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REVIEW

Endothelial nitric oxide synthase in the vascular wall: Mechanisms regulating its expression and enzymatic function

Michael Demosthenous a, c, Charalambos Antoniades a, c, *, Dimitris Tousoulis a, Marios Margaritis b, Kyriakoula Marinou b, Christodoulos Stefanadis a

a 1st Department of Cardiology, Athens University Medical School, Ilissas, Greece
b Department of Experimental Physiology, Athens University Medical School, Athens, Greece

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Tetrahydrobiopterin;
Atherosclerosis

Abstract  Endothelial nitric oxide synthase (eNOS) is the main source of nitric oxide (NO) in the vascular wall, a molecule with anti-inflammatory, antithrombotic, vasorelaxant, antioxidant and finally antiatherogenic properties. eNOS is expressed in vascular endothelium, and it uses L-arginine as a substrate, while it also requires the presence of multiple co-factors such as tetrahydrobiopterin (BH4), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and others. In the presence of BH4 deficiency, this enzyme becomes uncoupled, and it is turned into a source of superoxide radicals instead of NO. Therefore, under these conditions which are present in patients with advanced atherosclerosis, eNOS in human vascular endothelium is largely a source of reactive oxygen species, inducing in this way atherogenesis. Therefore, the aim of future therapeutic strategies targeting atherosclerosis through regulation of eNOS physiology, should take into account that up-regulation of this enzyme in the vascular wall may not lead to a respective increase of NO bioavailability and improvement of vascular homeostasis, but it may actually induce intravascular oxidative stress, if intracellular bioavailability of eNOS co-factors is not simultaneously elevated. In conclusion, eNOS plays a critical role in the regulation of vascular homeostasis, and it is a therapeutic target against atherogenesis.

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* Corresponding author.
E-mail address: antoniades@vodafone.net.gr (C. Antoniades).
c Authors equally contributed.

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Introduction

In 1980, Furchgott and Zawadzki\(^1\) demonstrated that the relaxation of vascular smooth muscle cells (VSMC) in response to acetylcholine is dependent on the anatomical integrity of the endothelium. The factor responsible for this intracellular relationship was named endothelium-derived relaxing factor (EDRF). In the late 1980s this factor was recognized as the free radical gas, nitric oxide (NO).

NO is synthesized by the enzyme NO synthase (NOS). The NOSs were first identified and described in 1989. NOSs catalyze NO biosynthesis through a reaction involving the conversion of L-arginine to L-citrulline.\(^2\) The enzyme, known as NOS, functions as a dimer consisting of two identical monomers, which can be functionally and structurally divided into two major domains. The first domain is C-terminal reductase domain and the second is N-terminal oxygenase domain.\(^3\)

The enzyme contains binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine nucleotide (FAD) and flavin mononucleotide (FMN) in close homology with cytochrome P-450 reductase, whereas the latter binds iron protoporphyrin IX (haem) and tetrahydrobiopterin (BH4), as well as the substrate L-arginine.

There are three distinct isofoms of NOS which differ both in structure and in function.\(^4\) Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and are referred to as Ca\(^{2+}\)-dependent enzymes (even though eNOS can be activated in a Ca\(^{2+}\)-independent manner).\(^5\) Inducible NOS (iNOS) is only expressed at high levels after induction by cytokines or other inflammatory agents, and its activity is independent of an increase of intracellular Ca\(^{2+}\).

The three NOS isoforms are characterized by regions of high homology (oxygenase and reductase domains) but at the same time each isoform exhibits distinctive features which reflect their specific in vivo functions.

The main source of endothelial NO is eNOS expressed by endothelial cells (ECs). Particular properties of eNOS which enable it to perform its specialized functions include Ca\(^{2+}\)-sensitivity and the post-translational modifications which mediate sub-cellular localization. These enable the enzyme to respond not only to neurohormonal agents but also to hemodynamic forces. Although eNOS was often referred to as constitutive NOS, a number of factors such as hypoxia, estrogens and exercise are now known to alter its expression. Since endothelial control of vascular tone is a sensitive and highly tuned process, these changes are likely to be important in cardiovascular function especially in pathophysiological situations.

In addition to the well-studied role of NO in the process of penile erection,\(^6\) non-adrenergic, non-cholinergic relaxation occurs in all vascular smooth cells as a result of the expression of nNOS in peripheral neurons.\(^7\) In terms of enzymatic function nNOS appears to differ from other NOS isoforms by its readiness to catalyze the uncoupled oxidation of NADPH. Few are known for the reaction mechanism, even though it will help to understand the damaging role of iNOS in brain ischemia.\(^8\)

iNOS expression can be induced by inflammatory mediators in most types of vascular cells, including ECs,\(^9\) cardiac myocytes,\(^10\) smooth muscle cells\(^11\) and macrophages.\(^12\) The expression of iNOS by macrophages and smooth muscle cells in atherosclerotic lesions has been taken as evidence for its determinant role in atherosclerosis.\(^12\) In addition iNOS expression is responsible for the impairment in eNOS-derived NO production in vessels treated with inflammatory mediators.\(^13\) On the other hand, iNOS expression may in some cases be protective, as shown by the iNOS-mediated suppression of allograft atherosclerosis, via the prevention of intimal hyperplasia.\(^14\)

Endothelial nitric oxide synthase (eNOS) gene

Although several vasoactive factors are produced by the endothelium, the principal and best characterized is NO,
produced by eNOS. The bioavailability of NO on the vessel wall is dependent on multiple factors such as the expression of eNOS, the presence of substrate and co-factors for eNOS, the phosphorylation status of eNOS, and the presence of reactive oxygen species (ROS) which can inactivate NO.

