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# DISSERTATION

Titel der Dissertation

„Modulation of the endothelial nitric oxide synthase system by natural compounds“

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Just one more thing...

(Lt. Columbo)

**Abstract**

The endothelium serves as a main regulator of vascular homeostasis by maintaining the balance between vasodilation and vasoconstriction, inhibition and stimulation of smooth muscle cell proliferation and migration, and thrombogenesis and fibrinolysis. Disruption of this balance is generally referred to as endothelial dysfunction, which is a key event in the development of atherosclerosis, the main pathology underlying cardiovascular diseases. A dysfunctional endothelium is mainly characterized by reduced endothelium-dependent vasodilation due to impaired endothelial nitric oxide synthase (eNOS) activity or decreased availability of its synthesis product, nitric oxide (NO).

Several natural products, found in the daily diet or in phytomedical preparations, have previously been discussed as modulators of eNOS function. Identification of so far unknown natural products, influencing NO availability and the elucidation of their mechanism of action provide valuable information for possible future therapeutic applications. In this work we investigated the molecular mechanism underlying an increased eNOS activity elicited by two selected compounds, ascorbate and DPPB (2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran).

Previous studies have shown that ascorbate (vitamin C) can stabilize and regenerate the essential eNOS cofactor tetrahydrobiopterin (BH4) after long-term treatment, thereby preventing the uncoupling of eNOS. Additionally, infusions of ascorbate were able to rapidly increase endothelium-dependent vasodilation in patients with different pathological conditions. In this study we found that the fast effect of ascorbate on eNOS activity is independent on BH4 stabilization. Instead, ascorbate was shown to rapidly enhance eNOS activity by inhibition of protein phosphatase 2A (PP2A), followed by an increase in activating AMP-activated protein kinase (AMPK) and eNOS phosphorylation patterns.

Several lignans isolated from the roots of the anti-inflammatory medicinal plant *Krameria lappacea* were investigated for their influence on eNOS activity and endothelial NO release. Among these compounds only DPPB was able to increase endothelial NO release. We could show that DPPB increases eNOS activity via raising intracellular $Ca^{2+}$ levels and increasing the $Ca^{2+}$/CaM-dependent kinase kinase β (CaMKKβ) and AMPK signaling.

**Zusammenfassung**

Das Endothelium is ein wichtiger Regulator für das Aufrechterhalten der vaskulären Homeostase. Es erhält das Gleichgewicht zwischen Vasodilation und Vasokonstriktion, Hemmung und Stimulierung der Proliferation und Migration glatter Muskelzellen, sowie Thrombogenese und Fibrinolyse. Ist diese Balance gestört, spricht man von endothelialer Dysfunktion, welche ein Schlüsselereignis in der Entstehung von Atherosklerose, die den meisten kardiovaskulären Erkrankungen zu Grunde liegt, ist. Das Hauptcharakteristikum endothelialer Dysfunktion ist eine verminderte Endothelium-abhängige Vasodilation auf Grund einer reduzierten Aktivität der endothelialen Stickstoffmonoxid-Synthase (eNOS) oder ihres Syntheseproduktes, Stickstoffmonoxid (NO).

Für einige Naturstoffe, die in der Nahrung oder Phytopharmaka enthalten sind, wurde eine modulierende Wirkung auf die eNOS gezeigt. Die Identifizierung von bisher unbekannten, eNOS aktivierenden Naturstoffen und die Aufklärung ihres Wirkungsmechanismus stellt eine wertvolle Information für zukünftige therapeutische Anwendungen dar. In dieser Arbeit wurden zwei ausgewählte Substanzen auf ihre Wirkungsweise im Bezug auf eNOS Aktivität untersucht.

Frühere Studien zeigten, dass Ascorbate (Vitamin C) den essentiellen eNOS Kofaktor Tetrahydrobiopterin (BH4) nach Langzeitstimulierung stabilisiert und regeneriert. Zusätzlich waren Infusionen von Ascorbate in der Lage, sehr schnell die Endothelium bedingte Vasodilation bei Patienten mit verschiedenen kardiovaskulären Krankheiten zu erhöhen. Hier konnten wir zeigen, dass dieser schnelle Effekt von Ascorbate unabhängig von der Stabilisierung von BH4 ist. Stattdessen scheint Ascorbate schnell die Protein Phosphatase 2A (PP2A) zu inhibieren und anschließend aktivierende AMP-aktivierte Protein Kinase (AMPK) und eNOS Phosphorylierungsmuster zu verstärken.

Mehrere Lignane, die aus den Wurzeln der entzündungshemmenden Pflanze *Krameria lappacea* isoliert wurden, wurden auf eine mögliche eNOS aktivierende oder NO erhöhende Wirkung untersucht. Unter den untersuchten Lignanen war nur DPPB (2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran) in der Lage, die NO Freisetzung zu steigern. Wir konnten zeigen, dass DPPB die eNOS Aktivität durch Steigern des intrazellulären $Ca^{2+}$ Levels und $Ca^{2+}$/CaM-abhängige Kinase Kinase β (CaMKKβ) und AMPK Signaltransduktion erhöht.

# Contents

# 1 Introduction

## 1.1 The endothelium

The endothelium is characterized as the inner lining of blood vessels. It consists of a monolayer of endothelial cells and has an estimated surface in the human body between 300 and 1000 $m^2$ [162; 270]. Therefore it is considered to be a main site for exchange between the blood and underlying tissue [5]. Endothelial cells are linked to the basal lamina, which provides a scaffold for endothelial cells towards the vessel lumen and for smooth muscle cells or pericytes towards the other side. Upon its discovery the endothelium was considered to be only a diffusion barrier, regulating the migration of molecules across the vasular wall. Today, endothelial cells are recognized to be structurally very diverse, dependent on the vascular bed, and to have multiple physiological functions [4]. They are known to be the main regulator of vascular tone, controlling the balance between vasodilation and vasoconstriction. Endothelial cells are also important regulators of wound healing, thereby adjusting blood fluidity, platelet aggregation, thrombogenesis and fibrinolysis. In addition, the endothelium regulates the ability of leukocytes to travel to surrounding tissues and the building of new vessels and remodeling of existing ones. Therefore it is also involved in metabolic and catabolic activities. Moreover, endothelial cells are able to regulate the proliferation of vascular smooth muscle cells (reviewed in [102]).
A dysfunctional endothelium is mainly characterized by an impaired endothelial nitric oxide production. This shifts the cells to a pro-thrombotic, pro-inflammatory and proliferative phenotype. Endothelial dysfunction marks the initial event for many cardiovascular diseases, such as atherosclerosis, hypertension and heart failure [67; 127].

## 1.2 The endothelial nitric oxide synthase

### 1.2.1 History of nitric oxide discovery

In 1980 Furchgott and Zawadski published a study which showed that the endothelial cell lining is necessary for acetylcholine induced relaxation of isolated blood vessels [115]. The relaxation observed was proposed to be dependent on the calcium induced release of the unidentified so called endothelium-derived relaxing factor (EDRF). This factor was shown to have a half-life of only a few seconds in solution, to target guanylyl cyclase in smooth muscle cells and to be inactivated by superoxide [129; 158; 281]. It took six years after this initial discovery until Furchgott and Ignarro independently proposed at the meeting "Mechanism of Vasodilatation" in Rochester, MN that this factor is nitric oxide (NO) [114; 160], although nitrovasodilators had already been known before [240]. Palmer et al. later demonstrated that the amount of NO released by endothelial cells is in the range of the biological activity of EDRF and that nitric oxide is produced from L-arginine by an NO-synthase [260; 261].

In 1998 Furchgott, Ignarro and Murad were awarded the Nobel prize in physiology and medicine for discovering that nitric oxide is an important signaling molecule in the cardiovascular system [251].

### 1.2.2 Nitric oxide synthase isoforms

In addition to its importance in the cardiovascular system, NO is involved in macrophage-induced responses to inflammatory stimuli, and signaling events in the central nervous system [178; 214]. These findings led to the discovery of three different isoforms of human nitric oxide synthases. Their genes are all located on different chromosomes and share 51 to 57 % homology [9].

#### 1.2.2.1 Neuronal nitric oxide synthase

The first nitric oxide synthase that was described was the neuronal nitric oxide synthase also termed nNOS or NOS-1 [36; 37]. It is expressed mainly in specific neurons of the brain [108]. Immunohistochemistry has revealed that nNOS is additionally expressed in many different tissues including the spinal cord, sympathetic ganglia, adrenal glands, peripheral nitrergic nerves (nerves expressing nNOS and releasing NO), epithelial cells of various organs, kidney macula densa cells, pancreatic islet

cells, vascular smooth muscle, and skeletal muscle cells [105]. Neuronal NOS is expressed constitutively, produces only low levels of NO and is regulated by $Ca^{2+}$ and calmodulin (CaM). nNOS contains no anchoring sites to hold it in the plasma membrane. However it contains a PDZ domain (also called DHR (Dlg homologous region) or GLGF (glycine-leucine-glycine-phenylalanine) domain) allowing it to interact with PDZ domains of other proteins. This determines the localization and activity of the enzyme. Physiological functions of nNOS are extensive. nNOS is important for synaptic plasticity and for cognitive processes. Independently of its activity in the central nervous system, it is involved in the regulation of blood pressure. In addition, NO produced by nNOS acts as an atypical neurotransmitter in the peripheral nervous system, mediating relaxing of gut peristalsis and penile erection. Moreover, it has been shown that low nNOS expression in vascular smooth muscle cells can provide some degree of vasodilation when eNOS is not functional [108]. Abnormal nNOS function has been linked to several neurodegenerative pathologies such as excitotoxicity following stroke, multiple sclerosis, Alzheimer's disease, and Parkinson's disease [309].

#### 1.2.2.2 Inducible nitric oxide synthase

The second nitric oxide synthase described was the inducible nitric oxide synthase also known as iNOS or NOS-2 [209; 359]. Inducible NOS is constantly active once induced, produces approximately 20 times higher levels of NO than the constitutive NOS isoforms and is not regulated by calcium [42]. As the name implies, this isoform is expressed only upon induction. It is mainly expressed in macrophages as response to inflammatory stimuli like bacterial lipopolysaccharide (LPS), cytokines and others, but has been found in almost every cell type when the correct stimulus was applied. The high amount of NO produced by iNOS can directly interfere with the DNA of target cells and cause strand breaks and fragmentation. In addition, NO can inhibit proteins which have iron in their catalytic centers, like complex I and II in the mitochondrial electron transport chain. These activities are the main cause for the cytotoxic effects of NO on microorganisms and tumor cells. However, it has to be considered that high levels of NO released by activated cells can also harm healthy cells. Furthermore, NO produced from iNOS has been reported to lead to septic shock [108].

#### 1.2.2.3 Endothelial nitric oxide synthase

The last nitric oxide synthase described was the endothelial nitric oxide synthase also termed eNOS or NOS-3 [268; 299]. Endothelial NOS, similar to nNOS, is constitutively active, produces only low levels of NO and is regulated by $Ca^{2+}$, CaM and many other factors described in detail in section 1.4. It is predominantly expressed in endothelial cells, but has also been found in other cell types such as platelets or cardiac myocytes. NO derived from eNOS has a crucial role in the regulation of vascular homeostasis. It serves as an important regulator of vasodilation and platelet aggregation and adhesion. Additionally, it inhibits leucocyte adhesion, vascular inflammation, and smooth muscle cell proliferation, regulates angiogenesis, and can activate endothelial progenitor cells (see section 1.4.7). Endothelial dysfunction is a hallmark for cardiovascular disease and definded as the impaired ability of eNOS to produce bioavailable NO. Interestingly, it has been suggested that gene therapy delivering eNOS hinders the progression of cardiovascular diseases at least in animal models [108].

### 1.2.3 Structure, catalytic function and localization

#### 1.2.3.1 Structure

The eNOS gene is located at chromosome 7 (7q35-36) in the human genome and contains 20,366 basepairs and 26 exons. It is transcribed in 1,203 amino acids and gives rise to a 133 kDa eNOS protein [9].

The eNOS enzyme consists of a C-terminal reductase domain and an N-terminal oxygenase domain. The reductase domain contains binding sites for the cofactors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotine adenine dinucleotide phosphate (NADPH), and the oxygenase domain for L-arginine, heme, and tetrahydrobiopterin (BH4). Both domains are linked with a binding site for CaM [9]. Additionally, the eNOS enzyme activity requires dimerization, to allow interaction of the reductase domain of one dimer with the oxygenase domain of the other dimer. For the formation of this homodimer, heme and a tetracoordinated zinc ion, that interacts with two cysteins from each monomer, are crucial. Moreover, BH4 and L-arginine have a stabilizing function for the already assembled dimer [14; 197]. Peroxynitrite can disrupt the zinc thiolate cluster [365], which leads to the release of the zinc ion and oxidation of the thiols. Furthermore, peroxynitrite can oxidize BH4 to dihydrobiopterin (BH2) [188].

### 1.2.3.2 Catalytic function

The reaction catalyzed by eNOS is an oxidation of one of the guanidino nitrogens of L-arginine in the presence of molecular oxygen [99]. This reaction has two steps. First, L-arginine is hydroxylated to $N^{\omega}$-hydroxy-arginine (NOHLA), which remains bound to the enzyme. Second, this intermediate product is oxidized to L-citrulline and NO. Both steps are dependent on $Ca^{2+}$/CaM and NADPH and are enhanced by BH4 [313]. When intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) levels increase CaM binds to the eNOS enzyme. Bound CaM then facilitates the flow of electrons from NADPH via FAD and FMN in the reductase domain to heme in the oxygenase domain [108]. There $O_2$ is reduced and L-arginine oxidized to L-citrulline and NO. This electron transfer leads to the binding of the ferric ($Fe^{3+}$) heme to $O_2$ and generation of a ferrous ($Fe^{2+}$)-dioxy heme. The ferrous heme then may receive a further electron from BH4 when the enzyme is in the coupled state or from the reductase domain in the uncoupled state. In the coupled reaction the protonated BH4 is oxidized to the trihydrobiopterin cation radical ($BH4^{\bullet+}$). The $Fe^{2+}$ heme and the oxidized BH4 can be recycled using electrons from NADPH via the reductase domain and the flavins of eNOS itself [107; 348]. Oxidized BH4 can alternatively be recycled by exogenous reducing agents such as ascorbic acid [331]. This reaction is schematically shown in Figure 1.1.

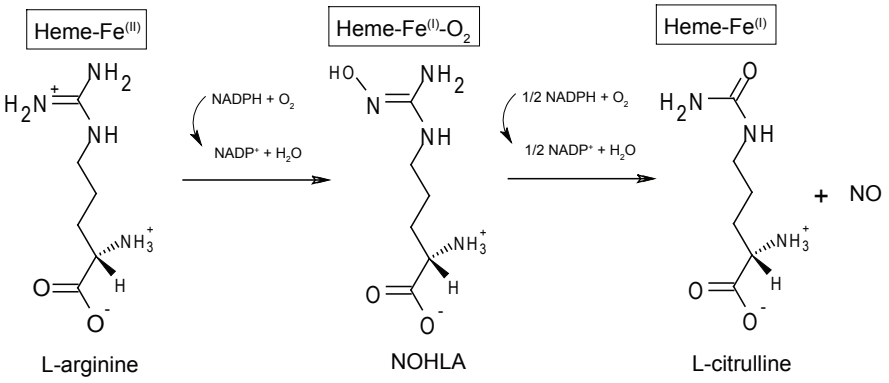


**Figure 1.1:** Reaction catalyzed by the eNOS enzyme

In summary the equation of the reaction catalyzed by eNOS is as follows:

$$1\,\text{L}-\text{arginine} + \tfrac{3}{2}\,\text{NADPH} + \tfrac{3}{2}\,\text{H}^+ + 2\,\text{O}_2 \longrightarrow 1\,\text{L}-\text{citrulline} + 1\,\text{NO} + \tfrac{3}{2}\,\text{NADP}^+ + 2\,\text{H}_2\text{O}$$

#### 1.2.3.3 Localization

The plasma membrane and the Golgi apparatus are the main sites of subcellular localization of eNOS in endothelial cells [254]. The targeting of eNOS from the Golgi apparatus to the plasma membrane involves two subsequent steps. First the N-terminal glycine of eNOS is irreversibly and cotranslationally myristoylated, followed by a reversible and posttranslational palmitoylation of the cysteine residues 15 and 26. This results in three acyl anchors that can fix eNOS to the caveolae at the plasma membrane. Myristoylation results in general membrane association of eNOS and palmitoylation targets eNOS specifically to the plasma membrane. Moreover depalmitoylation can occur at the plasma membrane and eNOS is then able to migrate to other cellular compartements. Repalmitoylation of eNOS takes place at the Golgi apparatus where it is subsequently redistributed [230; 254].

Caveolae are specialized subtypes of membrane rafts, which build flask-like invaginations in the plasma membrane and are enriched in cholesterol and sphingolipids. Caveolae are important structures for transcytosis and cellular signaling, since they allow the integration of extracellular signals into intracellular signaling cascades [75]. The structural scaffolding proteins of caveolae are termed caveolins. When eNOS is bound to caveolin-1, eNOS activity is abolished [168]. However, elevated $Ca^{2+}$/CaM levels can reverse this interaction, leading to the translocation of eNOS to the cytoplasm and its activation. Decreasing levels of $Ca^{2+}$/CaM cause the reassociation of eNOS with caveolin-1 and thereby its inactivation. Deletion of the caveolin binding site results in an active eNOS, uninhibitable by caveolin-1 [75]. The subcellular location with the highest eNOS activity is the plasma membrane followed by the cis Golgi [111; 126; 254]. Moreover, low activities are detectable in the trans Golgi [111], the cytosol [298], the mitochondria, and the nucleus [163].

Furthermore, reversible S-nitrosylation of eNOS at cysteine residues and proteins of the cytoskeleton are important for subcellular targeting of eNOS [230] (see section 1.3.5.1 and 1.4.3.6).

Additionally, two eNOS-associated proteins have been identified, that are crucial for eNOS trafficking. eNOS interacting protein (NOSIP) binds the C-terminus of the eNOS oxygenase domain and helps with the translocation of eNOS from the plasma membrane caveolae to intracellular membranes [177]. Caveolin-1 inhibts this interaction [72]. eNOS trafficking inducer protein (NOSTRIN) is stably expressed in endothelial cells and shuttles eNOS from the plasma membrane to intracellular vesicles in a caveolin-dependent manner [155; 286].

## 1.3 Nitric oxide signaling

Chemically nitric oxide is a gaseous, inorganic, uncharged but reactive radical which has only a few seconds half-life in a physiological environment as it is rapidly inactivated by superoxide or hemoglobin [330]. The unpaired electron occupies the $2p\pi^*$ antibonding orbital and is polarized towards nitrogen [85]. The fact that NO is a highly diffusible gas allows it to cross membranes and reach targets outside the location where it was generated. NO is highly reactive towards heme- or other metal-containing proteins and its radical property allows the generation of nitrating and nitrosylating agents that react with nucleophilic moieties like cysteine or tyrosine residues [330].

Endothelium-derived nitric oxide produced from eNOS has many physiological functions and thereby interacts with many intracellular target proteins [106] as detailed in the following sections.

### 1.3.1 Soluble guanylyl cyclase

The most prominent action of nitric oxide in the vasculature is the vasodilation of blood vessels due to the stimulation of soluble guanylyl cyclase and thus increased cyclic guanosine monophosphate (cGMP) production in vascular smooth muscle cells [150; 269; 274]. Guanylyl cyclase has a very high affinity to NO that allows sensitivity to picomolar concentrations of NO [21].

Soluble guanylyl cyclase is a heterodimeric enzyme composed of an α- and a β-subunit. The β-subunit contains a ferrous heme group ($Fe^{2+}$) that binds nitric oxide with great affinity. Binding of NO leads to a conformational change in the enzyme resulting in an increased catalytic activity. Activated soluble guanylyl cyclase catalyzes the conversion of guanosine-5'triphosphate (GTP) to cGMP [157]. cGMP then activates cGMP-dependent kinases such as cGKI and cGKII. cGMP produced in this manner interacts with phosphodiesterases, regulates cyclic adenosine monophosphate (cAMP) concentration and activates cyclic nucleotide gated cation channels [26; 284].

cGKI is mainly expressed in vascular smooth muscle cells, platelets and to a

lesser extent in the endothelium [150; 333]. Relaxation of vascular smooth muscle cells by nitric oxide is induced by activation of cGKI, that either leads to decreased $[Ca^{2+}]_i$ concentrations or altered calcium sensitivity of contractile proteins [150]. Activated cGKI in turn phosphorylates regulator of G-protein signaling-2 (RGS-2) and InsP3R-associated cGMP kinase substrate (IRAG) and thereby prevents inositol trisphosphate ($IP_3$) production and activation of its receptors [124; 321]. This leads to decreased $Ca^{2+}$ release from internal stores and inhibition of non-selective cationic channels and voltage-gated calcium channels. On the other hand potassium channels get stimulated leading to reduced $[Ca^{2+}]_i$ levels. Also, cGKI activates myosin light chain phosphatase leading to calcium desensitization [150].

## 1.3.2 Other targets containing ferrous heme

In addition to soluble guanylyl cyclase nitric oxide can interact with various other heme proteins, among them hemoglobin and cytochrome c oxidase.

### 1.3.2.1 Hemoglobin

Hemoglobin (specifically oxyhemoglobin and deoxyhemoglobin) rapidly reacts with nitric oxide *in vivo* [330]. Interestingly, the affinity of deoxyhemoglobin to NO is 10,000-fold greater than to molecular oxygen [332]. The reaction of NO with hemoglobin leads to its rapid removal and conversion to nitrate. However, a retardation mechanism prevents the consumption of NO in erythrocytes, such as an erythrocyte free zone in capillaries under laminar flow [201; 207]. Despite this retardation mechanism hemoglobin is reacting with a huge part of endothelial-derived NO and therefore crucial for its dynamics and bioavailability [330].

### 1.3.2.2 Cytochrome C oxidase (CcOx)

Cytochrome C oxidase (CcOx) is the last enzyme in the mitochrondrial electron transport chain. It catalyzes the reduction of molecular oxygen to water and the oxidation of cytochrome C during aerobic respiration [229]. These reactions are linked to the pumping of protons out of the mitochondrial matrix. CcOx contains a conserved bimetallic active site consisting of a heme iron and a copper ion [35]. NO

competes with oxygen for the binding to the ferrous heme of CcOx and thereby inhibits the enzyme. This leads to the reversible inhibition of mitochondrial respiration that increases with NO and decreases with oxygen concentrations [16]. NO-binding is a non-consuming reversible reaction, whereas $O_2$ binding leads to $O_2$ reduction to water. The affinity of CcOx for NO is much higher than for $O_2$. However, under normal physiological conditions $O_2$ concentrations are significantly higher than NO and therefore CcOx reacts more likely with $O_2$ [330]. Moreover, the reduction of $O_2$ is faster than NO dissociation, thus leading to a gradual increase in the nitrosyl CcOx up to the equilibrium concentration [16]. The cellular stress resulting from CcOx inhibition upon NO binding leads to an increase in the AMP/ATP ratio. This in turn activates AMP-activated protein kinase (AMPK), turning off anabolic pathways and initiating catabolic pathways. Under decreased $O_2$ availability AMPK leads to adenosine triphosphate (ATP) production, by enhancing glucose uptake and glycolysis [217].

These findings suggest that NO acts as an important regulator of oxygen sensitivity of respiration and plays a key role in the regulation of respiratory processes [41]. Additionally, CcOx is one of the most important targets of nitric oxide signaling as it can inhibit mitochondrial oxidative phosphorylation and act as a regulator of apoptosis and reactive oxygen species generation [40; 89].

### 1.3.3 Targets with non-heme iron sites

Besides heme proteins NO also interacts with metalloproteins containing iron-sulfur clusters. The iron is tetrahedrally coordinated and is bound by two sulfides and two other identical ligands, which are usually protein cysteine thiolates [300]. Binding of NO to the iron of the cluster results in the formation of various iron-nitrosyl species thereby leading to a conformational change and most often to the inhibition of the respective enzyme [79; 217].

Iron-sulfur clusters occur in proteins such as aconitase, ferredoxins, NADH dehydrogenase, mitochondrial complexes I, II and III and many more. They are important in multiple physiological processes such as energy metabolism, gene expression and iron homeostasis. Moreover, nitrosylation of the iron-sulfur cluster of these proteins has huge physiological consequences [300].

### 1.3.4 Molecular oxygen, superoxide and other radicals as targets of nitric oxide

#### 1.3.4.1 Molecular oxygen

Since $O_2$ has two unpaired electrons it is reactive towards other species with unpaired electrons including NO. When NO reacts with molecular oxygen nitrite ($NO_2^-$) is formed. As an intermediate of this reaction dinitrogen trioxide ($N_2O_3$) is generated [330]. This compound is important for N-nitrosation reactions and has been implicated in DNA base deamination [121]. Another intermediate in this reaction is nitrogen dioxide ($NO_2$), which is known to nitrate proteins, lipids and DNA [20]. These kind of reactions are more likely to occur in hydrophobic environments, like in membranes or hydrophobic cores of proteins, as NO and $O_2$ accumulate there [235].

#### 1.3.4.2 Superoxide

NO is the only known biomolecule that reacts faster with superoxide than superoxide dismutase [258]. Reaction of NO with superoxide leads to the production of peroxynitrite ($NO_3^-$) [330]. Peroxynitrite can create $CO_3^-$ radicals when reacting with $CO_2$, which is available in high concentrations in biological fluids, leading to protein-protein [227] and DNA-DNA cross-linking [360]. Additionally, peroxynitrite can interact with heme and thiol proteins having severe consequences for the development of pathologies [28; 84; 145].

#### 1.3.4.3 Radicals

Interaction of NO with radicals can result in antioxidant and pro-oxidant activities. NO is not a very strong oxidant and there are numerous radicals more reactive in a physiological environment [330]. Nevertheless, NO can potently inhibit lipid peroxidation [253] and protein oxidation [187] by interacting with lipid- and protein-derived radicals, thereby terminating radical chain reactions.

