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Nitric Oxide Signalling in Vascular Control and Cardiovascular Risk

Annette Schmidt

*Leibniz-Institute of Arteriosclerosis Research at the University of Muenster,
Germany*

1. Introduction

Nitric oxide – a free radical molecule – has been known for many decades, but only since its recognition as endothelium-derived relaxing factor (EDRF) the interest in the molecule has exponentially increased (Moncada, 1991). At the present time NO is an important messenger that regulates numerous functions and also participates in the pathogenesis of various diseases (Lloyd-Jones & Block, 1996). NO is generated from the conversion of arginine to citrulline in a multistep oxidation process by the NO-synthase (NOS), a NADPH-dependent enzyme that requires Calcium-Calmodulin, Flavinadeninedinucleotide, Flavinmononucleotide and Tetrahydro-L-biopterin as cofactors (Förstermann et al., 1994). Three isoforms of NOS have been identified. All isoenzymes, the neuronal NOS (nNOS), the inducible NOS (iNOS) and the endothelial NOS (eNOS) (Liu & Huang, 2008), are homodimers with subunits of 130 – 160 kDa. As major signalling molecule of the vascular system NO is generated by the constitutively expressed eNOS.

2. Endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) function

2.1 eNOS

The endothelium maintains the balance between vasodilation and vasoconstriction. NO generated by eNOS acts via cGMP-dependent pathway in a paracrine manner on neighbouring smooth muscle cells (SMC) diffusing radially from the production site. NO has a half-life of only a few milliseconds *in vivo* (**Tab. 1**) and rapidly reacts with iron of the heme moiety in the active site of the enzyme guanylate cyclase, stimulating it to produce the intracellular cGMP that in turn enhances the release of neurotransmitters resulting in SMC relaxation and vasodilation (**Fig. 1**). Acting via cGMP-independent pathways it is used in part to S-nitrosylation of intracellular or extracellular proteins (Castel & Vaudry, 2001; Mallis et al., 2001; Sun et al., 2001) or by inhibiting intraendothelial generated superoxide anions (Clancy et al., 1992).

2.2 NO functions

Beside its role as vasodilator various other activities of NO have been described: **(I)** NO prevents the expression of cell adhesion molecules thereby preventing leukocytes/monocytes adhering to vascular endothelium and their immigration into the

Compound	Blood/Plasma Levels, nmol/L	T1/2
Nitrate	20 000 - 50 000	5 - 8 hours
Nitrite	100 - 500	1 - 5 minutes
NO	<1	1- 2 milliseconds
Hb-NO	<1 - 200	15 minutes
S-nitroso-Hb	<1 - 200	--
S-nitroso-albumin	1 - 200	--

Table 1. Basal blood/plasma levels and half-lives of some NO-related compounds. Values are approximated from studies in human. For Hb-NO, S-nitroso-Hb and S-nitroso-albumin, no firm agreement about normal values has been reached, and reported values vary greatly. T1/2 for Hb-NO is from pig experiments, values for S-nitroso-Hb and S-nitroso-albumin are unknown (from J.O. Lundberg and E. Weitzberg, *Arterioscler Thromb Vasc Biol*, 2005;25:915-922).

Fig. 1. Nitric oxide signalling axis as therapeutic target in cardiac and vascular disorders. The endothelial eNOS catalyses the formation of NO from L-arginine through two sequential monooxygenation steps. The nitrogen atom of NO is derived from the guanodinogroup of the L-arginine side chain and the oxygen atom of NO derived from molecular oxygen. The cGMP generation in the vascular smooth muscle cell is catalysed by the soluble guanylate cyclase stimulated by the nitric oxide generated by the adjacent endothelial cell.

arterial wall. The monocytes accumulated in the arterial wall can promote local expression or activation of matrix metalloproteases, which decrease the strength of the cap by degrading collagen and other extracellular matrix components. Furthermore, activated macrophages kill neighbouring SMC by lytic damage leading to necrosis or by inducing apoptosis (Kockx et al., 1996, 1998). **(II)** NO reduces the influx of lipoproteins into the vascular wall and inhibits LDL oxidation. **(III)** NO inhibits DNA synthesis (Förstermann et al., 1994) and proliferation of SMC (Li & Förstermann, 2000; Li et al., 2002a). **(IV)** NO released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion (Busse et al., 1987; Radomski et al., 1987). **(V)** NO can react with superoxide anion $O_2^{\cdot -}$ forming the potent peroxynitrite ($ONOO^-$), which causes oxidative damage, nitration and S-nitrosylation of biomolecules. Furthermore, $ONOO^-$ oxidizes the NOS cofactor 5,6,7,8-tetrahydrobiopterin with the consequence of uncoupling NOS from NO synthesis thereby leading NOS to a superoxide producing proarteriosclerotic enzyme (Förstermann, 2006). **(VI)** Exogenous NO released from DETA/NO₂Oate causes overexpression of TGF-beta and extracellular matrix in cultured human coronary smooth muscle cells (A. Schmidt et al., 2003).

2.3 eNOS-independent sources of NO

The generation of NO is not restricted to NO-synthases. An endothelium-independent source of bioactive NO is the ingestion of dietary (inorganic) nitrate. Naturally occurring dietary nitrate (celery, cress, chervil, beetroot, spinach, rucula contain up to 250 mg NO/100 g fresh weight) elevate the tissue and blood plasma level of nitrite via bioconversion in the entero-salivary circulation. When nitrite is acidified, it yields HNO_2 , which decomposes to NO and other nitrogen oxides

Studies have indicated that acid-catalysed nitrite reduction to NO can also take place in blood vessels and tissues already at a moderately low pH and within nitrite concentrations normally present *in vivo*.