The three distinct genes for the human neuronal, inducible and endothelial NOS isoforms exist with a single copy each in the haploid human genome. The eNOS gene has 26 exons, 25 introns and it is located at 7q35–7q36 of chromosome 7. The protein consists of 1203 amino-acids and has a molecular weight of 133 kDa.

**Regulation of eNOS expression and NO availability**

The ability of eNOS to produce NO is essential to vascular homeostasis. Disturbance of this ability is a major contributor to the pathogenesis of vascular disease. In vivo studies have shown that expression of eNOS is vital to endothelial function and have led to the understanding that eNOS expression is subject to significant degrees of regulation by numerous physiological and pathophysiological stimuli via mechanisms that alter steady-state eNOS mRNA levels. Such stimuli involve shear stress, hypercholesterolemia, estrogens, sphingosine-1-phosphate, heat shock protein-90, transforming growth factor-β1, etc.

Laminar shear stress is one of the most potent regulators of eNOS mRNA expression. Laminar shear stress is a stimulus that has been shown to increase eNOS mRNA levels in vitro and in vivo. Transactivation of the eNOS promoter by laminar shear stress appears to be part of a negative feedback loop that involves NO. Laminar shear stress activates nuclear factor-κB (NF-κB), and in addition, simultaneously increases enzymatic production of NO. The activation of NF-κB leads to translocation of p50/p65 heterodimers to the nucleus. The binding of these factors to the eNOS promoter leads to an increase in eNOS transcription, increasing eNOS protein levels, further augmenting NO production. The increased NO production ultimately leads to nitrophosphorylation of p50 and inhibition of NF-κB, resulting in termination of eNOS transcription. Dekker et al. were the first to demonstrate increased expression of lung Kruppel-like factor (KLF2) in human umbilical vein ECs (HUVECs) exposed to sustained shear stress. Recently it was established that this transcription factor is involved in laminar shear-induced eNOS transcription. These investigators showed that KLF2 mRNA is present in the endothelium of healthy human aortas, exclusively in areas of high flow. Subsequently, it was shown that adenoviral over expression of KLF2 in HUVECs resulted in a dramatic induction of eNOS mRNA, protein, and enzyme activity.

Recently, it has been demonstrated that tumor necrosis factor-α (TNF-α) also has an effect on eNOS transcription. TNF-α decreased the activity of a human eNOS promoter construct that had been transiently transacted into bovine aortic ECs, in a time- and dose-dependent manner. Although the TNF-α-dependent inhibition of promoter activity appeared to involve the NF-κB cascade, the loci mediating the inhibition were mapped to two Sp1-binding sites positioned between −109 and −95 and −81 and −67 relative to the transcription starting site. Interestingly, mutations of either loci greatly suppressed basal promoter activity, but only the upstream locus (−109 to −95) showed a decrease in Sp1/Sp3 binding activity in response to TNF-α.

Cyclosporine A (CsA) has been shown to paradoxically increase NO production both in vivo and in vitro considering the fact that therapy with this agent is associated with hypertension. Bovine aortic ECs exposed to CsA had a threefold increase in the expression of eNOS mRNA which was time-dependent. Up-regulation of eNOS expression, induced by CsA has been proposed to be dependent on increased synthesis of ROS. Drummond et al. found that H2O2 increased eNOS mRNA levels up to fivefold in a time and dose-dependent manner. Analysis of the signaling cascade responsible for the effect of H2O2 on eNOS transcription established its dependence on Ca2+/calmodulin-dependent protein kinase II (CaMKII) and janus kinase (JAK2). Through activation of CaMKII, H2O2 appears to be the mediator of increased eNOS expression that occurs in response to oscillatory shear stress.

Lysophosphatidylcholine (lysoPC) has been shown to stimulate EC production of ROS and increases eNOS expression via enhanced transcription. Deletion analysis of human eNOS promoter-luciferase construct has identified Sp1 site at −104 to −90 and PEA3 sites at −40 to −24 as being involved in lysoPC-induced promoter activity. Gel-shift assays revealed that lysoPC augmented Sp1 binding activity. Subsequent analysis of the signaling events involved in the stimulatory effects of lysoPC on promoter activity, revealed a PI-3K-related pathway. In this pathway PI-3K activated JAK2, which in turn activated meiosis specific kinase 1 (MEK1). MEK1 stimulated Sp1 binding to the eNOS promoter through activation of Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) and subsequently increased protein phosphatase 2A activity. ERK1/2 exerted its effect through reducing the control of protein phosphatase 2A by casein kinase 2.

