Endothelial Nitric Oxide Synthase in Vascular Disease
From Marvel to Menace

Ulrich Förstermann, MD, PhD; Thomas Münzel, MD

Abstract—Nitric oxide (NO·) is an important protective molecule in the vasculature, and endothelial NO· synthase (eNOS) is responsible for most of the vascular NO· produced. A functional eNOS oxidizes its substrate L-arginine to L-citrulline and NO·. This normal function of eNOS requires dimerization of the enzyme, the presence of the substrate L-arginine, and the essential cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), one of the most potent naturally occurring reducing agents. Cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, or chronic smoking stimulate the production of reactive oxygen species in the vascular wall. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases represent major sources of this reactive oxygen species and have been found upregulated and activated in animal models of hypertension, diabetes, and sedentary lifestyle and in patients with cardiovascular risk factors. Superoxide ($O_2^{\cdot-}$) reacts avidly with vascular NO· to form peroxynitrite ($ONOO^-$). The cofactor BH_4 is highly sensitive to oxidation by $ONOO^-$. Diminished levels of BH_4 promote $O_2^{\cdot-}$ production by eNOS (referred to as eNOS uncoupling). This transformation of eNOS from a protective enzyme to a contributor to oxidative stress has been observed in several in vitro models, in animal models of cardiovascular diseases, and in patients with cardiovascular risk factors. In many cases, supplementation with BH_4 has been shown to correct eNOS dysfunction in animal models and patients. In addition, folic acid and infusions of vitamin C are able to restore eNOS functionality, most probably by enhancing BH_4 levels as well. **(*Circulation*. 2006;113:1708-1714.)**

Key Words: endothelium ■ arteriosclerosis ■ endothelium-derived factors ■ nitric oxide synthase ■ risk factors

The roughly 10^{14} endothelial cells of our vasculature protect us against atherosclerosis and thrombosis. A major weapon of endothelial cells to fight vascular disease is endothelial nitric oxide synthase (eNOS), an enzyme that generates the vasoprotective molecule nitric oxide (NO·). However, many of us unintentionally mistreat our endothelial cells. We expose them to risk factors such as cigarette smoke, high blood pressure, high glucose, or high lipids. Despite this abuse, our endothelium bears with us for some time, tries to maintain NO· production, and preserves vascular protection. However, the risk factors lead to excess production of superoxide ($O_2^{\cdot-}$; ie, they produce oxidative stress). $O_2^{\cdot-}$ reacts with NO· to form peroxynitrite, and vascular protection slowly vanishes. But that is only the beginning of the calamity. Our eNOS now enters into a vicious biochemical cycle. It changes its enzymology, starts making peroxynitrite ($ONOO^-$) itself, and eventually becomes an enzyme that generates only $O_2^{\cdot-}$. This brief review discusses how and when this happens and how it may be prevented.

Vascular Protection by eNOS-Derived NO·

eNOS, the predominant NOS isoform in the vasculature, is responsible for most of the NO· produced in this tissue.[1] Vascular NO· dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells.[1] NO· released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. NO· also can inhibit leukocyte adhesion to the vessel wall either by interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to form an adhesive bond with the endothelial cell surface or by suppressing CD11/CD18 expression on leukocytes. White cell adherence is an early event in the development of atherosclerosis; therefore, NO· may protect against the onset of atherogenesis. Furthermore, NO· has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells. The inhibition of platelet aggregation and adhesion protects smooth muscle from exposure to platelet-derived growth factor(s). Therefore, NO· also prevents a later step in atherogenesis, fibrous plaque formation. Based on the combination of those effects, endothelial NO· probably represents the most important antiatherogenic defense principle in the vasculature.[1]

NOS Structure and Structure-Related Enzymology

All NOS isoforms are modular enzymes. In intact NOS, a C-terminal reductase domain (which binds nicotinamide adenine dinucleotide phosphate [NADPH], flavin mononucleo-

From the Departments of Pharmacology (U.F.) and Internal Medicine II (Cardiology) (T.M.), Johannes Gutenberg University Hospitals, Mainz, Germany.

Correspondence to Ulrich Förstermann, MD, PhD, Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, 55131 Mainz, Germany. E-mail ulrich.forstermann@uni-mainz.de

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.602532**

tide [FMN], and flavin adenine dinucleotide [FAD]) is linked to the N-terminal oxygenase domain of the other monomer (Figure 1). As shown in Figure 1C, the oxygenase domain carries a prosthetic heme group. The oxygenase domain also binds (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen, and the substrate L-arginine.[1] Sequences located near the cysteine ligand of the heme are apparently also involved in L-arginine and BH_4 binding (Figure 2A). All 3 NOS isoforms possess a zinc-thiolate cluster formed by a zinc ion that is tetrahedrally coordinated to 2 CXXXXC motifs (1 contributed by each monomer) at the NOS dimer interface[2] (Figure 2A). Chemical removal of zinc from NOS or the possibility of expressing a zinc-deficient NOS that remained catalytically active demonstrated that the zinc in NOS is structural rather than catalytic. All NOS isozymes catalyze flavin-mediated electron transfer from the C-terminally bound NADPH to the heme on the N terminus. Calmodulin (on calcium-induced binding) increases the rate of electron transfer from NADPH via the reductase domain flavins to the heme center (Figures 1B and 1C). At the heme, the electrons are used to reduce and activate O_2. To synthesize NO·, the enzyme needs to cycle twice. In a first step, NOS hydroxylates L-arginine to N^{ω}-hydroxy-L-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes N^{ω}-hydroxy-L-arginine to L-citrulline and NO· (Figure 1C).[3] In human eNOS, Cys99, which is part of the zinc-thiolate cluster, is thought to represent (or largely contribute to) the binding site for BH_4; zinc itself does not contribute to BH_4 binding. Mutation of the homologous Cys331 in nNOS to alanine (C331A) led to an enzyme that lost its binding affinity for BH_4 and became catalytically incompetent.[4]