The NO generated by eNOS has a half-life ($T_{1/2}$) of 1-2 milliseconds and rapidly oxidizes to nitrate (NO_2^-). Nitrate however is not a final end product of NO metabolism but can be a substrate for NOS-independent regeneration to NO (Benjamin et al., 1994; Lundberg et al., 1994). Therefore other sources of nitrate in mammals can contribute to the formation of NO such as nitrate generated from commensal bacteria in the digestive tract or nitrate present in foodstuff. Thus, in a study of Milkowski (Milkowski et al., 2010) it was shown that the consumption of nitrite- and nitrate-rich food such as fruits, leafy vegetables, and cured meats along with antioxidants can compensate for any disturbance in endogenous NO. Regular intake of nitrate-containing food such as green leafy vegetables may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis. In several studies (Kapil et al., 2010a, 2010b; Tang et al., 2011) it was shown that nitrate supplementation or vegetable intake (such as beetroot juice) causes dose-dependent elevation in plasma nitrite concentration, elevation of cGMP concentration with a consequent decrease in blood pressure and reduction the risk of

ischaemic stroke. The collective body of evidence suggests that food enriched with nitrate and nitrite provide significant health benefits with very little risk. The weak and inconclusive data on the cancer risk of nitrite/nitrate and processed meats are far outweighed by the health benefit of restoring NO homeostasis via dietary nitrite and nitrate (Tang et al., 2011).

3. Regulation of eNOS activity

3.1 Phosphorylation

eNOS synthesizes NO in a pulsatile Ca^{2+} /calmodulin-dependent manner with eNOS activity markedly increasing when intracellular Ca^{2+} increases. Ca^{2+} induces the binding of calmodulin to the enzyme thus increasing the rate of electron transfer from NADPH to heme center (Hemmens & Mayer, 1998). However, eNOS can be activated by other stimuli as increased intracellular Ca^{2+} . The best-established stimulus is the shear stress of flowing blood, which can increase enzyme activity. This activation is mediated by phosphorylation of the enzyme (Fig. 2). The eNOS protein can be phosphorylated on several Ser, Thr and Tyr residues. Two main changes in enzyme function have been found. Phosphorylation of Ser¹¹⁷⁷ stimulates the flux of electrons within the reductase domain and increases the Ca^{2+} sensitivity of the enzyme (Fleming & Busse, 2003). Several protein kinases participate in phosphorylation of eNOS at Ser¹¹⁷⁷. These kinases include Akt, protein kinase A, 5'-AMP activated protein kinase and calmodulin-dependent kinase II. A negative regulatory site for phosphorylation is Thr⁴⁹⁵ under non-stimulated conditions probably by protein kinase C. Thr⁴⁹⁵ interferes with the binding of calmodulin to the calmodulin-binding domain. Dephosphorylation of Thr⁴⁹⁵ is associated with stimuli such as histamine and bradykinine both elevating intracellular Ca^{2+} concentration. Dephosphorylation of Thr⁴⁹⁵ has also been shown to favour eNOS uncoupling (Lin et al., 2003). Other phosphorylation sites including Ser¹¹⁴, Ser⁶³³ and some Tyr residues are not known to have major consequences for enzyme activity (Fleming & Busse, 2003; Fleming, 2010).

eNOS-associated proteins such as caveolin, heat shock protein 90 or eNOS interacting proteins provide the scaffold for the formation of the eNOS protein complex and its intracellular location (Fleming & Busse, 2003).

eNOS levels in endothelial cells can be regulated by changes in eNOS mRNA stability.

3.2 Enhancers of NO availability

Statins. Statins are a group of compounds which lower LDL-cholesterol, are inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A. Beside their lipid lowering property statins improve vascular relaxation, reduce vascular inflammation, reduce oxidative stress, decrease thrombosis and platelet aggregation (E. Schulz et al., 2004; Sowers, 2003). These beneficial effects of statins are in part mediated by an effect on eNOS because they can be blocked by L-NMMA (L-NG-monomethylarginine), an inhibitor of eNOS (John, et al., 1998; Rosenson & Tangney, 1998). Statins increase the expression of eNOS via Rho isoprenylation (Laufs et al., 1998) or posttranslational mechanism (Kureishi et al., 2000).

Superoxide dismutase (SOD). Superoxide dismutase has a key antioxidant role by dismutation of $\text{O}_2^{\cdot-}$ into oxygen and hydrogen peroxide. In humans, three forms of the enzyme are present (SOD1, SOD2 and SOD3). In the cardiovascular system, the action of extracellular SOD3 (Cu-Zn-SOD) lowers $\text{O}_2^{\cdot-}$ and maintains vascular NO levels (Jung et al., 2007).

