Nitric oxide is a principal factor involved in the antiatherosclerotic properties of the endothelium.1–3 NO interferes in vitro with key events in the development of atherosclerosis, such as monocyte and leukocyte adhesion to the endothelium4–7 as well as platelet–vessel wall interaction.8–20 NO also decreases endothelial permeability and reduces vessel tone, thus decreasing flux of lipoproteins into the vessel wall.11,12 Finally, NO has been shown to inhibit vascular smooth muscle cell proliferation and migration in vitro as well as in vivo.15–16 In agreement with these findings, inhibition of the NO-producing enzyme NOS III caused accelerated atherosclerosis in experimental models.17 Major risk factors for atherosclerotic vascular disease, such as hypercholesterolemia, diabetes, hypertension, and smoking, have been associated with impaired NO activity.18–22

In vivo, the activity of the L-arginine–NO pathway is a balance between the synthesis and breakdown of NO. At present, there are several reasons to believe that NO synthesis could indeed be impaired in hypercholesterolemia and atherosclerosis. In these conditions, there appears to be an uncoupling of the receptor–G, complex.23 The exact mechanism is not known, but a very intriguing hypothesis is that the altered lipid composition of the cell membrane may play a role in this phenomenon. Although there is some controversy regarding the effect of oxidized LDL on the transcription of the enzyme, there is evidence of reduced transcription and enhanced breakdown of NOS transcripts with increasing concentrations of oxidized LDL.24 Long-term stimulation with oxidized LDL may also lead to a decrease in the amount of the NOS protein through induction of cytokines.25 Finally, hypercholesterolemia is associated with increased circulating concentrations of ADMA, an endogenous inhibitor of NOS. This has been demonstrated in hypercholesterolemic rabbits and humans.26,27 This is particularly interesting because these observations suggest that administration of L-arginine may overcome a competitive inhibition of NOS.

In agreement with this theory, administration of L-arginine increases synthesis of NO by the vascular endothelium,28 improves NO-dependent vasodilation in conditions such as hypercholesterolemia and angina pectoris,18,29,30 and prevents development of atherosclerosis in LDL receptor knockout mice.31 These facts led to the concept that a reduction in NO synthesis is the primary process involved in endothelial dysfunction and that this reduction in NO synthesis is due to a reduced availability of the NOS substrate L-arginine. However, the intracellular concentration of L-arginine far exceeds the $K_m$ value of NOS, making less likely the possibility that the extracellular L-arginine concentration is rate limiting.32 It is also uncertain to what extent increased circulating levels of ADMA affect intracellular L-arginine availability. Finally, a recent study by Giugliano et al33 demonstrates that the effect of L-arginine on vasodilation is mediated in part by stimulation of insulin secretion.

Alternatively, reduced NO activity could be caused by enhanced catabolism. The in vivo half-life of NO is determined mainly by its reaction with oxyhemoglobin and superoxide.34 The reaction of superoxide and NO occurs at a diffusion-limited rate, with the production of the powerful oxidant peroxynitrite (ONOO$^-$). This reaction is more than three times faster than catabolism of superoxide by superoxide dismutase.34 Under physiological conditions, NO is probably formed in the picomolar to nanomolar range.35 Because peroxynitrite is formed optimally from equimolar concentrations of NO and superoxide,36 it is not very likely for peroxynitrite to achieve high concentrations in normal physiology. This is important because low concentrations of peroxynitrite have been shown to behave very similarly to NO: they can cause vasorelaxation,37–39 decrease platelet aggregation,40–42 reduce leukocyte adhesion to the vessel wall,41,42 exert cytoprotective effects,43 and in fact may act as an NO donor.44 By contrast, higher concentrations of peroxynitrite may be very toxic. It can form the cytotoxic peroxynitrous acid,43 cause hydroxyl radical toxicity,44 and cause protein fragmentation by nitration of amino acids.45 It can be postulated that such deleterious concentrations of peroxynitrite can be achieved in atherosclerotic lesions, in which superoxide generation is increased by endothelial oxidases such as xanthine oxidase.46,47 as well as oxidase systems in infiltrating leukocytes (see Fig 1), while at the same time NO production in atherosclerotic lesions may also be increased by induction of NOS II by cytokines.48 Moreover, hypercholesterolemia, as a risk factor for atherosclerosis, has been shown to impair the glutathione detoxification mechanism against peroxynitrite.49 In agreement, nitrotyrosine immunostaining, which has been advanced as a marker of peroxynitrite-mediated protein modification,34 is increased in human atherosclerotic plaques. Moreover, nitrosylation of LDL cholesterol isolated from atherosclerotic plaques is also largely increased,50 suggesting...
that enhanced peroxynitrite formation occurs in atherosclerosis and could contribute to lipid peroxidation in atherosclerosis. Taken together, these data indicate that catabolism of NO by its reaction with superoxide could be an important phenomenon in hyperlipidemia and atherosclerosis.