### 1.3.5 Covalent posttranslational modification of target proteins by nitric oxide

Covalent posttranslational modification of target proteins by NO occurs mostly at cysteine and tyrosine residues [217]. Three different modifications, directly or indirectly mediated by NO, are discussed in the following sections.

#### 1.3.5.1 S-nitrosylation

S-nitrosylation is a reversible posttranslational modification which leads to the formation of a nitrosothiol (R-S-N=O) by incorporation of a nitroso group to a cysteine thiol. S-nitrosylation is recognized to be an important mechanism for signal transduction, and is therefore highly regulated. Subcellular compartmentalization, site specificity and denitrosylation specificity are crucial for signaling involving S-nitrosylation [74].

S-nitrosylation is considered as a short-range signaling mechanism or "proximity based NO signaling" in contrast to long-range signaling like activation of soluble guanylyl cyclase where NO diffuses to vascular smooth muscle cells [218]. Nitrosylating agents have to be generated from NO, as NO itself is not considered to act as a nitrosylating species. Metal-catalyzed nitrosylation and to a lesser extent dinitrogen trioxide ($N_2O_3$), that is formed from NO auto-oxidation, generates S-nitrosylated species [130]. Tight regulation of subcellular compartmentalization leads to increased concentrations of nitrosylating species and target residue specificity can be achieved through the colocalization with target proteins and nitric oxide synthases [217].

There are two different denitrosylation mechanism discussed. On the one hand glutathione has been reported to reduce nitrosothiols leading to glutathionylated proteins and on the other hand thioredoxin can reduce nitrosothiols. Glutathione as well as thioredoxin can be regenerated by specific reductases and NADPH [134; 203].

#### 1.3.5.2 S-glutathionylation

Glutathione is the most abundant low-molecular-mass thiol inside cells. Reaction of glutathione with cysteine residues of proteins via formation of a disulfide bridge is called S-glutathionylation. S-glutathionylation can occur without the direct inter-

action with NO [217]. Two pathways have been suggested to link NO signaling and S-glutathionylation. First, glutathionylation is induced by peroxynitrite in several proteins [61; 255]. Second, an already nitrosylated protein can react with glutathione resulting in the S-glutathionylation of this protein [217]. S-glutathionylation is considered to be more stable than S-nitrosylation and seems to be a protective mechanism within the cell. Reaction of glutathione with a thiol guards it against oxidation to sulfinic or sulfonic acid and allows the enzymatic regeneration of the free thiol [118; 175].
For the posttranslational glutathionylation of eNOS see section 1.4.3.4.

#### 1.3.5.3 Tyrosine nitration

Tyrosine nitration describes the addition of a nitro group (-$NO_2$) to the phenolic ring of tyrosine residues to form a 3-nitrotyrosine residue. Peroxynitrite is the most common nitrating agent, although not the only one [1; 217]. Reactivity of peroxynitrite is pH dependent and therefore varies in different subcellular compartments [1].

Tyrosine nitration has been considered to be an irreversible modification, however non-enzymatic denitrating mechanisms may exist [1; 7]. It is therefore no classical signal transduction modification but changes the acitivity of proteins involved in signaling [307]. For example cytochrome C nitration leads to gain of function [151; 307] whereas nitration of the mitochondrial manganese superoxide dismutase (MnSOD) inhibits the enzyme [210]. Loss of function of MnSOD was described in several pathologies [357].

Nitration of fatty acids is also an important modification regarding pathophysiologies [103; 283]. Nitro-fatty acids are formed by the reaction of reactive nitrogen species with unsaturated fatty acids using nitrogen dioxide as nitrating agent. Nitration of oleate, lineolate, arachidonate and others has been reported. These nitrated fatty acids seem to have anti-inflammatory features as they can activate eNOS and heme oxygenase [173]. Reaction of nitro-fatty acids with thiols is termed nitroalkylation and another posttranslational modification involved in NO signaling [22].

## 1.4 Regulation of eNOS

Nitric oxide bioavailability is dependent on several regulatory factors such as the expression of eNOS, the availability of the substrate L-arginine and eNOS cofactors such as BH4, posttranslational modification of eNOS and the presence of ROS, which are able to inhibit eNOS activity [239; 281; 312; 349]. Additionally, tight temporal and spatial regulation of eNOS is necessary for NO functions, as its half-life is very short. In the following sections the different regulatory levels are discussed.

### 1.4.1 Regulation of the eNOS promoter

The eNOS promoter lacks a typical TATA box but has multiple potential cis-regulatory DNA sequences, including a CCAT box, Sp1 sites, GATA motifs, CACCC boxes, AP-1 and AP-2 sites, a p53 binding region, NF-1 elements, acute phase reactant regulatory elements, sterol regulatory elements, and shear stress response elements [215]. Interestingly, the human and the bovine promoter share 75 % homology suggesting a high evolutionary conservation of transcriptional regulation [337]. There are two regulatory regions involved in basal eNOS transcription. The positive regulatory domain I (PRDI) and II (PRDII), both located close to the transcriptional start site. These regions are recognition sites for transcription factors such as Sp1 and Ets-1 among others [171]. Interactions between these trans-acting factors and the regulatory domains is crucial for eNOS promoter activity. Moreover, a 269-nt enhancer sequence, located approximately 4700-nt upstream of the transcriptional start site, has been identified in the eNOS promoter [190].

Recently a polymorphism in the eNOS gene has been identified that gives rise to a 27-nt microRNA, which is able to inhibit eNOS transcription and thereby acts as a negative feedback regulator of eNOS expression [363].
Expression of eNOS mRNA is restricted to the vascular endothelium, therefore there needs to be a mechanism impairing eNOS expression in non-endothelial cells. It has been discovered that the eNOS promoter is more heavily methylated in non-endothelial cells, especially in the PRDI and PRDII regions, thus leading to the inhibition of promoter activity [49]. DNA methylation therefore seems to be a crucial mechanism for cell-type specific eNOS expression, probably also accompanied by histone modifications [93].

### 1.4.2 Posttranscriptional regulation of eNOS mRNA

Posttranscriptional regulation of eNOS happens on several levels, such as modification of the primary transcript, nucleocytoplasmic transport, subcellular localization, mRNA stability and translation efficiency [294]. In general, these regulatory modifications are mostly mediated by cis-acting RNA elements located in the 5'- and 3'-mRNA untranslated regions (5'-UTRs and 3'-UTRs). Both the primary and secondary structures of these elements and their recognition by trans-acting RNA binding proteins is necessary for posttranscriptional regulation [202; 267].

Several modulators of eNOS activity act on the transcriptional and posttranscriptional level. Transcriptional upregulation of eNOS expression has been reported for stimuli as diverse as laminar shear stress, hydrogen peroxide, transforming growth factor-β1 (TGF-β1), lysophosphatidylcholine, estrogen, and inhibition of histone deacetylase. Downregulation of eNOS transcription has been reported for instance, for tumor necrosis factor-α (TNF-α) (reviewed in [294]). Interestingly lysophosphatidylcholine, which is a component of oxidized low densitiy lipoprotein (oxLDL), stimulates reactive oxygen species production in endothelial cells and therefore may have a similar mechanism of action than hydrogen peroxide [183]. Moreover, increased eNOS expression has often been observed in combination with increased reactive oxygen species. It was hypothesized that the increase in eNOS expression is a compensatory mechanism to maintain bioavailable NO levels during increased reactive oxygen stress, and may explain increased NO levels, for instance, in hypertension and atherosclerosis [34; 191].

Furthermore, eNOS mRNA is regulated posttranscriptionally by modification of its stability. Stimuli that are known to act by increasing eNOS mRNA half-life are laminar shear stress, cell proliferation, hydrogen peroxide, statins and vascular endothelial growth factor (VEGF). Negative effectors of mRNA stability are among others oxLDL, TNF-α, lipopolysaccharide (LPS), hypoxia, thrombin and hypercholesterolemia (reviewed in [294]).

There are different mechanisms responsible for increased mRNA stability. For instance, interaction of ribonucleoproteins with the 3'-UTR mRNA can modify mRNA stability. A 51 kDa ribonucleoprotein, containing G actin, was suggested as a destabilizing factor in nonproliferating cells [295; 296]. As G actin is much more abundant in nonproliferating cells the inhibiting interaction of eNOS mRNA with the G actin

containing ribonucleoprotein is much more common in these cells. Additionally, the reduced G actin binding in proliferating cells corresponds to an increased interaction with the cytoskeleton [294].

Another example is a CU-rich 158-nt sequence at the 3'-UTR that was shown to be important for the regulation of mRNA stability [186]. Several other studies have identified cis-regulatory regions in the eNOS 3'-UTR thereby suggesting important RNA-protein interactions [11; 164].

Another important regulatory mechanism was found by Robb et al [278]. They discovered an antisense mRNA, also called sONE, that was complementary to 662-nt of the human eNOS mRNA. This RNA was shown to posttranscriptionally down-regulate eNOS expression in non-endothelial cells, as it was found in a range of cell types but not in vascular endothelial cells. For instance, sONE seems to repress eNOS expression in vasular smooth muscle cells. Therefore, sONE was suggested to be an important regulator of cell-specific expression of eNOS [278].

There is yet another mechanism leading to a more stable eNOS mRNA. An increased length in the 3'polyadenylated tail of eNOS transcripts leads to a more stable mRNA which has a prolonged half-life and is more actively translated. This mechanism has been reported in response to laminar shear stress, statins and hydrogen peroxide [344].

## 1.4.3 Posttranslational modification of eNOS protein

### 1.4.3.1 Phosphorylation

Phosphorylation is a well investigated mechanism for posttranslational regulation of eNOS activity. It allows fast changes in eNOS activity in response to diverse humoral, pharmacological or metabolic stimuli. There are five eNOS serine and threonine phosphorylation sites known, which are controlled by a still increasing number of kinases and phosphatases.

**Ser$^{1177}$ phosphorylation** Phosphorylation of eNOS at Ser$^{1177}$ is the best studied and most important eNOS phosphorylation site. Most stimuli activating eNOS are leading to an increase of phosphorylation at this site. Dephosphorylation seems to be

mainly performed by the phosphatase PP2A (protein phosphatase 2A). Whereas, several kinases have been implicated in the phosphorylation of this site such as Akt (protein kinase B), PKA (protein kinase A), AMPK, PKG (GKI), CaMKII ($Ca^{2+}$/CaM-dependent protein kinase II) and CHK-1 [239; 264]. These kinases in turn can be activated by numerous other factors like shear stress, VEGF (vascular endothelial growth factor), IGF-1 (Insulin-like growth factor 1), bradykinin, insulin, ATP, shingosine 1-phosphate, $H_2O_2$, IBMX (3-isobutyl-1-methylxanthine), estrogen, adiponectin, leptin, histamine, thrombin, ischemia, troglitazone, statins, 8-Br-cAMP [239]. Increased eNOS-$Ser^{1177}$ phosphorylation can affect eNOS activity by increasing $Ca^{2+}$/CaM binding or at resting levels of $[Ca^{2+}]_i$ [56; 236]. The eNOS-$Ser^{1177}$ phosphorylation site, located at the C-terminal tail, is jammed between two eNOS monomers thereby acting as autoinhibitory domain blocking the electron flow between the monomers. eNOS-$Ser^{1177}$ phosphorylation leads to a conformational change in the enzyme that is able to remove the autoinhibitory domain and increasing electron flow in the reductase domain and thereby enzyme activity [189].

**$Thr^{495}$ phosphorylation** Phosphorylation at $Thr^{495}$ decreases eNOS activity and is the most important negative regulatory site for eNOS activity. This phosphorylation site is located in the $Ca^{2+}$/CaM binding domain in between the reductase and the oxygenase domain [101]. Under normal physiological conditions eNOS is basally phosphorylated at this site. Phosphorylation is mediated by the kinases PKC (protein kinase C, unknown isoform), AMPK (only in vitro experiments) and Rho kinase [56; 220; 315]. The inhibitory effect of eNOS-$Thr^{495}$ on eNOS enzyme activity is due to its interference for binding of $Ca^{2+}$/CaM to eNOS [101]. Dephosphorylation of this site is performed by phosphatases like PP1 (protein phosphatase 1), PP2A and PP2B (protein phosphatase 2B, calcineurin), that appear to be activated upon stimulation with agonists that are mostly increasing $[Ca^{2+}]_i$ concentrations such as bradykinin, VEGF, $H_2O_2$ or ionomycin [139; 231; 324]. Interestingly, dephosphorylation at $Thr^{495}$ and phosphorylation at $Ser^{1177}$ have been shown to be coordinated in many studies [101; 139; 231; 324]. However, eNOS-$Thr^{495}$ dephosphorylation has also been described without additional increase in eNOS-$Ser^{1177}$ phosphorylation [291]. Moreover, in a study where eNOS-$Thr^{495}$ dephosphorylation was mimicked by mutation at this site to alanine, eNOS uncoupling and therefore superoxide production were promoted [204]. This finding suggests that eNOS-$Thr^{495}$

may have a critical role in eNOS uncoupling when calcium levels are low and therefore $Ca^{2+}$/CaM binding to eNOS is not increased.

**Ser$^{633}$ phosphorylation** eNOS-Ser$^{633}$ phosphorylation is enhancing eNOS activity. This phosphorylation site is located in the CaM autoinhibitory sequence in the flavin mononucleotide (FMN) binding region of the reductase domain [233]. Agonists such as VEGF, bradykinin, ATP, IBMX, 8-Br-cAMPK, statins and additionally shear stress that increase eNOS phosphorylation at Ser$^{1177}$ have also been described to increase phosphorylation at Ser$^{633}$. However, the onset of Ser$^{633}$ phosphorylation seems to be slower [239]. This phosphorylation has been shown to be performed by PKA and to be independent of an increase in $[Ca^{2+}]_i$ [32]. Which phosphatase performs the dephosphorylation at this site is not known. Altogether it has been suggested that eNOS-Ser$^{633}$ phosphorylation follows the initial $Ca^{2+}$-dependent increase in eNOS-Ser$^{1177}$ phosphorylation and maintains the enhanced eNOS activity [239].

**Ser$^{615}$ phosphorylation** This phosphorylation site is located in the CaM autoinhibitory sequence of the FMN binding domain like the Ser$^{633}$ site [233]. Phosphorylation at this site is transiently stimulated by agonists such as bradykinin, VEGF, ATP and statins through the kinases PKA and Akt [23; 137; 233]. An increase in eNOS-Ser$^{615}$ phosphorylation is suggested to enhance the eNOS sensitivity to $Ca^{2+}$/CaM or to modulate interactions of proteins with eNOS and regulation of other phosphorylation sites [23; 233]. Several eNOS agonists have been found to increase phosphorylation at this site but studies with mutants mimicking phosphorylation or dephosphorylation at Ser$^{615}$ have been controversial [23].

**Ser$^{114}$ phosphorylation** The eNOS-Ser$^{114}$ phosphorylation site is the only phosphorylation site located in the oxygenase domain of eNOS and is basally phosphorylated. The function of this phosphorylation site is still very controversial. Dephosphorylation of eNOS-Ser$^{114}$ by VEGF or troglitazone [59; 181] and phosphorylation by shear stress or high density lipoprotein (HDL) seem to be activating eNOS activity [80; 117]. Additionally, results from studies with phosphorylation or

dephosphorylation mimicking mutants have not been able to clarify the function of this phosphorylation site. Several kinases have been implicated to phosphorylate this site such as PKC, AMPK and cyclin-dependent kinase-5 (CDK-5). PP2B seems to dephosphorylate eNOS-Ser$^{114}$ [239].

**Tyrosine phosphorylation** In addition to serine and threonine phosphorylation, eNOS can be regulated by phosphorylation at two different tyrosine residues, Tyr$^{81}$ and Tyr$^{657}$ [95; 112]. eNOS phosphorylation at Tyr$^{81}$ is performed by Src kinase thereby increasing eNOS activity [112]. In contrast, Tyr$^{657}$ is phosphorylated by proline-rich tyrosine kinase 2 (PYK2), leading to a decrease in eNOS activity [95]. Interestingly, tyrosine phosphorylation seems to be well detectable in primary endothelial cells but seems to disappear in cultured cells [98; 119]. This may explain why these phosphorylation sites are not as extensivly studied.

#### 1.4.3.2 Acetylation

Another posttranslational modification important for eNOS function is the N-acetylation of the epsilon amino group of lysines. The addition of the acetyl group to lysine is performed by lysine acetyltransferases (KAT) using acetyl-CoA [8]. Deacetylation has been reported to be carried out by the class III $NAD^+$-dependent histone deacetylase SIRT1 (sirtuin 1). SIRT1 colocalizes with eNOS and deacetylation leads to an increase in eNOS activity. eNOS is constitutivly acetylated at Lys$^{497}$ and Lys$^{507}$ in the CaM binding domain of the enzyme. The acetylation in this region inhibits CaM binding and therefore has a similar function than the Thr$^{495}$ phosphorylation [221]. Another acetylation site is Lys$^{610}$, which has been shown to be acetylated by acetyl-salicylic acid (aspirin). Acetylation of this residue enhances eNOS activity by allowing increased binding of CaM to eNOS [169].

#### 1.4.3.3 Glycosylation

Addition of N-acetylglucosamine (GlcNAc) to serine or threonine residues leads to O-glycosylation. As this leads to the modification of the same residues as by phosphorylation, these two mechanism can compete with each other. Glycosylation is

relevant for the regulation of protein-protein interactions, protein turnover, subcellular localization and changes in activity. The enyzme performing the glycosylation is O-linked N-acetylglucosamine transferase (OGT) and β-N-acetylglucosaminidase (OGA) carries out the deglycosylation reaction [361]. Interestingly, there is only one enzyme for the glycosylation and one for the deglycosylation reaction. Therefore it seems likely that glycosylation is a regulator of signal intensities in dependance of the nutritional status of the cell. Hyperglycemia has been reported to increase GlcNAc concentrations resulting in the glycosylation of $Ser^{1177}$ of eNOS, thereby inhibiting eNOS activity [82].

#### 1.4.3.4 S-glutathionylation

For a detailed description of S-glutathionylation see section 1.3.5.2.

eNOS can be reversibly S-glutathionylated at $Cys^{689}$ and $Cys^{908}$ residues, located within the reductase domain. These glutathionylations lead to the uncoupling of eNOS, thereby resulting in increased superoxide production. S-glutathionylation of eNOS therefore seems to be an important mechanism for redox regulation of cellular signalling, endothelial function and vascular tone [53].

#### 1.4.3.5 Acylation

As already stated in section 1.2.3.3, eNOS is acylated at three residues to provide membrane association.

#### 1.4.3.6 S-nitrosylation

For a detailed describtion of S-nitrosylation see section 1.3.5.1.

S-nitrosylation of the cysteine residue 94 and 99 of eNOS have been shown to inhibit eNOS activity itself. These residues are located in the zinc tetrathiolate cluster at the eNOS homodimer interface and their nitrosylation was proposed to interfere with the dimer formation, the electron flow between the monomers or the substrate and cofactor binding. When eNOS is activated by agonists it is depalmitoylated as mentioned previously (section 1.2.3.3) and translocated away from the caveolae and the plasma membrane. There it is denitrosylated and thereby activated [90].

Additionally, S-nitrosylation has been reported of proteins directly interacting with eNOS, such as heat-shock protein 90 (Hsp90) (see section 1.4.4) [120]. Nitrosylation of a cysteine residue located within the interacting region with eNOS abolishes the ATPase activity of Hsp90 and the stimulating effect on eNOS itself. This suggests a negative feedback mechanism by which NO could regulate its own generation by a pseudo-allosteric mechanism [219].

### 1.4.4 Protein-protein interactions

Several protein-protein interactions are known to be important for eNOS enzyme regulation. In the following some of these proteins are introduced.

**Heat-shock protein 90 (Hsp90):** Hsp90 is an important molecular chaperone, responsible for the correct folding of numerous proteins, such as eNOS. Hsp90 is necessary for the insertion of heme into the immature eNOS protein [27]. Many eNOS agonists, such as VEGF, histamine, fluid shear stress and estrogen are able to increase the interaction of Hsp90 to eNOS [120]. Association of Hsp90 with eNOS is dependent on the agonist-induced tyrosine phosphorylation and leads to an increased binding of CaM to eNOS [138]. Additionally, nitrosative stress can lead to S-nitrosylation of Hsp90 (see section 1.4.3.6), thereby inhibiting eNOS activity [275].

Hsp90 binding to eNOS has been found to be crucial to Akt-dependent phosphorylation of eNOS-Ser$^{1177}$ [345]. Upon activation of Akt, binding of Hsp90 to eNOS leads to the switching from $Ca^{2+}$-dependent to $Ca^{2+}$-independent eNOS activation. This is achieved by the formation of a complex between Hsp90, Akt and CaM bound eNOS [319; 320].

**Calmodulin (CaM):** CaM was the first protein reported to be associated with eNOS [46]. The binding of calmodulin to the CaM-binding domain of eNOS is determined by several molecular interactions and also by the phosphorylation status of Thr$^{495}$ [101]. Other modifications such as the binding of Hsp90 and phosphorylation at Ser$^{1177}$ have also been shown to affect the association of these two proteins [128]. The association of CaM with its binding site activates eNOS by enabling the reductase domain to transfer electrons to the oxygenase domain [2]. Under low $Ca^{2+}$ concentrations CaM binding is inhibited by a 45-amino acid insert in the

FMN-binding domain, acting as an autoinhibitory loop. Rising $Ca^{2+}$ concentrations lead to the displacement of the insert and the binding of CaM and the activation of eNOS [66].

**PECAM-1 (platelet endothelial cell adhesion molecule; CD31):** PECAM-1 has been predominantly found at cell-cell contacts. PECAM-1 and eNOS colocalize at the plasma membrane where they are then associating [97]. It has been suggested that PECAM-1 is acting as a mechanoreceptor on the endothelial cell surface and regulates the response of endothelial cells on shear stress by allowing the interaction with tyrosine kinases, the tyrosine phosphatase SHP2 and the scaffolding protein Gab1 [58].

**Proteins important for the subcellular localization of eNOS:** Caveolins are the structural scaffolding proteins of the caveolae. Caveolin-1 binding to eNOS inhibits eNOS activity, by impairing the binding of calmodulin to eNOS, as mentioned in section 1.2.3.3. NOSTRIN and NOSIP are two proteins necessary for eNOS trafficking as mentioned earlier (section 1.2.3.3).

Additional proteins known to associate with eNOS include G protein-coupled receptors such as the angiotensin II AT1 receptor [167], polymerized actin [314], voltage-dependent anion channel 1 (VDAC1) [13] and many more.

### 1.4.5 Substrate availability

The substrate for the catalyzed eNOS reaction is L-arginine. Deficiency in the substrate availabilty leads to eNOS uncoupling resulting in the generation of superoxide instead of nitric oxide [108]. Interestingly, the mean plasma concentration of L-arginine is in the range of 100 µM and therefore far above the $K_m$ for eNOS [62]. Therefore, reduced L-arginine availability should not be an issue for eNOS activity. Moreover, endothelial cells are able to recycle the product of eNOS, L-citrulline, back to L-arginine and can generate L-arginine from protein breakdown [141; 301]. Despite these facts L-arginine supplementation has proven beneficial in conditions such as hypercholesterolemia, ischemia, and hypertension [30; 81; 149]. Therefore, this has been referred to as the "L-arginine paradox". An explanation for these observations may be that L-arginine locally competes with endogenous

analogues, like symmetric dimethyl-L-arginine (SDMA), asymmetric dimethyl-L-arginine (ADMA) or $N^G$-monomethyl-L-arginine (L-NMMA) [334]. ADMA and L-NMMA act as direct eNOS inhibitors and especially ADMA has been implicated in cardiovascular diseases [31]. An increase in ADMA reflects the activity and expression of the metabolizing enzymes dimethylarginine dimethylaminohydrolases (DDAH-1 and DDAH-2). Overexpression of DDAH-1 was described to enhance NO production in vivo, whereas its downregulation has been associated with endothelial dysfunction [68; 194].

Additionally the cationic amino acid transporter (CAT-1) may be a limiting factor for L-arginine availability as other amino acids such as L-lysine compete for the same transporter. This transporter is necessary for the L-arginine supply of eNOS [136].

Another factor involves the breakdown of L-arginine to L-ornithine and urea by arginase-II, an enzyme that competes with eNOS for the substrate. Arginase-II has been reported to be more active or to be higher expressed during cardiovascular diseases and suppression of arginase-II activity leads to enhanced vascular NO production [25; 238; 356].

### 1.4.6 Tetrahydrobiopterin (BH4) availability

Tetrayhydrobiopterin (BH4, 5,6,7,8-tetrahydrobiopterin) is an essential cofactor for eNOS, as previously described in section 1.2.3.2. Impaired availability of this cofactor leads to the uncoupling of eNOS. Then the electron transport to the ferrous ($Fe^{2+}$) heme-$O_2$ species occurs not fast enough for prevention of their oxidative decay. As a result superoxide is formed instead of NO. NO bioavailability is therefore reduced and endothelial dysfunction promoted [50].

It has been suggested recently that not only the amount of BH4 is crucial for endothelial function, but the ratio of the reduced BH4 to BH2 (7,8-dihydrobiopterin). eNOS is known to bind BH2 and BH4 with similar affinity, however, BH2 can replace bound BH4 leading to eNOS uncoupling [64; 65; 339]. Dihydrofolate reductase (DHFR) can reduce BH2 to BH4, thereby shifting the ratio towards BH4 and the coupled eNOS [289].

BH4 is synthesized via a *de novo* pathway from GTP in a three step reaction. The first reaction is catalyzed by GTP cyclohydrolase I (GTPCH I), and converts GTP to 7,8-dihydroneopterin triphosphate. This enzyme is rate-controlling for the biosynthesis of BH4. In the following step the enzyme 6-pyruvoyltetrahydropterin

synthase (PTPS) generates 6-pyruvoyltetrahydropterin, that is converted by sepiapterin reductase (SR) to BH4 in a final step [348]. Regulation of GTPCH occurs at transcriptional, translational and posttranslational levels [146]. Overexpression of GTPCH has been proven to augement NO availability in human endothelial cells [12]. Interestingly, the enzyme GTPCH feedback regulatory protein (GFPR) can reduce the amount of BH4 by catalyzing the reaction from BH4 to GTP [135].