$O_2^{\cdot-}$ Generation by eNOS and Enzyme Dimerization

The flow of electrons within NOS is tightly regulated. If disturbed, the ferrous-dioxygen complex dissociates, and $O_2^{\cdot-}$ is generated from the oxygenase domain instead of NO· (Figures 2B through 2D). This is referred to as NOS uncoupling.

In the recent literature, NOS-catalyzed reduction of molecular oxygen to $O_2^{\cdot-}$ has been attributed to the failure of the enzyme to form dimers. Indeed, it has been shown that monomers of NOS and even isolated reductase domains are sufficient for $O_2^{\cdot-}$ production (Figure 1A). However, the NADPH oxidase activity of such enzyme fragments is limited; the dimeric form has much higher enzymatic activity (Figure 1B). Studies with inhibitors of dimerization on inducible NOS have suggested that once a dimer is formed, there is little or no significant return to the monomer.[5] Most probably, this also applies to eNOS. Thus, uncoupling of oxygen reduction from NO· formation is unlikely to go along with significant monomerization of the enzyme in vivo.

Cardiovascular Risk Factors Cause Endothelial Dysfunction: Potential Mechanisms Involved

In the presence of cardiovascular risk factors, endothelial dysfunction frequently is encountered. Several molecular defects could account for reductions in endothelium-dependent vascular relaxation.

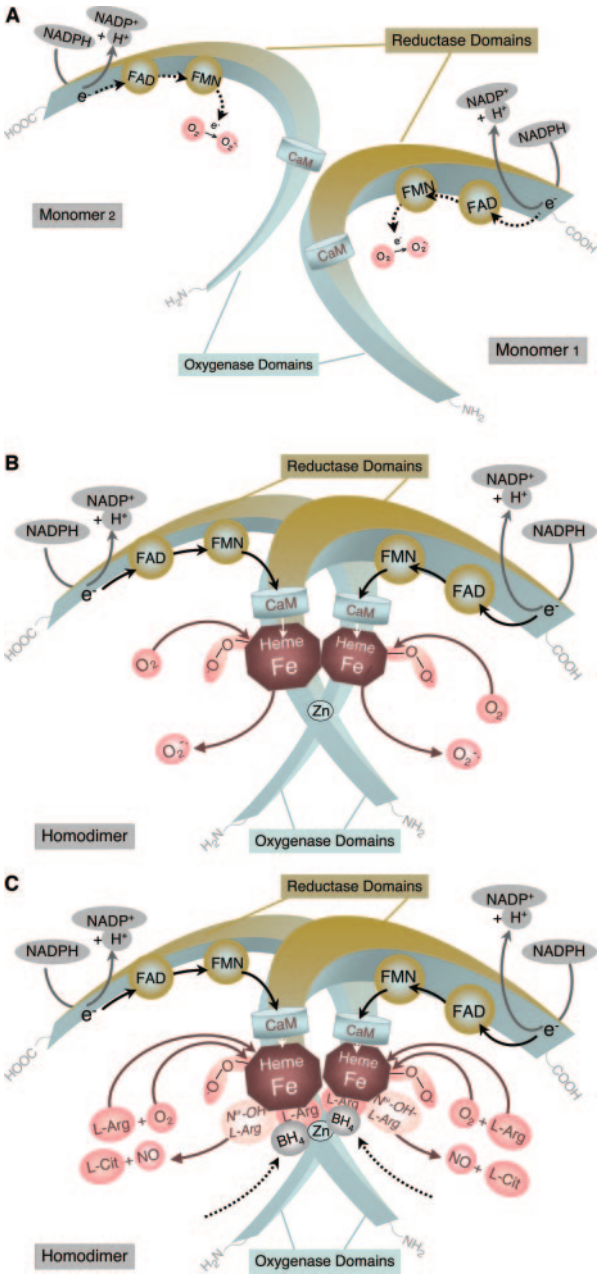

Figure 1. A, Basic structure of eNOS and scheme of NOS catalysis. All NOS enzymes are synthesized as monomers. Each subunit consists of a reductase domain and an oxygenase domain. Monomers and even isolated reductase domains are able to transfer electrons from NADPH to the flavins FAD and FMN and have a limited capacity to reduce molecular oxygen to $O_2^{\cdot-}$. Monomers and isolated reductase domains can bind calmodulin (CaM), which stimulates the electron transfer within the reductase domain. However, monomers are unable to bind the cofactor BH_4 or the substrate L-arginine and cannot catalyze NO· production. B, The presence of heme allows NOS dimerization; in fact, heme is the only cofactor that is absolutely required for the formation of active NOS dimers. Heme also is essential for the interaction between reductase and oxygenase domains and for the interdomain electron transfer from the flavins to the heme of the opposite monomer. NADPH oxidation rates are significantly enhanced in heme-containing substrate-free NOS dimers compared with monomers, consistent with a more effective $O_2^{\cdot-}$ production. C, When sufficient substrate L-arginine and cofactor BH_4 are present, intact NOS dimers couple their heme and O_2 reduction to the synthesis of NO·. L-Citrulline is formed as the byproduct; N^{ω}-hydroxy-L-arginine is an intermediate in the reaction.

