1. INTRODUCTION

Cardiovascular diseases with hypertension as a significant risk factor are the major contributors to global morbidity and mortality. The prevalence of hypertension appears to be around 30-45% of the population overall and escalates with an increase in age [1]. Environmental and genetical factors seem to play a role in the development of hypertension, but also several secondary factors like chronic kidney diseases, diseases of the thyroid gland, pheochromocytoma, sleep apnea, pharmacotherapy (e.g. corticosteroids), or aortic coarctation can cause hypertension which makes its genesis multifactorial. In only about 10% of the cases a cause can be identified (secondary hypertension), but in more than 90% no etiology can be found and this is referred to as essential hypertension [2]. *Vascular oxidative stress is considered* to be *a pathophysiological factor* that promotes *cardiovascular diseases* like *coronary artery disease, heart failure, diabetes, and hypertension* [3,4]. *It has been suggested, that the impairment of endothelial function at the level of resistance vessels may precede the development of cardiovascular disease and, therefore,* be *an additional risk factor* [5]. *For example, endothelial dysfunction at this level of vessels* has been *identified in normotensive young adults with a family history of hypertension* [6]. *Another clinical study* showed *that the magnitude of the impairment of endothelium function, as measured by flow-dependent vasodilation in the right forearm, may predict the onset of essential hypertension in postmenopausal women* [7]. Though, this study assessed *the dilator response of the brachial artery to increased flow. However, as a recent thorough review* pointed out [8], *hypertension* is *also likely* to induce *endothelial dysfunction associated with increased vascular oxidative stress. Therefore, the authors concluded, that the interconnection between endothelial dysfunction and essential hypertension is two-sided, that is, both may worsen and/or improve the other.*

1.1. Endogenous Nitric Oxide

In 1980, Robert F. Furchgott discovered that blood vessels relax only in response to the vasodilator acetylcholine if the endothelial layer is intact [9]. He postulated that the

This work was published in

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J Hypertens 2017;35(1):76-88. journals.lww.com/jhypertension/

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endothelium must be releasing a substance, which is responsible for this effect. This endothelium-derived relaxing factor (EDRF) was independently identified in 1987 by Louis Ignarro, Robert Furchgott and Salvador Moncada to be nitric oxide (NO) [10]. Two years later, the responsible enzyme was discovered to be the endothelial nitric oxide synthase (eNOS) [11].

1.2. ENDOTHELIAL NO-SYNTHASE

Within the endothelial layer, which lines the inner wall of the vasculature and forms the junction between blood and tissue, eNOS synthesizes NO. In this redox reaction L-arginine is specifically transformed into L-citrulline [12], depending on nicotinamide adenosine diphosphate (NADPH) and essential cofactors like heme, flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), Ca^{2+}/calmodulin, and (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4) (see fig. 1.1) [13].

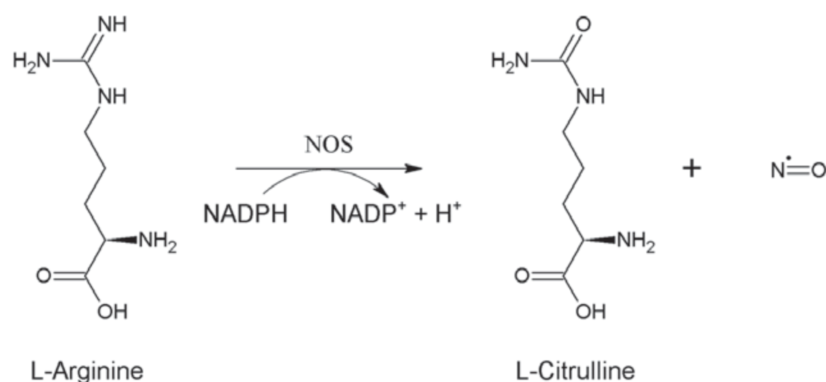

Fig. 1.1: *Scheme on the catalyzed reaction by nitric oxide synthases: L-Arginine is transformed into L-citrulline, and nitric oxide is formed.*

