12

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, Flavinmononcleotide 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-lifes 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).

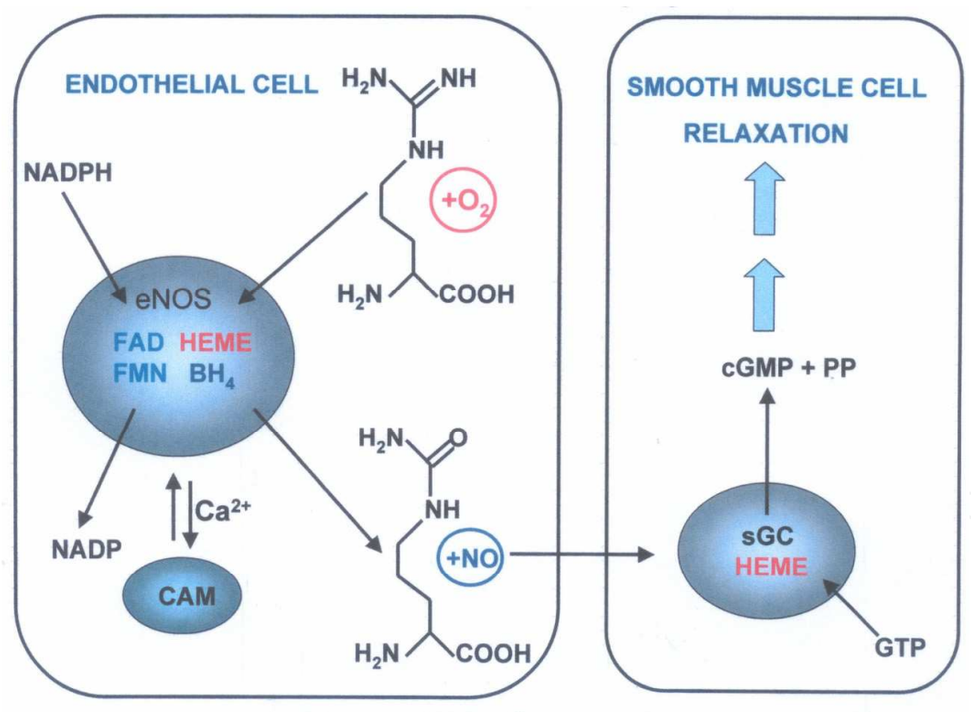


Fig. 1. Nitric oxide signalling axis as therapeutic target in cardiac and vascular disorders. The endothelial eNOS catalyses the formation of NO from L-arginine through two sequential monooxygenation steps. The nitrogen atom of NO is derived from the guanodinogroup of the L-arginine side chain and the 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 matrixmetalloproteases, 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/NONOate 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 und 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

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \quad (1)$$

$$2\,HNO_2 \rightarrow N_2O_2 + H_2O \quad (2)$$

$$N_2O_2 \rightarrow NO + NO_2 \quad (3)$$

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 mammalians 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 meals 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 Ser^{1177} 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 Ser^{1177}. These kinases include Akt, protein kinase A, 5′-AMP activated protein kinase and calmodulin-dependent kinase II. A negative regulatory site for phosphorylation is Thr^{495} under non-stimulated conditions probably by protein kinase C. Thr^{495} interferes with the binding of calmodulin to the calmodulin-binding domain. Dephosphorylation of Thr^{495} is associated with stimuli such as histamine and bradykinine both elevating intracellular Ca^{2+} concentration. Dephosphorylation of Thr^{495} has also been shown to favour eNOS uncoupling (Lin et al., 2003). Other phosphorylation sites including Ser^{114}, Ser^{633} 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 $O_2^{-\cdot}$ 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 $O_2^{-\cdot}$ and maintains vascular NO levels (Jung et al., 2007).