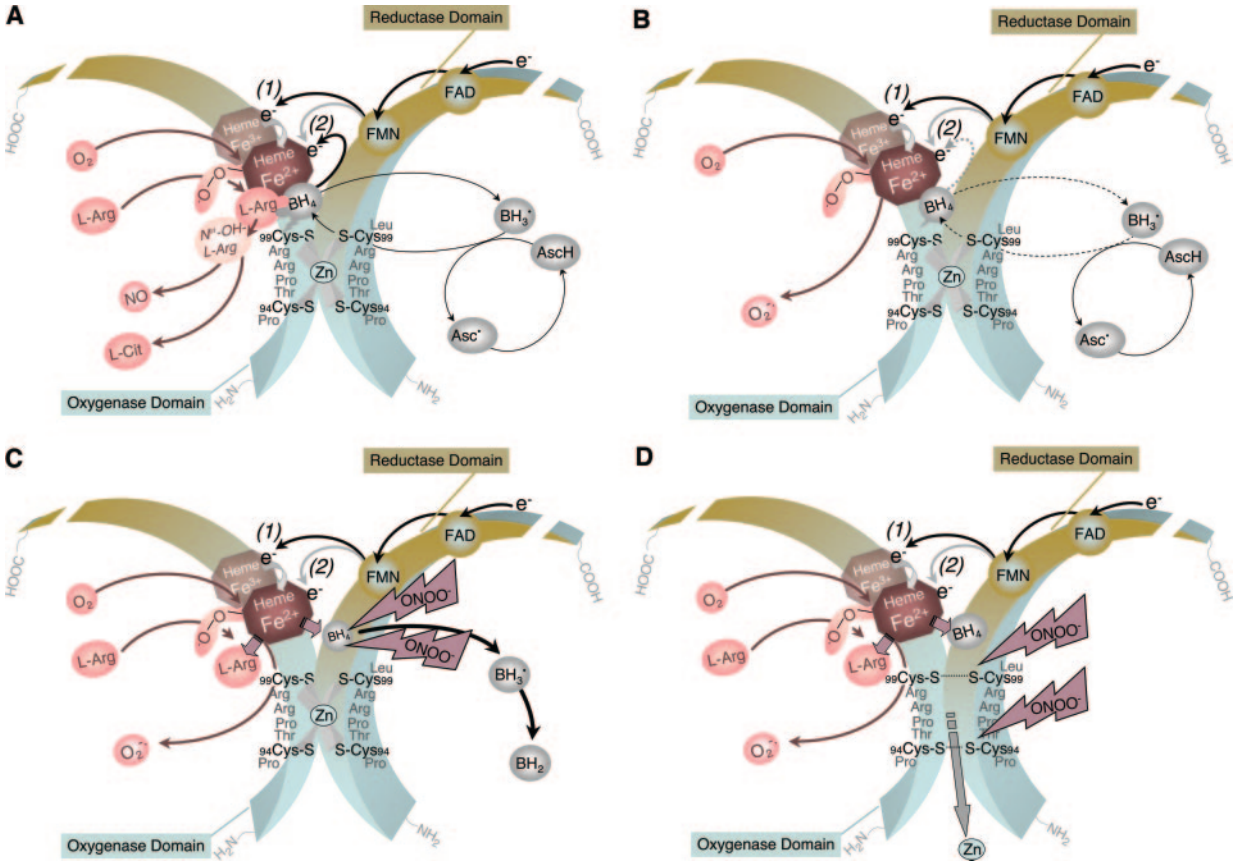

Figure 2. A, A closer look at the dimer interface of human eNOS. All NOS isoforms contain a zinc-thiolate cluster formed by a zinc ion coordinated in a tetrahedral conformation with pairs of CXXXXC motifs at the dimer interface. This site not only binds Zn but also is of major importance for the binding of the cofactor BH_4 and the substrate L-arginine. Interestingly, there is evidence for a mutual enhancement of BH_4 and L-arginine binding, which may be of therapeutic relevance in vivo. In human eNOS, Cys99 is essential for BH_4 binding; a Cys99Ala mutation reduces BH_4 binding by 90% and cuts catalytic activity to 20% (even at very high concentrations of added BH_4). Electron transfer from the reductase domain (1) enables NOS ferric (Fe^{3+}) heme to bind O_2 and form a ferrous (Fe^{2+})-dioxy species. This species may receive a second electron preferentially from BH_4 (or from the reductase domain) (2). This activates the oxygen and allows the catalysis of L-arginine hydroxylation. Recent observations indeed suggest that BH_4 acts as a 1-electron donor during reductive oxygen activation at the eNOS (rather than an allosteric activator). The nature of the resulting oxidized BH_4 has been identified by electron paramagnetic resonance as the trihydrobiopterin radical ($BH_3\cdot$) or trihydropterin radical cation protonated at N5 ($BH_3\cdot H^+$). The $BH_3\cdot$ radical (or radical cation) can be recycled to BH_4 by NOS itself (using an electron supplied by the flavins). Alternatively, there is evidence that ascorbate (which is present in cells in millimolar concentrations) can reduce the $BH_3\cdot$ radical back to BH_4. This function of ascorbate can explain its stimulation of eNOS activity. The $BH_3\cdot$ radical also can be disproportionate to the quinoid 6,7-[8H]-H_2-biopterin, which can be reduced by ascorbate back to BH_4. BH_4 is likely to play a redox role in the second reaction cycle, ie, the conversion of N^{ω}-hydroxy-L-arginine to NO. B, Significant $O_2^{\cdot-}$ production will occur when the effective concentrations of L-arginine fall below levels required to saturate the enzyme. In these circumstances, eNOS catalyzes the uncoupled reduction of O_2, leading to the production of $O_2^{\cdot-}$ (and/or H_2O_2). C, Oxidative stress is associated with endothelial dysfunction. Indeed, a BH_4 depletion of isolated arteries or rats in vivo produced endothelial dysfunction in a short period of time. Conversely, BH_4 is efficient in restoring endothelial dysfunction in a surprisingly wide range of experimental and clinical settings. Mechanistically, $O_2^{\cdot-}$ derived from the NADPH oxidases and/or xanthine oxidase (see above) may combine with NO· formed by a still-functional eNOS. This would lead to increased formation of $ONOO^-$. $ONOO^-$ has been shown to oxidize BH_4 to biologically inactive products such as $BH_3\cdot$ radical or 6,7-[8H]-H_2-biopterin (BH_2), thereby leading to an uncoupling