIGURE 27. The NO-mediated transcriptional increases of Mt1a, Znt1 and Ho-1 depend on the presence of "labile" zinc.

A,C,D) *EC were cultured in the presence of 3.3µM TPEN (△) at the concentration indicated or with 1 mM DETA/NO (◆) or DETA/NO+TPEN (▲). As a control, TPEN was titrated with equimolar concentrations of ZnSO4 (◇). At the time-points indicated the expression of Mt1, Znt1 and Ho1 was quantified by real time RT-PCR. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA (n=3; *p<0.05).* ***B)*** *EC were cultured in the presence of 5µM TPEN alone (lane 4) or in the presence of DETA/NO (lane 2) As a control, TPEN was titrated with equimolar concentrations of $ZnSO_4$ .After 6h of incubation the expression of Mt1a was quantified by real time RT-PCR. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. (n=3; *p<0.05).*

The NO-mediated up-regulation of Znt1 and Mt1a was completely abolished in the presence of respectively 3.3 M or 5µM TPEN. The presence of 3.3 µM TPEN limited the NO-mediated induction of Ho-1. Thus, the NO-mediated transcriptional increases of Mt1a, Znt1 and Ho1 depend on the presence of "labile" zinc

UNDER PRO-INFLAMMATORY CONDITIONS ZINC SUPPLEMENTATION ATTENUATES ENDOTHELIAL CELL ACTIVATION.

Next, the effect of zinc supplementation on cytokine-activated endothelial cells was analysed. Endothelial cells were incubated with pro-inflammatory cytokines (200 U/ml hIL-1β, 500 U/ml, hTNF-α and 100 U/ml rIFN-γ) for 24 hours. The expression of iNOS was measured as a marker of endothelial activation (Berendji *et al.*, 1997a; St Croix *et al.*, 2002). Under these conditions iNOS increased at the mRNA as well as the protein level (Fig.27 B, C). The activity of iNOS was assessed by measuring nitrite accumulation in culture supernatants. If zinc was added during cytokine-mediated endothelial activation a decrease of *iNos* mRNA as well as iNOS protein was observed and 60% less nitrite was measured in the supernatant.

To study the activity of the iNOS promoter under cytokine- and zinc-challenge, a reporter cell line was used. Experiments were performed using cells stably transfected with a construct containing the gene for firefly luciferase under control of the human iNOS promoter. Cytokines activate the promoter with a peak of luciferase activity at 6 hours. In the presence of zinc a 2 fold reduction of promoter activation is measured. Therefore, zinc inhibits iNOS transcription by inhibiting the activity of the promoter.

These experiments show that under pro-inflammatory conditions zinc supplementation attenuates endothelial cells activation.

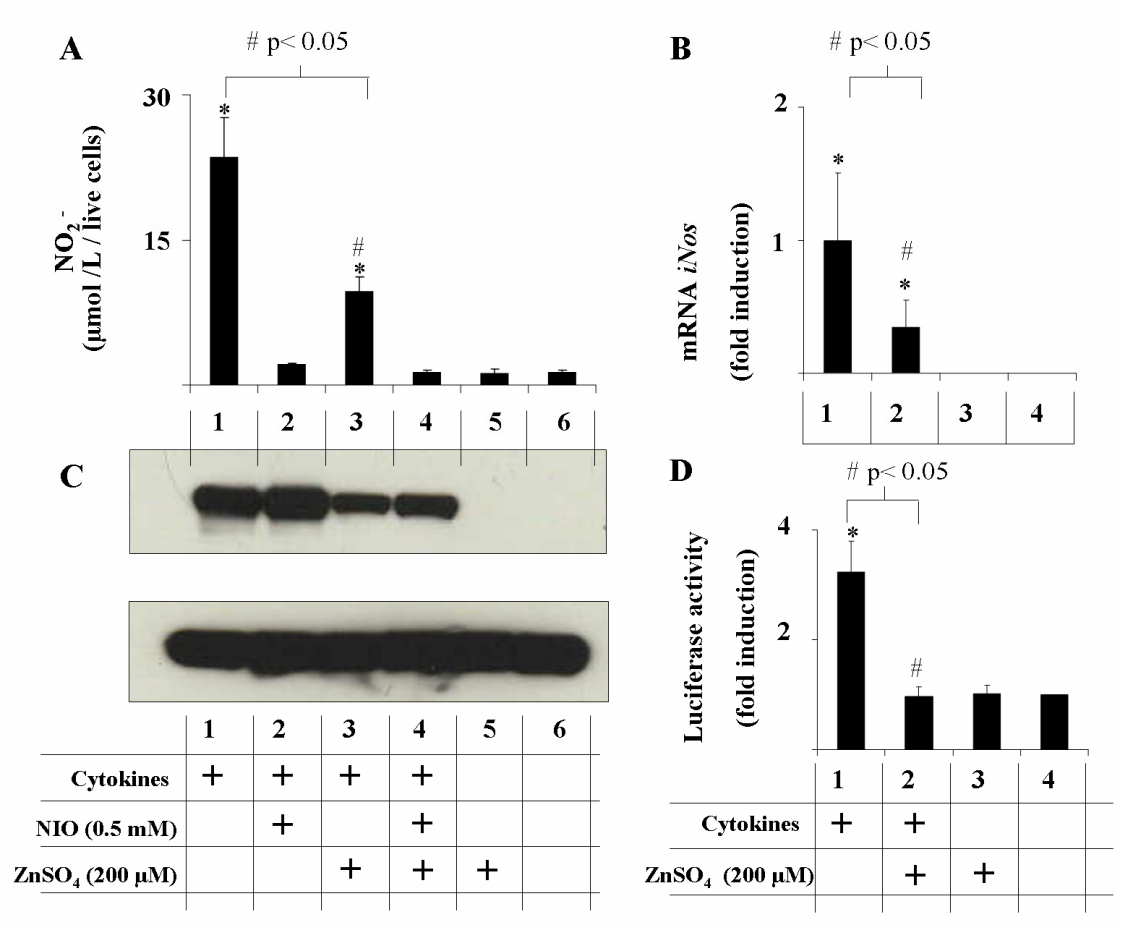

FIGURE 28. Under pro-inflammatory conditions zinc supplementation attenuates endothelial cells activation

A) *EC were incubated for 24 hours with a cytokine mixture (IL-1β 200 U/ml + IFN-γ 100 U/ml + TNF-α 500 U/ml) in the absence or in the presence of $ZnSO_4$. Nitrite concentration in culture supernatants was determined using the diazotization reaction and normalized to the protein concentration. Values represent the mean±SEM (n=6; *p<0.05).* ***B)*** *EC were treated as in A and then 15 µg of total protein extract was separated and analyzed by western blot, using anti-iNOS antibody and anti-β-actin antibody as a loading control.* ***C)*** *EC were treated as in A, then lysed and total mRNA was extracted. The levels of iNos mRNA (panel A) were measured by real time RT-PCR. Ct values from three independent experiments were averaged and the expressional levels relative to untreated cells were normalized against 18srRNA. (n=3; *p<0.05).* ***D)*** *A reporter cell line expressing luciferase under control of the human iNOS promoter activity was treated with the indicated additives for 6 h. After lysis, luciferase assay was performed. Values were normalised for the protein concentration and represent the mean±SEM (n=6; *p<0.05).*

In cytokine-treated cells zinc supplementation limits the increases in iNos mRNA, iNOS protein, and NO production as well as the iNOS promoter activation. Thus, under pro-inflammatory conditions zinc supplementation attenuates endothelial cells activation.

DISCUSSION

Within the vasculature, endothelial-derived NO, constitutively produced by eNOS, participates in a wide range of biological reactions to maintain the normal endothelial function and an anti-thrombotic vascular milieu. An impaired NO bioavailability represents the central feature of endothelial dysfunction and contributes to the development of vascular diseases such as atherosclerosis. Therefore, a protective and anti-atherogenic role has been attributed to eNOS-derived NO.

