Role of Cyclic Nucleotides in the Regulation of the Expression of Angiotensin II-Type 2 Receptors

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Tag der mündlichen Prüfung

DETECTED TO MY LATE SISTER AZIZA IBRAHIM EL AGOURI
MY GOD BLESS HER SOUL

Abbreviations

A	adenosine
A260	absorbance at 260 nm
A280	absorbance at 280 nm
A595	absorbance at 595 nm
ACE	angiotensin- converting enzyme
ACE2	angiotensin- converting enzyme releated carboxypeptidase
AC	adenylyl cyclase
Ang I	angiotensin I
Ang II	angiotensin II
ANOVA	analysis of variance
AT1 receptor	angiotensin II receptor type 1
AT2 receptor	angiotensin II receptor type 2
APS	ammoniumpersulfate
AP-1	activating protein -1
AkT	protein kinase B
AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
ATF2	activating transcription factor 2
bp	base pairs
BK	bradykinin
BKR-1	bradykinin receptor 1
BKR-2	bradykinin receptor 2
BSA	bovine serum
BAECs	bovine aortic endothelial cells
BW	body weight
BH_4	tetrahydrobiopterin
bENDs	brain endothelial cells
8- Br-cGMP	8- Bromo-guanosine 3, 5 cyclic monophosphate
cGMP	3'-5'-cyclic guanosine monophosphate

cAMP	3'-5'-cyclic adenosine monophosphate
CO	carbon monoxide
CO2	carbone dioxide
CRP	C – reactive protein
Ca^{2+}	bivalent calcium ion
C57B1/6	C57 black 6 laboratory mouse
C101A-eNOS-tg	transgenic mouse with an overexpression of a destabilized variant of eNOS
$CaCl_2$	calcium chloride
Ca. NO	catalogue number
CaM	calmodulin
cDNA	complementary DNA
CHF	Congistive Heart Failure
DEA/NO	Diethylamine NONate
DETA/NO	Diethylenetriamine /nitric oxide
DNA	Deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNA	desoxyribonuclec acid
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetra acetic acid
ERK	extracellular signal regulated kinase
eNOS	endothelial nitric oxide synthase
EDRF	endothelial derived relaxation factor
EGFR	epidermal growth factor receptor
ESC	Europien society of cardiology
et al	Latin: et alii (and others)
eNOS-ko	eNOS-knock-out mice
eNOS-tg	transgenic mouse with an endothelial specific overexpression of eNOS
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
G	guanosine

GC	Guanylyl cyclase
GAPDH	Glyceraldehyde 3 – phosphate dehydrogenase
GPCR	G- protein coupled receptor
Gln	Glutamine
Gly	Glycine
GTP	Guanosine triphosphate
HUVEC	human umbilical vein endothelial cell
His	Histidine
HF	Heart Failure
Hsp27	heat shock protein 27
iNOS	Inducible nitric oxide synthase
IP_3	Inositol-1,4,5-trisphosphat
IgG	Immunoglobulin G
Int	integration
i.p.	intraperitoneal
JAK	Janus activated kinase
K_2HPO_4	dipotassium hydrogen phosphate
kb	kilo base pairs
KCl	potassium chloride
kDa	kilodalton
KKS	kallikrein kinin system
L-NA	Nω-Nitro-L-arginine
L-NAME	L Nitroarginin- methyl ester
L-NMA	N^G-monomethyel-L-arginine
L- Arg	L- arginine
L- Cit	L- citrulline
$MgSO_4$	magnesium sulfate
mRNA	messenger RNA
MAPK	mitogen activated protein kinase
MKKs	MAP kinase kinase
MAPKAPK2	MAPK activated protein kinase 2

MAPKAPK3	MAPK activated protein kinase 3
6MB-cAMP	N^6- Monobutyryladenosine- 3', 5'- cyclic monophosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NaCl	sodium chloride
NaN_3	sodium azide
$NaNO_2$	sodium nitrite
Na+	monovalent sodium ion
NaF	sodium fluoride
NFKB	nuclear factor KB
NO	nitric oxide
NOS	nitric oxide synthases
nNOS	neuronal nitric oxide synthase
Na4P2O7	sodium diphosphate / sodium pyrophosphate
Na_2HPO_4	disodium hydrogen phosphate
$NaHCO_3$	sodium hydrogen carbonate
OD	optical density
ODQ	1 H- [1,2,4] oxadiazolo [4,3 - a] quinoxalin – 1 one
$ONOO^-$	Peroxynitrite
PCR	polymerase chain reaction
pH	negative decadic logarithm of the hydronium ion activity
PMSF	phenylmethanesulfonylfluoride
PAEC	porcine aortic endothelial cells
PAGE	polyacrylamide gel electrophoresis
PKG	cGMP - dependent protein kinase G
PKGI	cGMP - dependent protein kinase GI
PKGII	cGMP - dependent protein kinase GII
PKA	cAMP dependent protein kinase A
P38 MAPK	P38 mitogen – activated protein kinase
8-pcpT- cGMP	8-pCPT-cGMP 8(4- Chlorophenylthio) guanosine- 3', 5'- cyclic monophosphate
PDE – V	phosphodiesterase – V

PDGF	platelet derived growth factor
PAH	Pulmonary arterial hypertension
PRCP	prolycarboxypeptidase
Rp-8-pCPT-cGMP	Rp-8- (para- chlorophenylthio) guanosine-3',5'cyclic monophosphate
pH	negative decadal logarithm of $H3O^{+}$concentration
PIC	protease inhibitor cocktail
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinyliden difluoride
RAAS	renin angiotensin aldosterone system
RT	room temperature
RNA	ribonucleic acid
ROS	reactive oxygyen species
Rpm	Round per minute
RNA	ribonucleic acid
SOD	superoxide dismutase
SDS	sodium dodecyl sulfate
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SDS	sodium dodecyl sulphate
SEM	Standard Error Mean
sGC	soluble guanlate cyclase
SiL.	sildenafil
SMC	smooth muscle cells
SERCA	sarco/endoplasmatic reticulum Ca^{2+} -ATPase
SB 203580	(4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole)
Tris	tris (hydroxymethyl) aminomethane
TBS	tris buffered saline
TNFα	tumour necrosis factorα
Tween® – 20	polyoxyethlene (20) sorbitan monolaurate
TAE	tris-acetate-EDTA
TE	tris-EDTA
TyK2	tyrosine kinase2

Tempol®	4-Hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl
USA	United States of America
VSMC	vascular smooth muscle cell
v/v	volume / volume

INTRODUCTION

1. Introduction

1.1 History of Vascular NO-CGMP System

1.1.1 Endogenous nitric oxide

In 1980, Furchgott and Zawadzki discovered that endothelial cells produce an endothelium-derived relaxing factor (EDRF), in response to stimulation by acetylcholine, in vessels with intact endothelium (1, 1). In this study, vascular relaxation by acetylcholine and a number of other agonists were found to be dependent on the presence of endothelial cells, which, when stimulated by the agonists, release a diffusible, labile, non prostanoid substance that act on vascular smooth muscle (VSM) to produce relaxation. In 1987 Palmer et al and Ignarro et al separately, proved the EDRF to be nitric oxide (NO) (2),(3). A year later, Palmer also demonstrated that NO is synthesized from the amino acid L- arginine (4). All these work have contributed to the confirmation of NO as a signaling molecule in the cardiovascular system.

In 1992, the journal `Science' named NO as the molecule of the year. Six years later, Pfizer introduced Viagra as a drug with a mechanism of action (Phosphodiesterase inhibition) based on the NO-cGMP system, which revolutionized the management of erecticle dysfunction. In 1998, the importance of the NO discovery was recognized by awarding the Nobel price for Physiology and Medicine for the discovery of NO-cGMP signalling to the three US scientists Robert F. Furchgott, Louis Ignarro and Ferid Murad. By now, NO has been implicated in the pathogenesis of diseases, ranging from hypertension to septic shock and dementia (5, 5).

1.2 Biosynthesis of Nitric Oxide

1.2.1 Nitric Oxide synthase

Many reports supported the idea that mammalian cells synthesized nitric oxide (2). In 1989, it was reported that endothelial cells (EC) respond to mediators releasing EDRF such as ATP or bradykinin with a Ca^{2++}-dependent increase in cGMP (6). At present NO is synthesized enzymatically, in a two step process, via the formation of the N- hydroxyl L-arginine. The first isolation of a NOS was reported from brain (7). This enzyme neuronal NOS (nNOS) is found constitutively in a variety of cells in endothelial cells, and neurons (8). Inducible NOS (iNOS) is expressed in numerous cells after several hours of exposure to cytokines and / or microbial

products (9). Subsequently, endothelial nitric oxide synthase (eNOS) was isolated from bovine aortic endothelial cells. The human genes for NOS isoforms are officially categorized in the order of their isolation and characterization, thus the human genes encoding nNOS, iNOS, and eNOS are termed NOS1, NOS2 and NOS3 respectively.

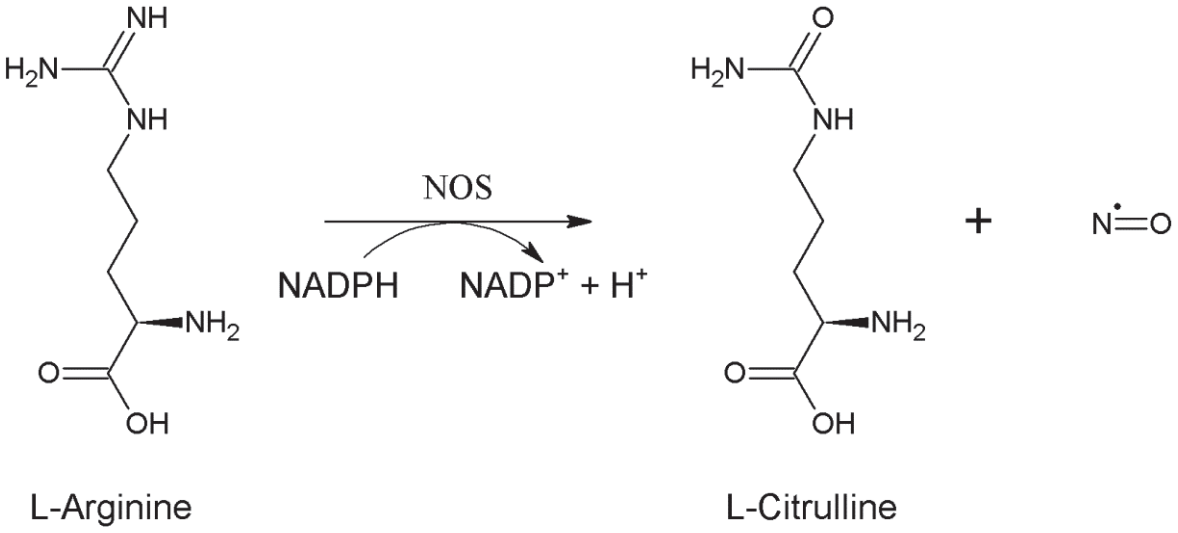


Figure 1.1
The formation of nitric oxide from L-arginine is carried out by the enzyme nitric oxide synthase. Endothelial homogenates form citrulline from L-arginine by a mechanism which is NADPH dependent and inhibited by L-NMMA (10, 10). NOS, in the presence of O_2 and the cofactors convert arginine to NO, with the formation of citrulline. Cofactors include NADPH, BH_4, FMN, and FAD. As NOS, cofactor, and L-arginine availability are all possible sites of pharmacologic intervention in this way. NO synthase from endothelial cells was inhibited by calmodulin-binding peptides and antagonists, an effect that was reversed by calmodulin and which suggests that the Ca^{2+} dependent stimulation of NO synthase in endothelial cells is mediated by calmodulin (11).

1.2.2 Structure and regulation of NOS isozymes

1.2.2.1 Structure of NOSs

The three distinct genes for the human neuronal, inducible and endothelial NOS isoforms have a similar genomic structure. NOSs exhibit a bidomain structure, N- terminal oxygenase domain containing binding sites for heme, BH4 and L- arginine is linked by a calmodulin (CaM) recognition site to a C- terminal reductase domain that contains binding sites for FAD,

FMN and NAPDH (12),(13). The oxygenase and reductase domains have been defined by limited proteolysis. The separate domains are catalytically active and reconstitution of the second step of NO synthesis has been achieved by combining the reductase and oxygenase domains of human eNOS and murine iNOS (14). L-arginine, NADPH and O_2 are substrates and NADP, citrulline and NO are products. However, they also have divergent regulatory properties. For the three isoforms, NO synthesis is dependent on the binding of the enzyme to calmodulin, a calcium regulatory protein. For eNOS and nNOS, the increase in resting intracellular Ca^{2+} concentrations (Ca^{2+}) is required to bind calmodulin, and consequently, to become fully activated, whereas iNOS is fundamentally different from eNOS and nNOS, as its activity is independent of Ca^{2+} (15).

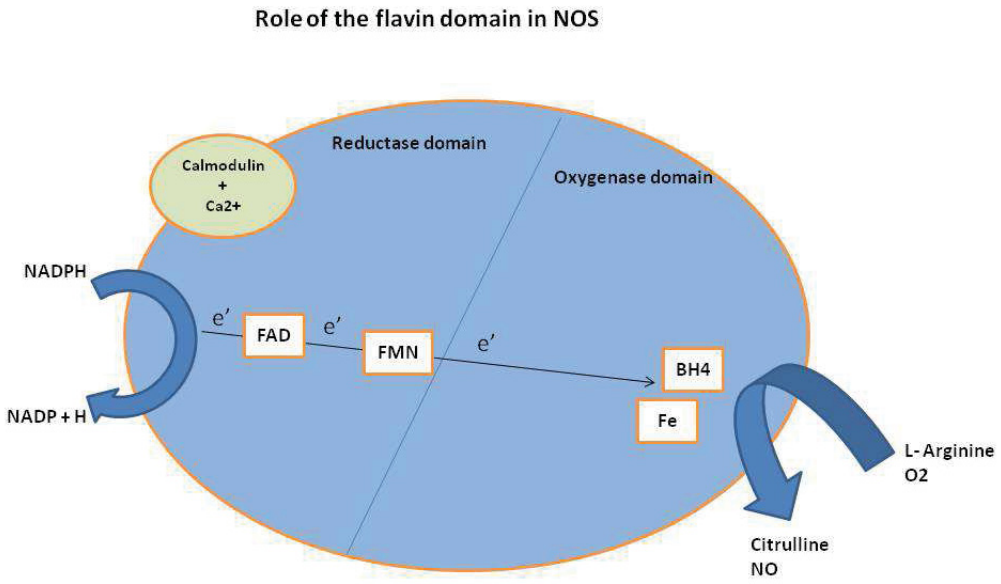


Figure 1.2

The formation of nitric oxide from L-arginine in the presence of O_2 and the cofactors. Cofactors include NADPH, BH_4, FMN, and FAD.

1.2.2.2 Regulation of NOSs

Cellular and tissue specific localization of the NOS isoforms can be regulated by transcriptional regulation. Calmodulin (CaM) was the first protein shown to interact with NOS and is necessary for the enzymatic activity of all three isoforms. The Ca^{2+} dependence of NO synthesis distinguishes the NOS isoforms, nNOS and eNOS having a much higher Ca^{2+} requirement than iNOS. CaM binding increases the rate of electron transfer from NADPH to the reductase domain flavins. Phosphorylation of the nNOS and eNOS isoforms has an effect on NOS activity. Fluid shear stress elicits phosphorylation of eNOS and an increase in calcium independent NOS activity (16). Studies showed that Ser1179 of eNOS is phosphorylated by protein kinase AKT which results in an increase in electron flux through the reductase domain and an increase in NO production (17). In 1999 Dimmeler and her colleagues demonstrated that the serine/threonine protein kinase AKT/PKB mediates a Ca^{2+} independent activation of eNOS, leading to increased NO production. Inhibition of the phosphatidylinositol-3-OH kinase/AKT pathway or mutation of the AKT site on eNOS protein at serine 1177 attenuates the serine phosphorylation and prevents the activation of eNOS. Mimicking the phosphorylation of Ser 1177 directly enhances enzme activity and alters the sensitivity of the enzyme to Ca^{2+}, increasing its activity at sub-physiological concentrations of Ca^{2+}. Thus, phosphorylation of eNOS by AKT represents an important Ca^{2+}-independent regulatory mechanism for activation of eNOS (18).

The serine/threonine protein kinase AKT can directly phosphorylate eNOS on serine 1179 and activate the enzyme, leading to NO production, while mutant eNOS (S1179) is resistant to phosphorylation and activation by AKT. Moereover, using adenovirus-mediated gene transfer, activated AKT increases basal NO release from endothelial cells (EC), and activation-deficient AKT attenuates NO production stimulated by vascular endothelial growth factor. Thus, eNOS is described as a AKT substrate linking signal transduction by AKT to the release of the NO (19).

1.3 Nitric Oxide Signaling

1.3.1 Cyclic Guanosine Monophosphate (cGMP)

Following the discovery of cAMP, research into the newly discovered second messenger cGMP was very active, with measurement of cGMP formation in many tissues and tissues homogenates, in response to numerous hormones and other agents. However molecular mechanisms of the second messenger formation were lacking, until the enzymes that generate cGMP were identified and characterized. A physiological function for cGMP was eventually found in experiments with preparation of smooth muscle cells (SMC), where an addition of inorganic nitrogen compounds as well as classical vasorelaxants, such as nitroglycerin or nitroprusside, to these tissue samples increased cGMP and caused relaxation (20),(21).

1.3.2 Synthesis of cGMP

There are two major families of guanylyl cyclase (GC), the particulate associated enzymes which are actually transmembrane receptors GC within their intracellular domains, and the soluble type that is activated by NO (22, 23).

1.3.2.1 Guanylate Cyclase Receptors (GCR)

This type of GC has a unique as well as an interesting structure, the polypeptide is a transmembrane receptor containing an extracellular ligand binding domain. Within this subfamily of guanylyl cyclase, there are a number of distinct members, each with their own specific ligand(s) (24). GC (A) binds atrial natriuretic peptide and brain natriuretic peptide, and is located primarily in the heart. GC (B) is located in neural tissues, where it specifically binds the related natriuretic peptide CNP that may function as a neurotransmitter. GC (C) is the intestinal epithelial isotype, its natural ligands are guanylin and uroguanylin, and is it activated by the E - Coli heat stable enterotoxin STa (25).

1.3.2.2 NO stimulates sGC

Soluble guanylyl cyclase (sGC) is an enzyme that catalyses the formation of guanosine 3,5 – monophosphate (cGMP) from guanosine triphosphate (GTP), and is found in tissues throughout the animal kingdom. Soluble GC is the receptor for (NO) in vascular smooth muscle cell (VSMC).

In the cardiovascular system, NO is endogenously generated by an endothelial NO synthase (eNOS) from L-arginine, which activates sGC in the adjacent VSMC to increase cGMP levels and induce relaxation. NO plays a major role in the regulation of vascular tone and blood pressure (26, 27).

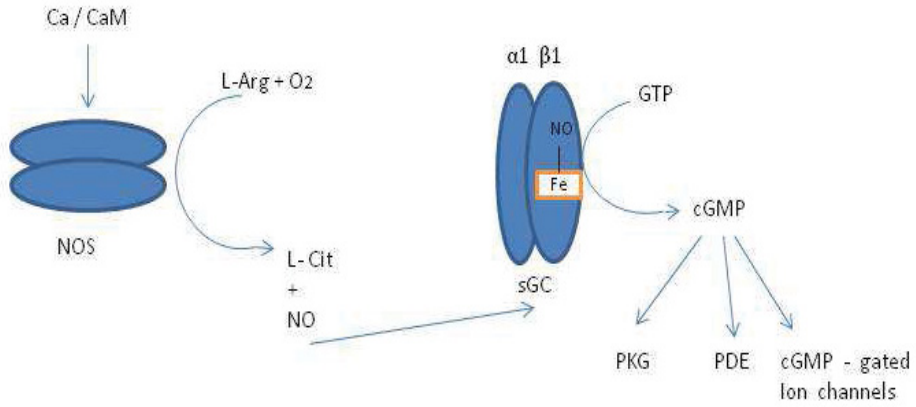


Figure 1.3 The nitric oxide / cyclic GMP signaling pathway

Ca2+ / calmodulin (CaM) complex binds nitric oxide synthase (NOS). NOS catalyzes the oxidation of L – arginine (L-Arg) to L – citrulline (L-Cit) and nitric oxide (NO). NO binds to the Fe II heme of α1β1 soluble guanylate cyclase (sGC) at a diffusion controlled rate. This binding event leads to significant increase in cGMP. cGMP then binds to and activates cGMP- dependent protein kinases (PKG), Phosphodiesterases (PDE) and ion- gated channels (28).

When NO released from the endothelium in response to physiologic stimuli, such as shear stress, NO binds to the normally reduced heme moiety of sGC, and increases the formation of cGMP from GTP, eventually leading to a decrease in intracellular calcium and vasodilation. Moreover, the NO-sGC-cGMP pathway is essential for controling a number of physiologic processes, including neuronal transmission, host defense, cell growth, and proliferation, and vascular and platelet homeostasis (29). Initial investigations into the role of NO were conducted, and it was found that nitrogen compounds such as sodium azide (NaN_3), sodium

nitrite ($NaNO_2$), hydroxylamine (NH_2OH), nitroglycerin ($C_3H_5N_3O_9$), and sodium nitroprusside (Na_2 $Fe(CN)_5NO$) were able to activate sGC. When tissues were homogenized and separated by centrifugation, GC activity was detected in particulate and soluble fractions. As NO was shown to rapidly activate sGC (27), it was hypothesized that the sGC activation may be due to the effect of NO or another substance that activate the enzyme. Moreover, these nitrogen-containing compounds were able to activate sGC, causing an increase in cGMP, and vascular relaxation. NO-sensitve GC is a heterodimer consisting of two different subunits termed α and β. The α_1 and β_1 subunits with molecular masses of 73 and 70 kDa, respectively, were cloned and sequenced. The α_3 and β_3 subunits of NO-sensitive GC represent human variants of the α_1 and β_1 subunits rather than different isoforms, and changes in the reading frame account for the differences in amino acid sequences (30). The occurrence of the α_2 subunit was demonstrated on the protein level in human placenta, and the β_2 subunits were identified as the dimerizing partners (31). Despite little sequence homology between the two α subunits in the N-terminal domains, no differences in catalytic activity, NO stimulation, or substrate affinity could be detected between the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers, the C-terminal peptide of the α_2 subunits defines the particular property of this subunits, the $\alpha_2\beta1$ heterodimer was shown to be localized to synaptic membranes (32). In accordance with a putative role in synaptic transmission, the highest expression of the α_2 subunits was found in brain. Furthermore, the β_1 subunit was detected at the presynaptic membrane of CA1 pyramidal cells, with nNOS being expressed in the postsynaptic side underlining the potential role of NO/cGMP signaling in the regulation of synaptic plasticity. Nevertheless, α_2 is not the only α subunit in brain; α_1 occurred in similar amounts. In all other tissues tested, the α_1 subunit was the major occurring α subunit (33). Like α_2 the β_1 subunit was identified by homology screening (34).

NO sensitive GC is a hemoprotein and presence of the prosthetic heme group is mandatory for the activation of the enzyme by NO (35). Removal of the heme group abolishes NO induced activation, which can be restored after reconstitution of NO sensitive GC with heme (36). The heme stoichiometry has been agreed to be one mole per mole GC heterodimer. The absorbance maximum at 430 nm is indicative for a five coordinated ferrous heme with a histidine as the axial ligand (37). In contrast to other hemoproteins such as hemoglobin or myoglobin, the heme of NO sensitive GC does not bind oxygen (38). The subunits α and β

subunits are both required for proper binding and orientation of the heme group. Sequential truncation showed that the subunits contribute unequally to heme binding with the β1 subunit playing a more important role (39), in addition, homodimers of the N terminal part of the β1 subunit expressed in bacteria were shown to bind heme in a manner similar to the wild type enzyme (40). The His-105 of the β1 subunit was identified as the heme coordinating residue (41). Mutation of this histidine led to the generation of an NO insensitive, heme depleted enzyme with intact basal activity. In addition, two conserved cysteines adjacent to the His-105 of the β1 subunit appear to play a role in the formation of the proper heme pocket. Mutation of these residues led to loss of enzyme bound heme and NO responsiveness, which could be regained after heme reconstitution (42).

A mong the three redox forms of NO, only the uncharged NO radical ($NO^{\cdot}$) has been shown to significantly activate NO sensitive GC (43). Activation of the enzyme by NO involves the binding to the enzyme's prosthetic heme group, which leads to the upto 200- fold activation of the enzyme (44). The heme group of NO sensitive GC exhibits an absorbance maximum at 431 nm, which is indicative of a five coordinated ferrous heme with a histidine as the axial ligand at the fifth coordinating position (37). The activation of NO sensitive GC, occur by binding of NO to the sixth coordination position of the heme results in a six coordinated NO-Fe^{2+}-His- complex.

1.3.2.3 Protein Targets for cGMP

1.3.2.3.1 Cyclic GMP dependent protein kinase

Regulation of cellular events by cGMP is achieved by the interaction of this nucleotide with several target proteins. One of the targets is a cyclic nucleotide- regulated protein kinase (PK), which is analogous to cAMP activation of the cAMP- dependent PK. The cGMP – regulated protein kinases could either be soluble or membrane enzymes. Protein Kinase G I (PKG I) is a soluble homodimer of 78- kDa subunit, two related types of PKGI (PKGIα and PKGIß) are comprised of monomers that arise from an alternative splicing of single gene, and differ only in N- terminal 100 amino acids (45). PKGI is most abundant in platelets, smooth muscle cell (SMC), and cerebellar Purkinje cells. PKGII is a membrane associated momomer

of 86 kDa, which has so far been located in only the small intestine epithelial brush border membrane. Activation of G kinase is selective, but not absolutely specific for cGMP as cAMP is also able to bind and activate the enzyme, PKGI has even less selectivity for cGMP after autophosohorylation (46).

1.3.2.3.2 cGMP- gated ion channels

A number of cation channels are gated by the binding of cGMP. These include the cation channel of the retina and the olfactory epithelial cation channel. These ion channels possess a single regulatory cGMP site that allows Ca^{2++} entry to the cell. In less defined cases, it is not always clear whether regulation of the ion channel by cGMP is through direct gating by cGMP binding or through phosphorylation by G kinase (47).

1.3.2.3.3 Cyclic nucleotide phosphodiesterases (PDE)

PDEs are a superfamily of enzymes that degrade cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). As many as 11 PDE families have been identified, many of which exist as splice variants. The cAMP specific enzymes include PDE4, -7 and -8. The cGMP specific PDEs are PDE5, -6 and -9, while PDE1, -2, -3, -10 and -11 use both nucleotides and induces processes including the proinflammatory mediator production and action, ion channel function, muscle contraction, learning, differentiation, apoptosis lipogenesis, glycogenolysis and gluconeogenesis. As essential regulators of the cyclic nucleotide signalling with diverse physiological functions, PDEs have become too recognized as important drug targets for the treatment of various diseases, such as heart failure, depression, asthma, inflammation and erectile dysfunction. cAMP and cGMP are ubiquitous second messengers responsible for transducing the effects of various extracellular signals, including hormones, light and neurotransmitters (48). These cyclic nucleotides are formed from ATP and GTP through the catalytic reactions of adenylyl cyclase and guanylyl cyclase, respectively. Adenylyl cyclase can be activated by forskolin and guanylyl cyclase by NO. The activity of cGMP is terminated by rapid conversion to GMP, which is catalysed by various PDEs. Of these, PDE V shows high specificity for Cyclic GMP. cGMP can also regulate the

activities of some PDE isoforms, such as the cGMP inhibited PDE (PDE III) and the cGMP activated PDE (PDE II). Through the inhibition of PDE III cGMP elevates the intracellular cAMP concentration and stimulates the PKA activity, the cGMP dependent activation of PDE II has the opposite effect (48).

1.4 Activity of nitric oxide (NO) in vascular system

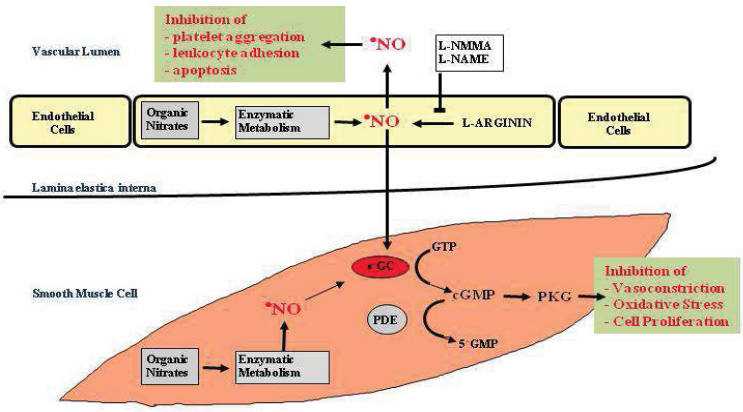


Figure 1.4 Effect of NO in vascular endothelial cells and smoth muscle cells.

1.4.1 Vasodilator effect of NO

NOS appears to be a homoeostatic regulator of numerous essential cardiovascular functions. NO dilates all types of blood vessels by stimulating sGC and increasing cGMP in smooth muscle cells (35). Vasodilation is the best documented activity of NO in the cardiovascular system, and this eventually lead to the discovery of EDRF 30 years ago (1). In blood vessels, NO is produced by the endothelium where it is a primary determinant of resting vascular tone through basal release, and cause vasodilation when synthesized in response to a wide range of vasodilator drugs. Following studies have shown that endogenous NO production is involved in the regulation of local vasomotion and blood pressure. Pharmacological inhibition of endogenous NO synthesis has been shown to induce a rise in blood pressure in man, and disruption of the eNOS gene cause mild hypertension in mice (49). Elevated blood pressure is a well known risk factor for the development of cardiovascular diseases, such as

strok and myocardial infraction, while a reduction of blood pressure is effective in reducing the morbidity and mortality of cardiovascular diseases. Thus, maintenance of normal blood pressure by endothelial NO may be considered as part of its vasoprotective action. The mechanism underlying NO induced vasodilation has been intensively investigated. The current knowledge suggests a central role for the cGMP dependent activation of PKG I which can phosphorylate different membrane proteins in the sarcoplasmic reticulum. It has been reported that PKGIcan phosphorylate phospholamban (50).

Phosphorylation of phospholamban (e.g by PKG I) favours the association of phospholamban monomers into pentamers and reverses the inhibition of sarco/endoplasmatic reticulum Ca^{2+} -ATPase (SERCA) (51). This initiates a rapid sequestration of intracellular calcium, which, in turn, also, or reduces the influx of extracellular Ca^{2+} into the sarcoplasmic reticulum. Ca^{2+} dependent K^{+} channels can also activate by NO, and increase the outward potassium current. Hyperpolarisation of the cell membrane decreases the effect of depolarising signals and induces vasodilation. It has been shown that this action of NO can both be independent and dependent on the activation of PKG I (52). cGMP dependent inhibition of voltage-gated calcium channels might also be involved in the mechanism of vasodilation induced by NO (53). The relative contribution of each of these PKG I and K^{+} channel dependent vasodialating mechanisms of NO, however, remains to be determined. The subsequent reduction of the intracellular Ca^{2+} concentration reduces the formation of the Ca^{2+}calmodulin myosin light chain kinase complex, this decreases the phosphorylation of Ser 19 in the myosin regulatory light chains and inhibits vasoconstriction (54).

1.4.2 Antiproliferative effects of NO

NO is a strong inhibitor of cell proliferation in many cell types. Both cGMP-dependent and cGMP-independent mechanisms seem to be involved in NO-mediated cytostasis (55).

Several proteins and molecular pathways have been proposed to directly or indirectly accounts for the NO antiproliferative effect. Among these, ribonucleotide reductase (56), ornithine decarboxylase and EGFR are inhibited by NO (57), whereas p38 MAPK and p53 are activated (58). Proliferation of smooth muscle cells (SMC) plays a key role in the narrowing of the lumen of blood vessels in restenosis and coronary artery diseases (59). In this process

vascular smooth muscle cells (VSMC), show substantial changes of their functions, such as the disappearance of contractile activity. These changes include a generation of matrix proteins and loss of myofibrils. Furthermore, the susceptibility to proliferating stimuli, such as PDGF, increases. These proliferating SMC can migrate into the intima and contribute to intial hyperplasia. Both the proliferation and the migration are controlled by various signaling molecules, such as Ang II, TNFα, and several growth factors (TGF-ß, FGF, PGDF). NO has been shown to inhibit SM proliferation.

The mechanism underlying the antiproliferative activity of NO is not yet fully understood. It has been suggested that the cGMP-dependent activation of PKA, partially mediated by the inhibition of the cGMP-inhibited-cAMP phosphodiesterase (PDE III), may contribute to this process. A similar pathway of the activation of PKA was reported to potentiate NO-induced inhibition of platelet aggregation, and to mediate the positive inotropic effects of NO (60).

It was suggested that NO-dependent activation of PKA can regulate the expression of cell cycle proteins. It is thought that such activation of PKA reduces smooth muscle proliferation by the inhibition of proto-oncogene serine/threonin kinase 1 (Raf-1) (61). Another potentially important mechanism underlying the antiproliferative effects of NO, which takes place independently of the cGMP generation, is the inhibition of ornithine decarboxylase and arginase (62). Although the NO donors effectively increased the cellular cGMP content, its modulation by an inhibition of sGC or PDEs had no effect on cell proliferation. In contrast, the inhibition of cell proliferation by NO donors was effectively reversed by putrescine but not by ornithine (63).