of NOS. D, In addition to BH_4 deficiency, another mechanism has been postulated by which $ONOO^-$ can lead to dysfunction of eNOS, namely the oxidation of the zinc-thiolate cluster of eNOS, which leads to a loss of zinc and a potential destabilization of the eNOS dimer. Consequently, $O_2^{\cdot-}$ production would increase at the expense of a decreased NO production. However, this mechanism may be closely related to BH_4 deficiency because Cys99 of eNOS also is part of the BH_4 binding site. AscH indicates ascorbic acid; Asc·, ascorbate radical.

Endothelial dysfunction could be due to decreased eNOS expression. However, several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression.[6] The increased expression of eNOS in vascular disease is likely to be a consequence of an excess production of H_2O_2. H_2O_2, the dismutation product of $O_2^{\cdot-}$, can increase eNOS expression through transcriptional and posttranscriptional mechanisms.[7]

On the other hand, an accelerated degradation of NO· (by its reaction with $O_2^{\cdot-}$) is likely to occur in vascular disease. NO· and $O_2^{\cdot-}$ react avidly to form $ONOO^-$, which in turn leads to eNOS uncoupling and enzyme dysfunction (see below and Figures 2C, 2D, and 3).

Cardiovascular Risk Factors and Vascular Disease Are Associated With Increased Levels of Reactive Oxygen Species

Cardiovascular risk factors increase the expression and/or activity of NADPH oxidases (NOX) in the vascular wall, thereby enhancing the production of reactive oxygen species

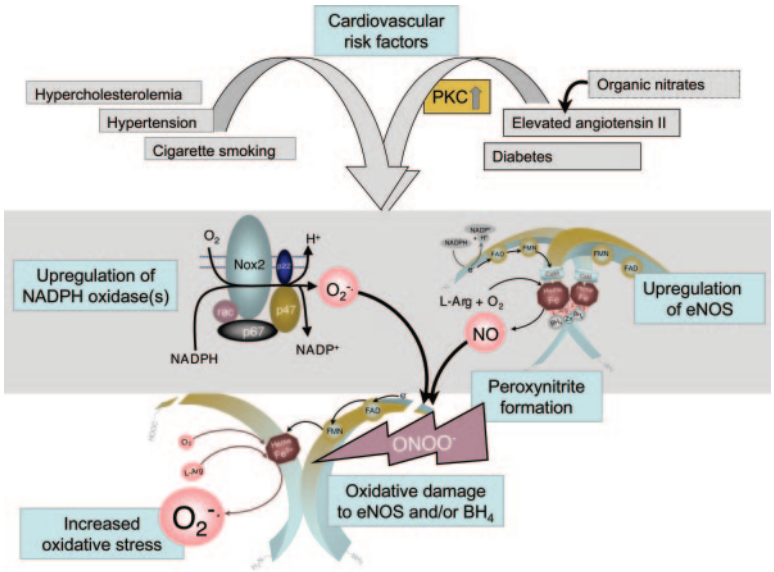

Figure 3. Potential mechanisms by which cardiovascular risk factors lead to endothelial dysfunction. In many types of vascular disease, NADPH oxidases and eNOS are upregulated in parallel. Their respective products rapidly recombine to form $ONOO^-$. This oxidizes BH_4, the essential cofactor of eNOS, and/or produces oxidative damage to the zinc-thiolate cluster of eNOS. Thus, O_2 reduction by eNOS is uncoupled from NO· formation, and a functional NOS is converted into a dysfunctional $O_2^{\cdot-}$-generating enzyme that contributes to vascular oxidative stress.

(ROS). Evidence for an activation of NOX has been provided in animal models of hypertension such as angiotensin II infusion[8] or spontaneously hypertensive rats (SHRs)[9] and models of diabetes mellitus.[10] In addition, experimental hypercholesterolemia is associated with an activation of NOX.[11] In atherosclerotic arteries, increased expression of gp91phox (Nox2) and Nox4 has been observed[12] (Figure 3). The stimulating effects of angiotensin II on the activity of these enzymes suggests that an activated (local or systemic) renin-angiotensin system can cause vascular dysfunction.[13] In addition, in hypercholesterolemia, local renin-angiotensin systems may be activated.[14] In vessels from hypercholesterolemic animals[15] and in platelets from hypercholesterolemic patients,[16] the AT_1 receptor has been found to be upregulated.

