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 endothelium\(^ {4-7}\) 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 al\(^ {33}\) 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 (\(\text{ONOO}^-\)). This reaction is more than three times faster than catabolism of superoxide by superoxide dismutase.\(^ {35}\) Under physiological conditions, NO is probably formed in the picomolar to nanomolar range.\(^ {36}\) 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,\(^ {38,40}\) reduce leukocyte adhesion to the vessel wall,\(^ {41}\) exert cytoprotective effects,\(^ {42}\) and in fact may act as an NO donor.\(^ {43}\) 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, nitrrosylation 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.\textsuperscript{24,51} 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\textsuperscript{53} 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.

**The Two Faces of NOS**

NOS III consists of a flavin-containing reductase domain and a heme-containing oxidase domain\textsuperscript{74} (Fig 2). NADPH reduces the flavin component of the reductase domain, but electron transfer to heme will not occur until Ca\textsuperscript{2+}/calmodulin is present. In the presence of Ca\textsuperscript{2+}/calmodulin, there is an electron transfer from NADPH to the heme moiety to reduce oxygen, which is used to oxidize L-arginine to NO and

**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.\textsuperscript{55} 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.\textsuperscript{55a} The NOS III itself (bottom) has binding sites for BH\textsubscript{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\textsubscript{4}.\textsuperscript{56}
The ability of BH$_4$ may therefore lead to the “paradoxical” defi-

Figure 3. Effects of BH$_4$ on serotonin (5-HT)-induced NO-

smediated vasodilation. BH$_4$ did not significantly alter vasodila-
tion in controls but significantly enhanced 5-HT–mediated vaso-
dilation in young patients with familial hypercholesterolemia (FH)
without macrovascular disease. There was no effect of BH$_4$ on
sodium nitroprusside (SNP)-induced endothelium-independent
vasodilation (reprinted with permission). 67

Citrulline. Another cofactor, BH$_4$, 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
isoinform, depletion of BH$_4$ results in uncoupling of oxygen
reduction and arginine oxidation, 57 thereby generating super-
oxide and subsequently hydrogen peroxide. 58,59 Regarding
recombinant NOS III, we recently also confirmed that addi-
tion of BH$_4$ increases NO production and reduces superoxide
generation by NOS III. 56,57 The exact mechanisms by which
BH$_4$ exerts these effects are not known. In NOS I, BH$_4$ appears
to play a major role in stabilizing the NOS in its active dimeric
form. 60,61 However, this allosteric role of BH$_4$ appears to be less
prominent for recombinant NOS III. 56,62

What are the implications for these enzyme kinetics for
impaired NO activity in vivo? Administration of BH$_4$ is
capable of restoring endothelium–dependent vasodilation in
experimental diabetes, 63 smoking, 64 and reperfusion injury. 65
Sepiapterin, which is converted intracellularly to BH$_4$ via the
salvage pathway, 66 also restores endothelial function. 66 We
recently demonstrated in hypercholesterolemic patients that
intra-arterial administration of BH$_4$ restores the impaired
NO-dependent vasodilation response to serotonin in these
patients. 67 (Fig 3). It has been suggested that there is cooperation
between the BH$_4$ and l-arginine binding site on NOS, 57
thereby reducing the $K_d$ for each other. A reduced bioavail-
ability of BH$_4$ may therefore lead to the “paradoxical” defi-
ciency of l-arginine. Interestingly, in vivo administration of
BH$_4$ also abolished the rate-limiting role of l-arginine in these
patients, lending further support for this hypothesis. 67

Such data suggest that conditions that are associated with
impaired NO activity and accelerated atherosclerosis are char-
terized by a reduced availability of BH$_4$. This obviously also
raises the question why BH$_4$ becomes rate limiting in such
conditions. NOS contains BH$_4$ as a tightly bound prosthetic
group, which does not undergo net oxidation during NO
synthesis. 68 Thus, when participating as a redox-active cofactor in
l-arginine oxidation, BH$_4$ shuttles its electron to l-arginine and
must be continuously recycled into its active reduced state
by NOS. 69–71 Several studies have shown that atherosclerosis is
associated with an increased cellular production of reactive
oxygen species. 66–67 This is also confirmed by in vivo observa-
tions in which the oxygen radical scavengers vitamin C and
probucol improved impaired endothelium–dependent vasodi-
lation in hypercholesterolemia and atherosclerosis. 72,73 It is thus
possible that the abnormal intracellular redox state in these
conditions, which is unfavorable for reduction of the oxidized
biopterin, impairs the endothelial recycling of BH$_4$. Studies
that measure BH$_4$ levels are required to address this issue
further.

Surprisingly, administration of exogenous BH$_4$, 74 and sepi-
apterin, 75 has also been associated recently with an inhibitory
effect on endothelium–dependent vasodilation in vitro. These
effects were noted with high levels of BH$_4$ (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, BH$_4$ is susceptible to auto-oxidation and the subse-
cquent production of superoxide radicals and oxidized bio-
p,74–77 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 BH$_4$
have been described as well. 56

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 peroxynitrite, which favor an antithrombotic environment.
However, during hyperlipidemia and athero-
sclerosis, it may contribute to the formation of oxidative stress
by a reduction in BH$_4$–dependent NO formation and unop-
posed 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 peroxynitrite in
atherosclerotic plaques. This concept further emphasizes the
role of redox state as a determinant of vascular integrity in
atherosclerosis.

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