1.4.3 Antioxidative effects of NO

Vascular oxidative stress with an increased production of reactive oxygen species (ROS) contributes to the pathophysiology of cardiovascular diseases. Oxidative stress is mainly caused by an imbalance between the activity of endogenous pro oxidative enzymes (such as NADPH oxidase, xanthine oxidase, or the mitochondrial respiratory chain) and anti oxidative enzymes (such as superoxide dismutase, glutathione peroxidise, heme oxygenase, thioredoxin peroxidise/peroxiredoxin, catalase, and paraoxonase) in favour of the former (64). Among the various ROS formed under these conditions, superoxide is presumably the

most important (65). As well known antioxidative effect of NO is the impairment of lipid oxidation. NO can potently inhibit the oxidation of free fatty acids, phosphatidylcholine and low density lipoprotein particles. In view of the proatherogenic effects of oxidized lipids, this antioxidative activity of NO is likely to be relevant. However, NO reacts very rapidly with superoxide to form peroxynitrite, which is a much stronger oxidant than superoxide itself. The carbonate adduct can rapidly form carbonate and nitrogen dioxide radicals (66). These radicals have been shown to have oxidized thiols to thiyl, sulfinyl and disulfide radicals. The oxidizing potential of peroxynitrite and the subsequently formed radicals facilitate lipid peroxidation, induce protein damage by tyrosine nitration, dityrosine formation and thioloxidation and reduce the antioxidative capacity of vascular cells by rapid oxidation of free undissociated thiols (67). In view of these reactions, it seems rather questionable that an increased generation of NO increases the antioxidative capacity of the vascular wall. However, the formation of peroxynitrite from superoxide competes with the formation of hydrogen peroxide catalysed by superoxide dismutases. Although superoxide reacts approximately 3–6 times faster with NO than with superoxide dismutase, the formation of $ONOO^-$ is outcompeted as the concentration of superoxide dismutase is much higher than that of NO. Thus, the physiologic concentrations of vascular NO, as generated by expression of eNOS or by therapeutic interventions, might exert its antioxidative effects in the presence of physiologic concentrations of superoxide dismutases (68). In contrast, very high concentrations of NO, as generated by iNOS, presumably promote peroxynitrite formation. NO has been identified as an inducer of endothelial hydroferritin synthesis (69). Ferritin binds free iron ions and may reduce oxidative cellular damage by preventing superoxide generation. This potent antioxidative enzyme is expressed in vascular SMC, and located at the outer cell membrane. In the vascular wall one third to half of the total superoxide dismutase is the extracellular type of the enzyme (70). The upregulation of the extracellular superoxide dismutase expresesion in vascular SMC may represent an important mechanism that prevents the superoxide mediated degradation of endothelial NO as it traverses between the two cell types. Likewise, the formation of peroxynitrite will be reduced, because higher amounts of superoxide dismutase favour the dismutation of superoxide to hydrogene peroxide. It was reported that a brief exposure of endothelial cells to the strong oxidant hydrogen peroxide can increase the expression and activity of eNOS (71), a mechanism that

might contribute to the antioxidative effects of NO. The antioxidative effects of NO are at least in part mediated by the induction of the expression of heme oxygenase-I, ferritin and extracellular superoxide dismutase. These activities of NO decrease both the superoxide levels and the formation of peroxynitrite in the vascular wall.

The pathophysiologic causes of oxidative stress are likely to involve changes in a number of different enzyme systems, most importantly, there is an upregulation of NADPH oxidases and eNOS. Together, they lead to an increased production of peroxynitrite ($ONOO^-$). This conveys oxidative damage to eNOS and/or its cofactor BH_4 leading to uncoupling of the enzyme. As a consequence, an increased production of ROS by uncoupled eNOS is likely to contribute significantly to vascular oxidative stress and endothelial dysfunction (64).

1.4.4 Antiplatelet effects of NO

Platelets play a vital role in vacular haemostasis. Their ability to aggregate and form a haemostatic plug must be carefully balanced against the fluid state of blood and to avoid thrombosis (72). NO is a potent vasodilator and inhibitor of platelet aggregation (73).

NO released towards the vascular lumen is a potent inhitor of platelet aggregation and adhesion to the vascular wall (73). Atherosclerotic changes are often followed by platelet hyperreactivity associated with thrombosis, myocardial infarction, and stroke. NO and NO donors stimulate cGMP production in human platelets, leading to activation of PKG and inhibition of platelet aggregation induced by agonists such as thrombin which increases the intracellular Ca^{2+} concentration (74). The NO-induced inhibition of platelet aggregation involves a decrease of the intraplatelet Ca^{2+} concentration. Similar to the mechanism of the NO-induced vasorelaxation, NO-induced inhibition of platelet aggregation involves phospholamban- and SERCA-dependent refilling of intracellular Ca^{2+} stores (75). In addition, cGMP indirectly activate PKA, since cGMP inhibits the breakdown of cAMP by PDE III (76). cGMP and cAMP are known to phosphorylate phospholamban, which then activates SERCA to enhance the sequestration of Ca^{2+} (77). NO and cGMP analogues act synergistically with cAMP-elevating agents, such as prostacyclin, to inhibit platelet aggregation (76). Both nucelotides -elevating agents inhibit platelet aggregation by a reduction of the intracellular Ca^{2+} concentration.

1.4.5 Antiadhesive effects of NO

NO can also inhibit leucocyte adhesion to the vessel wall by either interfering with the ability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leucocytes. Leucocyte adherence is an early event in the development of atherosclerosis, and therefore, NO may protectagainst the onset of atherogenesis (78).

Increased leucocyte adhesion is a major step in the pathogenesis of atherosclerosis (79). Leucocyte adhesion is critically dependent on the expression and appearance of various adhesion molecules on surface of vascular endothelial cells. Activation of redox sensitive transcription factors, such as NFkB and AP-1 are believed to play a key role in this process. Monocytes adhering to vascular cells migrate into the vascular wall, and increase further oxidant stress by releasing large amounts of ROS. Thus, a reduction of the adhesion molecule expression or inhibition of adhesion molecule function is considered vasoprotective. NO is an important endogenous mediator, which inhibits leukocyte adhesion. NO released by NO donors potently inhibit the expression of VCAM-1 (80), while the inhibition of endogenous NO synthesis has an opposite effect (81). In 1996 Khan and his colleagues provided evidence that this action of NO is mediated through the inhibition of NFkB expression and involves the antioxidative properties of NO (82). Oxidation of polyunsaturated fatty acids, such as linoleic acid to peroxidized metabolites, has been shown to be an important intermediate step in the cytokine-induced activation of the redox-sensitive transcription factor NFkB however, this oxidation can be markedly reduced by exogenous NO (80). Likewise, increased leukocyte adhesion induced by the inhibition of NO synthases was at least partially reversed by various intracellular oxygen radical scavengers (81). Thus, the mechanism of the antiadhesive action of NO most likely involves its antioxidative effects.

1.5 Consequence of Nitric Oxide Synthase Deficiency

NO is actually a gas that acts throughout the body as a messenger, sending and receving messages that regulate the activity of cells. NO instructs the body to perform certain key functions. Not only can NO relax and dilate the blood vessels, thereby ensuring that blood can efficiently nourish the heart and tissues of the entire body, it can also support healthy blood pressure levels. A deficiency of NO can have a dramatic impact on health.

Cardiovascular diseases are the leading causes of morbidity and untimely deaths in North America, and are associated with NO deficiency. Hypertension, stroke, atherosclerosis, heart attacks, diabetes, Alzheimer's disease, gastrointestinal ulcer and erectile dysfunction has been linked to NO -deficiency. A healthy vascular endothelium is essential to a healthy cardiovascular system because it is required for normal NO production and action. Vascular endothelial dysfunction leads to decreasing NO production and increased oxidative stress, creating a vicious cycle that promotes further endothelial dysfunction. Knockout mice serve as useful animal models for NOS deficiencies, and their phenotypes reflect the functions of each NOS isoform. nNOS knockout mice display enlarged stomachs because of abnormalities in pyloric relaxation (83). eNOS knockout mice lack the EDRF activity, and are hypertensive (49). iNOS knockout mice are more sensitive to certain infections, and are resistant to the sepsis induced hypotension (84).

1.6 Therapeutic Targeting of The Vascular NO-cGMP System

1.6.1 Direct activation of NO-cGMP system

1.6.1.1 Organic nitrates

Organic nitrates deliver NO directly to the vascular wall. These drugs are enzymatically denitrated to NO and denitrated metabolites (85). The enzymatic conversion involves a transfer of three electrons to the nitrate nitrogen, and is most likely to mediated by cytochrome P450 type enzymes such as CYP3A4 (86). The generation of NO from organic nitrates occurs in both vascular endothelial and smooth muscle cells. Therapeutic concentration of organic nitrates dilates preferentially venous vessels and conduit arteries, such as the left anterior descending artery, while higher doses decrease the peripheral resistance (85). Oral administration of the long acting organic nitrate pentaerythritol tetranitrate (PETN) to rabbits, who were fed a high cholesterol diet for 4 months, reduced the formation of aortic lesions and prevented the inhibition of endothelium dependent vasodilation (87). Nitroglycerin (GTN) has been one of the most widely used anti-ischemic drugs for more than a century. Given acutely, organic nitrates are excellent agents for the treatment of stable angina, acute myocardial infarction, chronic congestive heart failure, pulmonary edema, and severe arterial hypertension (88),(89).

1.6.1.2 Direct activators of soluble guanylate cyclase

In 1994, some researchers from Taiwan reported on the pharmacological activities of YC-1, a new benzylindazol derivative. They found that this drug activates the enzyme sGC independent of NO, but it can also potentiate the NO induced stimulation, presumably by stabilization of the enzyme's active configuration (90),(91). Although the potency of YC-1 is too low to consider this compound for clinical development, it served as a lead compound in the search of the new activators of sGC. This class of drugs is much more potent than YC-1 and display useful pharmacologic actions such as vasodilation and inhibition of platelet aggregation (92). The first sGC simulator to undergo clinical study was BAY 41-8543 (93). A clinical study was performed to evaluate the short-term safety profile of BAY 63-2561 (Riociguat) to determine the tolerability and efficacy in patients with moderate to severe pulmonary hypertension due to pulmonary arterial hypertension (PAH), distal chronic thromboembolic or PAH with mild to moderate interstitial lung disease (94). The administration of the sGC stimulator significantly improved pulmonary hemodynamic measurements and cardiac indices in patients with PH in a dose-dependent manner, to a greater extent than following administration of inhaled NO. Although riociguat had significant systemic blood pressure effects and showed no pulmonary selectivity, mean systolic blood pressure remained >110 mmHg (94).

1.6.1.3 Nitroaspirins

Nitroaspirins like NCX-4016 (2-(acetoxy)-3-[(nitroxy)methyl]) were designed to combine the pharmacologic effects of organic nitrates and acetylsalicylic acid in one drug. Basically, these drugs resemble the direct activators of sGC, with respect to the vasodilation and inhibition of platelet aggregation (95). Furthermore, it was anticipated that the liberation of NO from these drugs inhibit the initiation of peptic ulcer by aspirin and improve its therapeutic safety. (96). The liberation of NO from NCX-4016 is a metabolic and not a spontaneous process (97). This is not surprising since the generation of NO from a nitrate moiety requires a transfer of 3 electrons to the nitrate nitrogen. Presumably, NCX-4016 is metabolized by the same enzymes, the cytochrome P450 enzymes glutathione-s-transferase and mALDH than organic nitrates, and can act as an antiangiogenic drug (98).

1.6.1.4 L- Arginine

The amino acid L- arginine is the substrate of eNOS and is converted to citrulline and NO. Subsequent clinical studies demonstrated that L- arginine improves the endothelium-dependent vasodilation in hypercholesterolemic humans (99). It is believed that the beneficial effect of the administration of L-arginine are partially caused by a competition between this genuine amino acid and the derivative asymmetric dimethyl L- arginine (ADMA), which is an endogenous inhibitor of eNOS activity (100). The dose of L-arginine which increase the plasma concentrations by 20 fold can exert a variety of unsepecific effects such as increases in plasma insulin, prolactin and glucagon in healthy subjects and in patients with diabetes or essential hypertension (101). However, L- arginine might exert harmful effects in post myocardial infraction patients as concluded by Schulman et al. L-arginine when added to standard postinfarction therapies, does not improve vascular stiffiness measurements or ejection fraction and may be associated with higher postinfarction mortality. L- arginine, should not be recommended following acute myocardial infarction (102).

1.6.1.5 Phosphodiesterase inhibitors

By catalyzing cyclic nucleotide hydrolysis, the PDEs become important determinants that regulate the intracellular concentration and the biological actions of cAMP and cGMP (103). Intense biochemical and molecular genetic research has revealed the complexity and diversity of the PDE superfamily which contains at least 11 highly regulated and structurally-related gene families. The pharmacotherapeutic principle of isoform- selective PDE inhibition is the subject of intensive research in many different fields, including asthma, allergies, diabetes, restenosis and arthritis. The importance of cyclic nucleotide signaling and the molecular diversity of PDEs make these proteins interesting targets for selective intervention, including modulation of the NO- cGMP pathway. Sildenafil is an inhibitor of the cGMP – specific PDE V and it has been shown to be effective in the treatment of erectile dysfunction by elevating cGMP levels in the SMC of the corpus cavernosum (104). There is evidence indicating that the increase in erectile function induced by sildenafil involves cGMP – dependent inhibition of PDE III and the subsequent elevation of cAMP levels in the corpus

cavernosum (105). Inhibition of PDE V by sildenafil can induce severe hypotension when combined with NO- donating drugs such as organic nitrates but, under resting conditions, Sildenafil has minor effects on the vasculature as indicated by a lack of hemodynamic actions.

1.6.2 Indirect activators of NO- cGMP system

1.6.2.1 Inhibitors of cholesterol synthesis (Statins)

The inhibitors of Hydroxy Methyl Glutaryl – Coenzyme A Reductase (I- HMG- CoA-R) or statins have become the cornerstone of drug therapy that aim at reducing cardiovascular risk. Statins are the pharmacological group with the highest reduction power of the serum LDL cholesterol concentration, apart from their lipid and pleiotropic actions (106). The Inhibitors of cholesterol synthesis (Statins) such as Lovastatin have been shown to increase the expression of eNOS. Although statins act predominantly by reducing plasma cholesterol levels, it is possible that other effects of these drugs such as the stimulation of endothelial NO production contribute to their beneficial action in coronary artery disease (63).

1.6.2.2 Angiotensin Converting Enzyme inhibitors (ACE)

Angiotensin converting enzyme inhibitors such as captopril are another group of drugs showing the activation of the endothelial NO production (107). Inhibition of the carboxypeptiddase (angiotensin) converting enzyme results in reduced formation of angiotensin II from angiotensin I as well as reduced breakdown of bradykinin, a potent stimulator of the endothelial NO production. ACE inhibitors can increase vascular cGMP levels and induce NO dependent vasodilation mediated by the endothelium- derived kinin. These findings are consistent with a clinical study which showed that bradykinin contributes to the blood pressure- reducing effects of captopril (107). Large clinical trials demonstrated that angiotensin converting enzyme inhibitors reduce the mortality of patients with acute myocardial infraction or heart failure. Again, it is tempting to speculate that an increase of vascular NO levels during treatment with angiotensin converting enzyme inhibitors, contributes to the beneficial effects on mortality and morbidity of cardiovascular patients.

1.7 The Renin-Angiotensin Aldosterone System

The renin- angiotensin aldosterone system (RAAS) is now recognized as the body´s most powerful hormone system for controlling sodium balance, body fluid volumes, and arterial pressure.

1.7.1 Historical background on the RAAS

In 1898, Tigerstedt and Bergmann demonstrated the existence of a heat-labile substance in the crude extracts of rabbit renal cortex that caused a sustained increase in arterial pressure. They proposed the term “renin” for a presumed humoral pressor agent secreted by the kidney, a concept that was widely disputed or ignored until the classic studies of Goldblatt et al (108), published in 1934, which showed that renal ischemia, induced by clamping of the renal artery could induce hypertension. Shortly thereafter, it was shown that the ischemic kidney also release a heat-stable, short-lived pressor substance, in addition to renin. This finding eventually led to the recognition that renin’s pressor activity was indirect and resulted from its proteolytic action on a plasma substrate (eventually termed “angiotensinogen”) to liberate a direct-acting pressor peptide. In the early 1950s, during attempts at purification, Skeggs and colleagues discovered that this peptide existed in 2 forms, eventually termed as Ang I and II. In later work, they demonstrated that Ang I was cleaved by a contaminating plasma enzyme, termed “angiotensin-converting enzyme,” to generate the active pressor peptide Ang II. Soon after, the work of several investigators, culminated in the discovery that Ang II also stimulates the release of the adrenal cortical hormone aldosterone, a major regulator of sodium and potassium balance. These landmark discoveries established the concept that a single system, the RAAS, was involved in the regulation of blood pressure and fluid and electrolyte balance (109).

1.7.2 Physiology of the RAAS

The renin – angiotensin hormonal cascade begins with the biosynthesis of renin by the juxtaglomerular cells (JG) that line the afferent (and occasionally efferent) arteriole of the renal glomerulus. Renin is synthesized as a preprohormone and mature (active) renin is formed by proteolytic removal of a 43- amino- acid prosegment peptide from the N- terminal of prorenin, the proenzyme or renin precursor. Mature renin is stored in the granules of JG

cells and is released by an exocytic process involving stimulus- secretion coupling into the renal, and then, the systemic circulation. In addition to this regulated pathway, it appears that the kidney also releases unprocessed prorenin via a constitutive pathway. In fact, prorenin accounts for about 70% - 90% of the immunoreactive renin in the human circulation.

1.7.2.1 Angiotensinogen (Ang)

Angiotensinogen is a glycoprotein with intriguing structural similarities to the serine proteinase inhibitors, but with only one known function, to act as a substrate in the enzymatic generation of angiotensin peptides. It is the only known substrate for the enzyme renin. It is constitutively expressed in the liver and various other tissues, including the brain (110). Angiotensinogen is synthesized and secreted continuously by the liver. It is synthesis is stimulated by inflammation, insulin, estrogens, glucocorticoids, throid hormone and Ang II. There is a progressive relationship among the number of copies of the angiotensinogen gene, plasma level of anginotensinogen, and arterial blood pressure (111).

1.7.2.2 Renin

The protease renin is the key enzyme of the RAAS, which is relevant under both physiological and pathophysiological settings. Renin is synthesized, stored and secreted into the renal arterial circulation by the granular juxtaglomerular cells that lie in the wall of the afferent arterioles than enter the glomeruli. Renin is stored in granules within juxtaglomerular cells and is secreted by exocytosis (112). The kidney is the only organ capable of releasing enzymatically active renin.

The characteristic juxtaglomerular position is the best known site of renin generation, renin producing cells. In the kidney can varyin number and localization (113). Renin is also expressed in other tissues, for instance in the liver, heart, and blood vessels (114). Pro- renin is stored in vesicles, activated to renin, and then, released upon demand. The release of renin is under the control of cAMP (stimulatory) and Ca2+ (inhibitory) signalling pathways. The

biological action of prorenin are mediated by receptors for prorenin, Ang II, and angiotensin (1-7) (113). Both renin and prorenin are stored in the juxtaglomerular cells and when released circulate in the blood. The half-life of circulating renin is approximately 15 minutes. The concentration of proreninin the circulation is approximately 10 fold greater than that of the active enzyme. The secretion of renin from juxtaglomerular cells is controlled by 3 pathways, two acting locally within the kidney and the third acting through the centeral nervous system (CNS) and mediated by noradrenaline release from renal noradrenergic nerves. One internal mechanism controlling renin release is the macula densa pathway (Figure 1. 5). The macula densa lie adjacent to the juxtaglomerular cells and composed of specialized columnar epithelial cells in the wall of the portion of the cortical thick asscending limb that passes between the afferent and the efferent arterioles of the glomerulus.

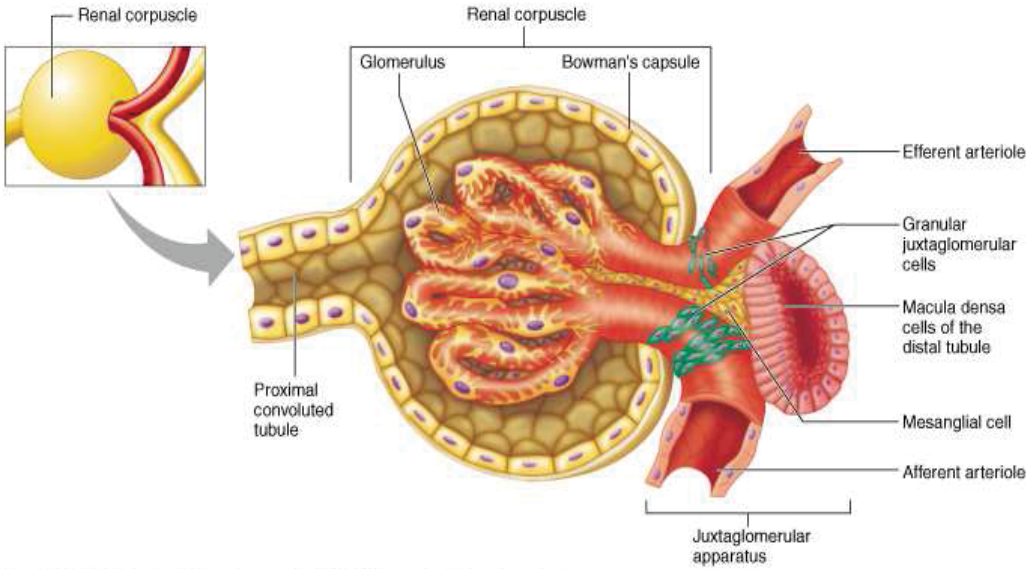


Figure 1.5 the macula densa pathway

(2001 Benjamin Cummings, an imprint of Alddlison Weseley Longman, inc.) (318)

A change in NaCl reabsorption by the macula densa results in the transmission to nearby juxtaglomerular cells of chemical signals that modify renin release. Increase in NaCl flux across the macula densa inhibit renin release, decrease in NaCl flux stimulate renin release. Both adenosine and prostaglandins mediate the macula densa pathway. The former is released when NaCl transport increases and the latter is released when NaCl transport decreases. Adenosine acting via the A1 adenosine receptor inhibits renin release, and prostaglandins stimulate renin release. There is role for inducible cyclooxygenase (COX-2) and nNOS in the mechanism of macula densa stimulated renin release. Although constitutive cyclooxygenase (COX-1) is the most abundant cyclooxygenase isoform in the mammalian kidney, Inducible COX-2 is the only cyclooxygenase form expressed in the macula densa, where it is up regulated by chronic dietary sodium restriction (115). Renin release induced by a low sodium diet is blunted by selective inhibition of COX-2 (116). Moreover, selective inhibition of COX-2 block macula densa mediated renin release in the isolated perfused juxtaglomerular preparation (117). In a similar manner, the expression of nNOS in the macula densa is up regulated by dietary sodium restriction (118), and selective inhibition of nNOS reduces renin release in response to chronic dietary sodium restriction (119). The nNOS and NO pathway may mediate increase in COX-2 expression induced by a low sodium diet (85). Together these findings suggest a biochemical interplay between COX-2 and nNOS in the regulation of macula densa mediated renin release. It is plausible that activation of macula densa mediated renin release by sodium depletion involves the following events. Up regulation of nNOS and COX-2 in the macula densa, increased biosynthesis of NO and peroxynitrite in the macula densa, peroxynitrite induced activation of COX-2 in the macula densa, increased prostaglandin production in the macula densa, and paracrine activation of prostaglandin receptor in neighboring juxtaglomerular cells (117). Although a change in NaCl transport by the macula densa is the key event that modulates the macula densa pathway, regulation of this pathway is more dependent on the luminal concentration of Cl- than Na+. NaCl transport into the macula densa is mediated by the Na-K+- 2 Cl symporter. Since the luminal concentration of Na+ at the macula densa usually is much greater than the level required for half maximal transport, physiological variation in luminal Na+ concentration at the macula densa have little effect on renin release. The second intrarenal mechanism controlling renin release is the intrarenal baroreceptor pathway. Increases and decreases in

blood pressure in the preglomerular vessels inhibit and stimulate renin release, respectively. The immediate stimulus to secretion is believed to be reduced tension within the wall of the afferent arteriole. Increases and decreases in renal perfusion pressure may inhibit and stimulate, respectively, the release of renal prostaglandins, which may mediate in part the intrarenal baroreceptor pathway. In support of this conclusion, COX-2 inhibition decrease renin secretion and blood pressure in renin-dependent renovascular hypertension (120).

The third mechanism, the β- adrenergic receptor pathway is mediated by the release of norepinephrine from postganglionic sympathetic nerves. Activation of β1 receptors on juxtaglomerular cells enhances renin secretion. The three mechanism regulating renin release are embedded in a physiological network. Increased renin secretion enhances the formation of angiotensin II, and Ang II stimulates AT1 receptors on Juxtaglomerular cells enhances to inhibit renin release, an effect termed short- lop negative feedback. Ang II also increases arterial blood pressure by stimulating AT1 receptors.

Increases in blood pressure inhibit renin release by activating high pressure baroreceptor thereby reducing renal sympathetic tone, Increasing pressure in the preglomerular vessels, and reducing NaCl reabsorotion in the proximal tubule, which increase tubular delivery of NaCl to the macula densa. The inhibition of renin release owing to Ang II induced increases in blood pressure has been termed negative feedback loop. The physiological pathways regulating renin release can be influenced by arterial blood pressure, dietary salt intake, and a number of pharmacological agents.

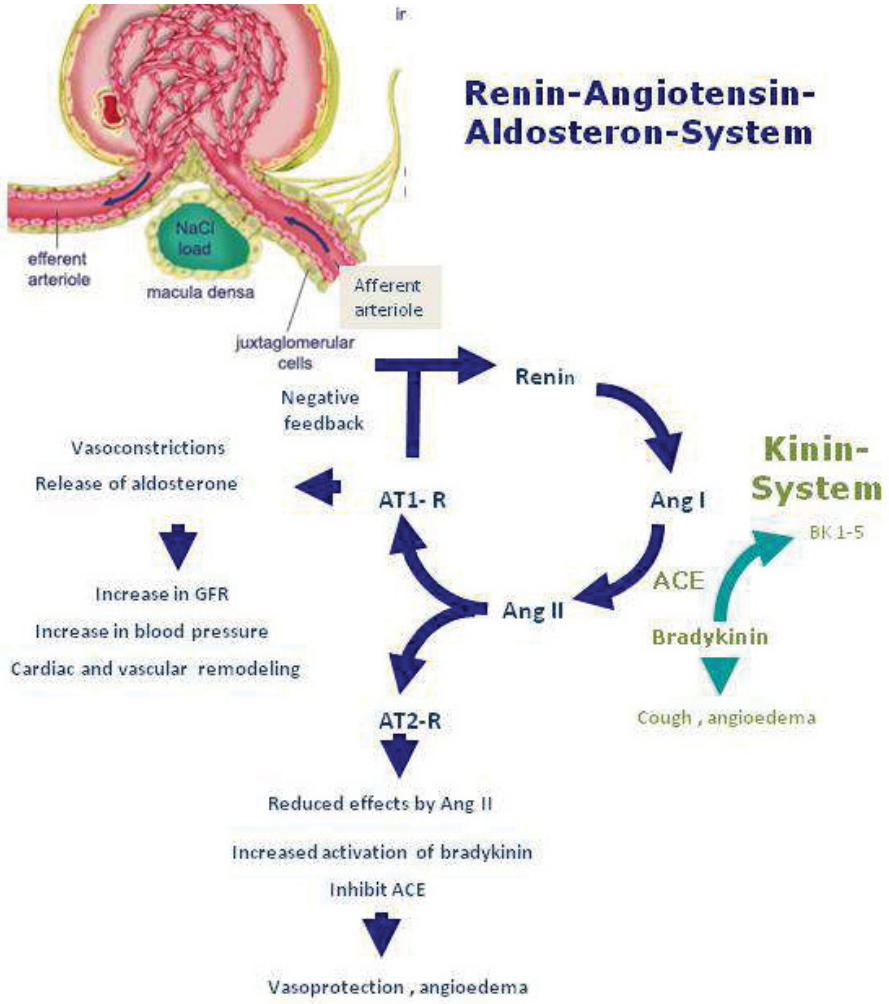


Figure 1. 6 Schematic overview of the renin angiotensin aldosterone system

1.7.2.3 Angiotensin I (Ang I)

Within the RAAS, the decapeptide angiotensin I (Ang I) may be viewed as the prohormone molecule from which biologicaly active peptides are derived by the hydrolytic action of enzymes bound to the plasma membrane of cells in the tissues. The active products generated from Ang I are Ang II and Ang (1-7). The mechanism controlling the formation and degradation of Ang II is important in determining its final physiological effect. Ang II is formed from the enzymatic cleavage of angiotensinogen to Ang I by the renin, with subsequent conversion of Ang I to Ang II by angiotensin converting enzyme (ACE).

The pharmacological profile of angiotensin (1-7) is distinct from that of angiotensin II, Angiotensin (1-7) does not cause vasoconstriction, aldosterone release, or facilitation of noradrenergic neurotransmission.

Angiotensin (1-7) releases vasopressin, stimulates prostaglandin biosynthesis, dilates some blood vessel and exerts a natriuretic action on the kidneys. Angiotensin (1-7) also inhibits proliferation of vascular smooth muscle cells (121). Ferrario and colleagues 1997 (122) proposed that angiotensin (1-7) serves as to counterbalance the action of angiotensin II. Carboxypeptidase, ACE2 cleavage one amino acid from either Ang I or Ang II, decreasing Ang II levels and increasing the metabolite Ang 1-7, which has vasodilator properties. The balance between ACE and ACE2 is an important factor to control Ang II levels (123).

1.7.2.4 Angiotensin converting enzyme (ACE)

ACE is a dipeptidyl peptidase transmembrane- bound enzyme. A soluble form of ACE in plasma is derived from the plasma membrane bound form by the proteolytic cleavage of its COOH – terminal domain. There are two distinct isoforms of ACE somatic and testicular. They are transcribed from a single gene at different initiation sites. The somatic form of ACE is a large protein (150-180 kDa) that has two identical catalytic domains and a cytoplasmic tail. ACE catalyses the conversion of Ang I to Ang II, in a substrate concentration dependent manner (124). ACE also degrades bradykinin, an potent vasoactive peptide. Inhibition of ACE by ACE inhibitors results in the decreased formation of Ang II and decreased metabolism of bradykinin, leading to systematic dilation of arteries and veins, and a decrease in arterial blood pressure. ACE is synthesized by the vascular endothelium, and also by several epithelial and neural cell types (125).

1.7.2.5 Angiotensin II

The actions of Ang II are mediated by specific heterogeneous populations of Ang II receptors (detailed description in chapter 1.8). Ang II is known to interact with at least two distinct Ang II receptors subtypes, designated AT1 and AT2 (126). The characterization of Ang II receptor subtypes was made possible by the discovery and development of selective nonpeptide Ang

II receptor blockers namely losartan (AT1 selective) and PD123319 (AT2 selective) **(127)**. The AT1 and AT2 receptors have little sequence homology. Most of the known biological effects of angiotensin II are mediated by the AT1 receptor. Although the AT2 receptor generally is conceptualizied as a cardiovascular protective receptor, its activation may contribute to cardiac fibrosis (128).

The AT2 receptor is distributed widely in fetal tissuees, but its distribution is more restricted in adult. In adults, some tissues contain primarily either AT1 receptors or AT2 receptors, whereas other tissuees contain the receptor subtypes in similar amounts. AT1 receptor mediates vasoconstriction by stimulation of sympathetic transmission and release of aldosterone. (129). AT2 receptor in mediating anti- proliferation, apoptosis, differentiation and vasodilatation (130). The mechanisms controlling the formation and degradation of Ang II are important in determining its final physiological effect. Ang II is formed by conversion of Ang I to Ang II by ACE. ACE releated ACE2 cleavage one amino acid from either Ang I or Ang II, decreasing Ang II levels and increasing the metabolite Ang 1-7, which has vasodilator properties. The balance between ACE and ACE2 is an important factor to control Ang II levels (123). Even though ACE is the primary enzyme leading to Ang II generation, in the heart, the majority of Ang I is converted by chymase (131). The tissue-specific effects of the increased Ang II level and the enhanced RAAS activity depend on the cellular expression and activation of AT1 receptor in cardiovascular and renal pathophysiology.

1.7.2.6 Aldosterone

The steroid hormone aldosterone, synthesized in the zone glomerulosa of the adrenal cortex, plays a pivotal role in electrolyte and fluid balance (132). Aldosterone, acting as a mineralocorticoid, is the final endocrine signal in the RAAS that targets epithelia in the kidney and colon, to regulate Na^+ (re)absorption and K^+ secretion. Ang II and K^+ promote aldosterone secretion. Blood pressure alters Ang II levels in a reciprocal fashion, and decreased blood pressure increases the production of Ang II. Thus, aldosterone secretion and its physiological action are under negative feedback control, as it responds positively to decreases in blood pressure and increases in K^+ and negatively to increases in blood pressure and decrease in K^+. The role of aldosterone in contributing to hypertension is suggested further by the broad antihypertensive effectiveness of mineralocorticoid antagonists in

treating unselected hypertensive individuals. Studies of spironolactone and eplerenone clearly demonstrate antihypertensive benefit that is not limited to patients with classical primary aldosteronism (133). Primary aldosteronism, also known as hyperaldosteronism (Conn's syndrome), is characterized by the overproduction of the mineralocorticoid hormone aldosterone by adrenal glands (134). Causing to lose K and retain Na, the exess Na in turn holds onto water increasing in the blood lead to increase blood volume and blood pressure, the people which has aldosteronism high risk to heart disease and stroke (135).

1.8 Angiotensin II Receptors

As mention above most effects of Ang II are mediated by specific receptors.

1.8.1 AT1 receptor

Any of the known physiological effects of Ang II are mediated by AT1 receptor. AT1 receptor is widely distributed in all organs, including the liver, adrenals, brain, lung, kidney, heart and vasculature. The AT1 receptor (40 kDa) belongs to the seven- helical membrane superfamily of G protein- coupled receptors. The human AT1 receptor gene has been mapped to chromosome 3 (136).