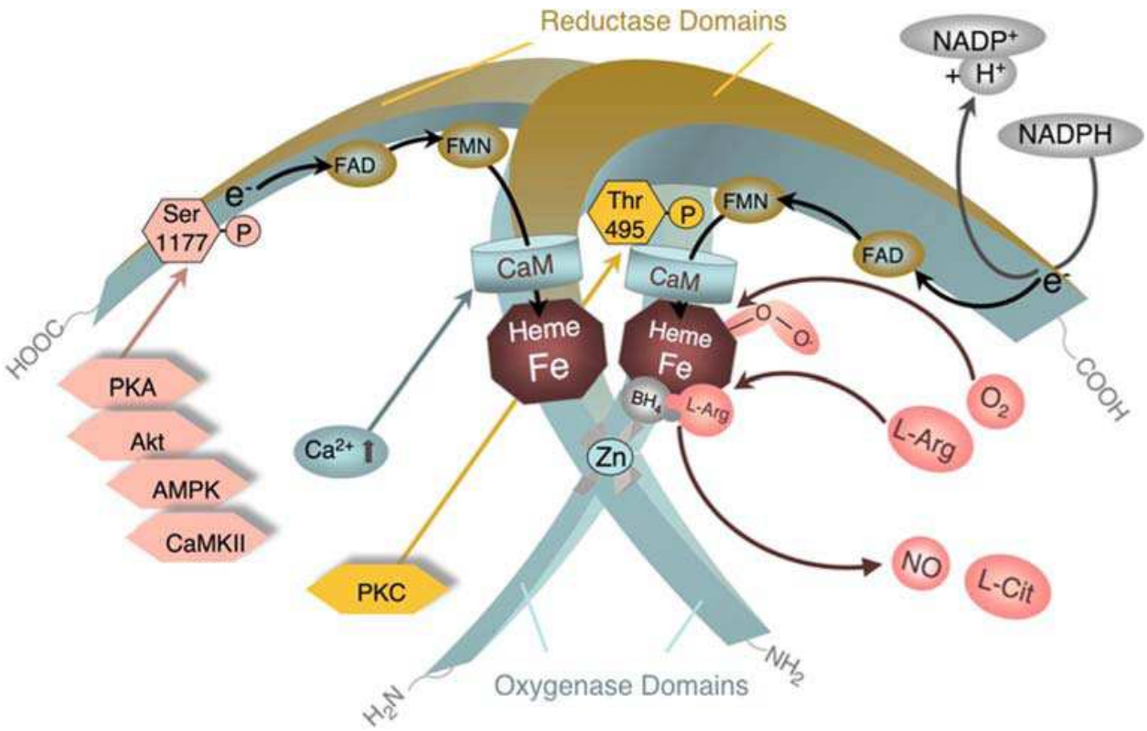


Fig. 2. Regulation of eNOS activity by intracellular Ca^{2+} and phosphorylation. An increase in intracellular Ca^{2+} (as produced by agonists such as histamine or bradykinin) leads to an enhanced binding of CaM (calmodulin) to the enzyme, which in turn displaces an auto-inhibitory loop and facilitates 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 Ser^{1177} (human eNOS sequence) in the reductase domain and Thr^{495} (human eNOS sequence) within the CaM-binding domain. In resting endothelial cells, Ser^{1177} 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 Ser^{1177} 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 Ser^{1177} is mediated by CaMKII. Phosphorylation of the Ser^{1177} residue increases the flux of electrons through the reductase domain and thus enzyme activity. The Thr^{495} residue of human eNOS tends to be constitutively phosphorylated in endothelial cells. Thr^{495} is a negative regulatory site, and its phosphorylation is associated with a decrease in enzyme activity. The constitutively active kinase that phosphorylates eNOS Thr^{495} is most probably protein kinase C (PKC). The phosphatase that dephosphorylates Thr^{495} 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 ($O_2^{-\cdot}$) (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 inbalance 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±14.6 μM (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

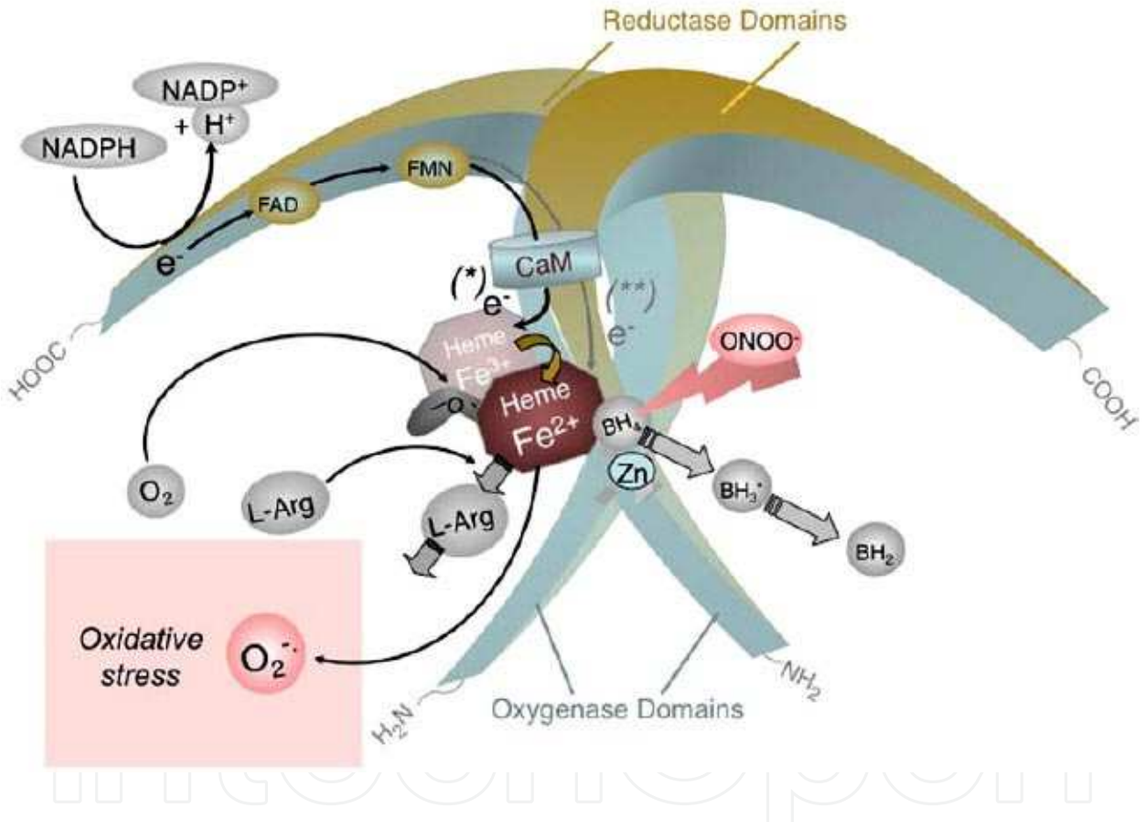


Fig. 3. Scheme of an endothelial NO synthase (eNOS) whose oxygen reduction is uncoupled from NO synthesis. Oxidative stress is associated with endothelial dysfunction. ONOO− can oxidize BH_4 to biologically inactive products such as trihydrobiopterin radical (BH_3 ·) or trihydrobiopterin radical cation protonated at N5 (BH_3 ·H^+). The BH_3 · 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 $O_2^{-\cdot}$ and hydrogen peroxide. Oxypurinol, an inhibitor of xanthine oxidase decreases $O_2^{-\cdot}$ 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. Epigentics 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 peroxinitrite (Laursen et al., 2001). Peroxynitrite has been shown to oxidize BH_4 to biological inactive products. Significant $O_2^{-\cdot}$ 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 O_2 leading to the production of $O_2^{-\cdot}$ and/or H_2O_2. 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 BH_4 deficiency, shortage of L-arginine or HSP 90, inhibitory phosphorylation of eNOS on Thr^{495} (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 BH_4 to BH_2 is probably a dominant factor, and BH_4 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.**