Xanthine oxidase is another potential source of ROS in vascular disease. The enzyme readily donates electrons to molecular oxygen, thereby producing $O_2^{\cdot-}$ and H_2O_2. Oxypurinol, an inhibitor of xanthine oxidase, has been shown to reduce $O_2^{\cdot-}$ production and improve endothelium-dependent vascular relaxations to acetylcholine in blood vessels from hyperlipidemic animals.[17] This suggests a contribution of xanthine oxidase to endothelial dysfunction in early hypercholesterolemia. Unlike NOX, however, the general importance of xanthine oxidase for endothelial dysfunction is uncertain. Whereas some investigators reported an improvement in endothelial dysfunction in hypercholesterolemic and diabetic patients with xanthine oxidase inhibitors,[18] other failed to show an effect with allopurinol.[19]

Uncoupled eNOS Contributes to Endothelial Dysfunction

Evidence for uncoupling of eNOS has been obtained in endothelial cells treated with low-density lipoprotein (LDL),[20] in $ONOO^-$-treated rat aorta,[21] and in isolated blood vessels from animals with pathophysiological conditions such as SHRs,[22] stroke-prone SHRs,[23] angiotensin II–induced hypertension,[24] hypertension induced with the mineralocorticoid deoxycorticosterone acetate (DOCA),[25] streptozotocin-induced diabetes,[10] or nitroglycerin tolerance.[26]

Importantly, NOS uncoupling has also been seen in patients with endothelial dysfunction resulting from hypercholesterolemia,[27] diabetes mellitus,[28] or essential hypertension[29]; in chronic smokers[30]; and in nitroglycerin-treated patients.[31]

This raises questions about the pathophysiological mechanism(s) leading to eNOS uncoupling in vascular disease. There is a growing body of evidence that vascular NOX plays a crucial role in the phenomenon of eNOS uncoupling in humans. The important hint came from experiments with NOX (p47phox)-knockout animals.[25] DOCA-salt–treated hypertensive mice showed an increased production of vascular ROS. This was significantly reduced by the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), demonstrating a marked contribution of uncoupled eNOS to oxidative stress in vascular tissue. p47phox-knockout animals showed much less oxidative stress on DOCA-salt treatment, and levels of ROS could no longer be reduced with L-NAME.[25]

Potential Role of L-Arginine in eNOS Uncoupling

Beneficial effects of L-arginine supplementation have been documented in both animal studies and humans under pathophysiological conditions such as hypercholesterolemia and hypertension.[32–34] This raises the question as to whether L-arginine concentrations can become critical as a substrate in vivo (Figure 2B). At first glance, this appears unlikely. The K_M of eNOS for L-arginine is ≈3 μmol/L[35]; normal L-arginine plasma concentrations are ≈100 μmol/L (even in pathophysiology, they hardly fall below 60 μmol/L); and there is up to a 10-fold accumulation of L-arginine within cells.[36] In addition, human endothelial cells can effectively recycle L-citrulline to L-arginine and can obtain L-arginine from protein breakdown.[37]

On the other hand, endothelial cells express arginases that can compete with eNOS for substrate and, if highly expressed, "starve" eNOS. Arginase exists in 2 isoforms; in human endothelial cells, arginase II seems to be the predominant isozyme.[38,39] Upregulated expression and activity of arginase II have been found in corpus cavernosum of diabetic

individuals[40] and in endothelium from the lung of pulmonary hypertensive patients.[41] Evidence for a role of increased enzymatic activity of arginase in endothelial dysfunction also has been provided in animal models of cardiovascular disease such as aging,[42] atherosclerosis,[38] endothelial dysfunction after ischemia-reperfusion,[43] and hypertension induced by aortic coarctation or high salt.[44,45] In apolipoprotein E–knockout mice, the expression of arginase II was unchanged compared with wild-type mice, but the activity of the enzyme was markedly increased.[38] Similarly, in human umbilical vein endothelial cells, arginase II enzymatic activity was enhanced after an 18- to 24-hour exposure to thrombin[38] or a 24-hour stimulation with inflammatory cytokines.[39]

Thus, a relative L-arginine deficiency in the vicinity of eNOS caused by excessive arginase activity is conceivable and could explain part of the beneficial effects of L-arginine supplementation. Effects of supplemental L-arginine also could be due to local competition with the endogenous eNOS inhibitor asymmetric dimethyl-L-arginine (ADMA)[46] (see below).

However, also nonsubstrate effects of L-arginine can contribute to these effects. These include potential direct radical scavenging properties of the guanidino nitrogen group or the cooperativeness between the L-arginine and BH_4 binding sites on NOS[4] (Figure 2A).

Potential Role of ADMA in eNOS Uncoupling

ADMA represents a novel independent predictor for all-cause cardiovascular mortality. The activities (not the expression) of both protein arginine N-methyltransferase (PRMT, type I)[47] and the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH)[48] are redox sensitive. In cultured endothelial cells, rat models, and humans, oxidative stress has been shown to increase the activity of PRMT(s) and decrease that of DDAH, thereby leading to increased ADMA concentrations.[46–48] Thus, an increased production of ROS could be the reason for increased ADMA levels. Elevated ADMA may inhibit NO· synthesis by eNOS or could even uncouple the enzyme, which would enhance oxidative stress.[46] However, it remains to be established whether ADMA concentrations reached in vivo (even in pathophysiology) are sufficient to effectively interact with eNOS.