In mammals, three isozymes of NO synthases (NOS) have been identified: inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS). All NOS are enzymes with a reductase domain at the carboxy-terminus and an oxygenase domain at the amino-terminus [14]. After binding of NADPH to the reductase domain [15], electrons are transferred via the cofactors FAD and FMN to molecular oxygen, bound to the iron atom

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in the heme center in the oxygenase domain. Binding of oxygen to the heme iron is enabled by the reduction of Fe^{3+} to Fe^{2+}. The electron flux itself depends on the binding of calmodulin [16]. Molecular oxygen is activated by reduction and reacts with the guanidine group of the substrate L-arginine. The intermediate N^{ω}-hydroxy-L-arginine is formed. In a second reaction cycle this interstate product serves as the substrate and is transferred into L-citrulline under separation of nitrogen from the guanidine group. Thus, NO is released [17].

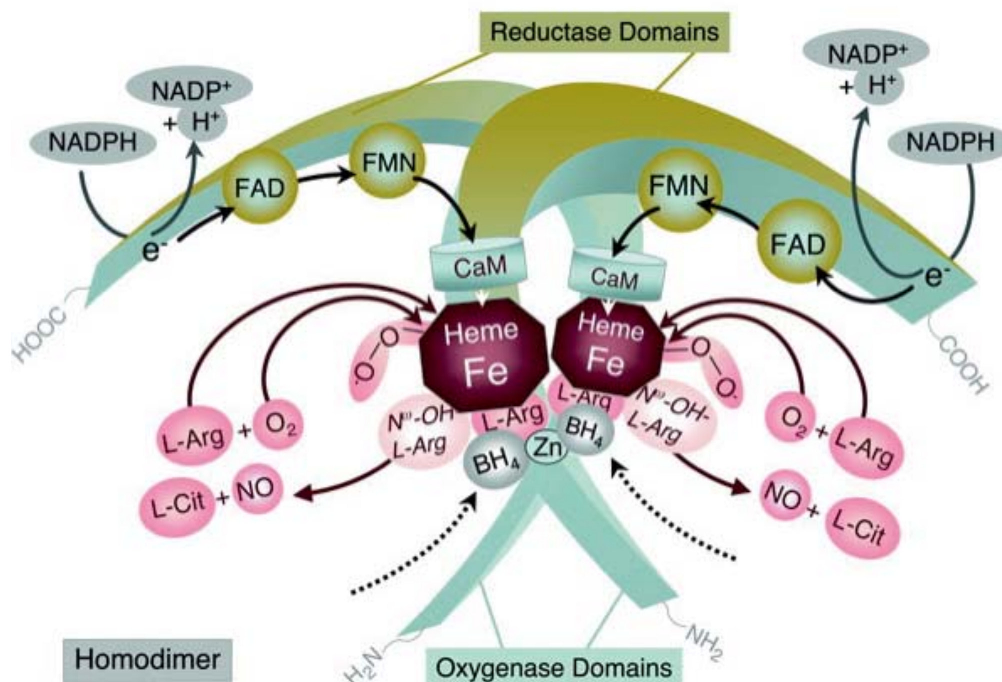

Fig. 1.2: *Scheme on the enzymatic reaction catalyzed by eNOS under normal conditions [18].*

The functional homodimer is stabilized by a zinc-cluster. As fig. 1.3 shows, the central zinc ion is coordinated in its tetrahedral conformation with pairs of symmetrically oriented cysteine residues: cysteines 96 and 101 of each monomer [19].