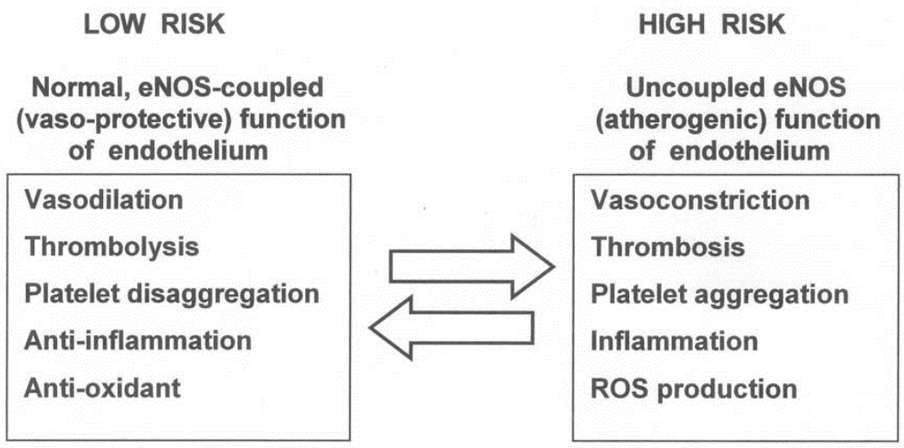


Table 2. The vaso-protective effect of eNOS is not restricted to the control of arterial dilation and constriction. The NO released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. The expression and formation of the alpha and beta component of various integrins 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.

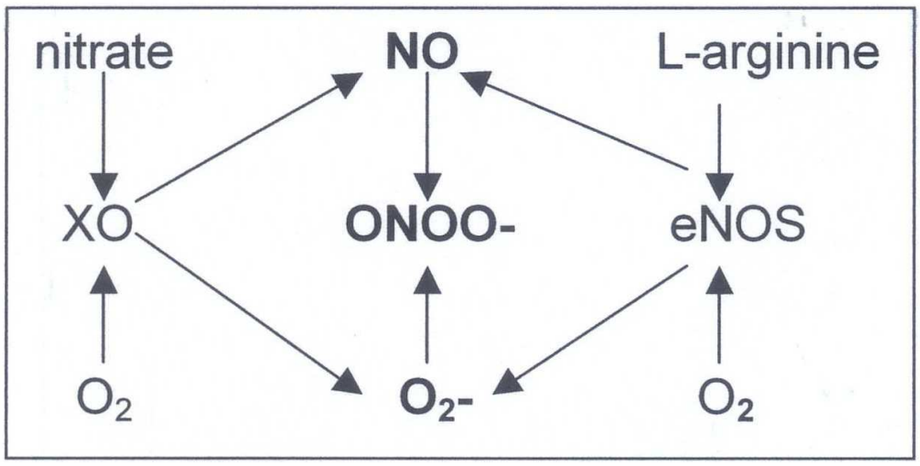


Fig. 4. XO and NOS are capable of generating either NO or superoxide (O_2) depending on the conditions. When the supply of L-arginine and oxygen is good, NOS makes NO, whereas the same enzyme may generate considerable amounts of superoxide when L-arginine or cofactors are limited. XO generates superoxide, 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-diolates 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 diethylentriamine. Diethylentriamine, the byproduct of detNO disintegration, is known to be effectiveless (Mooradian et al., 1995; Sarkar et al., 1997). detNO has been successful 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 µmol/L detNO. In short-term experiments in Western blot analysis detNO shows a clear increase of eNOS phosphorylation at Ser^{1177} 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 Ser^{1177} 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. Thr^{495} 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-^{3}H]arginine as indicator. The eNOS-mediated conversion of [^{3}H]arginine to NO and [^{3}H]citrulline was measured and the results are given in [^{3}H]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-^{3}H]arginine was added to the culture medium. The radioactivity of [^{3}H]citrulline formed by the NADPH-dependent NOS oxidoreductase is direct 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 [^{3}H]citrulline equivalents in accordance to the reduced Ser^{1177} 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 were 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

