Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis

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Abstract—Atherosclerosis is associated with an impairment of endothelium-dependent relaxations, which represents the reduced bioavailability of nitric oxide (NO) produced from endothelial NO synthase (eNOS). Among various mechanisms implicated in the impaired EDR in atherosclerosis, superoxide generated from dysfunctional eNOS has attracted attention. Under conditions in which vascular tissue levels of tetrahydrobiopterin (BH4), a cofactor for NO, are deficient or lacking, eNOS becomes dysfunctional and produces superoxide rather than NO. Experimental studies in vitro have revealed that NO from eNOS constitutes an anti-atherogenic molecule. A deficiency of eNOS was demonstrated to accelerate atherosclerotic lesion formation in eNOS knockout mice. In contrast, eNOS overexpression with hypercholesterolemia may promote atherogenesis via increased superoxide generation from dysfunctional eNOS. Thus, eNOS may have 2 faces in the pathophysiology of atherosclerosis depending on tissue BH4 metabolisms. An improved understanding of tissue BH4 metabolisms in atherosclerotic vessels is needed, which would help in developing new strategies for the inhibition and treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2004;24:998-1005.)

Key Words: endothelial nitric oxide synthase • atherosclerosis • tetrahydrobiopterin • superoxide • nitric oxide

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS), which requires Ca^{2+}/calmodulin, FAD, FMN, and tetrahydrobiopterin (BH4) as the cofactors. In the vessels, NO is produced from the endothelium by constitutive expression of the endothelial isoform of NOS (eNOS), which is activated by mechanical stress such as blood shear-stress and stimulation with agonists such as bradykinin and acetylcholine. NO has a variety of functions, but its action as the endothelium-derived relaxing factor (EDRF) is the most important for the maintenance of vascular homeostasis. An impairment of endothelium-dependent relaxations (EDR) is present in atherosclerotic vessels even before vascular structural changes occur and represents the reduced eNOS-derived NO bioavailability. Endothelial dysfunction as characterized by an impairment of EDR, and thereby reduced eNOS-derived NO bioactivity, is the critical step for atherogenesis. Among various mechanisms responsible for the impaired EDR, the increased NO breakdown by superoxide is important, and there is augmented production of superoxide in atherosclerotic vessels. Recently, it was revealed that under certain circumstances, eNOS becomes dysfunctional and produces superoxide rather than NO. The pathophysiological role of dysfunctional eNOS has attracted attentions in vascular disorders, including atherosclerosis. This review focuses on the role of dysfunctional eNOS on atherosclerotic vessels and refers to the possible role of dysfunctional eNOS on atherogenesis.

Impaired EDR in Atherosclerosis

All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension, and smoking are associated with impaired EDR. Although the underlying mechanisms of the reduced EDR are multifactorial, its most important cause is a derangements of the eNOS/NO pathway, which include the reduced activity and expression of eNOS, decreased sensitivity to NO, and increased degradation of NO by reaction with superoxide. Regarding the expression of eNOS at the vessel wall, it may be reduced in advanced atherosclerosis, possibly because of reduced transcription and/or increased instability of eNOS mRNA caused by cytokines. However, most animal models with atherosclerosis demonstrate the unchanged or rather augmented expression of eNOS, at least in early atherosclerosis, despite the presence of impaired EDR.