Within the endothelial cell, eNOS is locked in caveolae. After the influx of Ca^{2+}, eNOS is released [20]. Ca^{2+} binds to calmodulin and this construct to eNOS [21]. This activates

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the electron flux on eNOS, and NO can be released. If caveolin-1 binds to the reductase domain of eNOS, the electron flux is interrupted, and eNOS is inactivated and locked back in caveolae [22]. This step seems to be crucial as in mice with a caveolin-1 small interfering ribonucleic acid (siRNA)-induced knockdown plasma NO concentrations are 2.5 fold higher [23] and, in murine lung vascular endothelial cells with a caveolin-1 knockout, the deficiency leads to a 4-fold increase in NO production [24], both indicating a permanent eNOS activation. A recent study on endothelial-specific caveolin-1 knockout mice revealed increased eNOS expression, an enhancement of the NO-cGMP pathway, suppression of endothelium-dependent hyperpolarization-mediated responses in resistance vessels, coronary microcirculatory dysfunction and cardiac hypertrophy, and enhanced nitrative stress [25].

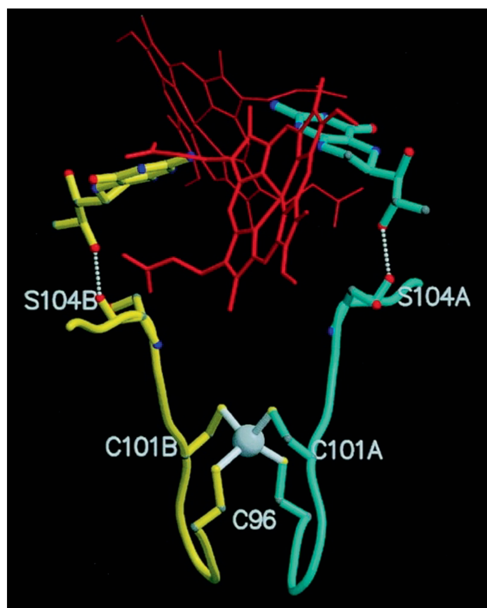

Fig. 1.3: *The ZnS_4 metal center and its relationship with tetrahydrobiopterin (red) [19]. The central zinc ion (grey) coordinates the dimerization two eNOS monomers (yellow and green) via the binding cysteines 96 and 101.*

An essential cofactor of eNOS is BH_4. It is one of the most potent naturally occurring reducing agents and is highly sensitive to oxidation by peroxynitrite [18] and modestly by superoxide anion radical ($O_2^{-\bullet}$) [26]. A lack of BH_4, as occurring in states of oxidative stress, is likely to be a cause for so-called eNOS-'uncoupling' [26]. Crucial for its binding within the oxygenase domain and, thus, the coupling of eNOS is the ability to coordinate two monomers via a Zn-finger [19]. If eNOS acts in the 'uncoupled' state, monomers can

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still bind calmodulin. But no binding of BH_4 or L-arginine is possible and eNOS cannot produce NO [14]. Rather, the electrons leak from their transport within the reductase domain or directly from the oxy-iron complex and are transferred to molecular oxygen, which is reduced to $O_2^{-\bullet}$ [27]. Studies have shown, that in these states, adding BH_4 or enhancing its regeneration by ascorbic acid can rescue the dimerization and, thus, the function of eNOS and shifts the reaction from producing $O_2^{-\bullet}$ back towards that of NO [18].

The expression eNOS 'uncoupling', as widely used in the literature, may mislead the reader, as it is not meant, that an isolated monomer can be active. Still two monomers have to be sterically associated in order to enhance oxidative stress by either an increased degradation of NO by its reaction with $O_2^{-\bullet}$, or a conversion of eNOS from an enzyme generating NO to one that produces $O_2^{-\bullet}$ [14]. In this work, the expression 'uncoupled' is used as in the literature for physiologically occurring states and solely employed in quotation marks to point out on this miswording. To distinguish the state of the genetically modified C101A-eNOS variant used in this study, this is referred to as 'destabilized' (see chapter 1.5).

eNOS is transcriptionally upregulated by certain growth factors (vascular endothelial growth factor, transforming growth factor beta 1) and hormons (insulin, estrogen). Another important stimulus is shear stress caused by the laminar flow of blood along endothelial cells. Additionally, oxygen radicals, e.g. hydrogen peroxide, and hypoxia as well as pharmaceutical drugs (statins) can enhance eNOS expression [28].