In both cell culture and animal studies, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG-CoA reductase; statins) have been shown to increase eNOS expression. In human saphenous vein ECs, simvastatin and lovastatin both increased eNOS mRNA and protein levels in a time- and dose-dependent manner. The mechanism responsible for this effect was found to be post-transcriptional; simvastatin treatment did not alter the rate of eNOS transcription but significantly prolonged eNOS mRNA half-life as assessed by actinomycin D transcriptional arrest studies. In addition, these studies showed that simvastatin and lovastatin both counteracted the downregulation of eNOS expression by hypoxia and oxLDL two stimuli known to decrease eNOS mRNA stability. By inhibiting HMG-CoA reductase, statins prevent the synthesis of isoprenoid intermediates in the cholesterol biosynthetic pathway. These intermediates serve as important lipid attachments for the post-translational modification and activity of a variety of signaling proteins. These proteins are members of the Rho GTPase family. Laufs and Liao found that Rho negatively regulates eNOS expression in human ECs; Rho inhibition increased eNOS expression, and Rho activation diminished eNOS expression. Furthermore, treatment of ECs with statins decreased the geranylgeranylation, membrane translocation, and GTP binding activity of Rho. Therefore, statins appear to upregulate eNOS expression by blocking Rho geranylgeranylation. Interestingly,
geranylgeranyl modification also appears to be involved in trichostatin A (TSA)-induced downregulation of eNOS expression. However, this latter mechanism is not dependent on Rho signaling. 36

TGF-β1 is a 25-kDa peptide that plays an important role in the pathogenesis of atherosclerosis, hypertension and angiogenesis. 36 Treatment of bovine aortic ECs with TGF-β1, increased eNOS mRNA in a time- and dose-dependent manner. 37 TGF-β1 increases eNOS transcription via recruitment of multiple transcription factors such as Smad2 and NF-1 to distinct cis-acting sequences. 37

Heat shock protein 90 (hsp90), modulates agonist-dependent eNOS activation. 38 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. 39

Sphingosine 1-phosphate (SP1) and lysophosphatidic acid activate endothelial G-protein-coupled S1P receptors (formerly called EDG receptors) and play key roles in vascular regulation 40 by stimulating AMP-activated protein kinase and the small GTP binding protein Rac1, critical determinants of the activity of eNOS.

The potentiating effect of estrogens on endothelium-dependent relaxations involves both genomic and non-genomic effects. 41,42 Phytoestrogens and selective estrogen receptor modulators also potentiate endothelium-dependent relaxations/vasodilatations. 42

High levels of cholesterol in humans affect endothelium-dependent relaxations/dilatations and the normalization of the cholesterol level with treatment restores endothelial function. 43 The effect of hypercholesterolemia is due to the combination of increased oxidative stress leading to a reduced bioavailability of NO, impairment of eNOS activity, and augmented levels of circulating asymmetric dimethylarginine (ADMA) 42 (Table 1).

### Common genetic polymorphisms of eNOS gene

To date more than 100 polymorphisms have been identified in, or in the vicinity of, the gene. More than 15 polymorphisms exist in the eNOS promoter that might influence mRNA transcription and reduce gene expression.

The −786T/C promoter polymorphism influenced transcriptional activity in vitro in a luciferase/reporter assay system and was associated with coronary arterial spasm in Japanese subjects. 44 In one study, ECs from subjects with the CC genotype exhibited reduced shear stress induced eNOS mRNA transcription, and vascular rings from such subjects had diminished endothelium-dependent vasodilatation. 45

Polymorphisms within the coding region of the eNOS gene could alter NOS enzymatic activity. One of the most studied NOS polymorphisms (G894T) within exon 7 is the only common polymorphism identified until now, that encodes an amino acid substitution-Glu298Asp (glutamate to aspartate position 298). 46 Two studies have shown that eNOS Asp298 is subjected to selective proteolytic cleavage in ECs and vascular tissues, that might account for reduced vascular NO generation. 47 Data from physiological studies have also been inconsistent. In some studies, individuals with the Asp298 allele exhibited altered vascular responses, for example a reduced blood pressure fall following exercise training, a lower basal blood flow and reduced vasodilatation to adenosine in the coronary arteries, an enhanced systemic pressure response to phenylephrine, a reduced flow mediated dilatation (FMD) of the brachial artery if concurrent smokers, impaired dilator responses to acetylcholine if hypertensive, or impaired enhancement of FMD during the first trimester of pregnancy. 46 Moreover, we have demonstrated that this polymorphism affects endothelial function in patients with atherosclerosis, 46 while it also plays a role in the regulation of systemic low-grade inflammation during myocardial infarction, an effect which is however disappeared in these patients under resting conditions, one year post-infarction. 49,50 The impact of this polymorphism on cardiovascular risk was also demonstrated in a large meta-analysis by Casas et al. 51 Despite the numerous data from clinical studies, some experimental data have questioned the association of this genotype with functional phenotype of eNOS. 52