Additionally, it has been reported that downregulation of GFRP in cultured endothelial cells leads to increased BH4 levels and GTPCH phosphorylation [199]. Bacterial lipopolysaccharide (LPS) and $H_2O_2$ are able to downregulate the expression of GFPR [161; 347].

Alternatively to the *de novo* synthesis pathway BH4 can be generated via the salvage pathway. Thereby, sepiapterin is converted into 7,8-dihydrobiopterin by sepiapterin reductase, followed by the reduction to BH4 by DHFR [249].

Taken together, supplementation of BH4 by addition of sepiapterin may be beneficial for endothelial function [262].

### 1.4.7 Endothelial function and dysfunction

Besides the function of eNOS in vasodilation it has also other important roles in the vasculature. eNOS-derived NO has been shown to inhibit platelet aggregation and platelet adhesion to the vascular wall [10; 45], to decrease the expression of the proinflammatory protein MCP-1 (monocyte chemotactic protein-1) [362], and to inhibit leukocyte adhesion by interfering with the binding of the leukocyte adhesion molecule CD11/CD18 to the endothelial cells or by suppressing CD11/CD18 expression [18; 182]. Furthermore, eNOS is critical for vascular remodelling in response to changes in blood flow [282], and for the prevention of endothelial cell apoptosis and stimulation of angiogenesis [78]. Another crucial function of eNOS is the inhibition of vascular smooth muscle cell proliferation, that is likely to be mediated by cGMP [122; 244]. All these functions of eNOS-derived NO contribute to its anti-atherosclerotic character.

Several mechanism have been linked to the uncoupling of eNOS, like reduced availability or oxidation of BH4, reduced availability of L-arginine, oxidative disruption of the zinc-thiolate cluster, and increased concentrations of asymmetric dimethyl-L-arginine (ADMA) [108]. A new mechanism for eNOS uncoupling is the

S-glutathionylation of conserved cysteine residues in the reductase domain during oxidative stress [53]. Oxidative stress is often enhanced in vascular diseases, like atherosclerosis. This can lead to the degradation of NO by superoxide to peroxynitrite or to the uncoupling of eNOS, so that the enzyme itself produces superoxide. Peroxynitrite, which is formed under oxidative stress from NO, can lead to the oxidation of BH4 to BH2, and disrupt the zinc-thiolate cluster of the eNOS enzyme [188; 365].

Additionally, hypertension and all forms of diabetes (type I and type II) have been associated with reduced NO bioavailability or impaired vasodilation, due to the increased production of superoxide [51].

Interestingly, once oxidative stress starts damaging eNOS a vicious cycle begins. Superoxide leads to the formation of peroxynitrite that further disrupts eNOS function leading to superoxide formation and so on, and so on. Upregulation of eNOS under these circumstances is very detrimental.

Endothelial dysfunction is regarded as the initial event for the onset of atherosclerosis. Atherosclerosis is in turn thought to be the underlying pathology of cardiovascular diseases, such as stroke, peripheral vascular disease and coronary heart disease [51]. Endothelial dysfunction is characterized by an impaired function of eNOS and NO availability [67]. A strong link between endothelial-derived NO and atherosclerosis is provided by studies showing that administration of L-arginine or BH4 reduces the progression of atherosclerotic lesions [6; 326]. This could be prevented by the addition of NOS inhibitors [343].

## 1.5 Natural products and their influence on eNOS

An increasing amount of studies show an influence of natural products on cardiovascular health. Moreover, natural products from dietary sources or derived from medicinal plants have been identified in the last years to have positive effects on eNOS activity or NO bioavailability. In general compounds taken up with the diet can easily reach the endothelium via the blood stream and are therefore interesting to investigate. Several natural products and dietary habits have been discussed as modulators of eNOS function such as grapes and red wine, resveratrol, black and green tea, cocoa, soy isoflavones and many more [291].

Many studies suggested a negative correlation between moderate red wine consumption and the occurance of cardiovascular diseases [69]. This effect has been attributed to the red wine polyphenols [73]. Moreover, synergistic effects between single polyphenols are likely [341]. Treatment with red wine polyphenols elicit a long-term and additionally a fast effect. Long-term treament was shown to upregulate eNOS expression, thereby increasing endothelial NO release [193; 342]. Furthermore, short-term effects are based on the rapid increase in $Ca^{2+}$ concentrations and eNOS-$Ser^{1177}$ phosphorylation via the activation of the PI3K/Akt pathway in cultured cells, resulting in fast NO-dependent vasodilation [288]. Red wine polyphenols have also been shown to increase reactive oxygen species production, such as superoxide and hydrogen peroxide [248]. It is possible that this moderate stress is triggering defense mechanisms protecting cells from future stress. Consumption of red wine had however not in all studies beneficial effects [243; 246; 364].

Grape juice, red grape polyphenol extract and grape skin extract have similar beneficial effects [15; 195; 305]. Oligomeric procyanidins seem to account for these effects which are also likely to be in part responsible for the effect of red wine [63]. Interstingly, the grade of oligomerization correlated with the degree of vasodilation elicited by these preparations [96].

The stilbene derivative resveratrol is another compound contained in red wine, but can also be found in grape skin, berries and peanuts. Both in vivo and in vitro studies showed improved vascular function upon resveratrol treatment [17; 277; 340; 341]. However, this was only in part due to increased NO availability [242; 273]. The eNOS stimulatory effect of resveratrol seems to be dependent on oestrogen receptors [176]. However, until now no studies in humans have been performed. The fact that the amount of resveratrol in red wine is only a few milligrams per litre and that it is metabolized rapidly in the blood suggests that resveratrol is not responsible for the beneficial effects of red wine [125; 256].

The consumption of black and green tee has been implicated with improved endothelial function [86; 165]. They contain different polyphenols dependent on their preparation and fermentation, among them (−)-epicatechin and epigallocatechin gallate (EGCG). (−)-Epicatechin increased NO production [265] and EGCG, a compound mainly contained in green tea, was shown to activate eNOS via the PI3K/Akt pathway [208].

(−)-Epicatechin is additionally contained in cocoa as well as oligomeric procyanidines, which are both responsible for the enhancing effect of cocoa preparations on

endothelium-dependent vasodilation and eNOS activity [88; 172; 293]. Interestingly, (−)-epicatechin serves as a building block for oligomeric procyanidins. However, the beneficial effect of cocoa products is attenuated by the high amount of sugar often contained in these products [91].

The soy isoflavone genistein was reported to enhance eNOS activity via PKA and eNOS-Ser$^{1177}$ phosphorylation after long-term treatment [205]. Another soy isoflavone, equol, rapidly enhances NO production in cultured endothelial cells in submicromolar concentrations via increasing $[Ca^{2+}]_i$ concentrations followed by enhanced eNOS-Ser$^{1177}$ phosphorylation [166]. Possibly, soy isoflavones mimic hormonal effects of estrogen, which is a known activator of eNOS [185; 346].

Pomegranate juice is also a rich source of polyphenols and has been reported to increase eNOS expression and prevent NO degradation via superoxide scavenging [70; 71; 159].

Oleic acid, oleanolic acid and polyphenols contained in olive oil are beneficial for endothelium-dependent vasodilation [109; 279; 280].

The polyunsaturated omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, contained in fish oil, directly increase endothelial NO release. This may be achieved by alteration of the lipid composition of the plasma membrane by these compounds, resulting in the release of eNOS from caveolin-1 [200].

The effect of caffeine on endothelial function is controversial. It has been reported that caffeine intake leads to increased $[Ca^{2+}]_i$ concentrations, thereby activating eNOS [140], but there are also studies indicating a decrease in endothelial function upon coffee consumption [263; 276].

The most abundant polyphenol in plants and therefore also in the diet is quercetin [212]. It was shown to elevate $[Ca^{2+}]_i$ levels and thereby eNOS activity [184]. However, besides numerous studies showing enhancing endothelium-dependent vasodilation upon quercetin treatment, there are also several studies showing no effect or even a negative effect of quercetin on NO availability (reviewed in [291]).

Other phytomedical preparations influencing endothelium-dependent vasodilation are *Ginkgo biloba* leaves, that increase eNOS expression and Ser$^{1177}$ phosphorylation [180]. Hawthorn extract is increasing endothelium-dependent vasodilation as well, probably dependent on the amount of oligomeric procyanidines contained [39]. Ginseng root aqueous extract activates eNOS via PI3K/Akt [174] and ursolic acid, contained in a *Salvia miltiorrhiza* water extract, strongly enhances eNOS expression

[310]. Interestingly, betulinic acid, contained in *Zizyphi spinosi* seeds, is upregulating eNOS expression and at the same time downregulates NADPH oxidase, thereby enhancing NO availability through both mechanisms [311].

Other compounds and preparations enhancing eNOS activity are ascorbic acid (see section 1.6.1), the lignan sesamol, contained in sesame seeds [54], an artichoke leaf extract [198], the citrus flavone hesperidin [206], the passion flower flavone chrysin [83], the legume flavanoid dioclein [196], cranberry [211] and watermelon juice [354] and many more.

## 1.6 Natural products used in this work

### 1.6.1 Ascorbate

Ascorbate is the deprotonated form of ascorbic acid (vitamin C, Figure 1.2). It acts as a water-soluble reducing agent and antioxidant in biological systems. Humans and other primates have lost the ability to synthesize vitamin C on their own due to a defect in L-gulono-1,4-lactone oxidase, the enzyme that catalyzes the conversion of L-gluconolactone into ascorbic acid. Therefore the diet is their only source of vitamin C to prevent the vitamin C deficiency disease, scurvy, and maintain general health [331]. In healthy, well-nourished, nonsmoking people mean plasma levels of ascorbate are between 50 to 60 µM. However, oral supplementation can increase plasma levels up to 100 µM [19; 38; 60; 317; 353]. Low levels of plasma ascorbate are common in different diseases linked to oxidative stress such as cancer, diabetes, cataract, HIV infection and sepsis or in smokers [29; 92; 116; 213; 257; 259; 323; 350]. In most cells ascorbate concentration is considerably higher than 50 µM. This is achieved by an active transport mechanism using sodium-dependent vitamin C transporters (SVCT) [335]. The final concentration of ascorbate inside cells was determined to be between 3 and 4 mM [216; 223]. Interestingly, the oxidized form of ascorbate, dihydroascorbate (DHA), is taken up by endothelial cells via GLUT-transporters and rapidly reduced to ascorbate inside the cells. However, DHA uptake seems not to be relevant for intracellular ascorbate concentrations as uptake competes with glucose which is approximately 1000 times more abundant [3; 77].

Ascorbate has many important functions in the body. Several studies have observed that ascorbate has beneficial effects on the prevention of atherosclerosis [3].

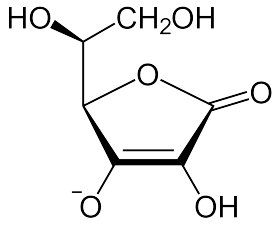


**Figure 1.2:** L-ascorbate

In cultured endothelial cells ascorbate was shown to stabilize BH4 after 24 hours [144; 153]. As mentioned earlier (see section 1.4.6) low levels of BH4 lead to the uncoupling of eNOS by promoting the transfer of electrons to molecular oxygen instead of L-arginine. The therapeutic potential of ascorbate to prevent eNOS uncoupling under conditions of oxidative stress has been investigated in several clinical studies. They showed that oral supplementation of vitamin C is mostly ineffective in this regard [308] whereas ascorbate infusions rapidly improved endothelium-dependent vasodilation in conditions such as diabetes [142; 327], hypertension [48; 148; 247; 306; 318], hypercholesterolemia [266; 328], experimental sepsis [336; 350] and smoking [143; 147; 287] without affecting healthy control groups.

It should be noted that the endothelial cell culture medium used in this study is not supplemented with ascorbic acid apart from traces contained in the FBS. The cells used are therefore under chronic lack of vitamin C which enhances oxidative stress and decreases eNOS activity likely due to BH4 deficiency [304]. Cultured endothelial cells are therefore a useful model for endothelial dysfunction in cardiovascular diseases.

Besides its importance for BH4 stabilization ascorbate acts as a cofactor and electron donor for several enzymes, such as prolyl and lysyl hydroxylases which are important for collagen synthesis and HIF-1α (hypoxia-inducible factor 1α) hydroxylation [43; 241]. In addition ascorbate is involved in the synthesis of norepinephrine, carnitine, cholesterol, several amino acids and peptide hormones [52]. Stabilization of collagen by ascorbate is crucial for the formation of connective tissue in the whole body which is responsible for the main symptoms of scurvy [3]. Additionally, ascorbate was shown to stabilize atherosclerotic plaques in apolipoprotein E (ApoE) knockout mice, through its ability to build up collagen [245].

Another important function of ascorbate is its action as antioxidant. The hydroxyl

groups can donate a hydrogen atom to different oxidants, including oxygen- and nitrogen-based free radicals, peroxides and superoxide [3; 44]. Furthermore, it can reduce ferric iron in dioxygenase enzymes [3]. Importantly, ascorbate can recycle oxidized vitamin E (α-tocopherol) [250].

Oxidized ascorbate itself can also be recycled inside the cell [3].

Here we investigated if stabilization of BH4 is the underlying mechanism for the rapid effects of ascorbate infusions in diseased patients or if additional mechanisms for the activation of eNOS are involved.

### 1.6.2 Lignans isolated from *Krameria lappacea*

Plant secondary metabolites from the benzofuran class are known to exhibit a broad range of bioactivites including anti-inflammatory [131; 152], cardio- and vaso-protective [113; 154], cytostatic [192], and antioxidant actions [170; 228]. However, there are no reports on eNOS activation by this compound class so far. The roots of *Krameria lappacea* (Dombey) Burdet et Simpson (syn. *K. triandra* Ruiz et Pavon), are a rich source of such compounds. Root preparations have been used traditionally in South America for the treatment of different inflammation-related complaints, including bowel complaints and inflammation of the throat. In the European medicine it has been used since the 18th century [47; 302; 303].

In this study we investigated eleven lignans (nine benzofuran lignans and two epoxy lignans) including the benzofran derivative 2-(2,4-dihydroxyphenyl)-5-(E)-propenylbenzofuran (DPPB, Figure 1.3) that were isolated from the dried roots of *K. lappacea*. Isolation of these compounds was perfomed by the group of Prof. Stuppner (Institut of Pharmacy/Pharmacognosy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Austria).

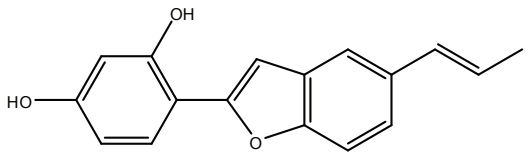


**Figure 1.3:** Structure of the benzofuran derivative DPPB (2-(2,4-dihydroxyphenyl)-5-(e)-propenylbenzofuran)

One study has been published identifying DPPB as the major active compound of

a standardized *K. triandra* root extract. There DPPB was shown to exert a potent cytoprotective effect on different cell lines exposed to stress.

Here we investigated if the isolated lignan derivatives act as activators of eNOS activity in cultured endothelial cells.

# 2 Material & Methods

## 2.1 Materials

## 2.2 Cell culture

For all experiments control cells were treated with an equal volume of solvent. When DMSO was used as solvent the concentration used did not exceed 0.1 % unless stated otherwise.

### 2.2.1 EA.hy926 cells

EA.hy926 cells were a kind gift from Dr. C.-J. S. Edgell, University of North Carolina, Chapel Hill (USA) [87]. This permanent human vascular cell line was created in 1983 by hybridization of primary human umbilical vein endothelial cells (HUVEC) and the human lung carcinoma cell line clone A549/8. This clone is deficient in hypoxanthine phosphoribosyltransferase (HPRT), which is used for selection with HAT medium (hypoxanthine-aminopterin-thymidine medium). HAT medium contains amongst others aminopterin which blocks de novo nucleotide synthesis. HPRT deficiency leads to loss of function in the alternative nucleotide synthesis pathway, the salvage pathway. Because of that A549/8 cells are not able to synthesize DNA in the presence of HAT medium and die. The primary HUVEC can only live for a limited number of replication cycles and will therefore die after some time in culture. Hybrid cells with restored HPRT function are therefore the only cells surviving in HAT medium for an extended passage number. The EA.hy926 cell line was confirmed to produce von Willebrand Factor (factor VIII related antigen) with the same morphological distribution as in their parental endothelial cells and to upregulate ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular adhesion molecule-1) and E-selectin expression upon stimulation with TNF-$\alpha$ [87; 325]. Futhermore

EA.hy926 cells grow faster, need less growth factors and can be used for more passages than HUVEC due to their immortality. Therefore they are a lot easier to cultivate. However, as a hybrid cell line differential responses and reactions can not be excluded. Keeping that in mind we confirmed all the key results obtained in EA.hy926 cells in HUVEC.

#### 2.2.1.1 Cultivation

EA.hy926 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) without phenol red containing 4.5 g/L glucose supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, HAT supplement (100 µM hypoxanthine, 16 µM thymidine, 0.4 µM aminopterin - for selection of hybridoma cells) and 10 % heat-inactivated fetal bovine serum (serum was heat-inactivated at 56℃ for 30 min). EA.hy926 cells were cultivated in an humidified incubator at 37℃ and 5 % $CO_2$.

**Thawing** A cryovial containing one million EA.hy926 cells was taken out of the liquid nitrogen and quickly defrosted in a 37℃ waterbath. The still frozen cells were poured into 10 ml complete growth medium. Then they were centrifuged at 210 g for 10 min to get rid of the DMSO contained in the freezing medium. The cell pellet was then resuspended in 20 ml complete growth medium and transferred to a 75 $cm^2$ flask. The medium was changed after three to four days and the cells reached confluency approximately after one week.

**Passaging** Cells were passaged when reaching confluency approximately after one week. The cells were washed with prewarmed PBS and subsequently trypsinized with T/E solution. Trypsinization was stopped with complete growth medium. Cells were then counted in the cell viability analyzer ViCell®. For cultivation in a 75 $cm^2$ flask 0.7 x $10^6$ cells were seeded and for a 175 $cm^2$ flask 1.6 x $10^6$ cells were seeded. The medium was changed after three to four days after passaging. EA.hy926 cells were used for experiments until reaching passage 26.

### 2.2.2 Human umbilical vein endothelial cells (HUVEC)

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza.

#### 2.2.2.1 Cultivation

HUVEC were grown in endothelial cell basal medium (EBM™) supplemented with 10 % heat-inactivated standardized fetal bovine serum (serum was heat-inactivated at 56℃ for 30 min), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 % amphotericin B and EGM™SingleQuots™, containing recombinant human epidermal growth factor, hydrocortisone, gentamicin sulfate, amphotericin B and 0.4 % bovine brain extract. Dishes and plates were coated with gelatine before cells were seeded. Thus, the culture dishes and plates were incubating with 0.1 % gelatine solution for 10 min at room temperature. HUVEC were cultivated in an humidified incubator at 37℃ at 5 % $CO_2$.

**Thawing** A cryovial containing one million HUVEC was taken out of the liquid nitrogen and quickly defrosted in a 37℃ waterbath. The still frozen cells were poured into 10 ml of complete growth medium. Cells were then centrifuged at 210 g for 10 min to get rid of the DMSO contained in the freezing medium. The cell pellet was resuspended in 20 ml complete growth medium and then seeded in a 10 cm dish. Usually the cells reached confluency after five days.

**Passaging** Cells were passaged when reaching confluency approximately after one week. The cells were washed with prewarmed PBS and subsequently trypsinized with T/E solution. Trypsinization was stopped with complete growth medium. Cells were then centrifuged at 210 g for 10 min and the resulting cell pellet was resusupended in complete growth medium. The concentration of the cell suspension was determined by counting in the cell viability analyzer ViCell®. Cells were seeded at a density of 0.2 x $10^6$ in a 10 cm dish and incubated for approximately one week until reaching confluency. HUVEC were used for experiments until reaching passage 6, since it was published before that they tend to loose their primary characteristics and responsiveness to various stimuli beyond this passage number [33].

### 2.2.3 Solutions and buffers for cell culture

| | | |
|---|---|---|
| Complete growth medium (EA.hy926) | DMEM with 4.5 g/L glucose | 500 ml |
| | L-glutamine | 2 mM |
| | penicillin | 100 U/ml |
| | streptomycin | 100 µg/ml |
| | heat-inactivated fetal bovine serum | 10 % |
| | HAT supplement: | |
| | hypoxanthine | 100 µM |
| | aminopterin | 0.4 µM |
| | thymidine | 16 µM |
| Complete growth medium (HUVEC) | EBM™ | 500 ml |
| | penicillin | 100 U/ml |
| | streptomycin | 100 µg/ml |
| | amphotericin B | 1 % |
| | heat-inactivated standardized fetal bovine serum | 10 % |
| | EGM™ SingleQuots™: | |
| | recombinant human epidermal growth factor | 0.5 ml |
| | hydrocortisone | 0.5 ml |
| | gentamicine sulfate & amphotericin B | 0.5 ml |
| | bovine brain extract | 0.4 % |
| PBS pH 7.4 | NaCl | 123 mM |
| | $Na_2HPO_4$ | 10 mM |
| | $KH_2PO_4$ | 3.2 mM |
| | $ddH_2O$ | ad 1000 ml |
| T/E solution | trypsin | 0.05% |
| | EDTA | 0.02 % |
| | in PBS | |
| 0.1% gelatine solution | gelatine | 0.1% |
| | in PBS | |

# 2.3 Quantification of endothelial NO release by 4,5-diaminofluorescein-2 (DAF-2)

## 2.3.1 Principle

Direct determination of endothelial NO release faces several difficulties. First of all the amount of NO released from endothelial cells is very low and additionally the half-life of NO is very short. Furthermore NO can rapidly be degraded by superoxide to form peroxynitrite. A very sensitive and specific method for determination of low amounts of NO was developed by Kojima et al. [179]. They generated fluorescence probes (diaminofluoresceins) which react with NO to the highly fluorescent triazolofluoresceins (DAF-Ts) by nitrosation and dehydration (Figure 2.1). The detection limit of this method is as low as 5 nM and therefore suitable for determination of NO produced from eNOS.

As NO can rapidly be degraded, eNOS enzyme activity does not necessarily correspond to the amount of bioavailable NO released from endothelial cells. Therefore it is important to determine endothelial NO release in addition to enzyme activity. In the course of this thesis the DAF-2 assay was optimized for 96-well plates and measurement in a plate reader.

In addition to DAF-2 cells were incubated with the calcium ionophore A23187. This allowed $Ca^{2+}$ ions to pass into the cells, thereby activating eNOS. To account for non-NO specific fluorescence the eNOS inhibitor L-NAME (L-$N^G$-nitroarginine methyl ester) was used. To normalize for seeding irregularities cell viability is measured using the resazurin conversion method.

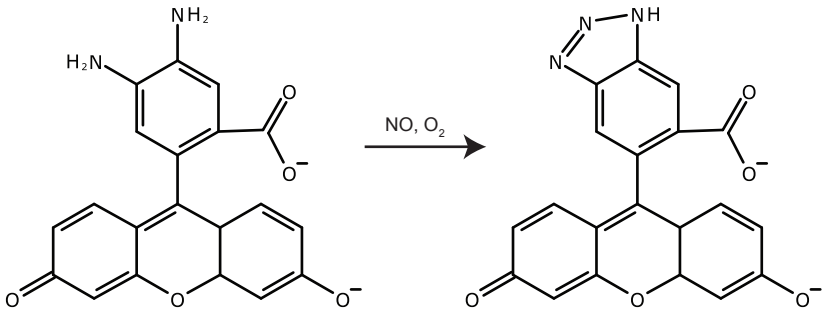


**Figure 2.1:** Reaction of DAF-2 to DAF-2T in the presence of $O_2$ and NO

### 2.3.2 Procedure

EA.hy926 cells were seeded in 96-well plates at a density of 2.5 x $10^4$ cells/well and were treated with test compounds at confluence after approximately 72 h. Cells were washed two times with $PBS^+$ containing 100 µM arginine and equilibrated 10 min in this buffer. Then the calcium ionophore A23187 was added to a final concentration of 1 µM and DAF-2 to a final concentration of 0.1 µM and the cells were incubated for 1 h at 37℃. Addition of L-NAME to a final concentration of 200 µM allowed correction for non-NO-specific fluorescence. The supernatant was transferred to a black 96-well plate and fluorescence was measured in a plate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence values were normalized to viable cells as determined by the resazurin conversion method [252]. For this, the cells were incubated with 0.1 mg/ml resazurin in PBS for 30 min before measuring the fluorescence at an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

### 2.3.3 Solutions and buffers

| | | |
|---|---|---|
| $PBS^+$ | NaCl | 137 mM |
| | KCl | 2.68 mM |
| | $Na_2HPO_4$ | 8.1 mM |
| | $KH_2PO_4$ | 1.47 mM |
| | $MgCl_2 \cdot 6\,H_2O$ | 0.5 mM |
| | $CaCl_2 \cdot 2\,H_2O$ | 0.68 mM |
| | $ddH_2O$ | ad 1000 ml |
| | sterile filtered | |

## 2.4 [$^{14}$C]L-arginine/[$^{14}$C]L-citrulline conversion assay

### 2.4.1 Principle

The eNOS enzyme catalyzes the reaction from L-arginine and molecular oxygen to L-citrulline and nitric oxide. As citrulline and NO are produced in equimolar

amounts by the enzyme, citrulline can be used as a surrogate marker for NO production. The principle of this assay is that radiolabeled [$^{14}$C]L-arginine is taken up by endothelial cells and converted to radiolabeled [$^{14}$C]L-citrulline by the eNOS enzyme. The ratio of this conversion over a given period of time can be used as an indirect measurement for eNOS enzyme activity. Metabolizing enzymes can however disturb this ratio [141; 237; 238].
With the addition of the calcium ionophor A23187 $Ca^{2+}$ ions from the buffer can pass through the cell membrane into the cells. This increase in $[Ca^{2+}]_i$ results in an increased activity of the eNOS enzyme and increased signal intensities. Differences in treatment groups can therefore be detected more reliably. After stopping the conversion reaction, cells are lysed and extracts are dried under vacuum. Amino acids are then extracted from the samples and separated with thin layer chromatography. Finally radioactivity can be detected by autoradiography.