The activity of the enzyme is regulated by Ca^{2+}-activated calmodulin, which enforces eNOS to synthesize NO in a pulsatile manner [14]. eNOS can be phosphorylated on several serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. Phosphorylation at Ser1177 activates eNOS by stimulating the electron flow within the reductase domain and increasing Ca^{2+} sensitivity. This can be elicited by shear stress and the activation of protein kinase A [14]. The serine residue at 1177 in humans corresponds to Ser1176 in murine and Ser1179 in bovine species. With elevated intracellular Ca^{2+} concentrations,

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dephosphorylation at Thr495 comes along with an increase of eNOS activity and the binding of calmodulin [14]. The phosphorylation by proline-rich tyrosine kinase 2 on Tyr657 directly inhibits eNOS [29]. In vitro studies have shown that this phosphorylation results in a complete loss of the ability of eNOS to generate L-citrulline, NO or $O_2^{-\bullet}$ [29,30].

1.3. eNOS Signaling

After its formation, the endogenous transmitter NO is able to diffuse freely across membranes and has several effects. The most prominent is vasodilation [20], but it also has an antiaggregatory effect on thrombocytes [31], an antiadhesive on leucocytes [32], and it is supposed to be antiproliferative [33] and antioxidative [34-36].

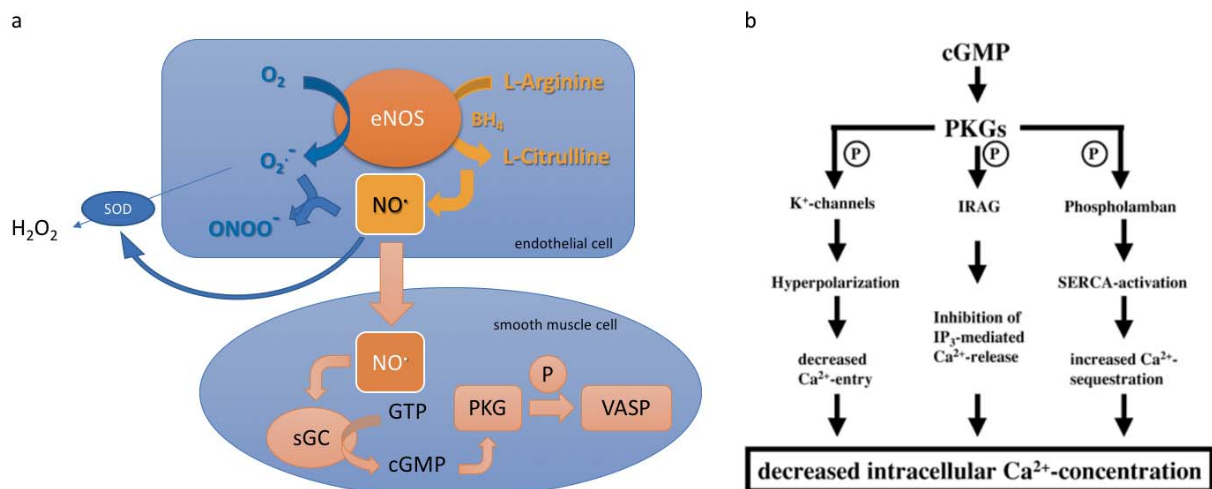

Fig. 1.4: *eNOS and NO/cGMP-signaling pathway: (a) After release of NO by endothelial cells the NO/cGMP-pathway is induced in smooth muscle cells. Here, upon activation of sGC, cGMP is formed, which stimulates cGMP-dependent protein kinases (PKG). One of the enzymes being phosphorylated and thus serving as a marker for eNOS activation is VASP. (b) Vasodilation is triggered by phosphorylation of three important proteins resulting in a decrease of the intracellular Ca^{2+} concentration [36].*

