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Cellular signaling and NO production

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Abstract: The endothelium can evoke relaxations (dilatations) of the underlying vascular smooth muscle, by releasing vasodilator substances. The best characterized endothelium-derived relaxing factor (EDRF) is nitric oxide (NO), which is synthesized by the endothelial isoform of nitric oxide synthase (eNOS). Endothelium-dependent relaxations involve both pertussis toxin-sensitive G_i (e.g. responses to serotonin, sphingosine 1-phosphate, alpha$_2$-adrenergic agonists and thrombin) and pertussis toxin-insensitive G_q (e.g. adenosine diphosphate and bradykinin) coupling proteins. eNOS undergoes a complex pattern of intracellular regulation, including post-translational modifications involving enzyme acylation and phosphorylation. eNOS is reversibly targeted to signal-transducing plasmalemmal caveolae where the enzyme interacts with a number of regulatory proteins, many of which are modified in cardiovascular disease states. The release of NO by the endothelial cell can be up- (e.g. by estrogens, exercise and dietary factors) and down-regulated (e.g. oxidative stress, smoking and oxidized low-density lipoproteins [oxyLDL]). It is reduced in the course of vascular disease (e.g. diabetes and hypertension). Arteries covered with regenerated endothelium (e.g. following angioplasty) selectively lose the pertussis-toxin sensitive pathway for NO-release which favors vasospasm, thrombosis, penetration of macrophages, cellular growth and the inflammatory reaction leading to atherosclerosis. The unraveling of the complex interaction of the pathways regulating the presence and the activity of eNOS will enhance the understanding of the perturbations in endothelium-dependent signaling that are seen in cardiovascular disease states, and may lead to the identification of novel targets for therapeutic intervention.

Short Title: NO and Endothelial Dysfunction

Keywords: diabetes, G-proteins, hypertension, nitric oxide

1. Introduction

Robert Furchgott demonstrated that the removal of the endothelial layer from isolated arteries prevents the *in vitro* dilator response to acetylcholine [Furchgott & Zawadzki, 1980]. Early bioassay studies demonstrated that the endothelial cells cause arterial relaxation by releasing a vasoactive substance(s) which was termed endothelium-derived relaxing factor (EDRF) [Furchgott & Zawadzki, 1980; Rubanyi et al., 1985]. The original EDRF [Furchgott & Zawadzki, 1980] stimulates soluble guanylyl cyclase in the vascular smooth muscle cells and thus increases the production of cyclic guanosine monophosphate (cyclic GMP) [see Furchgott & Vanhoutte, 1989; Lüscher & Vanhoutte 1990; Vanhoutte *et al*., 2009]. It is rapidly destroyed by superoxide anions [Rubanyi et al., 1986]. These experimental facts led to the proposal and the demonstration that EDRF is nitric oxide (NO) [see Vanhoutte, 2009]. Besides NO, a number of endothelium-derived factors (EDHFs) and the opening of gap junctions can cause NO-independent hyperpolarizations of the underlying vascular smooth muscle and thus endothelium-dependent relaxations [see Busse et al., 2002; Feletou & Vanhoutte 2006a, 2009]. When the ability of the endothelial cells to release NO [and to cause endothelium-dependent hyperpolarizations] is reduced, this appears to be the first step in the chain of events that leads to atherosclerosis and coronary disease [see Vanhoutte *et al.*, 2009]. Thus, endothelial dysfunction has become a hallmark and a predictor of cardiovascular disease. This article focuses on the cellular mechanisms leading to the release of endothelium-derived nitric oxide [EDNO] and how

reduction in such release plays a key role role in the genesis of vascular disease. For the survey of the original experimental literature, the reader will be referred mainly to more exhaustive previous review articles.

2. Nitric Oxide Protector of the vascular wall

As such, endothelium-dependent relaxations to acetylcholine are mainly of pharmacological interest since few blood vessels are innervated by cholinergic nerves. However, a number of more physiological stimuli [physical forces, circulating hormones (catecholamines, vasopressin, aldosterone), plasma constituents (thrombin, sphingosine 1-phosphate), platelet products (serotonin, adenosine diphosphate), and autacoids (histamine, bradykinin, prostaglandin E_4)] share with acetylcholine the ability to evoke the release of EDNO (Figure 1) [see Lüscher & Vanhoutte, 1990; Vanhoutte *et al.*, 2009]. NO plays a key role in the protection exerted by the endothelium against coronary disease. It is produced by the endothelial constitutive isoform of NO synthase (eNOS, NOS III) (Figure 2) [see Moncada, 1997; Dudzinksi et al., 2006; Dudzinski and Michel, 2007; Vanhoutte *et al*., 2009]. NO not only prevents abnormal constriction (vasospasm) of the coronary arteries, which favors intraluminal clot formation, but also inhibits the aggregation of platelets, the expression of adhesion molecules at the surface of the endothelial cells, and hence the adhesion and penetration of white blood cells (macrophages),

and the release and action of the vasoconstrictor and mitogenic peptide endothelin-1 (Figure 3). The protective release of NO is triggered by the local presence of thrombin and substances released by aggregating platelets. When this protective role of NO is curtailed, the inflammatory response that leads to atherosclerosis is initiated [see Vanhoutte *et al*., 2009].

The role played by the endothelial cells to protect against thrombin and platelet products by increasing the activity of eNOS has been demonstrated both *in vitro* and *in vivo* [see Harrison 2001; Vanhoutte *et al*., 2009]. Serotonin (5-hydroxytryptamine, 5HT) and adenosine diphosphate (ADP) are the two mediators released by aggregating platelets that can activate eNOS and thus augment the production of NO. Serotonin is the most important and stimulates 5-HT_{1D} serotonergic receptors of the endothelial cell membrane. ADP is a relatively minor contributor of the response to platelets and acts on P_{2y} purinoceptors (Figure 3). The serotonergic receptors and those for thrombin are coupled to the activation of eNOS through pertussis toxin-sensitive G_i-proteins, while the P_{2y}-purinoceptors are linked to the enzyme by Gq- proteins [see Vanhoutte *et al.*, 2009].If the endothelium is absent or dysfunctional such relaxations are no longer observed, and aggregating platelets induce constrictions (vasospasm), because they release the powerful vasoconstrictors thromboxane A_2 and serotonin.

The physiological importance of the endothelium-dependent relaxations to platelet products is obvious [see Feletou & Vanhoutte, 2006b; Vanhoutte *et al.*, 2009]. Thus, if platelet aggregation occurs in a coronary artery with a healthy endothelium the release by the platelets of serotonin

(and ADP) and the local production of thrombin will stimulate the endothelial cells with the resulting release of NO. The endothelial mediator will cause the underlying smooth muscle to relax, thus increasing blood flow and mechanically impeding the progression of the coagulation process. NO also exerts in synergy with prostacyclin an immediate feed-back inhibition on the platelets. When the endothelial barrier is interrupted by injury, the aggregating platelets can approach the vascular smooth muscle cells, and cause their contraction by releasing thromboxane A_2 and serotonin, initiating the vascular phase of hemostasis. The endothelium-dependent response to aggregating platelets is not present to the same extent in all arteries, but is the most prominent in the coronary and cerebral circulations [see Vanhoutte *et al*., 2009].