### Table 1 Factors affecting eNOS activity and expression.

<table>
<thead>
<tr>
<th>Positive Regulation</th>
<th>Expression</th>
<th>Negative Regulation</th>
<th>Expression</th>
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<tbody>
<tr>
<td>Activity</td>
<td>Laminar shear stress</td>
<td>Hypercholesterolemia</td>
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<tr>
<td>Laminar shear stress</td>
<td>Laminar shear stress</td>
<td>Ox-LDL</td>
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<td>S1P</td>
<td>LysoPC</td>
<td>ADMA</td>
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<td>Caveolin57</td>
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<td>Ang II92</td>
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<td>Caveolin57</td>
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<tr>
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<td>VEGF143</td>
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There are three distinct isoforms of NOS which are different both in their structure and function. Endothelial NOS (eNOS, NOS III, 2 x 134 kDa) and neuronal NOS (nNOS, NOS I, 2 x 160 kDa) are referred to as constitutively expressed, Ca2+-dependent enzymes although eNOS can be activated in a Ca2+-independent manner.5 iNOS (NOS II, 2 x 130 kDa) is expressed at high levels only after induction by several inflammatory agents, and its activity is independent of Ca2+ increase.

Of the three NOS isoforms, eNOS alone is acylated by both myristate and palmitate.53 eNOS is co-translationally and irreversibly myristoylated at an N-terminal glycine residue while palmitoylation occurs post-translationally and reversibly at cysteine residues Cys15 and Cys26.54 Dual acylation of eNOS is required for efficient localization to the plasmalemmal caveolae of ECs.55 Palmitoylation is dynamically regulated by agonist (e.g. bradykinin)-induced changes in intracellular Ca2+.56 eNOS is localized to the caveolae,55 which are microdomains of the plasmalemmal membrane that are implicated in a variety of cellular functions including signal transduction events. The caveolin proteins are the major coat proteins of caveolae and in ECs eNOS binds to caveolin-1, while in cardiac myocytes eNOS is associated with caveolin-3.57,58 Caveolin 1 and peptides from the caveolin-1 scaffold region directly inhibit eNOS activity and this interaction is regulated by Ca2+/CaM.57,58 A nine-amino-acid binding motif for caveolin has been identified in bovine eNOS, residues and deletion mutation of these amino-acids produced an active enzyme that was uninhibitable by caveolin-1.59 Additionally, an independent interaction between caveolin-1 and the reductase domain of eNOS has been described.60

eNOS enzymatic function

Biosynthesis of NO involves a two-step oxidation of L-arginine to L-citrulline, with concomitant production of NO. The reaction consumes 1.5 mol of NADPH and 2 mol of oxygen for every mol of L-citrulline formed. The proposed mechanism60,61,62 involves an initial hydroxylation of L-arginine leading to the formation of NG-hydroxyL-arginine, which can also act as a substrate for eNOS. This is followed by oxidation of the intermediate, using a single electron from NADPH63 to form L-citrulline and NO. Even though this scheme represents the reaction assumed to be catalyzed by eNOS, the enzyme has also the capability of catalyzing the production of superoxide anion (O2·−), depending on the conditions. The isolated reductase domain is able to transfer electrons from NADPH via the flavins FAD and FMN to cytochrome, while the oxygenase domain dimer can convert the reaction intermediate NG-hydroxyL-arginine to NO and L-citrulline.64 Hence, the two domains perform catalytically distinct functions. While the reductase domain itself is highly homologous to enzymes such as the NADPH:cytochrome P450 reductase, its dependence on the CaM-binding domain for efficient electron transfer is unique.55 In contrast, the binding sites for L-arginine, haem and BH4 in the oxygenase domain are less well characterized.

Even though the reductase and oxygenase domains are able to function independently under certain circumstances, NO synthase activity is carried out by the homodimer.

Haem plays an essential role in the dimerization of eNOS. In its absence, eNOS, and all the other isoforms exist in monomers, which are unable to bind BH4 or a substrate analog and do not catalyze the L-citrulline/NO production.66 Resolution of the crystal structure confirmed that the haem is bound via a proximal cysteine thiolate ligand.67 The formation of this bond has been suggested to be a key step in the process of dimerization.68 The coordination state of the haem can be unequivocally identified through the examination of the absorption spectrum. This depends on the spin state of the unpaired electrons of the haem iron, which in turn is related to the geometry of the haem ligands. When these electrons are in low spin state, reflecting a six coordinating haem, the maximum is observed at 394–397 nm. This form of eNOS is inactive. Upon binding L-arginine and its analogs, as well as BH4, the maximum shifts to 418 nm, indicative of a high-spin five coordinate haem, which is necessary for eNOS activity.

eNOS coupling and the role of vascular tetrahydrobiopterin regulation

The NOS cofactor BH4 is essential for the proper transport of electrons to L-arginine, known as "coupling" of the enzyme.69 BH4 bioavailability is dependent on both biosynthesis and oxidative degradation to dihydrobiopterin (BH2) and finally bioppterin (B). Reduced synthesis or oxidative inactivation of BH4 leads to reduced NO availability, because without this critical cofactor, eNOS becomes "uncoupled", resulting in the generation of superoxide radicals (O2·−) rather than NO from oxygen and NADPH, consequently leading to impaired endothelium-dependent vasorelaxation and endothelial dysfunction70 (Fig. 1).