1.8.1.1 Signal transduction of AT1 receptor

There are five classical signal transduction mechanisms for the AT1 receptor, activation of phospholipase A, phospholipase C, phospholipase D and L type, Ca^{2+} channels and inhibition of adenylyl cyclase. AT1 receptors activate a large array of signal transduction systems to produce effects that vary with cell type and that are a combination of primary and secondary responses (137). Activation of phospholipases A2 and D stimulates the release of arachidonic acid, the precursor molecule for the generation of prostaglandins (138). Ang II mediated stimulation of the AT1 receptor coupled to Gi protein can also inhibit adenylate cyclase in several target tissues, including liver, kidney and adrenal glomerulosa, thereby attenuating the production of the second messenger cAMP (139). AT1 receptor is also involved in the opening of Ca^{2+} channels and influx of extracellular Ca^{2+} into cells. This mechanism has been linked to Ang II mediated stimulation of aldosterone production and secretion, as well as vasoconstriction (140). In 1999 Touyz and his colleagues demonstrates that in VSMCs from human peripheral resistance arteries, Ang II induced contraction and associated second messengers are mediated via receptor of the AT1 subtype, signalling in part by tyrosine

kinases and ERKs. They also show that MEK may influence Ca^{2+} by modulating Ca^{2+} influx and intracellular Ca^{2+} mobilization. Their data thus show for the first time that in VSMCs from human small arteries, ERK-dependent pathways, which are characteristically involved in signalling cascades associated with cell growth, may also play an important role in Ang II mediated contraction. These results have important clinical significance, because small arteries are the vessels that play a critical role in regulating peripheral resistance and blood pressure (141).

1.8.1.2 Distribution and localization of AT1 receptors

AT1 receptor are primarily found in the brain, adrenals, heart, vasculature and kidney, and serve to regulate blood pressure and fluid and electrolyte balance. AT1 receptors have been demonstrated in the central nervous system of the rat, rabbit and human (142). AT1 receptors are localized mainly in the zona glomerulosa of medulla. In the heart, the highest density of AT1 receptor is found in the conducting system. Moreover, AT1 receptors in the vasculature, including the aorta, pulmonary and mesenteric arteries, are present in high levels on smooth muscle cells and low levels in the deventitia (142).

1.8.1.3 Function of AT1 receptors

Ang II stimulation of AT1 receptors in blood vessels causes vasoconstriction leading to an increase in peripheral vascular tone and systemic blood pressure (143). Ang II via activation of AT1 receptors is directly involved in the development of cardiac hypertrophy and heart failure, independently of blood pressure.

1.8.2 AT2 receptors

The AT2 receptors is characterized by its high affinity for PD123319, PD123177 and CGP42112, and very low affinity for losartan and candesartan (127). Ang II bind to the AT2 receptors with similar affinity as to the AT1 receptor. The AT2 receptor has been cloned in a variety of species, including human, rat and mouse (144). The AT2 receptors is also a seven transmembrane domain receptor, encoded by a 363 amino acid protein with a molecular mass of 41 kDa and shares only 34% sequence identity with the AT1 receptor (144).

The AT2 receptor gene is located on the X- chromosome. Therefore, male humans possess just a single copy of this gene. The human gene Xq22-q23 is similar to the gene in animals such as mouse and rats (145). The AT2 receptor protein consists of five potential N-glycosylation sites in the extracellular N- terminal domain and 14 cysteine residues. The second intracellular loop consist of a potential protein kinase C phosphorylation site and the cytoplasmic tail contains three consensus sequences for phosphorylation by protein kinase C and one phosphorylation site for cAMP dependent protein kinase (138). The third intracellular loop has been shown to be essential for AT2 (138).

1.8.2.1 Signaling pathways activated by the AT2 receptor

The AT2 receptor displays atypical signal transduction and G –protein – coupling mechanisms. AT2 activated three major cascades of intracellular events: 1) activation of protein phosphatases and protein dephosphorylation, 2) regulation of NO and stimulation of phospholipase A2 (PLA2) and 3) release arachidonic acid (AA). Depending on the cell type and signaling pathway examined, the AT2 receptor has been reported to couple to Gi (146). AT2 signaling pathways related to growth Inhibition, Na^+ transport and neuronal activation. AT2 receptor signalling pathways, including activation of protein phosphatases and protein dephosphorylation, NO –cGMP system, and phosphplipase A2 to release of arachidonic acid. In particular, stimulation of AT2 receptors lead to activation of various phosphatases such as protein tyrosine phosphatase, MAP kinase phosphattase1 (MKP- 1), SH2 domain containing phosphatase 1 (SHP-1) and serine / threonine phosphatise 2A (147), resulting in the inactivation of extracellular signal regulated kinase (ERK), opening of potassium channels and inhibition of T – type Ca^{2+} channels (147). Importantly, MAP kinase plays a major role in cellular proliferation and the AT2 receptor has been reported to block MAP kinase activation in rat neurons in culture by dephosphorylation of tyrosine phosphate by MAP-1 or serine/threonine phosphate by serine/ threonine phosphatase 2A (148). It is still unclear whether the AT2 receptor is coupled to a G-protein, but it has been reported that an inhibitory G- protein (GI) is linked to the AT2 receptor signalling mechanism (149). In the human uterine myometrium, the AT2 receptors is highly abundant but is down regulated during pregnancy, possibly due to sex hormones (150).

1.8.2.2 Clinical significance of AT2 receptors

Since the AT2 receptor is highly abundant in foetal tissues, it is believed to play an important role in foetal development. Some studies have demonstrated that the AT2 receptor is involved in the production of cGMP (151). Vasodilation and blood pressure regulation plays an important role for renal function. Although Ang II actions regulating blood pressure were mainly attributed to AT1 receptors, there is convincing evidence that AT2 receptors are also involved at cardiovascular and renal levels. AT2 receptor seems to functionally antahonize the Ang II actions mediated by the AT1 receptors, because blockade of the AT1 receptor, increase the plasma level of Ang II, and treatment with the AT receptors antagonists may lead to increased AT2 receptor stimulation (152). There are two general concepts regarding the mechanism of AT2 receptors. Experiments using AT2 receptor deficient mice, confirmed, firstly, Ang II regulates growth and apoptosis via the AT2 receptor, and secondary, in the aorta, heart and kidney, activation of the AT2 receptor stimulates the release of NO/cGMP and produce vascular relaxation. This effect could be indirectly mediated by the modulation of bradykinin release via PRCP (153). AT2 blocked the amiloride sensitive Na^+/H^+ exchanger, promoting intracellular acidosis in VSM cells and activating kininogenases induce ACE inhibition by AT2 inhibition of degradation Na^+/H^+ exchanger (154). A main physiological role of the AT2 receptor appears to be the inhibition of cell proliferation and the propagation of cell differentiation during tissue development. In the adult, these AT2 receptor actions seem to be reactivated throughout the structural tissue remodelling. By increasing apoptosis and inhibiting growth, AT2 receptor may counter regulate the growth stimulating Ang II effect meddiated by the AT1 receptor (155).

1.9 Drugs Targeting the RAAS

The role of angiotensin and aldosterone in some cardiovascular diseases have led to the development of several classes of RAAS inhibitors, ACE inhibitors, which block the conversion of Ang I to Ang II, Angiotensin II receptor type 1 (AT1) blockers (ARBs), mineralocorticoid receptor blockers (MRB) that inhibit aldosterone action via the MR receptor and renin

inhibitors that inhibit the activity of renin. Although, it is generally accepted that ACE inhibitors, ARBs, and MRB reduce the morbidity and mortality from diverse cardiovascular and renal conditions, their overall efficacy is limited leaving substantial unmet medical needs.

1.9.1 Angiotensin Converting Enzyme (ACE) inhibitors

ACE inhibitors reduce RAAS activation by blocking the conversion of Ang I to Ang II. The Ang II type I receptor predominantly mediates the pathological effects of Ang II, including vasoconstriction and other mechanisms which raise blood pressure as well as vascular hypertrophy, endothelial dysfunction, altherosclerosis, inflammation, and apoptosis (156). The ability to reduce levels of Ang II with orally effective inhibitors of ACE represents an important advance in the treatment of hypertension. The first orally active ACE inhibitors, captopril was introduced in 1977, and many other ACE inhibitors such as enalapril, ramipril, Lisinopril, quinapril and fosinopril are curently approved for the treatment of antihypertension and heart failure (157). The ACE inhibitors appear to confer a special advantage in the treatment of patient with diabetes, slowing the development and progression of diabetic glomerulopathy, they also are effective in slowing the progression of other forms of chronic renal disease, such as glomerulosclerosis, and many of these patients also have hypertension (157). ACE inhibitors are the preferred initial drugs in patients with hypertension and ischemic heart disease are candidates for treatment with ACE inhibitors, administration of ACE inhibitors in the immediate postmyocardial infarction period has been shown to improve ventricular function and reduce morbidity and mortality.

The attenuation of aldosterone production by ACE inhibitors also influences K+ homeostasis. There are several cautions in the use of ACE inhibitors in patients with hypertension. Patients starting treatment with these drugs should be explicitly warned to discontinue their use with the advent of any signs of angioedema. Due to the risk of severe fetal side effects, ACE inhibitors are contraindicated during pregnancy, a fact that should be communicated to women of childbearing age (158).

In most patients there is no appreciable change in glomerular filtration rate following the administration of ACE inhibitors. ACE inhibitors lower the blood pressure in most patients with hypertension. Following the initial dose of an ACE inhibitor, there may be considerable

fall in blood pressure in some patient, this response to the initial dose is a function of plasma renin activity prior to treatment (159). The potential for a large initial drop in blood pressure is the reason for using a low dose to initiate therapy, especially in patients who may have a very active renin angiotensin system supporting blood pressure, such as patient with diuretic induced volume contraction or congestive heart failure. With continuing treatment , there usually is a progressive fall in blood pressure that in most patients does not reach a maximum for several weeks (159). Most ACE inhibitors are approved for one daily dosing for hypertension. ACE inhibitors can be used alone or in combination with a diuretic. ACE inhibitors are a good choice for people who have heart attack or diabetes because, the medicine helps for reduce the work load on the heart and does not affect blood sugar level and may help to protect the kidneys (160),(161). ACE inhibitors suppress Ang II and aldosterone production, decrease sympathetic nervous system activity and potentiate the effects of diuretics heart failure. ACE is identical to kininase II, which degrades bradykinin, and thereby, lower the production of NO/cGMP and of vasoactive eicosanoids. These vasodilator mediators seem to oppose the effect of Ang II on the growth of vascular smooth muscle and cardiac fibroblasts and on production of extracellular matrix (162). Thus, the increased levels of bradykinin that result from ACE inhihition may play a role in the hemodynamic and re-modeling effects of ACE inhibitors.

1.9.2 Selective angiotensin II type 1 receptor blockade (ARBs)

The importance of Ang II in regulating cardiovascular function has led to the development of nonpeptide antagonists of the AT1 receptor for clinical use. Losartan is one of AT1 receptor antagonists, drugs have been approved for the treatment of hypertension (163). The angiotensin II receptor blockers (ARBs) available for clinical use bind to the AT1 receptor with high affinity. Although binding of ARBs to the AT1 receptor is competitive, the inhibition by ARBs of biological responses to Ang II often is insurmountable. ARBs potently and selectively inhibit, both in vitro and in vivo, most of the biological effects of Ang II (164). The combination of ACE1 and ARBs has been widely studied in patients with hypertension, nephropathy, diabetes, myocardial infarction, and heart failure (165). AT1 receptor antagonists should not be administered during pregnancy and should be discontinuedas soon

as pregnancy is detected. AT1 antagonists provided additive antihypertensive effect when used in combination with ACE inhibitors (166).

1.9.3 Aldosterone antagonists

This is a class of antihypertensive drugs that may offer unique advantages for people with heart failure and people with diabetic nephropathy. Mineralocorticoids cause retention of salt and water and increase the excretion of K+ and H+ by binding to specific mineralocorticoids receptors (167). The main action of aldosterone is to increase sodium re –absorption by the kidney, at the same time, it increase the ecretion of hydrogen and potassium ions. Aldosterone receptor antagonists block the effects of aldosterone, thus, decreasing sodium re –absorption and water retention by the kidneys, and consequently, lead to a decrease in blood pressure. Aldosterone antagonists are drugs given to people with moderate to severe chronic systolic heart failure and those with Conn-syndrome (167). The two aldosterone antagonists currently available on the market are spironolactone and eplerenone. Drugs such as spironolactone and eplerenone competitively inhibit the binding of aldosterone to the mineralocorticoids receptors (MR). As with the other K+ sparing diuretics, spironolactone often is coadministered with thiazide or loop diuretics in the treatment of edema and hypertension. Such combination result in increased mobilization of edema fluid while causing lesser perturbations of K+ hemeostasis. Spironolactone is particularly useful in the treatment of primary hyperaldosteronism and of refractory edema associated with secondary aldosteronism. Spironolactone is considered the diuretic of choice in patient with hepatic cirrhosis. Spironolactone, added to standard therapy, substantially reduces morbidity and mortality and ventricular arrhythmias (168) in patients with heart failure Eplerenone appears to be a safe and effective antihypertensive drug (169). One of the principal features of CHF is marked activation of the RAAS. In heart failure patients, plasma aldosterone concentrations may increase to as high 20 times the normal level. Antagonism oF aldosteron's actions is beneficial in patients with CHF (170). Patients with moderate to severe HF to treated with ≥25 mg daily of spironolactone and had a significant reduction in mortility and hospitalization for HF (170).

1.9.4 Renin inhibitors

Renin inhibitors are one of four classes of compounds that affect RAAS, ACE, ARBs and aldosterone receptor antagonists. It produce vasodilation by inhibiting the activity of renin, which is responsible for stimulating Ang II formatiom. Renin is a proteolytic enzyme that is released by the kidney in response to sympathetic activation, hypotension, and decreased sodium delivery to the distal renal tubule. Once released into the circulation, renin act on circulating angiotensinogen to form Ang I. These compounds were non peptidic renin inhibitors, had acceptable oral bioavailability and were potent enough for clinical use. The first renin inhibitors were synthesized already more than 40-years ago (171). The first drug in this class was aliskiren, which received a marketing approval in 2007 (172). As of January 2012, it is the only renin inhibitor on the market. Aliskiren is a renin inhibitor that was approved for the treatment of hypertension by and related disorders, alone or in combination with other antihypertensive agents (173). Aliskiren attenuates valsartan-induced increases in Ang II levels, thus preventing AT2 receptor-mediated cardioprotection by valsartan (174). Aliskiren is a renin inhibitor of a novel structural class that has been shown to be efficacious in hypertensive patients after once-daily oral dosing. In short-term studies, it was effective in lowering blood pressure either alone or in combination with valsartan and hydrochlorothiazide, and had a low incidence of serious adverse effects (175).

1.10 Aim of the studies

ACE inhibitors, such as captopril or enalapril and the angiotensin II type 1 receptor (AT1) antagonists developed later, such as losartan or valsartan, reduce mortality and morbidity in patients with heart attack and/or chronic heart failure. Therefore, such drugs are among the first choice options to treat these frequent cardiovascular diseases. When compared directly, AT1 antagonists, are better tolerated than ACE inhibitors as the incidence of both dry cough and angioedema is lower. The latter side effect is characterized by sometimes massive swellings of the mucosa and submucosa of the skin in head and neck region which can rapidly progress and is potentially life-threatening.

While experimental and clinical studies have provided good evidence that angioedema during therapy with ACE inhibitors is caused by the accumulation of bradykinin (176), which

is proteolytically cleaved by ACE, the mechanism of angioedema induced by AT1 antagonists remains unknown. However, it can be demonstrated that inhibition of AT1 in humans causes an increase in the plasma concentration of angiotensin II because a negative feedback mechanism inhibits the release of renin. Likewise, it has been shown that AT2 deficient mice have a four-fold increase in ACE activity as compared to transgene- negative littermates. Thus, a therapeutic blockade of AT1 is likely associated with increased stimulation of AT2 and possibly AT2-mediated inhibition of ACE. According to this hypothesis accumulation of bradykinin might underlie the reported development of angioedema induced by AT1 blockers.

Treatment with both types of drugs leads to an improvement of endothelial dysfunction and increase the bioavailability of the endogenous mediator nitric oxide (NO). NO is also released upon stimulation of bradykinin receptors. The regulation of the expression of AT2 is as yet poorly understood and no data exist on a possible effect of NO. The aim of this study was to investigate whether a molecular crosstalk from the NO-system to the renin angiotensin aldosterone system might contribute to the pathophysiology of angioedema induced by AT1-blockers.

MATERIALS AND METHODS

2. Materials and Methods

2.1 Substances and Solutions

All substances and solvents were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany), Merck KGaA (Darmstadt, Germany), and Carl Roth GmbH + Co. KG (Karlsruhe, Germany) in the best quality available. For the preparation of buffers and solutions, demineralized and freshly distilled water was used.

Bradford Reagent

0.02 % Coomassie Blue G 250

10 % Phosphoric acid

5 % Ethanol

Western blot transferbuffer

50 mM Tris

2 % SDS

10 % Glycerin

0.005 % Bromophenol blue

15 % ß- Mercaptoethanol

Stacking gel buffer

250 mM Tris / HCl

0.2 % SDS

pH 6.8

4 x Resolving gel buffer

150 mM Tris / HCl

0.4 % SDS

pH 8.8

10 x SDS – Running buffer

250 mM Tris / HCl

1.92 M Glycine

1 % SDS

pH 8.6

Western blot - buffer

25 mM Tris

200 mM Glycin 1

20 % Methanol

pH 8.5

TBS – buffer

20 mM Tris

150 mM NaCl

pH 7.6

TBST- buffer

20 mM Tris

150 mM NaCl

0.1 % Tween

pH 7.6

Cell lysis buffer (RIPA lysis buffer)

150 mM NaCl

50 mM Tris / HCl pH8.6

1 % NP- 40

0.1 % SDS

Stripping – buffer

5 mM Tris

2 % SDS

ß – Mercaptoethanol (1 : 33)

Lysis buffer (for protein with phosphatase inhibitors)

2.5 mM Na2P2O4

1 mM ß- Glycerolphosphat

1 mM Na3VO4

5 mM NaF

1 mM PMSF

0.04 % Proteinase Inhibitor Cocktail set 3 (Calbiochem, Darmstad).

Lysis buffer(For protein purification with phosphatase inhibitors)

5 mM Tris pH 7.6

1 mM EDTA

1 mM EGTA

2.5 mM Na2P2O4

1 mM ß- Glycerolphosphat

1 mM Na3 VO4

5 mM NaF

0.2 mM PMSF

0.1% Triton X-100

* PIC

10µg / mL PI M

*****PIC (Complete Protease Inhibitor Cocktail Tablets, Roche, Cat. 1169749800) was prepared as a 50 x stock solution by dissoving two tablets in 2 mL of dH2O according to the manufacturer instructions. The 50 x stock solution was diluted 1: 50 in the lysis buffer to receive the 1 x working concentration.

Coomassie Gel - Stain

Stock I	0.2 % Coomassie Brilliant blue G -250 in 90 % ethanol
Stock II:	20 % Acetic acid
Fixing solution:	40 % Ethanol
Destaining solution:	20 % Ethanol
	10 % Acetic acid
	70 % Distilled water

loading – buffer

50 % Glycerol

50 mM EDTA

0.1 % Bromophenol blue

to 50 ml 10 x TBE – buffer

OD measurement - buffer

50 mM Tris / HCl pH 7.5

1 mM EDTA

pH 8.0

2.2 Cell culture and incubation

2.2.1 Isolation of porcine aortic endothelial cells

The aortas of freshly slaughtered 7 to 9 month old pigs were obtained through the local slaughter house (Duisburg, Germany), and used for the preparation of the endothelial cells. Figure 2.1. The aorta was transported in sterile, autoclaved plastic bags with ice to avoid contamination by microorganism. The isolation and culturing, of pig aortas endothelial cells are primarily followed by the method described here. During the first step of the preparation, The aortas were cleaned of all adherent tissues by using a scissor, as showed in Figure 2.2. then, the outer parts of the aortas were disinfected with 80% alcohol, as showed in Figure 2.3. All branches of the intercostals arteries were closed by clamps, as shown in Figure 2.4. then, rinsed with 20 ml of PBS inside the aortas and the fluid was collected in the beaker. After that, 10 ml of dispase was injected into the aortas and they were incubated for 20 minutes in the incubator (TEMPP). Then the cell suspension was collected in a 50 ml falcon tube and rinsed with 20 ml of culture medium. After centrifugation at 200 X g for 5 minutes at room temperature, the supernatant was aspirated and discarded. The pellet was resuspended with 15 ml of appropriate culture medium M 199 containing 10% FCS (PAA, Kolbe), and then, the cell suspension was pipetted into a new 75 cm^2 cell culture flask to passage 4 which was used for incubation experiments.

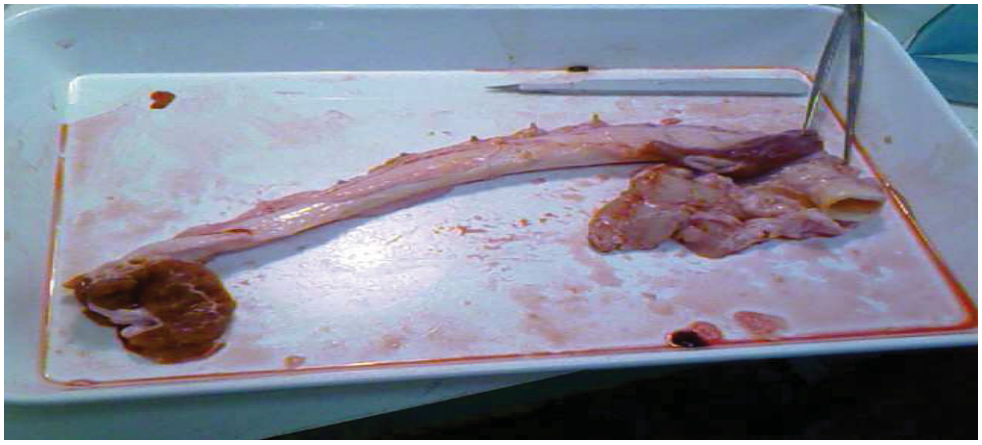


Figure 2.1: Aorta of the freshly slaughtered pigs for preparation of endothelial cells.

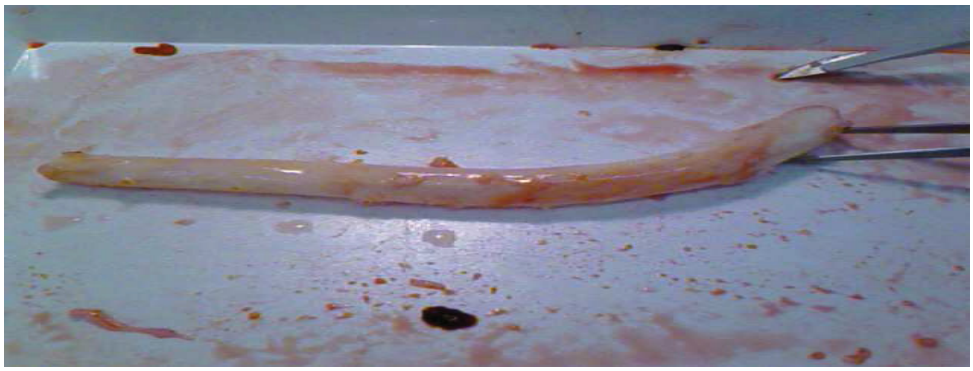


Figure 2.2: Aorta was cleaned of all adherent tissues by using scissor.

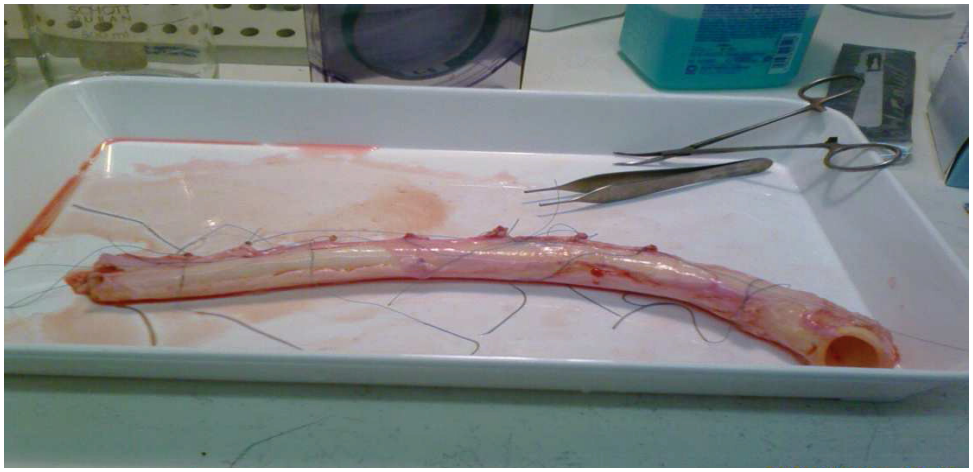


Figure 2.3: Outer parts of the aortas were disinfected with 80% alcohol.

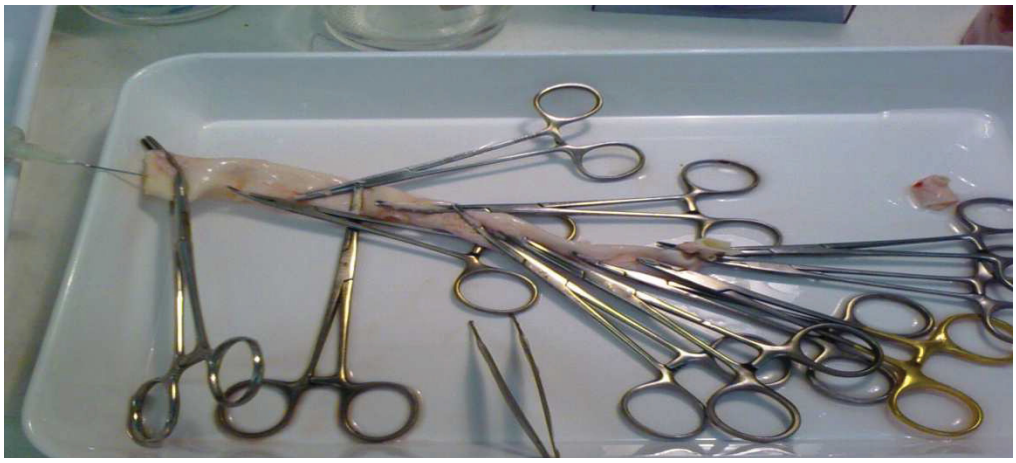


Figure 2.4: All branches of the intercostals arteries were closed by clamps.

2.2.2 Isolation of Human Venous Endothelial Cells from Umbilical Cord

Human umbilical vein endothelial cells (HUVEC), derived from apool of 10 individual women, were purchased from (ATCC® PCS-100-013, Wesel, Germany). The umbilical cords were disinfected externally with 80% alcohol, a cannula was fixed to one vessel and tightened by a pean-clamp, rinsed with 10 ml of PBS for two times, and the fluid was collected in a beaker. Then, the other end of the umbilical cord were occluded by another pean-clamp, after that 10 ml of dispase solution was injected into it and it was incubated for 15 minutes in the incubator(37 ºC). The cell susp ension was collected in a 50 ml falcon tube and rinsed with 20 ml of appropriate cell culture medium. Then, the suspension was centrifuged at 200 X g for 5 minutes at room temperature, and the supernatant was removed by a pipette, the pellet was re-suspended in 15 ml of cell culture M 199 medium and transferred to a new 75 cm^2 cell culture flask. Experiments with HUVEC were carried out at passage 4.

2.2.3 Mouse brain endothelial cells (bEND.3)

Brain endothelial mouse cells (bEND.3) were collected from the department of pharmaceutical chemistry II of the Pharmaceutical Institute, Bonn, Germany. And also obtained from ATCC® (Catalog NO. CRL- 2299) and the cell culture medium 500 ml bottles Dulbecco's Modified Eagle's medium (DMEM) + GlutaMAX™ (gibco® by Life Technologies™) as used for the maintenance of cell lines. 50 ml (Fetal calf Serum) gold (PAA, cölbe) and 5.5 ml 100 X times penicillin/streptomycin (PAA, cölbe) were added to the medium. Before pipetting the medium to the culture flask, the medium was warmed in 37 ºC in a water bath (GFL, Burgwedel). In new cell culture flask175 cm^2 flask pipet 30 ml or 15 ml in 75 $cm^º$ flask and added the bEND.3 cells to the culture flask. On the second day before starting the feeding, The cells were checked under a microscope (Phase – Contrast, Nikon) to make sure that there was no contamination in the culture cell flask. After determining the cell density by using the microscope, extract of the old medium and pipet 20 ml PBS were collected in bottles to wash culture flask. Then pipet 4 ml trypsin in 175 cm^2 cell culture flask, 2 ml trypsin in 75 cm^2 cell culture flask, trypsin incubation took from 3-5 minutes to release the cells. After the incubation period, we knocked on the bottle to shrink the cells and displace them from the bottle ground. One can control this process by the microscope. The cells were resuspended and they were pipeted it in a new falcon. The cells were centrifuged at 200 Xg

for 5 minutes in 22ºC. A medium supernatant was extracted by the pipette, the pellet was resuspended in 15 ml medium and transferred to the new cell culture flask with DMEM medium to passage 3 and passage 4 till passage 40. All experiments with bEND.3 were performed at passages 35-40.

2.3 Handling of the endothelial cell: PAEC, HUVEC and bEND.3 cell

2.3.1 Maintenance and culturing of the cell

The PAEC and HUVEC cells were maintained in M199 medium containing 10 % FCS (fetal calf serum) gold (PAA, cölbe) and 5.5 ml 100X penicillin/streptomycin (PAA, cölbe). The medium was warmed in 37 ºC in a water bath (GFL, Burgwedel) for every use and the cells were fed and supplemented with fresh medium for every two days. The cells were checked under a microscope (Phase – Contrast, Nikon) to ensure that there are no contamination cells.

2.3.2 Passaging of the cells

The medium and PBS was warmed in 37 ºC in a water bath and the cell density was determined by using the microscope; also the medium was removed. The cells were gently washed with 20 ml PBS to wash out the medium and dead cells. Then pipette out 4 ml trypsin for 175 cm^2 cell culture flask, 2 ml trypsin for 75 cm^2 cell culture flask and 1 ml trypsin for the petridish. The endothelial cells were treated with trypsin for 3-5 minutes to release the cells then, they were gently knocked on the bottom of the culture flask, so that they could be displaced from the bottle ground. The cells were re suspended and pipetted in a new falcon tube, and centrifuged at 200 X g for 5 minutes in 22 ºC. The M 199 medium supernatant was discarded and the pellet re suspended in 15 ml M 199 medium, and transferred into the new culture flask. This procedure was performed to all the passaging**.**

2.3.3 Scraping of the cells

The old cell culture medium was removed from the cell culture flask, and washed two times with PBS. To scrape out the cells, a 700 µl of cell lysis buffer was added to the cell culture flask, or 200 µl into the peteridish. Using a TC or a scraper (Orange Scientific), the cells were gently scraped and transferred to a new tube.

2.3.4 Lysis of the cells

After the scraping of the cells, the scraped cells with cell lysis buffer were transferred to an 1.5 eppendorf tube and placed on ice for 15 minutes in an ultrasonic circuit. Then, they were centrifuged at 100 X g for 15 minutes in 4 ºC, after the centrifugation, the supernatants were collected in new eppendorf tubes and stored at -80 ºC.

2.3.5 Treatment of the cells

After achieving 80% confluence of cells, the old medium was carefully removed and new fresh medium was added to the culture flask. The cells were treated with the listed substances (Table 1). The substances were also dissolved simultaneously with the solvent (PBS or DMSO) for control experiments. The cultured cells were treated with the substances at specific times and concentrations (see Table 1), and incubated in a humidified incubator at 37 ºC and 5 % CO2.

Table 1: Incubation of PAECs, HUVEC and bEND.3 cells with different substances at different time points

Incubation time	**1 hour**	**3 hour**	**6 hour**	**12 hour**	**24 hour**
NO - donor SNAP	1 µM 10 µM 100 µM	1 µM 10 µM 100 µM	1 µM 10 µM 100 µM	1 µM 10 µM 100 µM	
NO – donor DEA/NO	1 µM 10 µM 100 µM	1 µM 10 µM 100 µM	1 µM 10µM 100 µM	1 µM 10 µM 100 µM	
NO – donor DETA/NO		10 µM 100 µM 1 mM	10 µM 100 µM 1 mM	10 µM 100 µM 1 mM	10 µM 100 µM 1 mM
cGMP analogue 8-Br–cGMP		10 µM			
Analogue- cGMP 8 - pCPT - cGMP		1 µM 5 µM 10 µM			
Bradykinin		100 µM			
Activate of cAMP 6MB - cAMP		100 µM			

For the inhibitor treatment, the cells were preincubated with different inhibitors (sGC, PDE5, PKG and p38MAPK inhibitors) for 20 minutes or 30 minutes. Then, the substances were added to the same cell culture flask at different times and the cells were incubated in the humidified incubator at 37 ºC and 5 % CO2.