3. Intracellular regulation of endothelial nitric oxide synthase

3.1 Enzymatic function of eNOS

Endothelial nitric oxide synthase (eNOS) is a member of a family of three mammalian nitric oxide synthase ("NOS") isoforms that catalyze the oxidation of the terminal guanidino group of L-arginine to produce NO and L-citrulline (see reviews in Forstermann et al 1998; Shaul and Anderson 2004; Dudzinski et al., 2006). The three archetypal mammalian NOS isoforms are a] nNOS (NOS I) or neuronal NOS; b] iNOS (NOS II), an inducible NOS isoform expressed in a variety of activated tissues; and c] eNOS (NOS III) or endothelial NOS. They are the products of distinct genes, and share approximately 50-60% sequence identity. Although these isoforms possess similar overall enzymatic and chemical properties, they contain distinctive catalytic and regulatory properties which characterize them. Nitric oxide synthases are bifunctional enzymes:

the N-terminus exhibits homology principally to the other NOS isoforms, while the C-terminal domain has significant sequence homology to cytochrome P450 reductases. The NOS N-terminus binds tetrahydrobiopterin (BH4) and heme. L-arginine binds in the enzyme active site near heme, while molecular oxygen is directed to the ferrous heme iron. The binding sites for tetrahydrobiopterin and heme are localized along the interface of the monooxygenase domains of the dimeric, active form of NOS. Binding of heme and tetrahydrobiopterin, especially for iNOS, may be crucial in promoting dimerization and NOS catalytic activity. The C-terminal domain binds NADPH, FAD, and FMN cofactors [Steuhr, 1997]. The N- and C-terminal domains are linked by a short sequence that binds calmodulin, an allosteric effector that is essential for full NOS activity. The intact NOS functions as a mixed function monooxygenase / reductase enzyme, and has the capability to synthesize a number of molecules besides NO, most notably superoxide anions. In all NOS catalysis reactions, electrons are shuttled within the reductase domain sequentially from NADPH, to FAD, and finally to FMN. Calmodulin is believed to function in facilitating the flow of electrons from both the reductase domain to the monooxygenase domain as well as from FAD to FMN. Because electrons appear to flow from the reductase domain of one NOS monomer to the oxygenase domain of another NOS monomer, enzyme dimerization is required for full enzymatic activity (Forsterman et al 1998; Balligand and Feron 2009).

3.2 Post-Translational Modifications of the eNOS Protein

Endothelial eNOS undergoes a complex pattern of post-translational regulatory modifications that underly the dynamic regulation of enzymatic activity in response to physiological stimuli and pathophysiological conditions [See reviews in Fulton et al. 2001; Dudzinski et al. 2006; Sessa 2004].

3.2.1 *Acylation*: In quiescent cells, eNOS is targeted to invaginations of the plasmalemma called caveolae, which are membrane microdomains defined by the presence of the scaffolding protein caveolin. The caveolae are enriched in cholesterol and sphingolipids, and sequester diverse receptors and signaling proteins from a variety of signal transduction pathways, including G-protein coupled receptors, G proteins, growth factor receptors, calcium regulatory proteins, as well as eNOS. The targeting of eNOS may permit the enzyme to receive signals from these upstream signaling pathways and facilitate communication with downstream activators. The targeting of eNOS to caveolae is dependent on the irreversible co-translational myristoylation of its N-terminal glycine, followed by the dynamically regulated post-translational palmitoylation of the Cys15 and Cys26 residues. Myristoylation and palmitoylation comprise three acyl anchors that attach eNOS to the membrane. N-myristoylation of eNOS is catalyzed by an N-myristoyltransferase that recognizes a specific N-terminal consensus sequence. By contrast, no consensus sequence has been identified for thiopalmitoylation, and a family of palmitoyltransferases with varying substrate specificities and patterns of expression contribute to the process. In particular, palmitoyltransferase protein DHHC-21 may be implicated in the palmitoylation of eNOS. DHHC-21 co-localizes with the enzyme, but its specificity for eNOS palmitoylation has not been established. Depalmitoylation of eNOS is catalyzed by acyl protein thioesterase-1 (APT-1), an enzyme that also depalmitoylates the G protein Gαs. By contrast to the irreversible fatty acyl amide that links myristate to eNOS, the thiopalmitoyl bonds linking palmitate to eNOS are labile. Prolonged agonist stimulation of eNOS leads to depalmitoylation and intracellular translocation, possible serving as a mechanism for modulating eNOS signaling responses [Dudzinski and Michel 2007; Feron et al. 2006; Shaul 2002].

3.2.2 *Intracellular Calcium: Calmodulin Binding*: The activation of eNOS is critically dependent on the binding of the ubiquitous intracellular calcium regulatory protein calmodulin. Calmodulin binding to eNOS facilitates transfer of electrons between the enzyme's reductase and oxygenase domains, and simultaneously disrupts the inhibitory caveolin-eNOS interaction. A diverse group of agonists, including bradykinin, sphingosine 1-phosphate, and acetylcholine, activate a G protein-dependent signaling pathway that ultimately releases intracellular calcium stores. Receptor-dependent activation of phospholipase C (PLC) cleaves the cell membrane component phosphatidylinositol 4, 5-triphosphate into diacylglycerol (an activator of protein kinase C) and inositol 1, 4, 5-triphosphate (IP3), which binds to IP3 receptors, regulators of the intracellular calcium concentration densily located in caveolae [Fleming and Busse 2003].

3.2.3 *Phosphorylation: protein kinases and phosphoprotein phosphatases*: Phosphorylation and dephosphorylation networks complement acylation and calmodulin as major post-translational regulatory influences on eNOS activity [Sessa 2004; Dudzinski 2007]. Key serine and threonine residues in eNOS constitute regulatory loci: phosphorylations at Ser 1177 (primary sequence numbering corresponding to human eNOS), Ser 635, and Ser 617 promote increases in enzyme activity, while phosphorylation at Thr 495 and Ser 116 are inhibitory. Ser 1177 phosphorylation is catalyzed by numerous kinases, including kinase Akt (protein kinase B) as well as the cyclic AMP-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK), PKG, and calcium/calmodulin-dependent protein kinase II (CaM kinase II). The relative contributions of these different kinase pathways are not completely understood, but it is already obvious that different extracellular stimuli activate distinct kinase pathways leading to eNOS phosphorylation. For example, PKA appears to be the principal kinase that phosphorylates eNOS at ser1177 in response to hemodynamic shear stress [Balligand et al. 2009], while S1P- and VEGF-dependent phosphorylation of this same serine residue involves kinase Akt. An

additional level of complexity is revealed by the fact that beta3 adrenergic receptor signaling to eNOS in endothelial cells involves *both* PKA and Akt. Another critical kinase is the AMP-activated protein kinase, which has broad effects on endothelial metabolism and signaling [Fisslthaler and Fleming 2009]. AMPK can catalyze eNOS phosphorylation at ser1177, yet AMPK is also the principal kinase that phosphorylates eNOS at ser635[Chen et al. 2009]. Indeed, AMPK plays a key role in multiple phosphorylation pathways involving eNOS modulation, and AMPK is in turn regulated by upstream serine/threonine kinases including the calcium-calmodulin kinase kinase-beta [Levine et al. 2007]. In addition, tyrosine phosphorylation of eNOS Tyr 83 mediated by v-Src has been identified as another candidate eNOS post-translational modification [Fulton et al. 2005]. However, the extracellular signals that modulate phosphorylation at this site remain incompletely understood. In particular, it is unclear whether Tyr 83 phosphorylation is responsible principally for activating eNOS catalytic activity, or whether this site modulates signaling by serving as a binding site for proteins with Src homology (SH3) domains.