Inflammation and the correlated oxidative stress together with dyslipidemia have been indicated as main causes of endothelial dysfunction and of the onset of the atherosclerotic process. Under pro-inflammatory conditions, endothelial cells express iNOS, which produces high-output NO synthesis. Whether iNOS-derived NO exerts a deleterious or a protective role in endothelial cells is a matter of debate (Suschek *et al.*, 2004; Wassmann *et al.*, 2004). Particularly, as regards the induction of endothelial dysfunction and the development of the atherosclerotic plaque, the activation of iNOS may exert complex effects. For example, it has been hypothesized that NO-mediated induced cell death in macrophages and vascular smooth muscle cells may contribute to the development of the necrotic core in lesions (Boyle, 2005).

Using a treatment with H_2O_2 as a model for oxidative stress, it has been previously shown that ROS-induced endothelial cell death can be completely inhibited by the presence of endogenous high-output NO synthesis, as well as by exogenously applied NO through the inhibition of cytochrome c leakage, through the increases in bcl-2-gene expression, and through the inhibition of lipid peroxidation (Suschek *et al.*, 2001; Suschek *et al.*, 2003). The present study was focused on the transcriptional activity of high-output synthesis of NO. This study shows that the induction of long-term endogenous antioxidant defense mechanisms and, particularly, the GSH system may represent the major pathway by which NO limits oxidative injury and prevents endothelial cell death. Moreover, the data presented here clearly show that intracellular labile zinc is involved in the antioxidant activity of NO, pointing to a role of zinc as second-messenger in a novel NO-induced signaling pathway.

The figure 29 illustrates the effects mediated by NO, zinc supplementation, zinc deprivation on endothelial cells, and the involvement of GSH in the induced protective response.

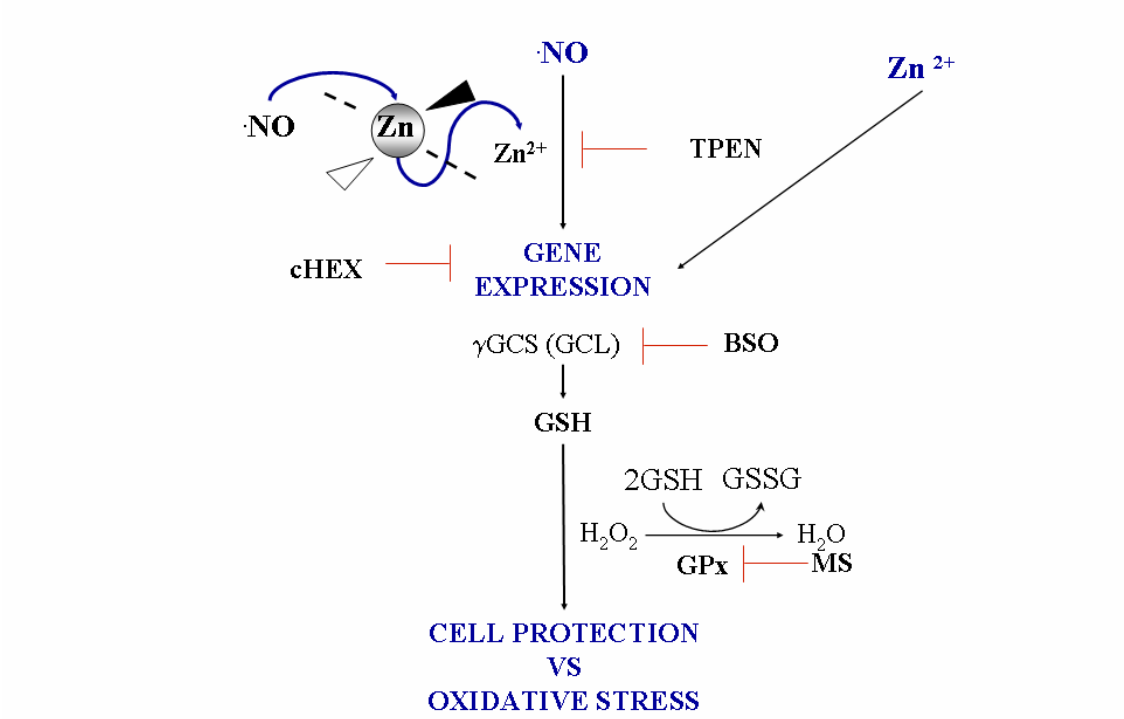

FIGURE 29. The antioxidant activity of NO and zinc in endothelial cells

NO and zinc induce the expression of GCL. The addition of the zinc chelator TPEN inhibits NO-mediated protection. If the de novo protein synthesis inhibitor cyclohexymide (cHEX) or the GCL inhibitor buthionine sulfoximide (BSO) are added during the pre-incubation of endothelial cells with NO or zinc, the protection against oxidative stress is inhibited. If glutathione peroxidase (GPx) is inhibited by mercaptosuccinate (MS) during the incubation with H_2O_2, the NO- and zinc-dependent protection activity is fully blocked, confirming the central role played by GSH in the protection of endothelial cells against oxidative stress. This demonstrates that the NO-mediated protective activity fully depends on labile zinc.

The antioxidant activity of nitric oxide in endothelial cells

High-output NO synthesis exerts short- or long-term antioxidant activity in endothelial cells. Among the short-term antioxidant effects of NO are the scavenging of radical species or the inhibition of the apoptotic cascade via S-nitrosation of target molecules, such as caspase-3 (Ascenzi *et al.*, 2001; Dimmeler *et al.*, 1997; Suschek *et al.*, 2001; Suschek *et al.*, 2003). On the other hand, NO exerts long-term protective effects mainly via transcriptional activation of protective proteins, such as HSP70, HO-1, and

VEGF (Suschek *et al.*, 1993). The NO-mediated long term protective response in endothelial cells and, particularly, the effects exerted by NO on the susceptibility of endothelial cells to oxidative stress were further analyzed here. A 24-hour treatment with peroxide was chosen as a model of oxidative stress. Because of their role as gate-keepers, endothelial cells are well equipped with highly efficient endogenous defense mechanisms to fight against oxidative stress. Therefore, they are expected to be particularly resistant to oxidative stress (Cai *et al.*, 2000). In fact, in the present study the lethal dose 50% (LD_{50}) of a 24-hour treatment with H_2O_2 is 1 mM. Cell death occurs mainly via apoptosis, as revealed by analyzing the presence of chromatin fragmentation by Hoechst staining and *in situ* nick translation.

The present study shows that NO protects endothelial cells against oxidative injury by inducing the adaptive response, which is one of the most important protective mechanisms present in mammalian cells and consists in the transcriptional induction of protective enzymes (Sies, 1993; Wassmann *et al.*, 2004). For instance, endothelial cells pre-incubated with exogenously applied NO, or with pro-inflammatory cytokines, inducing endogenous high-output NO-synthesis, are protected against peroxide-induced toxicity (Fig. 29). The observed NO-mediated protection fully depends on transcriptional activation of protective genes, as the addition of cyclohexymide, an inhibitor of protein *de novo* synthesis, completely abrogates the NO-induced resistance of endothelial cells against peroxide-induced toxicity (Fig. 29).

The ability of mammalian cells to produce antioxidant molecules is a fundamental endogenous defense mechanism against oxidative stress. The most abundant endogenously produced antioxidant molecule is the tripetide glutathione (γ-Glutamyl-cyteinyl-glycine; GSH) (Sies, 1999; Wu *et al.*, 2004). Total cellular GSH concentration (GSH + GSSG, 0.5–10 mmol/L) is determined by the rate of GSH *de novo* synthesis, as well as by the capacity of cells to recycle intracellular GSH. The rate limiting step of the *de novo* synthesis of GSH is catalyzed by glutamate-cysteine ligase (GCL) (Griffith, 1999; Lu, 2000). This enzyme is transcriptionally regulated and belongs to the family of the phase II detoxification enzymes, which plays a central role in the cellular adaptive response against oxidative stress (Sies, 1999; Wu *et al.*, 1998).