Table 2: Preincubation and incubation of PAEC with different inhibitors and NO donors

preincubation	**Time**	**Concentration**	**Incubation**	**Time**	**Concentration**
sGC inhibitor ODQ	30 min.	10µM	SNAP	3 hours	10µM
sGC inhibitor ODQ	30 min.	10µM	DEA/NO	3 hours	10µM
Phosphodiesterase inhibitor (Sildenafil)	20 min.	10µM	SNAP	3 hours 6 hours	10µM
PKG inhibitor (RP-pCPT-cGMP)	30 min.	20µM	DEA/NO	3 hours	10µM
P38MAPK inhibitor (SB 203580)	20 min.	10µM	SNAP	3 hours	10µM

2.4 Determination of the protein concentration

The Bradford method was first described by Dr Marion Bradford in 1976. This method is widely used to determine protein concentration. The assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 (Sigma, München) shifts from 465 nm to 595 nm during its binding to protein. Both hydrophobic and ionic interactions stabilizes the ionic form of the dye, causing a visible change of colour change. This assay is quite useful, since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. In addition to the standard liquid handling supply, a visible light spectrophotometer is needed with maximum transmission in the region of 595 nm on the border of the visible spectrum (DU640 spectrophotometer, Beckman, Krefeld). Bradford reagent can be prepared as follows: Dissolve 40 mg of Coomassie Brilliant Blue G-250 into 10 ml of 95 % ethanol and 20 ml 85 % (w/v) phosphoric acid. Add 150 ml of water once the dye has completely dissolved and filter through a filter

paper before use. If the Bradford reagent changes its colour from red-brown to blue filtration may have to be repeated. Calibration was performed with standard proteins and protein concentration was determined by a comparison to the colour response of the protein assay standards. Different concentrations of bovine serum albumin (BSA, Sigma, and München) were prepared for calibration of the measurement by pipetting, as shown in Table 3. Therefore, 50 µl of 10 mg/ml BSA stock solution was diluted with 450 µl Tris (5 mM, pH 7.6) to a working solution of 1 mg/ml, prior to the subsequent dilution procedures.

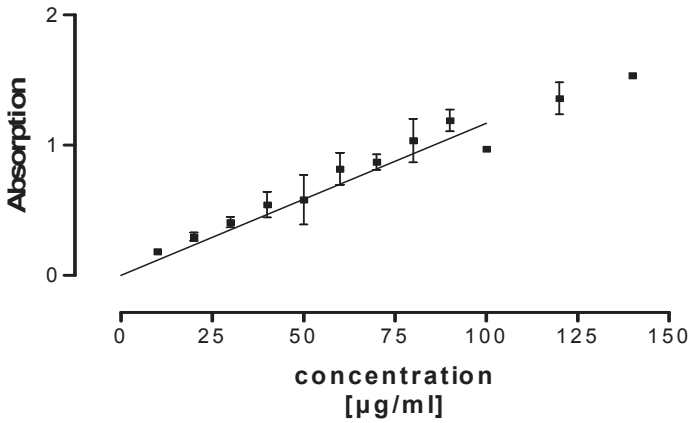
Table 3: Pipetting scheme for preparation of BSA calibration samples

Final concentration (µg/ml)	**Volume of BSA (µl)**	**Concentration of BSA (volume taken from)**	**Volume of tris (µl)**
140	100	1 mg/ml	614
120	100	1 mg/ml	733
100	50	1 mg/ml	450
90	50	1 mg/ml	505
80	50	1 mg/ml	575
70	200	140 µg/ml	200
60	200	120 µg/ml	200
50	200	100 µg/ml	200
40	100	80 µg/ml	100
30	100	90 µg/ml	200
20	50	100 µg/ml	200
10	50	100 µg/ml	450

2.4.1 Evaluation of the results of the dilution series

After completion of the measurement, the measured values were transferred into the computer program (Prism). The linear regression and the standard deviation were determined and the standard curve should be established.

The results of the measurements produced the following graph:



Linear equation of the curve **Y = 0.01167x ± 0.0003741**

The concentration of the protein was calculated using the following equation

C = (E – b) / m

E: Absorbance, **b**: Intercept, and **m**: Slope

2.4.2 Preparation of protein samples

The protein samples were prepared by diluting 20 µl of protein lysate in 180 µl Tris (5 mM, pH 7.6) to get 1 : 10 dilution. Subsequently five additional dilutions of 1 : 20, 1 : 50, 1 : 100, 1 : 200, 1 : 400 were prepared according to the pipetting scheme as shown in table 4.

Table 4: Pipetting scheme for sample preparation

Final dilution	Volume of Tris (µl)	Volume of sample (µl)
1 : 20	60	60
1 : 50	120	30
1 : 100	270	30
1 : 200	380	20
1 : 400	390	10

2.4.3 Photometric measurement

The samples were mixed thoroughly and 100 µl of each sample was transferred to a plastic cuvette (Sarstedt Cuvettes, 10 x 4 x 45 mm, Cat. 67 742) and mixed with 400 µl Bradford reagent. The samples were incubated for 5 min at room temperature and the A595 was measured in a spectrophotometer (Beckmann DU 640) against Tris as blank (Blan). Data analysis of the BSA calibration curve was performed with the computer software GraphPad Prism. Further data analysis of the protein samples based on the analysis of the GraphPad Prism software was calculated with Microsoft Excel.

2.4.4 Immunoblotting

The level of protein expression was determined by the western blotting analysis. A lysis was performed on the cells in RIPA buffer; they were denatured at 95 ºC for 5 minutes, and then, cleared by centrifugation at 13,200 rpm for 1 minute at room temperature. The proteins were loaded into 10 % SDS –polyacrylamide gel and separated in the electrophoresis chamber (BioRAD, München, Germany) by using 1x running buffer at 160 V for 90 minutes. A pre stained protein ladder (~ 11 – 250 kDa, Thermoscentific, RageRuler^{TM} plus Prestained Protein Ladder) was used as the molecular weight marker. Separated proteins were transferred to the PVDF membrane (Pore size 0.45µm, Millipore, Bedford, MA, USA). After the electrophoresis, the separated proteins were transferred from the inside of the gel onto the surface of a chemically inert PVDF membrane through a horizontal voltage application. Bounds on these membrane through hydrophobic interactions with the proteins they were accessible for subsequent analysis. The transfer was performed via a tank blotting procedure. The blotting cassette was opened and a sponge, soaked in a western-blot buffer, was placed on the black side of the cassette. The membrane was activated first for a few seconds in methanol for adequate protein binding. After activation, the membrane was equilibrated, in the western-blot buffer. Three Whatman® papers were placed on the sponge, also soaked in the western-blot buffer. The gel was placed on the wet papers carefully and the membrane (PVDF Western Blotting Membrane, Roche, Cat. No. 03010040001) was positioned onto the gel. Air bubbles between the gel and the membrane were diminished. The membrane was topped with three buffer-soaked Whatman® papers and a buffer-soaked sponge. The cassette

was closed and positioned in the cassette holder, so that the black side of the cassette showed to the black side of the holder. It was placed in the buffer tank and the tank was filled with the western-blot buffer. An icecooling unit was added to the tank for efficient system cooling, while it was running. The tank was arranged on a magnetic stirrer and a magnetic stir bar was added to the tank to ensure buffer homogeneity. The transfer was programmed for 90 minutes at 100 V. The cooling unit was changed after 45 minutes. The remaining binding sites of the membrane were blocked with a solution containing 5 % milk powder (Skim Milk Powder, Fluka, Cat. No. 70166-500G), in TBST. The membrane fragments were agitated for two hours in this solution at room temperature. Subsequently, the membranes were washed three times for 5 minutes with TBST. Following the blocking, the specific primary antibodies were added to the blocking solution and overnight incubation at 4 °C in the cold room was done. The next day, the membranes were washed four times for 5 minutes with TBST at room temperature. Following the washing step, the secondary antibody was incubated for one hour at room temperature. The secondary antibody recognizes the primary antibodies. The membranes were washed again four times for 5 minutes with TBST and placed on a wrapping film. Supersignal® West Dura, (Extended Duration substrate, Thermo Scientific) was prepared by mixing the two liquid components (Luminol/ Enhancer solution and stable peroxide solution), or ECL solution (Lumi- Light™ Western Blotting Substrate, Roche, Cat. NO. 12015200001) was prepared by mixing the two liquid components (Luminol/ Enhancer solution and stable peroxide solution) in 1 : 1 (v/v) ratio. Also 1 ml of the mixture was pipetted per membrane and incubated for 5 minutes at room temperature for chemiluminescence development. The incubated membranes were placed in a film exposure cassette (Kodak® BioMax® Cassette), and the signals were developed on a film (Amersham Hyperfilm® ECL High performance chemiluminescence Film, GE Healthcare, Cat. NO. 28906839) by using either an automatic develop unit (Dürr, XR24 NDT) or manually with developing and fixing a solution different exposure times were tried to end up in an optimal signal.

The protein expression was normalized to the housekeeping gene ß- actin after the stripping of membranes. After the signals were developed on the film autoradiography, the bands were quantified to calculate the relative expression of the target protein to actin expression. The quantification of the appropriate band density was accomplished by using a

densitometer (Gel Doc 1000, Bio – Rad, München). After the signals were developed on the film, they have to be quantified for a relative analysis of expression with regard to the housekeeping expression. The quantification was done with the computer software Quantity One® 4.2.3 (*Bio-Rad®*). The film was placed on a white light table and photographed with an installed camera from the gel documentation system (*Bio-Rad® Gel Doc™ 1000*). The signal intensities were measured in relation to a background, which had to be defined on the software desktop. The signals were marked individually, while the software calculated the signal intensities. The values were shown as uncalibrated optical density values.

Nitro cellulose membranes may be used for protein blotting, which is recommended for maximum performance. Pure cast nitrocellulose is generally preferred to support nitrocellulose. Protein should be transferred from gel to membrane by standared procedures. Block the membrane in Odyssey Blocking Buffer diluted 1:1 in PBS for 1 hour, be sure to use sufficient blocking buffer to cover the membrane. The membrane should not be exposed to Tween 20 until blocking is completed (Odyssey Blocking Buffer can be saved and re-used). Dilute primary antibody in Odyssey Blocking Buffer, optimum dilution depends on the antibody starting range of 1:1000 to 1:5000. Add 0.2% Tween 20 (to lower background) to the diluted antibody before incubation. Incubate the blot in primary antibody for 2 or 3 hours at room temperature or overnight in 4°C with gentle shaking. Wash membrane four times for 5 minutes each at RT in PBST with gentele shaking. Dilute the fluorescently-labelled secondary antibody in the Odyssey Blocking Buffer, and avoid prolonged exposure of the antibody vial light. Add tween 20 to the diluted antibody (1:10000). Incubate blot in secondary antibody for 60 minutes at RT with gentle shaking (protect it from light during the incubation). Wash membrane four times for 5 minutes each at RT in PBST with gentle shaking and protect it from light during the incubation. Rinse the membrane with PBS to remove Tween 20. The membrane is now ready to be scanned by (Odyssey Imagine System CLx, LI-COR, Bad Homburg, Germany). A comparative quantitative evaluation was performed with signals appearing on the same blot only. The quantified calculation for a relative analysis of expression is done with regard to actin or the vWF expression.

Table 5: Pipetting scheme for acrylamide – gel preparation

Substance	Volume for 10 % Separation gel	Volume for 4.5 % Stacking gel
Acrylamide / bisacrylamide (30%)	6.3 mL	2.4 mL
Separation gel buffer (4 x)	4.7	-
Stacking gel buffer (10 x)	-	4.0
dH2O	7.7	9.3
TEMED	18 µl	18 µl
APS (10 %)	180 µl	180 µl

Table 6: Primary antibodies used in this study

Antibodies & Source	Dilution	Manufacturrer
Anti – AT2 =Rabbit polyclonal IgG	1 : 1000	Sant Cruz Biotechnology
Anti – AT2 Rabbit polyclonal	1 : 500	Merck Millipore
AT1 rabbit polyclonal IgG	1 : 1000	Sant Cruz Biotechnology
Anti – Phospho MAPK Rabbit (Thr 180/Tyr 182)	1 : 300	Cell Signalling
P38 MAPK Rabbit	1 : 1000	Cell Signalling
Anti – Actin Rabbit	1 : 5000	Sigma, München
Mouse - Anti – eNOS	1 : 1000	BD- Transduction, Heidelberg
Von Willebrand Factor (VWF) polyclonal Rabbit – Anti - Human	1 : 1000	Dako

Table 7 : Secondary antibodies used in this study

Antibodies & Source	Dilution	Manufacturer
Immuno Pure Antibody Antigen – Rabbit IgG	1 : 20000	Thermo Scientific
Goat Anti – Rabbit - IgG	1 : 3000	Calbiochem
Goat Anti - Mouse	1 : 3000	Bio – Rad Munich
Stabilized Peroxidase Conjugated Goat – Anti- Rabbit	1 : 25000	Thermo Scientific
Goat anti-Rabbit IRDYe ® 800cw	1 : 10000	Odyssey LI – COR Biosciences
Goat anti – Mouse IRDYe ® 680LT	1 : 10000	Odyssey LI – COR Biosciences

2.5 Euthanasia and organ preparation

The euthanasia and organ preparation were already done prior to the start of the experiments. A day after the last drug administration, the animals were sacrificed by CO_2 inhalation and autopsied on ice for different organs and tissues. After the dissection and isolation of required organs and tissues, they were kept in a reagent tube and stored in liquid nitrogen until the end of the whole section. The samples were stored at -80 ºC.

2.6 Preparation and analysis of RNA

2.6.1 RNA isolation from Mouse brain endothelial cells (bEND.3)

For RNA isolation, a commercially available kit was used (RNeasy® Mini Kit, Qiagen®, Cat. NO. 74104). The 80 % to 90 % confluent cells were washed two times with 10 ml PBS and 700 µl cold lysis buffer (700 µl RTL buffer + 7 µl ß- mercaptoethanol) were added. The cells were scraped with a cell scraper (Neolab, Heidelberg) and transferred to an Eppendorf (-80 °C freeze). The cells were re suspended with the lysis buffer until all cell clumps were separated, to ensure a complete lysis of the cells. The cell lysate was centrifuged for 2 minutes at 13,000 rpm at room temperature, the supernatant was transferred into a new tube. One volume (approx. 700 µl) of 70 % (v/v) ethanol was added to the supernatant, re suspended thoroughly and transferred to the RNeasy® spin column placed into a 2 ml collection tube (provided with the kit) and centrifuged for 1 minute at 13,000 rpm at room temperature. The flow through was discarded, and subsequently, the on column DNAse digestion was performed, then, 350 µl of buffer RW1 was added to the spin column, and centrifuged for 1 minute at 13,000 rpm at room temperature and the flow through was discarded. The preparation of the DNAse working solution was prepared by adding 10 µl DNase I stock solution to 70 µl RDD buffer (RNase-free DNase set:Qiagen, Hilden). The 80 µl DNase mix was added to the spin column and incubated at room temperature for 15 minutes. Again 350 µl of buffer RWI was added to the column and centrifuged for 1 minute at 13,000 rpm at room temperature and the flow- through was discarded. Subsequently 500 µl of buffer RPE was added to the spin column, centrifuged 1 min at 13000 rpm at room temperature and the flow through was discarded. The spin column was placed on a new 1.5 ml collection tube and the washing with 500 µl RPE buffer was repeated and centrifuged for 2 minutes at 13,000 rpm at room temperature. At the final step, the collection tube with the flow through was discarded and the spin column was placed into a new 1.5 ml collection tube. 50 µl of RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 13,000 rpm at room temperature. The spin column was discarded and the concentration of the isolated RNA was measured.

2.6.2 Determination of the concentration of RNA

For RNA quantification, a spectrophotometric measurement of A_{260} was performed in an Eppendorf BioPhotometer by using disposable UV cuvettes (UVette® routine pack, Eppendorf, Cat. No. 952010069). The ratio from A260/A280 is a measure of the purity of the isolated RNA and should range from 1.8 to 2. Take the 45 µl OD measurement buffer into the 0.5 ml Eppendorf tube and add an additional 5 µl of the sample. A blank measurement was performed with the 50 µl OD measurement buffer. The dilution factor of 1 : 10 was entered in the photometer, and then, the sample was measured.

2.6.3 cDNA preparation

The real-time PCR system can only detect double-stranded nucleic acids; it is necessary to first reversely transcribe the isolated RNA into cDNA with the help of a reverse transcriptase (RNA-dependent DNA-polymerase). The purpose of converting RNA to cDNA is mainly for the analysis of the template mRNA because DNA is much more stable than RNA. Once RNA is converted to cDNA, the cDNA can be used for RT-PCR as a probe for the expression analysis and for cloning of the mRNA sequence. For transcription of the RNA into complementary cDNA, high capacity cDNA reverse transcription (Applied Biosystem, Weiterstadt) was used. The RT-PCR reaction was performed with1 µl of total RNA, 2.0 µl 10x RT Buffer, 0.8 µl 25x dNTP Mix (100 mM), 2.0 µl 10x RT

Random Primers, 1.0 µl MultiScribe Reverse Transcriptase, 1.0 µl RNase Inhibitor, 3.2 µl Nuclease-free H2O and the total reaction volume is 10 µl. Place the 2X RT master mix on ice and mix gently, and centrifuge shortly to collect the sample on the bottom of the tube. The samples were incubated as shown in Table 8 below.

Table 8: The program of the thermal cycler used for RT- PCR

	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 min	

2.6.4 Real Time PCR

Theory

Total RNA from mouse aortic tissue, HUVEC and bEND.3 was isolated using the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), and the cDNA synthesis was carried out as described above. The real time polymerase chain reaction, also called the quantitative real time polymerase chain reaction (qPCR) allows exponential amplification of short DNA sequences within a longer double stranded DNA molecule. For the real-time PCR, the required amount was reversely transcribed by the cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) and it was used according to the manufacturer's instructions. The DNA prepared under 2.3.6 served as the output strand primer were using the software Primer 3 Selected and ordered from MWG (Ebersberg) as fluorescent dye SYBR® Green was used as fluorescent dye. SYBR® Green was used, which intercalates between newly formed double- stranded nucleic acids. The principle of quantitative measurement method is based on the exponential increase of the fluorescence signal to increase of newly formed nucleic (Fig. 2.5) The date appearing concentration in which for the first time measurable DNA, called the Ct- value. This will be achieved more quickly, the more RNA is present from the beginning of amplification cycles.

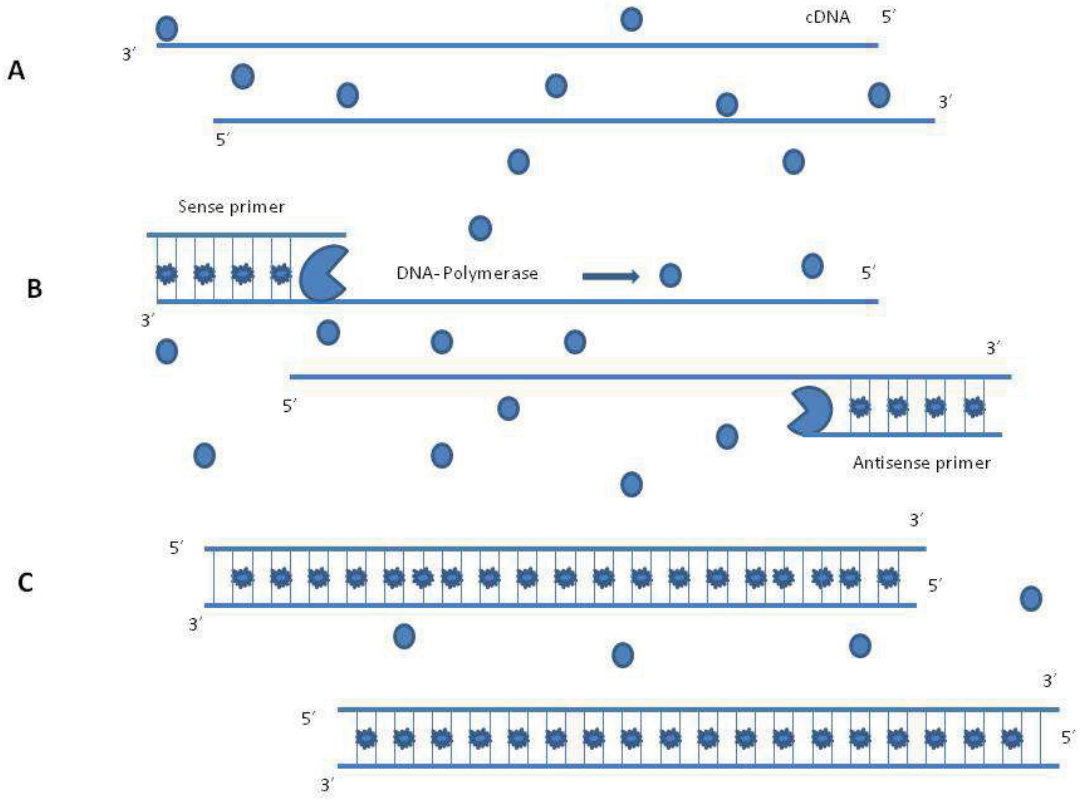


Figure 2.5: Schematic representation of the RT-PCR with SYBR® Green assay principle (peqlab, Erlangen). **A** denaturation: at 94 C, the denaturation is carried out both to individual strands double strange. **B** annealing and elongation: primers anneal to complementary and DNA polymerase prolongs the CDNA portion having complementary base pairs. **C**. SYBR® Green settles between newly formed double-stranded DNA and the result is an exponentially increasing fluorescence signal.

The target gene mRNA expression levels were determined by the real time PCR by using Sensi-mix SYBR® Green Reagent (Quantace, London, UK) and Quanti Tect Primer Assay (Qiagen, Hilden Germany) in the 7.300 real time PCR system (Applied Biosystems), according to the manufacturers´ instructions. The target gene expression levels of AT2 receptor were normalized to hypoxanthine guanine phosphoribosyl transferase (Hprt1). The primers, probes, and reaction mixtures, and the PCR conditions used for RT-PCR are summarized as in table 9. All materials were used obtained from Applied Biosystems (weiterstadt).

Table 9: The information of RT-PCR conditions for quantification of relative expression of mRNA of various genes in the tissues

	AT2	Hprt1
TagMan®Gene Expression Assay	Mm01341373	Mm00446968
Amplikon	86bp	65bp
Exon	2 - 3	6 - 7
Reactive approach	20 µl	20 µl
TagMan® Universal PCR Master Mix	10 µl	10 µl
Primer (10µM)	0.4 µl	0.4 µl
Sonde (10µM)	0.2 µl	0.2 µl
cDNA – Probe (100 - 500 ng)	9 µl	9 µl

Real time PCR run		
Polymerase activation	95 °C 10 sec	95 °C 10 sec
Denaturation	95 °C 15 sec	95 °C 15 sec
Annealing / Extension	95 °C 60 sec	95 °C 60 sec
Cycles	50	50

The AT2 mRNA expression levels relative to hprt1 were determined by using the ΔΔ- C_t - method, and effects of the treatment were expressed as fold change vs. control.

1. $C_{t\ GEN} - C_t$ Hprt1 = ΔC_t
2. $\Delta C_{t\ Probe} - \Delta C_t$ Calibrator = $\Delta\Delta C_t$

the formula according to The AT2 mRNA expression levels relative to hprt1 were determined by

using the ΔΔ- C_t - method, and effects of the treatment were expressed as fold change vs. control.

1. $C_{t\ GEN} - C_t$ Hprt1 = ΔC_t
2. $\Delta C_{t\ Probe} - \Delta C_t$ Calibrator = $\Delta\Delta C_t$

the formula according to (177) was the caculation of the relative

expresssion of the required gens.

3. Relative expression = $2^{-\Delta\Delta Ct}$

2.7 Statistical Analysis

All the basic statistical calculation was carried out in Software Graph Pad Prism (Version – 4, Graph Pad Software, San Diego). Data values are expressed as mean ± S.E.M of at least four different experiments and normalized to untreated controls. Statistical analysis utilized one-way analysis of variance (ANOVA) followed by Neuman – Keuls test used for comparison with more than two groups. $P \leq 0.05$ (*) was accepted as significant.

RESULTS

3. Results

3.1 Cell culture

Endothelial cells were isolated from porcine aortic endothelial cells (PAECs) and human umbilical vein endothelial cells (HUVECs), and established in primary culture after subcultivation; the cells showed an endothelial morphology. In addition, a cell line, derived from mouse brain endothelial cells was used (bEND.3).

3.1.1 Porcine Aortic Endothelial Cells (PAEC)

The phenotype of endothelial cells is maintained throughout the subcultivation. This type of endothelial cell culture has been widely used as a model to study the role of endothelium in many physiological and pathological conditions. PAECs were harvested from fresh porcine aortas and selected on the basis of morphological and phenotypical features. A light microscope image of PAECs, shows the confluence of the growth (**Figure 3.1)**.

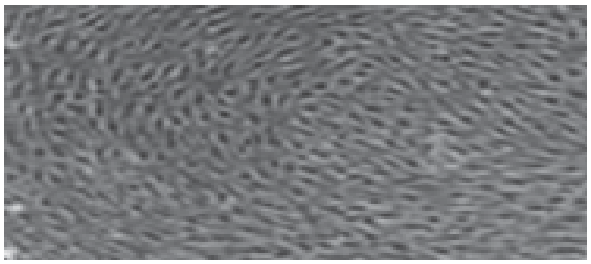


Figure 3.1: Example of the porcine aortic endothelial cells in culture shows the confluent monolayers of endothelial cells derived from the different porcine aortas. Cells were seeded in M199 medium containing serum and cultured for 7-10 days until confluent growth reached.

3.1.2 Mouse brain endothelial cell (bEND.3)

The cell line bEnd.3 cells (Brain Endothelium Mouse) were obtained from ATCC® Catalog NO. CRL- 2299The. This type of endothelial cell culture has been widely used as a model to study the role of endothelium in many physiological and pathological conditions. bEND.3 were harvested from mouse brain endothelial cells, and selected on the basis of morphological and phenotypical features. A light microscope image of bEND.3, shows the confluence of the growth (**Figure 3.2)**.

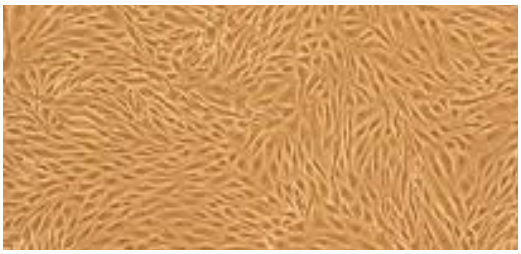


Figure 3.2: Example of the mouse brain endothelial cells in culture, shows the confluent monolayers of endothelial cells derived from the brain mouse cells. Cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) and cultured for 7-10 days until confluent growth reached.

3.1.3 Human Umbilical Vein Endothelial Cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) are derived from the endothelium of veins of the umbilical cord. They are used as a laboratory model system for physiological and pharmacological investigations. HUVEC were harvested from fresh human umbilical cords, and selected on the basis of morphological and phenotypical features. A light microscope image of HUVEC, shows the confluence of the growth (**Figure 3.3)**.

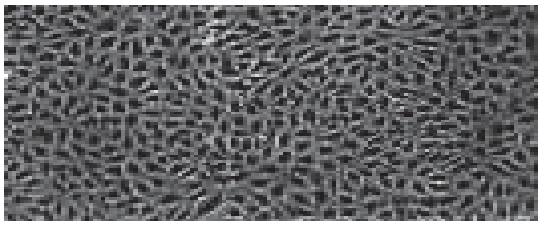


Figure 3.3: Example of the human umbilical vein endothelial cells (HUVEC) in culture. Shown are confluent monolayers of endothelial cells derived from different umbilical cords. Cells were seeded M199 containing 10% serum and cultured for 7-10 days until confluent growth reached.

3.1.4 Characterization of PAECs by staining for endothelial-specific proteins

The glycoprotein von Willebrand factor (vWF) and the cytosolic protein eNOS are the well studied markers for the endothelial cells. Thus, we used the western blot to demonstrate the expression of these proteins in our cell culture, which demonstrates the specificity of the cultured cell lines (**Figure 3.4**). Similarly, this procedure has been used to characterize bEND.3 cultures **(Figure 3.5).**

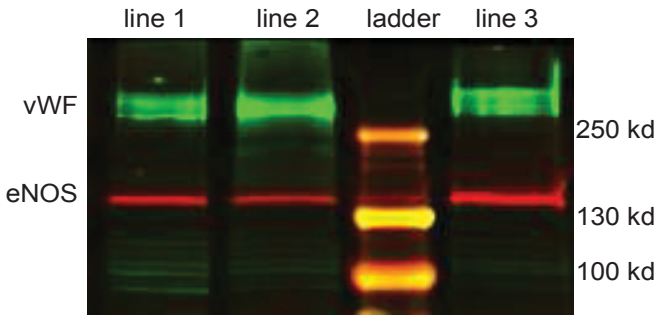


Figure 3.4: Characterization of the porcine aortic endothelial cells by staining for the endothelial-specific proteins van Willebrand factor (vWF) and endothelial NO-synthase. Homogenates of cell lines (40 µg of total protein) from 3 different porcine aortas were loaded on an SDS- PAGE gel, blotted and stained with appropriate antibodies (see Methods).

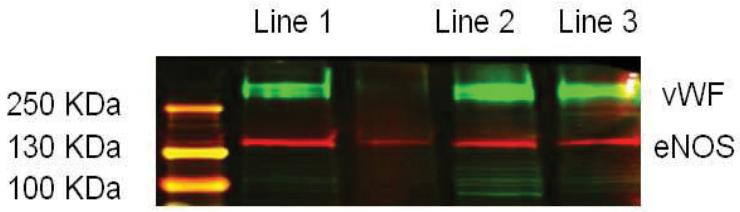
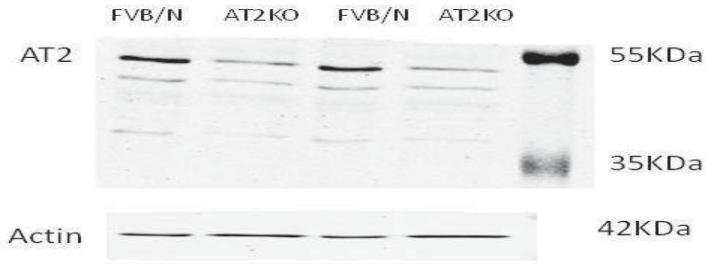


Figure 3.5: Characterization of the bEND.3 cells by staining for the endothelial-specific proteins van Willebrand factor (vWF) and the endothelial NO-synthase. Homogenates of cell lines (40 µg of total protein) from 3 different bEND.3 cells were loaded on an SDS- PAGE gel, blotted and stained with appropriate antibodies (see Methods).

3.2 Validation of the anti–AT2 receptor antibodies

Two different antibodies were used to detect AT2 protein expression. These antibodies have been evaluated using AT2 knockout mice and the results were confirmed by quantification of AT2 mRNA content using real-time PCR. Surprisingly, the initial experiments reveal that the millipore antibody gave protein signals at the expected size in aortic homogenates of AT2 knockouts. suggests that this polyclonal antibody is not highly specific for AT2 protein (**Figure 3.6**). But, the AT2 signal was much lower in AT2 in this knockout strain.

A



B

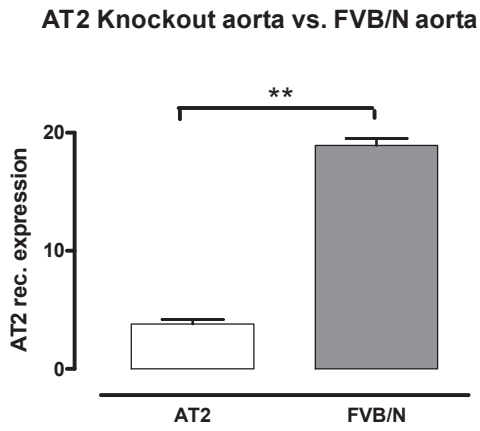


Figure 3.6: A Representative western blot examples of the AT2 receptor expression in in aortic tissue of AT2 knockouts and transgene negative littermates (mouse strain FVB/N). **B** Using the millipore antibody the AT2 signal was much lower with AT2 knockout mouse strain.

Therefore, it was evaluated if AT2 mRNA is expressed in aortic tissue of these AT2 knockouts. The results of a comparison of AT2 mRNA in AT2 knockouts and their appropriate transgene negative littermates (mouse strain FVB/N) showed an AT2 mRNA expression in AT2 knockouts reaching about 15 % of the expression measured in FVB/N (**Fig. 3.7**). Similar results were obtained in kidney tissue (**Figure 3.8**).

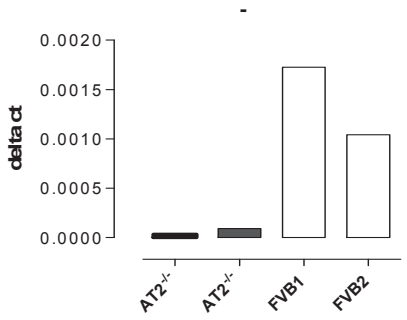


Figure 3.7: The results of a comparison of AT2 mRNA (gene: agtr2) in AT2 aorta knockouts and their appropriate transgen negative littermates (mouse strain FVB/N) demonstrated an AT2 mRNA expression in AT2 knockouts reaching about 15 % of the expression measured in FVB/N .