The relative contribution of protein phosphatases versus phosphoprotein kinases in eNOS regulation also remains uncertain. Thus, depending on the specific phosphorylation site, dephosphorylation of eNOS can either activate or inhibit enzyme activity. Several phosphatases, including serine-threonine protein phosphatase 1 (PP1), serine-threonine protein phosphatase 2A (PP2A), and calcineurin participate in eNOS regulation. A pathway involving the phosphatase calcineurin leads to dephosphorylation of Ser 116 and to the activation of eNOS. The immunosuppressive drug cyclosporine inhibits calcineurin, preventing VEGF-induced Ser 116 dephosphorylation and thus offering a potential mechanism to explain the mechanism of cyclosporine-induced hypertension. [Fleming and Busse 2003, Dudzinksi et al 2006, Govers and Rabelink 2001]

3.2.4 S-*Nitrosylation*: Reversible S-nitrosylation provides an additional level of *in vivo* dynamic receptor-mediated post-translational control of the eNOS enzyme in endothelial cells. Indeed, eNOS appears to be the source of the NO required for its own S-nitrosylation, implying a spatial mechanism of specificity in nitrosylation of Cys 94 and Cys 99 given the approximately thirty cysteine residues in eNOS. S-nitrosylation of eNOS leads to enzyme inhibition, whereas denitrosylation is associated with an increased enzyme activity. Treatment of cultured endothelial cells or intact blood vessels with eNOS agonists promotes rapid and reversible de-nitrosylation of eNOS, temporally associated with enzyme activation. The return to basal eNOS enzyme activity following agonist treatment is associated with the re-nitrosylation of eNOS. These findings imply some component of temporal selectivity in nitrosylation reactions given that eNOS is denitrosylated during the period of maximal NO production. Like phosphorylation and acylation, subcellular localization affects eNOS S-nitrosylation and may help generate the apparent temporal selectivity. Membrane targeting is required for S-nitrosylation, as shown by experiments in which eNOS S-nitrosylation is virtually abolished in the myristoylation-deficient mutant (Myr^-) recombinant eNOS [Erwin et al. 2006] as compared to the hyper-nitrosylation of the membrane-tethered fusion protein CD8-Myr^- eNOS. The subcellular dependence of nitrosylation may reflect distinct chemical environments that favor or disfavor nitrosylation (Lamas et al. 2001).

3.3 Protein-protein interactions in eNOS regulation

3.3.1 *Caveolin*: Caveolins are ~22kDa intrinsic membrane proteins [see reviews in Gratton et al. 2004, Shaul 2003, Shaul 2002]. Caveolin-1 and caveolin-2 are ubiquitously expressed and

abundant in endothelial cells, while caveolin-3 is a muscle-specific isoform expressed in cardiomyocytes and skeletal muscle. Robust protein-protein interactions lead to the binding of caveolar-localized eNOS with caveolin-1, although this protein-protein interaction is apparently not necessary for the localization of eNOS to caveolae [Gonzalez et al. 2004]. Caveolin tonically inhibits eNOS in quiescent cells both by impeding the signaling of caveolae-targeted receptors that transduce eNOS-stimulatory signals as well as by inhibiting calmodulin binding to eNOS. Diseases in which caveolins have been implicated include atherosclerosis, hypertension, cardiomyopathy, diabetes, and oncogenesis.

3.3.2 *Heat Shock Proteins*: Heat shock protein 90 (hsp90), a chaperone involved in protein trafficking and folding, modulates agonist-dependent eNOS activation [Sessa 2004]. Hsp90 binding stimulates eNOS activity, and also affects eNOS specific activity by binding to kinase Akt. This interaction may be dynamically regulated by hsp90 S-nitrosylation (Martinez-Ruiz et al, PNAS 102: 8525, 2005) and by statins. Among other effects on eNOS signaling pathways, statins appear to stimulate eNOS phosphorylation by Akt; one Akt-dependent effect of statins involves tyrosine phosphorylation of hsp90, which in turn facilitates hsp90 binding and activation of Akt.

3.3.3 *Actin Cytoskeleton*: Dynamic structural changes in the actin cytoskeleton impact on eNOS via links to caveolar membrane domains and caveolar membrane associated proteins [see reviews in Su et al. 2003; Skidgel 2002). For example, shear stress may transduce its effect on eNOS via actin-based cellular architecture. Furthermore, caveolin and caveolae may use the actin network to reversibly translocate between plasmalemma and the intracellular vesicles. Connections between plasmalemmal caveolae and the cytoskeleton have been identified in many cellular systems, and may play a key role in the involvement of the cytoskeleton-associated GTPase Rac1 in eNOS activation. Modulation of Rac1 and other members of the Rho GTPase

family by statins may represent another important pathway for the control of eNOS signaling pathways by these therapeutic agents [Du et al. 2001].

3.3.4 *NOSIP and NOSTRIN*: Two eNOS-associated proteins identified using yeast two-hybrid screening include the eNOS Interacting Protein NOSIP [Schilling et al. 2006], and the eNOS TRafficking INducer protein NOSTRIN [Icking et al. 2005; see review in Dudzinski 2007]. NOSIP is a 34kDa protein that binds the carboxy-terminal of the eNOS oxygenase domain and assists in the translocation of eNOS from the plasma membrane caveolae to intracellular membranes. Association of eNOS and NOSIP is inhibited by caveolin-1. NOSTRIN is robustly expressed in the endothelium. Overexpression of NOSTRIN can promote the translocation of eNOS from the plasma membrane to intracellular vesicles, with a concomitant reduction in eNOS enzyme activity. NOSTRIN-dependent shuttling of eNOS is caveolin-dependent and likely reflects specialized endocytosis of caveolar endosomes involving the actin cytoskeleton.

4. Modulation of the protective role of Nitric Oxide

4.1. Up-regulation

4.1.1 *Shear Stress*: Both acute and chronic increases in flow, and the resulting increasing force of shearing (shear stress) of the blood on the endothelial cells, augment the expression and the activity of eNOS, and thus the release of EDRF/NO (Figure 2) [Rubanyi et al., 1986; Miller & Vanhoutte, 1988; Davies, 1995; Busse & Fleming, 2003; Balligand 2009]. The immediate effect of an increase in shear stress on the release of NO explains flow-mediated dilatation (FMD), a phenomenon commonly used to estimate endothelial function in people. In the porcine and

human coronary circulation, flow-dependent vasodilatation involves the local production of bradykinin that stimulates the release of NO through a Gq-dependent mechanism (Figure 2) [see Vanhoutte *et al*., 2009]. The chronic effect of shear stress is due to an up-regulation of eNOS and a greater activation (phosphorylation) of the enzyme, leading to a larger release of NO for each given stimulation, explaining the beneficial effects of regular exercise on endothelial function [see Vanhoutte *et al*., 2009].