GSH exerts its antioxidant activity by directly scavenging radical species, as well as being a co-factor in reactions catalyzed by antioxidant enzymes such as glutathione peroxidase, which reduces H_2O_2 to H_2O. Of interest, the NO treatment leads to an increase of total GSH. Moreover, the measured GSH content of the cells defines the susceptibility of endothelial cells to oxidative stress. In fact, a direct correlation between the GSH levels measured before the treatment with peroxide and the cell viability after the treatment was observed.

The rate of GSH *de novo* synthesis and the expression of GCL control the GSH levels of the cells and, consequently, their susceptibility to oxidative stress, as previously shown in bovine aortic endothelial cells (Moellering *et al.*, 1999). For example, stimuli such as exogenously applied NO or $ZnSO_4$, which increase the expression of GCL, increase the GSH content of the cells and the resistance of endothelial cells to oxidative stress. Moreover, after the addition of buthionine sulfoximine (BSO), a specific inhibitor of GCL, the total GSH concentration strongly decreases and the sensitivity to peroxide-induced toxicity increases. Thus, NO contributes to the adaptation of endothelial cells to oxidative stress by controlling the GSH *de novo* synthesis (Fig.32).

A further confirmation of the central role played by GSH in the NO-mediated protection is that an inhibitor of glutathione peroxidase, added during the H_2O_2 treatment, completely inhibits the protective effect of the pre-incubation with NO (Fig. 29). Inhibiting the GSH-independent enzyme catalase, which like glutathione peroxidase catalyses the reduction of H_2O_2 to H_2O in mammalian cells, in a GSH-independent fashion, no effects were seen. Therefore, the NO-induced protection of endothelial cells depends on the activation of the GSH redox cycle, as depicted in Fig. 29.

Some authors propose that NO contributes to the depletion of GSH macrophages and neurons, also via inhibiting GCL or by the formation of GSNO (Canals *et al* 2003). Interestingly, the effect of NO on the GSH level of the cell appears to depend on the cell type considered and on its sensitivity towards NO and oxidative stress (Berendji *et al.*, 1999a). In particular, if mammalian endothelial cells of bovine or rat origin, as presented in this study, are treated with NO donors, a NO-dependent increase of GSH is observed (Moellering *et al.*, 1999).

In cytokine-treated cells, the increase of *Gclc* does not appear to correspond to an increase in GSH levels, but does correspond to increased cell viability after H_2O_2 treatment. The lack of an increase in the GSH levels of the cells could be considered a consequence of the increased production of ROS during inflammation because of the activation of oxidant enzymes, such as xantine oxidase and NADP(H) oxidase (Cai *et al.*, 2000). The resulting oxidative stress has been correlated to GSH depletion (Berendji *et al.*, 1999a).

Interestingly, the data presented here show that iNOS-derived NO plays a protective role also under pro-inflammatory conditions. In fact, if during the cytokine treatment the activity of iNOS is inhibited, the GSH levels and cell viability after the peroxide treatment strongly decrease. These findings clearly demonstrate that, under pro-inflammatory conditions, endogenous NO synthesis counterbalances the GSH loss determined by the cytokine-induced oxidative stress by inducing the *de novo* synthesis of GSH. Therefore, iNOS-derived NO contributes to maintain constant cellular GSH levels.

The study of the protective activity exerted by iNOS and its relationship to the GSH levels of the cells may have important implications in the understanding of the pathophysiology of endothelial cells under pro-inflammatory conditions and may elaborate correct strategies of prevention. For example the lack of substrate i.e., L-arginine or the excessive consummation of important co-factors, as well as the presence of peroxynitrite could lead to the iNOS enzyme uncoupling, a condition where the enzyme, instead of protecting against oxidative stress, contributes to it by producing ROS (Cai *et al.*, 2000).

The antioxidant activity of zinc in endothelial cells

Little is known about the requirements and functions of zinc in maintaining the integrity of the vasculature (Beattie *et al.*, 2004). A growing set of evidence indicates that zinc is required for cellular growth, the cellular repair processes, the exertion of antioxidant activity, and the stabilisation of bio-membranes (Powell, 2000). An inadequate zinc concentration in plasma or vascular tissues may, therefore, be involved not only in the initiation of cell injury, in the increase of oxidative stress and of the inflammatory response, but also in the lack of protection against apoptosis (Beattie *et al.*,

2004). As already mentioned, endothelial cell injury and dysfunction have been indicated as one of the major causes of vascular diseases (Cai *et al.*, 2000).

Under physiological conditions zinc is redox inert and it exerts its antioxidant activity directly (via stabilisation of –SH groups, protecting them from oxidant-mediated reduction) as well as indirectly (via activating the transcription of antioxidant genes) (Powell, 2000). Zinc is, in fact, a well known transcriptional activator (Beyersmann *et al.*, 2001). It is able to induce the expression of proteins responsible for its storage, e.g., *Mt1a*, or for zinc transport, e.g., *Znt1*, as also shown here (Andrews, 2001; Heuchel *et al.*, 1994; Langmade *et al.*, 2000). As a consequence of the zinc-mediated induction of genes controlling zinc homeostasis, cells are able to buffer even high increases in zinc concentration, explaining why zinc, unlike other metals like Fe^{2+} or Mg^{2+} and other of the IIB series, is non-toxic up to extremely high concentrations (Vallee *et al.*, 1993). In this system after a 24-hour treatment with $ZnSO_4$, the LD_{50} is 0.6 mM.

Zinc-mediated transcriptional regulation mainly involves the activation of the zinc sensor transcription factor MTF-1, which binds to metal responsive elements (MREs) present in the promoter of its target genes (Andrews, 2001). In addition, zinc activates the transcription of many other genes, as shown by microarray analysis (Cousins *et al.*, 2003). Interestingly, among the zinc-responsive genes are antioxidant genes belonging to the family of the phase II detoxifying enzymes, such as HO-1 and *Gclc*, as has been shown here.

Therefore, similarly to what was observed in the NO-mediated response, zinc supplementation exerts long-term antioxidant effects also by inducing the adaptive response. In fact, endothelial cells, pre-incubated with non-toxic zinc concentrations for 24 hours, were fully protected against peroxide–induced toxicity (Fig. 29). The addition of the protein synthesis inhibitor cyclohexymide blocked zinc-induced protection, showing that the transcriptional activity of zinc is involved in the observed protective effects. Similarly to NO, zinc supplementation increases the expression of *Gclc* gene and the production of the GCL enzyme and, consequently, the increase of total GSH levels of the cells. If GCL is inhibited during the zinc treatment by addition of buthionine sulfoximine, a decrease of total GSH and of cell viability under oxidative stress is observed (Fig. 29).

Zinc depletion, instead, increased the susceptibility of endothelial cells to peroxide-induced cell death, as also previously observed *in vitro* and *in vivo* (Ho *et al.*, 2002; Oteiza *et al.*, 2000; Oteiza *et al.*, 1995; Verstraeten *et al.*, 2004). Here, zinc depletion was achieved either by culturing the cells for 3 days in a zinc-free medium or by treating them for 24 hours with a non-toxic concentration of the membrane-permeable zinc chelator TPEN, which is highly zinc specific and is commonly used to chelate intracellular labile zinc (Cousins *et al.*, 2003; Outten *et al.*, 2001; Spahl *et al.*, 2003; St Croix *et al.*, 2004; St Croix *et al.*, 2002). The effectiveness of the TPEN treatment in depleting the cells from "labile" zinc was controlled by measuring the expression of the zinc transporter-1 (*Znt1*), which represents an excellent marker for the intracellular zinc status (Liuzzi *et al.*, 2004). Cells treated with TPEN for 24 hours showed a strong down-regulation of *Znt1*. Because of the effectiveness, the rapidity, and the convenience of the treatment, TPEN was then chosen as a method for zinc depletion. Again, the increased susceptibility of endothelial cells to oxidative stress after zinc depletion can be attributed to the effects exerted by the treatment on the expression of *Gclc*: the TPEN treatment leads to a strong down-regulation of *Gclc* and a decrease of total GSH levels in resting endothelial cells.