### 2.4.2 Procedure

HUVEC or EA.hy926 cells were seeded in 6-well plates at a density of 0.1 x $10^6$ cells/well or 0.5 x $10^6$ cells/well, respectively, and treated with test compounds when reaching confluency approximately after 72 h. All further steps were performed in the same manner for both cell types.
Cells were washed and equilibrated in HEPES buffer for 10 min at 37°C. Then the cells were incubated with 10 µl 0.32 µM of [$^{14}$C]L-arginine solution (349 mCi/mmol) for 10 min before 1 µM of the calcium ionophore A23187 was added for exactly 15 min. The reaction was stopped by putting the plates on ice. The cells were now washed twice with ice-cold PBS and then lysed with 800 µl 96 % (v/v) ice-cold ethanol for 15 min while shaking on ice and 10 min while shaking at room temperature. 600 µl of the supernatant were transferred to a fresh reagent tube. 900 µl $H_2O$ were added to the cells and they were again incubated for 15 min at room temperature while shaking. Then the remaining supernatant was transferred to the reagent tubes. The cell extracts were then dried under vacuum in a SPD 1010 SpeedVac (Thermo Savant) and the resulting pellets were resolved in 35 µl water/methanol (1:1, v/v). Amino acids were extracted by alternating shaking and vortexing of the samples. The extracts were then centrifuged for 20 min at 867 g and 20 µl of the supernatant were applied to thin layer chromatography plates (Polygram SIL N-HR, Machery-Nagel). Separation of [$^{14}$C]L-arginine from [$^{14}$C]L-citrulline was

performed in the solvent system water:chloroform:methanol:ammonium hydroxide 25 % (2:1:9:4, v/v/v/v). The plates were analyzed by autoradiography in a phosphoimager (BAS-1800II, Fujifilm). AIDA software (raytest) was used for densitometric analysis. ENOS activity was determined as the percentage of [$^{14}$C]L-citrulline in relation to the total radioactivity recovered and normalized to the untreated control.

### 2.4.3 Solutions and buffers

| | | |
|---|---|---|
| HEPES buffer pH 7.4 | HEPES | 10 mM |
| | NaCl | 145 mM |
| | KCl | 5 mM |
| | $MgSO_4$ | 2 mM |
| | α- D(+)-glucose | 10 mM |
| | $CaCl_2 \cdot 2\,H_2O$ | 1.5 mM |
| [$^{14}$C]L-arginine solution | [$^{14}$C]L-arginine | 349 mCi/mmol |
| | 1:10 diluted in $ddH_2O$ | |

## 2.5 Determination of eNOS mRNA levels by quantitative real-time PCR

### 2.5.1 Principle

Analysis of mRNA levels in cells requires multiple steps. First of all RNA has to be isolated from cells, then the mRNA is converted to cDNA by reverse transcription, followed by quantitative real-time polymerase chain reaction (qRT-PCR) as a final step. The PCR reaction consists of several steps that are repeated in cycles. First the double-stranded DNA has to be denatured at 95℃, then primers are annealed at a lower temperature (dependent on primer sequence). Finally the DNA is elongated by a heat-stable DNA polymerase. The principle of PCR is that each cycle doubles the amount of DNA, thereby leading to an exponential increase in PCR products. Using quantitative real-time PCR it is possible to monitor the amplification process by measurement of the fluorescence of a DNA double-strand-specific dye. Analysis of the resulting fluorescence curves serves as a measure for the originally present mRNA levels in the sample. To control for the specificity and quality of the PCR reaction

a melting point analysis can be performed. Therefore the samples are heated up constantly until a drop in fluorescence occurs. This signals the denaturation of the DNA strands. The melting temperature is specific for a given DNA sequence and allows to check for the accuracy of the measurement.
As an internal control for normalization of RNA levels 18S ribosomal RNA (rRNA) was used. This RNA is suitable as it is expressed constantly and in the same extent as the target RNA.

## 2.5.2 Procedure

EA.hy926 cells were seeded at a density of 1.1 x $10^6$ cells per 6 cm dish and treated with test compounds when reaching confluency approximately after 72 h.

### 2.5.2.1 RNA extraction

For extraction of RNA from EA.hy926 cells the peqGOLD Total RNA Kit (peqlab) was used according to the manufacturer's instructions. EA.hy926 cells were lysed with 400 µl of RNA lysis buffer. DNA was removed with DNA removing columns. 70% ethanol was added to the lysates before vortexing vigourously. Then the lysates were applied to RNA binding columns and washed three times. Afterwards the columns were dried by centrifugation and the RNA was eluted with 30 µl RNAse free $H_2O$. To control the quality of the isolated RNA 9 µl of RNA were mixed with 1 µl loading buffer. This mixture was loaded on a 1 % agarose gel. The agarose gel was stained with SYBR® Green (Invitrogen) and as a running buffer 0.5 % TBE was used. Bands were visulized by UV transillumination at a gel documentation system (Biostep).

### 2.5.2.2 First-strand cDNA synthesis

For reverse transcription of the isolated RNA to complementary DNA (cDNA) the Superscript™ First-Strand Synthesis System from Invitrogen was used. 500 ng RNA were mixed with 200 ng random hexamers and 1 µl 10 mM dNTPmix (desoxyribonucleotides) and filled up with DEPC-treated water to a volume of 10 µl. This mixture was incubated at 65°C for 5 min to allow denaturation of the RNA and cooled on ice for at least 1 min. Meanwhile 2 µl of 10x RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT and 1 µl RNaseOUT ribonuclease inhibitor (40 U/µl) per sample were mixed. 9 µl of this reaction mixture were added to each sample and

incubated at room temperature for 2 min to allow annealing of the primers. Then 1 µl of Superscript™ II Reverse Transcriptase (50 U/µl) was added and the samples were left at room temperature for 10 min. The tubes were then transferred to 42℃ and incubated for 90 min. The reaction was terminated by inactivation of the enzyme at 70℃ for 15 min. 1 µl of RNase H was added to each tube and incubated for 20 min at 37℃ to break down the remaining RNA in the samples and thereby avoid interference with the following quantification step.

#### 2.5.2.3 Quantitative real-time PCR

For analysis of mRNA isolated from cells and converted to cDNA in the previous steps quantitative real-time polymerase chain reaction (qRT-PCR) was used. For this purpose the LightCycler® 480 real-time PCR system was used including the LightCycler® 480 instrument and the LightCycler® 480 SYBR Green I Master reagent.
cDNA was diluted 1:10 for the measurement of eNOS mRNA and 1:20 for the detection of 18S rRNA. 2 µl of every cDNA were mixed with 4 µl $H_2O$, 7.5 µl SYBR Green Mastermix, 0.75 µl of 15 µM primer solution for each primer. See table 2.2 for primer sequences. Every cDNA sample was measured in triplicate. The qRT-PCR was performed as stated in Table 2.1.

| **step** | **T[℃]** | **time** |
|---|---|---|
| Denaturation | 95 | 10 min |
| Cycling (50 cycles) | 95 | 5 sec |
| | 61 | 5 sec |
| | 72 | 15 sec |
| Melting | 95 | 5 sec |
| | 60 | 10 sec |
| | 97 | 0.5 sec |
| Cooling | 40 | ∞ |

**Table 2.1:** qRT-PCR conditions

| Gene | Direction | Sequence (5' to 3') |
|---|---|---|
| eNOS | Forward | CAC TGT GAT GGC CGA GCG AAG GTT G |
| | Reverse | GCT TAG GGA GAG CGA GCT GGT GTT |
| 18S | Forward | GAA TTG ACG GAA GGG CAC CAC CAG |
| | Reverse | GTG CAG CCC CGG ACA TCT AAG G |

**Table 2.2:** Primer sequences used for qRT-PCR

### 2.5.3 Solutions and buffers

| | | |
|---|---|---|
| TBE 10x pH 8.0 | Tris-base | 890 mM |
| | boric acid | 890 mM |
| | EDTA | 20 mM |
| | $ddH_2O$ | ad 1000 ml |
| 10x DNA loading buffer | bromphenol blue | 0.6 µM |
| | glycerol | 50 % |
| | $ddH_2O$ | ad 10 ml |

## 2.6 Protein detection by Western Blotting

### 2.6.1 Principle

Protein detection by Western Blotting requires several steps. First of all cells have to be lysed and proteins solubilized. For the solubilization of membrane proteins, such as eNOS, the usage of a strong detergent, like CHAPS, in the lysis buffer is advantageous. Additionally membranes can be ruptured by sonication or in an ultrasonic bath. Proteins are denatured by boiling and disulfide bonds are reduced with β-mercaptoethanol. In addition, the proteins are negatively charged with the detergent sodium dodecylsulfate (SDS), in a uniform mass-charge ratio, which allows separation of proteins by their molecular weight. Proteins are separated by discontinuous gel electrophoresis using a polyacrylamide gel. Afterwards they are transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes are then blocked with bovine serum albumin or milk to prevent unspecific binding of the antibody to the membrane. Then membranes are incubated with primary antibody solutions which are directed against the protein of interest. To visualize the interactions of this antibody with the membrane a secondary antibody is used. Secondary

antibodies bind the primary antibody and are usually conjugated to a reporter enzyme, like horeseradish peroxidase. When provided with a certain detection buffer antibodies coupled with horseradish peroxidase can oxidize the substrate luminol thus producing a luminescence signal which can be related to the amount of protein bound to the membrane.

## 2.6.2 Procedure

### 2.6.2.1 Whole cell protein lysates

EA.hy926 cells or HUVEC were seeded in 6-well plates at a density of 0.5 x $10^6$ cells/well or 0.1 x $10^6$ cells/well, respectively, and treated with test compounds when reaching confluency approximately after 72 h. For preparation of whole cell protein lysates cell layers were washed twice with ice-cold PBS and put on ice. Then 100 µl lysis buffer were added to each well before cells were scraped. The lysates were transferred to reagent tubes and incubated on ice for 15 min. Afterwards they were put into a ultrasonic bath for 30 sec followed by centrifugation at 4℃ for 40 min at 16,090 g.

5 µl of the supernatant were used for the determination of protein concentration by the Bradford method. EA.hy926 cell lysates were diluted 1:20 and HUVEC lysates 1:10 before samples were mixed 1:20 with Bradford reagent (1:2.7 dilution of Roti®-Quant). Protein concentration was measured at 595 nm at a plate reader after 5 min incubation on a shaker. Samples and BSA standards (2.5 - 25 µg/ml final BSA concentration) were measured in triplicate.

The remaining supernatant was mixed with 3x sample buffer and boiled for 5 min at 95℃ to denature the proteins. Samples were then frozen until loaded on a gel.

### 2.6.2.2 SDS-PAGE

The separation of proteins was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli. Gels were prepared with Rotiphorese® Gel 30 (containing 30 % acrylamid solution with 0.8 % bisacrylamid at the ratio 37.5 : 1) and a Mini-PROTEAN® 3 Cell System. The final concentration of polyacrylamide (PAA) in the resolving gel was 7.5 - 12 % dependent on the size of the target proteins. In every lane of the gel the same amount of protein was loaded. For EA.hy926 cells usually 30 µg of protein was loaded and for HUVEC 8

| Protein | kDa | Dilution | diluted in | Source | Company |
|---|---|---|---|---|---|
| eNOS | 140 kDa | 1:2500 | 1x TBS-T | mouse | BD |
| phospho-eNOS-Ser$^{1177}$ | 140 kDa | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| phospho-eNOS-Thr$^{495}$ | 140 kDa | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| AMPK | 62 kDa | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| phospho-AMPK-Thr$^{172}$ | 62 kDa | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| Akt | 60 kDa | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| phospho-Akt-Ser$^{473}$ | 60 kDa | 1:1000 | 5% BSA in 1x TBS-T | rabbit | Cell Signaling |
| HA-Tag | - | 1:1000 | 5 % BSA in 1x TBS-T | rabbit | Cell Signaling |
| iNOS | 130 kDa | 1:1000 | 2 % milk in 1x TBS-T | mouse | R&D |
| nNOS | 160 kDa | 1:1000 | 5 % milk in 1x TBS-T | rabbit | Cell Signaling |
| tubulin | 55 kDa | 1:1000 | 1x TBS-T | mouse | Santa Cruz |
| actin | 42 kDa | 1:1000 | 1x TBS-T | mouse | MPBio |
| rabbit IgG | - | 1:1000 | 5 % milk in 1x TBS-T | goat | Cell Signaling |
| mouse IgG | - | 1.6:1000 | 1x TBS-T | goat | MPBio |

**Table 2.3:** Antibodies used for Western Blotting

- 20 µg, dependent on the available amount. Proteins were seperated at 25 mA per gel for approximately 80 min.

#### 2.6.2.3 Western Blotting

Proteins were transferred to a PVDF membrane with the Mini Trans-Blot® Electrophoretic Transfer Cell System at 100 V for 90 min. Afterwards the membrane

was blocked with 5 % fat-free milk powder in 1x TBS-T for 2 hours and washed three times with 1x TBS-T for 10 min.

#### 2.6.2.4 Protein detection

Primary antibodies were diluted as listed in Table 2.3. Membranes were incubated with primary antibody solutions over night at 4℃. Then the membranes were washed three times with 1x TBS-T for 10 min before incubation for 2 h with the respective horseradish peroxidase conjugated secondary antibody solution at room temperature. The membranes were then again washed three times with 1x TBS-T for 10 min and incubated in 10 ml homemade ECL-solution for 1 min. Protein bands were visualized in the luminescent image analyzer LAS-3000 and quantified by densitometric analysis with the AIDA software (raytest).

### 2.6.3 Solutions and buffers

| **solutions and buffers** | | |
|---|---|---|
| lysis buffer Stock pH 7.5 | Tris-HCl | 50 mM |
| | CHAPS | 20 mM |
| | EDTA | 0.5 mM |
| | EGTA | 0.5 mM |
| | DTT | 2 mM |
| | glutathione | 7 mM |
| | glycerol | 10 % |
| | $ddH_2O$ | ad 25 ml |
| phosphatase inhibitor stock 20x | NaF | 0.2 M |
| | $Na_3VO_4$ | 40 mM |
| | $Na_4P_2O_7 * 10\,H_2O$ | 0.1 M |
| | $ddH_2O$ | ad 5 ml |
| lysis buffer (prepared freshly before use) | lysis buffer stock | 637 µl |

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| **solutions and buffers** | | |
|---|---|---|
| | complete™ protein inhibitor 25x | 28 µl |
| | phosphatase inhibitor stock 20x | 35 µl |
| Western blot sample buffer 3x | Tris-HCl pH 6.8 | 187.5 mM |
| | SDS | 0.2 M |
| | glycerol | 30 % |
| | bromphenol blue | 0.2 mM |
| | $ddH_2O$ | ad 1000 ml |
| | β-mercaptoethanol (added shortly before use) | 15 % |
| resolving gel | PAA (Rotiphorese® Gel 30 - 30 % PAA) | 7.5 - 12 % |
| | Tris-HCl pH 8.8 | 375 mM |
| | SDS | 0.1 % |
| | TEMED | 0.1 % |
| | APS | 0.05 % |
| | $ddH_2O$ | ad 7.5 ml |
| stacking gel | PAA (Rotiphorese® Gel 30 - 30 % PAA) | 5 % |
| | Tris-HCl pH 6.8 | 125 mM |
| | SDS | 0.1 % |
| | TEMED | 0.2 % |
| | APS | 0.1 % |
| | $ddH_2O$ | ad 3.75 ml |
| SDS-Running buffer 10x | Tris-base | 248 mM |
| | glycine | 1.9 M |
| | SDS | 35 mM |
| | $ddH_2O$ | ad 1000 ml |

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| **solutions and buffers** | | |
|---|---|---|
| Blotting buffer 5x | Tris-base | 125 mM |
| | glycine | 971 mM |
| | $ddH_2O$ | ad 1000 ml |
| Blotting buffer 1x | Blotting buffer 5x | 200 ml |
| | methanol | 200 ml |
| | $ddH_2O$ | ad 1000 ml |
| TBS-T pH 8.0 10x | Tris-base | 248 mM |
| | NaCl | 1.9 M |
| | Tween-20 | 1 % |
| | $ddH_2O$ | ad 1000 ml |
| homemade ECL-solution | Tris-base pH 8.5 | 100 mM |
| | luminol | 1.24 mM |
| | p-coumaric acid | 0.2 mM |
| | $H_2O_2$ | 0.018 % |
| | $ddH_2O$ | ad 10 ml |

## 2.7 Transient transfection of cells

### 2.7.1 Principle

Introduction of nucleic acids into mammalian cells by transient transfection is an important technique not only for cell signaling studies. In this way small interfering RNAs (siRNA), targeting a gene for knockdown, or DNA plasmids carrying a gene for overexpression studies or a mutated gene isoform, can be transfected into cells. Several different approaches for transfection are available, using chemicals or physical methods or even viruses. For the transfection of plasmids into HUVEC we used a lipid-based transfection system from Promega (FuGENE® HD). Most often proteins from expression vectors are tagged, for instance with a HA-tag (human influenza hemagglutinin tag) for easier detection with antibodies or with a poly(His) tag used for affinity purification. Protein tags can have other purposes as well, like helping with the correct folding of the protein or adding a fluorescence epitope.
For the knockdown of a target protein siRNA transfection into HUVEC was performed using the Oligofectamine transfection system from Invitrogen. SiRNAs can

bind their complementary mRNAs in cell, thereby leading to the degeneration of the target mRNA.

### 2.7.2 Overexpression of the catalytic subunit of protein phosphatase 2A (PP2Ac)

Isolation of the plasmids was performed using the PureYield™ Plasmid Midiprep System according to the manufacturer's instructions. 5 ml bacterial pre-cultures were prepared and after 7 h used to inoculate 200 ml LB medium for overnight growth. These overnight cultures were pelleted at 8,068 g for 10 min and resuspended in 6 ml Resuspension Solution. Then 6 ml lysis solution were added. These mixtures were inverted several times and incubated for 3 min at room temperature before 10 ml Neutralization Solution was added. Again the tubes were inverted and left at room temperature for 3 min. The lysates were then centrifuged for 15 min at 20,500 g. Meanwhile column stacks were assembled by nesting a PureYield Clearing Column into the top of a PureYield Binding Column. These column stacks were placed onto the vacuum manifold. After centrifugation the lysates were poured into the PureYield Clearing Column and incubated for 3 min. Then vacuum was applied until all liquid has passed through both columns. The columns were washed with 5 ml Endotoxin Removal Wash and 20 ml of Column Wash Solution. Then the membranes were dried by applying vacuum for 30 sec. The Binding Columns were removed and placed on the Eluator device. The DNA was eluted with 1 ml of nuclease free water and the concentration of DNA was measured spectrophotometrically. The sequence of the DNA was checked by restriction analysis.

HUVEC were seeded in 6-well plates at a density of 0.5 x $10^6$ cells/well and transfected 1 day later with 1 µg of an expression vector for the catalytic subunit of protein phosphatase 2A (pCMV-HA-PP2Ac), kindly provided by Dr. Verin (Medical College of Georgia, Atlanta, GA, USA)[322] or empty control vector (pCMV) using the FuGENE® HD transfection reagent according to the manufacturer's instructions. For one well 100 µl Opti-MEM® were mixed with 2 µg vector and 4 µl FuGENE® HD transfection reagent. This mixture was left on room temperature for 15 min. Meanwhile cells were washed with PBS and 1.5 ml Opti-MEM® were added to every well. Then the FuGENE®HD / DNA mixture was added to the wells. After four hours cells were washed with PBS and 2 ml of complete growth medium were added

per well. One day later cells were treated with test compounds and further used for experiments. Successfull overexpression was confirmed by Western Blot analysis.

### 2.7.3 siRNA-mediated knockdown of AMPKα

HUVEC were seeded in 6-well plates at a density of 0.3 x $10^6$ cells/well and transfected 1 day later with 33 pmol AMPKα siRNA or scrambled control (Stealth RNAi negative control, Medium GC control from Invitrogen) using the Oligofectamine™ transfection reagent. First 10 µl 20 µM siRNA solution were mixed with 155 µl Opti-MEM® and separatly 8 µl Oligofectamine transfection reagent and 17 µl Opti-MEM® were mixed. Both tubes were left at room temperature for 10 min. Then the contents of the tubes were mixed and again incubated for 15 min at room temperature. Meanwhile cells were washed with PBS and 800 µl Opti-MEM® per well were added. The Oligofectamine™/siRNA mix was added to the wells and incubated for 5 h. Then 1 ml complete growth medium was added. This time point was taken as 0 h after transfection. At the next day the medium was changed. 72 h after transfection cells were used for experiments. Successfull knockdown of AMPKα was confirmed by Western Blot analysis.

## 2.8 Ascorbate uptake assay

### 2.8.1 Principle

Determination of ascorbate uptake into endothelial cells is relatively easy to perform. Cells are incubated for a particular period of time with radiolabeled ascorbate and then the radioactivity that enters the cells is measured in a liquid scintillation counter. Ascorbate is taken up into endothelial cells mainly by the SVCT2 transporter (Sodium-dependent Vitamin C Transporter). However when ascorbate is oxidized to dehydroascobate (DHA) it can be transported by glucose transporters and is reduced to ascobate inside the cells. Addition of reduced glutathione to the incubation buffer keeps ascorbate in its reduced state and addition of glucose serves as a competitor to DHA for the glucose transporter and acts as an additional mechanism to prevent the influence of DHA on the ascorbate uptake measured.

### 2.8.2 Procedure

EA.hy926 cells or HUVEC were seeded in 12-well plates at a density of 0.16 x $10^6$ cells/well or 0.08 x $10^6$ cell/well, respectively, and were treated with test compounds when reaching confluency approximately after 72 h. Cells were washed twice with KRH buffer and incubated for the indicated time points at 37°C with KRH buffer containing 5 mM D-glucose, 0.5 mM glutathione, and 100 µM [1-$^{14}$C]L-ascorbic acid. The supernatant was aspirated and the cell layer was washed twice with ice-cold KRH buffer before the cells were treated for 30 min with 0.5 ml 0.05 N NaOH in PBS. 350 µl of the cell lysate was added to 5 ml Ultima Gold liquid scintillation fluid. The radioactivity of duplicate samples was measured in a Packard TRI-CARB 2100TR liquid scintillation analyzer after at least 1 h, to allow decay of chemiluminescence. Results were normalized to protein content of the cells as determined by the Bradford method like described in section 2.6.2.1.

### 2.8.3 Solutions and buffers

| | | |
|---|---|---|
| KRH buffer | HEPES | 20 mM |
| (Krebs-Ringer-HEPES) | NaCl | 128 mM |
| pH 7.4 | KCl | 5.2 mM |
| | $NaH_2PO_4$ | 1 mM |
| | $MgSO_4$ | 1.4 mM |
| | $CaCl_2$ | 1.4 mM |

## 2.9 Determination of intracellular $Ca^{2+}$ concentration

### 2.9.1 Principle

An easy method to determine intracellular $Ca^{2+}$ levels uses cell permeable fluorescent indicators, e.g. Fluo-3-AM. These indicators can cross the cell membrane due to their ester structure. Esterases inside the cell then hydrolyze the ester to yield the $Ca^{2+}$ indicator. Upon binding of $Ca^{2+}$ an increase in fluorescence can be measured in a flow cytometer.

### 2.9.2 Procedure

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined with the intracellular calcium indicator Fluo-3-AM. Therefore EA.hy926 cells were seeded at a density of 0.5 x $10^6$ cells/well in a 6-well plate and treated with test compounds when reaching confluency approximately after 72 h. Cells were washed once with PBS and trypsinzed with 600 µl T/E solution per well. The trypsinization was stopped after approximately 5 min with 1.2 ml Stop-Medium (PBS with 5 % heat-inactivated fetal bovine serum). The cells were transferred to FACS vials and centrifuged at 130 g for 5 min. Then the cells were washed once with PBS and resuspended in 500 µl 2 µM Fluo-3-AM solution in PBS. The vials were incubated at 37°C for 45 min protected from light. Afterwards cells were pelleted and washed twice with PBS. As a last step the cell pellets were resuspended in 500 µl PBS and read immediately in the flow cytometer (FL-1 channel; excitation wavelength: 495 nm, emission wavelength: 519 nm).

### 2.9.3 Solutions and buffers

| | | |
|---|---|---|
| Flow cytometer buffer pH 7.37 | NaCl | 0.14 M |
| | $KH_2PO_4$ | 1.9 mM |
| | $Na_2HPO_4$ | 17 mM |
| | KCl | 3.8 mM |
| | LiCl | 10 mM |
| | $NaN_3$ | 3 mM |
| | $Na_2EDTA$ | 1 mM |
| | $ddH_2O$ | ad 1000 ml |

## 2.10 Determination of Cell Viability

### 2.10.1 Principle

Cell viability assays are widely used for the determination of cytotoxic effects in cells treated with a given substance. Several different systems are used including

cytolysis or membrane leakage assays and assays testing for mitochondrial or caspase activity. In this work two different assays were used. The LDH (lactate dehydrogenase) release assay tests for LDH that is released into the culture medium when cells lose their membrane integrity either due to apoptosis or necrosis. LDH activity can be measured by a colorimetric reaction with a provided substrate.
The resazurin conversion assay measures the mitochondrial activity of cells. When cells are healthy they can convert resazurin to resorufin. The fluorescent resorufin can then be measured and taken as indicator of cell viability.