Fig. 2. Regulation of eNOS activity by intracellular Ca^{2+} and phosphorylation. An increase in intracellular Ca^{2+} (as produced by agonists such as histamine or bradykinin) leads to an enhanced binding of CaM (calmodulin) to the enzyme, which in turn displaces an auto-inhibitory loop and facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. There are several potential phosphorylation sites in eNOS, but most is known about the functional consequences of phosphorylation of Ser¹¹⁷⁷ (human eNOS sequence) in the reductase domain and Thr⁴⁹⁵ (human eNOS sequence) within the CaM-binding domain. In resting endothelial cells, Ser¹¹⁷⁷ is usually not phosphorylated. Phosphorylation is induced when the cells are exposed to fluid shear stress, estrogens, VEGF, insulin, or bradykinin. The kinases responsible for phosphorylation depend on the primary stimulus. Shear stress elicits the phosphorylation of Ser¹¹⁷⁷ by activating protein kinase A (PKA), estrogen and VEGF phosphorylate eNOS mainly via Akt, insulin probably activates both Akt and the AMP-activated protein kinase (AMPK), and the bradykinin-induced phosphorylation of Ser¹¹⁷⁷ is mediated by CaMKII. Phosphorylation of the Ser¹¹⁷⁷ residue increases the flux of electrons through the reductase domain and thus enzyme activity. The Thr⁴⁹⁵ residue of human eNOS tends to be constitutively phosphorylated in endothelial cells. Thr⁴⁹⁵ is a negative regulatory site, and its phosphorylation is associated with a decrease in enzyme activity. The constitutively active kinase that phosphorylates eNOS Thr⁴⁹⁵ is most probably protein kinase C (PKC). The phosphatase that dephosphorylates Thr⁴⁹⁵ appears to be protein phosphatase1. (Figure and legend from U. Förstermann, Pflügers Arch - Eur J Physiol, 2010;459:923-933)

Catalase. Catalase decomposes hydrogen peroxide to water and oxygen. Overexpression of catalase has protective effects in the cardiovascular system such as delayed development of arteriosclerosis (Yang et al., 2004) and inhibition of angiotensin II-induced aortic wall hypertrophy (Zhang et al., 2005).

Glutathion peroxidase (GPx). Several isoenzymes of GPx were found in mammals, the isoenzyme 1 being most abundant. In patients with coronary artery disease the activity of red blood cell GPx1 is inversely associated with the risk of cardiovascular events (Blankenberg et al., 2003). In ApoE-deficient mice, the deficiency of GPx1 leads to arteriosclerotic lesion progression (Torzewski et al., 2007).

Heme oxygenase (HO). In break down of heme CO, biliverdin and free ferrous iron are formed. The biliverdin is converted to bilirubin, which has radical-scavenging properties (Jiang et al., 2006). The carbon monoxide has antiproliferative and anti-inflammatory as well as vasodilatory properties (Morita, 2005).

Thioredoxin (Trx). Thioredoxin seems to exert most of its ROS-scavenging properties through Trx peroxidase (peroxiredoxin), which uses endogenous SH groups as reducing equivalents. Thioredoxin is present in endothelial- and vascular smooth muscle cells. It exerts its ROS-scavenging properties through Trx peroxidase. Trx scavens ROS and nitric peroxide, ONOO⁻ (Yamawaki et al., 2003).

Paraoxonase (PON). The PON family of enzymes acts as vascular antioxidant defense and protects against coronary artery disease (Aviram et al., 1998). The PON1 and PON3 enzymes are synthesized in the liver and circulate in plasma associated with the high-density lipoprotein (HDL) fraction. The capacity of HDL in decreasing HDL and LDL lipid peroxidation largely depends on its PON1 content (Aviram et al., 1998). Deletion of the PON1 gene increases oxidative stress in mouse macrophages and aortae (Rozenberg et al., 2005). The enzyme has been shown to reduce ROS in human endothelial cells, vascular smooth muscle cells, and fibroblasts (Horke et al., 2007).

4. eNOS – A multiple cofactors-dependent enzyme

eNOS is a homodimer protein and consists of two subunits: **(I)** the alpha reductase domain which is able to transfer electrons from NADPH to FAD and FMN and can bind calmodulin for stimulation of electron transfer. It has a limited capacity to reduce molecular oxygen to superoxide (O₂⁻) (Stuehr et al., 2001). **(II)** The oxygenase domain of eNOS is unable to bind the cofactor 5,6,7,8-tetrahydrobiopterin or L-arginine and can not catalyse NO production. The presence of heme allows for NOS dimerization and is the only cofactor that is essential for NOS for the interaction and coupling reductase and oxygenase domains. eNOS monomers are unable to bind the 5,6,7,8-tetrahydrobiopterin or the L-arginine and can not catalyse NO production. Under pathological conditions the molecular oxygen is no longer coupled to L-arginine reduction but results in the production of superoxide. This phenomenon is referred to eNOS uncoupling (Förstermann & Münzel, 2006; Li et al., 2002b).

5. eNOS uncoupling

5.1 Molecular mechanisms leading to eNOS uncoupling

Various mechanisms can contribute to eNOS uncoupling (**Fig. 3**). Their imbalance causes eNOS dysfunction and cardiovascular risk. This has been shown by numerous clinical studies and for experimental animals. **(I)** Inhibition of eNOS activity. A lack or deficiency of eNOS disrupted at the calmodulin binding site resulted in enhanced arteriosclerosis or peripheral coronary arteriosclerosis and aortic aneurism in ApoE/eNOS double knock out mice (Chen et al., 2001; Hodgin et al., 2002; Knowles et al., 2000; Kuhlencordt et al., 2001).