Very intriguing are observations that NOS III itself can be an important source of endothelial superoxide production in hypercholesterolemia. Pritchard et al found that endothelial cells that were incubated with LDL released superoxide, which could be largely inhibited by the NOS inhibitor L-NAME. In fact, superoxide production by the endothelium fell below control levels during administration of L-NAME, suggesting that there is continuous superoxide production by NOS III. In other words, NOS III is both an NO- as well as a superoxide-producing enzyme. To understand this double action of NOS III, one has to take a closer look at its biochemistry.

### Figure 1. Proposed scheme of catabolism of NO by superoxide (O$_2^-$). Under physiological conditions, endothelial NOS III produces NO as well as superoxide (see text). NO may react rapidly with superoxide generated by mitochondria and membrane-bound oxidase systems such as xanthine oxidase (XO) to form peroxynitrite (ONOO$^-$), which at low concentrations has very similar actions to NO. In atherosclerosis (bottom), the NOS III produces less NO and more superoxide, while at the same time superoxide generation by XO and possibly NADPH oxidases is also increased. In addition, there is increased superoxide formation by leukocytes through, for example, myeloperoxidases (MPO) and lipoygenase (lipox). If this situation is accompanied by induction of NOS II, concomitant increments in NO and superoxide release by NOS II may lead to the formation of high concentrations of peroxynitrite, a condition that has been associated with cellular toxicity.

### Figure 2. NO is produced by NOS III, which incorporates molecular oxygen into the substrate L-arginine. NOS III is present as a homodimer. The NOS III also undergoes posttranslational acylation (myristoylation and palmitoylation), which appears to be essential for its activity, by anchoring the enzyme to the cell membrane. The NOS III probably binds to distinct domains of the plasma membrane, called caveolae, which may serve as sites for the sequestration of receptor-coupled signaling proteins and which are tethered to the cytoskeleton. Recently, it has been shown that interaction of NOS III with caveolin-1 has an inhibitory effect on enzyme activity, while this interaction may be reversed by calcium-calmodulin activation. The NOS III itself (bottom) has binding sites for BH$_4$, L-arginine, and heme. Electrons, donated by NADPH, are transported toward the oxidase domain. Heme may reduce molecular oxygen, leading to the formation of superoxide. The electrons are donated to the aminoguanidine group of L-arginine, leading to the formation of NO and L-citrulline. This reaction is dependent on the presence of BH$_4$.

### The Two Faces of NOS

NOS III consists of a flavin-containing reductase domain and a heme-containing oxidase domain (Fig 2). NADPH reduces the flavin component of the reductase domain, but electron transfer to heme will not occur until Ca$^{2+}$/calmodulin is present. In the presence of Ca$^{2+}$/calmodulin, there is an electron transfer from NADPH to the heme group. In the presence of L-arginine, electrons flow to the heme moiety to reduce oxygen, which is used to oxidize L-arginine to NO and...
citrulline. Another cofactor, BH₄, has been postulated to play an important role in whether the electron flow in the enzyme can be directed to L-arginine. Indeed, in the (neural) NOS I isoform, depletion of BH₄ results in uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide and subsequently hydrogen peroxide. Regarding recombinant NOS III, we recently also confirmed that addition of BH₄ increases NO production and reduces superoxide generation by NOS III. The exact mechanisms by which BH₄ exerts these effects are not known. In NOS I, BH₄ appears to play a major role in stabilizing the NO in its active dimeric form. However, this allosteric role of BH₄ appears to be less prominent for recombinant NOS III.