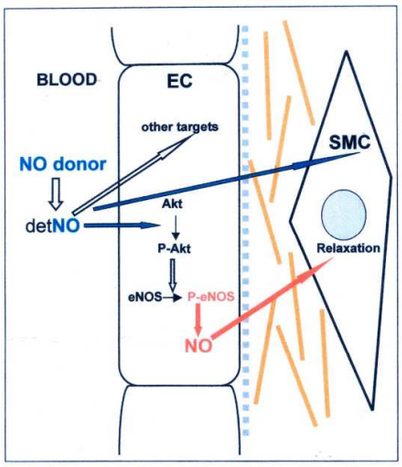


Fig. 5. Scheme of the new mechanism of action of the NO-donor DETA/NONOate in cultured human vascular endothelial cells. During short-term exposure the exogenous NO donor enhances the phosphorylation of the protein kinase Akt (PKB) that in turn activates eNOS of the endothelial cells by increasing its phosphorylation of Ser^{1177} leading to a higher release of the physiological endogenous NO as judged by the conversion of [^{3}H]arginine to [^{3}H]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 G_o-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 [^{3}H]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 [^{3}H]leucine incorporation into total cellular protein. After 48 h incubation of subconfluent cultures in the presence of [4,5-^{3}H]leucine the incorporated radioactivity was 24.8 x 10^3 dpm/10^5 control cells and 34.6 x 10^3 dpm/10^5 cells exposed to detNO. The concomitant upregulation of p21 refers to a block at the G_1-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 µmol/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-BH_2, sepiapterin reductase catalyses the following reaction

$$\text{7,8-dihydrobiopterin} + NADP^+ \rightleftharpoons \text{tetrahydrobiopterin} + NADPH + H^+$$

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-BH_4 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-BH_4 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 BH_4 and stimulating the endogenous regeneration of BH_2 back to BH_4, sepiapterin can be reduced in cells by sepiapterin reductase to BH_2 and further dihydrofolate reductase to form BH_4, 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 BH_4 synthesis by stimulating GTP cyclohydrolase1 expression or activities. Statins, angiotensin converting enzyme inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor prevent BH_4 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 peroxinitrate 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.

10. Acknowledgment

The author wishes to thank Prof. Dr. E. Buddecke, Muenster, for critical discussion and revising the manuscript.

11. References

Antoniades, C., Shirodaria, C., Crabtree, M., Rinze, R., Alp, N., Cunnington, C., Diesch, J., Tousoulis, D., Stefanadis, C., Leeson, P., Ratnatunga, C., Pillai, R., & Channon, K.M. (2007). Altered plasma versus vascular biopterins in human atherosclerosis reveal relationships between endothelial nitric oxide synthase coupling, endothelial function, and inflammation. *Circulation*, 116(24):2851-2859

Aviram, M., Rosenblat, M., Bisgaier, C.L., Newton, R.S., Primo-Parmo, S.L., & La Du, B.N. (1998). Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest*, 101:1581–1590

Bendall, J.K., Alp, N.J., Warrick, N., Cai, S., Adlam, D., Rockett, K., Yokoyama, M., Kawashima, S., & Channon, K.M. (2005). Stoichiometric relationships between endothelial tetrahydrobiopterin, endothelial NO synthase (eNOS) activity, and eNOS coupling in vivo: insights from transgenic mice with endothelialtargeted GTP cyclohydrolase 1 and eNOS overexpression. *Circ Res*, 97(9):864-871

Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M., & McKenzie, H. (1994). Stomach NO synthesis. *Nature,* 368:502

Billecke, S.S., D'Alecy, L.G., Platel, R., Whitesall, S.E., Jamerson, K.A., Perlman, .L., & Gadegbeku, C.A. (2009). Blood content of asymmetric dimethylarginine: new insights into its dysregulation in renal disease. *Nephrol Dial Transplant*, 24(2):489-496

Blankenberg, S., Rupprecht, H.J., Bickel, C., Torzewski, M., Hafner, G., Tiret, L., Smieja, M., Cambien, F., Meyer, J., Lackner, K.J., & AtheroGene Investigators. (2003). Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *N Engl J Med*, 349(17):1605–1613

Boyle, J.J., Weissberg, P.L., & Bennett, M.R. (2002). Human macrophage- induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas–L interactions. *Arterioscler Thromb Vasc Biol*, 22:1624–1630

Busse, R., Luckhoff, A., & Bassenge, E. (1987). Endotheliumderived relaxant factor inhibits platelet activation. *Naunyn-Schmiedeberg's Arch Pharmacol*, 336:566–571

Castel, H., & Vaudry, H. (2001). Nitric oxide directly activates GABA (A) receptor function through a cGMP/protein kinase-independent pathway in frog pituitary melanotrophs. *J Neuroendocrinol,* 13:695–705

Chen, J., Kuhlencordt, P.J., Astern, J., Gyurko, R., & Huang, P.L. (2001). Hypertension does not account for the accelerated atherosclerosis and development of aneurysms in

male apolipoprotein e/endothelial nitric oxide synthase double knockout mice. *Circulation,* 04(20):2391-2394

Clancy, R.M., Leszczynska, P., Piziak, J., & Abramson, S.B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on NADPH oxidase. *J Clin Invest,* 90:1116–1121

Closs, EI., Scheld, J.S., Sharafi, M., & Förstermann, U. (2000). Substrate supply for nitric-oxide synthase in macrophages and endothelial ells: role of cationic amino acid transporters. *Mol Pharmacol*, 57:68–74

Cooke, J.P. (2004). Asymmetrical dimethylarginine: the Uber marker? *Circulation*, 109(15): 1813-1818

Crabtree, M.J., Smith, C..L, Lam, G., Goligorsky, M.S., & Gross, S.S. (2008). Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS. *Am J Physiol Heart Circ Physiol*, 294:H1530-1540

de Nigris, F., Mancini, F.P., Balestrieri, M.L., Byrns, R., Fiorito, C., Wiiliams- Ignarro, S., Palagiano, A., Crimi, E., Ignarro, L.J., & Napoli, C. (2008). Therapeutic dose of nebivolol, a nitric oxide-releasing beta-blocker, reduces atherosclerosis in cholesterol-fed rabbits. *Nitric Oxide*, 19(1):57–63

d'Uscio, L.V., Milstien, S., Richardson, D., Smith, L., & Katusic, Z.S. (2003). Longterm vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res*, 92:88-95

d'Uscio, L.V., & Katusic, Z.S. (2006). Increased vascular biosynthesis of tetrahydrobiopterin in apolipoprotein E-deficient mice. *Am J Physiol Heart Circ Physiol,* 290:H2466-2471