4.1.2 *Estrogens and Gender*: The reintroduction of physiological levels of estrogens in ovariectomized animals augments endothelium-dependent relaxations [see Vanhoutte *et al*., 2009]. The potentiating effect of estrogens on endothelium-dependent relaxations involves both genomic (Figure 2) and non-genomic effects [see Miller & Duckles, 2008; see Vanhoutte *et al*., 2009]. Phytoestrogens and selective estrogen receptor modulators (SERMs) also potentiate endothelium-dependent relaxations/vasodilatations [see Vanhoutte *et al*., 2009]. In coronary arteries, the potentiating effect of chronic treatment with estrogens is seen only with stimuli that activate Gi-coupled receptors on the endothelial cells and is counteracted by the chronic administration of progesterone [Miller & Vanhoutte, 1991]. It is likely that this potentiating effect of estrogens on NO release, presumably resulting from lower oxidative stress, helps to explain why endothelium-dependent relaxations are more pronounced in arteries from female than male animals and why pre-menopausal women are protected against coronary disease. The

opposing effects of progesterone helps to explain why hormone replacement therapy has not always had the expected beneficial cardiovascular effects [see Vanhoutte *et al*., 2009].

4.1.3 *Sphingolipids and Lysophospholipids*: A class of serum-borne lipid mediators including sphingosine 1-phosphate (S1P) and lyosphosphatidic acid activate endothelial G protein-coupled S1P receptors (formerly called EDG receptors) play key roles in vascular regulation [Hla 2001] stimulate AMP-activated protein kinase (AMPK) the small GTP binding protein Rac1, a critical determinant of the activity of eNOS.

4.1.4 *Diet*: The chronic intake of ω_3-unsaturated fatty acids potentiates endothelium-dependent relaxations and has antiatherogenic properties. The same holds true for the intake of flavonoids and other polyphenols of vegetal origin [see Vanhoutte *et al*., 2009].

4.2. Down-regulation:

4.2.1 *Oxygen-derived free radicals:* Several enzymes in the endothelial cells can produce superoxide anions. They include NADPH oxidase, xanthine oxidase, cyclooxygenase and eNOS itself, when it is uncoupled by lack of substrate (L-arginine) or shortage of the essential co-factor tetrahydrobiopterin [see Kojda & Harrison 1999; Fleming et al., 2005]. Superoxide anions can be dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) which can act as an EDHF and contribute to endothelium-dependent relaxations [see Feletou & Vanhoutte, 2006a, 2009], or been broken down by catalase. However, superoxide anions also scavenge NO avidly with the resulting formation of peroxynitrite which reduces the bioavailability of NO [see Kojda

& Harrison, 1999; Rida, 2004]. Hence, increases in oxidative stress lead to reduced endothelium-dependent relaxations, and antioxidants acutely improve such responses *in vitro* and *in vivo* both in animals and humans. However, chronic treatment with antioxidants usually fails to improve endothelial function in people [see Vanhoutte *et al*., 2009].

4.2.2 *Aging*: Both in animals and in humans, increasing age reduces the ability of the endothelium to elicit endothelium-dependent vasodilatations *in vitro* and *in vivo* [see Vanhoutte *et al*., 2009]. This has been attributed to increased activity of arginase [competing with eNOS for the common substrate arginine], augmented production of superoxide anions reducing NO bioavailability, reduced expression/presence and/or activity of eNOSand ultimately NO lesser release of [see Vanhoutte *et al*., 2009].

4.3.3 *Hypercholesterolemia*: Both in animals and in humans, high fat diets reduce endothelium-dependent relaxations/dilatations and the normalization of the cholesterol level with treatment restores endothelial function. The effect of hypercholesterolemia is due to the combination of increased oxidative stress leading to a reduced bioavailability of NO, impairment of the turnover rate of eNOS and augmented levels of circulating asymmetric dimethyl arginine (ADMA, an endogenous inhibitor of eNOS) [see Vanhoutte *et al*., 2009]. In addition, lipoproteins appear to reduce eNOS targeting to plasmalemmal caveolae as well the interactions of the enzyme with caveolin and other regulatory proteins that influence its activity [Feron and Balligand, 2006].

4.3. Cardiovascular disease

4.3.1 *Hypertension*: Endothelium-dependent relaxations are reduced in i arteries harvested from hypertensive animals, and responses to endothelium-dependent vasodilators are decreased in hypertensive humans. This blunting, which can be corrected by an appropriate treatment probably reflects the premature aging of the vasculature exposed chronically to the increased arterial blood pressure [see Vanhoutte *et al*., 2009]. In the spontaneously hypertensive rats and in the essential hypertensive human the reduction of endothelium-dependent relaxations/vasodilatations is caused mainly by the concomitant release of endothelium-derived vasoconstrictor prostanoids (endothelium-derived contracting factors; EDCF) rather than to a reduced release of NO [see Vanhoutte *et al*., 2005, 2009; Vanhoutte and Tang, 2009].

4.3.2 *Diabetes*: Insulin resistance and diabetes cause an impairment of arterial endothelium-dependent relaxations in animals and humans, presumably due to the chronic exposure to hyperglycemia [see De Vriese et al., 2000; Vanhoutte *et al*., 2009]. The endothelial dysfunction of diabetes has been attributed to a relative deficiency of reduced tetrahydrobiopterin, associated with inceased eNOS “uncoupling” and ROS production [Katucic 2009; Fleming and Busse 2003; Katusic 2001; Lubos et al. 2008; Moens and Kass 2006; Thomas et al. 2008; Wolin 2009], increased activity of arginase competing with eNOS for the common substrate, arginine, elevated

levels of the endogenous inhibitor ADMA, augmented production of superoxide anions and thus increased formation of peroxynitrite, and quenching of NO by advanced glycosylation products [see Vanhoutte, 2008; Vanhoutte *et al*., 2009].

4.3.3 *Coronary Disease*: Individuals at increased risk of coronary heart disease exhibit impaired dilatations in response to endothelium-dependent vasodilators both in the peripheral and coronary circulations. Both in animals and humans, the presence of such endothelial dysfunction predicts the severity of the outcome, in particular the occurrence of myocardial infarction [see Vanhoutte *et al*., 2009].

5. Regenerated endothelium

After maturation, endothelial cells remain quiescent for years before aging and apoptotic programming initiate their turnover, although the latter is accelerated by cardiovascular risk factors (obesity, smoking, hypertension and diabetes). When apoptotic endothelialcells die, they are replaced rapidly by regenerated endothelial cells [see Vanhoutte *et al*., 2009]. Regenerated endothelial cells are dysfunctional (Figure 4). Thus, coronary arteries covered with regenerated endothelium exhibited a marked blunting of the endothelium-dependent relaxations to agonists using the G_i-protein signaling cascade (aggregating platelets, serotonin or thrombin) and the remaining response is no longer inhibited by pertussis toxin. By contrast, relaxations evoked by ADP and bradykinin, which both depend on the Gq-signaling cascade are normal, illustrating the

ability of the regenerated endothelial cells to generate NO. These observations imply a selective dysfunction of the Gi-dependent responses in regenerated endothelial cells. This selective dysfunction is reduced by the chronic intake of ω_3-unsaturated fatty acid, and exacerbated by a high fat diet which results in the occurrence of typical atherosclerotic lesions only in the part of the coronary artery covered with regenerated endothelium [see Vanhoutte *et al*., 2009]. Thus, the selective dysfunction of regenerated endothelial cells appears to be an initial step allowing the atherosclerotic process.