What are the implications for these enzyme kinetics for impaired NO activity in vivo? Administration of BH₄ is capable of restoring endothelium-dependent vasodilation in experimental diabetes, smoking, and reperfusion injury. Sepiapterin, which is converted intracellularly to BH₄ via the salvage pathway, also restores endothelial function. We recently demonstrated in hypercholesterolemic patients that intra-arterial administration of BH₄ restores the impaired NO-dependent vasodilation response to serotonin in these patients (Fig 3). It has been suggested that there is cooperation between the BH₄ and L-arginine binding site on NOS, thereby reducing the Kₘ for each other. A reduced bioavailability of BH₄ may therefore lead to the “paradoxical” deficiency of L-arginine. Interestingly, in vivo administration of BH₄ also abolished the rate-limiting role of L-arginine in these patients, lending further support for this hypothesis.

Such data suggest that conditions that are associated with impaired NO activity and accelerated atherosclerosis are characterized by a reduced availability of BH₄. This obviously also raises the question why BH₄ becomes rate limiting in such conditions. NOS contains BH₄ as a tightly bound prosthetic group, which does not undergo net oxidation during NO synthesis. Thus, when participating as a redox-active cofactor in L-arginine oxidation, BH₄ shuttles its electron to L-arginine and must be continuously recycled into its active reduced state by NO. Several studies have shown that atherosclerosis is associated with an increased cellular production of reactive oxygen species. This is also confirmed by in vivo observations in which the oxygen radical scavengers vitamin C and probucol improved impaired endothelium-dependent vasodilation in hypercholesterolemia and atherosclerosis. It is thus possible that the abnormal intracellular redox state in these conditions, which is unfavorable for reduction of the oxidized bioprotein, impairs the endothelial recycling of BH₄. Studies that measure BH₄ levels are required to address this issue further.

Surprisingly, administration of exogenous BH₄ and sepiapterin has also been associated recently with an inhibitory effect on endothelium-dependent vasodilation in vitro. These effects were noted with high levels of BH₄ (10 to 100 μmol/L) but not found with dihydrobiopterin and appeared reversible with superoxide dismutase, suggesting that these effects were mediated by superoxide anion. Indeed, in the presence of oxygen, BH₄ is susceptible to auto-oxidation and the subsequent production of superoxide radicals and oxidized bioprotein. However, these data cannot be easily extrapolated to the in vivo situation due to the different in vivo redox status, which actually determines if a compound acts as an antioxidant or a prooxidant. For example, direct antioxidant effects of BH₄ have been described as well.

Conclusions

On the basis of these observations, we propose that NOS III has a dual role in the pathogenesis of atherosclerosis: under normal conditions, it generates low concentrations of NO and probably peroxy nitrite, which favor an antiatherosclerotic environment. However, during hyperlipidemia and atherosclerosis, it may contribute to the formation of oxidative stress by a reduction in BH₄-dependent NO formation and unopposed superoxide formation by the enzyme. Particularly in the setting of local induction of NOS II, this could favor the development of local toxic concentrations of peroxy nitrite in atherosclerotic plaques. This concept further emphasizes the role of redox state as a determinant of vascular integrity in atherosclerosis.

Acknowledgments

We thank Erik Stroes for his comments and the Dutch Heart Foundation and the Royal Dutch Academy of Sciences for their support.

References

24. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990;87:1620–1624.
detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. Circ Res. 1997;80:894–901.


52. Deleted in proof.


Atherosclerosis and the Two Faces of Endothelial Nitric Oxide Synthase
Robert M. F. Wever, Thomas F. Lüscher, Francesco Cosentino and Ton J. Rabelink

Circulation. 1998;97:108-112
doi: 10.1161/01.CIR.97.1.108

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/1/108

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/