De novo production of BH4 is reliant upon three enzymes: GTP cyclohydrolase I (GTPCH), 1,6-pyruvoyl-tetrahydrobiopterin synthase, and sepiapterin reductase.71 The initial step in BH4 synthesis is the conversion of GTP to dihydroneopterin triphosphate by GTPCH, which is the rate-limiting enzyme in this biosynthetic pathway and is encoded by the GCH1 gene.72 This gene is characterized by two different haplotypes, termed X and O, which are defined by 3 polymorphisms: rs8007267G_A, rs3783641A_T, and rs10483639C_G (X haplotype: A, T, G; O haplotype: any other combination).73 The presence of the X haplotype in patients with multivessel CAD has been associated with lower levels of both plasma BH4 and total bioppterins (which is the sum of BH4, BH2, and B, reflecting the overall biosynthetic activity of GTPCH), as well as significantly lowers vascular GCH1 mRNA expression and vascular BH4 levels.74 This effect was not only observed in the XX genotype but also in the XO genotype, suggesting that the X haplotype may be an important and common factor in regulating circulating bioppterin levels in patients with CAD.

Several in vitro studies suggested that GCH1 expression in human macrophages and endothelial cells is induced by proinflammatory cytokines, resulting in an elevation of intracellular BH4 in endothelial cells.75,76 However, this effect was only observed after simultaneous exposure to
high concentrations of multiple cytokines, which is not as likely to occur in a clinical setting. In contrast, we have shown that in patients with CAD, systemic inflammatory stimuli can cause an elevation in plasma biopterins lead to a decrease in vascular biopterins, which are mainly located in the endothelium.77 Plasma biopterins are positively correlated with CRP levels and inversely associated with endothelial function, whereas vascular BH4 was associated with maintained eNOS coupling, reduced vascular O2 and improved endothelial function. These findings delineate a discordant regulation of circulating versus vascular biopterins, thus linking systemic inflammation with endothelial dysfunction and oxidative stress.

Oxidative inactivation of BH4 probably occurs through reaction with ROSs generated within the vessel wall, mainly peroxynitrite (ONOO⁻) and O2⁻.77 The resulting uncoupling of eNOS leads to generation of O2⁻ which react with NO to form ONOO⁻, further oxidizing BH4 in a feed-forward mechanism. Local intravascular administration of BH4 to smokers, hypertensives or hypercholesteroleemics can reinstate normal endothelial function, whereas BH4 has no effect on healthy vessels at the same dose.70 This observation suggests that BH4 availability may be limited in diseased vessels. In addition, we have previously reported that intravenous administration of 5-methyl-tetrahydrofolate (5-MTHF), the circulating form of folic acid, lead to an amelioration of eNOS function, improving NO bioavailability and reducing vascular oxidative stress in arteries and veins of CAD patients, both in vivo and ex vivo.78 Our findings suggest that although 5-MTHF is a rather weak superoxide scavenger, it may act by preventing ONOO⁻-induced oxidation of BH4 in the vascular wall and increasing intracellular BH4 bioavailability. Similarly, oral folate supplementation improved endothelial function in CAD patients, partly through improved BH4 bioavailability.79 This beneficial effect was similar between patients receiving high and low-dose supplementation, suggesting that it is mediated through an increase in vascular 5-MTHF levels rather than plasma 5-MTHF levels, which are dose-dependent.80 In a very recent study, we demonstrated that the functional C677T polymorphism in the methyl tetrahydrofolate reductase (MTHFR) gene, which is associated with lower plasma and vascular 5-MTHF levels, exerts a direct effect on vascular BH4 levels, eNOS coupling and NO bioavailability in human vessels in vivo.81 These results propose that physiological variations of vascular and plasma 5-MTHF levels could reflect endothelial function in humans. In addition, Lemarie et al.82 showed that MTHFR deficiency was associated with increased ROS production and reduced NO generation in endothelial progenitor cells (EPCs), heterozygous for the gene deletion MTHFR (+/-). Furthermore, treatment of EPCs with sepiapterin, a precursor BH4 significantly reduced ROS and improved NO production.