Primary cultures derived from regenerated endothelial cells are characterized by accelerated senescence, reduced expression and activity of eNOS, greater production of oxygen-derived free radicals by the endothelial NADPH oxidase, greater uptake of modified low-density lipoprotein cholesterol (LDL) and larger generation of oxidized LDL (oxyLDL). By contrast, the presence of Gi-proteins is comparable to that observed in primary cultures derived from native endothelium. The genomic changes observed in cultures of regenerated endothelial cells are in line with those phenotypic and functional changes. These experimental findings prompt the conclusion that the augmented presence of oxyLDL is responsible for the selective loss in Gi-protein mediated responses in regenerated endothelial cells and thus of their inability to respond to serotonin and thrombin [Figure 2] [see Vanhoutte *et al*., 2009]. Obviously, this is not the only deleterious effect of oxygen-derived free radicals and oxyLDL which play a central role in the atherosclerotic process (Figure 5) [Thomas et al. 2008; Stocker & Keany, 2004]. Other factors

include inhibition of the expression, reduced activation (dephosphorylation) and uncoupling of eNOS and an enhanced activity of arginase, which competes with NO for the common substrate arginine (Figure 6) [Katusic, 2007; Vanhoutte 2008; see Vanhoutte *et al*., 2009]. In addition, a greater production of superoxide anions will reduce the bioavailability of NO and increase the levels of peroxynitrite [Kojda & Harrison 1999; Fleming et al., 2005; Cai 2005; Schroder and Eaton 2008; Thomas et al. 2008; Wolin 2009]. Endogenous mediators, other than the increased presence of oxyLDL, may accelerate or contribute to the atherosclerotic process. This may be in particular the case for the emergence of fatty acid-binding proteins in regenerated endothelial cells [Lee et al., 2007; Furuhashi & Hotamisligil, 2008]. Whatever the cause of the dysfunction, regenerated endothelial cells cannot produce enough NO in response to platelets and thrombin, and this NO deficiency permits the inflammatory reaction leading to atherosclerosis [Aikawa & Libby, 2004].

6. Conclusions:

In native, healthy endothelial cells a number of stimuli (e.g. serotonin from aggregating platelets, sphingosine 1-phosphate, and thrombin) activate eNOS causing the release of NO, which relaxes the vascular smooth muscle that surrounds them. NO, in synergy with prostacyclin, further inhibits platelet aggregation. It also reduces the endothelial expression of adhesion molecules and thus the adhesion and penetration of leukocytes (macrophages). The endothelial mediator

also prevents the proliferation of vascular smooth muscle cells and limits the formation of oxy LDL. Aging and certain lifestyle factors (e.g. lack of exercise, Western diet, smoking), or certain diseases (e.g. diabetes and hypertension) result in a lesser release of NO and an acceleration of the turnover of the apoptotic process in the endothelium. The apoptotic endothelial cells are replaced by regenerated ones. However, such regenerated cells are dysfunctional, senescent, and incapable of producing the required amounts of NO, which facilitates the inflammatory response leading to the formation of atherosclerotic plaques.

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References:

1. Aikawa, M. & Libby, P. 2004. The vulnerable atherosclerotic plaque Pathogenesis and therapeutic approach. *Cardiovasc Path.* **13**, 125-138.
2. Balligand, J. L., O. Feron, et al. (2009). "eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues." Physiol Rev 89(2): 481-534.
3. Berka, V., Wu, G., Yeh, H.C., et al. 2004. Three different oxygen-induced radical species in endothelial nitric-oxide synthase oxygenase domain under regulation by L-arginine and tetrahydrobiopterin. *J Biol Chem.* 279:32243-51.
4. Busse, R., Edwards, G., Félétou, M., Fleming, I. & Vanhoutte, P.M. 2002. EDHF: Bringing the concepts together. *Trends in Pharmacol Sci*. **23**, 374-380.
5. Busse, R., Fleming, I. 2003. Regulation of endothelium-derived vasoactive autacoid production by hemodynamic forces. *Trends in Pharmacol Sci.* **24**, 24-29.
6. Cai, H. (2005). "Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences." Cardiovasc Res 68(1): 26-36.

7. Cai, H., K. K. Griendling, et al. (2003). "The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases." Trends Pharmacol Sci 24(9): 471-8.

8. Chen, Z., I. C. Peng, et al. (2009). "AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633." Circ Res 104(4): 496-505.

9. Davies, P.F. 1995. Flow-mediated endothelial mechanotransduction. *Physiol Rev.* **75**, 519-560.

10. De Vriese, A.S., Verbeuren, T.J., Van de Voorde, J., Lameire, N.H. & Vanhoutte, P.M. 2000. Endothelial dysfunction in diabetes. *Br J Pharmacol.* **130,** 963-974.

11. Deanfield, J. E., J. P. Halcox, et al. (2007). "Endothelial function and dysfunction: testing and clinical relevance." Circulation 115(10): 1285-95.

12. Dudzinski, D. M. and T. Michel (2007). "Life history of eNOS: partners and pathways." Cardiovasc Res 75(2): 247-60.

13. Dudzinski, D. M., J. Igarashi, et al. (2006). "The regulation and pharmacology of endothelial nitric oxide synthase." Annu Rev Pharmacol Toxicol 46: 235-76.

14. Erwin, P. A., A. J. Lin, et al. (2005). "Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells." J Biol Chem 280(20): 19888-94.

15. Erwin, P. A., D. A. Mitchell, et al. (2006). "Subcellular targeting and differential S-nitrosylation of endothelial nitric-oxide synthase." J Biol Chem 281(1): 151-7.

16. Félétou, M. & Vanhoutte, P.M. 2006a. *EDHF: The Complete Story.* CRC Taylor and Francis, Boca Raton, pp. 1-298.

17. Félétou, M. & Vanhoutte, P.M. 2006b. Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol.* **291**, H985-H1002.

18. Félétou, M. & Vanhoutte, P.M. 2009. EDHF: An Update. *Clin Sci.* **in press**.

19. Feron, O. and J. L. Balligand (2006). "Caveolins and the regulation of endothelial nitric oxide synthase in the heart." Cardiovasc Res 69(4): 788-97.

20. Feron, O., F. Saldana, et al. (1998). "The endothelial nitric-oxide synthase-caveolin regulatory cycle." J Biol Chem 273(6): 3125-8.

21. Feron, O., F. Saldana, et al. (1998). "The endothelial nitric-oxide synthase-caveolin regulatory cycle." J Biol Chem 273(6): 3125-8.

22. Fisslthaler, B. and I. Fleming (2009). "Activation and signaling by the AMP-activated protein kinase in endothelial cells." Circ Res 105(2): 114-27.

23. Fleming, I. and R. Busse (2003). "Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase." Am J Physiol Regul Integr Comp Physiol 284(1): R1-12.

24. Forstermann, U., Boissel, J.-P., and Kleinert, J. (1998). "Expressional control of the 'constitutive' isoforms of nitric oxide synthase" FASEB J 12: 773-790/

25. Fulton, D., J. E. Church, et al. (2005). "Src kinase activates endothelial nitric-oxide synthase by phosphorylating Tyr-83." J Biol Chem 280(43): 35943-52.

26. Fulton, D., J. P. Gratton, et al. (2001). "Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough?" J Pharmacol Exp Ther 299(3): 818-24.

27. Furchgott, R.F. & Vanhoutte, P.M. 1989. Endothelium-derived relaxing and contracting factors. *FASEB J.* 3, 2007-2017.