(II) eNOS uncoupling factors such as hypercholesterolemia, diabetes, smoking, hypertension are associated with endothelial dysfunction. Evidence for uncoupling of eNOS has been obtained in endothelial cells treated with LDL (Pritchard et al., 1995) in peroxynitrite-treated rat aorta (Laursen et al., 2001) and in spontaneously hypertensive rats (Li et al., 2006), in human diabetes (Heitzer et al., 2000) and streptozotocin-induced diabetic rats (Hink et al., 2001). (III) Arginine deficiency. L-arginine – the physiological substrate of eNOS – is a constituent amino acid and present in human blood plasma in a concentration of $113.6 \pm 14.6 \mu\text{M}$ (Psychogios et al., 2011). A decrease of L-arginine induced in hypercholesterolemia below physiological levels favours eNOS uncoupling and formation of ROS. Beside L-arginine the asymmetric dimethylated form of arginine (ADMA) is a major

Fig. 3. Scheme of an endothelial NO synthase (eNOS) whose oxygen reduction is uncoupled from NO synthesis. Oxidative stress is associated with endothelial dysfunction. ONOO^- can oxidize BH_4 to biologically inactive products such as trihydrobiopterin radical ($\text{BH}_3^\cdot$) or trihydrobiopterin radical cation protonated at N5 ($\text{BH}_3^\cdot\text{H}^+$). The $\text{BH}_3^\cdot$ radical can be converted to the quinonoid 6,7-[8H]-H2-biopterin (BH_2), which also lacks biological activity. When ONOO^- overwhelms the cell's capacity to re-reduce these products to BH_4 , eNOS “uncouples” and reduces oxygen to O_2^- , but does not synthesize NO anymore. eNOS then contributes to oxidative stress in the cell. (Figure and legend from U. Förstermann, *Pflügers Arch - Eur J Physiol*, 2010;459:923-933)

component of blood plasma in a concentration of 0.4-0.8 μM (Billecke et al., 2009) and acts as an endogenous inhibitor of eNOS. ADMA is formed by dimethylation of protein-bound L-arginine and released by proteolysis. ADMA acts as a local competitor of L-arginine (Cooke, 2004; Maas, 2005). Arginase is an ubiquitous enzyme which catalyses the degradation of arginine to ornithine and urea. Two isoenzymes are found in mammals. Arginase I catalyses the final step of the urea cycle in liver. Arginase II is a mitochondrial enzyme that functions in L-arginine homeostasis and can be dysregulated by ox LDL (Ryoo et al., 2006) resulting in eNOS uncoupling. **(IV)** 5,6,7,8-tetrahydrobiopterin deficiency. 5,6,7,8-tetrahydrobiopterin deficiency causes eNOS dysfunction and uncoupling (Moens & Kass, 2006), if the primary function of 5,6,7,8-tetrahydrobiopterin such as both allosteric and redox function, the improvement, the binding affinity of L-arginine for eNOS and providing the second electron to the heme of eNOS are missing. These alterations have the consequence that the reduction of molecular oxygen still occurs at the heme site of eNOS but oxidation of the guanidine nitrogen of L-arginine is prevented so that the reduced oxygen is converted by the uncoupled eNOS to superoxide instead of NO and citrulline (Gao et al., 2007; Xia et al. 1998). Even the partially oxidized 5,6,7,8-tetrahydrobiopterin - the 7,8-tetrahydrobiopterin (BH_2) - has no eNOS cofactor activity and is unable to prevent superoxide formation of eNOS (Gao et al., 2007). In addition, BH_2 probably competes with BH_4 for eNOS binding. Therefore the ratio BH_4/BH_2 is important for eNOS activity (Shinozaki et al., 1999; Vasquez-Vivar et al., 2002). Apparently a diminished BH_4/BH_2 level rather than BH_4 deficiency is a molecular trigger for eNOS uncoupling (Crabtree et al., 2008). Normally the majority of BH_4 is present in vascular endothelial cells (Antoniades et al., 2007; Katusic, 2001) in a concentration of 1.40 pM/ 10^6 cells. Intracellular BH_4 concentration has been found under hypercholesterolemic conditions thus aortic BH_4 levels are decreased by 50% in hypercholesterolemic ApoE knockout mice compared with wild-type mice (Ozaki et al., 2002), but also discrepant results are described (d'Uscio et al. 2003; d'Uscio & Katusic, 2006) apparently depending on the degree of hypercholesterolemia and differences in the level of oxidative stress. The tissue level of BH_4 is determined by the balance of biosynthesis from GTP via de novo synthesis by GTP cyclo hydrolase (GCH-1) or by the salvage pathway from BH_2 back to BH_4 and degradation by oxidation of BH_4 to BH_2 (T.S. Schmidt & Alp, 2007) - a process that can be rapidly accelerated by peroxynitrite (Landmesser et al., 2003; Laursen et al., 2001; Zou et al. 2002).

The oxidase-mediated stress of BH_4 can be increased by several ROS producing enzyme systems such as NADPH oxidase that plays a major role in vascular cells (Förstermann, 2008; Harrison et al., 2003; Schnabel & Blankenberg, 2007), by xanthine oxidase, cytochrome P450 monooxygenase and enzymes of the respiratory chain. Xanthine oxidase is generated from xanthine dehydrogenase by proteolysis. This enzyme is another potential source of ROS in vascular disease. The enzyme readily donates electrons to molecular oxygen, thereby producing $\text{O}_2^{\cdot-}$ and hydrogen peroxide. Oxypurinol, an inhibitor of xanthine oxidase decreases $\text{O}_2^{\cdot-}$ production and improves endothelium-dependent vascular relaxation to acetylcholine in blood vessels from hyperlipidemic animals (Ohara et al., 1993). This suggests a contribution of xanthine oxidase to endothelial dysfunction in early hypercholesterolemia. Experimental evidence suggests that endothelial cells themselves can express xanthine dehydrogenase (xanthine oxidase) and that this expression is regulated in a redox sensitive way depending on endothelial NADPH oxidase (McNally et al., 2003).