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All these effects including vasodilation are mostly mediated by activation of soluble guanylyl cyclase (sGC). This activation leads to formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), which, in turn, activates cGMP-dependent proteinkinase G (PKG) (see fig. 1.4). This PKGs then phosphorylate different enzymes yielding in the effects mentioned above. cGMP is deactivated by phosphodiesterase V under formation of guanosine monophosphate (GMP). In addition to the classical NO-sGC-cGMP signaling, NO can also exert its regulatory effects via post-translational protein modification and changes in function through S-nitrosylation of cysteine thiols [37] .

1.4. Endothelium-Dependent Vasodilation

One of the effects of released NO upon activation of eNOS is vasodilation. The activation of PKG by cGMP seems to play a central role here: Phosphorylation of either phospholamban, 1,4,5-inositoltriphosphate receptor associated cGMP kinase substrate (IRAG) or potassium channels result in decreased intracellular Ca^{2+} concentration. In dephosphorylated states, phospholamban monomers inhibit sarcoplasmatic reticulum adenosine triphosphatase (SERCA) by binding to its cytoplasmic and membrane domains which causes a Ca^{2+} pump aggregation. Upon phosphorylation by PKG, phospholamban pentamers are formed and the inhibition of SERCA is reversed. This results in an increased sequestration of intracellular Ca^{2+} and in turn diminishes the influx of extracellular Ca^{2+} into the sarcoplasmatic reticulum. By phosphorylation of IRAG, 1,4,5-inositoltriphosphate (IP_3)-mediated Ca^{2+}-release is inhibited. And, by activation of Ca^{2+}-dependent potassium channels, the increase of outward K^+ current is increased which leads to a cell membrane hyperpolarization. Besides, a cGMP-independent effect of NO on potassium channels in smooth muscle cells has been described, too [38]. Taken together, all these mechanisms result in a decrease of intracellular calcium concentrations and a diminishing effect of depolarizing signals, which lead to an impairment of actin/myosin-interactions within smooth muscle cells and finally result in vasodilation. Other effects of vascular NO generation include antiaggregatory,

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antiadhesive, antioxidative and antiapoptotic effects, all of which confer vasoprotection and endothelial dysfunction [36].

1.5. Oxidative Stress

We know that rats with hereditary hypertension have increased $O_2^{-\bullet}$ levels and show an impaired endothelium-dependent relaxation [39]. Already more than 30 years ago, it was postulated that $O_2^{-\bullet}$ seems to be involved in the breakdown of EDRF [40]. The imbalance of $O_2^{-\bullet}$ and NO is associated with endothelial dysfunction. There are indications, that, in this situation, eNOS activity could be reduced, which may partly be dependent on the NO-cGMP-pathway [40].

In resistance vessels, endothelium-dependent vasorelaxation to acetylcholine is different from that of conductance vessels like *aorta or coronary arteries. Resistance vessels possess many options to compensate for a loss of NO produced by eNOS by other endothelium-dependent vasodilators like prostaglandins, by neuronal NOS expression or by endothelium-derived hyperpolarizing factor* [41-44].

Certainly, increased vascular oxidative stress is linked to a conversion of eNOS activity, leading to an 'uncoupled' state in which eNOS produces more $O_2^{-\bullet}$ and less NO. In addition to the deficiency of the cofactor BH_4, mechanisms underlying this process include the depletion of L-arginine, the accumulation of endogenous asymmetrical dimethylarginine, and eNOS-S-glutathionylation [14]. In detail, the catalytic domains of eNOS consist of a flavin-containing NADPH-binding reductase and a heme-binding oxygenase, which is the binding site for the substrate L-arginine and the redox labile cofactor BH_4 [45]. *There are several sources of $O_2^{-\bullet}$ in vascular smooth muscle and endothelial cells* [46].