The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia. Pro-atherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) and lysophosphatidylcholine, inhibit signal transduction from receptor activation to eNOS activation. Hypercholesterolemic serum and LDL upregulate caveolin abundance, augments caveolin–eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells. Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) and N-monomethylarginine (NMA) are also revealed to be involved in the mechanisms of reduced EDR in atherosclerosis.
The accelerated degradation of NO by increased superoxide from vessel wall is demonstrated as another important mechanism of the reduced EDR in hyperlipidemia and atherosclerosis. Superoxide production from atherosclerotic vessels is augmented in human and animal models with atherosclerosis. The endothelium is important as a source of superoxide production, and its denudation decreases superoxide production from vessels with atherosclerosis but has no effects in normal vessels without atherosclerosis. Animal models of hyperlipidemia and atherosclerosis demonstrate an excess vascular superoxide flux that is linked to reduced NO bioactivity. As an evidence for the involvement of superoxide in the impaired EDR in atherosclerotic vessels, the restoration of EDR by antioxidants and superoxide dismutase has been shown. In rabbit aortas with high-cholesterol diet-induced atherosclerosis, the impaired vasodilatory responses to acetylcholine and A23187 were restored by chronic treatment with polyethylene-glycolated SOD. Antioxidants improve EDR in human and animal models with atherosclerosis. In particular, vitamin C is effective in the restoration of EDR associated with most risk factors for atherosclerosis, including hypercholesterolemia, hypertension, diabetes mellitus, and smoking.

**Superoxide Production From Vessels**

Superoxide is produced by a variety of enzymes, including xanthine oxidase, cyclooxygenase, and NADPH oxidase. Among them, NADPH oxidase plays a major role in vascular cells. In normal vessels, NADPH oxidase is present in adventitial fibroblasts. In atherosclerotic vessels, increased expression of subcomponents of NADPH oxidase has been found. In the early stage of atherosclerosis, superoxide seems to be produced from NADPH oxidase localized in the endothelium; in advanced atherosclerosis, vascular smooth muscle cells serve as the major source of NADPH oxidase-derived superoxide.

However, in vitro biochemical studies demonstrated that NO can independently produce superoxide under certain conditions. The catalytic mechanisms of NO involve flavin-mediated electron transport from C-terminal–bound NADPH to the N-terminal heme center, where oxygen is reduced and incorporated into the guanidine group of L-arginine, giving rise to NO and L-citrulline. The eNOS-mediated superoxide generation is primarily regulated by BH4 availability. In the presence of suboptimal concentrations of BH4, activation of NO leads to “uncoupling of NO” and subsequent production of superoxide. In “uncoupled NO,” electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to L-arginine; thereby, production of superoxide occurs. The ability of NO to produce superoxide was first demonstrated in neuronal NO (nNOS) and then extended to eNOS. In the recombinant bovine eNOS, the heme moiety was identified as the main source for superoxide production. In endothelial cells, a close link between cellular BH4 levels and NO synthesis was demonstrated, suggesting that an optimal concentration of BH4 is essential for NO production. The precise role of BH4 in the formation of NO is not completely understood, but it is postulated that BH4 donates electrons from the reductase domain to the ferrous–dioxygen complex in the oxygenase domain. It is also demonstrated that addition of exogenous BH4 increases NO production and decreases superoxide production from endothelial cells. As mentioned later in this article, there is an interaction between NADPH oxidase and eNOS, and it is thought that superoxide produced by NADPH is involved in the uncoupling of eNOS.

**Exogenous BH4 and eNOS Function**

It has been demonstrated in clinical and animal studies that acute administration of BH4 improves endothelial dysfunction associated with hypercholesterolemia, atherosclerosis, hypertension, and cigarette smoking. These data have been presented as evidence for the presence of “uncoupled eNOS,” which produces superoxide rather than NO, leading to impaired EDR. Laursen et al clearly demonstrated the production of superoxide from eNOS. In apolipoprotein E-knockout (apoE-KO) mice, they showed the increased vascular superoxide production from the endothelium, which was associated with impaired EDR. Incubation of vessels with sepiapterin, a precursor to BH4, improved EDR and decreased superoxide production.