Esper, R.J., Nordaby, R.A., Vilarino, J.O., Paragano, A., Cacharrón, J.L. & Machado, R.A. (2006). Endothelial dysfunction: a comprehensive appraisal. *Cardiovasc Diabetol*, 5:4 doi:10.1186/1475-2840-5-4

Fleming, I. & Busse, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol*, 284:R1–12

Fleming, I. (2010). Molecular mechanisms underlying the activation of eNOS. *Pflügers Arch - Eur J Physiol*, 459:793-806

Förstermann, U., Closs, EI., Pollock, JS., Nakane, M., Schwarz, P., Gath, I., & Kleinert, H. (1994). Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension,*.23(6, Part 2):1121-1131

Förstermann, U., & Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation,* 113:1708-1714

Förstermann, U. (2006). Janus-faced role of endothelial NO synthase in vascular disease: uncoupling of oxygen reduction from NO synthesis and its pharmacological reversal. *Biol Chem*, 387:1521–1533

Förstermann, U. (2008). Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med*, 5:338-349

Förstermann, U. (2010). Nitric oxide and oxidative stress in vascular disease *Pflügers Arch - Eur J Physiol*, 459:923–939

Gao, Y.T., Roman, L.J., Martasek, P., Panda, S.P., Ishimura, Y., & Masters, B.S. (2007).Oxygen metabolism by endothelial nitric-oxide synthase. *J Biol Chem*, 282:28557- 28565

Gharavi, N.M., Baker, N.A., Mouillesseaux, K.P., Yeung, W., Honda, H.M., Hsieh, X., Yeh, M., Smart, E.J., & Berliner, J.A. (2006). Role of endothelial nitric oxide synthase in the regulation of SREBP activation by oxidized phospholipids. *Circ Res*, 98(6):768-776

Harrison, D., Griendling, K.K., Landmesser, U., Hornig, B., & Drexler, H. (2003). Role of oxidative stress in atherosclerosis. *Am J Cardiol*, 91:7A-11A

Heitzer, T., Krohn, K., Albers, S., & Meinertz, T. (2000). Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. *Diabetologia,* 43:1435-1438

Hemmens, B. & Mayer, B. (1998). Enzymology of nitric oxide synthases. *Methods Mol Biol,* 100:1–32

Herman, A. G., & Moncada, S. (2005). Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis. *Eur Heart J,* 26(19):1945–1955

Hink, U., Li, H., Mollnau, H., Oelze, M., Matheis, E., Hartmann, M., Skatchkov, M., Thaiss, F., Stahl, R.A.K., Warnholtz, A., Meinertz, T., Griendling, K., Harrison, D.G., Förstermann, U., & Münzel, T. (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res*, 88(2):E14-E22

Hodgin, J.B., Knowles, J.W., Kim, H.S., Smithies, O., & Maeda, N. (2002). Interactions between endothelial nitric oxide synthase and sex hormones in vascular protection in mice. *J Clin Invest,* 109:541-548

Horke, S., Witte, I., Wilgenbus, P., Kruger, M., Strand, D., & Förstermann, U. (2007). Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation. *Circulation*, 115:2055–2064

Hrabie, J.A., Klose, J.R., Wink, D.A., & Keefer, L.K. (1993). New nitric oxide- releasing zwitterions derived from polyamines. *J Org Chem*, 58:1472–1476

Ishikawa, K., Kanamasa, K., Ogawa, I., Takenaka, T., Naito, T., Kamata, N., Yamamoto, T., Nakai, S., Hama, J., Oyaizu, M., Kimura, A., Yamamoto, K., Aso, N., Arai, M., Yabushita, & H., Katori, Y. (1996). Long-term nitrate treatment increases cardiac events in patients with healed myocardial infarction. Secondary Prevention Group. *Jpn Circ J*, 60(10):779-788

Ishimaru, R.S., Leung, K., Hong, L., & LaPolt, P.S. (2001). Inhibitory effects of nitric oxide on estrogen production and cAMP levels in rat granulose cell cultures. *J Endocrinol*, 168:249–255 ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group. (1995). ISIS-4: arandomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. *Lancet*, 345:669–685

Jiang, F., Roberts, S.J., Datla, S., & Dusting, G.J. (2006). NO modulates NADPH oxidasefunction via heme oxygenase-1 in human endothelial cells. *Hypertension* 48:950–957

John, S., Schlaich, M., Langenfeld, M., Weihprecht, H., Schmitz, G., Weidinger, G., &Schmieder, R.E.(1998). Increased bioavailability of nitric oxide after lipidlowering therapy in hypercholesterolemic patients: a randomized, placebo-controlled, double-blind study. *Circulation*,211–216

Jung, O., Marklund, S.L., Xia, N., Busse, R., & Brandes, R.P. (2007). Inactivation of extracellular superoxide dismutase contributes to the development of high-volume hypertension. *Arterioscler Thromb Vasc Biol*, 27:470–477

Kapil, V., Milsom, A.B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F.,Arghandawi, S., Pearl, V., Benjamin, N., Loukogeorgakis, S., Macallister, R., Hobbs, A.J., Webb, A.J., & Ahluwalia, A. (2010a). Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*, 56(2):274-281

Kapil,V., Webb, A.J., & Ahluwalia, A. (2010b). Inorganic nitrate and the cardiovascularsystem. *Heart*, 96(21):1703-1709