All these cited cofactors required for regulation eNOS activity depend on the physiological transcription and translation of the corresponding genes. These processes, however, are regulated by epigenetics. Epigenetics refer to chromatin-based pathways including three distinct but highly interrelated mechanisms: DNA methylation, Histone density and posttranslational modifications. These factors together offer new perspectives on transcriptional control paradigm in vascular endothelial cells and provide a molecular basis for understanding how the environment impacts the genome to modified function and disease susceptibility (Yan et al., 2010).

5.2 Mechanisms leading to a loss of function of eNOS

Oxidative stress is associated with endothelial dysfunction. Mechanistically, superoxide derived from NADPH oxidases and/or xanthine oxidase may combine with NO formed by a still functional eNOS. This would lead to increased formation of peroxynitrite (Laursen et al., 2001). Peroxynitrite has been shown to oxidize BH₄ to biological inactive products. Significant O₂^{•-} production also occurs when concentrations of L-arginine fall below the levels required to saturate the enzyme. In these circumstances eNOS catalysis the uncoupled reduction to O₂ leading to the production of O₂^{•-} and/or H₂O₂. Whether L-arginine concentration ever becomes critical as a substrate *in vivo* appears questionable since the K_m of eNOS for L-arginine is ~3 μM while the L-arginine plasma concentration is ~100 μM and a ~10-fold accumulation of L-arginine within cells (Closs et al., 2000).

5.3 eNOS uncoupling in arteriosclerosis

Under cardiovascular risk factors such as diabetes, hypertension, smoking, the enzymatic reduction of molecular oxygen by eNOS is no longer used for L-arginine conversion to citrulline and NO, but the uncoupling of oxidase and reductase chain of eNOS produced ROS via the NADPH domains. The cardiovascular risk factors initiate the eNOS uncoupling and this can occur before arteriosclerotic lesions can be detected. The eNOS uncoupling can be triggered by various mechanisms which include BH₄ deficiency, shortage of L-arginine or HSP 90, inhibitory phosphorylation of eNOS on Thr⁴⁹⁵ (see above) eNOS redistribution to the cytosolic fraction of the cell, oxidation of the zinc-thiolate cluster in eNOS or elevated ADMA levels (Sud et al. 2008). Among all of these mechanisms the reaction BH₄ to BH₂ is probably a dominant factor, and BH₄ deficiency seems to be the primary cause for eNOS uncoupling in pathophysiology. Some researchers have postulated that eNOS may exist in two separate pools: a coupled form and an uncoupled form. The coupled enzyme is associated with the membrane and is readily accessible to the "signalome" for activation and NO production, whereas the uncoupled enzyme may reside in the cytosol and produces superoxide (Gharavi et al., 2006; Sullivan et al., 2006). In eNOS overexpressing mice for example, there is clear evidence for eNOS uncoupling (i.e. eNOS-mediated ROS production). In the same mice, however, NO-generating activity is elevated 2-fold when compared with wild-type mice (the total eNOS protein levels are elevated 8-fold) (Bendall et al., 2005). Thus, it is possible that coupled eNOS and uncoupled eNOS may exist in the same tissue at the same time.

The principle mechanisms of vascular protection by eNOS-derived NO and the consequences of endothelial dysfunction and the concomitant eNOS uncoupling are listed in **Tab. 2**.

Table 2. The vaso-protective effect of eNOS is not restricted to the control of arterial dilation and constriction. The NO released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. The expression and formation of the alpha and beta component of various integrins including the cell adhesion molecules ICAM and VCAM can also be inhibited and reduce the transendothelial migration of macrophages and T-lymphocytes known to be an early event in the development of arteriosclerosis and characteristic for its inflammatory phases. The uncoupled eNOS leads to an excessive production of superoxide ($O_2^{\cdot-}$) and in turn to the formation of highly toxic peroxynitrite (Förstermann & Münzel, 2006).

6. eNOS-independent production of reactive oxygen species in vascular disease

Beside the eNOS there are several enzymes that can produce ROS in the endothelial cells: NADPH oxidase, xanthine oxidase, and enzymes of the mitochondrial respiratory chain are of major importance.

NADPH oxidases. Several isoforms of ROS producing NADPH oxidase are present and active in the vascular wall. In arteriosclerotic arteries the NADPH oxidase subunits NOX 2 and NOX 4 (Sorescu et al., 2002) have been identified.

Xanthine oxidase (XO). Increased cholesterol levels have been shown to stimulate the release of xanthine oxidase from the liver into the circulation. The circulating xanthine oxidase than can associate with endothelial glycosaminoglycans (White et al., 1996) however endothelial cells themselves can express xanthine oxidase and the expression is regulated in a redox sensitive pathway depending on endothelial NADPH oxidase (McNally et al., 2003) (Fig. 4).