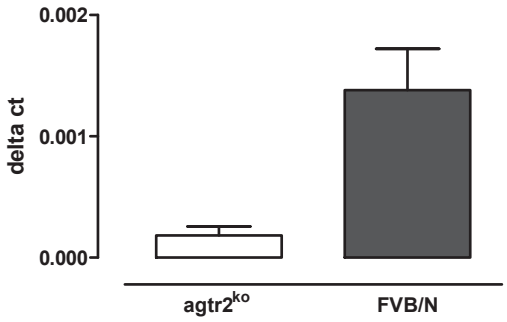
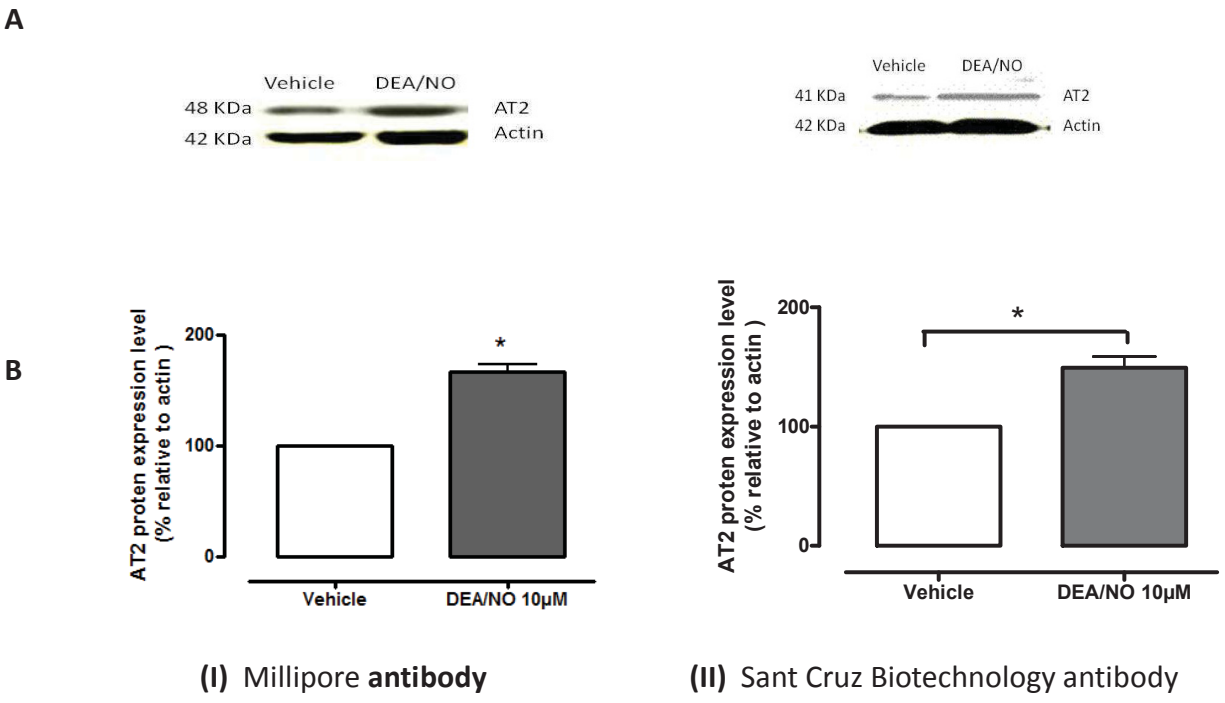


Figure 3.8: Similar results were obtained in kidney tissue (**Figure 3.8**). Comparison of AT2 mRNA in AT2 knockouts and their appropriate transgene negative littermates (mouse strain FVB/N) demonstrated an AT2 mRNA expression in AT2 knockouts reaching about 15 % of the expression measured in FVB/N.

Using the millipore antibody it has been shown that PAECs responds to NO with an increase of AT2 expression **(Figure 3.9 I).** A similar result was obtained when blot membranes were stained with another polyclonal antibody (Sant Cruz Biotechnology) designed to bind to AT2 protein on western blot membranes (**Figure 3.9 II).** This result confirms the specificity of the millipore antibody and shows that the other antibody is also suitable for the detection of AT2 expression. In addition we have observed similar results in bEND.3 cells as well (**Figure 3.10**). A quantitation of the upregulation of AT2 by other NO-donors is given in the following chapter 3.2.



(I) Millipore **antibody** **(II)** Sant Cruz Biotechnology antibody

Figure 3.9 I & II: A Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and vehicle (-) incubated in PAECs . B The relative expression of AT2 is calculated using the protein band intensity illustrated NO- induced up regulation of the AT2 receptor after 3 h incubation with DEA / NO in comparison to vehicle (vehicle = 100%). All blots were normalized to actin. All values are presented as mean ± SEM .(*P< 0.05 vs vehicle) n = 6-7 experments.

A

B

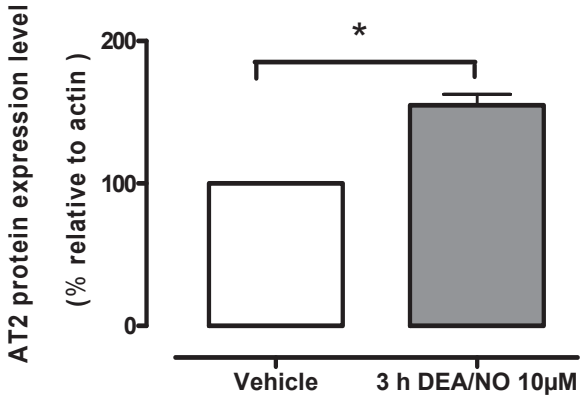


Figure 3.10: **A** Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and vehicle (-) incubated in bEND.3 cells. **B** Using the Sant Cruz Biotechnology antibody the results illustrated NO – induced up regulation of the AT2 receptor after 3h incubation with DEA/NO in comparison to vehicle (vehicle = 100%) All the blots were normalized to actin. All value is presented as mean ± SEM (*P< 0.05 vs vehicle) n = 6-7 experments.

3.3 Effect of NO on AT2 mRNA expression in bEND.3 and HUVEC

To analyse whether the up-regulation in the AT2 protein levels reflected a transcriptional regulatory event, we determined the AT2R mRNA by RT-PCR in the bEND.3 cells incubated with 10 µM NO- donor DEA / NO for 3 hours and 6 hours. A Significant (*p<0.0001) increase of the aortic AT2R mRNA was observed in the bEND.3 cells exposed to DEA / NO for 3 hours and 6 hours **(Figure 3.11),** the same results were obtained in the HUVEC incubated with 10 µM DEA / NO for 3 hours as a significant (*p<0.0341) increase of AT2 mRNA was observed in HUVEC (**Fig. 3.12**).

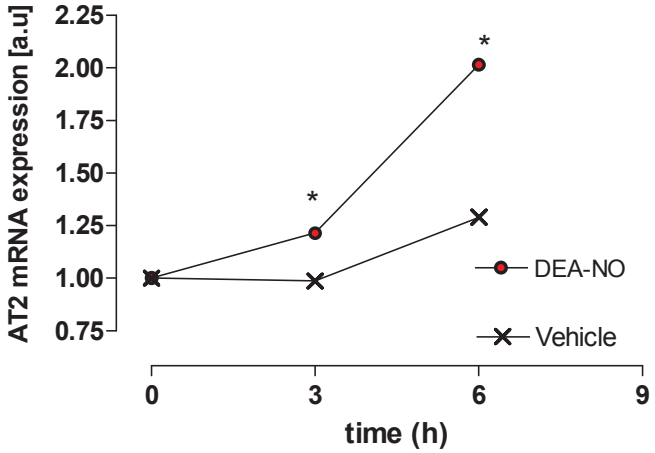


Figure 3.11: AT2R mRNA quantification by quantitative PCR in mouse brain endothelial cells (bEND.3) normalized to GAPDH levels. AT2R mRNA significantly (*p<0.0001) increased in the b. END3 cells exposed to DEA / NO for 3 hours and 6 hours compared with controls.

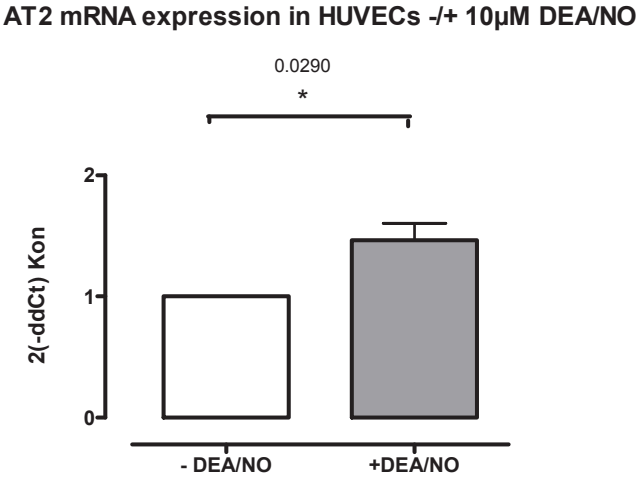


Figure 3.12: AT2R mRNA quantification by quantitative PCR in human umbilical vein endothelial cells (HUVEC) normalized to GAPDH levels. AT2R mRNA significantly (*p<0.0290, n= 5) increased in the HUVEC cells exposed to 10µM DEA/NO for 3 hours compared with controls.

3.4 Effect of NO on the AT2 mRNA stability in bEND.3

To study whether the upregulation of AT2 mRNA in response to NO is mediated by transcriptional or translational mechanisms or both, de novo transcription was blocked by exposing bEND.3 with actinomycin D and cells were subsequently incubated with DEA/NO (10 µmol/l) to determine AT2 mRNA expression. In another set of experiments cells were treated with DEA/NO alone (10 µmol/l). There was a rapid decay of AT2 mRNA to about 50 % of the initial value within the first 3 h in all groups studied (**Figure 3. 13**). Actinomycin D completely inhibited the increase of AT2 mRNA induced by DEA/NO, while DEA/NO itself did not change the half-live of AT2 mRNA that was observed following inhibition of transcription. These data suggest that the NO-induced increase of AT2 mRNA content in endothelial cells is the result of an elevated transcription rate, while increased mRNA stability is unlikely involved.

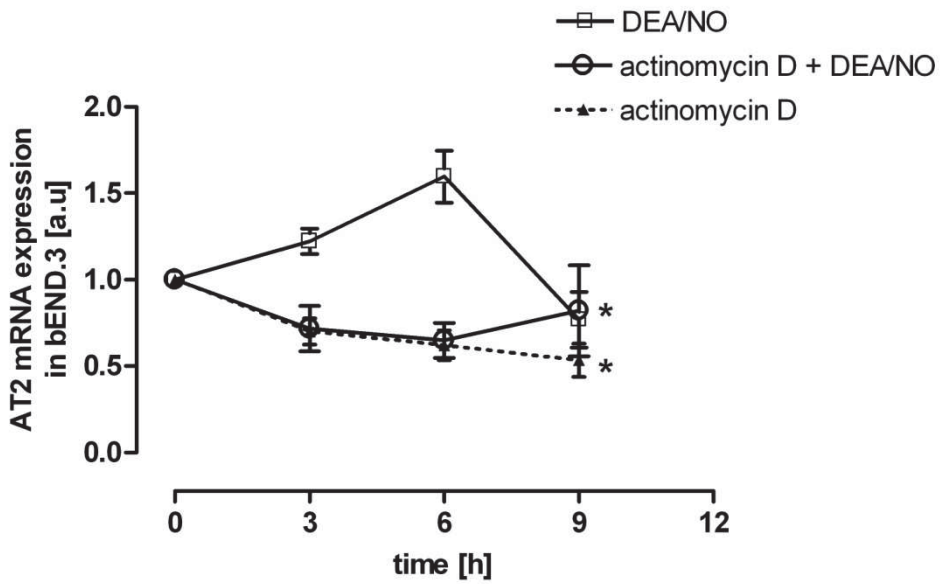


Figure 3.13: Effect of NO on AT2 mRNA stability in bEND.3**.** Messenger RNA levels were determined by quantitative realtime PCR in bEND.3 following inhibition of transcription with actinomycin D (10 µg/ml, 30 min) either alone or in the presence of 10 µmol DEA/NO and compared to the effects of 10 µmol DEA/NO alone at the indicated time points (*P<0.001, two-way ANOVA, n=6).

3.5 Studies on the effect of NO induced AT1–receptor -regulation by porcine aortic endothelial cells (PAEC)

We studied the AT1 receptor expression as control to elucidate whether the NO induced up-regulation of the AT2 receptor also led to a change in the AT1 receptor expression. Therefore, PAEC were incubated for 3 hours with the NO- donor SNAP (10 µM) to determine AT1 –receptor expression by western blot shown in **Figure 3.14**, the AT1 receptor expression in the cells with SNAP treated and untreated cells remained unchanged (students t –Test , P >0.05, n= 6).

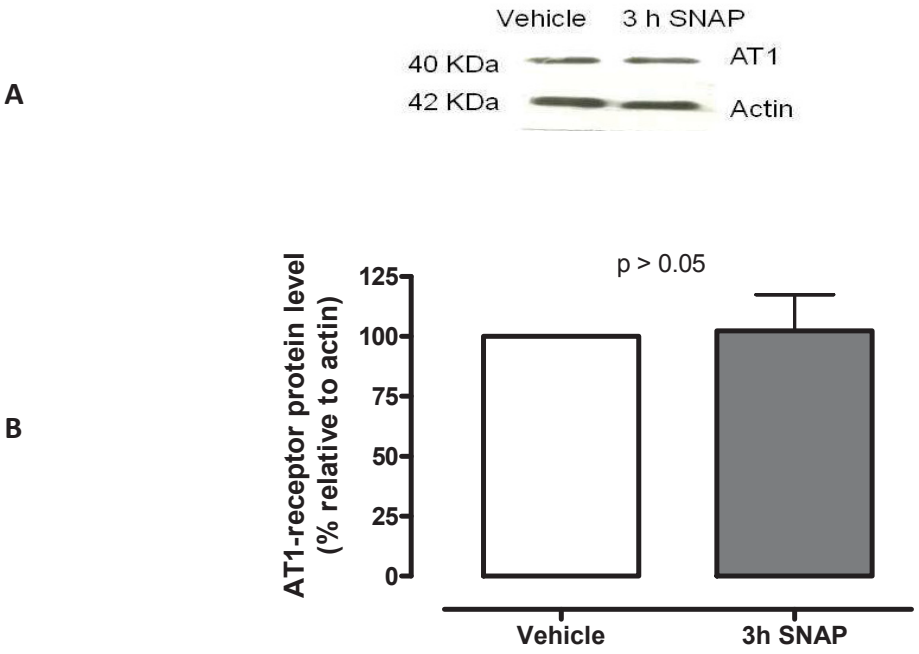


Figure 3.14: A representative western blot of the AT1-receptor expression in SNAP treated in PAECs compared to vehicle (vehicle = 100%). **B** Quantitative evaluation of western blots showed no change in AT1 receptor expression in response to SNAP in PAECs compared to vehicle. All the blots normalized to actin (students t Test, *P >0.05, n=5).

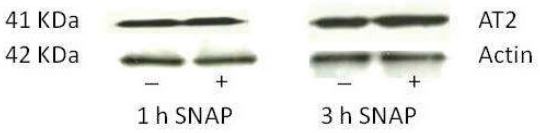
3.6 Studies on the effect of NO induced AT2–receptor up-regulation by endothelial cells

To study the involvement of the NO- cGMP signal transduction for the NO – induced AT2 receptor up-regulation and elucidate the mechanism of the NO – induced upregulation of AT2 expression, the primary cells were incubated with NO at different time intervals and the samples were collected.

3.6.1 Incubation of PAECs with the NO donor SNAP

Cells incubated with the NO donor SNAP (10 µM) and DMSO (0.005) served as the control, for different times 1 hour and 3 hours, while the results showed a significant upregulation of the AT2 receptor after 3 hour of incubation (one – way ANOVA, P<0.001, n= 7) (**Figure 3.15**). The results suggests that the AT2 receptor expression is up-regulated by NO in a time dependent manner.

A



B

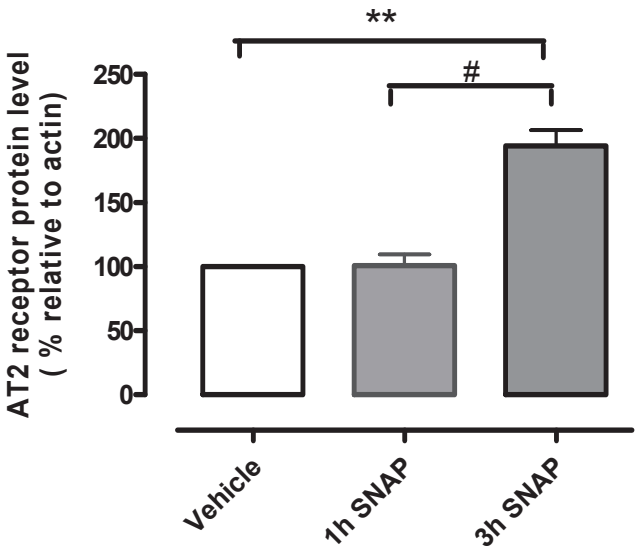


Figure 3.15: **A** Representative western blot examples of the AT2 receptor expression in SNAP (+) and vehicle (-) incubated in PAECs. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with SNAP in comparison to vehicle (vehicle = 100%). All blots were normalized to actin. All values are presented as mean ± SEM (** P<0.001 vs vehicle, # P<0.05 vs 1 h SNAP).

3.6.2 Incubation of PAECs with NO donor DEA/NO

DEA / NO showed a similar stimulatory effect on the expression of AT2 protein in PAEC. The cells were incubated with the NO donor DEA / NO for 1 hour and 3 hours, in a concentration of 10 µM, the AT2 receptor expression was examined. The results showed a significant up-regulation of AT2 receptor after 3 hour of incubation (one – way ANOVA, P<0.001, n= 6((**Figure 3.16**).

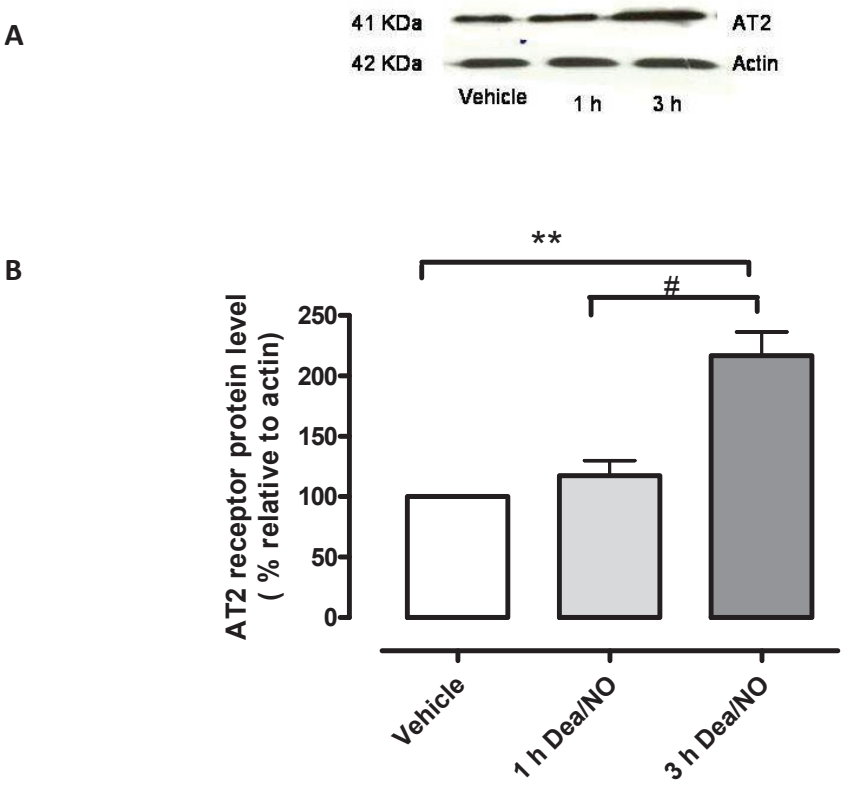
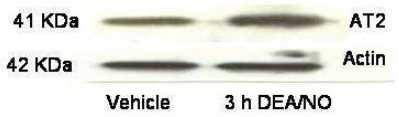


Figure 3.16: A Representative western blot examples of the AT2 receptor expression in DEA / NO(+) and vehicle (-) incubated in PAECs. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with DEA / NO in comparison to vehicle (vehicle = 100%). All blots were normalized to actin. All values are presented as mean ± SEM. (** P<0.001 vs vehicle, # P<0.05 vs 1 h DEA / NO).

3.6.3 Incubation of bEND3 cells with the NO-donor DEA/NO

To investigate the effect of AT2 receptor in **bEND3** cells, the cells were incubated with the NO donor DEA / NO 10 µM for 3 hours the AT2 receptor expression was examined. The results showed a significant increased of the AT2 receptor after 3 hours of incubation (**Figure 3.17**) (*P<0.05, 199.2 ± 26.93, n = 6).

A



B

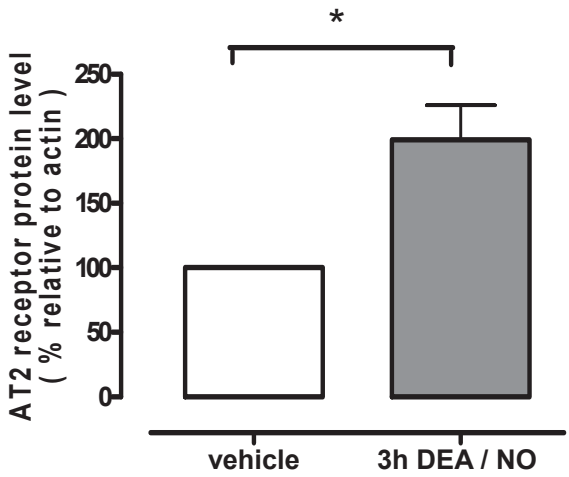
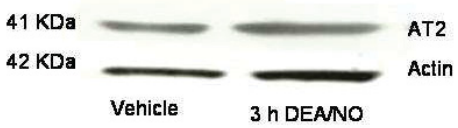


Figure 3.17: A Representative western blot examples of the AT2 receptor expression in DEA / NO(+) and vehicle (-) incubated in bEND.3 cells. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with DEA / NO in comparison to vehicle (vehicle = 100%). All the blots were normalized to actin. All values are presented as mean ± SEM. (* P<0.05 vs vehicle).

3.6.4 Incubation of HUVEC cells with the NO-donor DEA/NO

To investigate the effect of the AT2 receptor in HUVEC, the cells were incubated with the NO donor DEA / NO 10 µM for 3 hours. The results shows that there is a significant increase of the AT2 receptor expression after 3 hours of treatment (**Figure 3.18**). (*P<0.05, 174.1 ± 8.768, n = 6).

A



B

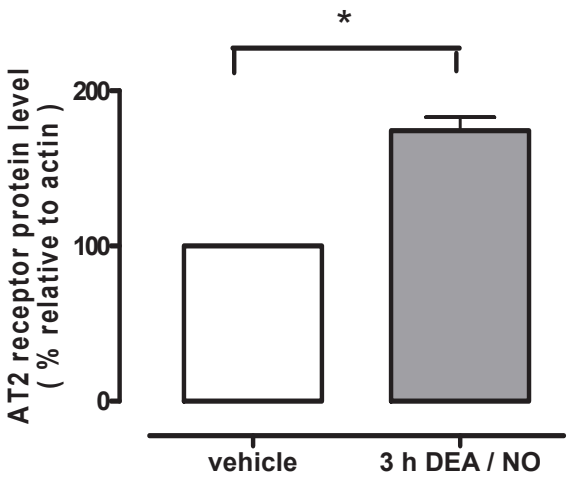


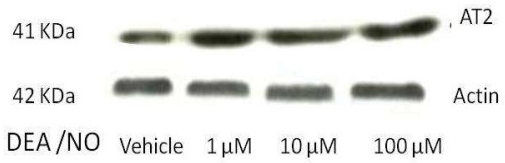
Figure 3.18: A Representative western blot examples of the AT2 receptor expression in DEA / NO(+) and vehicle (-) incubated in HUVECs. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with DEA / NO in comparison to vehicle (vehicle = 100%). All the blots were normalized to actin. All values are presented as mean ± SEM. (* P<0.05 vs vehicle).

3.7 Time and concentration dependency of NO donor DEA/NO and DETA/NO in PAEC and bEND.3 cells

3.7.1 Incubation of PAEC with NO donor DEA/NO

The cells were treated with concentration of 1 µM, 10 µM and 100 µM DEA / NO at different time points for 1 and 3 hours. Results showed a significant up-regulation of AT2 receptor after 3 hours of DEA / NO incubation (**Figure 3.20**).

A



B

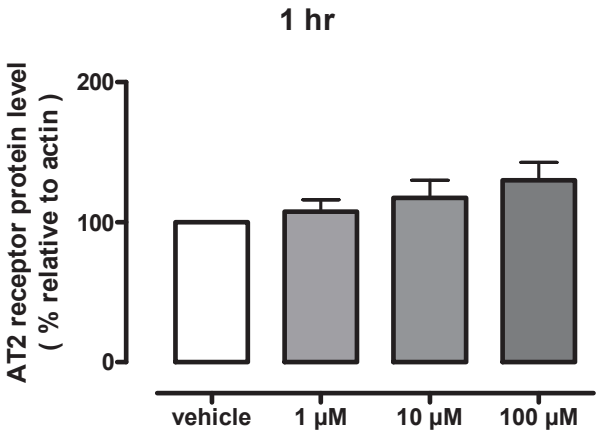
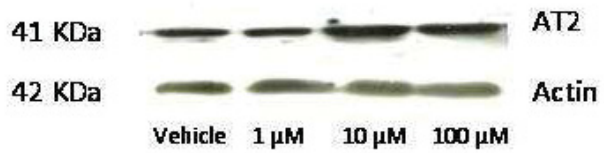


Figure 3.19: A Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and vehicle (-) incubated in PAECs. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 1 hour of incubation with DEA/NO in comparison to vehicle (vehicle =100%). All the blots were normalized to actin. All values are mean ± SEM. (no significant difference vs vehicle).

A



B

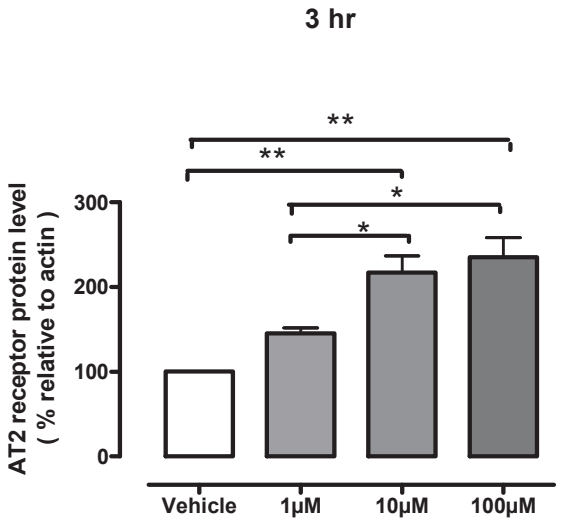


Figure 3.20: **A** Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and the vehicle (-) incubated in PAECs. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with DEA / NO in comparison to vehicle (vehicle =100%). All blots were normalized to actin. All values are mean ± SEM (** P<0.001 vs vehicle, * P<0.05 vs 1 µM).

3.7.2 Incubation of bEND.3 cells with the NO donor DEA/NO

The bENDs cells were incubated with the DEA / NO for 1 hour, 3 hours and 6 hours, in different concentrations of 1 µM, 10 µM, and 100 µM, respectively. The results showed a significant up-regulation of the AT2 receptor after 3 hours and 6 hours of the DEA / NO incubation (**Figure 3.22** and **Figure 3.23**).

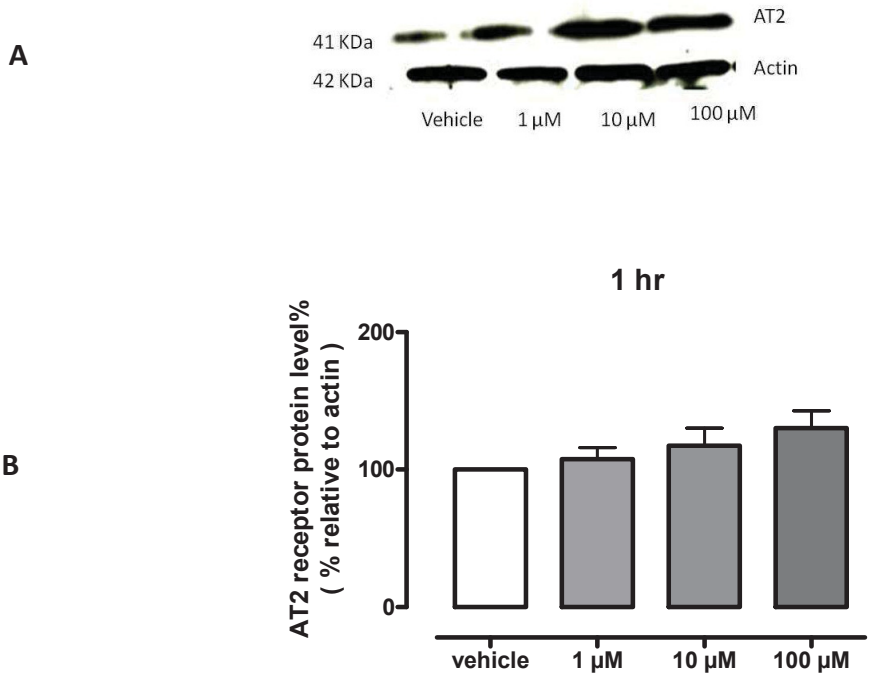


Figure 3.21: A Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and the vehicle (-) incubated in bEND.3 cells. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 1 hour of incubation with DEA / NO in comparison to vehicle (vehicle =100%). All blots were normalized to actin. All values are mean ± SEM (no significant difference vs vehicle).

A

B

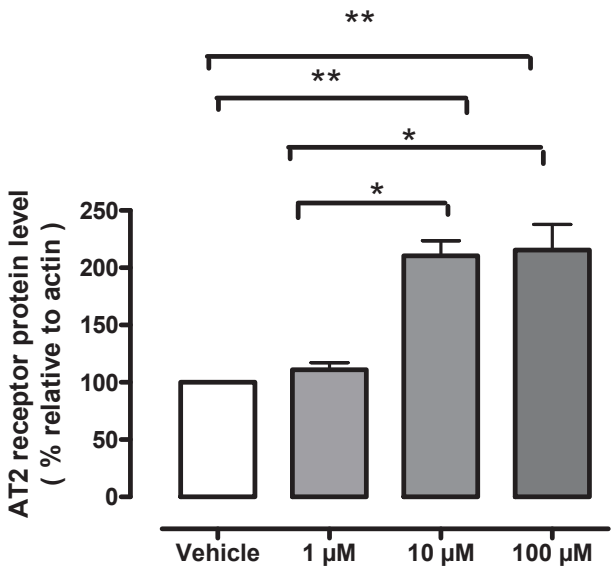


Figure 3.22: A Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and vehicle (-) incubated in bEND.3 cells. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours of incubation with DEA /NO in comparison to vehicle (vehicle =100%). All blots were normaized to actin. All values are mean ± SEM (** P<0.001 vs vehicle, * P<0.05 vs 1 µM).

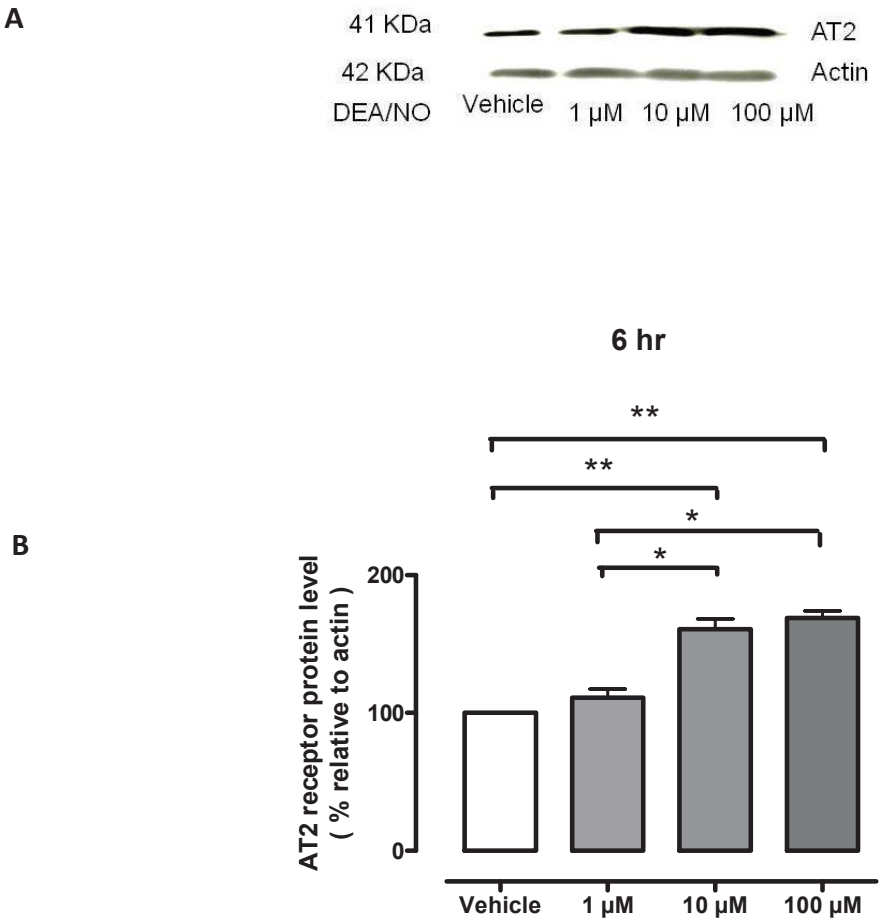
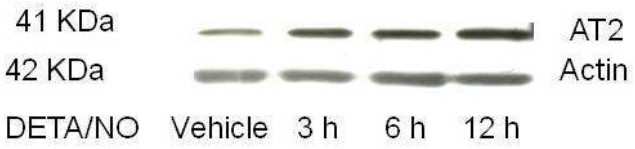


Figure 3.23: A Representative western blot examples of the AT2 receptor expression in DEA / NO (+) and vehicle (-) treated in bEND.3 cells. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 6 hours of incubation with DEA / NO in comparison to vehicle (vehicle =100%). All blots were normalized to actin. All values are mean ± SEM (** P<0.001 vs vehicle, * P<0.05 vs 1 µM).

3.7.3 Incubation of PAEC cells with the NO donor DETA/NO

The treatment of PAEC with DETA/NO for 3 hours, 6 hours, and 12 hours, with different concentration of 10 µM, 100 µM, and 1 mM, respectively, leads to the increased up-regulation of the AT2 receptor expression which is directly proportional to the concentration of DETA / NO (**Figure 3.24, Figure 3.25** and **Figure 3.26**).