28. Furchgott, R.F. & Zawadzki, J.V. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* **288**, 373-376.

29. Furuhashi, M. & Hotamisligil, G.S. 2008. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nature Rev.* **7**, 489-503.

30. Gonzalez, E., A. Nagiel, et al. (2004). "Small interfering RNA-mediated down-regulation of caveolin-1 differentially modulates signaling pathways in endothelial cells." J Biol Chem 279(39): 40659-69.

31. Govers, R. and T. J. Rabelink (2001). "Cellular regulation of endothelial nitric oxide synthase." Am J Physiol Renal Physiol 280(2): F193-206.

32. Govers, R. and T. J. Rabelink (2001). "Cellular regulation of endothelial nitric oxide synthase." Am J Physiol Renal Physiol 280(2): F193-206.

33. Gratton, J. P., P. Bernatchez, et al. (2004). "Caveolae and caveolins in the cardiovascular system." Circ Res 94(11): 1408-17.

34. Harrison, D. G. (1997). "Cellular and molecular mechanisms of endothelial cell dysfunction." J Clin Invest 100(9): 2153-7.

35. Hess, D. T., A. Matsumoto, et al. (2005). "Protein S-nitrosylation: purview and parameters." Nat Rev Mol Cell Biol 6(2): 150-66.

36. Hla, T., M. J. Lee, et al. (2001). "Lysophospholipids--receptor revelations." Science 294(5548): 1875-8.

37. Icking, A., S. Matt, et al. (2005). "NOSTRIN functions as a homotrimeric adaptor protein facilitating internalization of eNOS." J Cell Sci 118(Pt 21): 5059-69.

38. Jain, M. K. and P. M. Ridker (2005). "Anti-inflammatory effects of statins: clinical evidence and basic mechanisms." Nat Rev Drug Discov 4(12): 977-87.

39. Katusic, Z. S. (2001). "Vascular endothelial dysfunction: does tetrahydrobiopterin play a role?" Am J Physiol Heart Circ Physiol 281(3): H981-6.

40. Katusic, Z. S., L. V. d'Uscio, et al. (2009). "Vascular protection by tetrahydrobiopterin: progress and therapeutic prospects." Trends Pharmacol Sci 30(1): 48-54.

41. Katusic, Z.S. 2007. Mechanisms of endothelial dysfunction induced by aging: Role of arginase I. *Circ Res.* **101**, 640-641.

42. Kojda, G. & Harrison, D. 1999. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res.* **43**, 562-571.

43. Konig, P., J. Dedio, et al. (2005). "NOSIP and its interacting protein, eNOS, in the rat trachea and lung." J Histochem Cytochem 53(2): 155-64.

44. Stamler, J.S., Lamas, S., and Fang, F.C. (2001) “Nitrosylation: the prototypic redox-based signaling mechanism.” Cell 106:675-683

45. Lee, M.Y.K., Tse, H.F., Siu, C.W., Zhu, S.G., Man, R.Y.K. & Vanhoutte, P.M. 2007. Genomic changes in regenerated porcine coronary arterial endothelial cells. *Arterioscler Thromb Vasc Biol.* **27**, 2443-2449.

46. Levine, Y. C., G. K. Li, et al. (2007). "Agonist-modulated regulation of AMP-activated protein kinase (AMPK) in endothelial cells. Evidence for an AMPK -> Rac1 -> Akt -> endothelial nitric-oxide synthase pathway." J Biol Chem 282(28): 20351-64.

47. Li, J. M. and A. M. Shah (2004). "Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology." Am J Physiol Regul Integr Comp Physiol 287(5): R1014-30.

48. Li, P. L. and E. Gulbins (2007). "Lipid rafts and redox signaling." Antioxid Redox Signal 9(9): 1411-5.

49. Loscalzo, J. and G. Welch (1995). "Nitric oxide and its role in the cardiovascular system." Prog Cardiovasc Dis 38(2): 87-104.

50. Lubos, E., D. E. Handy, et al. (2008). "Role of oxidative stress and nitric oxide in atherothrombosis." Front Biosci 13: 5323-44.

51. Lüscher, T.F. & Vanhoutte, P.M. 1990. *The Endothelium: Modulator of Cardiovascular Function.* CRC Press, Inc., Boca Raton, pp. 1-228.

52. Martinez-Ruiz, A., Villanueva L., et al, 2005. S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities Proc Natl Acad Sci USA102: 8525-8530.

53. Miller, V.M. & Duckles, S.P. 2008. Vascular actions of estrogens: functional implications. *Pharmacol Rev.* **60**, 210-241.

54. Miller, V.M. & Vanhoutte, P.M. 1988. Enhanced release of endothelium-derived factors by chronic increases in blood flow. *Am J Physiol.* **255**, H446-H451.

55. Miller, V.M. & Vanhoutte, P.M. 1991. Progesterone and modulation of endothelium-dependent responses in canine coronary arteries. *Am J Physiol* **261**, R1022-R1027.

56. Moens, A. L. and D. A. Kass (2006). "Tetrahydrobiopterin and cardiovascular disease." *Arterioscler Thromb Vasc Biol.* 26(11): 2439-44.

57. Moncada, S. 1997. Nitric oxide in the vasculature: physiology and pathophysiology. *Ann N Y Acad Sci.* **811**, 60-67.

58. Radi R. 2004. Nitric oxide, oxidations, and protein tyrosine nitration. *Proc Natl Acad Sci USA.* 101: 4003–4008.

59. Rubanyi, G.M. & Vanhoutte, P.M. 1986. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor(s). *Am J Physiol.* **250**, H822-H827.

60. Rubanyi, G.M., Lorenz, R.R. & Vanhoutte, P.M. 1985. Bioassay of endothelium-derived relaxing factor(s). Inactivation by catecholamines. *Am J Physiol.* **249**, H95-H101.

61. Rubanyi, G.M., Romero, J.C. & Vanhoutte, P.M. 1986. Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol.* **250**, H1145-H1149.

62. Schilling, K., N. Opitz, et al. (2006). "Translocation of endothelial nitric-oxide synthase involves a ternary complex with caveolin-1 and NOSTRIN." Mol Biol Cell 17(9): 3870-80.

63. Schroder, E. and P. Eaton (2008). "Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations." Curr Opin Pharmacol 8(2): 153-9.

64. Sessa, W. C. (2004). "eNOS at a glance." J Cell Sci 117(Pt 12): 2427-9.

65. Shaul, P. W. (2002). "Regulation of endothelial nitric oxide synthase: location, location, location." Annu Rev Physiol 64: 749-74.

66. Shaul, P. W. (2003). "Endothelial nitric oxide synthase, caveolae and the development of atherosclerosis." J Physiol 547(Pt 1): 21-33.

67. Skidgel, R. A. (2002). "Proliferation of regulatory mechanisms for eNOS: an emerging role for the cytoskeleton." Am J Physiol Lung Cell Mol Physiol 282(6): L1179-82.

68. Steuhr DJ. 1997. Structure-function aspects in the nitric oxide synthases. Ann Rev Pharmacol Toxicol. 37:339–59

69. Stocker, R. & Keaney, J.F.Jr. 2004. Role of oxidative modifications in atherosclerosis. *Physiol Rev.* **84,** 1381-1478.