Therefore zinc depletion might exert toxic effects not only by caspase-3 activation, or by oxidative DNA damage as suggested before, but also by affecting the GSH *de novo* synthesis and the NO-mediated cyto-protective response (Ho *et al.*, 2002; Truong-Tran *et al.*, 2001).

Zinc participates to the antioxidant activity of nitric oxide

As already discussed, the results presented in this study demonstrate that the ability of NO to induce endothelial cell adaptation to oxidative stress depends on NO-mediated transcriptional regulation. NO can induce gene expression either via the activation of guanylate cyclase-dependent signaling pathways or via S-nitrosation (Bogdan, 2001b; Kröncke, 2003). It has been shown that high-output NO synthesis induces intracellular zinc redistribution in endothelial cells and in other cell types, by inducing zinc release from zinc clusters. Thus, a role of zinc in the NO-mediated gene expression regulation has been put forward (Berendji *et al.*, 1997a; Kröncke *et al.*, 1994; Spahl *et al.*, 2003; St Croix *et al.*, 2004; St Croix *et al.*, 2002).

In the present study, the NO-induced transcription leads to the protection of endothelial cells against peroxide-induced toxicity. In order to define whether zinc plays a role in the NO-mediated response, it has been examined whether zinc depletion might impair the NO-mediated response. Zinc depletion by TPEN fully abolishes NO-induced expression of *Gclc,* the increase of GSH levels and the ensuing resistance to peroxide-derived toxicity (Fig. 29). On the other hand, the TPEN-mediated effects can be fully reversed by addition of equimolar concentration of zinc, confirming the specificity of those observations. Moreover, the effects observed for exogenously applied NO are fully reproduced if TPEN is added during the treatment with cytokines, confirming the central role played by zinc in the NO-mediated cyto-protection.

The participation of zinc in the NO-mediated signaling and cyto-protective activity could have important implications in the response of endothelial cells to oxidative stress during inflammation and, therefore, in the pathogenesis of vascular diseases. First, this study demonstrates that the expression of iNOS plays a major role in the defense of endothelial cells against oxidative stress by regulating the GSH levels of the cells. Second, this study shows that zinc deficiency impairs the NO-mediated long-term protective response, consisting in the increased expression of GCL and the increased production of GSH. Here it has been shown for the first time that zinc deficiency can be an additional and important factor contributing to the imbalance between protective and damaging effects under pro-inflammatory conditions. Thus, zinc deficiency is likely implicated in the development of endothelial dysfunction, not only by increasing the sensitivity of endothelial cells to oxidative stress, but mainly because zinc deficiency impairs the NO-mediated cyto-protective response to oxidative stress.

The ability to control and induce the antioxidant status of endothelial cells, for example by controlling the zinc status or by targeting the antioxidant defence mechanisms with specific drugs activating protective enzymes or transcription factors, may represent a future approach in preventing the development of cardiovascular diseases.

Nitric oxide exerts antioxidant effects via the activation of the transcription factor Nrf2

This study shows that the NO- and zinc-mediated induction of GCL regulate the GSH levels of the cells and, consequently, the sensitivity of endothelial cells to peroxide-induced toxicity. As mentioned before, GCL belongs to the Phase II detoxifying enzyme class, together with NADPH quinine oxidoreductase (NQO1), glutathione S-transferase Ya subunit, heme oxygenase, and other antioxidant enzymes (Talalay *et al.*, 2003; Wakabayashi *et al.*, 2004). Each member of this class confers protection against oxidants and xenobiotics, also by participating in the adaptive response (Wakabayashi *et al.*, 2004). The mechanism of transcriptional activation of these enzymes has been studied mainly in mouse models and human cells. It has been proposed that they are coordinately regulated at the transcriptional level by the nuclear factor, erythroid-derived 2, like (Nrf2) (Chan *et al.*, 1999; Chan *et al.*, 1996; Jaiswal, 2004; Moi *et al.*, 1994). The transcription factor Nrf2 recognizes the antioxidant responsive element (ARE), which has been identified in the promoter of phase II response genes (Chan *et al.*, 1999; Chan *et al.*, 1996; Otterbein *et al.*, 2002; Sies, 1993; Wasserman *et al.*, 1997). *Nrf2* knock-out mice are viable and live to adulthood, although they exhibit an increased sensitivity to oxidative stress and a reduced basal expression of phase II genes and, in particular, of *Gclc* (Chan *et al*., 1999; Dinkova-Kostova *et al*., 2001; Wakabayashi *et al*., 2004). Nrf2 is activated by antioxidants, electrophiles such as NO, and heavy metals (e.g., zinc), but the activation mechanism is currently under discussion. It appears that Nrf2 is retained in the cytoplasm by the inhibitor Keap-1 (Kelch-like ECH-associated protein). In the presence of electrophiles, Nrf2 is released from the inhibitor and translocates into the nucleus, where it activates the transcription of ARE-target genes (Itoh *et al.*, 2003; Nguyen *et al.*, 2005; Wakabayashi *et al.*, 2004).

Using confocal microscopy it has been observed that, under basal conditions, Nrf2 is localized mainly in the cytoplasm of rat aortic endothelial cells. Moreover, after treatment with tert-butyl-hydroquinone (tBHQ), a classical Nrf2 inducer, nuclear staining is detected, indicating Nrf2 translocation into the nucleus. Of interest is that nuclear staining is also detected if cells were treated with exogenously applied NO or with $ZnSO_4$, indicating that both NO and zinc induce the translocation of Nrf2 into the nucleus.

The involvement of Nrf2 activation in the activation of NO-dependent transcriptional regulation and, particularly, in the regulation of *Gclc* expression was confirmed by using RNA interference. After silencing *Nrf2* expression, the basal level of *Gclc* mRNA strongly decreases. The addition of Nrf2 inducers, such as NO, zinc, or cytokines, does not exert any effect. Therefore, identically to what has been described before in mouse and human cell lines-based experiments, in primary rat aortic endothelial cells the *Gclc* expression is controlled by Nrf2.ly

The expression of the antioxidant phase II enzyme HO-1 was also analyzed. Since this gene has been previously confirmed as an Nrf2 target in rat models, it served as an excellent control (Alam *et al.*, 2003; Alam *et al.*, 1999). Indeed, the silencing of Nrf2 abrogated the transcription of *Ho-1*, the same effects observed for *Gclc*.

On the other hand, studies indicate that in mice, the *Gclc* gene is also a target for Nrf2-mediated transcriptional regulation. These studies have revealed that MREs are present in the promoter of *Gclc* and that a strongly reduced basal expression of *Gclc* is observed in *Mtf-1* knock-out mice (Lichtlen *et al.*, 2001). To verify if MTF-1 plays a role in the activation of *Gclc* in the model under study, the silencing of *Mtf-1* was also performed. If *Mtf-1* is silenced, no effect is found on *Gclc* transcription, while strong effects are exerted on the basal expression, as well as on the zinc-mediated induction of typical MTF-1 target genes, such as *Mt1a* and *Znt1* (Andrews, 2001; Heuchel *et al.*, 1994; Langmade *et al.*, 2000). Therefore, in the cells under study the NO-mediated transcriptional regulation of *Gclc* is dominantly regulated by Nrf2.