A



B

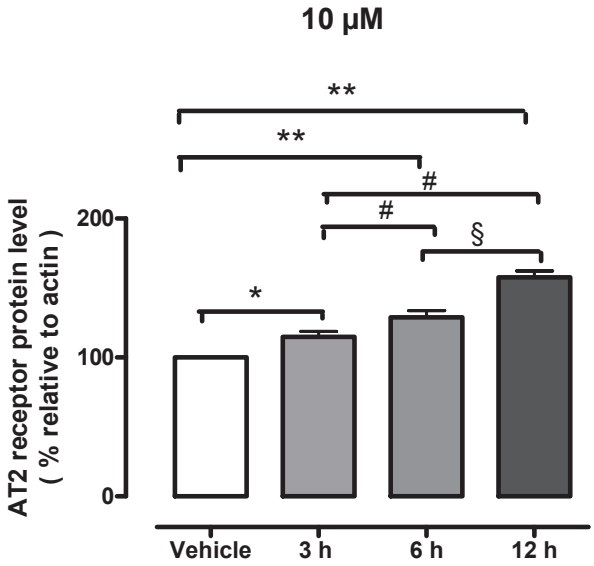
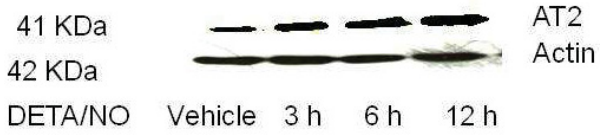


Figure 3.24: A Representative western blot example of the AT2 receptor expression in DETA / NO (+) 10 µM and vehicle (-) incubated in PAECs for 3 hours, 6 hours and 12 hours. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours, 6 hours and 12 hours of incubation with DETA /NO in comparison to vehicle (vehicle =100%). All the blots were normalized to actin. All values are mean ± SEM. (* $P<0.05$ vs vehicle, ** $P<0.001$ vs vehicle, # $P<0.05$ vs 3 h, § $P<0.001$ vs 6).

A



B

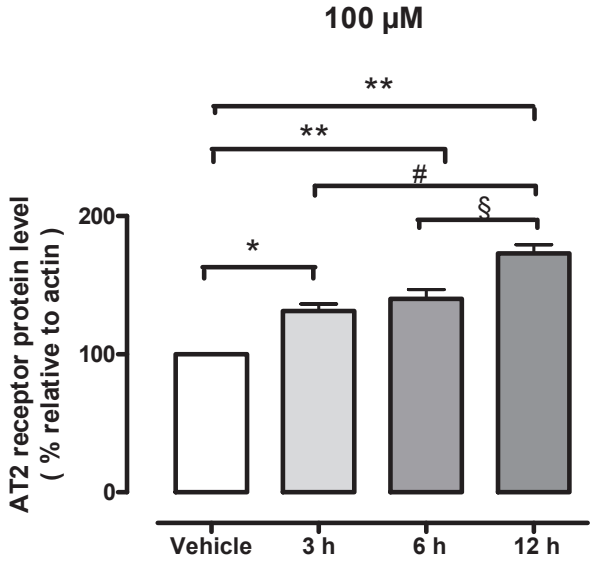


Figure 3.25: A Representative western blot example of the AT2 receptor expression in DETA / NO (+) 100 µM and vehicle (-) incubated in PAECs for 3 hours, 6 hours and 12 hours. **B** Quantitative evaluation showed NO – induced up-regulation of the AT2 receptor after 3 hours , 6 hours and 12 hours of incubation with DETA / NO in comparison to vehicle (vehicle =100%). All the blots were normalized to actin. All values are mean ± SEM. (* P<0.05 vs vehicle, ** P<0.001 vs vehicle, # P<0.05 vs 3 h, § P<0.001 vs 6h).

A

B

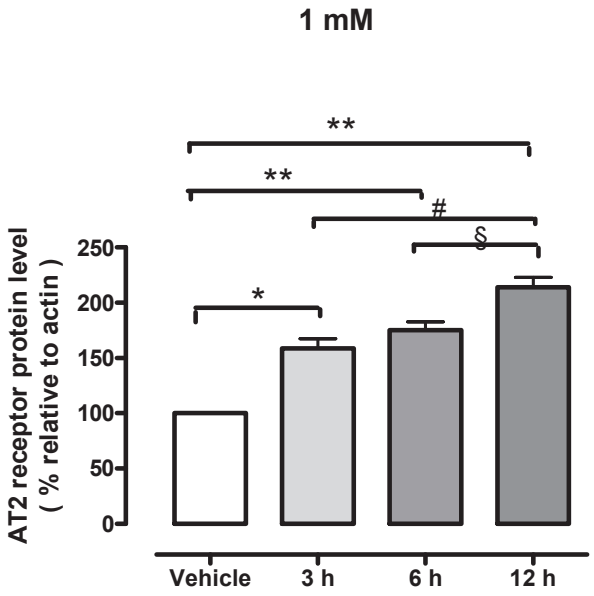


Figure 3.26: A Representative western blot example of the AT2 receptor expression in DETA / NO (+) 1 mM and vehicle (-) incubated in PAECs for 3 hours, 6 hours and 12 hours. **B** Quantitative evaluation showed NO –induced up-regulation of the AT2 receptor after 3 hours, 6 hours and 12 hours of incubation with DETA / NO in comparison to vehicle (vehicle =100%). All blots were normalized to actin. All values are mean ± SEM. (* $P<0.05$ vs vehicle, ** $P<0.001$ vs vehicle, # $P<0.05$ vs 3 h, § $P<0.001$ vs 6h).

3.7.4 Incubation of bEND.3 cells with NO donor DETA/NO

The time and concentration dependent AT2 expression with NO- stimulation has already been shown. We have performed the long-term exposure of bEND.3 cells with the long-lasting NO releasing substance DETA / NO for 3 hours, 6 hours, 12 hours and 24 hours, which resulted of in continuous up-regulation the of AT2 receptor protein levels (p< 0.001 vs vehicle), as shown in **(Figure 3.27).**

A

B

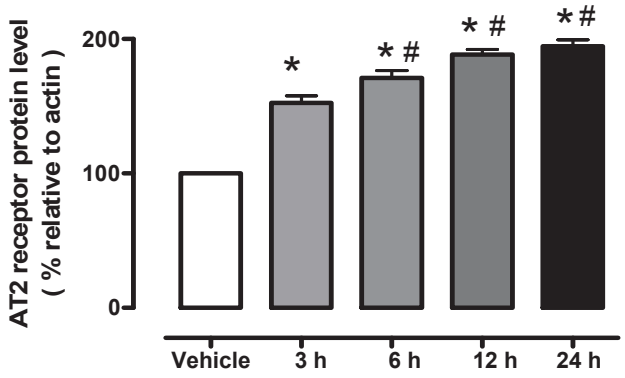


Figure 3.27: A The western blot example of the AT2 receptor expression in DETA / NO (+) 100 µM and the vehicle (-) incubated in bEND.3 cells for 3 hours, 6 hours, 12 hours and 24 hours. **B** Quantitative evaluation showed NO –induced up-regulation of the AT2 receptor after 3 hours, 6 hours, 12 hours and 24 hours of incubation with DETA / NO in comparison to vehicle (vehicle =100%). All blots were normalized to actin. All values are mean ± SEM. (* P<0.0001 vs vehicle, # P< P<0.05 vs 3 hours).

3.8 Effect of the sGC inhibitor (ODQ)

To investigate the underlying mechanism mediating NO-dependent upregulation of AT2, PAEC were incubated with the soluble GC inhibitors ODQ (10 µM), while ODQ itself had no effect, it completely inhibited AT2 upregulation induced by SNAP **(Figure 3.28).** (one way ANOVA, p< 0.001, n=6-7).

A

B

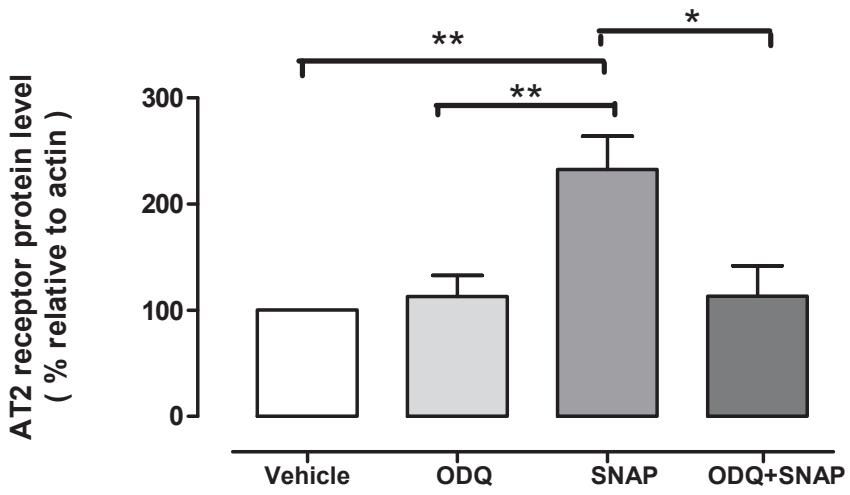


Figure 3.28: A Representative western blot example of the AT2 receptor expression in ODQ, SNAP, and ODQ and SNAP together incubated in PAECs and vehicle (vehicle = 100%). **B** Quantitative evaluation showed ODQ inhibited the NO –induced up-regulation of the AT2 receptor expression in response to SNAP. All the blots were normalized to actin. All values are mean ± SEM. (** P<0.001 vs vehicle, * P<0.05 vs SNAP).

3.8.2 Incubation of PAEC with ODQ and DEA/NO

To investigate the underlying mechanism mediating NO-dependent upregulation of AT2, PAEC were incubated with the soluble GC inhibitors ODQ (10 µM), while ODQ itself had no effect, it completely inhibited AT2 upregulation induced by DEA/NO **(Figure 3.29).** (one way ANOVA, p< 0.001, n=6).

A

B

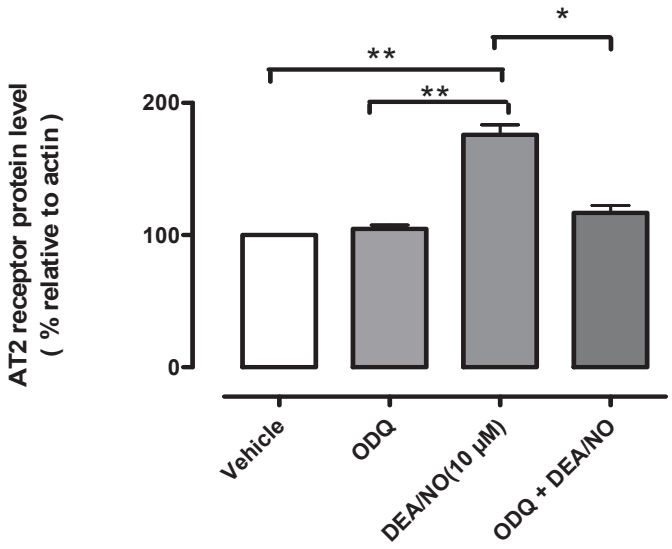


Figure 3.29: A The western blot example of the AT2 receptor expression in ODQ , DEA / NO and ODQ and DEA/NO together incubated in PAECs cells and vehicle (vehicle = 100%). **B** Quantitative evaluation showed ODQ inhibited the NO –induced up-regulation of AT2 receptor expression in response to DEA/NO. All the blots were normalized to actin. All values are mean ± SEM. (** P<0.001 vs vehicle, * P<0.05 vs DEA / NO).

3.9 Effect of Sildenafil

To further substantiate the important role of cGMP for the NO-dependent upregulation of AT2, PAECs were incubated for 3 hours with sildenafil. This drug is an fairly specific inhibitor of PDE V which increases the steady-state levels of cGMP. Thus, it was investigated whether sildenafil incubation results in upregulation of AT2 and whether sildenafil potentiates the effect of SNAP. As shown in **Figure 3.30**, there was a significant and comparable increase of AT2 expression following a 3 hours incubation with SNAP. Moreover, a combination of both drugs resulted in a stronger increase of the AT2 expression than the effect achieved with either of the drugs. Likewise, a similar pattern of AT2 upregulation was observed following a 6 hours incubation period (Significant, P< 0.05) **(Figure 3.30).** These data confirm the view that the NO-induced upregulation of AT2 is indeed dependent on cGMP.

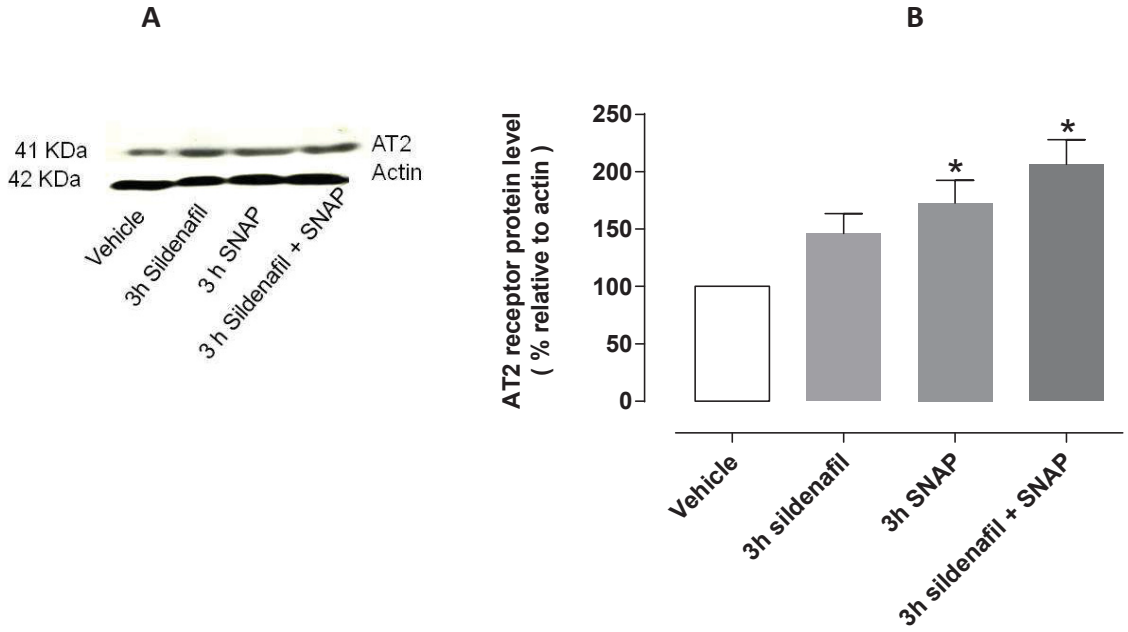
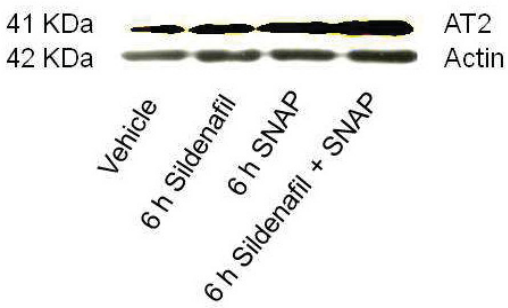


Figure 3.30: A The western blot example of the AT2 receptor expression in Sildenafil, SNAP and Sildenafil and SNAP together treated in PAECs and vehicle (vehicle = 100%). **B** Quantitative evaluation showed sildenafil induced up-regulation of AT2 receptor expression. All blots were normalized to actin. All values are me an ± SEM . (P value = 0.003).

A



B

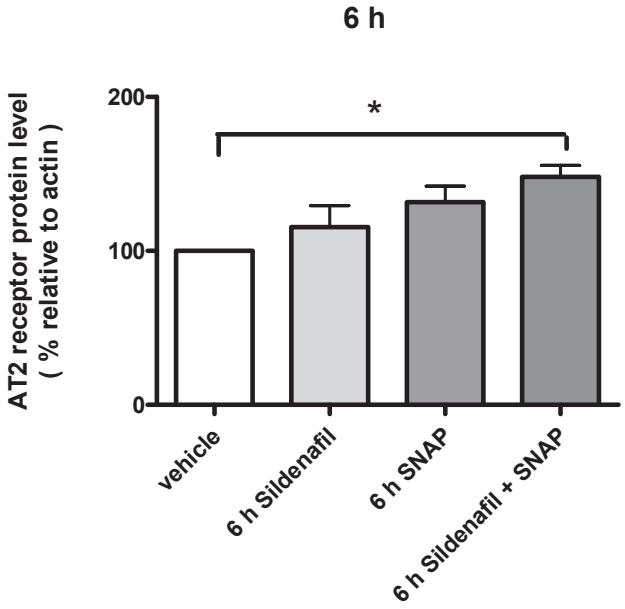


Figure 3.31: A The western blot example of the AT2 receptor expression in Sildenafil, SNAP and Sildenafil and SNAP together treated in PAECs and vehicle (vehicle = %). **B** Quantitative evaluation showed sildenafil induced up- regulation of the AT2 receptor expression. All the blots were normalized to actin. All values are mean ± SEM. (*P<0.05 vs vehicle).

3.10 Effect of cGMP analogues

According to the important role of cGMP for the NO-induced upregulation of AT2, PAECs were incubated with the cell permeable cGMP analogues 8-Br-cGMP and 8-pCPT-cGMP for 3 hours and the expression level of AT2 was assessed by westernblot.

As shown in **Figure 3.32**, the less specific cGMP analogue 8-Br-cGMP induced an up regulation of AT2. Likewise, 8-pCPT-cGMP, which is known to be much more specific for PkG over PKA, showed an up regulation of AT2 similar in magnitude to that observed with 8-Br-cGMP (**Figure 3.33).** Thus, activation on PKG seems to be a crucial step in mediating the effect of NO on the AT2 expression.

A

B

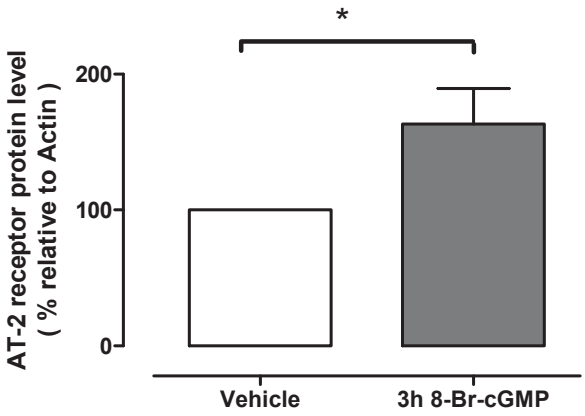
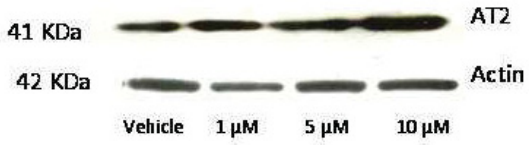


Figure 3.32: A Representative western blot example of the AT2 receptor expression with 8- Br-cGMP incubated in PAECs cells and vehicle (vehicle = 100%). **B** Quantitative evaluation showed that 8Br-cGMP increased the AT2 receptor expression. All the blots were normalized to actin. All values are mean ± SEM .(*significant difference to the vehicle, *P<0.05 vs vehicle, 163.5 ± 25.79, n = 6).

A



B

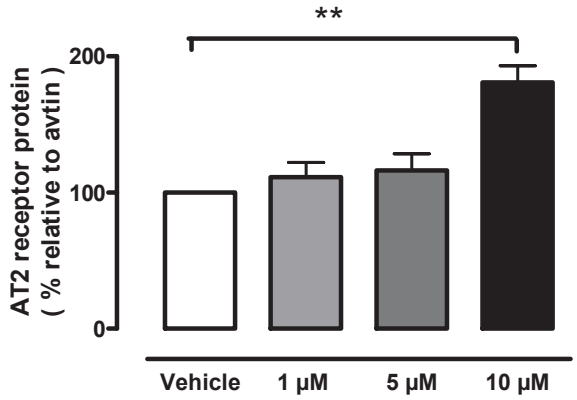
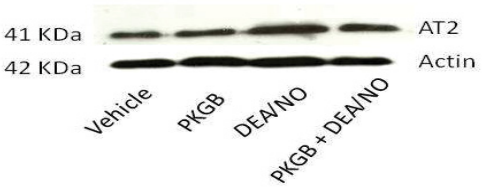


Figure 3.33: A The western blot example of the AT2 receptor expression with 8- pCPT-cGMP incubated in PAECs (vehicle = 100%). **B** Quantitative evaluation showed 8-pCPT-cGMP induced up-regulation of AT2 receptor after 3 hours of incubation with different concentration 1, 5 and 10 µM. All the blots were normalized to actin. All values are mean ± SEM (**P < 0.001 vs vehicle, n= 7).

3.11 Incubation of PAEC with PKG inhibitor

To investigate the effect of the AT2 receptor in porcine aortic endothelial cells, the cells were incubated with the PKG inhibitors (**RP –pCPT cGMP**) 20 µM and DEA / NO 10 µM for 3 hours. while inhibition of PKG with the inhibitor RP-pCPT-cGMP reversed the upregulation of AT2 induced by DEA/NO (Figure 3.34).

A



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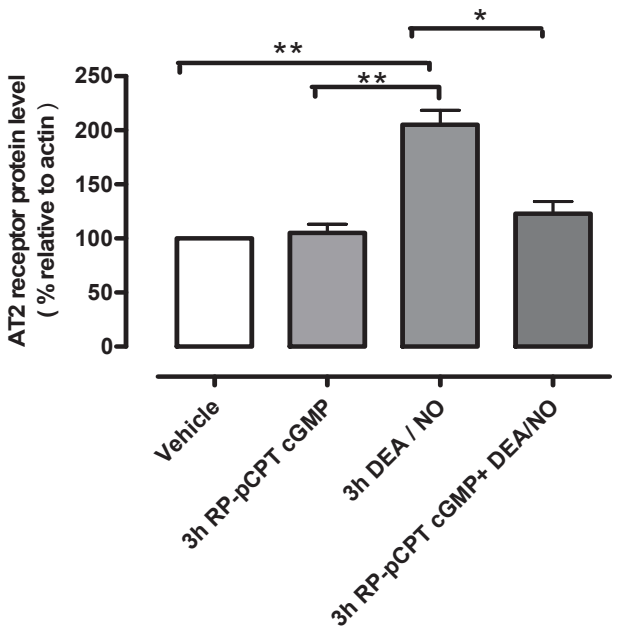
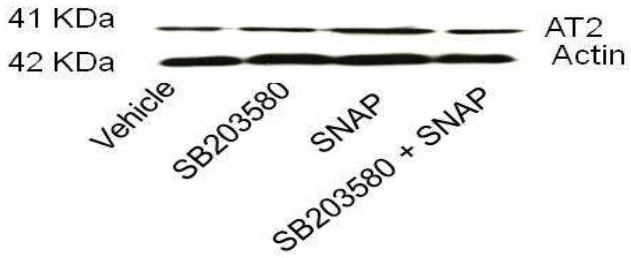


Figure 3.34: A The western blot example of the AT2 receptor expression in PAECs incubated with the PKG inhibitor and with DEA/NO (vehicle = 100%). **B** Quantitative evaluation showed PKG inhibitor decrease the AT2 receptor expression in response to DEA/NO. All the blots were normalized to actin. All the values are mean ± SEM. (** P<0.001 vs vehicle * P<0.05 vs 3 h DEA / NO).

3.12 Incubation of PAEC with P38 MAPK inhibitor

To investigate the effect of AT2 receptor in porcine aortic endothelial cells, the cells were treated with P38 inhibitors (**SB203580**) 10µM and the NO donor SNAP 10 µM for 3 hours and the AT2 receptor expression was examined. The increased bioavailability of NO was associated with a significant increased AT2 receptor expression, and this effect decreased in the PAEC cells pre incubated with the P38 inhibitor (SB203580) (**Figure 3.35**).

A



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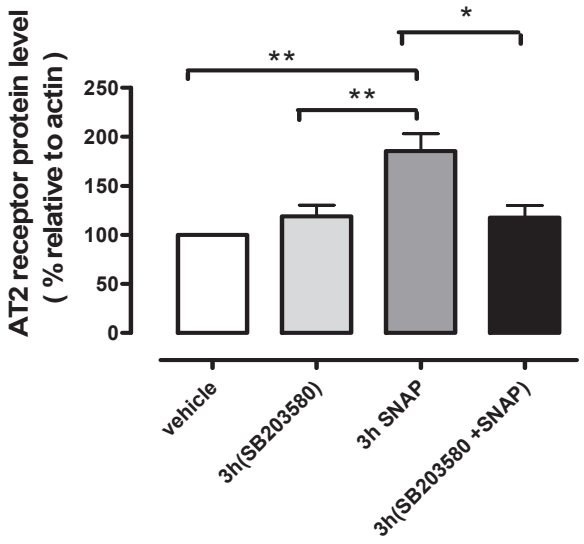


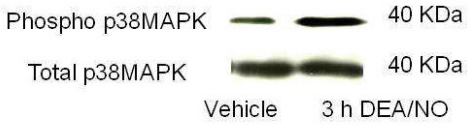
Figure 3.35: A Representative western blot example of the AT2 receptor expression in PAECs incubated with P38 MAPK inhibitor and SNAP (vehicle = 100%). **B** Quantitative evaluation showed that the P38 MAPK inhibitor decreases AT2 receptor expression in response to SNAP. All the blots were normalized to actin. All values are mean ± SEM. (** P<0.001 vs vehicle, * P<0.05 vs SNAP).

3.13 Investigation of P38 MAPK phosphorylation

3.13.1 Incubation of PAEC cells with the NO donor DEA/NO

To investigate which transcription factors are activated by NO, the PAEC cells were lysed and blotted for the measurement of phosohorylated and total P38 MAPK. The PAEC were incubated with the NO donor DEA / NO for 3 hours, representive blot for phospho P38 MAPK and total P38 MAPK are shown (**Figure 3.36**). DEA/NO (10 µM), significantly increased phosphorlyation P38 MAPK, (students t- Test, P= 0.0156, n = 7).

A



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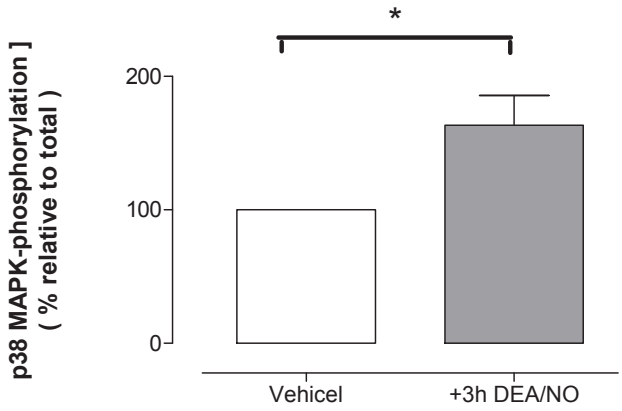


Figure 3.36: A Representative western blot for phosphorylated the P38 MAPK expression (40KDa) and total P38MAPK expression in DEA / NO incubated in PAECs compared to vehicle (vehiclel = 100%). **B** Quantitative evaluation showed increase of the P38 MAPK phosphorylation in response to DEA / NO. All the blots were normalized to total protein All values are mean ± SEM. (students t Test, *P < 0.05 vs vehicle, n= 5).

3.13.2 Incubation of PAEC cells with 8-pCPT-cGMP

To investigate the p38MAPK phosphorylation, the cells were lysed and blotted to measure the phosphorylated and total p38 MAPK. The PAEC cells were incubated for 3 hourswith 8-pCPT- cGMP, which is an activator of the cGMP dependent protein kinase, the representive blot for phospho- p38 MAPK and total p38MAPK are shown (**Figure 3.37**), 8-pCPT- cGMP with the 10 µM concentration significantly increased the phosphorlyated p38 MAPK, (*P < 0.05, 241.7 ± 14.20, n = 6).

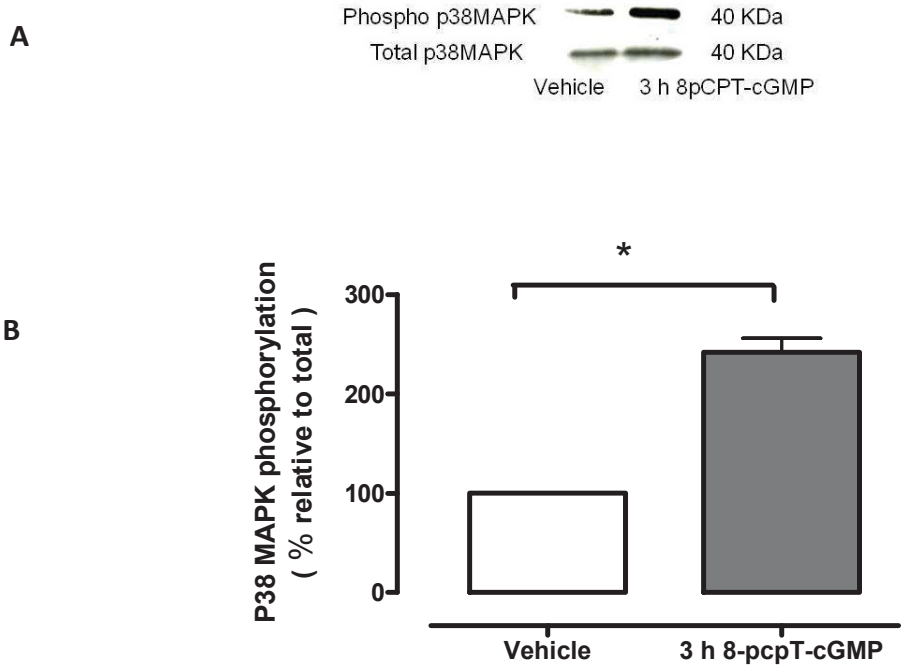
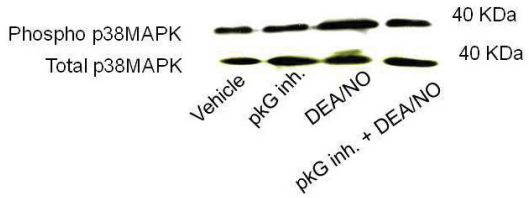


Figure 3.37: A representative western blot example of P38 MAPK phosphorylation in 8-pCPT-cGMP and vehicle incubated PAECs (vehicle = 100%). **B** Quantitative evaluation showed 8-pCPT-cGMP increases P38 MAPK phosphorylation. All the blots were normalized to total protein, All values are mean ± SEM. (*P < 0.05 vs vehicle).

3.13.3 Incubation of PAEC cells with PKG inhibitors

To investigate the effect of the P38 MAPK phosphorylation in porcine aortic endothelial cells, the cells were treated with the PKG inhibitors (**RP –pcpT cGMP**) 20µM and the NO donor DEA/NO 10 µM for 3 hours and examined for the P38 MAPK phosphorylation. The results showed that an increased bioavailability of NO was associated with a significantly increased p38 MAPK activation, and this effect was decreased in the pre incubated cells with the PKG inhibitor (**Figure 3.38**).

A



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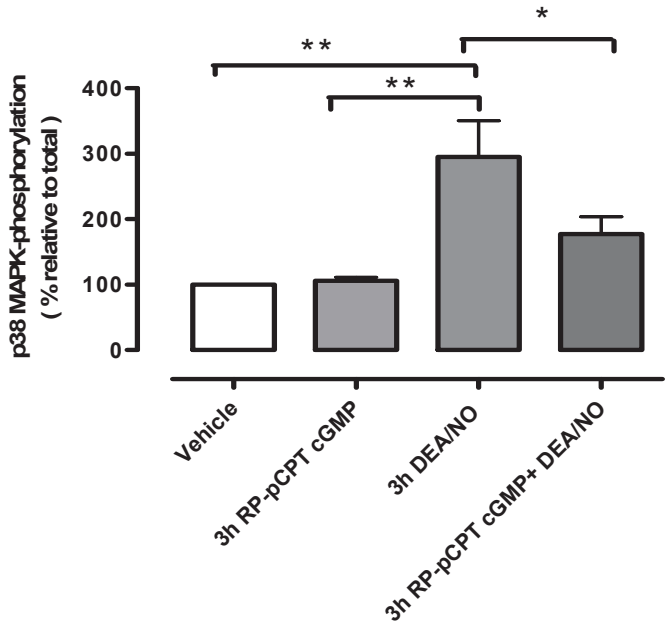
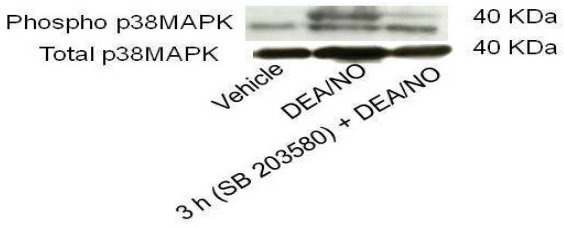


Figure 3.38: A Representative western blot for the phosphorylated P38 MAPK expression (40KD) and total P38 MAPK expression in PKG inhibitor and DEA/NO treated PAECs compared to vehicle (vehicle = 100%). **B** Quantitative evaluation showed that increased P38 MAPK phosphorylation in response to DEA / NO is decreased in the presence of the PKG inhibitor. All the blots were normalized to total, All values are mean ± SEM. n = 6-8 (** P<0.001 vs vehicle, * P<0.05 vs DEA / NO).

3.13.4 Incubation of PAEC cells with P38 MAPK inhibitors (SB203580)

To investigate the effect of the P38 MAPK phosphorylation in the PAEC cells, the cells were treated with the P38 inhibitors (**SB203580**) 10µM and the NO donor DEA / NO 10 µM for 3 hours and the P38MAPK phosphorylation was examined. The results showed that the increased NO bioavailability was associated with a significantly increased p38 MAPK activation, and this effect decreased in the pre incubated cells with the P38 inhibitor (SB203580) (**Figure 3.39**).