Katusic, Z.S. (2001). Vascular endothelial dysfunction: does tetrahydrobiopterin play a role?*Am J Physiol Heart Circ Physiol,* 281: H981-986

Keefer, L.K., Nims, R.W., Davies, K.M., & Wink, D.A. (1996). 'NONOates' (1-substituteddiazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. *Meth Enzymol*, 268:281–293

Knowles, J.W., Reddick, R.L., Jennette, J.C., Shesely, E.G., Smithies, O., & Maeda N. (2000).Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest*, 105:451-845

Kockx, M.M., de Meyer, G.R., Muhring, J., Bult, H., Bultinck, J., & Herman, A.G. (1996).Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol-fed rabbits. *Atherosclerosis*, 120:115–124

Kockx M.M. (1998). Apoptosis in the atherosclerotic plaque: quantitative and qualitativeaspects. *Arterioscler Thromb Vasc Biol*, 18:1519–1522

Kuhlencordt, P.J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T.H., Hajjar, R., Picard,M.H., & Huang, P.L. (2001). Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation,* 104(4):448-454

Kumar, V., Hong, S.Y., Maciag, A.E., Saavedra, J.E., Adamson, D.H., Prud' homme, R.K., Keefer, L.K., & Chakrapani, H. (2010). Stabilization of the nitric oxide (NO) prodrugs and anticancer leads, PABA/NO and double JS-K, through incorporation into PEG-protected nanoparticles. *Mol Pharm*, 7(1):291–298

Kureishi, Y., Luo, Z., Shiojima, I., Bialik, A., Fulton, D., Lefer, D.J., Sessa, W. C., & Walsh, K. (2000). The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med,* 6:1004–1010

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E., &Harrison, D.G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*, 111(8):1201-1209

Laufs, U., La Fata, V., Plutzky, J., & Liao, J.K. (1998). Upregulation of endothelial nitric oxidesynthase by HMG CoA reductase inhibitors. *Circulation,* 97:1129–1135

Laursen, J.B., Somers, M., Kurz, S., McCann, L., Warnholtz, A., Freeman, B.A., Tarpey, M., Fukai, T., & Harrison D.G. (2001). Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*, 103(9):1282-1288

Lefer, D.J., & Lefer, A.M. (1988). Studies on the mechanism of the vasodilator action ofnicorandil. *Life Sci,* 42:1907–1914

Li, H., & Förstermann, U. (2000). Nitric oxide in the pathogenesis of vascular disease. *JPathol*, 190:244-254

Li, H., Wallerath, T., & Förstermann, U. (2002a). Physiological mechanisms regulating theexpression of endothelial-type NO synthase. *Nitric Oxide,* 7:132-147

Li, H., Wallerath, T., Münzel, T., & Förstermann, U. (2002b). Regulation of endothelial-typeNO synthase expression in pathophysiology and in response to drugs. *Nitric Oxide*, 7:149-164

Li, H., Witte, K., August, M., Brausch, I., Godtel-Armbrust, U., Habermeier, A., Closs, El.,Oelze, M., Münzel, T., & Förstermann, U. (2006). Reversal of endothelial nitric oxide synthase uncoupling and up-regulation of endothelial nitric oxide synthase expression lowers blood pressure in hypertensive rats. *J Am Coll Cardiol,* 47(12):2536-2544

Li, H., & Förstermann, U. (2009). Prevention of Atherosclerosis by Interference with theVascular Nitric Oxide System. *Curr Pharm Des*, 15(27):3133-3145

Lin, M.I., Fulton, D., Babbitt, R., Fleming, I., Busse, R., Pritchard, K.A. Jr., & Sessa, W.C. (2003). Phosphorylation of threonine 497 in endothelial nitric-oxide synthase

coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J Biol Chem*, 278: 44719–44726

Lloyd-Jones, IM., & Block, KD. (1996). The vascular biology of nitric oxide and its role inatherogenesis. *Annu Rev Med,*.47:365–375

Liu, VW., & Huang, PL. (2008). Cardiovascular roles of nitric oxide: a review of insightsfrom nitric oxide synthase gene disrupted mice. *Cardiovasc Res,*.77(1):19-29

Lundberg, J.O, Weitzberg, E., Lundberg, J.M., & Alving, K. (1994). Intragastric nitric oxideproduction in humans: measurements in expelled air. *Gut.* 35:1543–1546

Lundberg, J.O., & Weitzberg, E. (2005). NO Generation From Nitrite and Its Role in VascularControl. *Arterioscler Thromb Vasc Biol,* 25(5):915-922

Maas, R. (2005). Pharmacotherapies and their influence on asymmetric dimethylargine(ADMA). *Vasc Med*, 10(Suppl 1): S49-57

Mallis, R.J., Buss, J.E., & Thomas J.A. (2001). Oxidative modification of H-ras: S-thiolationand S-nitrosylation of reactive cysteines. *Biochem J,* 355:145–153

McNally, J.S., Davis, M.E., Giddens, D.P., Saha, A., Hwang, J., Dikalov, S., Jo, H., Harrison,D.G. (2003). Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. *Am J Physiol Heart Circ Physiol*, 285:H2290–H2297

Milkowski, A., Garg, H.K., Coughlin, J.R. & Bryan, N.S. (2010). Nutritional epidemiology inthe context of nitric oxide biology: A risk–benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide*, 22(2):110-119

Moens, A.L., & Kass, D.A. (2006). Tetrahydrobiopterin and cardiovascular disease.*Arterioscler Thromb Vasc Biol,* 26:2439-2444