Multiple mechanisms have been proposed to explain the inactivation of Keap-1 by electrophiles and the consequent Nrf2 release. Nrf2 can inactivate Keap-1 indirectly, by serine-phosphorylation, or directly, by interaction with specific cysteines (Itoh *et al.*, 2004). Recently, a zinc cluster has been identified in Keap-1 (Dinkova-Kostova *et al.*, 2005). On the basis of *in vitro* structural studies, it has been proposed that the presence of zinc within the zinc cluster is necessary for the inhibitory activity of Keap-1 and, therefore, electrophiles that are capable of releasing the zinc from this cluster might, indeed, inactivate Keap-1 (Dinkova-Kostova *et al.*, 2005). Provided that these *in vitro* observations will be confirmed in cell culture systems and *in vivo,* the participation of

zinc in Nrf2-activation would become central. Thus, zinc, as signaling molecule, might play a central role in pathways controlling the stress response.

The zinc sensor transcription factor MTF-1 is a target of nitric oxide

Highly conserved proteins able to sense little variations of labile zinc have been isolated from organisms ranging from bacteria to mammals (Andrews, 2001; Outten *et al.*, 2001). One of those is MTF-1, which regulates the expression of genes responsible for zinc homeostasis, such as the genes for the zinc-binding protein metallothionein and for the zinc exporter *Znt1* (Andrews, 2001; Heuchel *et al.*, 1994; Langmade *et al.*, 2000). MTF-1 contains six zinc finger structures, of which the first binds zinc with low affinity. After activation through zinc binding, MTF-1 is translocated from the cytoplasm into the nucleus, where it binds to the MREs present on the promoter of its target genes (Saydam *et al.*, 2001; Smirnova *et al.*, 2000). The present study confirms that the expression of *Znt1* is fully MTF-1 and zinc dependent, since the silencing of MTF-1 or the treatment with the zinc-chelator TPEN produce strong down-regulation of *Znt1*.

In contrast to our understanding of the mechanisms by which MTF-1 senses zinc ions, we know very little about how it senses non-metal inducers, such as NO. It has been shown that H_2O_2, heat shock, and low pH activate the translocation of MTF-1 into the nucleus. However, among the non-metal inducers, only H_2O_2 activates the MTF-1 binding to the MREs and the expressional activation (Dalton *et al.*, 1996; Saydam *et al.*, 2001). It is conceivable that oxidants may cause redistribution of zinc in the cell leading to the activation of MTF-1, or utilise specific co-activators of MTF-1 and/or activate signal transduction cascades that activate MTF-1 via post-translational modifications, such as phosphorylation (LaRochelle *et al.*, 2001; Palmiter, 1994; Spahl *et al.*, 2003; Stitt *et al.*, 2006).

Interestingly, under the experimental conditions used in this study, NO induces the expression of MTF-1 target genes. The observed induction involves the activation of MTF-1. In fact, after the silencing of MTF-1, the NO-induced expression of *Znt1* and the 6-hour peak of transcription of *Mt1a* are fully abrogated. In cytokine-treated cells, the NO-dependent transcriptional induction is also abrogated by MTF-1 silencing, which confirms the results obtained using exogenously applied NO.

According to the results presented in this study, NO induces the activation of MTF-1 via labile zinc redistribution. The present study demonstrates that the NO-mediated induction of *Znt1* is not only MTF-1-dependent but also strictly zinc-dependent. In fact, if the zinc chelator TPEN is added during the treatment with DETA/NO, no induction of *Znt1* is measured, confirming a role of labile zinc in the NO-mediated activation of MTF-1. Even if the addition of 3.3 µM TPEN does not affect either the basal levels of *Mt1a* mRNA, or the NO-mediated activation of *Mt1a* mRNA, greater concentrations of TPEN (5 µM) decrease the basal levels of *Mt1a* mRNA, as well as fully inhibit the NO-mediated activation. These results confirm previous findings obtained in mouse aortic endothelial cells (Spahl *et al.*, 2003). The fact that the transcription of *Mt1* is affected only by greater concentration of TPEN could be related to the activation of back-up mechanisms. In fact, metallothionein is the most important zinc storage protein and plays a central role, not only in zinc homeostasis, but also in the control of the antioxidant response.

Since MTF-1 itself contains zinc finger structures, the possibility that oxidants would target also the cysteines belonging to MTF-1 exists. If this would be the case, the activation of MTF-1 via zinc redistribution could not take place. It has been proposed that a system able to "repair" the oxidized -SH group should exit in the cells. A putative candidate for this function is the thioredoxin reductase system, composed of the protein thioredoxin, the enzyme thioredoxin reductase and NADPH. It has been shown that the thioredoxin system is able to restore the -SH groups of protein such as tubulin (Landino *et al.*, 2004), as well as can release NO from GSNO (Nikitovic *et al.*, 1996). Further research is needed to investigate the molecular mechanism for the activation of target molecules by oxidants- and by NO-mediated zinc redistribution.

Zinc supplementation exerts anti-inflammatory effects in endothelial cells

Atherosclerosis is considered as an inflammatory disease (Libby, 2002; Stocker *et al.*, 2004). It has been shown that inflammatory mediators induce the recruitment of inflammatory cells and contribute to the production of oxidative stress by inducing the expression of oxidative enzymes, mainly via the activation of the transcription factor NFκB(Wassmann *et al.*, 2004). The activation of NFκB may contribute to the onset of endothelial dysfunction and vascular disease (Rainer de Martin *et al.*, 2000). In fact,

activated NFκB has been identified in endothelial cells, smooth muscle cells, and macrophages within human atherosclerotic lesions (Bourcier *et al.*, 1997; Brand *et al.*, 1996). In addition, in atherosclerosis-prone mice, NFκB is activated in response to a high fat, high cholesterol diet (Liao *et al.*, 1993). Moreover, the formation of atherosclerotic lesions in mice has been correlated to the induction of inflammatory genes and activation of transcription factors belonging to the NFκB family (Liao *et al.*, 1994).

In the present study, the induction of iNOS was considered as a marker of endothelial cell activation and, particularly, of NFκB activation(Xie *et al.*, 1994). The present study shows that under pro-inflammatory conditions zinc supplementation decreases NO production in endothelial cells. Since a decrease of iNOS at the protein as well as the mRNA level was observed, therefore zinc inhibits the cytokine induced iNOS expression. Similar results were previously obtained in mouse keratinocytes (Yamaoka *et al.*, 2000). In rat intestine zinc deficiency increase the expression of iNOS (Cui *et al.*, 2003). Zinc supplementation directly inhibits the cytokines-mediated activation of the iNOS promoter, as also confirmed by using a reporter cell line stably transfected with the human iNOS promoter.

The inhibitory effect exerted by zinc on NFκB activation has been extensively demonstrated, and the participation of MT in this inhibitory effect has also been proposed (Connell et al., 1997; Hennig et al., 1999; Jeon et al., 2000; Kim et al., 1999a; Kim et al., 2003; Kim et al., 1999b). Therefore, zinc-mediated inhibition might represent one of the feedback mechanisms controlling the inflammatory response (Fig. 30).Under pro-inflammatory conditions, the NFκB activation produces endothelial activation corresponding to the expression of inflammatory genes, such as pro-inflammatory cytokines, and contributing to the propagation of the inflammatory response. In activated endothelial cells the expression of iNOS is induced and high-output synthesis of NO is produced. Then, iNOS-derived NO may induce zinc redistribution from zinc clusters. Since it has been shown that zinc inhibits NFκB activation and/or binding to the DNA, NFκB might be a target of NO-mediated zinc redistribution (Jeon et al., 2000; Shumilla et al., 1998). If this is the case, the NO-mediated zinc redistribution might contribute to limiting an excessive propagation of the inflammatory response by inhibiting or limiting endothelial cell activation (Fig. 30).