As in the study of Laursen et al, sepiapterin has been shown to restore endothelial function in acute studies, however, sepiapterin may not always be effective when vessels are exposed to it for a long time. Sepiapterin is an oxidized BH4 analogue that generates BH4 by enzymatic reduction of sepiapterin reductase and dihydrofolate reductase. It is reported that relatively long-term (6 hours) incubation of hyperlipidemic rabbit vessels with sepiapterin resulted in a further derangement of vasodilatory response to endothelium-dependent agonists. In addition, incubation of canine cerebral arteries with high levels of sepiapterin was shown to reduce EDR significantly, despite an increase in vascular BH4 levels. It is revealed that a high concentration of sepiapterin can serve as a pro-oxidant and thereby oxidizes BH4 to dihydrobiopterin (BH2). Sepiapterin may increase BH2 rather than BH4 in the tissues, and the increased BH4 levels potentially compete with BH4 for eNOS binding and worsen eNOS uncoupling.

**Vascular Pteridine Metabolism in Atherosclerosis**

The presence of eNOS dysfunction as a mechanism of impaired endothelial function seems to be well-recognized now. However, only limited information is available on pteridine metabolism in the vessel wall in diseased states. In normal vascular tissue, >60% of total BH4 is present in the endothelium. Endothelial cells from diabetic BioBreeding (BB) rats have a marked reduction in BH4 contents. In the insulin resistance rat model induced by high-fructose diet, a modest reduction of BH4 levels in the aortas was associated with impaired EDR. Furthermore, as compared with control rats, the levels of 7,8-dihydrobiopterin and biopterin, the oxidized form of BH4, were increased in the aortas of diabetic BB rats. Plasma BH4 levels were decreased in SHR with established hypertension. Recently, it was reported that BH4 content was reduced and the content of oxidized forms of BH4 was increased in vessels from mice with deoxycorticosterone (DOCA)-salt hypertension.
Regarding hyperlipidemia and atherosclerosis, Vasquez-Vivar et al reported that BH4 levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits.\textsuperscript{65} We have also demonstrated the BH4 levels in the aortas were decreased \(\approx 50\%\) in apoE-KO mice with marked hypercholesterolemia compared with normocholesterolemic wild-type mice.\textsuperscript{66} In contrast, d’Uscio et al reported that in the aortas of apoE-KO mice with moderate hypercholesterolemia, BH4 levels were increased by \(\approx 1.8\)-fold compared with those in control mice.\textsuperscript{64}

The tissue levels of BH4 are determined by a balance between its production and degradation. As shown in Figure 1, BH4 is synthesized from GTP via a de novo pathway by the rate-limiting enzyme guanosine 5’-triphosphate (GTP) cyclohydrolase I (GTPCH I). Alternatively, the synthesis of BH4 can occur via a so-called salvage pathway, which uses BH2 as a substrate. Therefore, the reduced activity or expression of GTPCH I results in the decreased BH4 levels in the tissue. In the insulin resistance rat model, Shinozaki et al reported that GTPCH I activity in the aorta was significantly lower than that of control rats.\textsuperscript{67} We also found the reduced vascular GTPCH I activity in apoE-KO mice fed a “high-cholesterol diet” (S Kawashima et al, article under submission). Although the activity of GTPCH I is augmented by inflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\), which are activated in atherosclerotic vessels, GTPCH I gene expression is reduced by oxidized LDL.\textsuperscript{68–70} The mechanisms of the reduced GTPCH I activity in the aortas of apoE-KO mice are currently under investigation. However, the tissue levels of BH4 are also determined by their gradation, namely by their oxidation to 7,8-dihydrobiopterin.\textsuperscript{38} Studies in vitro showed that BH4 can be rapidly oxidized by reactive oxygen species such as peroxynitrite.\textsuperscript{52,69} In DOCA-salt hypertensive mice, it was demonstrated that superoxide produced by NADPH oxidase led to the formation of peroxynitrite in reaction with NO, which induced uncoupling of eNOS. With elevated oxidative stress, the oxidation of BH4 is enhanced and vascular tissue levels of 7,8-dihydrobiopterin increase. Therefore, the discrepant results in vascular BH4 levels in hyperlipidemia and atherosclerosis can be at least partly explained as caused by the difference in the levels of oxidative stress. The studies of Vasquez-Vivar et al and ours were conducted in animals with severe hypercholesterolemia, which is likely associated with high oxidative stress, and d’Uscio et al used animals with mild hypercholesterolemia.\textsuperscript{58,63,64}