### 2.10.2 LDH release assay

For determination of LDH release the CytoTox96® Non-Radioactive Cytotoxicity Assay from Promega was used. Therefore, EA.hy926 cells were seeded in 96-well plates at a density of 2.5 x $10^4$ cells/well and treated with test compounds at confluence after approximately 72 h. For maximal LDH release, cells were lysed with 10 µl of lysis solution (10x) per 100 µl medium for 45 min prior to harvesting supernatants. Then the plate was centrifuged for 5 min at 250 g. 50 µl of the cell supernatants were transferred to a new plate and 50 µl reconstituted substrate mix were added per well. Then absorbance was measured at 490 nm in 5 min cycles to assure that the values are not already saturated.

### 2.10.3 Resazurin conversion assay

EA.hy926 cells were seeded in 96-well plates at a density of 2.5 x $10^4$ cells/well and treated with test compounds at confluence after approximately 72 h. The medium of the cells was changed to 0.1 mg/ml resazurin in PBS and incubated for 30 min at 37°C. Then fluorescence was measured at an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

## 2.11 Determination of BH4 levels

BH4 levels were determined in collaboration with Prof. Ernst Werner (Medical University of Innsbruck, Austria). Levels were quantified by HPLC after oxidation with iodine in acid and base as described [144], by methods modified from Fukushima

and Nixon [110]. In short, cells were homogenized with an microhomogenizer in dd $H_2O$ with 5 mM DTT and centrifuged at 13,000 g for 10 min at 4 ℃. To 100 µl supernatant, 20 µl of a 1:1 (v/v) mixture of 0.1 M HCl and iodine (0.1 M in 0.25 M KI) or 0.1 M NaOH and iodine (0.1 M in 0.25 M KI) was added and incubated for 60 min in the dark. 20 µl 0.1 M HCl were then added to the alkaline solution and insoluble material removed from both incubations by centrifugation for 5 min at 13,000 g. Then 20 µl freshly prepared ascorbic acid (100 µM in dd$H_2O$) were added to both incubations and the mixtures were analyzed on an Agilent 1200 HPLC System. 20 µl of the final mixture were injected onto a Nucleosil 10 SA column isocratically eluted with 100 mM potassium phophate buffer, pH 3.0, at a flow rate of 1.5 ml/min at 35 ℃. Biopterin was detected by fluorescence with an excitation wavelength of 350 nm and an emission wavelength of 440 nm an a detection limit of 1 nmol/L. The amount of tetrahydrobioperin was calculated from the difference between the oxidation in acid and base, respectively. Values were normalized to the protein content of extracts determined by the Bradford method.

## 2.12 Determination of extracellular hydrogen peroxide ($H_2O_2$) levels

### 2.12.1 Principle

In the presence of peroxidase, the Amplex® red reagent reacts with $H_2O_2$ in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. The fluorescence of resorufin can easily be measured in a plate reader.

### 2.12.2 Procedure

Extracellular $H_2O_2$ levels were determined with the Amplex® red assay according to the manufacturer's instructions. EA.hy926 cells were seeded in 12-well plates at a density of 0.16 x $10^6$ cells/well until reaching confluence after approximately 72 h. Cells were washed twice with prewarmed PBS. Then 300 µl complete Amplex® red reagent were added and cells were equilibrated for 5 min in an incubator. Cells were then stimulated as indicated and incubated for 60 min. Addition of 150 U Catalase allowed correction for non-catalase-blockable fluorescence. 80 µl of every treatment (duplicates) were transferred to a 96-well plate and fluorescence was measured with

an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

### 2.12.3 Solutions and buffers

| | | |
|---|---|---|
| KRPG buffer (Krebs-Ringer-Phosphate-Glucose) pH 7.35 | NaCl | 145 mM |
| | $Na_2HPO_4$ | 57 mM |
| | KCl | 4.86 mM |
| | $CaCl_2$x2 $H_2O$ | 0.54 mM |
| | $MgSO_4$x7 $H_2O$ | 1.22 mM |
| | glucose | 5.5 mM |
| | $ddH_2O$ | ad 500 ml |
| complete amplex red reagent | horse radish peroxidase | 0.2 U/ml |
| | amplex red reagent | 1 mM |
| | KRPG buffer | ad 3.75 ml |

## 2.13 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 4.03. One-way or two-way ANOVA was used for comparison of different treatment groups and Student's $t$ test for comparison of two groups. $p$ values $< 0.05$ were considered significant. In figures with bar graphs, these show means ± SEM of at least three independent experiments unless stated otherwise.

# 3 Results and discussion

## 3.1 Ascorbate stimulates endothelial nitric oxide synthase enzyme activity by rapid modulation of its phosphorylation status

### 3.1.1 Results

#### 3.1.1.1 Rapid increase in eNOS activity by ascorbate is independent of chemical stabilization of BH4

Previous studies have shown that ascorbate stabilizes the eNOS cofactor BH4 in endothelial cells, thereby enhancing eNOS enzyme activity [144; 153]. To confirm these results we performed time-course experiments in EA.hy926 cells and HUVEC. Cells were treated with 100 µM ascorbate for 30 min to 24 h. In both cell types ascorbate treatment led to a time-dependent gradual increase in eNOS enzyme activity (Figure 3.1). Already 0.5 h after treatment an increase was detectable and got significant after 2 h in EA.hy926 cells and after 4 h in HUVEC.

The administration of increasing concentrations of ascorbate showed that already 5 µM were enough for a significant increase in eNOS enzyme activity. Concentrations from 10 to 100 µM saturated the activating effect of ascorbate (Figure 3.2).

As mentioned before, previous studies showed that ascorbate is able to stabilize the cofactor BH4 after 24 h [144; 153]. To elucidate whether BH4 stabilization upon ascorbate treatment is also a gradual effect, BH4 levels were measured in collaboration with Prof. Ernst Werner (Medical University of Innsbruck, Austria). Ascorbate was able to increase BH4 levels after 24 h in EA.hy926 cells, however shorter incubation periods showed no influence (Figure 3.3). Total biopterin levels did not change.

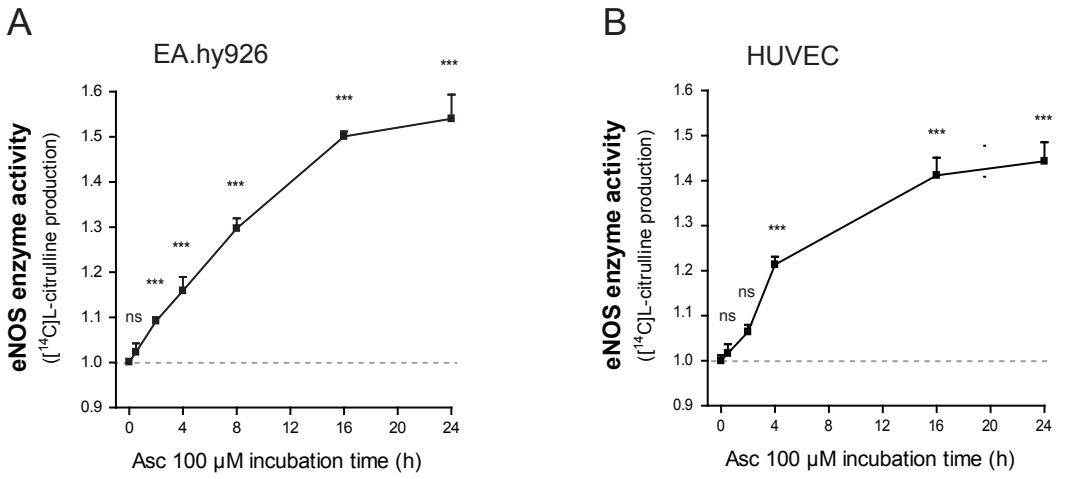


**Figure 3.1:** Ascorbate increases eNOS enzyme activity time-dependently. (A) EA.hy926 cells or (B) HUVEC were treated with 100 µM ascorbate (Asc) for 0.5 to 24 h. Then eNOS enzyme activity was determined. [$^{14}$C]L-citrulline production was normalized to the untreated control (***, $p < 0.001$; ns, not significant; mean ± SEM, n = 3). Figure A by Christoph A. Schmitt [290]. Figure B obtained with the technical assistance of Daniel Schachner.

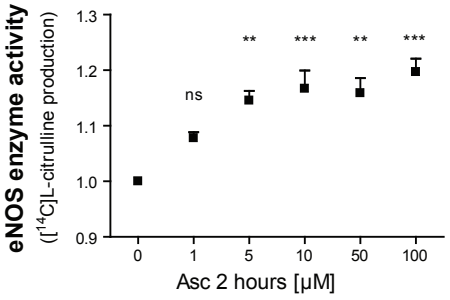


**Figure 3.2:** Concentration-dependent effect of ascorbate on eNOS enzyme activity. EA.hy926 cells were treated for 2 h with 0 to 100 µM ascorbate (Asc) and eNOS activity was determined (***, $p < 0.001$; **, $p < 0.01$; ns, not significant; mean ± SEM, n = 3). Figure obtained with the technical assistance of Daniel Schachner.

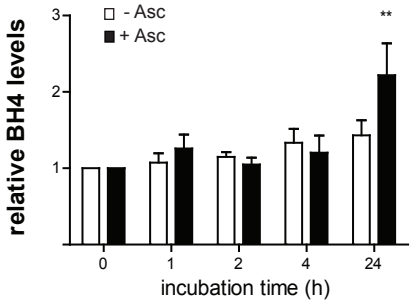


**Figure 3.3:** Time-dependent effect of ascorbate on endothelial BH4 levels. EA.hy926 cells were treated with 100 µM ascorbate (Asc) for 0 to 24 h. Intracellular BH4 levels were quantified by HPLC after oxidation with iodine in acid and base. The values obtained (pmolBH4/µg cellular protein) were normalized to the cellular BH4 level at time point 0 (**, $p < 0.01$; mean ± SEM, n = 3).

Upon supplementation of the BH4 precursor sepiapterin ascorbate was still able to increase eNOS-Ser$^{1177}$ phosphorylation (Figure 3.4), leading to the conclusion that BH4 stabilization had no influence in the rapid effect of ascorbate on eNOS enzyme activity and phosphorylation changes (see next chapter).

#### 3.1.1.2 Rapid activation of eNOS by ascorbate is associated with changes in eNOS phosphorylation

Changes in eNOS enzyme activity are usually accompanied by changes in the eNOS phosphorylation pattern. An increased phosphorylation at Ser$^{1177}$ and a decreased phosphorylation of Thr$^{495}$ are indicating an increase in eNOS enzyme activity and seem to be coordinated together [239]. The Ser$^{1177}$ residue is located in the reductase domain close to the carboxy-terminus of the enzyme and phosphorylation at this site increases the electron flux in the reductase domain thereby resolving an autoinhibitory loop and activating eNOS. Phosphorylation of the Thr$^{495}$ in the $Ca^{2+}$/CaM binding domain interferes with binding of $Ca^{2+}$/CaM to eNOS.
To confirm if the results from figure 3.1 are correlated to an activating phosphorylation pattern of eNOS, EA.hy926 cells and HUVEC were treated with 100 µM ascorbate and the eNOS phosphorylation status was determined after 0 to 60 min incubation. In both cell types ascorbate elicited the same activating pattern. Already after 5 min a significant reduction in eNOS-Thr$^{495}$ phosphorylation was detectable

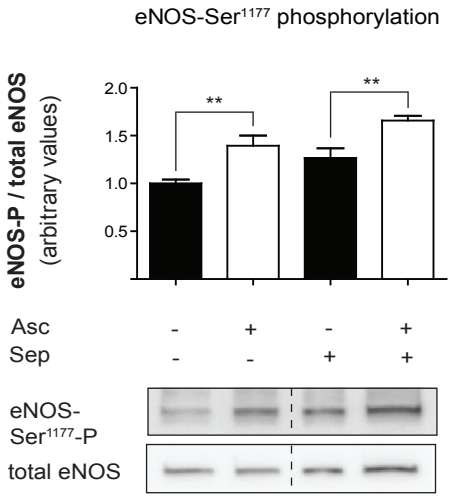


**Figure 3.4:** Supplementation of sepiapterin does not abolish the effect of ascorbate on eNOS phosphorylation. HUVEC were pretreated with 10 µM sepiapterin (Sep) for 30 min and then incubated with 100 µM ascorbate (Asc) for 60 min as indicated. (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown. The dashed line indicates that interjacent lanes on the same membrane were cut out (**, $p < 0.01$; mean ± SEM, n = 3).

and after 30 min the increase in eNOS-Ser$^{1177}$ phosphorylation was significant (Figure 3.5).

Furthermore, ascorbate elicited a dose-dependent change in eNOS phosphorylation in EA.hy926 cells when treated with concentrations of ascorbate, ranging from 0 to 100 µM (Figure 3.6).

In comparison with already well-known activators of eNOS, changes induced by treatment with ascorbate are in the same range (Figure 3.7).

As already mentioned in the introduction, plasma levels of ascorbate are in the range of 50 µM in humans [353]. Therefore the question arose whether eNOS is chronically stimulated at a basal level under physiological conditions. We compared the response of scorbutic (normal, not Asc supplemented) and non-scorbutic (supplemented with 50 µM Asc) endothelial cells to ascorbate treatment. The basal activation of eNOS, as determined by eNOS enzyme activity and eNOS-Ser$^{1177}$ phosphorylation, was increased in the Asc-supplemented cells compared to the non-supplemented cells. However, additional exogenous administration of ascorbic acid was able to further elevate these parameters of eNOS activity (Figure 3.8). Thus

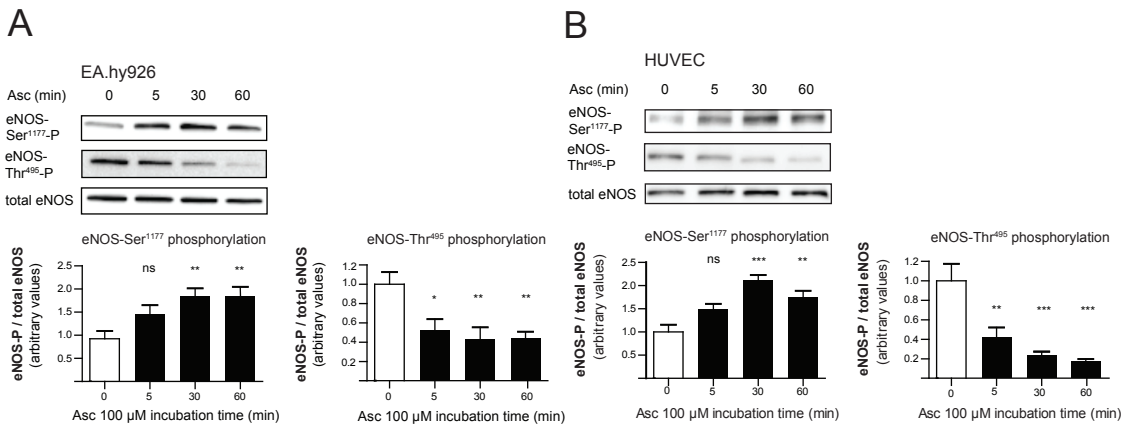


**Figure 3.5:** Ascorbate rapidly changes the eNOS phosphorylation pattern. (A) EA.hy926 cells and (B) HUVEC were treated with 100 µM ascorbate (Asc) for 5 to 60 min. (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities are normalized to tubulin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; mean ± SEM, n = 5 for (A) and n = 3 for (B)). Experiments for Figure A and B were in part performed by Christoph A. Schmitt [290].

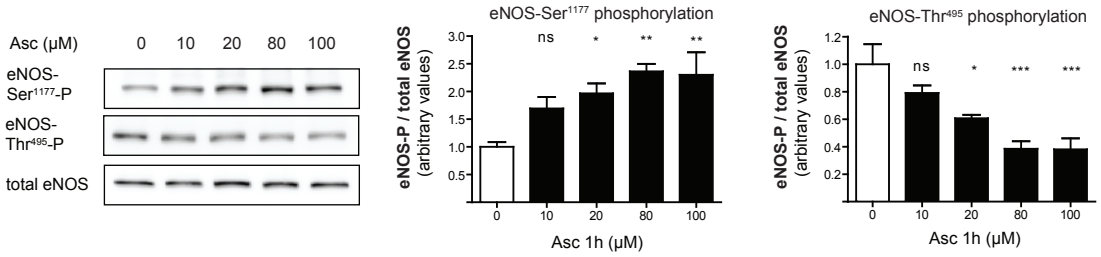


**Figure 3.6:** Ascorbate dose-dependently changes eNOS-Ser$^{1177}$ and eNOS-Thr$^{495}$ phosphorylation. EA.hy926 cells were treated for 1 h with 0 to 100 µM ascorbate (Asc). (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; mean ± SEM, n = 4)

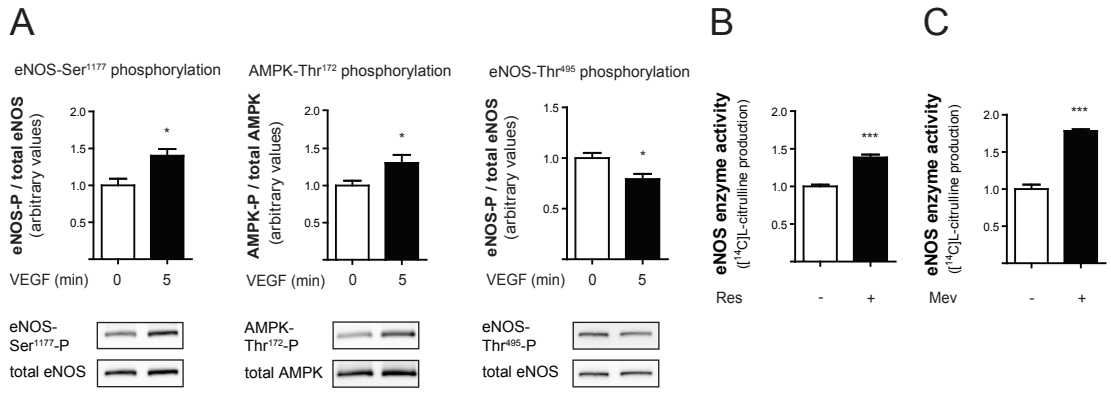


**Figure 3.7:** ENOS activation by known activators. (A) EA.hy926 cells were treated with 50 ng/ml VEGF for 5 min. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; mean ± SEM, n = 3). EA.hy926 cells were treated with (B) 30 µM resveratrol (Res) or (C) 3 µM mevastatin (Mev). Then eNOS enzyme activity was determined (***, $p < 0.001$; mean ± SEM, n = 3 for (B) and n = 5 for (C)).

treatment with ascorbate still has an effect in non-scorbutic cells and not only in ascorbate-deficient cells.

#### 3.1.1.3 Ascorbate has no influence on cellular $H_2O_2$ levels or Akt activity

It was shown previously that ascorbate can promote $H_2O_2$ formation in cell culture medium [123; 132] and interstitial fluids after infusion [55]. In endothelial cells $H_2O_2$ in µM concentration can activate the phosphatidylinositol-3-kinase (PI3K) that in turn activates Akt and eNOS further downstream [324]. These two findings led us to the hypothesis that ascorbate may trigger an elevation of extracellular $H_2O_2$ concentration and thereby an increase in eNOS enzyme activity. However, the pretreatment of endothelial cells with catalase, an enzyme necessary for the decomposition of $H_2O_2$ to $H_2O$ and oxygen, did not alter the effect of ascorbate on eNOS enzyme activity (Figure 3.9A).

Determination of extracellular $H_2O_2$ levels showed that ascorbate produced less than 0.25 µM $H_2O_2$ and therefore $H_2O_2$ seems to be very unlikely to play a role in the rapid ascorbate effect (Figure 3.9B). Moreover, inhibition of PI3K by wortmannin had no effect on the enhanced eNOS-Ser$^{1177}$ phosphorylation after ascorbate treatment (Figure 3.9C) and ascorbate treatment did not elicit an activation of Akt kinase (Figure 3.9D). Altogether these data suggested that neither $H_2O_2$ nor the

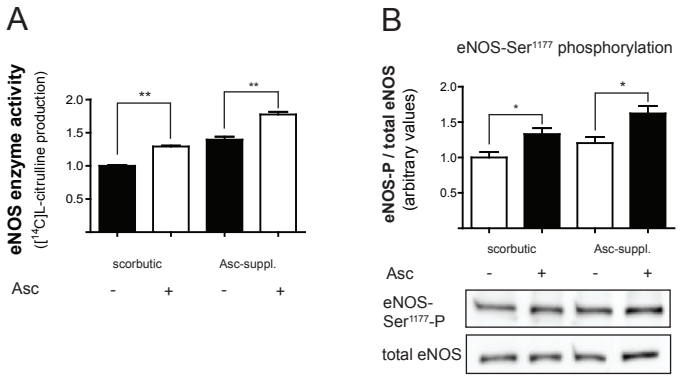


**Figure 3.8:** Exogenous addition of ascorbic acid elevates eNOS enzyme activity and eNOS-Ser$^{1177}$ phosphorylation in both scorbutic and non scorbutic cells. Endothelial cells were incubated in normal (not Asc supplemented) medium (scorbutic) or medium supplemented with 50 µM ascorbate (Asc-supplemented) at least for 7 days. Then 100 µM ascorbate (Asc) were added (on top) for 4 h for (A) and for 1 h for (B) and (A) eNOS activity or (B) eNOS phosphorylation was determined (*, $p < 0.05$; **, $p < 0.01$; mean ± SEM, n = 4 for (A) and n = 2 for (B)). Figure A obtained with the technical assistance of Daniel Schachner.

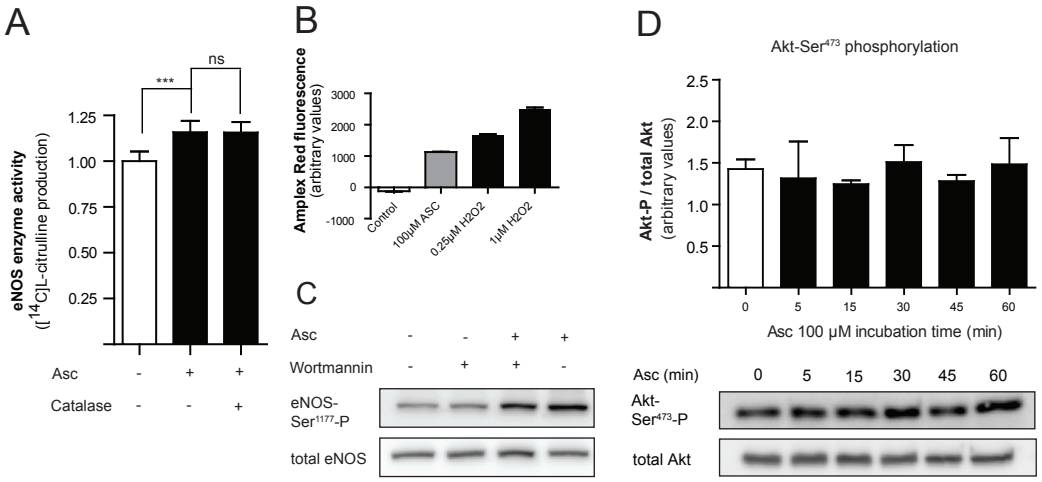


**Figure 3.9:** Ascorbate has no influence on extracellular $H_2O_2$. (A) EA.hy926 cells were pretreated with 300 U catalase for 35 min and then incubated with 100 µM ascorbate (Asc) for 4 h. Then eNOS enzyme activity was determined (***, $p < 0.001$; ns, not significant; mean ± SEM, n = 4). (B) EA.hy926 cells were treated with 100 µM ascorbate (Asc), 0.25 µM or 1 µM $H_2O_2$ as indicated. For determination of extracellular $H_2O_2$ levels an Amplex® red assay was performed. Supernatants of untreated cells were used as controls (mean ± SEM, n = 1). (C) EA.hy926 were pretreated with 0.1 µM wortmannin for 30 min and then incubated with 100 µM ascorbate (Asc) for 30 min. (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (n = 3). (D) EA.hy926 cells were treated with 100 µM ascobate (Asc) for 5 to 60 min. (Phospho-) Akt protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (mean ± SEM, n = 3). Figure C by Christoph A. Schmitt [290]. Figure A and B obtained with the technical assistance of Daniel Schachner.

PI3K pathway is involved in the rapid effect of ascorbate on eNOS enzyme activity.

#### 3.1.1.4 Modulation of eNOS phosphorylation is dependent on cellular uptake of ascorbate

To elucidate whether an intracellular rise in ascorbate is necessary for the change in eNOS-Ser$^{1177}$ upon ascorbate treatment, we determined ascorbate uptake in endothelial cells. Endothelial cells were able to take up ascorbate very rapidly with a linear increase over time. Already after 5 min $\sim$ 500 pmol/mg protein ascorbate were taken up and after 1 h $\sim$ 4 nmol/mg protein were measured. There was no difference detectable between EA.hy926 cells and HUVEC (Figure 3.10A and 3.10B).

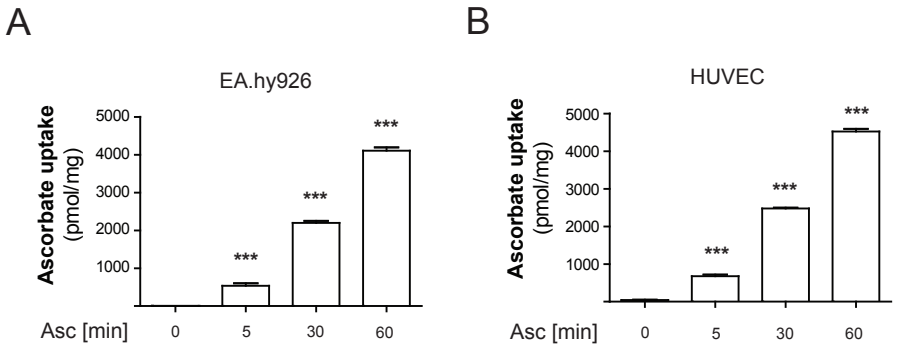


**Figure 3.10:** Intracellular ascorbate uptake into endothelial cells. (A) EA.hy926 cells or (B) HUVEC were treated with 100 µM [1-$^{14}$C]L-ascorbic acid (Asc) for 5 min to 1 h. Then ascorbate uptake was performed as described. The measured radioactivity was normalized to cellular protein content (***, $p < 0.001$; mean ± SEM, n = 4).