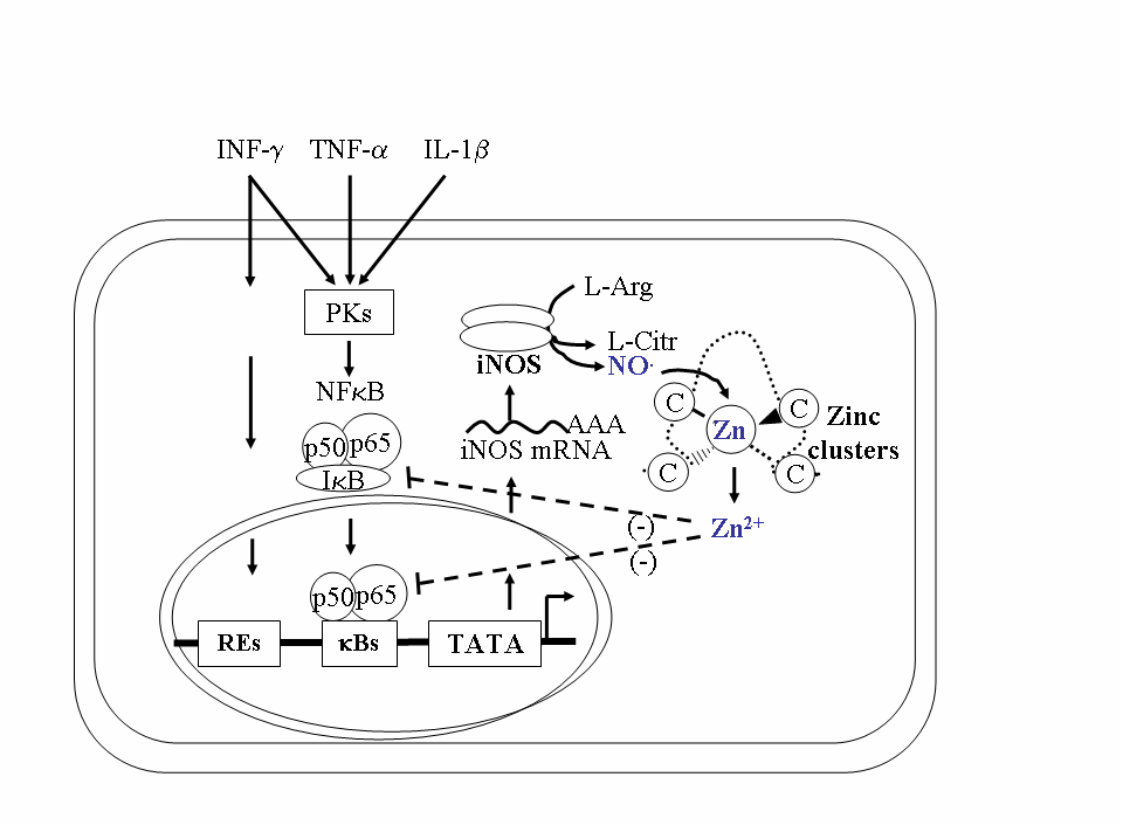

FIGURE 30 Zinc-mediated inhibition of iNOS expression as a negative feedback mechanism controlling the inflammatory response.

Under pro-inflammatory conditions the NFκB activation produces the expression of inflammatory genes such as pro-inflammatory cytokines, which contribute to the propagation of the inflammatory response. In activated endothelial cells, iNOS is also induced and high-output NO synthesis is generated. Then, iNOS-derived NO induces zinc release from zinc clusters. The zinc released could then counteract an excessive activation of the response by limiting NFκB activation.

Interestingly, it has been shown that not only does zinc supplementation inhibit NFκB activation, but also that zinc deficiency exerts a permissive effect on the expression of inflammatory genes and exacerbates chronic inflammation (Connell *et al.*, 1997; Hennig *et al.*, 1999). Therefore, the maintenance of an adequate zinc status could be an important clinical strategy to prevent the development of cardiovascular diseases (Beattie *et al.*, 2004).

Uncovering the role of inflammatory pathways in the pathogenesis of atherosclerosis has raised the possibility that future treatments may target effectors of inflammation directly to add to the benefit of current therapies (Call *et al.*, 2004). In addition, the anti-inflammatory properties of drugs currently used in the cure and/or

prevention of atherosclerosis have been recently uncovered(Call *et al.*, 2004; Martin *et al.*, 1994). In particular, the inhibitory effects exerted by anti-inflammatory glucocorticoids, saliciliate, and statins on NFκB activation have been considered as a part of their mechanism of action.(Hishikawa, 2002; Pande *et al.*, 2005) The clinical application of specific NFκB activation inhibitors appears infeasible, because of the central role played by NFκB in the in a variety of cellular functions. However, the flourishing discovery of novel, low-molecular weight inhibitors shows that the search for molecules decreasing or inhibiting NFκB activation has been actively undertaken by the pharmaceutical industry (Meng, 2004; Pande *et al.*, 2005).

Summary and conclusions

NO and its chemically-related products show different reactivity towards various targets, exerting pleiotropic effects in biological systems (Kröncke *et al.*, 1997; Kröncke *et al.*, 1998; Kröncke *et al.*, 1995). The NO-mediated pathways are classified as GC-dependent pathways, in which NO reacts with the Fe^{2+}-heme of GC and activates the enzyme to produce cGMP and GC-independent pathways, in which NO acts primarily through S-nitrosation of target molecules and the formation of S-nitrosothiols (Denninger *et al.*, 1999; Jia *et al.*, 1996; Stamler, 1994; Stamler *et al.*, 1992). The S-nitrosation of cysteine residues forming zinc-sulfur clusters in proteins results in zinc release (Berendji *et al.*, 1997b; Kröncke, 2001a; Tartler *et al.*, 2000). The observed destruction of zinc sulfur clusters was initially considered as a damaging effect of NO (Kröncke *et al.*, 1994). Since the NO-mediated destruction of zinc sulfur clusters is reversible (Kröncke *et al.*, 2002), it has been proposed that it could represent a mechanism of transcriptional regulation (Jacob *et al.*, 1998; Kröncke, 2003; Kröncke, 2001b; Kröncke *et al.*, 2002). In addition it has been shown that oxidants could induce the transfer of zinc from zinc-clusters to target enzymes regulating their activity (Jacob *et al.*, 1998). Therefore, it has been hypothesised that the zinc released by NO exerts a signalling role, similarly to Ca^{2+}.

A growing amount of evidence, mainly obtained through experiments of zinc supplementation and/or depletion, recognizes zinc as a signaling molecule in eukaryotic cells, because of its activity on membrane receptors, transduction proteins, or transcription factors (Beyersmann *et al.*, 2001). In particular, highly specific zinc sensor

molecules, able to sense small variations of zinc concentration, have been isolated in organisms ranging from bacteria to mammals (Andrews, 2001).