It has been proposed that in addition to the absolute availability of BH4, the ratio of BH4/7,8-dihydrobiopterin, the ratio of reduced and oxidized biopterin, is important for determining the rates of NO production versus uncoupled superoxide formation from eNOS.\textsuperscript{60,70} Only the completely reduced (tetrahydro) form of biopterin supports NOS coupling of NADPH oxidation to NO synthesis. Partially oxidized analogues of BH4 enhance rates of superoxide formation from purified eNOS in the presence of saturating L-arginine concentration.\textsuperscript{58} Therefore, oxidative stress causes “uncoupling” of eNOS not only by decreasing BH4 levels but also by increasing the ratio of BH4/7,8-dihydrobiopterin. Then, generation of superoxide and peroxinitrite from dysfunctional (uncoupled) eNOS induces a further reduction of BH4 availability.\textsuperscript{54}

The mechanism of the improvement of endothelial dysfunction by vitamin C includes its effects on BH4.\textsuperscript{71–73} Vitamin C not only scavenges superoxide but also enhances NO synthase activity. Vitamin C increases the K\textsubscript{max} of NOS enzyme without any effects on L-arginine. It is postulated that, by its reductase capacity, vitamin C chemically stabilizes BH4, but a recent study of Kuzkaya et al showed that vitamin C reduces the intermediate product of the reaction between peroxynitrite and BH4, BH3, back to BH4.\textsuperscript{74} Saturated ascorbic acid levels in endothelial cells are necessary to protect BH4 from oxidation to provide optimal condition for cellular NO synthesis.

**eNOS and Atherogenesis**

As described, it seems to be established now that in hyperlipidemia and atherosclerosis, eNOS is dysfunctional and produces superoxide, which is implicated in endothelial dysfunction and impaired EDR. However, only limited information is available on how eNOS dysfunction affects atherogenesis. A substantial body of evidence in vitro suggests that eNOS-derived NO acts as anti-atherogenic molecule.\textsuperscript{75–78} NO from eNOS inhibits leukocyte–endothelial adhesion, vascular smooth muscle migration and proliferation, and platelet aggregation, all of which are important steps in atherogenesis. Although the exact mechanisms are still not well defined and although there is still some controversy, chronic treatment with L-arginine, a substrate for NOS, inhibits atherosclerotic lesion formation in animal models of atherosclerosis, such as diet-induced atherosclerosis models of rabbits and LDL-receptor knockout mice.\textsuperscript{79,80} On the contrary, NOS inhibitors like L-NAME significantly accelerate atherosclerotic lesion development, suggesting that inhibition of endogenous NO synthesis facilitates the progression of atherosclerosis.\textsuperscript{81,82} Although little information is available for NOS gene transfer in atherosclerotic lesion formation, local adenovirus-mediated nNOS gene transfer to atherosclerotic carotid arteries rapidly reduces adhesion molecule expression and inflammatory cell infiltration in cholesterol-fed rabbits, indicating an anti-atherogenic role of endogenous NO in vivo.\textsuperscript{83}
eNOS Gene Engineered Mice as a Tool to Study the Role of eNOS in Atherogenesis