Additionally if endothelial cells were pretreated with phloretin, a compound used to block the sodium-dependent vitamin C transporter (SVCT2; SLC23A2) [222; 338] the uptake of ascorbate was reduced by 59 % (Figure 3.11A). Moreover, phloretin abolished the effect of ascorbate on eNOS-Ser$^{1177}$ phosphorylation (Figure 3.11B).

The application of ouabain, another compound used to block ascorbate uptake, instead of phloretin had similar results (Figure 3.11A and 3.11B). Altogether these data suggests that ascorbate is rapidly taken up by endothelial cells and subsequently increased eNOS-Ser$^{1177}$ phosphorylation.

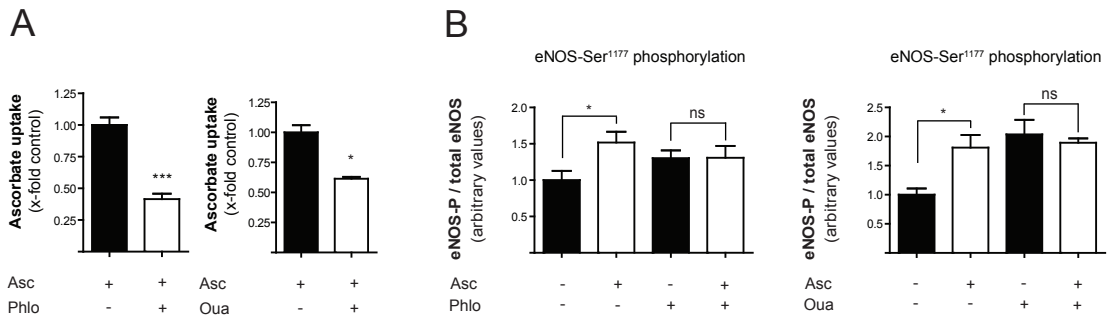


**Figure 3.11:** Ascorbate uptake is necessary for the modulation of eNOS phosphorylation. (A) EA.hy926 cells were pretreated with 10 µM phloretin or 10 µM ouabain for 30 min and then incubated with 100 µM [1-$^{14}$C]L-ascorbic acid (Asc). Ascorbate uptake was determined as described. The measured radioactivity was normalized to cellular protein content (*, $p$ 0.05; ***, $p < 0.001$; mean ± SEM, n = 3). (B) EA.hy926 cells were pretreated with 10 µM phloretin or 10 µM ouabain for 30 min and then incubated with 100 µM ascorbate (Asc) for 1 h. (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3).

#### 3.1.1.5 Ascorbate promotes phosphorylation of eNOS-Ser$^{1177}$ via activation of AMPK-Thr$^{172}$

Several kinases are located upstream of eNOS. One of them is the AMP-activated protein kinase (AMPK). AMPK has been reported to phosphorylate eNOS at the Ser$^{1177}$ residue [56]. To investigate a possible influence of AMPK on the ascorbate effect we used compound C, an AMPK inhibitor. When endothelial cells were pretreated with compound C ascorbate was not able to increase eNOS-Ser$^{1177}$ phosphorylation (Figure 3.12A). Interestingly compound C alone led to an unexpected increase in eNOS-Ser$^{1177}$ phosphorylation probably due to off-target effects or compensatory mechanisms. Therefore we performed an additional experiment using a siRNA approach. Downregulation of AMPK by siRNA completely blocked the effect of ascorbate on eNOS-Ser$^{1177}$ phosphorylation (Figure 3.12B).

Next we investigated whether treatment of endothelial cells with ascorbate is activating AMPK. Therefore AMPK phosphorylation at Thr$^{172}$ was investigated after treatment with ascorbate for up to 1 h. Indeed ascorbate led to a significant increase of AMPK-Thr$^{172}$ phosphorylation already after 30 min in EA.hy926 cells as well as in HUVEC (Figure 3.13A and 3.13B). This increase in AMPK phosphorylation was also dose-dependent, reaching significance at 20 µM of ascorbate (Figure 3.13C).

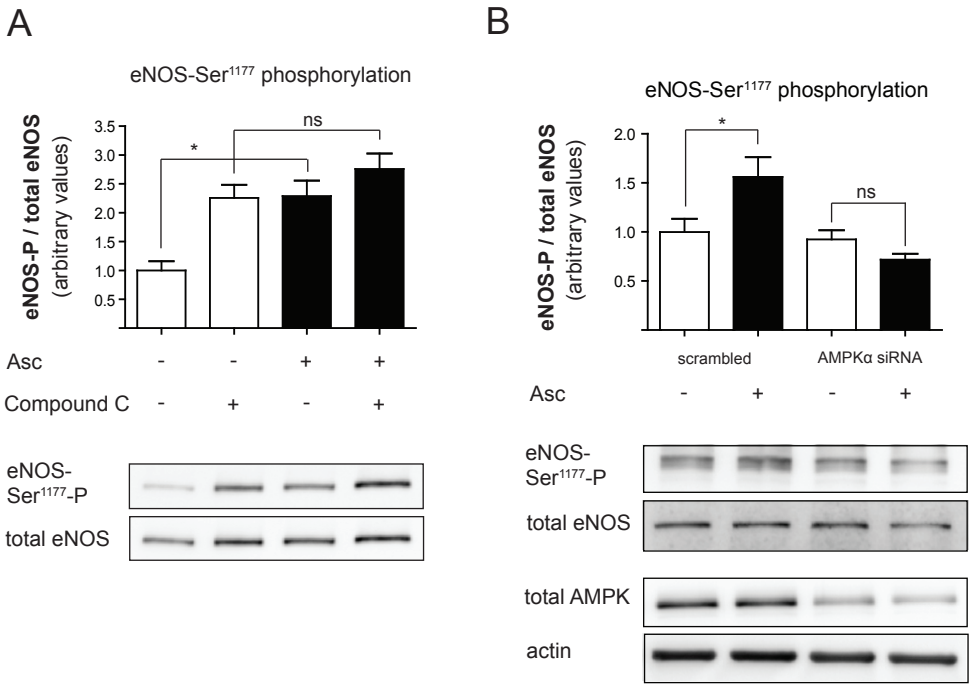


**Figure 3.12:** Inhibition of AMPK abrogates the effect of ascorbate on eNOS-Ser$^{1177}$ phosphorylation. (A) EA.hy926 cells were pretreated with 20 µM compound C for 30 min and then incubated with 100 µM ascorbate (Asc) for 1 h. (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3). (B) HUVEC were transfected with AMPKα siRNA or scrambled control before treated with 100 µM ascorbate (Asc) for 1 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 4).

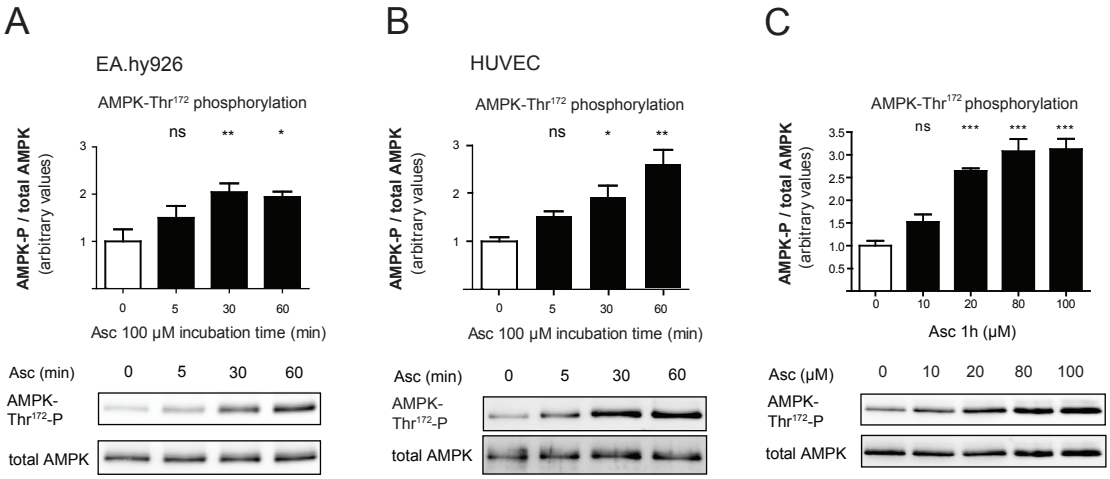


**Figure 3.13:** Ascorbate increases AMPK phosphorylation in a time and dose-dependent manner. (A) EA.hy926 cells or (B) HUVEC were treated with 100 µM ascorbate (Asc) for 5 min to 1 h. (Phospho-) AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown. (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM, n = 3).(C) EA.hy926 cells were treated with 0 to 100 µM ascorbate (Asc) for 1 h. (Phospho-) AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown. (***, $p < 0.001$; ns, not significant; mean ± SEM, n = 4).

These data suggests that the fast activation of AMPK by ascorbate is necessary for the modulation of eNOS phosphorylation and activation.

#### 3.1.1.6 CaMKKβ may be involved in the activation of AMPK upon ascorbate treatment

To elucidate how phosphorylation of AMPK comes about we next assayed $Ca^{2+}$/CaM-dependent protein kinase kinase β (CaMKKβ), a known activator of AMPK [94]. Pre-incubation with the CaMKKβ inhibitor STO 609 completely abolished activation of AMPK and diminished eNOS-Ser1177 phosphorylation in response to ascorbate (Figure 3.14A). Again STO 609 led to a basal increase in eNOS-$Ser^{1177}$ phosphorylation, that may be due to off-target effects. We also tried to knockdown CaMKKβ using a siRNA approach. Unfortunately we were only able to manage a maximal knockdown of $\sim$ 30 % , which in fact was accompanied by a trend to decreased eNOS and AMPK phosphorylation upon Asc exposure (Figure 3.14B). However, due to the only moderate decrease in CaMKKβ protein levels, these data are not very convincing. Based on these findings, we could not find a clear connection between the activation of AMPK upon ascorbate treatment and CaMKKβ.

#### 3.1.1.7 Ascorbate inhibits PP2A

Protein phosphatase 2A (PP2A) is a regulator of eNOS and AMPK activity as it can dephosphorylate eNOS at $Ser^{1177}$ and AMPK at $Thr^{172}$ [231; 351]. Okadaic acid (OA) is a selective inhibitor of PP2A when used in concentrations below 1 µM [234]. Interestingly, endothelial cells treated with okadaic acid elicited a strikingly similar eNOS and AMPK phosphorylation pattern in comparison to ascorbate treated cells (Figure 3.15).

Furthermore, okadaic acid increased eNOS enzyme activity in a dose-dependent manner and endothelial NO release is upregulated upon treatment with 10 nM okadaic acid for 20 h (Figure 3.16). Endothelial NO release was, however, only measured twice and therefore no statistics could be calculated.

These results suggested that ascorbate may act as inhibitor of PP2A. To study this further we overexpressed the catalytic subunit of PP2A (PP2Ac) in HUVEC.

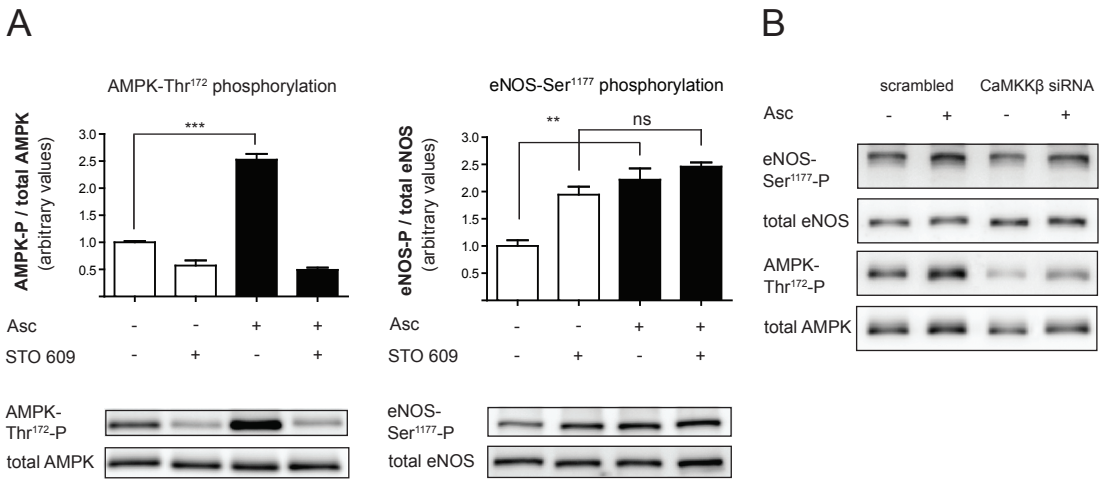


**Figure 3.14:** CaMKKβ may be involved in the activation of AMPK upon ascorbate treatment.(A) EA.hy926 cells were pretreated with 20 µM STO 609 and incubated with 100 µM ascorbate (Asc) for 1 h as indicated. (Phospho-) AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (**, $p < 0.01$; ***, $p < 0.001$; ns, not significant; mean ± SEM, n = 3). (B) HUVEC were transfected with CaMKKβ siRNA or scrambled control before treated with 100 µM ascorbate (Asc) for 1 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis.

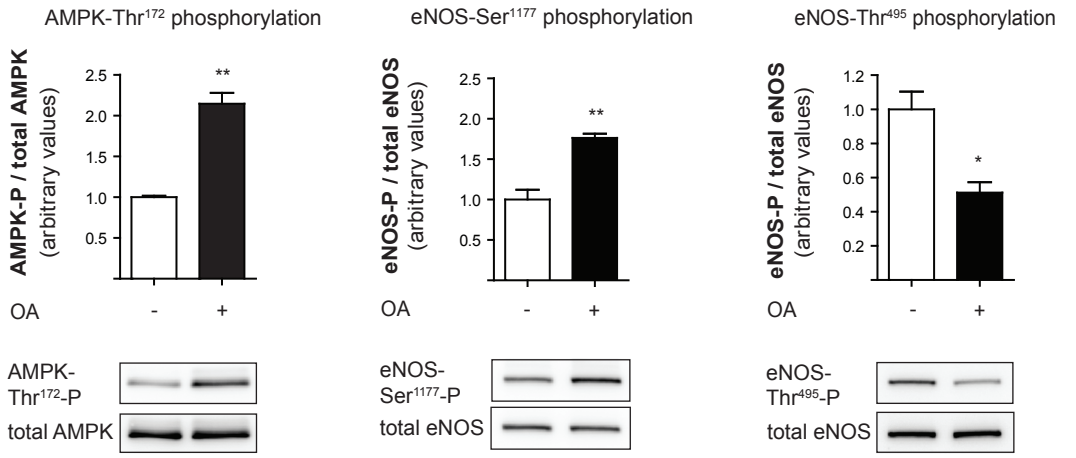


**Figure 3.15:** Okadaic acid elicits a strikingly similar phosphorylation pattern in comparison to ascorbate. EA.hy926 cells were treated with 30 nM okadaic acid (OA) for 1.5 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM, n = 3). Figure obtained with the technical assistance of Daniel Schachner.

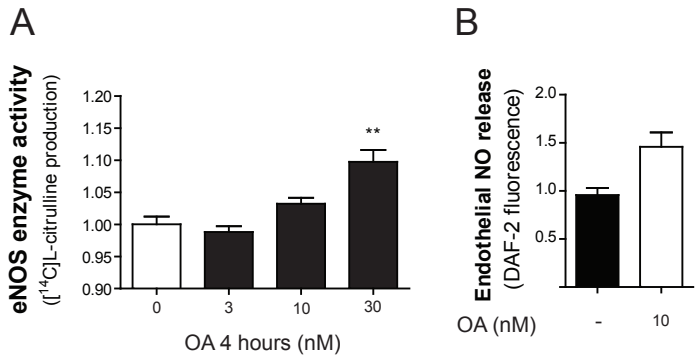


**Figure 3.16:** Okadaic acid increases eNOS enzyme activity and endothelial NO release. (A) EA.hy926 cells were treated with 0 to 30 nM okadaic acid (OA) for 4 h and eNOS activity was determined (**, $p < 0.01$; mean ± SEM, n = 3). (B) EA.hy926 cells were treated with 10 nM okadaic acid (OA) for 20 h and endothelial NO release was measured (mean ± SEM, n = 2). Figure A obtained with the technical assistance of Daniel Schachner.

This overexpression completely abrogated the increase in eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ phosphorylation upon ascorbate treatment (Figure 3.17).

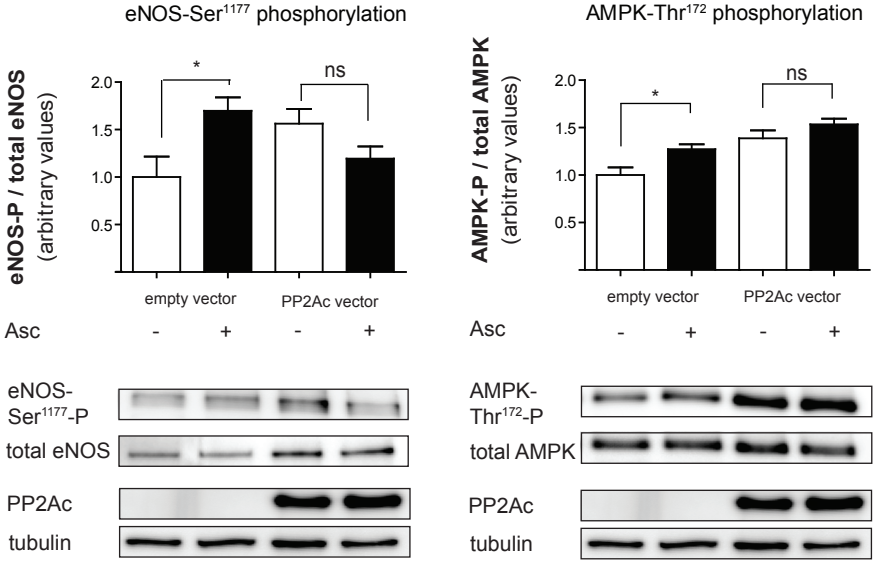


**Figure 3.17:** Overexpression of PP2A abrogates the increase in phosphorylation of eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ upon ascorbate treatment. HUVEC were transfected with empty vector or HA-tagged PP2Ac expression vector and then treated with 100 µM ascorbate (Asc) for 1 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3).

Moreover, overexpression of PP2Ac blocked the ascorbate induced increase in eNOS enzyme activity (Figure 3.18).

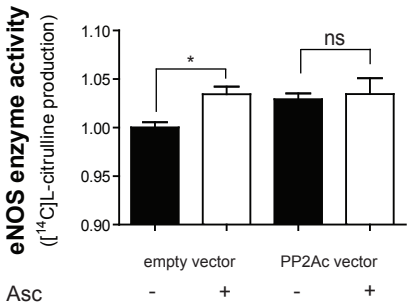


**Figure 3.18:** Overexpression of PP2A blocks the ascorbate induced increase in eNOS enzyme activity. HUVEC were transfected with empty vector of HA-tagged PP2Ac expression vector and treated with 100 µM ascorbate (Asc) for 4 h. Then eNOS activity was determined (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3). For better visualization of the small but significant effect the x-axis starts at 0.9.

Interestingly, the response of transfected HUVEC treated with ascorbate was weaker when compared to the untransfected control. This may be due to the stress of the transfection. However, the effect of ascorbate on eNOS and AMPK phosphorylation as well as on eNOS activity was abolished when PP2Ac was overexpressed. Taken together this data strongly links inhibition of PP2A activity or activation to the rapid effect of ascorbate on AMPK and eNOS in endothelial cells.

## 3.1.2 Discussion

In this study we showed a novel mechanism where ascorbate rapidly enhances eNOS activity in cultured endothelial cells via changes in eNOS phosphorylation. This fast response was dependent on changes on PP2A and AMPK activity, however it proved to be independent of BH4 stabilization. Interestingly, the rapid effect of ascorbate on eNOS enzyme activity was in the same range as for well known activators such as VEGF.

Long-term treatment of endothelial cells in culture with ascorbate (24 hours) has previously been shown to increase eNOS enzyme activity by stabilizing the essential eNOS cofactor BH4 [144; 153]. However, whether the fast increase in eNOS activity upon ascorbate treatment, as observed in cultured endothelial cells, or the rapid increase in endothelial-dependent vasodilation after ascorbate infusion is dependent

on stabilization of BH4 had not yet been investigated to the best of our knowledge.

In this study we could show that an increase in intracellular ascorbate led to enhanced eNOS-Ser$^{1177}$ phosphorylation and concomitantly decreased eNOS-Thr$^{495}$ phosphorylation within 30 minutes after ascorbate treatment. With the help of pharmacological inhibitors and an siRNA knockdown approach, AMPK was found to be the responsible upstream kinase for eNOS-Ser$^{1177}$ phosphorylation. The Akt/PI3K pathway was not involved in these effects. Overexpression of PP2A blocked eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ phosphorylation, thereby suggesting that ascorbate may act via inhibition of PP2A activity or PP2A activation. Moreover, the pharmacological inhibition of PP2A by okadaic acid led to a similar phosphorylation pattern of eNOS and AMPK. Taking into account that PP2A is known to dephosphorylate both AMPK and eNOS we hypothesized that ascorbate elicits its effect on eNOS activity at least in part by inhibition of PP2A activation or activity. This suggestion is supported by two studies in which ascorbate enhanced endothelial barrier function in an experimental model of sepsis by inhibition of PP2A activation [133] and increased NO formation [224]. This recently observed importance of PP2A in endothelial health may support PP2A as a novel therapeutic target.

PP2A is known to be activated by oxidative stress, therefore ascorbate may interfere with PP2A activation via scavenging of reactive oxygen species and reducing oxidative stress [133]. In cultured endothelial cells oxidative stress is often increased when ascorbate levels in the culture medium are low [304]. As this is also the case with patients, who suffer from endothelial dysfunction, this could explain the rapid effects of ascorbate infusions on patients with endothelial dysfunction and the lack of such rapid effects on patients with normal endothelial function. So far, the molecular mechanism of how the generation of reactive oxygen species is rapidly prevented is not known. It is possible that ascorbate acts as a general antioxidant so that preferred oxidation of ascorbate maintains stores of the intracellular antioxidant glutathione and thereby counteracts prooxidant signaling [225; 226]. Therefore, we investigated if ascorbate treatment can reduce intracellular reactive oxygen species, using oxidized dihydrofluorescein. Suprisingly, ascorbate had no effect on intracellular reactive oxygen species levels in our experimental setting (unpublished data from our lab). This result suggests that ascorbate is not acting via modulation of reactive oxygen species levels.

In addition to the rapid effects, ascorbate can stabilize the cofactor BH4 after long-term treatment as stated before. This leads to the prevention of eNOS uncoupling

and thereby reduces the amount of oxidative stress generated [304].

It has been published that ascorbate as well as other antioxidants are able to generate hydrogen peroxide in cell culture medium which can then activate eNOS [132; 324]. However, in our experimental settings only nanomolar amounts of hydrogen peroxide were measurable in the medium, which is two to three orders of magnitude below the concentrations needed to activate eNOS [324]. Moreover, the addition of catalase, the enzyme responsible for hydrogen peroxide breakdown, to the medium did not prevent the fast increase in eNOS activity upon ascorbate treatment.

Our data provided a mechanism for the increased phosphorylation of eNOS-Ser$^{1177}$ after ascorbate treatment, but the signaling events necessary for eNOS-Thr$^{495}$ dephosphorylation could not be identified. It is known that PP2A can directly dephosphorylate eNOS at Thr$^{495}$, however, suprisingly inhibition of PP2A with okadaic acid did not increase the phosphorylation at this site [239]. This suggests that PP2A is predominantly acting on eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ and not on eNOS-Thr$^{495}$ as previously shown [231; 292]. eNOS-Thr$^{495}$ was shown to be selectively altered in at least one study but other studies suggest a concomitant regulation with eNOS-Ser$^{1177}$ phosphorylation [100; 292]. In order to understand the underlying mechansim of this coordinated regulation, further research is required.

PP2A is known to dephosphorylate CaM, thereby inactivating calcium/calmodulin dependent kinases [272]. This could place PP2A inhibition upstream of the apparently calcium-independent activation of CaMKKβ upon treatment with ascorbate. CaMKKβ, in turn, may lead to activation of AMPK which then positively affects eNOS-Ser$^{1177}$ phosphorylation and eNOS activity. However, our data did not show a clear connection between the activation of AMPK upon ascorbate treatment and CaMKKβ.

Taken together, this study describes a novel mechanism for the rapid activation of eNOS by ascorbate independent of BH4 stabilization. Ascorbate was shown to modulate PP2A and AMPK in order to change the phosphorylation pattern of eNOS, thereby activating the enzyme (Figure 3.19). Activation of eNOS by ascorbate is therefore divided into two phases. First, rapid changes in eNOS phosporylation increase eNOS activity within minutes. Second, long-term effects of ascorbate are achieved by stabilization of BH4 after one day. One has to keep in mind that this study was only performed in cell culture models. Whether these mechanisms take

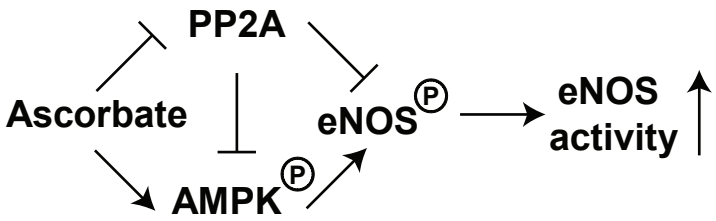


**Figure 3.19:** Proposed mechanism underlying the rapid eNOS activation by ascorbate. Inhibition of PP2A by ascorbate leads to an increase in AMPK, and eNOS activity. Full arrows indicate activation, blocked lines inhibition.

place *in vivo* as well needs further investigation.