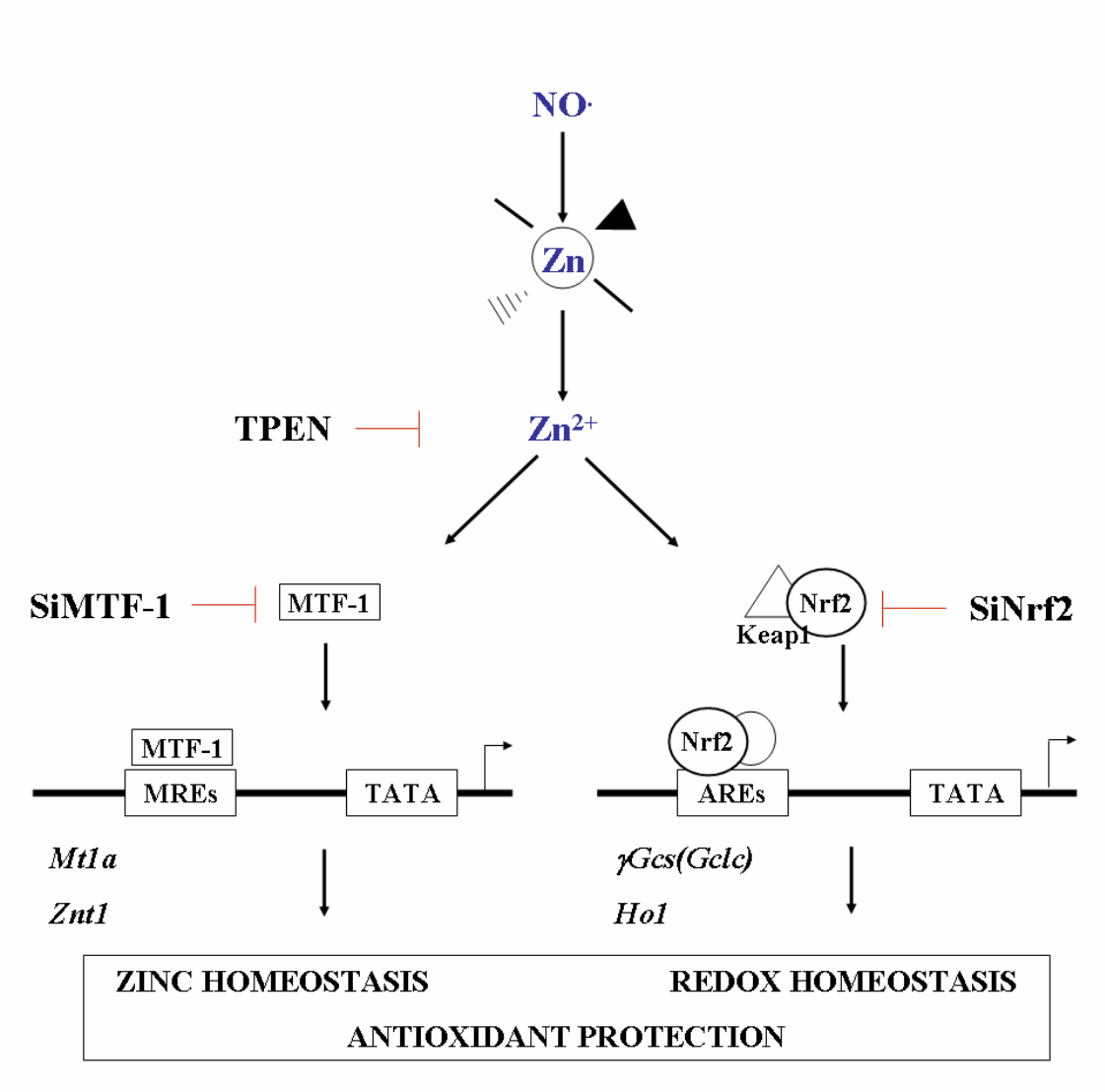

FIGURE 31. Targets of the NO-mediated zinc-release.

NO controls the expression of Gclc via the transcription factor Nrf2. The NO-mediated induction of Gclc is fully zinc-dependent and controls the GSH levels of endothelial cells, as well as their susceptibility to oxidative stress.
NO induces the expression of MTF-1 target genes in a zinc-dependent fashion. The zinc sensor MTF- is responsible for the control of cellular zinc homeostasis and is involved in the cellular response to oxidative stress.

A second-messenger like function of zinc is feasible only if an endogenous mechanism for zinc release exists in the cells. Provided that *in vivo* the zinc released by NO effectively exerts a signaling function and is a reversible mechanism (as some publication have indicated), the NO-mediated S-nitrosation of zinc clusters would represent the endogenous mechanism responsible for the intracellular release of zinc and for its signaling role (Kröncke *et al.*, 2002; Spahl *et al.*, 2003; St Croix *et al.*, 2002; Stitt *et al.*, 2006). Such sequence of events would constitute a novel signaling pathway.

The present study identified two distinct targets of the NO-mediated zinc release, comprising the Nrf2-target gene *Gclc* and MTF-1 target genes (Fig. 31). The NO-mediated induction of *Gclc* is completely zinc dependent and controls the GSH levels of endothelial cells, as well as their susceptibility to oxidative stress, as discussed. This pathway may play a major role in the response of endothelial cells to oxidative stress under pro-inflammatory conditions. Moreover, it may provide a reason for the possible correlation between zinc deficiency and increased susceptibility to endothelial dysfunction and cardiovascular diseases, as proposed recently (Beattie *et al.*, 2004; Lee *et al.*, 2005a).

Another possible target of the zinc released by NO is the zinc sensor MTF-1, responsible for the control of cellular zinc homeostasis and involved in the cellular response to oxidative stress, via regulating the expression of metallothioneins (Fig. 31). The expression of MTF-1 target genes might be considered a sort of feedback mechanism to restore homeostasis after zinc release. In addition, given the central role played by zinc and metallothionein in the response to oxidative stress (Maret, 2003; Palmiter, 1998), the activation of MTF-1 target genes should be considered as part of the NO-mediated defencive response.

An interrelation between the Nrf2 pathway and the MTF-1 pathway can be assumed. In fact, both Nrf-2 and MTF-1 driven transcription are activated by heavy metals (e.g., zinc) and by oxidative stress (Zhang *et al.*, 2003). In addition, active ARE elements are also present in the promoter of *Mt1*. Future research should be addressed to delineate the interrelations between the pathways governed by MTF-1 and Nrf2 and the role of NO-mediated zinc release in their activation.

In addition, also the transcription factor NFκB might be a target of NO-mediated zinc redistribution. For instance, zinc supplementation limited endothelial cells activation, as determined through the analysis of promoter activation of the expression and of the enzymatic activity of iNOS, a well known NFκB target gene. Therefore, the zinc-mediated inhibition of NFκB might represent a feedback mechanism that limits an excessive propagation of the inflammatory response by inhibiting or reducing endothelial cell activation.

In conclusion, the objective of this study was to analyse the long-term protective activity of iNOS derived NO in endothelial cells and the involvement of zinc in the NO-mediated effects. In particular, this study focused on the hypothesis that NO-mediated zinc redistribution could be a novel mechanism of signal transduction leading to cellular protection against oxidative stress. This study shows that iNOS-derived NO protects endothelial cells against oxidative stress via activating the transcription of protective genes in a zinc dependent fashion. Two targets of NO-mediated zinc release has been identified here, i.e., Nrf2 and MTF-1. In fact, NO induces the expression of Nrf2-target genes, such as *HO-1* and *Gclc*, which play a major role in mounting the adaptive response to noxious stimuli. This produces an increase of GSH levels of the cells, which substantially contributes to protecting the cells against oxidative stress. Moreover, NO induces the expression of MTF-1 target genes, which not only regulate zinc homeostasis, but are also involved in the antioxidant protection of the cells. A third putative target of NO-mediated zinc redistribution could be the transcription factor NFκB, responsible for endothelial activation. Thus, by inhibiting the NFκB activation zinc might exert a sort of feedback mechanism that limits the excessive propagation of the inflammatory response.

Since chronic inflammation and particularly the induced production of ROS plays a major role in the onset of endothelial dysfunction and vascular diseases, uncovering the role of the central players of inflammatory pathways, such as iNOS-derived NO and zinc, could contribute to elaborate new approaches in the prevention and cure of endothelial dysfunction. For example, the maintenance of the endothelium in an adequate zinc status might contribute to limiting oxidative stress, to permitting a correct functionality of the

endogenous defence mechanisms (such as the NO-mediated protection), and to limiting an excessive activation of the endothelium during inflammation.