Recently, eNOS gene-engineered mice have been used to clarify more directly the role of eNOS/NO system on atherogenesis. Knowles et al first demonstrated that a genetic lack of eNOS resulted in enhanced atherosclerosis in association with hypertension in apo E/eNOS double-knockout mice, which were produced by crossing apo E-KO mice with eNOS knockout (eNOS-KO) mice.84 Based on the positive correlation between blood pressure and the size of atherosclerotic lesions in aortas, they suggested that an elevation of blood pressure was responsible for the increases in the lesion size in these mice. More recently, their group reported that the hypertensive and atherogenic effects of eNOS deficiency in apoE-KO mice depended on the presence of endogenous sex hormones.85 By use of gonadectomized apo E/eNOS double-knockout mice, they suggested that in the absence of sex hormones, eNOS had little effect on blood pressure and atherogenesis, although which hormones were responsible for these effects were not identified. Kuhlencordt et al also reported that eNOS deficiency promoted atherosclerosis in apo E/eNOS double-knockout mice.86 Fed with a “Western-type” diet, apo E/eNOS double-knockout mice showed significant increases in aortic lesion area, which were associated with peripheral coronary atherosclerosis and aortic aneurysm formation. Later, they showed that these changes were not inhibited by hydralazine treatment, which reduced blood pressure to the levels comparable to those of apoE-KO mice and concluded that hypertension did not account for the accelerated atherosclerosis and aortic aneurysm formation.87 Therefore, although the participation of elevated blood pressure and sex hormones remains to be further clarified, these reports indicated that the absence of endogenous eNOS-derived NO caused by the lack of eNOS gene accelerates atherosclerosis.

In contrast, recently Shi et al reported the paradoxical reduction of atherosclerotic lesion size in high-cholesterol diet-induced atherosclerosis in eNOS-KO mice compared with wild-type mice.88 They fed mice a “high-cholesterol diet” for 12 weeks and then examined the lesion size in the aortic sinus. They found that eNOS-KO mice had much smaller aortic sinus lesions than did wild-type mice. 1-NAME, the NO inhibitor, reduced LDL oxidation by endothelial cells from wild-type mice but not from eNOS-KO mice. Based on these findings, they speculated that eNOS may contribute to the oxidation of LDL under the circumstance of hypercholesterolemia, and that the absence of eNOS-mediated LDL oxidation may lead to the reduction of atherosclerotic lesion formation in eNOS-KO mice. They did not refer to the mechanisms of eNOS-mediated LDL oxidation, but it is very likely that superoxide from the dysfunctional eNOS was involved in the mechanisms. This study raised the possibility that eNOS may act to accelerate atherogenesis under certain conditions such as hypercholesterolemia.

We have examined the effects of eNOS overexpression on atherosclerotic lesion formation with the use of transgenic (eNOS-Tg) mice that overexpress eNOS mainly in the endothelium.89,90 We crossed eNOS-Tg mice with apo E-KO mice and fed them a “high-cholesterol diet.” Unexpectedly, the atherosclerotic lesion areas were significantly larger in eNOS-overexpressing apo E-KO (apo E-KO/eNOS-Tg) mice compared with control apo E-KO mice.63 In apoE-KO/eNOS-Tg mice, we found the presence of eNOS dysfunction, demonstrated by lower NO production relative to eNOS protein levels and enhanced superoxide production in the endothelium. We also found decreased vascular BH4 levels and increased 7,8-dihydrobiopterin levels in apo E-KO/eNOS-Tg mice. Therefore, chronic overexpression of eNOS does not inhibit, but rather accelerates atherosclerosis under hypercholesterolemia. In contrast, van Haperen et al also crossed apo E-KO mice with another line of eNOS transgenic mice that they created and reported that atherosclerotic lesion size was reduced by eNOS overexpression.91 Regarding the mechanisms, they cited the reductions of blood pressure and plasma cholesterol levels. In their study, eNOS overexpression was associated with 20- to 25-mm Hg reduction in mean blood pressure and a ∼15% decrease in plasma cholesterol levels. Although the difference in promoter by which eNOS was targeted to the endothelium is possibly involved, the discrepancy between their study and ours can be explained at least partly by a difference in the balance between NO and superoxide production from the endothelium. The increase of plasma cholesterol levels achieved by the “Western-type” diet that they used was much modest compared with that we achieved by feeding a “high-cholesterol” diet. Therefore, it is speculated that oxidative stress in the hypercholesterolemic mice of van Haparen et al was not increased as much as that in our model, although they did not describe oxidative stress and eNOS function in their model.