A



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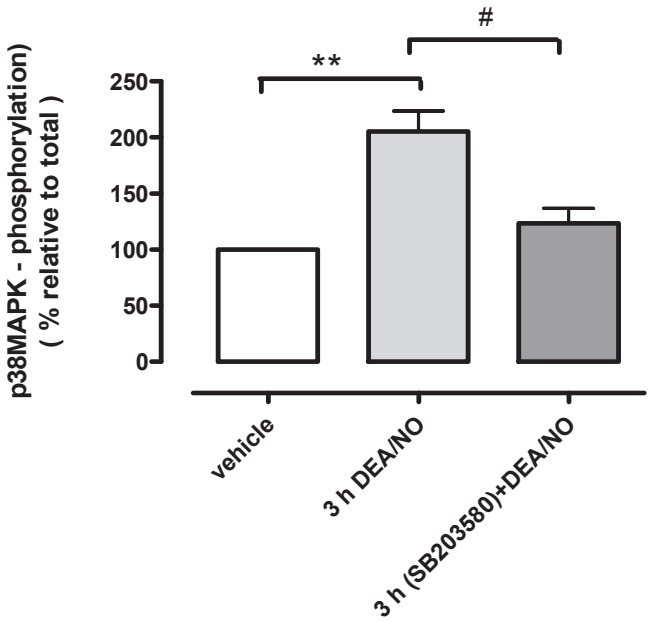
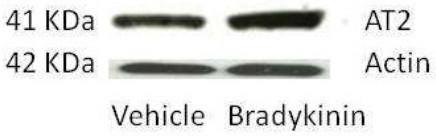


Figure 3.39: A Representative western blot for the phosphorylated P38 MAPK expression (40KDa) and total P38 MAPK expression in SB 203580 and DEA / NO incubated in PAECs compared to vehicle (vehicle = 100%). **B** Quantitative evaluation showed increase of P38 MAPK phosphorylation in response to DEA / NO decreased in the presence of p38 MAPK inhibitor. All the blots were normalized to total protein. All the values are mean ± SEM. (** P<0.001 vs vehicle, # P<0.001 vs DEA / NO).

3.14 Incubation of PAEC with bradykinin

The PAEC cells were treated for 3 hours with 100 µM of bradykinin to study the expression of the AT2 receptor. The results showed that there is a significant increase in the AT2 receptor expression (students t –Test, *P < 0.05, 244.0 ± 18.66 n = 6).

A



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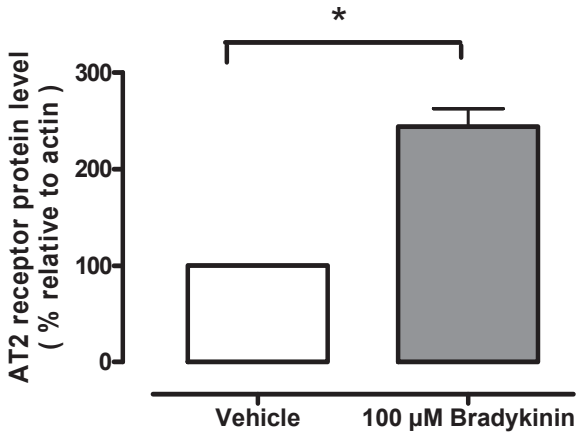


Figure 3.40: A Representative western blot for the AT2 receptor expression in bradykinin (+) and vehicle (-) treated PAECs. **B** Quantitative evaluation showed up-regulation of the AT2 receptor after 3 hours of incubation with bradykinin in comparison to vehicle (vehicle = 100%). All the blots were normalized to actin. All values are presented as mean ± SEM (*P< 0.05 vs vehicle).

3.15 NO-dependent upregulation of AT2 in transgenic mice

Transgenic mice overexpressing eNOS ($eNOS^{tg}$) and their negative littermates ($eNOS^{n}$) were used to study the NO-dependent changes of AT2 expression in-vivo. To confirme the NO-dependency of AT2 upregulation, mice underwent pharmacologic inhibition of NOS by L-nitroarginine (L-NA) treatment (1.5 mg/ml) dissolving in water for 3 weeks. This chronic intervention resulted in a reduced aortic AT2 protein expression in $eNOS^{n}$ and $eNOS^{tg}$ mice as compared to placebo-treated $eNOS^{n}$ and $eNOS^{tg}$ mice (Figure 3.41), respectively. These data strongly suggest that NO-dependent regulation of vascular AT2 expression is operative in-vivo.

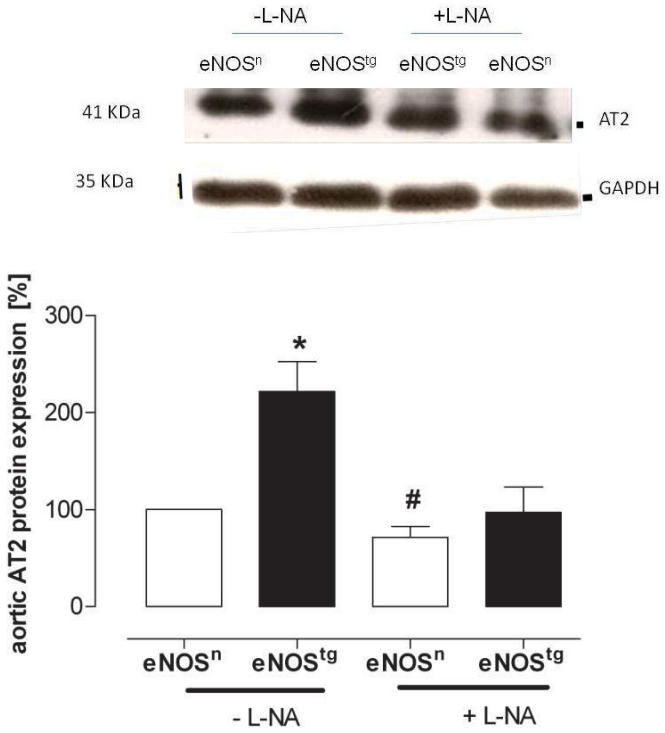


Figure 3.41: evaluation of AT2 protein expression in $eNOS^{n}$ and $eNOS^{tg}$ mice treated with the NOS-blocker L-NA as compared to placebo-treated $eNOS^{n}$ and $eNOS^{tg}$ mice (n=7, each). Data are means ± s.e.m. (*P<0.01 vs. $eNOS^{n}$, $eNOS^{tg}$; #P<0.05 vs. placebo-treated $eNOS^{n}$ using one-way-ANOVA and Newman-Keuls Multiple Comparison Test).

3.16 Incubation of PAEC with activator of the cAMP dependent kinase

To study the effect of the AT2 receptor expression in porcine aortic endothelial cells, the cells were treated with an activator of cAMP dependent kinase 6MB – cAMP and the PKA activator (6MB - cAMP) with 100µM for 3 hours, and the AT2 receptor expression was examined (**Figure 42**). The result showed a significant increase in the AT2 receptor expression, compared to vehicle (students t –Test, *P < 0.05, 179.8 ± 18.36 n= 6).

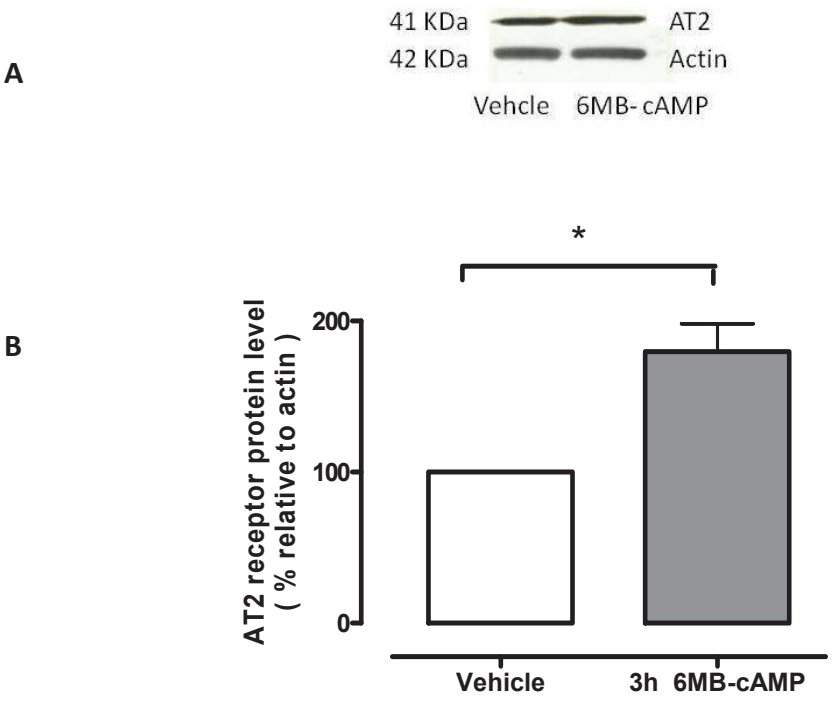


Figure 3.42: A Representative western blot examples of the AT2 receptor expression in PKA activator (+) and vehicle (-) incubated in PAECs. **B** Quantitative evaluation showed up-regulation of the AT2 receptor after 3 hours of incubation with 6MB - cAMP in comparison to vehicle (vehicle = 100%). All the blots were normalized to actin. All values are presented as mean ± SEM. (*P< 0.05 vs vehicle).

DISCUSSION

4. Discussion

This study aimed to investigate the mechanism of action of NO-induced upregulation of AT2. Endothelial cells of different species were subjected to increasing concentrations of NO-donors for different time intervals, and changes of AT2 mRNA and protein were monitored by RT-PCR and westernblot. It was found that NO increased AT2 by the classical NO-cGMP pathway, i.e. activation of soluble guanylyl cyclase, generation of cGMP, and activation of protein kinase G which resulted in phosphorylation of p38MAPK. While NO increased AT2 mRNA, it did not change the stability of AT2 mRNA suggesting a transcriptional mechanism. In mice, endothelial-specific overexpression of eNOS was found to stimulate, while treatment with a NOS-blocker decreased the AT2 expression. These results demonstrate a novel activity of endothelial NO signalling that has direct impact on the vascular renin angiotensin system. It is assumed that these cellular signalling events contribute to maintenance of vascular health, and thus may decrease pathophysiologic mechanisms leading to cardiovascular disease such as coronary artery disease, hypertension, heart failure, or stroke. On the other hand, NO-induced increase of AT2 may be invoved in the development of known side effect of cardiovascular drugs like ACE inhibitors and AT1 blockers.

4.1 Characteristics of endothelial cells

Endothelial cells are a lining layer of cells or tissue (endothelium), especially the interior of the blood vessels. In fact, the endothelium lines the entire circulatory system including the heart and all types of blood vessels (178). Endothelial cells form the interface between the lumen and wall of the vessel. The endothelium or the endothelial cells as a unit mainly form a selective barrier between the lumen and the surrounding organs or tissues. In addition, the formation of new blood vessels, blood clotting, control of blood pressure, and many more functions are either assisted or performed by the endothelial cells (179),(180). Endothelial cells play a wide variety of critical roles in the control of vascular function. Indeed, since the early 1980s, the accumulating knowledge of the endothelial cell structure as well as of the functional properties of the endothelial cells shifted their role from a passive membrane or barrier to a complex tissue with complex functions adaptable to needs specific in time and location. Hence, it participates to all aspects of the vascular homeostasis but also to

physiological or pathological processes like thrombosis, inflammation, or vascular wall remodeling.

4.1.1 Porcine aortic endothelial cells (PAECs)

The culture of primary endothelial cells (ECs) was first reported by Jaffe et al in 1973 (181). PAECs are porcine cells selected on the basis of morphological, phenotypical, and functional features similar to primary cells for in vitro study (182). These cells have been maintained in culture for many passages without signs of senescence, and with preserved characteristics of primary endothelial cells as shown in figure 3.1. PAECs are more close to human endothelial cells, and are easy to handle. The disadvantage is that the porcine genome is not completely sequenced, and no sequence for AT2 is available. The main problem to be solved is the presence of contaminating non-endothelial cells which easily overgrow in the endothelial cells in culture and, hence, influence the outcome of the experiments. Furthermore, the presence of endothelial marker such as endothelial nitric oxide (eNOS) and von Willebrand factor (vWF) are used as a marker for PAECs (183).

4.1.2 Human umbilical vein endothelial cells (HUVECs)

We cultured endothelial cells from human umbilical veins and to identify them unequivocally as endothelial cells according to morphologic, immunohistologic, and serologic criteria (181). Human umbilical vein endothelial cells (HUVECs), derived from foetal tissues, have been a major source of primary ECs, mainly because umbilical cords are readily available. HUVECs have been used to study a range of important pathophysiological processes, and they may not be representative of adult endothelium. HUVECs may be used to study the cell signaling in human cells, which allows extrapolation of data from mouse and porcine endothelial cells to human physiology. This technique has been widely used, (14,027 studies have been published in this regard, since 1973).

A number of previous studies have characterized endothelial cells from both aorta and human umbilical vein (184). The cultured HUVECs were attached completely after 24 hours, and became confluent after 5 days in vitro. Also, 48 hours after the culture, they grew in the form of colonies of homogenous and closely opposed cells. Confluent HUVECs during the third passage had a polygonal cobblestone appearance. Only endothelial cells contained

Weibel-Palade bodies which are storage granules containing vWF and these bodies were encountered in endothelial cells, especially those of umbilical veins (185). Furthermore, the presence of vWF in specific storage organelles in endothelial cells was widely used as a marker for the endothelial nature of cells they were not present in fibroblasts, smooth muscle cells or other tissue components. It is reasonable to conclude from these observations that the cells isolated from umbilical veins and grown in culture were, indeed, endothelial cells (186).

4.1.3 Immortalized mouse brain endothelial cell line (bEND.3)

Mouse brain endothelial cells (bEND.3) were provided by the department of pharmaceutical chemistry II of Pharmaceutical Institute, Bonn, Germany. The cells were cultured in Dulbecco's modified Eagle's medium and used for the maintenance of immortalized cell lines. bENDs are fast growing cells used in an exterimently for mRNA stability, because mouse genome is known and easy to handle. Immortalized mouse brain endothelial cell lines are well characterized by their expression of endothelial cell specific proteins, vWF and eNOS.

4.2 The expression of endothelial markers vWF and eNOS in bENDs and PAEC

The vascular endothelium is involved in the production of many mediators important for cardiovascular pathophysiology. One such substance, which is synthesised by and stored in endothelial cells, is vWF (185). Another is eNOS which expressed in endothelial cells (187). Western blot in PAECs and bENDs demonstrated the expression of eNOS and vWF **(Figure 3.4 and Figure 3.5)**. They were detected by immunostaining with specific monoclonal mouse antibody and polyclonol rabbit antibody, against the membrane receptor for vWF and eNOS. All endothelial cells expressed vWF which is considered as the most reliable marker for ECs, and expressed also another endothelial marker eNOS (187).

4.2.1 von Willebrand factor (vWF)

vWF is a large glycoprotein that is found in blood plasma, platelet α-granules and subendothelial connective tissue (188). vWF plays a central role in haemostasis. It is defective in von willebrand disease and is involved in a large number of other diseases. Its

plasma levels are increased in a large number of cardiovascular, neoplastic and connective tissue diseases. It is synthesized in megakaryocytes and endothelial cells, and stored in platelet α-granules and in EC (189). It mediates the adhesion of platelets to subendothelial connective tissue, and binds to the blood clotting factor VIII. vWF deficiency or dysfunction (vWF disease) lead to bleeding (189). The vWF gene is located near the tip of the short arm of human chromosome 12 at 12p13.3 (190). The structure function relationship of vWF has led to a clinically useful classification of vWF based on pathophysiologic mechanism (188). vWF is involved in a large number of other diseases, including thrombotic thrombocytopenic purpura, Heyde's syndrome, and possibly hemolytic-uremic syndrome (188). Thus, the mechanism of cell-type-specific transcriptional regulation of the vWF gene is central to studying the basis of endothelial-cell-specific gene expression (191).

4.2.2 Endothelial NOS (eNOS)

Nitric oxide synthases (eNOS) is clearly localized in various types of arterial and venous endothelial cells as well as in capillaries that is why we used it in this study to identified the endothelial cells. It generates NO in blood vessels and regulates vascular function (192). The gene coding for eNOS is located on chromosome 7. eNOS is associated with (caveolae) a component of plasma membranes surrounding cells, and the membrane of golgi bodies within cells (192). eNOS is a 133-kDa protein with 1,203 amino acids that was identified initially in vascular endothelial cells. eNOS is essential in the signaling for vascular endothelial growth factor (VEGF), which is required for the development and function of endothelial cells (193). eNOS contributes importantly to the endothelium-dependent vascular functions, including vasodilation and angiogenesis. eNOS expression during endothelial cell differentiation and development may help to characterize the mechanisms by which NO influences endothelial cell development and function (194). The cytosolic protein eNOS is a well studied marker for endothelial cells. The western blot is used to demonstrate the expression of these protein in endothelial cells PAEC and bEND.3 as shown **in figure 3.4** and **3.5.**

4.3 Validation of anti-AT2 receptor antibodies

Two different antibodies were used to detect the AT2 protein expression. These antibodies have been evaluated by using AT2 knockout mice, and the results were confirmed through the quantification of AT2 mRNA content by using a real-time PCR. Surprisingly, the initial experiments revealed that the Millipore antibody gave faint protein signals at the expected size in aortic homogenates of AT2 knockouts, suggesting that this polyclonal antibody is not highly specific for AT2 protein (**Figure 3.6**). However, the AT2 signal was much lower, which could be explained by the fact that the antibody might be specific when the differences in protein expression are evaluated. Nevertheless, it might be possible that there is some protein expression of AT2 in this knockout strain. Thus, it was evaluated whether AT2 mRNA is expressed in the aortic tissue of these AT2 knockouts as well. Indeed, the results of a comparison of AT2 mRNA in AT2 knockouts and their appropriate transgene negative littermates (mouse strain FVB/N) demonstrated a small AT2 mRNA expression in AT2 knockouts, reaching about 15% of the expression measured in FVB/N (**Figure 3.7**). The reason for this apparent mRNA expression in AT2 knockouts is not known. However, similar results were obtained in kidney tissue (**Figure 3.8**). Altogether, these data suggest that the Millipore antibody can be used to detect differences in AT2 protein in mice and cells. By using the Millipore antibody, it has been shown that PAECs respond to NO with an increase of the AT2 expression(**Figure 3.9. I).**,. A similar result was obtained when blot membranes were stained with the polyclonal antibody (Sant Cruz Biotechnoogy), designed to bind to AT2 protein on western blot membranes (**Figure 3.9. II).** These results confirm the specificity of the Millipore antibody and shows that the other antibody is also suitable for the detection of AT2 expression.

4.4 Detection of AT2 mRNA and protein

The effect of NO on AT2 mRNA and protein expression was observed in mice in vivo as well. We determined the significance of NO for upregulation of AT2 by investigation of AT2 expression in eNOStg mice (195). The increase of AT2 expression detected in this strain could be completely reversed by chronic inhibition with L-NA, demonstrating the importance of endogenous NO formation for AT2 expression. Moreover, L-NA decreased AT2 expression in C57Bl/6, thereby suggesting a significance for vascular physiology.

In this study, a real-time reverse transcription PCR was used. The reverse transcription polymerase chain reaction (RT-PCR) is one of the many variants of polymerase chain reaction or PCR. This laboratory technique is widely used in molecular biology, in order to produce multiple copies of a particular DNA sequence, through a process called 'amplification'. The difference of RT-PCR from traditional PCR is that RNA is first transcribed in reverse into its DNA complement, which utilizes the reverse transcriptase. The new complementary DNA containing the reversed transcription will then be amplified by using the traditional PCR or real-time PCR. The real-time PCR is known as quantitative real-time PCR or qPCR. qPCR is considered as the most powerful and sensitive analysis technique for genetic studies. It is currently involved in the quantitative gene expression analysis. The RNA expression in this study has been analysed by using techniques like real-time PCR; this method traditionally use the reference or housekeeping gene to control for errors between samples **(see chapter 2.6.4)**. Likewise, the AT2 mRNA expression increased after the DEA/NO incubation ($p<0.0001$) of bEND.3 cells and HUVECs ($p<0.05$) **(Chapter 3, Figure 3.11 and Figure 3.12).** These observations indicate that the upregulation of the AT2 receptor, by an increased NO bioavailability and the NO cGMP pathway, was involved in the AT2 receptor activation.

4.5 Gene expression mechanism (mRNA stability)

mRNA stability appears to be a key regulator in controlling the expression of many proteins (196), and plays an important role in regulating the expression of genes. The determination of mRNA stability was done using actinomycin D. This is a polypeptide that can inhibit transcription through tight and specific binding to double-helical DNA. It has been extensively used as a highly specific transcript inhibitor. The stability of mRNA is used to determine the AT2 mRNA expression by a quantitative real-time PCR in bEND.3. The cells were treated with transcriptional inhibitors actinomycin D (197). It does this by interfering with the elongation of the growing RNA chains by the RNA polymerase enzyme (198). Nucleolar (ribosomal) RNA synthesis is particularly sensitive to the presence of actinomycin D, and this probably accounts for its pharmacological activity as well as its extreme toxicity to mammalian cells (199). As shown in **(Figure 3.13)** Actinomycin D completely inhibited the increase of AT2 mRNA induced by DEA/NO, while DEA/NO itself did not change the half-live of AT2 mRNA that was observed following inhibition of transcription. These data suggest that

the NO-induced increase of AT2 mRNA content in endothelial cells is the result of an elevated transcription rate, while increased mRNA stability is unlikely involved.

4.6 NO donors

NO donors, capable of producing a sustained release with a wide range of half-lives as well as with a predictable estimated dose, have become useful tools to study the biological properties of NO in cells and vivo models. The specific advantages of NO donors, such SNAP, DEA/NO and DETA/NO, include varied half-lives and spontaneous release of NO, depending on the compound (200). SNAP is a stable analogue of endogenous S-nitroso compounds. It is a NO donor in vitro and in vivo. The unique NO-releasing properties of SNAP make it an ideal agent for studying the pharmacological and physiological actions of NO. Spontaneous release of NO from nitrosothiols is catalyzed by metal ions (201). SNAP is a nitrosothiol that does not release NO in solutions free of even trace amounts of heavy metals, such as copper or iron, while in vivo (or in isolated organs) this membrane permeable nitrosothiol is likely undergoing trans-nitrosylation with endogenous thiols, such as reduced glutathione to form nitrosoglutathione which spontaneous release NO at physiologic pH (202). In contrast, DEA/NO rapidly degrades, yielding the highest concentration of NO, and NO release was completed after 6-7 minutes. SNAP showed a similar time course of NO release but low concentration of NO. Thus, the ability of SNAP to release NO is dependent on the availability of free tissue thiols. For this reason, the spontaneous release of NO in buffer solutions, as well as activation of isolated sGC is approximately 10 times lower with SNAP than with DEA/NO, while both drugs are equipotent vasodilators (203). At present, NONO ates such as DEA/NO are not used clinically, although they have been tested frequently in experimental models of cardiovascular diseases. DEA/ NO spontaneously dissociates in a pH-dependent, first-order process, with a half-life of 2 minutes and 16 minutes at 37°C and 22-25°C, pH 7.4, respectively, to liberate 1.5 moles of NO per mole of parent compound (Cayman Chemical Sheet). DETA /NO possesses a half-life of approximately 24 hours. DETA/NO spontaneously release NO as a free radical in aqueous solution, at a rate of two moles of NO per mole of DETA/NO. In contrast, the thiol-based donor SNAP contributes only 1 mole of NO per mole of donor, and does so as a nitrosonium species (NO+) to other thiols (204). DETA/NO was

chosen as the nitric oxide donor for the cell line adaptations due to its long half-life time and higher delivery rate of NO, relative to other donors (205).

4.7 Molecular mechanism underlying NO-induced AT2 expression

Among vascular mediator systems, the NO-cGMP, the kallikrein-kinin and the renin-angiotensin systems are of major importance. The new finding of this investigation is the crucial involvement of NO in the regulation of AT2 expression. The data provide significant evidence to demonstrate a new crosstalk between these vascular signaling cascades which is likely relevant for vascular physiology. Furthermore, our findings suggest that NO-dependent AT2 expression may play a role for vascular pathologies such as endothelial dysfunction and/or AT1-blocker-induced angioedema. The upregulation of AT2 by NO is predominantly mediated by the classical NO-cGMP pathway, i.e. activation of sGC, generation of cGMP and activation of PKG. The mechanism of action of NO induced upregulation of AT2 protein expression in different species of endothelial cells were subject to increasing concentration of the NO donors SNAP, DEA/NO and DETA/NO for different time intervals. The study it was found that NO increased AT2 receptor by the NO-cGMP pathway. Angiotensin II (Ang II) is a vasoactive peptide that regulates blood pressure and fluid homeostasis, and is likely to play a key role in the pathogenesis of cardiovascular diseases in humans (206). The octapeptide Ang II binds, with approximately equal affinity, to two receptors sub-types— AT1 receptor and AT2 receptor. The two G-protein-coupled receptors are important components of the renin angiotensin aldosterone system (RAAS) which is involved in the pathophysiology of various cardiovascular and neurological disorders (206). In normotensive rats, stimulation of AT_2 receptors causes vasodilation via the local production of bradykinin in resistant arteries of the rat mesentery arterles in a flow-dependent manner (207).

The AT1 receptor is the predominant receptor subtype in the adult vasculature, whereas the AT_2 receptor is predominant during fetal development and declines after birth (208). Several studies showed that the AT2 receptor is upregulated in cardiovascular tissues under pathological conditions such as myocardial infarction, heart failure, hypertension, and vascular injury (209). However, it is unconfirmed that the kinin/cGMP system contributes to the signaling cascade of the AT_2 receptor in the rat aorta, because of a documented

reduction in bradykinin-induced vasodilation responsiveness in the rat (210),(211). In vitro and vivo studies, have been shown that there is a cross-connection between the AT2 receptor and the bradykinin 2 receptor activation, leading to an increase in NO production, and experimental studies in vascular endothelial cells showed an increase in NO production, caused by Ang II mediated receptor activation (212).

This AT2 receptor activation presumably led to a stimulation of the bradykinin 2 receptor, and subsequently, mediated through NO release by increasing cGMP (213). NO regulates the vascular tone by activating soluble guanylate cyclase. In the present study, it has been hypothesized that NO itself could regulate the AT2 receptor expression. The assumption was confirmed by different experimental findings. The NO-mediated upregulation of AT2 receptor was demonstrated in vitro experiments by using three different endothelial cells lines, PAECs, HUVECs, and bEND.3. Incubation of these cells with different NO-donors SNAP and/or DEA/NO significantly increased AT2 protein expression at different time intervals **(Figures 3.15, 3.16, 3.17 and 3.18)**. Activation of AT2 is known to counter regulate many effects mediated by Ang II type 1 receptor (AT1) and thus is viewed as vaso protective target for future therapeutic interventions (214),(215, 215). For example, activation of AT2 was shown to induce the prekallikrein activator prolylcarboxypeptidase (216),(217) and thereby increases generation of bradykinin and subsequently endothelial NO. AT2 was also shown to inhibit degradation of bradykinin by the angiotensin converting enzyme (ACE) (218). Hence, activation of AT2 stimulates the kallikrein kinin as well as the NO system in vascular endothelial cells by increasing the levels of bradykinin (219). In conclusion, the results suggest that NO is involved in the regulation of AT2 expression. This evidence supports the assumption that the AT2 receptor acts as part of a vasodilatory pathway in the vasculature through the kinin/NO/cGMP system. The results obtained in the present study are the first to show that AT2 receptor upregulation is mediated by NO. Incubation of PAECs for 3 hours with SNAP resulted is no change in AT1 protein expression **(Figure 3.14),** In contrast, downregulation of AT1 receptor protein expression in HUVECs was observed after incubation with SNAP (220).

4.7.1 Soluble guanylate cyclase inhibitor (ODQ)

To test the possibility that the effect of NO on AT2 expression was mediated through the stimulation of sGC, endothelial cells were incubated with either the guanylate cyclase inhibitors [1-H-[1, 2, 4]oxadiazolo[quinoxalin-1-one] (ODQ) or the phosphodiesterase inhibitor sildenafil. This was done to reduce the cellular cGMP level by ODQ or increase the cellular cGMP level by sildenafil, respectively. Soluble guanylate cyclase (sGC) is the principal physiological receptor for NO. When activated, sGC catalyses the synthesis of cyclic GMP, which modulates the activity of several intracellular targets including cGMP dependent kinases (221). cyclic nucleotide gated ion channels and phosphodiesterase (222). ODQ is used as a specific inhibitor of sGC in pharmacological studies of the NO/cGMP pathway (223). ODQ has been used extensively as a specific inhibitor of sGC to examine the specificity of sGC activation, as induced by NO donors (224). Therefore, in this study we examined whether ODQ abrogates the upregulatory action of NO donors like SNAP and DEA/NO on AT2 expression in endothelial cells.

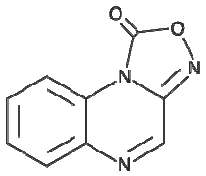


Figure 4. 1. Structure of ODQ

ODQ potently and selectively inhibits the NO stimulated guanylate cyclase activity. The inhibitory mechanism of ODQ has been characterized for the purified enzyme as a slow, irreversible oxidation of the sGC haem iron (225),(226). It binds to the sGC heme group, and thereafter, causes oxidation of the ferrous heme iron to the ferric (Fe^{3+}) form. Ferric sGC has low affinity for NO (151) and thus NO activity is prevented.

The inhibitor of sGC, ODQ, appears to act on the purified enzyme and similarly in cells, except that, in cells the inhibition is reversible a property that may contribute to the complex kinetics of the onset of inhibition (225),(226). The identification of a selective inhibitor of soluble guanylate cyclase, the oxadiazoloquinoxaline derivative, ODQ **(Fig.4.1)** provide the means to investigate the importance of the cGMP pathway in nitric oxide signal transduction (225). ODQ is useful in investigating the function of the cGMP pathway in NO signal transduction. Two other compounds are often referred to, and used, as guanylate cyclase

inhibitors, namely LY-83583 and methylene blue.

In reality, LY-83583 was originally reported to stimulate the guanylate cyclase activity, but, paradoxically, to lower the tissue cGMP levels non specifically and by an unknown mechanism (227). Although under certain assay conditions the inhibition of guanylate cyclase has been observed, LY-83583 more potently inhibits the release of NO (228). Methylene blue is actually one of the weak guanylate cyclase inhibitors that more effectively generates superoxide anions and inhibits NO synthase (229). Methylene blue was found to inhibit the stimulation of soluble guanylyl cyclase by NO and nitrovasodilators in cell- free systems (230) as well as to block smooth muscle relaxation induced by nitrovasodilators or acetylcholine (231). The compound has been extensively used as a selective inhibitor of soluble guanylyl cyclase to demonstrate the involvement of cGMP accumulation in vascular relaxation, platelet aggregation and neurotransmission (232). In summary, the use of ODQ is appropriate to inhibit sGC and thus the NO-cGMP signalling system.

4.7.2 Phosphodiesterases Inhibitor

The cGMP pool in the cell is tightly controlled by the contribution of cyclic nucleotide phosphodiesterases (PDEs). PDEs are enzymes with important roles in intracellular signalling, they hydrolyse the phosphodiester bonds of cAMP and cGMP, second messengers that activate cAMP and cGMP dependent protein kinases (PKs) A and G respectively (233).

21 PDE gene have been cloned and are classified into 11 families according to their sequence homology, biochemical, and pharmacological properties (48). The PDEs vary in their substrate specificity for cAMP and cGMP PDE5, PDE6 and PDE9 are specific for cGMP, PDE4,PDE7 and PDE8 are specific for cAMP, and PDE1, PDE2, PDE10 and PDE11 have mixed specificity for cAMP and cGMP (234). PDE5 selectively hydrolyzes cGMP, and inhibition increases cellular state levels of cGMP. PDE5 was originally identified, isolated and characterized from platelets (235) and later from lung (236). However, this PDE received little attentions until it was discovered to be a regulator of vascular smooth muscle contraction and more importantly the target of drugs such as sildenafil **(Figure 4.3).** PDE5 is characterized by a relative specificity for cGMP hydrolysis at low substrate level and by the presence of high affinity binding sites for cGMP. These binding sites are known as to be on the N-terminal regulatory GAF domains of the enzyme. Only one PDE5 gene has been

discovered to date, PDE5A, although several variants under the control of differentially regulated promoters has been identified (Figure 4. 2) (48).

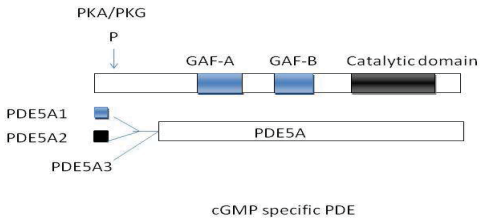


Figure 4.2. The cGMP specific PDE. Binding of cGMP to the high affinity GAF –A domain activates the enzyme

The structural basis for the high affinity cGMP binding to PDE5A was solved when it was found to have two highly homologous GAF domain (GAF-A and GAF-B) (48). In contrast with PDE2, high affinity cGMP binding occurs only to the GAF-A domain of PDE5 (237). In the present study, the upregulation of AT2 receptor after incubation of endothelial cells with SNAP, in the presence of phosphodiesterase inhibitor (sildenafil) for 3 hours and 6 hours was shown as in (**Figure 3.30 and Figure 3.31),** Inhibition of PDE5 potentiates the actions of NO by preventing the degradation of cGMP and increase in intracellular cGMP contents.