Role of BH_4 in eNOS Uncoupling

NO· and L-citrulline production by eNOS in endothelial cells correlates closely with the intracellular concentration of BH_4,[49] and supplementation with BH_4 is capable of correcting eNOS dysfunction in several types of pathophysiology. In isolated aortas from prehypertensive SHRs, BH_4 supplementation diminished the NOS-dependent generation of $O_2^{\cdot-}$.[22] Administration of BH_4 restored endothelial function in animal models of diabetes[50] and insulin resistance,[51] as well as in patients with hypercholesterolemia,[27] diabetes mellitus,[28] and essential hypertension[29] and in chronic smokers.[30]

Intracellular BH_4 levels depend on the balance of its de novo synthesis and its oxidation/degradation. BH_4 is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of BH_4[21,52] (Figure 2C). Thus, oxidation of BH_4 may be the common cause of eNOS dysfunction in vascular pathophysiology. In agreement with this concept, BH_4 levels have been found to be decreased in the aorta of insulin-resistant rats,[53] in plasma of SHRs compared with age-matched Wistar-Kyoto rats,[54] in aorta of hypercholesterolemic apolipoprotein E–knockout mice,[21] and in DOCA-salt–treated hypertensive rats.[25]

It is important to note that particularly $ONOO^-$, the direct reaction product of NO· and $O_2^{\cdot-}$, is able to oxidize BH_4. Recently published studies revealed that $ONOO^-$ oxidizes BH_4 to the BH_3· radical, which can be re-reduced to BH_4 by NOS itself or by appropriate chemical reducing agents such as ascorbic acid (vitamin C)[55,56] (Figure 2A). Thus, the improvement in endothelial function seen with infusions of vitamin C[57–59] may involve mechanisms beyond mere protection of NO from inactivation by free oxygen radicals. Because of an enhanced regeneration of BH_4,[55,56] ascorbic acid can "recouple" eNOS and enhance its enzymatic activity.

Improvement in Endothelial Dysfunction by Folic Acid

Folic acid has proved effective in reversing endothelial dysfunction in animal models of cardiovascular disease and in patients with cardiovascular risk factors.[60–62] Recent studies have indicated that folates possess stabilizing effects on the heme-containing oxygenase domain of eNOS.[63] First, folates may rescue or stabilize BH_4 by stimulating the endogenous regeneration of quinoid BH_2 to BH_4. This can recouple the eNOS enzyme, thereby increasing NO production. Second, folates as reduced pteridines, have potent antioxidant properties per se and can directly scavenge the $O_2^{\cdot-}$ produced by an uncoupled eNOS. Third, folates may interact with the pteridine-binding site in NOS. This can enhance the binding of BH_4, leading to a facilitated electron transfer from the reductase domain or BH_4 itself to the catalytic heme center.

Oxidation of the Zinc-Thiolate Cluster in eNOS May Lead to Enzyme Uncoupling

Zou et al[64] have put forth an alternative concept potentially explaining eNOS uncoupling. They showed that the exposure of the isolated enzyme to $ONOO^-$ leads to a disruption of the zinc-thiolate cluster, resulting in an uncoupling of the enzyme (Figure 2D). BH_4 was oxidized at concentrations 10- to 100-fold higher than those needed to disrupt the zinc-thiolate complex. From these findings, the authors suggested that the principal mechanism of uncoupling is the oxidation of the zinc-thiolate center rather than BH_4 oxidation.[64] However, it should be kept in mind that Cys99 in the thiolate center of eNOS is also essential for BH_4 binding (Figure 2A); its oxidation would damage the BH_4 binding site (Figure 2D) with similar consequences for the enzyme as oxidation of the cofactor itself. In addition, it is not clear whether a loss of zinc from eNOS ever occurs in intact cells in vivo.

Potential Clinical Interventions to Restore Normal eNOS Function

On the basis of the pathophysiology mentioned above, there are several possible approaches to restore eNOS functionality

(ie, recouple eNOS) in the clinical situation. These include the intra-arterial infusion of the eNOS cofactor BH_4 as demonstrated by studies in chronic smokers,[30] diabetics,[28] hypercholesterolemic patients,[27] and hypertensive individuals.[29]

Folic acid increases intracellular BH_4 levels and has been used successfully to restore endothelial function in patients with hypercholesterolemia,[60] diabetes mellitus,[61] or hyperhomocysteinemia.[62] Folic acid also prevented or reversed eNOS dysfunction in nitroglycerin-treated patients[31] and in healthy volunteers with postprandial endothelial dysfunction.[65]

In addition, infusions of high doses of vitamin C have been found to improve endothelial function acutely.[57–59] The exact mechanism of action of ascorbic acid is unknown, but as detailed above, vitamin C also is likely to recouple eNOS (Figure 2A).

Conclusions

Oxidative stress and endothelial dysfunction in the coronary and peripheral circulation have important prognostic implications for subsequent cardiovascular events. As detailed here, an increased production of ROS by uncoupled eNOS contributes markedly to this pathophysiology (Figure 3).

Disclosures

None.