## 3.2 2-(2,4-dihydroxyphenyl)-5-(E)-propenylbenzofuran (DPPB) promotes endothelial nitric oxide synthase activity in human endothelial cells

### 3.2.1 Results

#### 3.2.1.1 DPPB is the only compound isolated from K. lappacea that stimulates endothelial NO release and increases eNOS enzyme activity

Eleven lignan derivatives were isolated from the dried roots of *K. lappacea* by the group of Prof. Stuppner (Institut of Pharmacy/Pharmacognosy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Austria) (Figure 3.20) [24] and we investigated these compounds for their ability to influence endothelial NO release.

Compound **6** (DPPB) was the only lignan that led to a significant increase in endothelial NO release. Whereas treatment with compounds **1**, **5**, and **9** led to a decrease. All the other compounds had no significant effect in this assay (Figure 3.21A). Furthermore we tested the eleven lignans for possible cytotoxic effects, using the LDH release assay and the resazurin conversion assay. None of the compounds had a negative effect on endothelial cell viability (Figure 3.22A and 3.22B). Compounds **6**, **7**, **8**, and **11** were further investigated for their ability to alter eNOS enzyme activity as they were the most promising compounds regarding the NO release assay, although only compound **6** elicited a significant increase. Interestingly, again only compound **6** (DPPB) significantly increased eNOS enzyme activity (Figure 3.21B).

Consequently we chose to further investigate the effect of DPPB. Treatment of endothelial cells with 10 µM DPPB led to a $\sim$ 2-fold increase in endothelial NO release when compared to the solvent control (Figure 3.23A). As a positive control 1 µM mevastatin was used, which is known to upregulate eNOS expression and thus increases NO release [285; 297]. DPPB did also elicit a dose-dependent increase in eNOS enzyme activity in EA.hy926 cells, reaching $\sim$ 1.2-fold activity at 10 µM

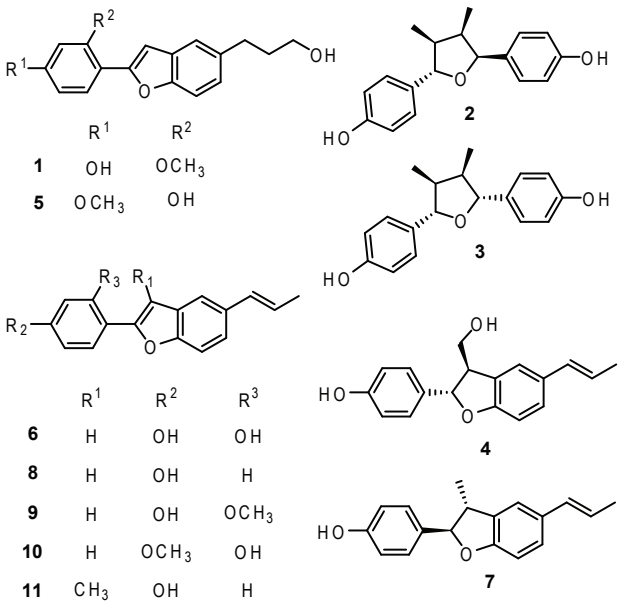


**Figure 3.20:** Structures of the eleven lignans isolated from *K. lappacea*. 5-(3-hydroxypropyl)-2-(2-methoxy-4-hydroxyphenyl)benzofuran (**1**), (−)-larreatricin (**2**), *meso*-3,30-didemethoxynectandrin B (**3**), (2S,3S)-2,3-dihydro-3-hydroxymethyl-2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran (**4**), 2-(2-hydroxy-4-methoxyphenyl)-5-(3-hydroxypropyl)benzofuran (**5**), 2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran (**DPPB**; **6**), (+)-conocarpan (**7**), 2-(4-hydroxyphenyl)-5-(*E*)-propenylbenzofuran (**8**), rataniaphenol III (**9**), rataniaphenol I (**10**), and rataniaphenol II (**11**).

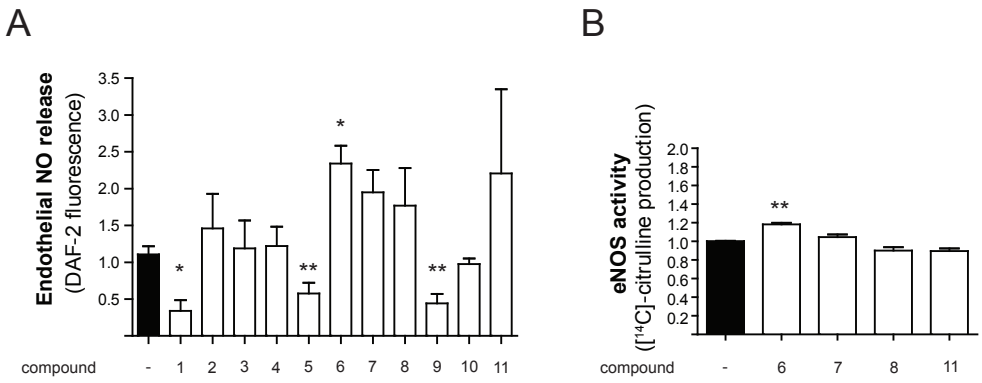


**Figure 3.21:** Action of the eleven lignans in endothelial NO release and eNOS activity assays. EA.hy926 cells were treated with 10 µM of the indicated compound for 24 h. Then (A) endothelial NO release or (B) eNOS enzyme activity was determined (*, $p < 0.05$; **, $p < 0.01$; mean ± SEM, n = 3 for (A) and n = 4 for (B)).

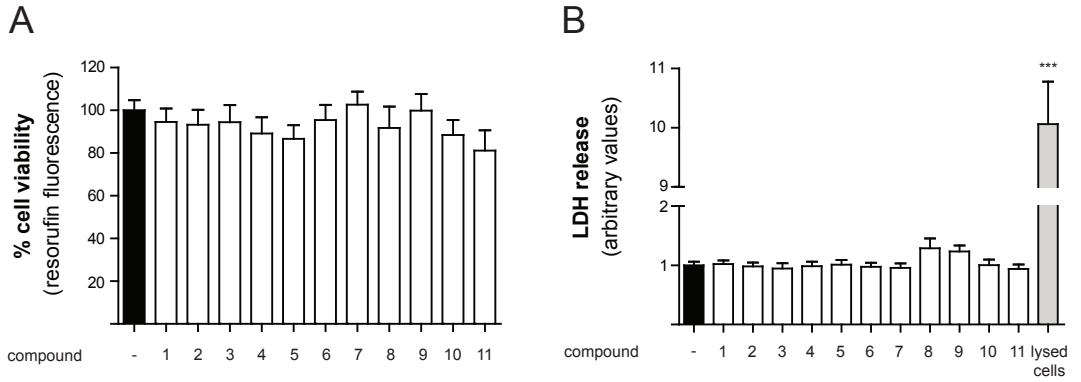


**Figure 3.22:** None of the eleven lignans had a cytotoxic effect on endothelial cells. EA.hy926 cells were treated with 10 µM for 24 h. Then (A) Resazurin conversion assay or (B) LDH release assay was performed. Values were normalized to the solvent control (***, $p < 0.001$; mean ± SEM, n = 3 for (A) and n = 4 for (B)).

DPPB when compared to the solvent control (Figure 3.23A). In HUVEC 10 µM DPPB had a similar effect (Figure 3.23B). As a positive control in this assay we used ascorbic acid, which is known to stabilize the eNOS cofactor BH4 thereby leading to an increase in eNOS enzyme activity [144; 153].

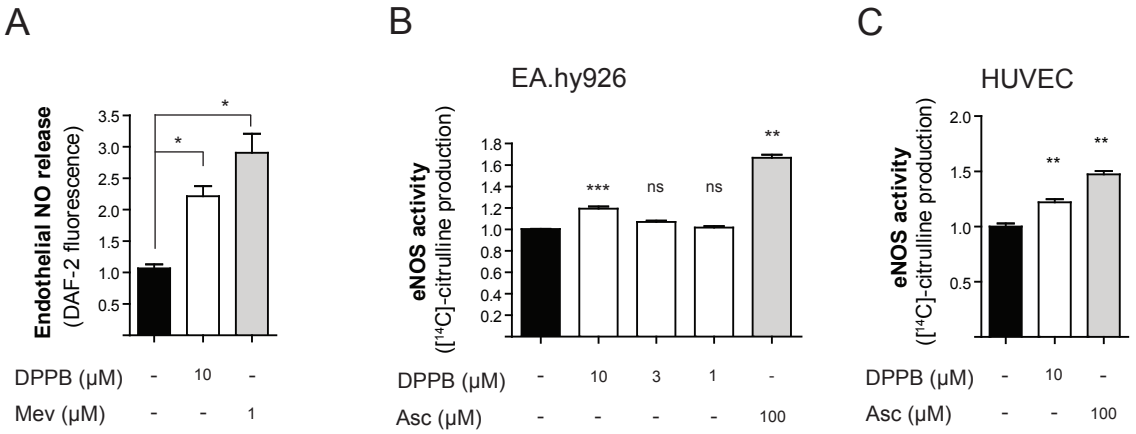


**Figure 3.23:** DPPB increases endothelial NO release and eNOS enzyme activity. (A) EA.hy926 cells were treated with 10 µM DPPB for 24 h. Mevastatin (Mev, 1 µM) was used as positive control. Then endothelial NO release was measured (*, $p < 0.05$; mean ± SEM, n =3). (B) EA.hy926 cells or (C) HUVEC were treated with the indicated concentrations of DPPB for 24 h. Ascorbic acid (Asc, 100 µM) was used as positive control. Then eNOS enzyme activity was determined (**, $p < 0.01$; ***, $p < 0.001$; ns, not significant; mean ± SEM, n =3).

### 3.2.1.2 DPPB changes the eNOS phosphorylation pattern

ENOS enzyme activity is upregulated by increased phosphorylation at $Ser^{1177}$ or dephosphorylation at $Thr^{495}$. These two phosphorylation sites have been reported to be controlled concomitantly [239]. Treatment of EA.hy926 cells with 10 µM DPPB led to a time-dependent increase in eNOS phosphorylation at $Ser^{1177}$ and dephosphorylation at Thr495, corresponding to an enhanced eNOS enzyme activity (Figure 3.24A). After 24 h treatment the effects of DPPB were strongest. Therefore this condition was used in the following experiments. Similar results were obtained with HUVEC when 10 µM DPPB for 24 h were used (Figure 3.24B). Interestingly, DPPB did not alter total eNOS protein levels (Figure 3.24) and moreover, had no effect on eNOS mRNA levels (Figure 3.25), suggesting a direct stimulatory action of DPPB on eNOS enzyme activtiy.

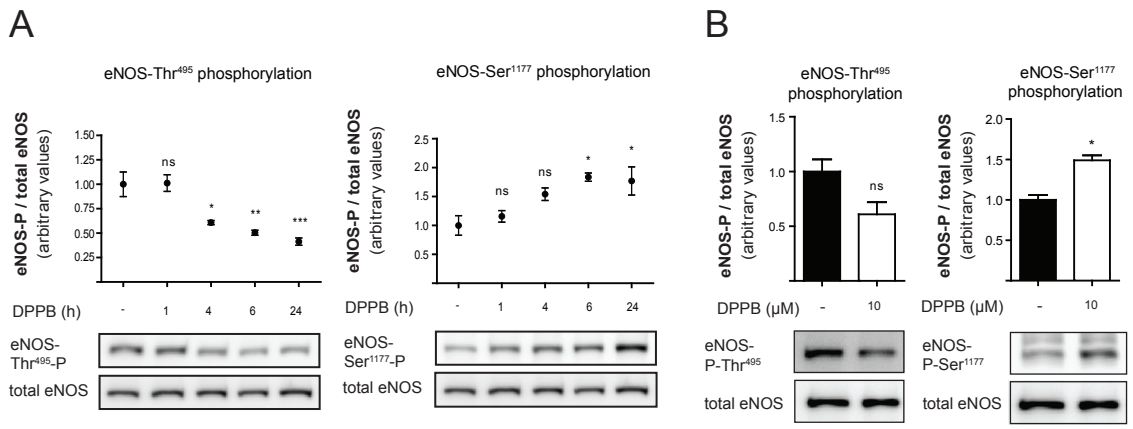


**Figure 3.24:** Changes in eNOS phosphorylation upon DPPB treatment. (A) EA.hy926 cells or (B) HUVEC were treated with 10 µM DPPB for 0 to 24 h in (A) and for 24 h in (B). (Phospho-) eNOS protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin or actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; mean ± SEM, n = 3)

Furthermore we investigated DPPB for its action on neuronal NOS (nNOS) and inducible NOS (iNOS) protein levels. However, no differences between DPPB treated and solvent treated cells were detectable (Figure 3.26). For the detection of nNOS rat brain extract was used as positive control, and for iNOS DLD-1 cells (colorectal adenocarcinoma cell line) treated with a cytomix (kindly provided by Prof. Klein-

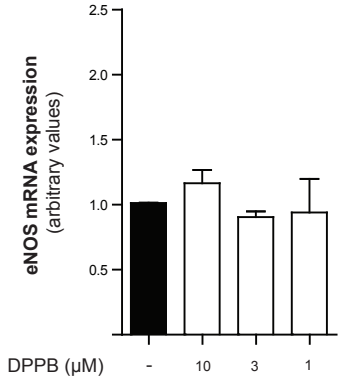


**Figure 3.25:** DPPB does not influence eNOS mRNA levels. EA.hy926 cells were treated with 10 to 1 µM DPPB for 24 h and eNOS mRNA levels were determined (n = 2).

ert, University of Mainz, Germany) was used as positive control.

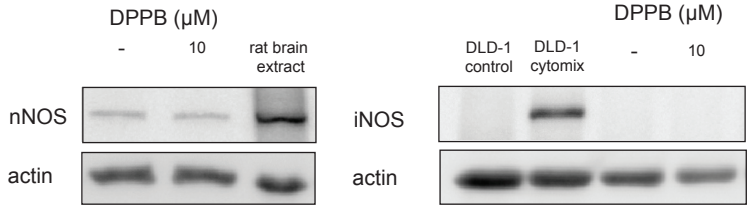


**Figure 3.26:** Expression levels of nNOS and iNOS in EA.hy926 cells. EA.hy926 cells were treated with 10 µM DPPB for 24 h. Rat brain extract was used as a positive control for nNOS and as a positive control for iNOS we used DLD-1 cells, either untreated (negative control) or treated with cytomix (DLD-1 cell lysates were a kind gift from Prof. Kleinert). NNOS and iNOS protein levels were determined by Western blot. One representative blot is shown (n = 3).

#### 3.2.1.3 DPPB does not affect Akt but increases the phosphorylation of AMPK-Thr$^{172}$

Two of the most studied kinases known to activate eNOS are AMP-activated kinase (AMPK) and Akt kinase [56; 232]. Both are directly catalyzing the phosphorylation of eNOS-Ser$^{1177}$, thus activating the enzyme. Treatment of EA.hy926 cells with DPPB time-dependently increased AMPK phosphorylation at Thr$^{172}$, thus suggesting an increased AMPK activity (Figure 3.27A). However, Akt kinase was not influenced by DPPB, as determined by Akt-Ser$^{473}$ phosphorylation status (Figure 3.27A). Similar results were obtained in HUVEC (Figure 3.27B).

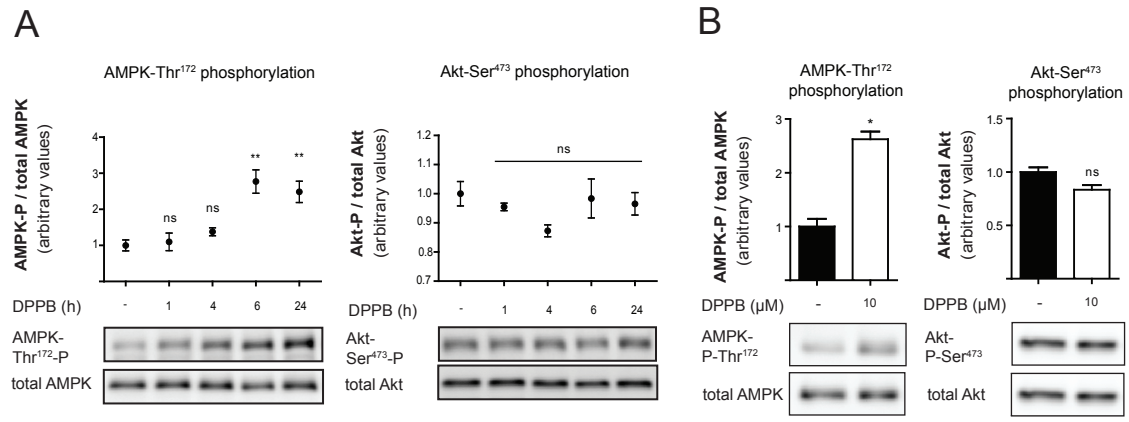


**Figure 3.27:** Changes in AMPK and Akt phosphorylation upon DPPB treatment. (A) EA.hy926 cells or (B) HUVEC were treated with 10 µM DPPB for 0 to 24 h in (A) and for 24 h in (B). (Phospho-) AMPK and Akt protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin or actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM, n = 3)

#### 3.2.1.4 Increased eNOS-Ser$^{1177}$ phosphorylation upon DPPB treatment is dependent on AMPK and CaMKKβ

Next we investigated whether AMPK is directly involved in the enhancing effect of DPPB on eNOS activity. Treatment of endothelial cells with compound C, an inhibitor of AMPK, abolished the increase in eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ phosphorylation (Figure 3.28A). However, compound C alone increased the phosphorylation at these sites. This is probably due to off-target effects or compensatory mechanisms in endothelial cells upon treatment with compound C. Additionally we used a siRNA approach to downregulate AMPK expression. HUVEC transfected with AMPKα siRNA were no longer able to enhance eNOS-Ser$^{1177}$ phosphorylation upon DPPB treatment (Figure 3.28). Interestingly, transfection with AMPKα siRNA also increased eNOS-Ser$^{1177}$ phosphorylation without any treatment when compared to cells transfected with scrambled control. This is probably caused due to off-target effects of the siRNA.

To strengthen these results we studied the effect of DPPB on eNOS enzyme activity in the presence of compound C. Compound C completely blocked the increase in eNOS enzyme activity upon DPPB treatment (Figure 3.29A). This strongly links the enhancing effect on eNOS activity by DPPB to AMPK.

Next we studied how AMPK is activated by DPPB. Therefore we investigated

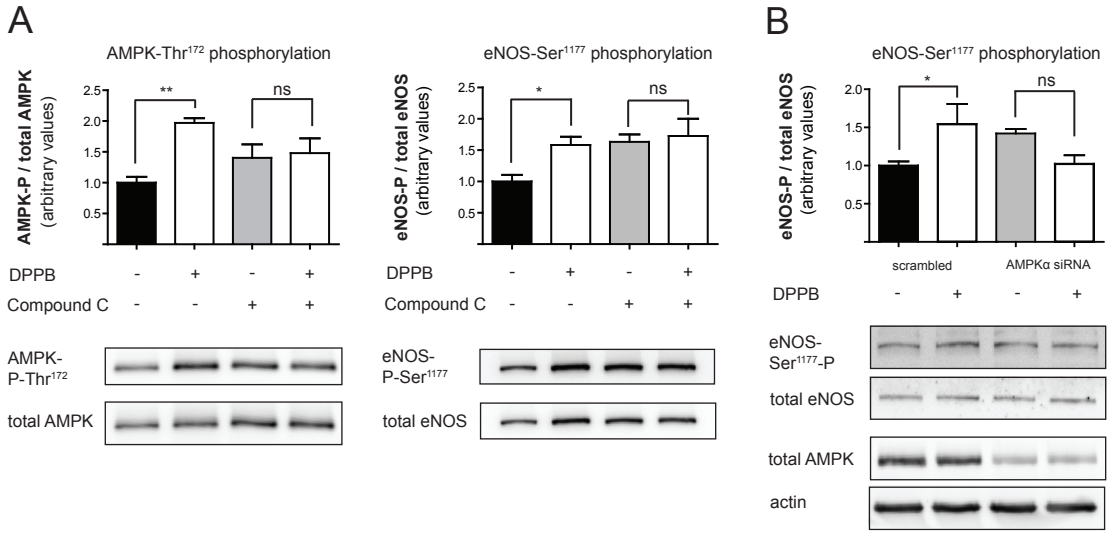


**Figure 3.28:** ENOS activity is enhanced by DPPB in an AMPK dependent fashion. (A) EA.hy926 cells were pretreated with 10 µM compound C for 30 min and then incubated with 10 µM DPPB for 24 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; **, $p < 0.01$; ns, not significant; mean ± SEM, n = 3). (B) HUVEC were transfected with AMPKα siRNA or scrambled control before treated with 10 µM DPPB for 24 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3).

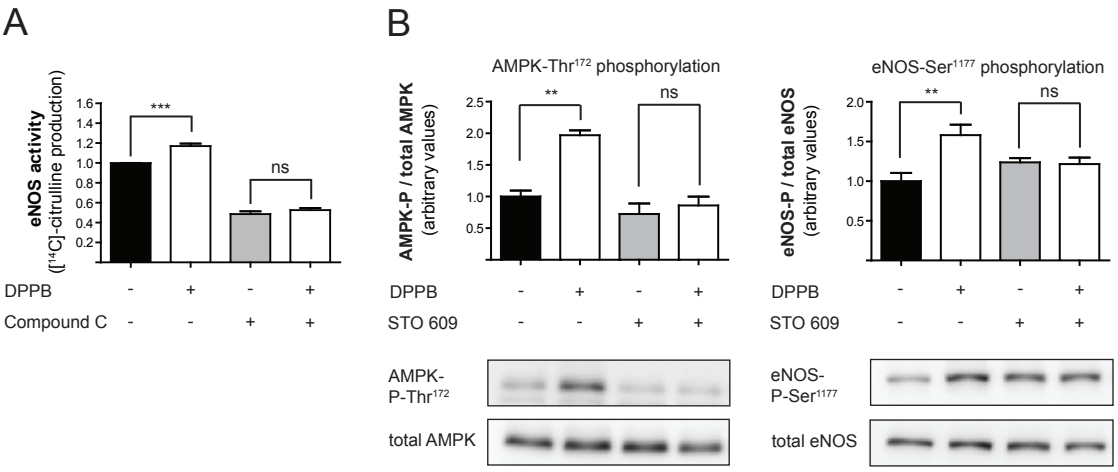


**Figure 3.29:** AMPK and CaMKKβ are involved in the stimulating effect of DPPB on eNOS-Ser$^{1177}$. (A) EA.hy926 cells were pretreated with 10 µM compound C for 30 min and then incubated with 10 µM DPPB for 24 h. Then eNOS enzyme activity was determined (*, $p < 0.05$; ns, not significant; mean ± SEM, n = 3). (B) EA.hy926 cells were pretreated with 10 µM STO 609 and then incubated with 10 µM DPPB for 24 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to tubulin and expressed as fold untreated control. One representative blot is shown (**, $p < 0.01$; ns, not significant; mean ± SEM, n = 3).

the influence of CaMKKβ, an upstream kinase of AMPK [94; 352]. Treatement of EA.hy926 with STO 609, an inhibitor of CaMKKβ, abolished the increase in eNOS-Ser$^{1177}$ and AMPK-Thr$^{172}$ phosphorylation, suggesting an involvement of CaMKKβ in the DPPB-induced activation of eNOS (Figure 3.29B).

### 3.2.1.5 DPPB enhances intracellular $Ca^{2+}$ levels

An increase in intracellular calcium ($[Ca^{2+}]_i$) can lead to the activiation of CaMKKβ as well as eNOS [230; 316]. Therefore we next investigated whether DPPB treatment leads to an increase in $[Ca^{2+}]_i$. Indeed, DPPB led to a dose-dependent increase in $[Ca^{2+}]_i$ levels in endothelial cells (Figure 3.30A).

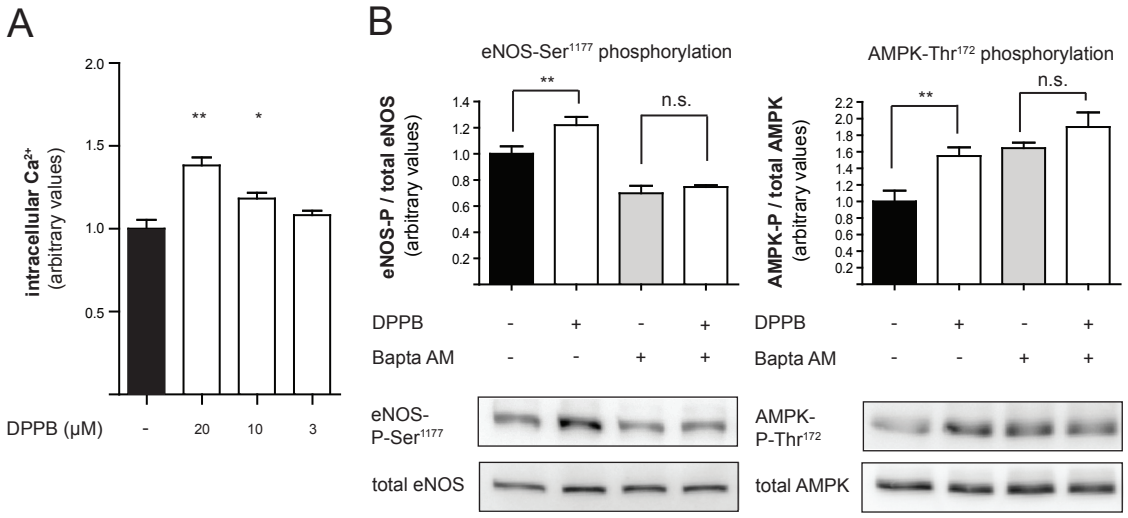


**Figure 3.30:** DPPB induced changes in eNOS-Ser$^{1177}$ phosphorylation are dependent on increased intracellular $Ca^{2+}$ levels. (A) EA.hy926 cells were incubated with 20 to 3 µM DPPB for 24 h. Intracellular $Ca^{2+}$ levels were measured (*, $p < 0.05$; **, $p < 0.01$; mean ± SEM, n =3). (B) EA.hy926 cells were treated with 10 µM DPPB for 24 h and with 30 µM Bapta AM for 2 h. (Phospho-) eNOS and AMPK protein levels were determined by Western blot and subsequent densitometric analysis. Band intensities were normalized to actin and expressed as fold untreated control. One representative blot is shown (**, $p < 0.01$; ns, not significant; mean ± SEM, n = 6).