ADMA is a naturally occurring amino acid that circulates in plasma, is excreted in urine, and is found in tissues and cells.83,84 ADMA is synthesized when arginine residues in proteins are methylated by the protein arginine methyltransferases (PRMTs), while dimethylaminohydrolase (DDAH) catabolises ADMA. Importantly, ADMA is an endogenous inhibitor of eNOS, and has been associated with eNOS uncoupling; in the presence of BH4 deficiency it induces uncoupling while in the presence of excessive BH4 it behaves as an inhibitor of the enzyme.

**Figure 1** The role of tetrahydrobiopterin (BH4) and asymmetrical dimethylarginine (ADMA) on endothelial nitric oxide synthase (eNOS) coupling: The NOS cofactor BH4 is essential for the proper transport of electrons to L-arginine, known as “coupling” of the enzyme. Reduced synthesis or oxidative inactivation of BH4 leads to reduced NO availability, since without this critical cofactor, eNOS becomes “uncoupled”, resulting in the generation of superoxide radicals (O2⁻) rather than NO. ADMA is synthesized when arginine residues in proteins are methylated by the protein arginine methyltransferases (PRMTs), while dimethylaminohydrolase (DDAH) catabolises ADMA. Importantly, ADMA is an endogenous inhibitor of eNOS, and has been associated with eNOS uncoupling; in the presence of BH4 deficiency it induces uncoupling while in the presence of excessive BH4 it behaves as an inhibitor of the enzyme.

The understanding of eNOS molecular biology as well as the mechanisms that regulate its expression and activity has turned the therapeutic targeting of several vascular
Angiotensin converting enzyme inhibitors and angiotensin receptor blockade

As it is well-known angiotensin II (Ang II) exerts pro-oxidant, proinflammatory and proliferative effects on the vasculature mainly via the constitutively expressed angiotensin II type 1 receptor (AT1R).91 Angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) exert their effect on endothelial function partly via the increase of NO bioavailability. Further to its well known atherogenic and proinflammatory properties, Ang II contributes to the ROS pool by activating NADPH oxidase activity resulting to the reduction of NO bioavailability.92 One of the mechanisms by which ACEi exert their effect on NO availability increase is the reduction of ROS generation. As it was demonstrated by Lob et al. ACEi reduced the Ang II stimulated intracellular production of ROS.93 Another mechanism, which probably is responsible for the effect of ACE inhibition on NO availability is the prevention of bradykinin breakdown, thereby increasing kinin-dependent nitric oxide release mediated by the B2-kinin receptor. Several animal studies demonstrated that the effects of ACEi on NO release are blocked by B2-kinin receptor antagonists.94,95

Blockade of the angiotensin II AT1 receptor reduces the vascular signaling and the production of ROS that suppress nitric oxide activity. As Taddei et al.96 demonstrated, can-desartan inhibited the vascular production of ROS in ApoE-deficient mice. In addition, the synergic use of an ACEi metabolite and an ARB resulted in an increase of bradykinin and cGMP in sodium depleted rats.97 Moreover, a five-week treatment with olmesartan suppressed the elevation of blood pressure and the reduction of phosphorylated eNOS-Ser(1177) in spontaneously hypertensive rats (SHR) resulting to the elevation of eNOS activity.98 Tumor necrosis factor-alpha (TNF-alpha) is known to decrease eNOS expression and is an important mediator of endothelial dysfunction. Kataoka et al. induced a TNF-α decrease in eNOS expression in HUVECs. Pretreatment of HUVECs with AT1 receptor blockers (olmesartan or candesartan) restored the TNF-alpha-dependent reduction of eNOS.99

Aliskiren

Aliskiren is the first in a new class of orally effective rennin inhibitors for the treatment of hypertension. Early clinical trials in hypertensive patients showed that this drug provided antihypertensive efficacy comparable to those of the ARBs losartan and irbesartan.100,101 By using a catheter-type NO sensor, Imanishi et al.102 demonstrated that aliskiren administration increases Ach-induced and basal plasma NO concentrations in Watanabe heritable hyperlipidemic (WHHL) rabbits. Moreover, aliskiren/valsartan cotreatment increased the ACh-induced and basal plasma NO concentrations to a significantly greater extent than either aliskiren or valsartan alone. The results of this study could provide an experimental rationale for the combined application of ARBs and renin inhibitors in the treatment of hypertension and related cardiovascular diseases.102

Table 2 Therapeutic strategies targeting eNOS expression and function.