70. Su, Y., S. Edwards-Bennett, et al. (2003). "Regulation of endothelial nitric oxide synthase by the actin cytoskeleton." Am J Physiol Cell Physiol 284(6): C1542-9.

71. Thomas, S. R., P. K. Witting, et al. (2008). "Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities." Antioxid Redox Signal 10(10): 1713-65.

72. Vanhoutte, P.M. 2008. Arginine and arginase: eNOS double crossed? *Circ Res.* **102**, 866-868.

73. Vanhoutte, P.M. & Tang, E.H.C. 2008. Endothelium-dependent contractions: when a good guy turns bad. *J Physiol.* **586**: 5295-5303.

74. Vanhoutte, P.M. 2009. How we learned to say NO. *Arterioscl Thromb Vasc Biol.* **29**: 1156-1160.

75. Vanhoutte, P.M., Félétou, M. & Taddei, S. 2005. Endothelium-dependent contractions in hypertension. *Br J Pharmacol.* **144**, 449-458.

76. Vanhoutte, P.M., Shimokawa, H., Tang, E.H.C. & Félétou, M. 2009. Endothelial dysfunction and vascular disease. *Acta Physiol*. **196**: 193-222.

77. Wolin, M. S. (2009). "Reactive oxygen species and the control of vascular function." Am J Physiol Heart Circ Physiol 296(3): H539-49.

Figure Legends:

Figure 1. Some of the neurohumoral mediators that cause the release of endothelium-derived relaxing factors (EDRF) through activation of specific endothelial receptors (circles). E, epinephrine; AA, arachidonic acid; Ach, acetylcholine; ADP, adenosine diphosphate; α = alpha adrenergic receptor; AVP, arginine vasopressin; B, kinin receptor; ET, endothelin, endothelin-receptor; H, histaminergic receptor; 5-HT, serotonin (5-hydroxytryptamine), serotoninergic receptor; M, muscarinic receptor; NE, norepinephrine; P, purinergic receptor; T, thrombin receptor; VEGF, vascular endothelial growth factorVP, vasopressin receptor [From Vanhoutte *et al*., 2009. By permission].

Figure 2. Schematic of possible mechanisms by which production of nitric oxide is regulated in endothelial cells. Nitric oxide is produced through enzymatic conversion of L-arginine by nitric oxide synthase (endothelial or type III, eNOS). The transcription of this enzyme is regulated genomically by hormones and growth factors. Stability of eNOS mRNA is modulated by statins and hormones. eNOS enzyme activity requires calcium, calmodulin, nicotinamide adenine dinucleotide phosphate (NADPH), and 5, 6, 7, 8-tetra-hydrobiopterine (BH_4). Enzyme activity is regulated by complexing to these proteins in microdomains of the endothelial cell. Association with this complex of heat shock protein 90 (HSP 90) increases enzyme activity. Stimulation of specific receptors on the endothelial surface (R) complexed with guanine nucleotide regulatory proteins, which are sensitive to pertussis toxin (G_i) or insensitive to pertussis toxin (G_q), activate intracellular pathways that modulate eNOS activity posttranslationally through heat shock protein 90 or AKT-phosphorylation. Association of eNOS with caveolin-1 or glycosylation of the enzyme reduces activity. A metabolite of L-arginine, asymmetric dimethyl arginine (ADMA) decreases production of the nitric oxide through competitive binding to eNOS. Thus, this endogenous amine may be a risk factor for the development of cardiovascular disease. **+**, indicates stimulation; -, indicates inhibition; **?**, indicates those pathways in which the regulation is unknown [From Vanhoutte *et al*., 2009. By permission].Figure 3. Postulated G-protein mediated signal transduction processes in a normal, native endothelial cell. Activation of the cell causes the release of nitric oxide (NO), which has important protective effects in the vascular wall. Abbreviations: 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins [From Vanhoutte *et al*., 2009. By permission].

Figure 4. Effects of oxidized low-density lipoproteins (OXLDL) in a regenerated endothelial cell, resulting in the reduced release of nitric oxide (NO). Abbreviations: 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins [From Vanhoutte *et al*., 2009. By permission].

Figure 5. Mechanisms of oxLDL-induced impairment of endothelial NO production. The NO synthase (NOS) uses L-arginine to generate NO. NO production could be attenuated in the presence of oxLDL by interfering with the supply of L-arginine to the enzyme through endogenous competitive inhibitors such as asymmetrical dimethyl-L-arginine (ADMA) as well as degradation of arginine through arginase. NOS expression and specific activity are decreased by oxLDL through RhoA and PKC. NO bioavailability is reduced by an oxLDL-mediated activation of the NADPH oxidase, which leads to superoxide anion (O_2^-) formation. This process facilitates the generation of peroxynitrite ($ONOO^-$), which subsequently oxidizes tetrahydrobiopterin (BH_4) of NOS, leading to NOS uncoupling [Berka *et al*., 2004]. Uncoupled NOS itself produces O_2^-, further promoting the process of BH_4 oxidation. [From Vanhoutte *et al*., 2009. By permission].

Figure 6. Overview of principal eNOS post-translational modifications. eNOS undergoes dual acylation: co-translational N-myristoylation at glycine 2 and reversible post-translational

thiopalmitoylation at cysteine 15 and cysteine 26. [The numbering scheme used here reflects the amino acid sequence of bovine eNOS, which has been extensively characterized.] eNOS-derived NO promotes S-nitrosylation at cysteines 96 and 101, leading to enzyme inhibition (signified by the red arrow). Phosphorylation at serine 116 and threonine 497 leads to enzyme inhibition, and phosphorylations at serines 617, 635 and 1179 and at tyrosine 83 generally increase enzyme activity. Other eNOS post-translational modification sites have been more recently described, and are being actively investigated. References are in the text.