References

1. Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase isozymes: characterization, purification, molecular cloning, and functions. *Hypertension*. 1994;23:1121–1131.
2. Raman CS, Li H, Martasek P, Kral V, Masters BS, Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell*. 1998;95:939–950.
3. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem*. 2001;276:14533–14536.
4. Martasek P, Miller RT, Liu Q, Roman LJ, Salerno JC, Migita CT, Raman CS, Gross SS, Ikeda-Saito M, Masters BS. The C331A mutant of neuronal nitric-oxide synthase is defective in arginine binding. *J Biol Chem*. 1998;273:34799–34805.
5. McMillan K, Adler M, Auld DS, Baldwin JJ, Blasko E, Browne LJ, Chelsky D, Davey D, Dolle RE, Eagen KA, Erickson S, Feldman RI, Glaser CB, Mallari C, Morrissey MM, Ohlmeyer MH, Pan G, Parkinson JF, Phillips GB, Polokoff MA, Sigal NH, Vergona R, Whitlow M, Young TA, Devlin JJ. Allosteric inhibitors of inducible nitric oxide synthase dimerization discovered via combinatorial chemistry. *Proc Natl Acad Sci U S A*. 2000;97:1506–1511.
6. Li H, Wallerath T, Münzel T, Förstermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. *Nitric Oxide Biol Chem*. 2002;7:149–164.
7. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000;86:347–354.
8. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone. *J Clin Invest*. 1996;97:1916–1923.
9. Li H, Witte K, August M, Brausch I, Gödtel-Armbrust U, Habermeier A, Closs EI, Oelze M, Münzel T, Förstermann U. Reversal of eNOS uncoupling and upregulation of eNOS expression lowers blood pressure in hypertensive rats. *J Am Coll Cardiol*. In press.
10. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A, Meinertz T, Griendling K, Harrison DG, Förstermann U, Münzel T. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res*. 2001;88:E14–E22.
11. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Bohm M, Meinertz T, Munzel T. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*. 1999;99:2027–2033.
12. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of Nox family proteins in human atherosclerosis. *Circulation*. 2002;105:1429–1435.
13. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.
14. Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation*. 1996;94:2756–2767.
15. Vergnani L, Hatrik S, Ricci F, Passaro A, Manzoli N, Zuliani G, Brovkovych V, Fellin R, Malinski T. Effect of native and oxidized low-density lipoprotein on endothelial nitric oxide and superoxide production: key role of L-arginine availability. *Circulation*. 2000;101:1261–1266.
16. Nickenig G, Baumer AT, Temur Y, Kebben D, Jockenhovel F, Bohm M. Statin-sensitive dysregulated AT1 receptor function and density in hypercholesterolemic men. *Circulation*. 1999;100:2131–2134.
17. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*. 1993;91:2546–2551.
18. Cardillo C, Kilcoyne CM, Cannon RO 3rd, Quyyumi AA, Panza JA. Xanthine oxidase inhibition with oxypurinol improves endothelial vasodilator function in hypercholesterolemic but not in hypertensive patients. *Hypertension*. 1997;30:57–63.
19. O'Driscoll JG, Green DJ, Rankin JM, Taylor RR. Nitric oxide-dependent endothelial function is unaffected by allopurinol in hypercholesterolaemic subjects. *Clin Exp Pharmacol Physiol*. 1999;26:779–783.
20. Pritchard KA Jr, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res*. 1995;77:510–518.
21. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*. 2001;103:1282–1288.
22. Cosentino F, Luscher TF. Tetrahydrobiopterin and endothelial function. *Eur Heart J*. 1998;19(suppl G):G3–G8.
23. Kerr S, Brosnan MJ, McIntyre M, Reid JL, Dominiczak AF, Hamilton CA. Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. *Hypertension*. 1999;33:1353–1358.
24. Mollnau H, Wendt M, Szocs K, Lassegue B, Schulz E, Oelze M, Li H, Bodenschatz M, August M, Kleschyov AL, Tsilimingas N, Walter U, Forstermann U, Meinertz T, Griendling K, Munzel T. Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. *Circ Res*. 2002;90:E58–E65.
25. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*. 2003;111:1201–1209.
26. Münzel T, Li H, Mollnau H, Hink U, Matheis E, Hartmann M, Oelze M, Skatchkov M, Warnholtz A, Duncker L, Meinertz T, Förstermann U. Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. *Circ Res*. 2000;86:E7–E12.
27. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest*. 1997;99:41–46.
28. Heitzer T, Krohn K, Albers S, Meinertz T. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with type II diabetes mellitus. *Diabetologia*. 2000;43:1435–1438.
29. Higashi Y, Sasaki S, Nakagawa K, Fukuda Y, Matsuura H, Oshima T, Chayama K. Tetrahydrobiopterin enhances forearm vascular response to acetylcholine in both normotensive and hypertensive individuals. *Am J Hypertens*. 2002;15:326–332.
30. Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T, Munzel T. Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. *Circ Res*. 2000;86:E36–E41.