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<th>Therapeutic agent</th>
<th>Comment/selected most important mechanisms</th>
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<td>ACEi/AT1R blockers</td>
<td>Improvement of intracellular redox state, preventing oxidative degradation of co-factors.93 Increase of eNOS activity by increasing eNOS phosphorylation at Ser1177 and bradykinin levels.94 Increase of eNOS expression by suppressing TNF-α activity.99</td>
</tr>
<tr>
<td>Statins</td>
<td>Reduction of overall ROS pool, preventing the oxidation of co-actors and improving coupling.105 Increase eNOS expression, by stabilizing mRNA and reducing Nuclear factor kappaB and other transcriptional pathways.106 Increase eNOS activity by modifying P13-kinase — Akt protein kinase pathway.106 Improve eNOS coupling by affecting eNOS phosphorylation at Ser1177.114</td>
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<td>TZDs</td>
<td>Modify heat shock protein 60-eNOS interaction, affecting eNOS activity.114 Reduce gp91phox expression and modify NADPH-oxidase activity; by reducing overall cellular oxidative stress status improve eNOS coupling/activity and stimulate its expression.116</td>
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<td>Folic acid</td>
<td>Reduce homocysteine, therefore exert intracellular antioxidant properties; stimulate eNOS expression.80 Scavenge peroxynitrite (and less superoxide) directly, therefore protecting the oxidation of BH4 and improving eNOS coupling.78</td>
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<tr>
<td>BH4</td>
<td>Improve eNOS coupling and activity.147</td>
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<tr>
<td>Vitamins C and E</td>
<td>Antioxidant properties. Improves the activity/coupling of eNOS by improving intracellular BH4 bioavailability.125</td>
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<td>Polyphenolic antioxidants</td>
<td>Antioxidant properties. Reduce NADPH oxidase activity, increase eNOS activity.126-128 Endothelin ETA receptor blockade. Increases NO/L-arginine availability.138</td>
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<tr>
<td>ET-antagonists</td>
<td>Endothelin ETA receptor blockade. Increases NO/L-arginine availability.138</td>
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<tr>
<td>Aliskerin</td>
<td>Aliskerin is the first in a new class of orally effective rennin inhibitors for the treatment of hypertension. Early clinical trials in hypertensive patients showed that this drug provided antihypertensive efficacy comparable to those of the ARBs losartan and irbesartan.100,101 By using a catheter-type NO sensor, Imanishi et al.102 demonstrated that aliskiren administration increases Ach-induced and basal plasma NO concentrations in Watanabe heritable hyperlipidemic (WHHL) rabbits. Moreover, aliskiren/valsartan cotreatment increased the ACh-induced and basal plasma NO concentrations to a significantly greater extent than either aliskiren or valsartan alone. The results of this study could provide an experimental rationale for the combined application of ARBs and renin inhibitors in the treatment of hypertension and related cardiovascular diseases.102</td>
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renowned for their "pleiotropic" lipid lowering-independent effects. HMG-CoA reductase inhibitors improve endothelial function in many ways. NO bioavailability is increased not only by statins-induced reduction in ROS production but also by direct effects on eNOS enzyme. It has been suggested that statins upregulate eNOS expression and activity\(^\text{103}\) and prolong eNOS mRNA half-life\(^\text{104}\) by a post-transcriptional mechanism involving inhibition of geranylgeranylation of Rho GTPase, and stabilization of eNOS mRNA.\(^\text{32,35}\) Statin-induced activation of phosphatidylinositol 3-kinase — Akt protein pathway also increases NO production and inhibits endothelial cell apoptosis.\(^\text{104}\) In addition, several statins inhibit endothelial superoxide formation by reducing the activity of NADPH oxidase. This is partly due to the prevention of the isoprenylation of p21pnox, which is critical for NADPH oxidase assembly.\(^\text{105}\)

We have recently demonstrated that atorvastatin has a direct effect on vascular superoxide generation in human vein grafts, mainly by reducing NADPH-oxidase activity as a result of the reduction of membrane translocation of p67pnox and Rac1, subunits of the enzyme.\(^\text{106}\) Furthermore simvastatin treatment increases the number of functionally active endothelial progenitor cells as well as SOD activity,\(^\text{107}\) while statins also increase GCH1 mRNA expression in endothelial cells and elevate intracellular BH4.\(^\text{108}\)

In addition statins, by suppressing the expression of pro-inflammatory mediators, reduce circulating ADMA plasma levels and improve NO bioavailability, an ability alien to antioxidant vitamins, mainly in diabetic subjects.\(^\text{109}\)

**Thiazolidinediones**

Many antidiabetic agents are currently in use for treatment of hyperglycemia, however the insulin-sensitizing thiazolidinediones (or TZDs) seem to have additional beneficial effects on vascular wall. Thiazolidinediones are agonists of the peroxisome proliferators-activated receptor gamma (PPAR-\(\gamma\)). PPAR-\(\gamma\) is a nuclear hormone receptor which is involved in the modulation of the expression of several genes that affect glucose and lipid metabolism. In vitro evidence suggests that PPAR-\(\gamma\) is also expressed in vascular smooth muscle cells and endothelial cells.\(^\text{110}\) One report demonstrated that ciglitazone and a PPAR-\(\gamma\) synthetic ligand can stimulate the release of NO from endothelial cells.\(^\text{110}\) However Linscheid et al. demonstrated that rosiglitazone reduces vascular O\(_2\)-production in human arteries and veins, both in vivo and ex vivo.\(^\text{110}\) Oral folic acid administration (0.4 mg/day or 5 mg/day) seven weeks before scheduled coronary by-pass grafting, resulted into improved endothelial function and reduced vascular O\(_2\)-generation due to an improvement of eNOS coupling.\(^\text{79,12}\) Therefore, homocysteine-lowering treatment with folates may improve eNOS coupling both by reducing intracellular HCy in vascular endothelium and by acting as a direct scavenger of peroxynitrite radicals in endothelial cells. Furthermore, we have recently shown that treatment with folic acid induces a significant improvement of aortic distensibility and reduces indexes of arterial stiffness such as pulse wave velocity.\(^\text{79}\) Of course treatment with folates can be useful only in the presence of intracellular 5-MTHF deficiency, such as in the presence of 677 TT genotype in the MTHF reductase (MTHFR).\(^\text{81}\)