As mentioned, increasing evidence demonstrates the presence of eNOS dysfunction in hyperlipidemia and atherosclerosis. It is conceivable that dysfunctional eNOS may promote atherogenesis under certain pathological conditions that alter the balance between eNOS protein levels and tissue pteridine metabolism. Under pathological conditions with severe hyperlipidemia, there exists an increase in oxidative stress, which determines the extent of eNOS uncoupling and the resultant generation of superoxide from eNOS. In contrast to NO, superoxide is a pro-atherogenic molecule, and antioxidants have been demonstrated to inhibit atherosclerotic lesion formation.92 The marked increase in superoxide in association with decreased NO production would promote atherogenesis. However, it is totally unclear whether acceleration of atherogenesis by dysfunctional eNOS occurs only under a specific condition with severe hypercholesterolemia or whether it may take place under other pathological conditions with elevated oxidative stress. The role of eNOS dysfunction on atherogenesis needs further studies (Table).

Therapeutic Implication

It is important to define a therapeutic intervention for atherosclerosis from the standpoint of dysfunctional eNOS. Although the role of BH4 in the regulation of eNOS function is still not well understood, supplementation with exogenous BH4 is effective for the treatment of endothelial dysfunction. We found that supplementation with BH4 inhibits atherosclerotic lesion formation in apo E-KO mice.63 Although the detailed mechanisms are unclear, it is conceivable that in
addition to the simple removal of superoxide by its antioxidant effect, exogenous BH4 improved pteridine metabolism at the vessel wall and led to restore normal eNOS function. However, the effect of sepiapterin on atherosclerosis lesion formation has not been reported yet and it may not be effective. It is necessary to further clarify pteridine metabolism in the tissues, particularly in the vascular wall. GTPCH could be a rational target to augment endothelial BH4 and normalize eNOS activity in endothelial dysfunction. As for the strategy for augmenting GTPCH activity, GTPCH I gene transfer in vitro to human endothelial cells augments intracellular BH4 levels in association with an increase in enzymatic activity of eNOS to produce NO.93 Recently, Alp et al generated transgenic mice overexpressing GTPCH I solely in the tissue was sufficient to maintain normal eNOS function. The beneficial effects of GTPCH I gene transfer was also confirmed by a very recent study of Zheng et al, who reported that ex vivo gene transfer of human GTPCH I to the aortic segments from DOCA-salt hypertensive rats reversed BH4 deficiency in the vascular tissue and improved EDR.95

The anti-atherogenic property of drugs may also be evaluated from the standpoint of their effects on GTPCH. Statins are shown to increase eNOS protein levels in endothelial cells. Hattori et al demonstrated that statins increased GTPCH I mRNA in vascular endothelial cells and led to an elevation of intracellular BH4 levels.96 These effects may be partly responsible for the anti-atherogenic action of statins.

However, simply augmenting NOS protein levels under pathological conditions such as hyperlipidemia may not increase NO but instead augment superoxide production, resulting in detrimental rather than beneficial effects. Therefore, a strategy directed at increasing NOS protein levels in association with maintaining its enzymatic activity is needed.97,98 (Table 1, Figure 2)

**Summary**

It is now being widely recognized that eNOS becomes dysfunctional and produces superoxide rather than NO in hyperlipidemia and atherosclerosis. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in atherosclerotic vessels. It seems to be widely accepted that eNOS with normal function inhibits atherogenesis by producing NO. However, although further studies are needed, recent reports on eNOS gene-engineered mice raised the possibility that dysfunctional eNOS may serve to promote atherosclerotic lesion formation under severe hypercholesterolemia (Figure 2). For the development of eNOS dysfunction, an abnormality in BH4 metabolism in vascular tissue seems to be fundamental. However, little is known about BH4 metabolism in vascular tissue, particularly in diseased states including atherosclerosis. We need an improved understanding of tissue BH4 metabolism in atherosclerotic vessels in relation to conditions in which eNOS dysfunction develops. It would be intriguing to know whether dysfunctional eNOS participates in the pathogenesis of vascular disorders other than atherosclerosis.

**References**

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