Figures

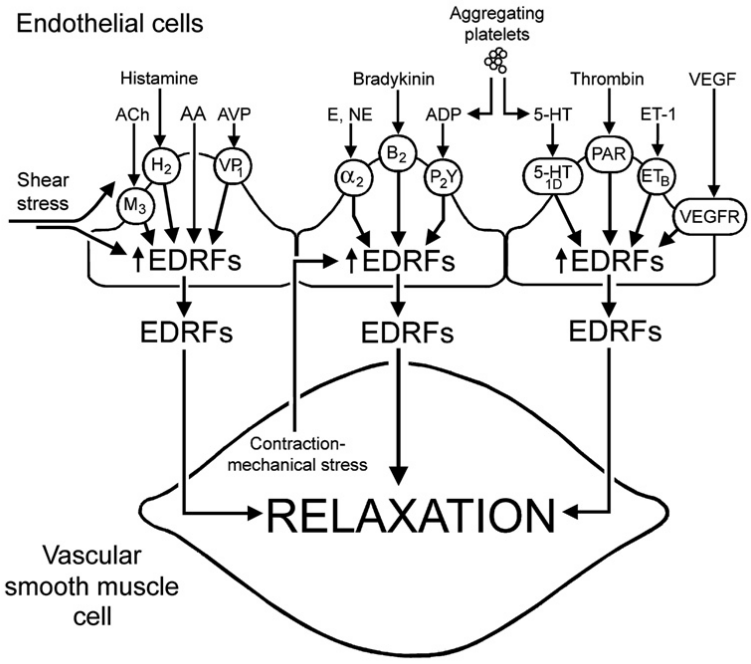

Figure 1

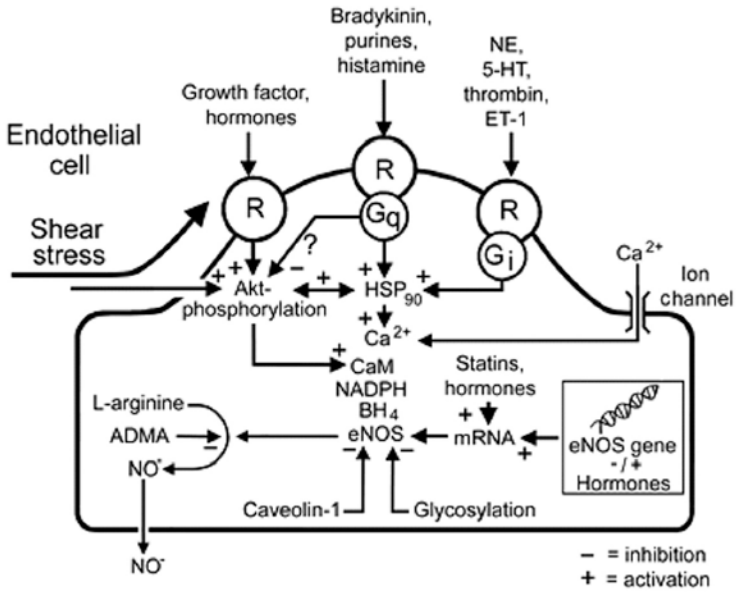

Figure 2

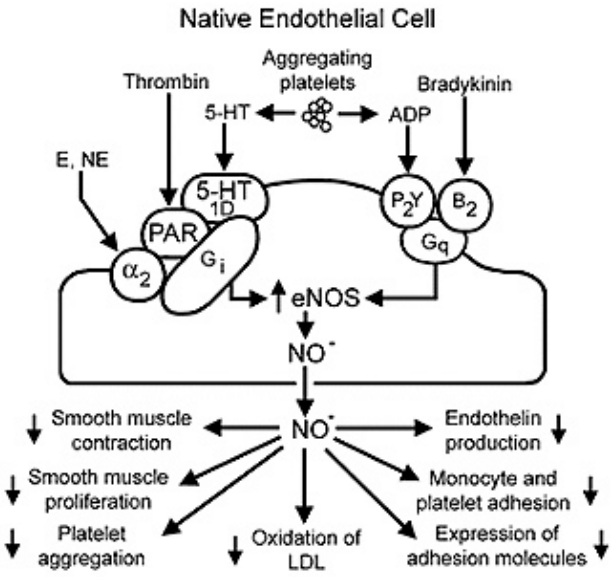
Native Endothelial Cell
Thrombin
Aggregating platelets
Bradykinin
5-HT
ADP
E, NE
5-HT 1D
PAR
P_2Y
B_2
G_q
G_i
α_2
eNOS
$NO^{\cdot}$
Smooth muscle contraction
Endothelin production
Smooth muscle proliferation
Monocyte and platelet adhesion
Platelet aggregation
Oxidation of LDL
Expression of adhesion molecules

Figure 3

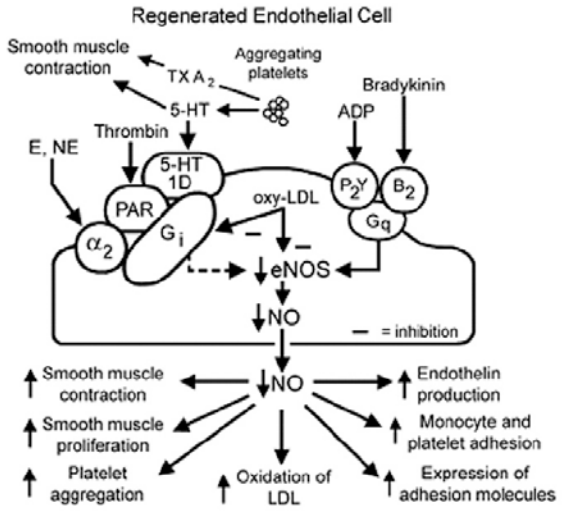

Figure 4

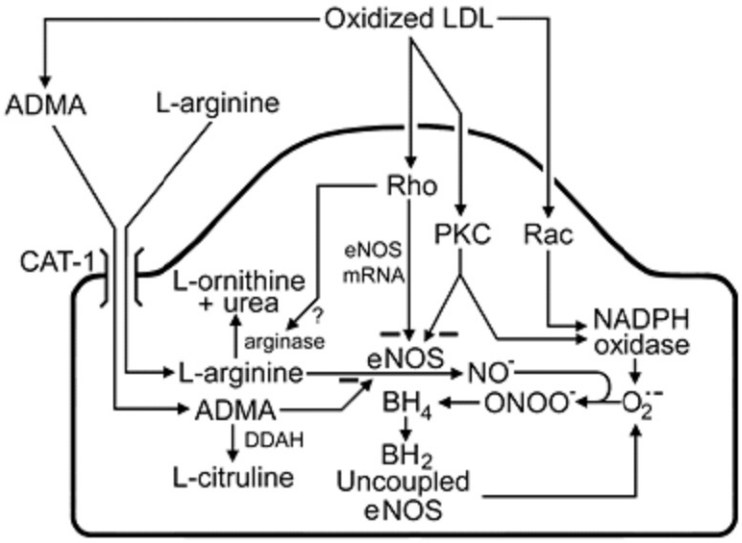

Figure 5

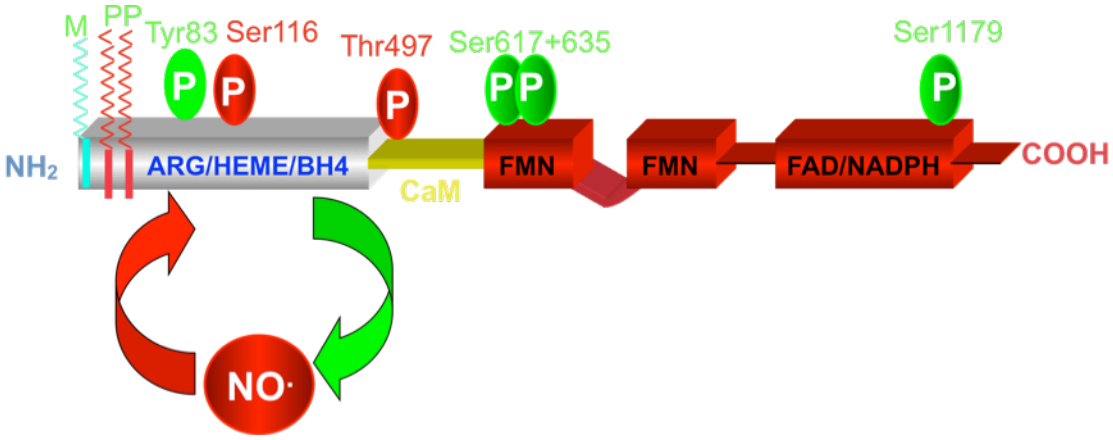
M
PP
Tyr83 Ser116
Thr497
Ser617+635
Ser1179
P
P
P
P
P
P
NH2
ARG/HEME/BH4
CaM
FMN
FMN
FAD/NADPH
COOH
NO·

Figure 6