Respiratory chain of the mitochondria. The molecular oxygen is consumed by mitochondria thereby forming $O_2^{\cdot-}$. Evidence has been provided that some cardiovascular diseases are associated with mitochondrial dysfunction (Ramachandran et al., 2002) and the mitochondrial production of ROS may be linked to the development of early arteriosclerotic lesions.

Fig. 4. XO and NOS are capable of generating either NO or superoxide (O_2^-) depending on the conditions. When the supply of L-arginine and oxygen is good, NOS makes NO, whereas the same enzyme may generate considerable amounts of superoxide when L-arginine or cofactors are limited. XO generates superoxide, for example, during reperfusion after ischemia, whereas nitrite reduction to NO occurs preferentially during hypoxia. NO generation from XO can be beneficial and works as a backup system to supply NO during hypoxia when NO synthesis from NOS is compromised. Detrimental effects of these 2 enzyme systems can also be foreseen, for example, in a situation in which NO and superoxide are generated simultaneously and react to form potentially harmful peroxynitrite. (Figure and legend from J.O. Lundberg and E. Weitzberg, *Arterioscler Thromb Vasc Biol*, 2005;25:915 – 922)

7. Factors protecting against eNOS uncoupling and oxidative stress

7.1 Nitric oxide donors

NO-delivering drugs (NO donors) are used for their potential therapeutic benefit in coronary heart disease risk patients (D.J. Lefer & A.M. Lefer, 1988) by increasing coronary blood flow and dilating coronary arteries. Several studies have described the action of NO donors on vascular smooth muscle cells (Sarkar et al., 1997; A. Schmidt et al. 2003; Young et al., 2000). The pathway leading to NO formation differs among individual NO donor classes: indirect NO donors such as organic nitrates (nitroglycerol, isosorbide mononitrate, isosorbide dinitrate) require enzymatic catalysis, other NO donors require interaction with thiols to release NO, some have to undergo oxidation or reduction. In contrast, direct NO donors generate NO non-enzymatically. Examples are nicorandil, SIN-1 (the active metabolite of molsidomine) and the group of 1-substituted diazen-1-ium-1,2-diols that releases NO spontaneously with a half-life from minutes to hours (Mooradian et al., 1995).

7.2 The NO donor DETA/NONOate

The compound (Z)-1-[2-Aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (in the following detNO) belongs to the class of direct NO donors. Under cell culture conditions detNO releases spontaneously NO with a half-life of about 20 h at 37° C in a strictly first order reaction (Hrabie et al., 1993; Keefer et al., 1996; Mooradian et al., 1995), thereby

disintegrating to two NO and diethylenetriamine. Diethylenetriamine, the byproduct of detNO disintegration, is known to be ineffective (Mooradian et al., 1995; Sarkar et al., 1997). detNO has been successfully used (Boyle et al., 2002; Ishimaru et al., 2001; A. Schmidt et al., 2003). In experimental studies (A. Schmidt et al., 2008) on cultured endothelial cells exogenously applied NO released from the NO donor detNO has a dual function in the regulation of eNOS expression. During short-term exposure of endothelial cells, exogenous detNO enhances the phosphorylation of the protein kinase Akt that in turn activates eNOS of endothelial cells by increasing its phosphorylation leading to a higher release of endogenous NO.

Phosphorylation can be achieved by exposure of human vascular endothelial cells to 150 $\mu\text{mol/L}$ detNO. In short-term experiments in Western blot analysis detNO shows a clear increase of eNOS phosphorylation at Ser¹¹⁷⁷ after a short lag phase, detectable 20 min after detNO addition. The phosphorylation is mediated by the protein kinase Akt that is converted into p-Akt within 10 min after addition of detNO in a concentration-dependent manner. The phosphorylated Akt increases in turn Ser¹¹⁷⁷ phosphorylation of eNOS. This phosphorylation cascade could be reverted by preincubation of the cells with the PI-3 kinase inhibitor LY294002 that prevents phosphorylation of both Akt and eNOS. Thr⁴⁹⁵ is constitutively phosphorylated in all endothelial cells (Fleming & Busse, 2003) and is a negative regulatory site, i.e. phosphorylation leads to a decrease of eNOS activity. The release of endogenous NO in response to exogenous detNO was confirmed by L-[2,3,4,5-³H]arginine as indicator. The eNOS-mediated conversion of [³H]arginine to NO and [³H]citrulline was measured and the results are given in [³H]citrulline equivalents. A statistically significant increase of endogenous NO production after 20 and 30 min exposure to detNO is shown. N-nitro-L-arginine methyl ester HCl (NAME), a competitive NOS inhibitor, verifies the reaction conditions of the assay. Taking this reaction sequence into account, the effect of the NO donor could be considered partially a trigger for the acceleration of endogenous NO production that finally effects vasodilation via the physiologic pathway. This leads to the hypothesis of a potential switch from an exogenously applied to an endogenously generated NO stimulation (Fig. 5).