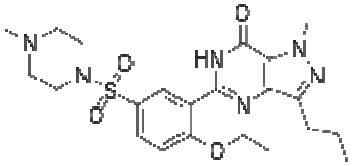


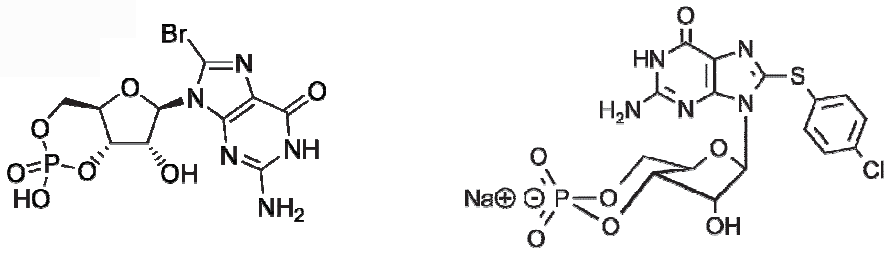
Figure 4. 3. Structure of Sildenafil

The mechanism of action of sildenafil involves the protection of cGMP from degradation by the cGMP specific PDE5 in endothelial cells. NO in the endothelial cells binds to sGC receptors, which results in increased level of cGMP, thus, leading to increase in AT2 expression by raising the cellular cGMP content. Because cGMP hydrolytic activity is also attributable to PDE1 and PDE3, Vandeput et al suggested that the effect of sildenafil on cGMP hydrolysis were due to inhibition of both PDE5 and PDE1 in the left ventricles of normal and failing mouse heart (238). Sildenafil or other PDE5 inhibitors have suggested to

increase cGMP, activate PKG, and prevent and reverse cardiac hypertrophy induced by pressure overload (239). Zaprinast was the first agent developed with some selective PDE5 inhibitory activity (240). Tadalafil and vardenafil are two more recent example of selective PDE5 inhibitors used to treat erectile dysfunction (241). PDE5 inhibitors may have utility in the treatment of pulmonary hypertension (242).

4.7.3 Protein Kinase G activity

In an attempt to directly demonstrate the role of PKG activity, we repeated the experiments by incubation of PAECs with the membrane permeable cGMP analog 8-Br-cGMP and 8-pCPT-cGMP which are specific activators of cGMP-dependent protein kinase.



8-Br-cGMP 8-pCPT-cGMP

Figure 4. 4 Structure of 8-Br-cGMP and 8-pCPT-cGMP

8-Br-cGMP is an analogue of the natural signal molecule cGMP in which the hydrogen in position 8 of the heterocyclic nucleobase is replaced by bromine. It is an activator of cGMP-dependent protein kinase type I α with preferential binding to its slow exchanging site, showing increased activation potential compared to the parent compound cGMP. 8-bromo-cGMP is known to mimic most intracellular effects of cGMP (243). The compound is a bad activator of both, type I and II of cAMP-dependent protein kinase with more than two order of magnitudes difference to PKG. 8-Br-cGMP is also a potent cGMP agonist for cGMP-dependent ion channels, with 10 times higher potency compared to cGMP. In comparison to cGMP, 8-Br-cGMP is degraded by cyclic nucleotide-dependent phosphodiesterases much more slowly, however, it is not completely stable. So it is possible that disturbing metabolites can appear, especially during long term incubation experiments (243). In this study we incubated PAECs with highly lipophilic 8-pCPT-cGMP. This is an analogue of the

natural signal molecule cGMP in which the hydrogen in position 8 of the heterocyclic nuclobase is replaced by the lipophilic para-chlorophenylthio moiety. The cGMP analogue 8-pCPT-cGMP is an activator of PKG I-α, I-β and type II of PKG, but not of PDE (244).

Direct stimulation of PKG in PAEC with 8-Br-cGMP or 8-pCPT-cGMP resulted in a concentration dependent increase of AT2 protein expression **(Figure 3.32, Figure 3.33),** while inhibition of PKG with the inhibitor RP-pCPT-cGMP (PKGB) prevented the upregulation of AT2 induced by DEA/NO **(Figure 3.34).**

Rp-8-pCPT-cGMP antagonise the activation of the cGMP- dependent PKG by 8-Pcpt- cGMP without affecting cAMP dependent protein kinase or cGMP regulated phosphodiesterases (245). Rp-8-pCPT-cGMP is an analogue of the parent second messenger cyclic GMP in which the hydrogen in position 8 of the nucleobase is replaced by the lipophilic 4-chlorophenylthio moiety. In addition, the equatorial one of the two exocyclic oxygen atoms of the cyclic phosphate moiety is modified by sulphur as shown in **Figure 4. 5.**

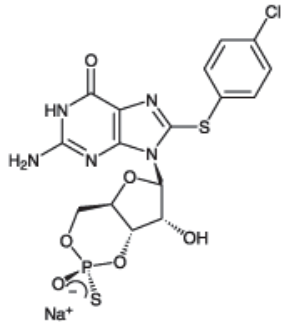


Figure 4. 5. Structure of Rp-8-pCPT-cGMPS

Rp-8-pCPT-cGMP is a competitive inhibitor of the PKG isozymes type Iα, I ß and II, with strong preference for PKG type II (82). Rp-8-pCPT-cGMP is lipophilic and therefore effectively in pentetrates the cell membrane and to reach an intracellular concentration sufficient to inhibit the cGMP- dependent protein kinase. RP-8-pCPT-cGMP, may be a useful tool to study the role of cGMP in vitro and in intact cells.

4.7.4 Phosphorylation of P38 MAPK

DEA/NO induced Thr^{180}/Tyr^{182} phosphorylation of P38 MAPK **(Figure 3.36)** was inhibited by the PKG-inhibitor **(Figure 3.38).** Increased a P38 MAPK phosphorylation could be mimicked by direct stimulation of PKG using 8-pCPT-cGMP **(Figure 3.37).** Of note, inhibition of P38

MAPK (SB 203580) resulted in a strong reduction of DEA/NO-induced P38 MAPK phosphorylation **(Figure 3.39).** These data suggest that upregulation of AT2 by NO is mediated by the classical NO-sGC-cGMP-pathway and involves phosphorylation of the transcription mediator p38 MAPK.

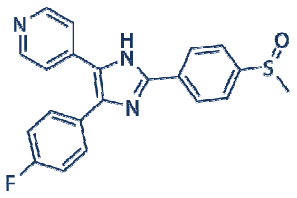


Figure 4. 6. Structure of SB 203580

SB 203580 is a selective inhibitor of p38 MAPK (246),(247). This compound **(Figure 4.6)** inhibits the activation of MAPKAPK-2 by P38 MAPK and subsequent phosphorylation of HSP27 (248). SB203580 inhibits P38 MAPK catalytic activity by binding to the ATP-binding pocket and thereby specifically inhibits its enzymatic activity (249). Upon phosphorylation on Thr^{180} and Tyr^{182}, e.g by the upstream kinase MKK6, P38 MAPK is activated resulting in binding of ATP (250),(248). Activated P38 MAPK phosphorylates several substrates including MAPK activated protein kinase 2 (MAPKAPK2) and activating transcription factor 2 (ATF2) (251). This phosphorylation results in activation of the kinase activity of MAPK. There are at least three families of MAPKs that differ in the sequence and size of the activation loop, the extracellular signal regulated kinases (ERKs) have a TEY motif, the c- Jun N – terminus kinases (JNKs) or stress- activated protein kinase (SAPKs), which have a TPY motif, and the P38 family, which has a TGY motif (252). However, some more studies indicate some differences in the MKKs that activate these two MAPKs, since P38 MAPK is activated by MKK3 and MKK6 (253). A common feature of these homologues is the presence of a 12 amino acid activation loop comprising the TGY motif. P38 MAPK isoforms are 60-70% identical to each other. Most of these kinases are widely expressed except P38γ, which shows the highest expression in skeletal muscle. Among the upstream kinases, MKK3 and MKK6 activate one or more of P38 isoforms. MKK3 has been shown to selectively activate P38α and P38γ (254). P38 MAPK play an important role in integrating various stress signals from the extracellular environment

and conveying these signals to the cytoplasm and nucleus by phosphorylating a variety of substrates. SB 203580 is the best studied member of the pyridinylimidazole class of P38 MAPK inhibitors that selectively inhibits P38 MAPK and its widespread use has helped elucidate the role of P38 MAPK in various physiological processes (255). MAPKAPK2 and MAPKAPK3 have been identified as physiological substrates for P38 MAPK, since treatment of cells with SB 203580 inhibited the activation of MAPK K2 (248).

One of the most characterized functional targets of the MAPK family is the transient phosphorylation of the transcription factor complex that regulates the c-fos promoter. Both c-fos and c-jun are components of redox-sensitive transcription factor AP-1. Considerable experimental evidence supports the notion that the induction of oxidative stress activates signaling pathways involving various members of the MAPK family (256).

Several transcription factors have been shown to be phosphorylated and subsequently activated by P38α. These transcription factors include activating transcription factor -2 (ATF-2), ATF-1, SRF, Sap 1, CHOP, p53, MEF2C, MEF2A (257, 257). The various transcription factor have unique modes of action. Phosphorylation of P53 by P38 may be play a role in P53 dependent transcription (257). An important cis-element, AP-1 binding site, appears to be influenced by the P38 pathway in several different mechanisms. ATF2, a substrate of p38 can form heterodimers with Jun family transcription factors can thereby directely associate with AP-1 binding site. Since it is well established that induction of c-fos, a component of AP-1, is SRE dependent by involving c-fos upregulation, the P38 pathway indirectly regulates AP-1 activity. P38 is likely to be involved in c-Jun expression by regulating MEF2A and 2C activity (258). Participation in c-Jun induction may be another way by which the P38 pathway regulates AP-1 activity. It is known that ERK and JNK mediate another component of the TCF called ELK-1.

Nrf2 and Nrf1 first described as NF-E2 related factors (259), are transcription factors which form heterodimers with each other (260). Regulation of heme oxygenase-1(HO-1) induction by Nrf2 dependent antioxidant response element (ARE) activation has been described in several cell types (261).

Nrf2 nuclear translocation was dependent on MAPK activation. While NO donor increased MAPK phosphorylation, blocked by PD98059 and SB 203580 which are ERK and P38 inhibitors, decreased NO stimulated Nrf2 translocation. The mechanism by which MAPK

pathways regulated Nrf2 translocation is not yet elucidated. However, other investigators have demonstrated that phosphorylation plays a role in Nrf2 translocation and /or ARE activation (262),(263). ERK and P38 pathways may regulate nitric oxide mediated adaptive responses in vascular endothelium via translocation of Nrf2 and activation of the ARE (263). Another group of transcription factors that may fall under the control of P38 is the C/EBP family of transcription factors. CHOB 10 , a member of the C/EBP family, is known to be involved in the regulation of cell growth and differention (258). In vitro assays reveal that CHOP 10 is phosphorylated by P38. In vivo assays further support the notion of such interaction as the P38 inhibitor, SB 203580, abolishes the stress induced phosphorylation of CHOP 10 (264). Many of the transcriptional events stimulated by P38 MAPK may also be mediated by the activation of MK2 and MSK1, direct targets of P38. Previous studies with vascular endothelial cells have demonstrated P38 MAPK activity is dependent on reactive oxygen species (ROS) production and activation of Hsp27 (heat shock protein 27) (265). MAPKAP K2/K3 is activated rather poorly by P38γ and P38δ (248),(266). P38β has been proposed to phosphorylate activating transcripyion factor (ATF)2 with higher efficiency than P38α (267). Following phosphorylation of MAPAP kinase 2 nuclear P38 is exported to the cytoplasm in a complex with MAPKAPK2. The cytoplasm translocation of MAPKAPK2 requires phosphorylation by P38 without a requirement for MAPKAPK2 activity (268). MAPKAPK2 serves as an effector of P38 by phosphorylating substrates and as a determinant of cellular localization of P38. Some report suggested that inhibition of P38 activation may be an important mechanism contributing to the effectiveness of SB 203580 (269). The P38 MAPK inhibitors are efficacious in several disease models, including inflammation (potent inhibitors of the synthesis of pro inflammatory cytokines), arthritis (reduces joint edema in the collagen induced arthritis) and other joint diseases, septic shock, and myocardial injury (270). Thus, P38 MAPK can serve as a target for novel drug development for neural diseases. P38 MAPK inhibitors have been studied extensively in both preclinical experiments and clinical trials for inflammatory diseases and a new P38 MAPK inhibitors are now being tested in phase II clinical trials for neuropathic pain and depression and possible future applications of P38 MAPK inhibitors as therapeutic agents in neural diseases. (271).

4.7.5 NO-dependent upregulation of AT2 in transgenic mice

L-arginine analogues are widely used inhibitors of nitric oxide synthase (NOS) activity both in vitro and in vivo **(272).** L-NMA is a product of the degradation of arginine- methylated proteins, occurs naturally in living organisms, it is widely used in both acute and long term in vitro and in vivo experiments **(273).** L-NMA has been used widely as a general tool to decrease NO bioavailability or to establish the NO dependency of a physiological process (274). L-NA is a specific inhibitor of the NO formation from L- arginine in endothelial cells. It is useful tool to demonstrate the molecular mechanism of mammalian NO synthesis (275), (276). The theory of insufficient NO production in human essential hypertension, led to creation of an animal model of human hypertension due to NO deficiency, achieved by long term L-NAME treatment of experimental animals. Long term administration of NOS inhibitor in relatively high doses induced NO deficient hypertension in normotensive rats and this model became widely used tool for investigation of the NO involvement in cardiovascular disease (277, 278). Regarding vascular function, higher doses of L-NAME administered for 3-6 weeks reduced relaxant response of the aorta, femoral artery and small mesenteric arteries to carbachol or acetylcholine in vitro (279). In addition, the doses of 10 to 100 mg of L-NAME per kg/day led to dramatic decrease of cGMP content in the aorta of normotensive rats (280). The response of normotensive and hypertensive organism to the same dose of L-NAME differs and, apparently, the sensitiveity of NOS/NO pathway is altered under hypertensive conditions (281).

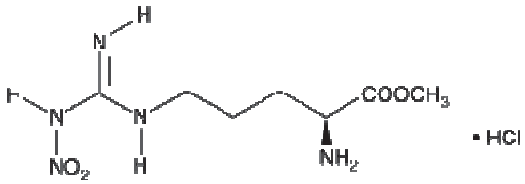


Figure 4.7 Chemical structure of L-NAME

Mice overexpressing eNOS ($eNOS^{tg}$) and their negative littermates ($eNOS^{n}$) were used to study the NO-dependent changes of AT2 expression in-vivo (195),(282). The endothelial specific overexpression of eNOS was visualized by confocal microscopy and the 3.5 fold higher eNOS protein expression was associated with a strong blood pressure reduction

indicating functional activity (195). To verify the NO-dependency of AT2 upregulation, mice underwent pharmacologic inhibition of NOS by L-nitroarginine (L-NA) treatment (1.5 mg/ml) for 3 weeks (283, 284). This chronic intervention resulted in a reduced aortic AT2 protein expression in $eNOS^{n}$ and $eNOS^{tg}$ mice as compared to placebo-treated $eNOS^{n}$ and $eNOS^{tg}$ mice **(Figure 3.41),** respectively. These data strongly suggest that NO-dependent regulation of vascular AT2 expression is operative in-vivo.

4.7.6 Bradykinin

The kallikrein kinin system (KKS) is an endogenous metabolic cascade, which results in the release of vasoactive kinin (bradykinin-related peptides). This complex system includes the precursors of kinins known as kininogens and mainly tissue and plasma kallikreins (285). Kinins are a family of peptides, including bradykinin, kallidin and methionyl-lysyl-bradykinin of which kallidin and methionyl-lysyl-bradykinin are converted very rapidly into bradykinin via the action of aminopeptidases which are present in the plasma and urine (286). The bradykinin B1 and B2 receptors are G-protein coupled receptors (GPCR) that interact primarily via the G proteins $G\alpha_{q/11}$ and $G\alpha_{i/0}$ also independently of G proteins through intracellular effectors (287). The human bradykinin receptor type 2 (BKR-2) gene is located on chromosome 14q32 whereas the human bradykinin receptor type 1 (BKR-1) has been mapped to chromosome 14q32.1-q32.2 (288). At the amino acid level, the BKR-1 and BKR-2 share only 36% sequence homology (289).

Bradykinin (BK) is a 9-amino acid peptide and the amino acid sequence of bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. It is present in several tissues like blood vessels, pancreas, gut, salivary, kidney, spleen, adrenal and neutrophils (290). BK is one of the most potent vasodilators, binding to endothelial as it is capable to BKR-2 leads to NO production, prostacyclin formation, elevated intra xcellular Ca^{2+} and the formation of hyperpolarizing factor, triggering vasodilation and increased vascular permeability (291).

The physiological role of BK has been identified in the 1980s, when different selective antagonists of BKR-1 and BKR-2 were discovered (292). BK was shown to dilate peripheral and coronary vessels, decrease arterial blood pressure in normotensive animals, and exert

antithrombogenic, antiproliferative and antifibrogenic effects (293). BK is also shown to be involved in the cardioprotective effect of preconditioning on myocardial ischaemia injury (294). Furthermore, local accumulation of BK may lead to the activation of proinflammatory peptides and local release of histamine, including a cough reflex hypersensivity (295). The evidence which linking BK to pathophysiological processes that induce tissue damage and inflammation, hyperaemia, leakage of plasma proteins, bone resorption induced by inflammation and pain. Many of these activitors follow stimulation of BKR-1 (296). BK is also involved in the production of pain and hyperalgesia by direct activation of BKR-2 receptors on primary nonmyelinated sensory neurones and thus, participates in the direct pain response (297). BK has been shown to increase the release of insulin from pancreatic β-cells through the increase of intracellular calcium in response to hyperglycaemia, and enhance insulin-dependent glucose transport (298).

The decreased degradation of BK contributes to the beneficial effects of ACEi in cardiovascular patients (299). BK-1 is expressed only as a result of tissue injury, and play a role in chronic pain and inflammation. Receptor activation leads to an increase in cytosolic Ca^{2+}, ultimately resulting in chronic and acute inflammatory responses (300). The B_2 receptor is a GPCR, coupled to G_q and G_i. G_q stimulates phospholipase C to increase intracellular free calcium and G_i inhibits adenylate cyclase. Furthermore, the receptor stimulates the PKG pathways. The B_2 receptor is constitutively expressed and mediates BK effects such as vasodilation. The B_2 receptor can form a complex with ACE, and this is thought to play a role in cross-talk between the RAAS and the KKS. The heptapeptide angiotensin (1-7) also potentiates BK action on B_2 receptors (301). Although it is well established that binding of BK to BK-2 receptor can activate formation of endothelial NO (302, 302). It was so far unknown that NO signals back to the KKS involving the RAAS.

The role of BK and B2 receptor in the mediation of AT2 receptor dependent vascular NO production has been determined by several investigators. Ang II induced NO production by the coronary microvessels of the canine heart is blocked by both icatibant and aprotinin, a kallikrein inhibitors (213). In this regard preliminary data demonstrating that treatment of PAEC with 100 µmol/l BK results in a 2-fold increase of AT2 protein expression (**Figure 3.40**). The activation of the BKR-2 on endothelial cells leads to an activation of phospholipase C gamma via a transient tyrosine phosphorylation, followed by an increased formation of

inositol 1,4,5- triphosphate (IP_3) and diacylgcerol (303). As a consequence of elevated IP_3 cocentration, intracellular calcium rises by liberalization from internal stores or by an increased Ca^{2+} influx (304), finally leading to an activation of the Ca^{2+} sensitive eNOS. In addition , the elevated intracellular Ca^{2+} activates the Ca^{2+} sensitive phospholipase $A_{2,}$ which hydrolyses membrane phospholipids liberating arachidonic acid, and this is the rate limiting step in the synthesis of PGI_2 (304). Beside these calcium regulated signalling pathways, it depend on phosphorylation. BK activates Tyk2 of the JAK family, resulting in subsequent tyrosine phosphorylation and nuclear translocation of STAT3 (305). BK also activates PKA leading to an acute increase of NO due to a phosphorylation of eNOS at Ser^{1179} (306, 306). BK primarily activates constitutively expressed B2 initiating activation of eNOS and subsequent generation of NO. BK primarily activates constitutively expressed B2 initiating activation of eNOS and subsequent generation of NO. Another clinical outcome might be the contribution to angioedema induced by BK which include hereditary forms as well as those caused by drugs such as ACE-inhibitors and AT1-blockers (307). Activation of B2 is likely involved in hereditary angioedema and angioedema induced by ACE-inhibitors as evidenced by clinical studies using the B2 antagonist icatibant (308, 309). In contrast, angioedema induced by AT1-blockers which occurs at frequency of 0.1-0.2 % (310) are not fully explained mechanistically. However, these very selective drugs inhibit AT1 mediated reduction of renin release and thereby increase circulating angiotensin II (219) which has free access to AT2 and might contribute to the development of angioedema by inhibition of ACE.

Some studies on the functions of AT2 receptor have demonstrated that the AT2 receptor plays a protective role via the BK/NO/cGMP pathway in ischemic diseases of the cardiovascular and renal tissues (311), also play an important role in the protective action of ACE (287). In addition to ACE, which is a major link between the RAAS and KKS, the AT2 receptor appears to be a link between these 2 systems.

4.7.7 Activator of cAMP-dependent protein kinase (6-MB-cAMP)

The second messenger cAMP is important in many biological processes. It is synthesized by adenylyl cyclase (AC), through cyclic nucleotide gated ion channels and the exchange factor for Rap, Epac, also cAMP targets (312). AC is activated by a range of signaling molecules through the activation of adenylyl cyclase stimulatory G (G_s) protein-coupled receptors and inhibited by agonists of adenylyl cyclase inhibitory G (G_i)-protein-coupled receptors. cAMP is used for intracellular signal transduction, such as transferring into cells the effects of hormones like glucagon and adrenaline, which cannot pass through the plasma membrane. cAMP also binds to and regulates the function of ion channels such as the HCN channels and a few other cyclic nucleotide binding proteins (313).

The two signaling pathways (cAMP/ cGMP), often exert opposing influences on cardiac function, in part as a consequence of the opposing effects of PKA and PKG mediated phosphorylation on target proteins. A separate level of cross talk between the cAMP and cGMP signaling pathway involves the activity of PDEs **(see chapter 1.3.2.3.3.)**. In the heart, cGMP acts as a regular of the activity of cAMP hydrolyzing PDEs such that the intracellular concentration of cGMP can increase the intracellular concentration of cAMP by inhibition of PDE III (314). The second messengers cAMP/cGMP are important regulators of cardic function (315). cAMP and cGMP are hydrolyzed exclusively by cyclic nucleotide PDEs, which therefore have a key role in regulating the signal conveyed by cAMP and cGMP. An emerging principle of cyclic nucleotide signaling in platelets is the high degree of interconnection between activating and cAMP/cGMP dependent inhibitory signaling pathways at all levels, including cAMP/cGMP synthesis and breakdown, and PKA/PKG mediated substrate phosphorylation (76, 316). Furthermore, defects in cAMP/cGMP pathways might contribute to platelet hyperreactivity in cardiovascular disease. Most of the inhibitory function of cAMP/cGMP in platelets can be attributed to phosphorylation of substrate proteins by PKA and PKGI. 6-MB-cAMP is an analogue of the natural signal molecule cAMP in which one of the hydrogen atoms of the amino group in position 6 of the purine nucleobase is replaced by a butyrate group.

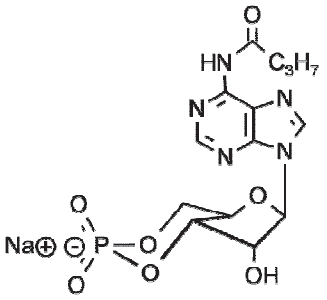


Figure 4. 8. Structure of 6MB cAMP

6-MB-cAMP is a selective PKA agonist, which activate cAMP-dependent protein kinase, and only slowly metabolized by mammalian cyclic nucleotide-responsive phosphodiesterases. Due to its site selectivity it is often used as a partner for selective stimulation of PKA type I by synergistic pairs of cAMP analogues. The substitution with a butyryl group results in considerably higher lipophilicity and membrane permeability compared to cAMP (Biolog-life science instituite 2013). In this study the upregulation of AT2 protein expression in PAECs incubated with 100µM 6-MB-cAMP was due to increased the PKA activity **(Figure 3.42).** Bradykinin also activates PKA leading to an acute increase of NO due to a phosphorylation of eNOS at Ser^{1179} (306).

4. 8 Relevance of the results for human physiology and pathophysiology

The results demonstrate a novel vasoprotective activity for endothelial NO and show for the first time that the endothelial NO signalling has a direct impact on the vascular renin angiotensin system. It is assumed that activation of these cellular signalling events contribute to maintaining health, and thus, may decrease pathophysiologic mechanisms leading to cardiovascular diseases, such as coronary artery disease, hypertension, heart failure, or stroke. On the other hand, the NO-induced increase of AT2 may be involved in the development of the bradykinin-dependent angioedema attacks, which are either of hereditary origin or occur as a well-known side effect of cardiovascular drugs, such as ACE inhibitors and AT1-blockers.

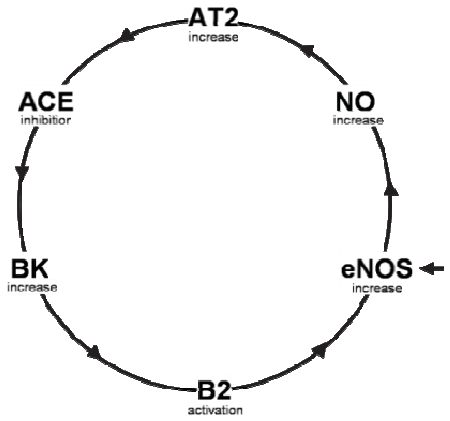


Figure 4. 9. Scheme of the signaling loop postulated following the discovery of upregulation of AT2 protein and function by vascular NO formation. Increased activation of eNOS and/or treatment with NO-donors upregulate AT2 expression and activity thus lead to in inhibition of ACE-activity. This will result in decreased formation of angiotensin II, while degradation of BK is reduced. BK primarily activates constitutively expressed B2 initiating activation of eNOS and subsequent generation of NO. The arrow depicts activation of this loop by interventions which increase vascular NO bioavailability such as exercise training. Other interventions which are known to increase vascular NO-bioavailability include treatment with statins or organic nitrates (317) but whether these interventions increase endothelial AT2 expression remains to be demonstrated.

In summary, these findings allow postulating the existence of a signaling loop in vascular endothelial cells (**Figure 4. 9**). Accordingly, increased activation of eNOS and/or treatment with NO-donors increase AT2 expression and activity thereby lead to in inhibition of ACE-activity. This will decrease the formation of angiotensin II and inhibit degradation of BK. Another mechanism which increases BK formation following activation of AT2 is induction of prolylcarboxypeptidase and/or decreasing intracellular pH and subsequent activation of kallikrein (154, 216, 217). BK primarily activates constitutively expressed B2 initiating activation of eNOS and subsequent generation of NO. Another clinical outcome might be the contribution to angioedema induced by bradykinin which include hereditary forms as well as those caused by drugs such as ACE-inhibitors and AT1-blockers (307). Activation of B2 is likely involved in hereditary angioedema and angioedema induced by ACE-inhibitors as evidenced by clinical studies using the B2 antagonist icatibant (308, 309). In contrast, angioedema induced by AT1-blockers which occurs at frequency of 0.1-0.2 % (310) are not fully explained mechanistically. However, these very selective drugs inhibit AT1 mediated reduction of renin release and thereby increase circulating Ang II (219) which has free access to AT2 and might

contribute to the development of angioedema by inhibition of ACE. Some preliminary clinical data of this study demonstrating a diminished activity of ACE in patients treated with an AT1-blocker confirm these previous findings. Thus, direct activation of AT2 using specific low molecular agonists might show angioedema as a side effect (318).

5. SUMMARY

There are some vascular signalling interactions between the vascular endothelial NO system and the renin-angiotensin aldosterone system (RAAS), for example activation of AT2 receptors lead to the generation of cGMP. The aim of these studies was to investigate whether endothelial nitric oxide (NO) has any influence on the expression of angiotensin (Ang) type 1 (AT1) and type 2 (AT2) receptors. The interaction between the RAAS and the NO/cGMP system was identified. NO donors increased the expression of the angiotensin II (Ang II) type 2 receptorss (AT2). In striking contrast, the expression of the AT1 receptor did not change. The interaction was confirmed by investigations in primary cell culture. The experiments obtained in vitro with different endothelial cells and in vivo with transgenic animals, show that the NO/cGMP and the MAPK signal transduction pathways are likely involved in the underlying mechanism. Primary cultures of porcine aortic endothelial cells (PAEC) and human umbilical vein endothelial cells (HUVEC) and the cell line brain mouse endothelial cells (bEND.3) were used. The incubation of these cells with different NO donors such as SNAP (S-nitroso-N-acetyl-D-L-pencilliamin), and/or DEA/NO (DiethylamineNONate) and DETA/NO (Diethylenetriamine /nitric oxide) and significantly increased AT2 protein expression as determined by western blot. Likewise, AT2 mRNA expression was determined by RT PCR in bEND.3 cells. AT2 mRNA expression was increased after DEA/NO incubation. AT2 mRNA stability remained unchanged suggesting a transcriptional mechanism. Studies on time and concentration dependently AT2 following NO stimulation revealed that long-term exposure of bEND3 cells with the 24 hours NO releasing compound DETA/NO resulted in continuous upregulation of AT2 protein levels which plateaued at 12 hours. Furthermore, co-incubation of PAEC with the soluble guanylate cyclase (sGC) inhibitor ODQ (oxidiazolol quinoxalin) or protein kinase G (PKG) inhibitor (RP-pCPT cGMP) abolished NO- induced AT2 upregulation. Treatment of PAEC with the PDE-V inhibitor sildenafil, the protein kinase G (PKG) activators, 8-pCPT-cGMP (8 para chlorophenylthioguanosin – cGMP) and 8-Br-cGMP (8- Bromo-guanosine 3, 5 cyclic monophosphate) resulted in significantly increased AT2 protein expression.

In addition, treatment of PAEC with either DEA/NO or 8-pCPT-cGMP increased phosphorylation of p38 MAPK, a kinase known to be involved in NO/cGMP signalling. Accordingly, co-incubation of PAEC with DEA/NO and the p38 MAPK inhibitor (SB203580) abolished NO induced AT2 upregulation and phosphorylation of p38 MAPK.

These results suggest that NO induced vascular AT2 upregulation is mediated by activation of the NO/cGMP pathway including stimulation of sGC and PKG and subsequent induction of p38 MAPK phosphorylation. In addition, a significant increase in AT2 protein expression was detected in a transgenic mouse line overexpressing eNOS in an endothelial-dependent manner and of a magnitude that strongly reduced blood pressure. Furthermore, this increase was not only completely abolished by the NO synthase inhibitor L- NAME but strongly reduced so that there was no difference to L-NAME treated transgene negative littermates anymore.

The upregulation of AT2 receptors by NO is a newly discovered interaction between the RAAS and the NO system. The importance of this new pathway for the development of endothelial dysfunction and/or the pathophysiology of cardiovascular disease such as coronary artery disease, hypertension, heart failure, or stroke has to be clarified. On the other hand, NO-induced increase of AT2 may be invoved in the development of known side effect of cardiovascular drugs like AT1 blockers, i.e. angioedema.

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7. Publications

Abstracts

• Dao V.T., Suvorava T., Kocgirli O., **Agouri S.,** Oppermann M., Balz V., Gojda G. In-vivo-Effekt von endogenem NO und Pentaerythrityltetranitrat auf die myokardiale AT-2- Rezeptor-Expression ***Naunyn Schmiedebergs Arch Pharmacol*** (2009) 379 (Suppl 1): 152. **(oral presentation).**

• V. T. Dao, O. Kocgirli, M. Oppermann, **S. Agouri**, T. Suvorava, V. Balz, G. Kojda. Effect of endogenous NO and pentaerythritol tetranitrate on myocardial AT-2 receptor expression in-vivo. ***Clin Res Cardiol*** 98, (Suppl) 1, April 2009**. (oral presentation).**

• V T Dao, O Kocgirli, **S. Agouri**, M Oppermann, T Suvorava, V Balz, G Kojda. Effect of endogenous NO and pentaerythritol tetranitrate on myocardial AT-2 receptor expression in-vivo. ***Eur J Cardiovasc PrevRehabil*** 2009, 16 (Supp 1): S87. **(oral presentation).**

• V.T. Dao, T. Suvorava, O. Kocgirli, **S. Agouri,** M. Oppermann, V. Balz, G. Kojda. Effect of nitric oxide on the AT-2 receptor expression in-vivo. ***European Heart Journal*** (2009) 30 (Abstract Supplement), 361. **(Poster presentation).**

• **Agouri S.,** Dao V.T**.**, Kocgirli T., Oppermann M, Suvorava T., Kojda G. NO-Donor-induzierte cGMP-abhängige AT2-Rezeptorexpression in porvinen Aortenendothelzellen. ***Naunyn Schmiedebergs Arch Pharmacol*** (2010) 381(Suppl 1):35 S143**. (Poster presentation).**

• **Agouri S.,** Dao V.T**.**, Kocgirli T., Oppermann M, Suvorava T., Kojda G. Nitric oxide (NO) induces AT2 receptor expression in a cGMP-dependent manner. **Eur J Cardiovasc PrevRehabil** (2010) voume 18,(suppl 1): S 29 P 152. **(Moderated Poster presentation).**

• **S. Agouri.,** Dao V.T**.**, T Kocgirli., M Oppermann., Suvorava T., Kojda G. NO- donor induced cGMP- dependent AT2 receptor expression in porcine aortic endothelial cells and human umbilical vein endothelial cells. **Eur J Cardiovasc PrevRehabil** (2011) volume 18, (Supplement 1) S 22 P 183. **(Poster presentation).**

• **Agouri S.,** Bisha M**.,** Dao V.T**.**, Suvorava T., Kojda G. Phosphp p38 MAPK dependent upregulation of vascular AT2 receptor expression by nitric oxide.. ***Naunyn Schmiedebergs Arch Pharmacol*** (2012) 385(Suppl 1): S3 – S4. **(Poster presentation).**

• Vu Thao-vi Dao[*]., **S. Agouri[*]., ** M Bisha and G. Kojda. cGMP- dependent upregulation of angiotensin II type 2 receptors in human, porcine and mouse endothelial cells**. Bradykinin Symposium Berlin 2012. (oral presentation).**
http://www.bradykinin-symposium.de/index.php?id=1212

Prize

• Winning Moderated Poster Presentation in the Basic Science section. **Europrevent (2010) Prage.**
http://www.escardio.org/congresses/europrevent-2010/scientific/Pages/abstracts.aspx#Moderated_poster_prizes_winners

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