31. Gori T, Burstein JM, Ahmed S, Miner SE, Al-Hesayen A, Kelly S, Parker JD. Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: a human in vivo study. *Circulation*. 2001; 104:1119–1123.
32. Imaizumi T, Hirooka Y, Masaki H, Harada S, Momohara M, Tagawa T, Takeshita A. Effects of L-arginine on forearm vessels and responses to acetylcholine. *Hypertension*. 1992;20:511–517.
33. Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet*. 1991;338:1546–1550.
34. Rossitch E, Alexander E, Black PM, Cooke JP. L-Arginine normalizes endothelial function in cerebral vessels from hypercholesterolemic rabbits. *J Clin Invest*. 1991;87:1295–1299.
35. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*. 1991;88: 10480–10484.
36. Closs EI, Scheld JS, Sharafi M, Forstermann U. Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol*. 2000;57:68–74.
37. Simon A, Plies L, Habermeier A, Martine U, Reining M, Closs EI. Role of neutral amino acid transport and protein breakdown for substrate supply of nitric oxide synthase in human endothelial cells. *Circ Res*. 2003;93:813–820.
38. Ming XF, Barandier C, Viswambharan H, Kwak BR, Mach F, Mazzolai L, Hayoz D, Ruffieux J, Rusconi S, Montani JP, Yang Z. Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction. *Circulation*. 2004;110:3708–3714.
39. Bachetti T, Comini L, Francolini G, Bastianon D, Valetti B, Cadei M, Grigolato P, Suzuki H, Finazzi D, Albertini A, Curello S, Ferrari R. Arginase pathway in human endothelial cells in pathophysiological conditions. *J Mol Cell Cardiol*. 2004;37:515–523.
40. Bivalacqua TJ, Hellstrom WJ, Kadowitz PJ, Champion HC. Increased expression of arginase II in human diabetic corpus cavernosum: in diabetic-associated erectile dysfunction. *Biochem Biophys Res Commun*. 2001;283:923–927.
41. Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, Thunnissen FB, Farver C, Hazen SL, Jennings C, Dweik RA, Arroliga AC, Erzurum SC. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J*. 2004;18:1746–1748.
42. Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, Hare JM. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation*. 2003;108:2000–2006.
43. Hein TW, Zhang C, Wang W, Chang CI, Thengchaisri N, Kuo L. Ischemia-reperfusion selectively impairs nitric oxide-mediated dilation in coronary arterioles: counteracting role of arginase. *FASEB J*. 2003;17: 2328–2330.
44. Zhang C, Hein TW, Wang W, Miller MW, Fossum TW, McDonald MM, Humphrey JD, Kuo L. Upregulation of vascular arginase in hypertension decreases nitric oxide-mediated dilation of coronary arterioles. *Hypertension*. 2004;44:935–943.
45. Johnson FK, Johnson RA, Peyton KJ, Durante W. Arginase inhibition restores arteriolar endothelial function in Dahl rats with salt-induced hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2005;288: R1057–R1062.
46. Sydow K, Munzel T. ADMA and oxidative stress. *Atheroscler Suppl*. 2003;4:41–51.
47. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation*. 2002;106:987–992.
48. Böger RH, Sydow K, Borlak J, Thum T, Lenzen H, Schubert B, Tsikas D, Bode-Böger SM. LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases. *Circ Res*. 2000;87: 99–105.
49. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter H. Pteridine biosynthesis in human endothelial cells: impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem*. 1993;268:1842–1846.
50. Pieper GM. Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. *J Cardiovasc Pharmacol*. 1997;29:8–15.
51. Shinozaki K, Nishio Y, Okamura T, Yoshida Y, Maegawa H, Kojima H, Masada M, Toda N, Kikkawa R, Kashiwagi A. Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. *Circ Res*. 2000;87: 566–573.
52. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun*. 1999;263:681–684.
53. Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, Toda N, Kikkawa R. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O_2^- imbalance in insulin-resistant rat aorta. *Diabetes*. 1999;48:2437–2445.
54. Hong H-J, Hsiao G, Cheng T-H, Yen M-H. Supplementation with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. *Hypertension.* 2001;38:1044–1048.
55. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem*. 2003;278: 22546–22554.
56. Werner ER, Gorren AC, Heller R, Werner-Felmayer G, Mayer B. Tetrahydrobiopterin and nitric oxide: mechanistic and pharmacological aspects. *Exp Biol Med*. 2003;228:1291–1302.
57. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001;104:2673–2678.
58. Heitzer T, Just H, Munzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation*. 1996;94:6–9.
59. Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest*. 1996;97:22–28.
60. Verhaar MC, Wever RM, Kastelein JJ, van Dam T, Koomans HA, Rabelink TJ. 5-Methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation*. 1998;97:237–241.
61. van Etten RW, de Koning EJ, Verhaar MC, Gaillard CA, Rabelink TJ. Impaired NO-dependent vasodilation in patients with type II (non-insulin-dependent) diabetes mellitus is restored by acute administration of folate. *Diabetologia*. 2002;45:1004–1010.
62. Woo KS, Chook P, Lolin YI, Sanderson JE, Metreweli C, Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. *J Am Coll Cardiol*. 1999;34:2002–2006.
63. Verhaar MC, Stroes E, Rabelink TJ. Folates and cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2002;22:6–13.
64. Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest*. 2002;109:817–826.
65. Wilmink HW, Stroes ES, Erkelens WD, Gerritsen WB, Wever R, Banga JD, Rabelink TJ. Influence of folic acid on postprandial endothelial dysfunction. *Arterioscler Thromb Vasc Biol*. 2000;20:185–188.

Endothelial Nitric Oxide Synthase in Vascular Disease: From Marvel to Menace

Ulrich Förstermann and Thomas Münzel

Circulation. 2006;113:1708-1714
doi: 10.1161/CIRCULATIONAHA.105.602532
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539