7.3 Long-term application of detNO and other NO donors

In contrast an exposure of endothelial cells to detNO for 24 and 48 h reduces the eNOS protein content as compared with controls. Densitometry revealed a reduced eNOS protein content after 24 h and 48 h. Real-time RT-PCR confirmed the reduced transcription of eNOS-specific mRNA. For direct determination of the reduced eNOS enzyme activity after long-term exposure to detNO, [2,3,4,5-³H]arginine was added to the culture medium. The radioactivity of [³H]citrulline formed by the NADPH-dependent NOS oxidoreductase is directly proportional to the NO produced and released by the endothelial cells. Under these conditions the results show a significant reduction of NO production expressed as [³H]citrulline equivalents in accordance to the reduced Ser¹¹⁷⁷ phosphorylation of eNOS. Taken together, these results emphasize a limitation of NO donors as long-term therapeutics owing to the inhibition of eNOS synthesis. However, whether exogenous NO donors are operative and effective in a similar way also in humans is still uncertain. In numerous clinical studies the outcome of repeated administration of indirect or direct NO donors to patients with coronary artery disease was ambiguous and the potential benefit of long-acting nitrates has remained controversial. Pathways leading to NO formation differ significantly among individual NO donor classes. In the Fourth International Study of

Fig. 5. Scheme of the new mechanism of action of the NO-donor DETA/NONONOate in cultured human vascular endothelial cells. During short-term exposure the exogenous NO donor enhances the phosphorylation of the protein kinase Akt (PKB) that in turn activates eNOS of the endothelial cells by increasing its phosphorylation of Ser¹¹⁷⁷ leading to a higher release of the physiological endogenous NO as judged by the conversion of [³H]arginine to [³H]citrulline. The NOS-released NO reaches the smooth muscle cell and effects vasodilation.

Infarct Survival (ISIS-4), there was no significant reduction in five-week mortality and no survival advantage (ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group, 1995). Chronic administration of long acting nitrates in patients with healed myocardial infarction resulted in an increased number of patients with cardiac events (Ishikawa et al., 1996) and an increased risk of cardiac deaths occurred in CAD patients with long acting nitrates (Nakamura et al., 1999). Furthermore, a study on 19 healthy volunteers documented that isosorbide mononitrate given over 7 days impaired endothelial function due to formation of free radicals (Thomas et al., 2007). In total, epidemiological evidence indicates that chronic administration of long acting nitrates increase rather than decreases fatal and non-fatal events (Ishikawa et al., 1996; Nakamura et al., 1999). This view is confirmed by experiments on human vascular endothelial cells, which show detNO-induced cell cycle arrest and hypertrophy. Cultured quiescent EC released from the G₀-phase by seeding at a low density re-enter the cell cycle and proliferate up to confluence. In this phase detNO causes a dose-dependent suppression of proliferation of EC indicated by a decreased incorporation of [³H]thymidine and a cell cycle arrest. The antiproliferative effect of detNO was associated with a remarkable increase of cell protein content that continued up to a 2-3-fold amount of control cells within 3 days while the cell number indicates an inhibition of cell proliferation and shows neither increase nor decrease. The elevated total cell protein was the result of *de novo* synthesis indicated by measurements of [³H]leucine incorporation into total cellular protein. After 48 h incubation of subconfluent cultures in the presence of [4,5-³H]leucine the incorporated radioactivity was 24.8×10^3 dpm/ 10^5 control cells and 34.6×10^3 dpm/ 10^5 cells exposed to detNO. The concomitant upregulation of p21 refers to a block at the G₁-phase of the cell cycle. The detNO-induced metabolic alterations convert the cells into a hypertrophic phenotype. Measurements of cell volume show an increase from 2.49 ± 0.18 up to 3.38 ± 0.36 (200 μ mol/L detNO) fL /cell.

The inhibition of proliferation is cytostatic but not cytotoxic as evaluated by cell death determination and is reversible. A quantitative determination of mono- and oligonucleosomes revealed no significant apoptotic cell death in detNO-pretreated cells. When the medium of detNO-induced growth-arrested cells is replaced by a standard medium, cell proliferation recovers within the following 48 h with continuous increase of cell number.

7.4 Antioxidant compounds potentially protecting against vascular oxidative stress

Important antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, heme oxygenase (HO), and the thioredoxin (Trx) peroxidase and perhaps also paraoxonases (PON) (see Chapter 3.2).

Pentaerythritol tetranitrate (PETN) is a NO donor that does not induce significant nitrate tolerance and reduces oxidative stress (probably by inducing heme oxygenase). **Sepiapterin** can be reduced in cells by sepiapterin reductase (SR) to 7,8-BH₂, sepiapterin reductase catalyses the following reaction

Midostaurin, betulinic acid and ursolic acid upregulate eNOS and concomitantly decrease NADPH oxidase expression (Li & Förstermann, 2009).

AVE9488 and AVE3085, a new class of eNOS enhancers, upregulated the promotor activity of eNOS *in vitro* and *in vivo*. Application to wild type and ApoE-knockout mice over 12 weeks enhanced vascular eNOS expression at mRNA and protein level (Xue et al., 2010). The hybrid NO-releasing prodrug PABA/NO can be stabilized as nanoparticle with significant stability in mice circulation over 24h (Kumar et al., 2010).

Nebivolol - a NO-releasing beta-blocker induced a consistent increase of aortic eNOS expression rabbits receiving high-cholesterol diet (de Nigris et al., 2008). NO-releasing S-nitrosothiol-modified xerogels are capable of generating NO for up to 2 weeks (Riccio et al., 2009). These new generation of NO donors might be a rational approach to develop a new generation of antiatherogenic and anti-inflammatory NO donors. AVE9488 and AVE3085 are eNOS transcription enhancers that reverse eNOS uncoupling and preserve eNOS functionality.