Further research is needed to, first, investigate the molecular mechanism for the activation of target molecules by NO-mediated zinc redistribution and, second, to determine the relevance of this novel signalling pathway *in vivo*.

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CURRICULUM VITAE

PERSONAL DATA

Age:	28
Nationality:	Italian
Date of Birth:	Sept 4, 1977
Place of Birth:	Milano, Italy
Marital Status:	Single
Spoken Languages	**Italian** (mother tongue); **English** (fluent); **German** (very good to fluent); **French** (good)

EDUCATION

Sept.1983-June 1988	Primary School (5 years) in Milano, Italy (with the best grade)
Sept.1988-June 1991	Secondary School (3 years) in Milano, Italy (with the best grade).
Sept 1991-June 1996	High School Liceo Ginnasio "A. Manzoni" in Milano, Italy
June 1996	**Maturitá Classica** (equivalent to A-Level) (July 1996) **Grade: 52/60**

UNIVERSITY EDUCATION

Sept 1996-Dec. 2001	Study of **Biotechnology applied to Pharmacy** (5 years)**,** within the Faculty of Pharmacy at the University of Milan, Milano, Italy
Jun. 2000-Mar. 2002	Scientific work and preparation of the dissertation in the laboratory for the study of Atherosclerosis, Milano, Italy, under the supervision of PD Dr. Giulia Chiesa.(Group Leader **Dr. Prof Cesare Sirtori)**
March 2002	**Laurea** in **Biotechnology applied to Pharmacy (**equivalent to Master of Science**)** **Grade: 110/110 cum laude** **Dissertation:** The apolipoprotein A-I and the mutant apoA-I Milano: evaluation of anti-atherogenic effect in a transgenic animal model
Mar. **-**Oct.2002	Research fellow at the Cystic Fibrosis Unit- DIBIT (S. Raffaele Hospital), Milano, Italy
From Nov. 2002	**PhD student** in Life Science (Candidate Doctor in Rerum Natura) at Heinrich-Heine-University, Duesseldorf, Germany. Scientific work and preparation of the dissertation in the Research Group Immunobiology, Heinrich-Heine-University, Düsseldorf, Germany (Group Leader **Prof. Dr. Victoria Kolb-Bachofen**)

RESEARCH EXPERIENCE

June 2000 – March 2002
Laboratory for the study of Atherosclerosis, Milano, Italy (Group Leader **Dr. Giulia Chiesa)**
Position: Graduating student
Dissertation: The apolipoprotein AI and the mutant apoAI Milano: evaluation of anti-atherogenic effect in a transgenic animal model
Evaluation of the anti-atherogenic effect of the human protein apoAI Milano in mice under high fat diet conditions through biochemical and immunological methods.
Creation of a suitable double transgenic mice model for studying atherosclerosis starting from two transgenic mice lines. Genotyping (by PCR and western blot) and maintenance of the line.

March 2002 – October 2002
Cystic Fibrosis Unit- DIBIT (S. Raffaele Hospital), Milano, Italy
Position: Research fellow
Project title: Establishment and analysis of Animal Models of acute *Pseudomonas Aeruginosa* pulmonary infection.
Analysis of role of white blood cells or alveolar macrophages on pulmonary acute infection made by intra-tracheal injection of P.Aeruginosa in C57BL/6 mice
Genotyping and maintenance of a knock in mice line (CFTR K-in).

Nov. 2002 – Present time
Research Group Immunobiology, Heinrich-Heine-University, Düsseldorf, Germany (Group Leader **Prof. Dr. Victoria Kolb-Bachofen**, Assistant Professor **Dr. Christoph Suschek**)
Position: PhD student
Project title: A new stress signalling pathway involving nitric oxide and zinc leads to inherent cellular protection against oxidative injury.

AWARDS

Research fellowship (April 2002-November 2002)
Institute for Experimental Treatment of Cystic Fibrosis, S. Raffaele Hospital, Milano, Italy.

PUBLICATIONS AND PRESENTATIONS

Publications:

Parolini C, Chiesa G, Gong E, Caligari S, **Cortese MM**, Koga T, Forte TM, Rubin EM.Apolipoprotein A-I and the molecular variant apoA-I(Milano): evaluation of the anti-atherogenic effects in knock-in mouse model. **Atherosclerosis.** 2005 Dec;183(2):222-9

Cortese MM and Kolb-Bachofen V. Efficient siRNA transfection and low cytotoxicity allow study of nitric oxide-mediated effects in primary endothelial cells. **QIAGEN News,** 2005 Nov;3, e12

Manuscripts in preparation:

Cortese MM, Suschek CV, Wetzel W., Kolb-Bachofen V.
High-output synthesis of nitric oxide protects endothelial cells against oxidative stress in a zinc dependent fashion via modulation of the transcription factor Nrf2

Cortese MM, Suschek CV, Kolb-Bachofen V.
Zinc exerts antioxidant activity on endothelial cells via increasing the total cellular GSH levels.

Oral and poster presentations:

Cortese MM, Suschek CV, Kolb-Bachofen V. Nitric oxide and zinc cooperation in transcription modulation. **Oral presentation** at the "First joint French-German NO-meeting (NO: from chemistry to therapeutics)", Strasbourg, France, October 2-4, 2003

Cortese MM, Suschek CV, Kolb-Bachofen V. Nitric oxide cytoprotection depends on the presence of zinc. **Oral presentation** at the 10.Workshop "Mechanismen der Zell-und Gewebeschädigung" (Mechanisms of cell and tissue protection), Xanten, Germany, December 11 -13, 2003

Cortese MM, Suschek CV, Kolb-Bachofen V. A new stress signalling pathway deduced from the integrated study of effects mediated by zinc(II) ions and nitric oxide. **Poster presentation** in the workshop on chemical biology Aventis [i]lab initiative "Molecules as modulators: system biology challenges Chemistry", Eberbach Monastery, Eltville, Germany, January 29-31, 2004,

Cortese MM, Suschek CV, Kolb-Bachofen V**.** A new stress signalling pathway involving nitric oxide and zinc leads to inherent cellular protection against oxidative injury. **Oral presentation** at the first international conference "Molecular research in environmental medicine" Düsseldorf, Germany, March 18-20th, 2004

Cortese MM, Suschek CV, Kolb-Bachofen V**.** A direct expressional link between nitric oxide synthesis, zinc and glutathione: new stress signaling pathway. **Oral presentation** at 9.NO-forum der deutschsprachigen Länder**,** Mainz, October 5-6 2004

Cortese MM, Suschek CV, Kolb-Bachofen V. A direct expressional link between high-output nitric oxide synthesis, zinc and glutathione: new stress signaling pathway**. Oral presentation** at 2.Xantener gespräch zur Medizinisch-Biologishen Chemie "Von molekulare Wechselwirkungen zu Lebensvorgängen", Xanten, Germany, December 9-11, 2004

Cortese MM, Suschek CV, Liebmann J., Guo Z., Kolb-Bachofen V. High-output nitric oxide synthesis during inflammation protects endothelial cells from oxidative stress via a zinc-dependent signaling pathway. **Oral presentation** at the 13.Atherosklerose-Symposium, Erfurt, 17-18 June 2005

Cortese MM, Suschek CV, Kolb-Bachofen V. High-output nitric oxide synthesis increases cellular GSH levels and protects endothelial cells from oxidative stress via a zinc dependent signaling pathway. **Oral presentation** at the 10.NO-forum NO-forum der deutschsprachingen Länder, Köln, Octrober 6-7th, 2005

Cortese MM, Suschek CV, Kolb-Bachofen V. High-output nitric oxide synthesis increases cellular GSH levels and protects endothelial cells from oxidative stress via a zinc dependent signalling pathway. **Oral presentation** at 11.Workshop "Mechanismen der Zell-und Gewebeschädigung" (Mechanisms of cell and tissue protection), Xanten December 8 -10, 2005