**Polyphenolic antioxidants and angioxidant vitamins**

Epidemiological evidence suggests that, dietary polyphenolic antioxidants (fruit, vegetables, wine, grape juice, etc) have a negative correlation with the incidence of cardiovascular disease.\(^\text{124}\) Even though most polyphenols are only mild antioxidants, some can reduce the activity of pro-oxidative NADPH-oxidas, and others can stimulate antioxidative enzymes and eNOS.\(^\text{125}\)

High doses of vitamin C have been found to improve endothelial function in humans.\(^\text{126–129}\) Some other studies demonstrated that long-term oral treatment with vitamin C also improved endothelial function in humans.\(^\text{130}\) Despite all that, long-term epidemiological studies with oral vitamin C treatment failed to support a role for vitamin C in reducing cardiovascular morbidity or mortality.\(^\text{131}\)

Even though vitamin E has antioxidant properties, large studies discourage the use of vitamin E for the prevention of cardiovascular events.\(^\text{132}\)

**Endothelin receptor blockade**

The 21-amino acid peptide endothelin-1 (ET-1) is the predominant isoform of the endothelin peptide family, which includes ET-2, ET-3, and ET-4. It exerts various biological effects, including vasoconstriction and the stimulation of cell proliferation in tissues both within and outside of the
cardiovascular system. ET-1 activates Gi-protein — coupled, 7-transmembrane domain receptors. Five ET receptors have been cloned. Mammals possess ETA\textsuperscript{133} and ETB receptors.\textsuperscript{134}

Studies in L-NG-nitroarginine methyl ester hypertension suggest that ET-1 is linked to the dysfunction of the L-arginine/NO pathway\textsuperscript{35,136} because ETA-selective\textsuperscript{137} but not combined ET blockade\textsuperscript{137} improves endothelial function, independent of blood pressure. Thus, selective inhibition of ETA receptors improves the endothelial L-arginine/NO pathway, which agrees with observations in humans.\textsuperscript{138}

**Tetrahydrobiopterin**

Despite the well documented role of BH\textsubscript{4} in human vessels function, treatment with BH\textsubscript{4} was infeasible until recently not only due to the high cost of such treatment, but also because of the high instability of BH\textsubscript{4} molecule: BH\textsubscript{4} compound readily undergoes aerobic oxidation\textsuperscript{139} and has a shelf-life of less 8 h at room temperature.\textsuperscript{140} However a stable tablet formulation of BH\textsubscript{4} has been developed recently, wherein the compound retains at least about 98% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin after 6 months.\textsuperscript{141} This tablet form makes BH\textsubscript{4} oral administration a possible strategy in the treatment of atherosclerosis. Alternative strategies could aim at the intracellular oxidation of BH\textsubscript{4} (e.g. by folate administration) or increasing BH\textsubscript{4} vascular levels by upregulating GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme for BH\textsubscript{4} synthesis.\textsuperscript{27} Moreover, chronic treatment with oral BH\textsubscript{4} of hypercholesterolemic patients reversed endothelial dysfunction and decreased plasma levels of isoprostanes, a marker of oxidative stress.\textsuperscript{142} However the efficacy of such promising strategies at a clinical level remains to still be confirmed by large clinical trials.

**Conclusions**

It has now become apparent that numerous physiological and pathophysiological stimuli can modulate eNOS expression. Several in vitro and in vivo studies tried to investigate the expression pattern of eNOS gene. Data from the most recent studies suggested that there is a complex regulatory mechanism of eNOS expression, at both transcriptional and post-transcriptional level. More recent studies tried to define the regulatory mechanisms involved in eNOS expression including signaling pathways and cis-trans interaction. Finally, by understanding eNOS molecular biology it is becoming more and more feasible the designation of new therapeutical strategies mainly targeting eNOS expression and eNOS coupling, essential in the treatment of various diseases such as atherosclerosis and coronary artery disease. It is therefore widely believed that eNOS function regulation is a therapeutic target in atherosclerosis since this enzyme not only regulates endothelial NO bioavailability, but it also plays a crucial role in the regulation of vascular redox, affecting in this way multiple redox-sensitive transcriptional pathways in the human vascular wall.

**References**


eNOS regulation and vascular function