Moncada, S., Palmer, RM., & Higgs EA. (1991). Nitric oxide: physiology, pathophysiology,and pharmacology. *Pharmacol Rev,* 43(2):109–142

Mooradian, D.L., Hutsell, T.C., & Keefer, L.K. (1995). Nitric oxide (NO) donor molecules:effect of NO release rate on vascular smooth muscle cell proliferation in vitro. *J Cardiovasc Pharmacol*, 25:674–678

Morita, T. (2005). Heme oxygenase and atherosclerosis. *Arterioscler Thromb Vasc Biol*, 25: 1786–1795

Nakamura, Y., Moss, A.J., Brown, M.W., Kinoshita, M., & Kawai, C. (1999). Longterm nitrateuse may be deleterious in ischemic heart disease: A study using the databases from two large-scale postinfarction studies. Multicenter Myocardial Ischemia Research Group. *Am Heart J*, 138:577-585

Ohara, Y., Peterson, T.E., & Harrison, D.G. (1993). Hypercholesterolemia increasesendothelial superoxide anion production. *J Clin Invest*, 91:2546–2551

Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui,H., Sakurai, H., Yoshida,Y., Masada, M., & Yokoyama, M. (2002). Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J Clin Invest*, 110(3):331-340

Pritchard, K.A., Jr., Groszek, L., Smalley, D.M., Sessa, W.C., Wu, M., Villalon, P., Wolin,M.S., & Stemerman, M.B. (1995). Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res*, 77(3):510-518

Psychogios, N., Hau, D.D., Peng, J., Guo, A.C., Mandal, R., Bouatra, S., Sinelnikov, I.,Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., Hollander, Z., Pedersen, T.L., Smith, S.R., Bamforth, F., Greiner, R., McManus, B., Newman, J.W., Goodfriend, T., & Wishart, D.S. (2011). The human serum metabolome. *PLoS One*,16;6(2):e16957

Radomski, M.W., Palmer, R.M., & Moncada, S. (1987). The anti- aggregating properties ofvascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol,* 92:639–646

Ramachandran, A., Levonen, A.L., Brookes, P.S., Ceaser, E., Shiva, S., Barone, M.C., &Darley-Usmar, V. (2002). Mitochondria, nitric oxide, and cardiovascular dysfunction. *Free Radic Biol Med*, 33:1465–1474

Riccio, D.A., Dobmeier, K.P., Hetrick, E.M., Privett, B.J., Paul, H.S., & Schoenfisch, M.H. (2009). Nitric oxide-releasing S-nitrosothiol-modified xerogels. *Biomaterials*, 30:4494–502

Rosenson, R.S., & Tangney, C.C. (1998). Antiatherothrombotic properties of statins:implications for cardiovascular event reduction. *JAMA*, 279:1643–1650

Rozenberg, O., Shih, D.M., & Aviram, M. (2005). Paraoxonase 1 (PON1) attenuatesmacrophage oxidative status: studies in PON1 transfected cells and in PON1 transgenic mice. *Atherosclerosis*, 181:9–18

Ryoo, S., Lemmon, C.A., Soucy, K.G., Gupta, G., White, A.R., Nyhan, D., Shoukas, A.,Romer, L.H., & Berkowitz, D.E. (2006). Oxidized low-density lipoprotein-dependent endothelial arginase II activation contributes to impaired nitric oxide signaling. *Circ Res*, 99(9):951-960

Sarkar, R., Gordon, D., Stanley, J.C., & Webb, R.C. (1997). Cell cycle effects of nitric oxide onvascular smooth muscle cells. *Am J Physiol*, 272:H1810–1818

Schmidt, A., Geigenmüller, S., Völker, W., Seiler, P., & Buddecke, E. (2003). Exogenousnitric oxide causes overexpression of TGF-beta1 and overproduction of extracellular matrix in human coronary smooth muscle cells. *Cardiovasc Res,* 58: 671–678

Schmidt, A., Bilgasem, S., Lorkowski, S., Vischer, P., Völker, W., Breithardt, G., Siegel, G., &Buddecke, E. (2008). Exogenous nitric oxide regulates activity and synthesis of vascular endothelial nitric oxide synthase. *Eur J Clin Invest*, 38(7):476–485

Schmidt, T.S., & Alp, N.J. (2007). Mechanisms for the role of tetrahydrobiopterin inendothelial function and vascular disease. *Clin Sci (Lond),* 113:47-63

Schnabel, R., & Blankenberg, S. (2007). Oxidative stress in cardiovascular disease: successfultranslation from bench to bedside? *Circulation*, 116:1338-1340

Schulz, E., Anter, E., & Keaney, J.F. Jr. (2004).Oxidative stress, antioxidants, and endothelialfunction. *Curr Med Chem*, 11:1093–1104

Schulz, R., Kelm, M., & Heusch, G. (2004). Nitric oxide in myocardial ischemia/reperfusioninjury. *Cardiovasc Res,* 61(3):402– 413

Shinozaki, K., Kashiwagi, A., Nishio, Y., Okamura, T., Yoshida, Y., Masada, M., Toda, N., & Kikkawa, R. (1999). Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O2- imbalance in insulin-resistant rat aorta. *Diabetes*, 48(12):2437-2445

Sorescu, D., Weiss, D., Lassegue, B., Clempus, R.E., Szocs, K., Sorescu, G.P., Valppu, L.,Quinn, M.T., Lambeth, J.D., Vega, J.D., Taylor, W.R., & Griendling, K.K. (2002). Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation*, 105:1429–1435