#### 3.2.1.6 Chelation of intracellular $Ca^{2+}$ abolishes the activating effect of DPPB on eNOS-$Ser^{1177}$ and AMPK-$Thr^{172}$ phosphorylation

To strengthen our hypothesis, that the activation of AMPK and eNOS by DPPB may be caused by an increase in $[Ca^{2+}]_i$, we treated EA.hy926 cells with the $Ca^{2+}$ chelator, Bapta AM. Chelation of $Ca^{2+}$ blocked the enhancing effect of DPPB on eNOS-$Ser^{1177}$ and AMPK-$Thr^{172}$ phosphorylation, suggesting that $Ca^{2+}$ ions are necessary for the action of DPPB (Figure 3.30B). Again Bapta AM alone led to basally increased AMPK-$Thr^{172}$ phosphorylation, similar to compound C. However, as for compound C DPPB could not increase the phophorylation any further.

Taken together, these data strongly suggests a link between increased endothelial NO release and the observed increase in $[Ca^{2+}]_i$ upon DPPB treatment.

### 3.2.2 Discussion

In this study we show that the benzofuran derivative DPPB increases eNOS activity and also NO availability in cultured endothelial cells by increasing $[Ca^{2+}]_i$ levels, leading to the activation of eNOS via $Ca^{2+}$/CaM, CaMKKβ and AMPK.

Secondary metabolites from the benzofuran class are known to exhibit a broad range of bioactivities (see section 1.6.2 and [131; 152; 154; 170; 192]), but an influence on eNOS activity or nitric oxide availability had not been shown so far. Out of eleven benzofuran derivatives isolated from *K. lappacea* [24], we could identify DPPB as the only compound which promotes eNOS activity in cultured human endothelial cells.

The mechanism underlying the increase in endothelial NO release upon DPPB treatment involves increased $[Ca^{2+}]_i$ concentrations. Typically an increase in $[Ca^{2+}]_i$ can be divided into two phases. In the first phase $[Ca^{2+}]_i$ levels rise transiently until the intracellular $Ca^{2+}$ stores are depleted. This triggers the second phase, which is characterized by a sustained increase in $[Ca^{2+}]_i$, that is due to $Ca^{2+}$ entry from the extracellular space through plasma membrane channels [104; 271]. Upon treatment of endothelial cells with DPPB we could detect a late rise of $[Ca^{2+}]_i$ but no fast transient increase. This suggests a mechanism independent on the depletion of $Ca^{2+}$ stores. Store-operated $Ca^{2+}$ channels (SOCCs) are located at the plasma membrane and act as the main route for cellular $Ca^{2+}$ entry. Treatment of endothelial cells with DPPB probably activates SOCCs at the plasma membrane leading to an in-

crease in $[Ca^{2+}]_i$ without involving the intracellular calcium stores. Interestingly, S-nitrosylation of transient receptor potential (TRP) proteins, acting as SOCCs, has been reported to induce $Ca^{2+}$ entry, thereby further sustaining increased $[Ca^{2+}]_i$ levels and enhanced eNOS activity [358].

eNOS activation via an increase in $[Ca^{2+}]_i$ levels is a well known mechanism. Protein-protein interaction of eNOS with the intracellular calcium sensor CaM enables the electron transfer from NADPH at the reductase domain of the enzyme to the heme center at the oxygenase domain, thereby activating the enzyme [230]. CaM acts as a well-established and important mediator of calcium signaling. Several different target proteins are regulated by the binding of CaM, thereby affecting numerous cellular functions [57]. CaMKK$\beta$ is such a target protein of CaM. Interestingly, CaMKK$\beta$ translates the increase in $[Ca^{2+}]_i$ to the phosphorylation of downstream targets such as AMPK [94; 352]. Besides its importance in metabolism AMPK is also crucial for the maintenance of endothelial function and redox balance [355]. Therefore, AMPK acts also as anti-atherosclerotic modulator by influencing various signaling pathways important in the increase of NO bioavailability, attenuated free radical generation, and activation of angiogenic factors [94]. Additionally, activated AMPK has been shown to exert anti-inflammatory effects as it reduces the activation of NF-$\kappa$B in endothelial cells [76]. Furthermore, AMPK inhibits the proliferation of vascular smooth muscle cells, thus supporting anti-atherosclerotic functions [156]. The increase in eNOS activity upon activation of AMPK was achieved by enhanced phosphorylation of eNOS-Ser$^{1177}$ [239].

Our data revealed a strong increase in AMPK-Thr$^{172}$ phosphorylation upon DPPB treatment, concomitant with eNOS-Ser$^{1177}$ phosphorylation. This finding led us to the suggestion that eNOS is not only activated via a direct protein-protein interaction with $Ca^{2+}$/CaM, but additionally via a pathway involving $Ca^{2+}$/CaM-dependent CaMKK$\beta$, and AMPK activation and finally increased eNOS-Ser$^{1177}$ phosphorylation. Application of pharmacological inhibitors of CaMKK$\beta$ and AMPK (STO 609 and compound C, respectively) and knockdown of AMPK by siRNA confirmed a causal connection between these pathway components.

These approaches successfully blocked the DPPB-induced increase in eNOS-Ser$^{1177}$ phosphorylation, resulting in the activation of eNOS. Phosphorylation of eNOS-Ser$^{1177}$ was accompanied by a decrease in eNOS-Thr$^{495}$ phosphorylation. The eNOS-Thr$^{495}$ phosphorylation site is located in the CaM binding domain of the enzyme. Therefore reduced phosphorylation at this site correlates with a stronger interaction

of CaM and eNOS. This is due to interference of the phosphorylated residue with CaM binding to the CaM binding domain of eNOS [97; 101].

Phosphorylation of eNOS at $Ser^{1177}$ is performed by kinases, such as Akt and the already mentioned AMPK. Several studies have observed crosstalk between the signaling of the Akt and AMPK pathway, therefore it is difficult to determine which effects are directly influenced by AMPK activation [94]. However, we could not detect any modulation of Akt upon treatment with DPPB. Similar results have been shown for the regulation of eNOS by several agonists of G protein-coupled receptors such as bradykinin or histamine. They elicit their function independent of the PI3K/Akt pathway but are dependent on an increase in $[Ca^{2+}]_i$ in their signaling in endothelial cells [97; 329].

Taken together, this data shows that the benzofuran derivative DPPB, isolated from the medicinal plant *K. lappacea* increases eNOS activity and NO availability in cultured endothelial cells. This is mediated by a mechanism involving increased $[Ca^{2+}]_i$ concentrations and increased activity of CaMKKβ and AMPK (Figure 3.31). Moreover this data presents DPPB as an interesting natural compound regarding the treatment of cardiovascular diseases. Further studies would be required to determine the suitability of DPPB for therapeutical applications, especially regarding the metabolism of the compound and its toxicity and bioavailability *in vivo*.

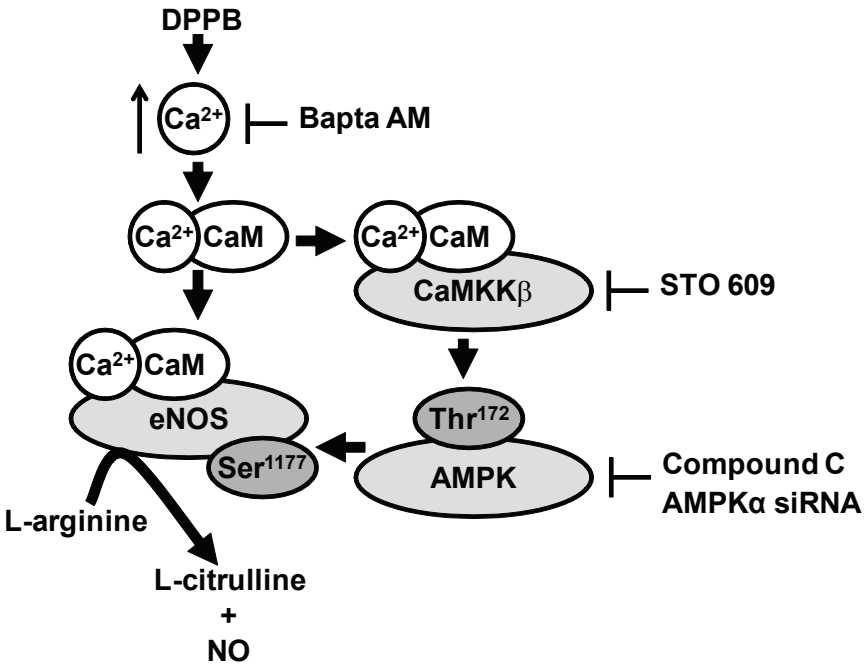


**Figure 3.31:** Scheme of the proposed mechanism by which DPPB increases eNOS activity. Treatment of endothelial cells with DPPB results in an increase in $[Ca^{2+}]_i$ and enhanced $Ca^{2+}$/CaM signaling, thereby activating CaMKKβ , AMPK and finally eNOS. This leads ultimately to increased NO production. The inhibitors used in this study (Bapta AM, STO 609, AMPKα siRNA and compound C) and their place of action is indicated.

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# 5 List of Figures

# 6 Appendix

## 6.1 Material Suppliers and stock solutions used

**Table 6.1:** Different materials, their suppliers and stock solutions used

| name | stock solution | supplier |
|---|---|---|
| DMEM with 4.5 g/l glucose | - | Lonza, Verviers, Belgium |
| L-glutamine | 200 mM in 0.85 % NaCl | Lonza, Verviers, Belgium |
| penicillin/streptomycin mixture | 10,000 U/ml penicillin; 10,000 U/µl streptomycin | Lonza, Verviers, Belgium |
| trypsin | 2.5 % 10x in HBSS without Ca, Mg | Cambrex Bio Science, Verviers, Belgium |
| HAT-supplement | 50x in BSS (balanced salt solution) | Biochrom AG, Berlin, Germany |
| FBS Superior Standardized | - | Biochrom AG, Berlin, Germany |
| FBS | - | Gibco via Invitrogen, Paisley, UK |
| EBM™ | - | Lonza, Verviers, Belgium |
| EGM™ SingleQuots™ | - | Lonza, Verviers, Belgium |
| A23187 (free acid) | 10 mM in DMSO | Alexis Biochemicals, Lausen, Switzerland |
| DAF-2 | 5 mM in DMSO | Alexis Biochemicals, Lausen, Switzerland |

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| **name** | **stock solution** | **supplier** |
|---|---|---|
| [$^{14}$C]L-arginine | 1:10 dilution in $ddH_2O$ | New England Nuclear, Boston, MA, USA |
| [1-$^{14}$C]L-ascorbic acid | 100 mM in 0.1 mM acetic acid in $ddH_2O$ | New England Nuclear, Boston, MA, USA |
| L-arginine | 100 mM in $ddH_2O$ | Fluka, Sigma-Aldrich, Vienna, Austria |
| L-NAME | 1 M in $ddH_2O$ | Sigma-Aldrich, Vienna, Austria |
| Resazurin | 10 mg/ml in PBS | Sigma-Aldrich, Vienna, Austria |
| Ascorbic acid | 100 mM in $ddH_2O$ pH 7.4 adjusted with NaOH | Sigma-Aldrich, Vienna, Austria |
| Wortmannin | 100 µM in DMSO | Sigma-Aldrich, Vienna, Austria |
| Sepiapterin | 100 mM in DMSO | Sigma-Aldrich, Vienna, Austria |
| Phloretin | 10 mM in EtOH (always prepared fresh) | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Ouabain | 10 mM in EtOH (always prepared fresh) | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Compound C | 10 mM in DMSO | Sigma-Aldrich, Vienna, Austria |
| Okadaic acid | 300 µM in DMSO | Alexis Biochemicals, Lausen, Switzerland |
| Mevastatin | 3 mM in DMSO | Sigma-Aldrich, Vienna, Austria |
| Resveratrol | 100 mM in DMSO | Sigma-Aldrich, Vienna, Austria |
| STO 609 | 10 mM in DMSO | Sigma-Aldrich, Vienna, Austria |
| Bapta AM | 30 mM in DMSO | Tocris, Bristol, UK |

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| **name** | **stock solution** | **supplier** |
|---|---|---|
| rat brain extract | 2.5 µg/µl in western blotting buffer | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| SYBR® Green I Nucleic Acid Gel Stain | 10,000 x | Invitrogen, Paisley, UK |
| peqGOLD Total RNA kit | - | peqlab Biotechnologie, Erlangen, Germany |
| eNOS primers | 300 µM in $ddH_2O$ | Invitrogen, Paisley, UK |
| 18S rRNA primers | 300 µM in $ddH_2O$ | Invitrogen, Paisley, UK |
| Roti®-Quant Bradford reagent | 5 x | Carl Roth, Karlsruhe, Germany |
| Immun-Blot® PDVF membrane | - | Bio-Rad Laboratories, Hercules, CA, USA |
| Gel blotting paper | - | Whatman plc, Kent, UK |
| Polygram SIL N-HR thin layer chromatography plates | - | Machery-Nagel, Markus Bruckner Analysentechnik, Linz, Austria |
| FuGENE®HD transfection reagent | - | Promega, Madison, WI, USA |
| Opti-MEM® | - | Invitrogen, Paisley, UK |
| AMPKα siRNA | 20 µM in $ddH_2O$ | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Oligofectamine | - | Invitrogen, Paisley, UK |
| Stealth RNAi negative control, Medium GC | 20 µM in $ddH_2O$ | Invitrogen, Paisley, UK |
| Fluo-3-AM | 442 µM in DMSO | Invitrogen, Paisley, UK |
| Amplex red reagent | 10 mM in DMSO | Invitrogen, Paisley, UK |
| Hydrogen peroxide 30 % | 200 mM in $ddH_2O$ | Carl Roth, Karlsruhe, Germany |
| Catalase | 600 U/µl in $ddH_2O$ | Sigma-Aldrich, Vienna, Austria |
| Horse radish peroxidase | 200 U/ml in PBS | Fluka, Sigma-Aldrich, Vienna, Austria |

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| **name** | **stock solution** | **supplier** |
|---|---|---|
| Superscript™ First-Strand Synthesis system | - | Invitrogen, Paisley, UK |
| CytoTox96® Non-Radioactive Cytotoxicity Assay | - | Promega, Madison, WI, USA |
| PureYield™ Plasmid Midiprep System | - | Promega, Madison, WI, USA |
| ViCell® cell viability analyzer | - | Beckman Coulter, Vienna, Austria |
| LAS-3000 Luminescent Image Analyzer | - | Fujifilm, Düsseldorf, Germany |
| BAS-1800II | - | Fujifilm, Düsseldorf, Germany |
| Mini-PROTEAN® 3 Cell system | - | Bio-Rad Laboratories, Hercules, CA, USA |
| Power Supply PowerPac HC | - | Bio-Rad Laboratories, Hercules, CA, USA |
| Mini Trans-Blot® Electrophoretic Transfer Cell system | - | Bio-Rad Laboratories, Hercules, CA, USA |
| LightCycler® 480 real-time PCR system | - | Roche Diagnostics, Vienna, Austria |
| Tecan Genios Pro | - | Tecan, Grödig, Austria |
| Tecan Sunrise | - | Tecan, Grödig, Austria |
| Ultima Gold liquid scintillation fluid | - | PerkinElmer, Waltham, MA, USA |
| Packard TRI-CARB 2100TR liquid scintillation analyzer | - | PerkinElmer, Waltham, MA, USA |
| SPD 1010 SpeedVac Thermo Savant | - | Thermo Scientific, Langenselbold, Germany |

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| **name** | **stock solution** | **supplier** |
|---|---|---|
| FACSCalibur | - | BD Bioscciences, Franklin Lakes, NJ, USA |
| Biostep Gel documentation system DH-30/32 | - | Biostep, Jahnsdorf, Germany |

## 6.2 Abbreviations

**A**

| | |
|---|---|
| A | adenine |
| ADMA | asymmetric dimethyl-L-arginine |
| Akt | protein kinase B |
| AMPK | AMP-activated protein kinase |
| ApoE | apolipoprotein E |
| ATP | adenosine triphosphate |

**B**

| | |
|---|---|
| BH2 | 7,8-dihydrobiopterin |
| BH4 | 5,6,7,8-tetrahydrobiopterin |

**C**

| | |
|---|---|
| C | cytosine |
| $[Ca^{2+}]_i$ | intracellular calcium |
| CaM | calmodulin |
| CaMKII | $Ca^{2+}$/CaM-dependent protein kinase II |
| CaMKKβ | $Ca^{2+}$/CaM-dependent kinase kinase β |
| cAMP | cyclic adenosine monophosphate |
| CAT-1 | cationic amino acid transporter 1 |
| CcOx | cytochrome C oxidase |
| CDK-5 | cyclin-dependent kinase 5 |
| cGK | cGMP-dependent kinase |
| cGMP | cyclic guanosine monophosphate |

**D**

| | |
|---|---|
| DAF-2 | 4,5-diaminofluorescein-2 |
| DAF-T | triazolofluorescein |
| DDAH | dimethylarginine dimethylaminohydrolase |

| | |
|---|---|
| DHA | dehydroascorbate |
| DHFR | dihydrofolate reductase |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | dimethylsulfoxide |
| DPPB | 2-(2,4-dihydroxyphenyl)-5-(E)-propenylbenzofuran |
| DTT | dithiothreitol |

**E**

| | |
|---|---|
| EDRF | endothelium-derived relaxing factor |
| EGCG | epigallocatechin gallate |
| eNOS | endothelial nitric oxide synthase |

**F**

| | |
|---|---|
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |

**G**

| | |
|---|---|
| G | guanine |
| GlcNAc | N-acetylglucosamine |
| GFPR | GTPCH feedback regulatory protein |
| GTP | guanosine-5'triphosphate |
| GTPCH I | GTP cyclohydrolase 1 |

**H**

| | |
|---|---|
| HA-tag | human influenza hemaglutinin-tag |
| HAT-medium | hypoxanthine-aminopterin-thymidine medium |
| HDL | high density lipoprotein |
| HIF-1α | hypoxia-inducible factor 1α |
| HPLC | high-performance liquid chromatography |
| HPRT | hypoxanthine phosphoribosyltransferase |
| Hsp90 | heat-shock protein 90 |
| HUVEC | human umbilical vein endothelial cell |

**I**

| | |
|---|---|
| IBMX | 3-isobutyl-1-methylxanthine |
| ICAM-1 | intercellular adhesion molcule 1 |
| IGF-1 | insulin-like growth factor-1 |
| iNOS | inducible nitric oxide synthase |
| IP3 | inositol triphosphate |
| IRAG | InsP3R-associated cGMP kinase substrate |

**K**

| | |
|---|---|
| KAT | lysin acetyltransferase |
| kDA | kilodalton |

**L**

| | |
|---|---|
| LDH | lactate dehydrogenase |
| L-NAME | L-$N^G$-nitroarginine methylester |
| L-NMMA | $N^G$-monomethyl-L-arginine |
| LPS | lipopolysaccharide |

**M**

| | |
|---|---|
| MCP-1 | monocyte chemotactic protein-1 |
| MnSOD | manganese superoxide dismutase |

**N**

| | |
|---|---|
| NADPH | nicotine adenine dinucleotide phosphate |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NOHLA | Nω-hydroxy-arginine |
| NOS-1 | neuronal nitric oxide synthase |
| NOS-2 | inducible nitric oxide synthase |
| NOS-3 | endothelial nitric oxide synthase |
| NOSIP | eNOS interacting protein |

| | |
|---|---|
| NOSTRIN | eNOS trafficking inducer protein |

**O**

| | |
|---|---|
| OGA | β-N-acetylglucosaminidase |
| OGT | O-linked N-acetylglucosamine transferase |
| oxLDL | oxidized low density lipoprotein |

**P**

| | |
|---|---|
| PAA | polyacrylamide gel electrophoresis |
| PAGE | polyacrylamide |
| PECAM-1 | platelet endothelial cell adhesion molecule, CD31 |
| PI3K | phosphoinositol 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PP1 | protein phosphatase 1 |
| PP2A | protein phosphatase 2A |
| PP2B | protein phosphatase 2B |
| PRD | positive regulatory domain |
| PTPS | 6-pyruvoyltetrahcdropterin synthase |
| PVDF | polyvinylidene difluoride |
| PYK2 | proline-rich tyrosine kinase |

**Q**

| | |
|---|---|
| qPCR | quantitative real-time polymerase chain reaction |

**R**

| | |
|---|---|
| RGS-2 | regulator of G-protein signaling-2 |

**S**

| | |
|---|---|
| SDMA | symmetric dimethyl-L-arginine |
| SDS | sodium dodecyl sulfate |
| SIRT1 | sirtuin 1 |

| | |
|---|---|
| SOCC | store-operated $Ca^{2+}$ channel |
| SR | sepiapterin reductase |
| SVCT | sodium-dependent vitamin C transporter |

**T**

| | |
|---|---|
| T | thymine |
| TGF-β1 | transforming growth factor-β1 |
| TNF-α | tumor necrosis factor-α |
| TRP | transient receptor potential |

**U**

| | |
|---|---|
| UTR | untranslated region |

**V**

| | |
|---|---|
| VDAC-1 | voltage-dependent anion channel 1 |
| VECAM-1 | vascular adhesion molecule-1 |
| VEGF | vascular endothelial growth factor |

## 6.3 Publications

### Original publications

- Ladurner A*, Schmitt CA*, Schachner D, Atanasov AG, Werner ER, Dirsch VM, Heiss EH. Ascorbate stimulates endothelial nitric oxide synthase enzyme activity by rapid modulation of its phosphorylation status. *Free Radical Biology and Medicine*, 52(10):2082-2090, May 2012.

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- Baumgartner L, Sosa S, Atanasov AG, Bodensieck A, Fakhrudin N, Bauer J, Del Favero G, Ponti C, Heiss EH, Schwaiger S, Ladurner A,Widowitz U, Della Loggia R, Rollinger JM, Werz O, Bauer R, Dirsch VM, Tubaro A, Stuppner H. Lignan Derivatives from Krameria lappacea Roots Inhibit Acute Inflammation in Vivo and Pro-inflammatory Mediators in Vitro. *Journal of Natural Products*, 74(8):1779-1786, July 2011.

- Fakhrudin N*, Ladurner A*, Atanasov AG, Heiss EH, Baumgartner L, Markt P, Schuster D, Ellmerer EP, Wolber G, Rollinger JM, Stuppner H, Dirsch VM. Computer-aided discovery, validation, and mechanistic characterization of novel neolignan activators of peroxisome proliferator-activated receptor gamma. *Molecular Pharmacology*, 77(4):559-566, April 2010.

* equal contribution to the study

### Poster presentations

- Ladurner A, Schmitt CA, Schachner D, Atanasov AG, Werner ER, Dirsch VM, Heiss EH. Ascorbate stimulates eNOS enzyme activity by rapid modulation of its phosphorylation status. 3rd EMBO Conference Series on Cellular Signaling & Molecular Medicine, Cavtat, Dubrovnik (Croatia), 2012.

- Ladurner A, Schmitt CA, Schachner D, Atanasov AG, Werner ER, Dirsch VM, Heiss EH. Ascorbate stimulates eNOS enzyme activity by rapid modulation of its phosphorylation status. 77. Jahrestagung der Deutschen Gesellschaft für

Experimentelle und Klinische Pharmakologie und Toxikologie e.v. (DGPT), Frankfurt am Main (Germany), 2011.

- Fakhrudin N, Ladurner A, Atanasov AG, Heiss EH, Baumgartner L, Markt P, Schuster D, Ellmerer E, Wolber G, Rollinger J, Stuppner H, Dirsch VM. Discovery and mechanistic characterization of neolignans as PPARγ agonists. 35th Congress of the Federation of European Biochemical Societies (FEBS), Gothenburg (Sweden), 2010.

- Baumgartner L, Sosa S, Fakhrudin N, Ladurner A, Atanasov AG, Heiss EH, Widowitz U, Schuster D, Rollinger JM, Wolber G, Bauer R, Dirsch VM, Tubaro A, Stuppner H. Evaluation of the antiinflammatory properties of secondary metabolites from Ratanhiae radix. International Symposium: Drugs from Nature Targeting Inflammation, Innsbruck (Austria) 2010.

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## 6.5 Curriculum Vitae

### Personal information

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| Name: | Angela Ladurner |
| Present address: | Am Tabor 11/19, 1020 Wien |
| Date and place of birth: | January 29, 1984 in Bregenz, Austria |
| Nationality: | Austrian |

### Academic education

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|---|---|
| since 12/2008 | PhD thesis at the Department of Pharmacognosy, University of Vienna under the supervision of Univ.-Prof. Dr. Verena Dirsch with the title: "Modulation of the endothelial nitric oxide synthase system by natural compounds"<br>Position as University assistant in training |
| 2002 - 2008 | Studies of Molecular Biology at the University of Vienna.<br>Diploma thesis at the Departement of Microbiology and Immunology under the supervision of Dr. Angela Witte with the title: "Characterization of the inversion reaction of ΦCh1 and the establishment of a transformation system for *Natrialba magadii*" |

### Scientific stays

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| 06/2006 | Internship at the Universität des Saarlandes, under the supervision of Prof. Dr. Elmar Heinzle within the project "Metabolic and Genetic Engineering of *Corynebacterium glutamicum* for the production of L-methionine". |

### Teaching

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|---|---|
| 2008 - 2012 | Assistant in practical trainings for students in botanical microscopy and microbiology courses (Identitäts- und Reinheitsprüfung biogener Arzneimittel, Allgemeine Mikrobiologie) |
| 05/2007, 12/2007, 05/2008 | Tutor in the course "Molekularbiologisches Laborarbeiten", University of Vienna |
| 06/2007, 02/2008, 06,2008 | Tutor in the course "Molekularbiologie II", FH Campus Vienna Molecular Biotechnology |