1.6. eNOS and Blood Pressure

The specific role of endothelial oxidative stress in the regulation of blood pressure before overt hypertension occurs – that is, under otherwise healthy conditions – remains

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uncertain. One study showed that increased $O_2^{-\bullet}$ generation, induced by a very strong eight-fold endothelial-specific overexpression of bovine eNOS and presumably caused by a shortage of the essential cofactor BH_4, does reduce blood pressure, and concomitant overexpression of guanosine triphosphate cyclohydrolase 1 to increase the endothelial generation of BH_4 in a double transgenic strain did change $O_2^{-\bullet}$ levels but had no effect on blood pressure [47]. *However, in both transgenic mouse models, there is clear evidence of an impairment of vascular NO signaling, which* may also have an *impact on blood pressure. For example, the magnitude of hypotension observed in another mouse model characterized by endothelial-specific overexpression of eNOS, that is, a reduction of systolic blood pressure of about 15 mmHg, is already achievable by a 3.3-fold overexpression of bovine eNOS. In this model, no impairment of NO signaling, and no increase of vascular oxidative stress is evident* [37,48].

eNOS significantly contributes to the regulation of blood pressure as, in four different strains of eNOS-deficient mice (eNOS-KO) [49-52], hypertension is the most evident phenotype. It is generally assumed that the lack of vasodilation by endothelial NO, more precisely, the lack of endothelium-dependent NO-induced vasodilation, is an important underlying cause [49,50] for hypertension in eNOS-KO. Obviously, other physiologic systems cannot compensate the absence of eNOS in these animals. Similarly, after treatment with NOS inhibitors like L-N^G-monomethyl arginine or L-NA, rabbits [53], mice [49,54], and humans [53,55] develop hypertension. Among all three NOS isoform, eNOS appears to play the key role in blood pressure regulation, as nNOS-deficient mice are normotensive [56], and triple e/i/nNOS-knockouts have similar hypertension to that in single eNOS- and double e/nNOS-deficient animals [57].

1.7. Aim of the Study

The aim of this study is to investigate the influence of eNOS dimer stability on the regulation of blood pressure. In the states in which eNOS is ‘uncoupled’, one mechanism is that the balance between NO and $O_2^{-\bullet}$ shifts towards $O_2^{-\bullet}$ [26]. *It remains to be*

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elucidated whether a partial impairment of the catalytic function of eNOS and, thus, the generation of endothelial oxidative stress in otherwise healthy conditions might support the development of hypertensive disease states like essential hypertension.

A mutant eNOS was generated in this work group *in which one of the two essential cysteines that are required for the coordination with the central Zn-ion, correct dimer formation and normal activity* [19] *is replaced by alanine [bovine eNOS destabilized by the replacement of cysteine* (Cys) *101 to alanine* (Ala) *(C101A-eNOS)]*. *The expression of this mutant eNOS in human embryonic kidney cells* 293 (HEK 293) *resulted in a substantial reduction of* L-*citrulline formation in cell homogenates and similar data are obtained following the investigation of purified C101A-eNOS. At the same time,* $O_2^{-\bullet}$ *generation detected by electron spin resonance was strongly increased* [58]. *This mutant eNOS was used to generate a novel transgenic mouse model [mice with endothelium-specific overexpression of destabilized C101A-eNOS (C101A-eNOS-tg)] characterized by endothelial-specific overexpression of C101A-eNOS on a C57BL/6 background. In this novel transgenic mouse strain, largely increased vascular oxidative stress* can be observed *but no impairment of vascular NO signaling, suggesting that this transgenic model might help to clarify whether oxidative stress induced by the impairment of the catalytic function of eNOS may have an impact on blood pressure. To accomplish this, another newly generated mouse model characterized by an about 2.4-fold increase in endothelial-specific overexpression of normal bovine eNOS, is used for comparison purpose* (eNOS-tg).

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