Angiotensin II type 1-receptor blockers (ARBs), estrogens and erythropoietin (EPO) enhance (6R)-5,6,7,8-BH₄ synthesis by stimulating GCH1 expression or activity (Li & Förstermann, 2009.)

Angiotensin-converting enzyme (ACE) inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor aliskiren prevent (6R)-5,6,7,8-BH₄ oxidation by decreasing the expression and/or activity of NADPH oxidase.

8. Clinical implications

Cardiovascular risk factors cause oxidative stress that alters the endothelial cells capacity and leads to endothelial dysfunction. The term „endothelial dysfunction“ is used to refer to an incompetence of endothelial cell-dependent vasorelaxation resulting from eNOS

uncoupling but a molecular or biochemical basis for biomarkers indicating uncoupled eNOS has not been established. A biomarker is a characteristic that is objectively measured and evaluated as an indicator for normal or pathogenic processes or pharmacological response to a therapeutic intervention. As biomarkers for cardiovascular diseases oxLDL, CRP, IL-6, fibrinogen, TNF-alpha, MMP-9, MPO and cell adhesion molecules have been proposed (Vasan, 2006). Indirect biomarkers for eNOS uncoupling are a number of pharmaceuticals that have been shown to act as vaso-protective agents. Such agents listed by Förstermann (Förstermann, 2010) are: pentaerythritol tetranitrate, a NO donor that does not induce significant nitrate tolerance and reduces oxidative stress probably by inducing heme oxygenase 1, L-arginine stimulates NO release from eNOS, folic acid may improve eNOS functionality by stabilising BH₄ and stimulating the endogenous regeneration of BH₂ back to BH₄, sepiapterin can be reduced in cells by sepiapterin reductase to BH₂ and further dihydrofolate reductase to form BH₄, midostaurin, betulinic acid and ursolic acid upregulate eNOS and concomitantly decrease NADPH oxidase expression, AVE9488 and AVE3085 are eNOS transcription enhancers that reverse eNOS uncoupling and preserve eNOS functionality, statins, angiotensin II type 1-receptor blockers, estrogens and erythropoietin enhance BH₄ synthesis by stimulating GTP cyclohydrolase1 expression or activities. Statins, angiotensin converting enzyme inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor prevent BH₄ oxidation by decreasing the expression and/or activity of NADPH oxidase. All these compounds are secondary biomarkers indicating a pharmacological response to a therapeutic intervention.

Clinically, endothelial function can be assessed by invasive or non-invasive techniques (for review see Esper et al., 2006). These techniques evaluate the endothelial functional capacity depending on the amount of NO produced and the resulting vasodilation effect. The percentage of vasodilation with respect to the basal value represents the endothelial functional capacity. A non-invasive technique most often used is the transient flow-modulate endothelium-dependent post-ischemic vasodilation performed on conductance arteries such as the brachial, radial or femoral arteries. This vasodilation is compared with the vasodilation produced by NO donors. The vasodilation is quantified by measuring the arterial diameter with high-resolution ultrasonography. Laser-Doppler techniques are used to consider tissue perfusion. There is no doubt that endothelial dysfunction contributes to the initiation and progression of arteriosclerosis and could be considered an independent vascular risk factor.

9. Conclusion

Nitric oxide produced in vascular endothelial cells by the nitric oxide synthase is a major signalling molecule for maintaining vascular homeostasis. The nitric oxide synthase - constitutionally expressed by endothelial cells - is a dimeric enzyme molecule depending on multiple cofactors for its physiological activity and optimal endothelial function. Any imbalance of reductase and NADPH oxygenase or deficient supply of the enzyme substrate L-arginine or of cofactors leads to an upregulation of endothelial nitric oxide synthase and oxygenase activity with the consequence of an uncoupling of the nitric oxide synthase and production of detrimental reactive oxygen species and/or highly toxic peroxynitrate instead of nitric oxide. The resulting endothelial dysfunction implies a high cardiovascular risk. Several drugs reverting endothelial nitric oxide synthase uncoupling and/or improving endothelial dysfunction are in clinical use. Nitric oxide delivering drugs (NO donors) show

potential therapeutical benefit and are used to relief or prevent acute episodes of angina pectoris by activating the endothelial nitric oxide synthase – a new mechanism found for the NO donor DETA/NONOate. However, a long-term administration of NO donors has been found to reduce endothelial nitric oxide synthase of endothelial cells drastically (in cell culture experiments). This could be the basis for development of a new generation of NO donors that mimics the low continuous pulsatile stress-induced release of endogenous nitric oxide.

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11. References

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Cardiovascular risk factors contribute to the development of cardiovascular disease from early life. It is thus crucial to implement preventive strategies addressing the burden of cardiovascular disease as early as possible. A multidisciplinary approach to the risk estimation and prevention of vascular events should be adopted at each level of health care, starting from the setting of perinatology. Recent decades have been marked with major advances in this field, with the emergence of a variety of new inflammatory and immune-mediated markers of heightened cardiovascular risk in particular. The current book reflects some of the emerging concepts in cardiovascular pathophysiology and the shifting paradigm of cardiovascular risk estimation. It comprehensively covers primary and secondary preventive measures targeted at different age and gender groups. Attention is paid to inflammatory and metabolic markers of vascular damage and to the assessment of vascular function by noninvasive standardized ultrasound techniques. This is a must-read book for all health professionals and researchers tackling the issue of cardiovascular burden at individual and community level. It can also serve as a didactic source for postgraduate medical students.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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