Sowers, J.R. (2003). Effects of statins on the vasculature: implications for aggressive lipidmanagement in the cardiovascular metabolic syndrome. *Am J Cardiol*, 91:14B–22B

Stuehr, D., Pou, S., & Rosen, G.M. (2001). Oxygen reduction by nitric- oxide synthases. *J BiolChem*, 276:14533–14536

Sud, N., Wells, S.M., Sharma, S., Wiseman, D.A., Wilham, J., & Black, S.M. (2008).Asymmetric dimethylarginine inhibits HSP90 activity in pulmonary arterial endothelial cells: role of mitochondrial dysfunction. *Am J Physiol Cell Physiol*, 294:C1407-1418

Sullivan, J.C., Pollock, J.S. (2006). Coupled and uncoupled NOS: separate but equal?Uncoupled NOS in endothelial cells is a critical pathway for intracellular signaling. *Circ Res*, 98:717-719

Sun, J., Xin, C., Eu, J.P., Stamler, J.S., & Meissner, G. (2001). Cysteine-3635 is responsible forskeletal muscle ryanodine receptor modulation by NO. *Proc Natl Acad Sci USA*, 98:1158–1162

Tang, Y., Jiang, H., & Bryan, N.S. (2011). Nitrite and nitrate: cardiovascular risk-benefit andmetabolic effect. *Curr Opin Lipidol*, 22(1):11-15

Thomas, G.R., DiFabio, J.M., Gori, T., & Parker, J.D. (2007). Once daily Therapy WithIsosorbide-5-Mononitrate Causes Endothelial Dysfunction in Humans. *J Am Coll Cardiol*, 49:1289–1295

Torzewski, M., Ochsenhirt, V., Kleschyov, A.L., Oelze, M., Daiber, A., Li, H., Rossmann, H., Tsimikas, S., Reifenberg, K., Cheng, F., Lehr, H.A., Blankenberg, S., Förstermann, U., Münzel, T., & Lackner, K.J. (2007). Deficiency of glutathione peroxidase-1 accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*, 27:850–857

Vasan, R.S. (2006). Biomarkers of Cardiovascular Disease: Molecular Basis and PracticalConsiderations. *Circulation*, 113:2335-23362

Vasquez-Vivar, J., Duquaine, D., Whitsett, J., Kalyanaraman, B. & Rajagopalan, S. (2002).Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler Thromb Vasc Biol,* 22:1655-1661

White, C.R., Darley-Usmar, V., Berrington, W.R., McAdams, M., Gore, J.Z., Thompson, J.A.,Parks, D.A., Tarpey, M.M., & Freeman, B.A. (1996). Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc Natl Acad Sci USA*, 93:8745–8749

Xia, Y., Tsai, A.L., Berka, V., & Zweier, J.L. (1998). Superoxide Generation from endothelialnitric-oxide synthase. A a2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem*, 273:25804-25808

Xue, H.M., He, G.W., Huang, J.H., & Yang, Q. (2010). New strategy of endothelial protectionin cardiac surgery: use of enhancer of endothelial nitric oxide synthase. *World J Surg*, 34:1461–1469

Yamawaki, H., Haendeler, J., & Berk, B.C. (2003). Thioredoxin: a key regulator ofcardiovascular homeostasis. *Circ Res*, 93:1029–1033

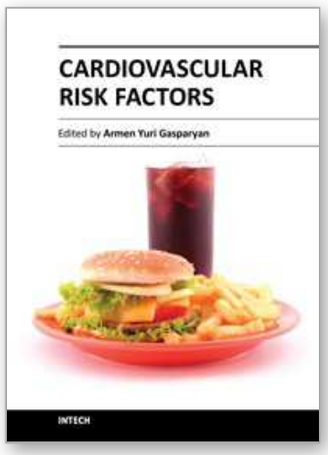
Yan, M.S., Matouk, C.C., & Marsden, P.A. (2010). Epigenetics of the vascular endothelium. *JAppl Physiol*, 109(3):916-926

Yang, H., Roberts, L.J., Shi, M.J., Zhou, L.C., Ballard, B.R., Richardson, A., & Guo, Z.M.(2004). Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. *Circ Res*, 95:1075–1081

Young, D.V., Serebryanik, D., Janero, D.R., & Tam, S.W. (2000). Suppression of proliferationof human coronary artery smooth muscle cells by the nitric oxide donor, S-nitrosoglutathione, is cGMP-independent. *Mol Cell Biol Res Commun*, 4:32–36

Zhang, Y., Griendling, K.K., Dikalova, A., Owens, G.K., & Taylor, W.R. (2005). Vascularhypertrophy in angiotensin II-induced hypertension is mediated by vascular smooth muscle cell-derived H2O2. *Hypertension*. 46:732–737

Zou, M.H., Shi, C.,& Cohen, R.A. (2002). Oxidation of the zinc- thiolate complex anduncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest*, 109:817-826



Cardiovascular Risk Factors
Edited by Prof. Armen Gasparyan

ISBN 978-953-51-0240-3
Hard cover, 498 pages
Publisher InTech
Published online 14, March, 2012
Published in print edition March, 2012

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.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Annette Schmidt (2012). Nitric Oxide Signalling in Vascular Control and Cardiovascular Risk, Cardiovascular Risk Factors, Prof. Armen Gasparyan (Ed.), ISBN: 978-953-51-0240-3, InTech, Available from: http://www.intechopen.com/books/cardiovascular-risk-factors/nitric-oxide-signalling-in-vascular-control-and-cardiovascular-risk

INTECH
open science | open minds

InTech Europe
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

IntechOpen