Malfunction of Vascular Control in Lifestyle-Related Diseases: Endothelial Nitric Oxide (NO) Synthase/NO System in Atherosclerosis

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Abstract. Nitric oxide (NO) from the endothelial NO synthase (eNOS) is believed to be implicated in the development and progression of atherosclerosis. The impaired endothelium-dependent vasodilatory response (EDR) has been demonstrated in vessels exposed to hypercholesterolemia and atherosclerosis. The extent of impairment serves as a predictor of future progression of atherosclerosis. As to the mechanisms of impaired EDR, increased production of superoxide is important. Recently it was revealed that eNOS becomes dysfunctional and produces superoxide rather than NO under conditions in which vascular tissue levels of tetrahydrobiopterin (BH4), a co-factor for eNOS, are deficient or lacking. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in various vascular disorders including atherosclerosis. Regarding the role of eNOS in atherogenesis, experimental studies in vitro have revealed that NO from eNOS constitutes as an anti-atherogenic molecule. In eNOS-knockout mice, eNOS deficiency augments atherosclerotic lesion formation, although the effects may be partly due to the associated hypertension. However, in eNOS-transgenic mice (eNOS-Tg) crossbred with apolipoprotein E-deficient mice (apoE-KO/eNOS-Tg), we found the accelerated lesion formation in association with increased superoxide production from vessels compared with apoE-KO mice. The vascular tissue levels of BH4 were reduced and BH2, an oxidized form, levels were increased. Chronic administration of exogenous BH4 or overexpression of GTPCH-1, a rate limiting enzyme for BH4 synthesis, restored the lesion to the levels comparable to apoE-KO mice. Therefore, eNOS may have two faces in the pathophysiology of atherosclerosis depending on tissue BH4 levels.

Keywords: nitric oxide (NO), superoxide, endothelial NO synthase, atherosclerosis, tetrahydrobiopterin

Introduction

In vessels, nitric oxide (NO) is constitutively produced from the endothelium by the endothelial type NO synthase (eNOS), which is activated by mechanical stresses such as blood flow-mediated shear stress and stimulation with vasoactive substances such as bradykinin and acetylcholine. NO from the endothelium controls vascular tone, inhibits monocyte and leukocyte adhesion to the endothelium, inhibits platelet aggregation, and decreases endothelial permeability (1). Endothelial production of NO also inhibits vascular smooth muscle cell migration and proliferation. Because of these actions, NO produced by eNOS has been regarded as an anti-atherogenic molecule, and, in contrast, superoxide is regarded as a pro-atherogenic molecule and counteracts those actions of NO (2).

As the superoxide producing enzyme, NAD(P)H oxidase is important in vascular tissue (3, 4), but recent studies revealed that eNOS itself may produce superoxide rather than NO under pathological conditions such as hyperlipidemia (5, 6). Although studies on eNOS-gene-deficient mice seem to support the idea that lack of eNOS promotes atherogenesis, there still remains controversy on the role of eNOS on atherosclerotic lesion formation under hyperlipidemia (7, 8). Recently
we demonstrated that overexpression of eNOS under hypercholesterolemia paradoxically accelerated atherosclerotic lesion formation in apolipoprotein E-deficient (apoE-KO) mice and showed that augmented production of superoxide from eNOS was related to the mechanisms (9). This mini-review focuses on the role of eNOS as a superoxide-producing enzyme and two faces of eNOS in the pathophysiology of atherosclerosis depending on tissue tetrahydrobiopterin (BH₄) levels.

**NO and superoxide in atherosclerotic vessels**

Among a variety of functions of NO produced by eNOS, the action as the endothelium-derived relaxant factor (EDRF) is most important for vascular function. An impairment of the endothelium-dependent vasodilatory response (EDR) is present in atherosclerotic vessels even before vascular structural changes ensue (10, 11). Risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking, are associated with impaired EDR. The impaired EDR represents the reduced eNOS-derived NO activity. The underlining mechanisms of the reduced NO synthesis include the reduced activity and expression of eNOS, decreased sensitivity of vascular smooth muscle to NO, and increased degradation of NO by reaction with superoxide (12). Among these mechanisms, both the impaired enzymatic activity of eNOS and the accelerated degradation of NO by superoxide play central roles in the reduced NO synthesis in atherosclerosis and hyperlipidemia. Regarding the reduced eNOS activity, proatherogenic lipids, such as oxidized low-density lipoprotein (ox LDL) and lysophosphatidylcholine, are shown to inhibit signal transduction from receptor activation to eNOS activation (13). Hypercholesterolemic serum as well as LDL up-regulates caveolin abundance, augments the caveolin-eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells (14). Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) are also involved in the mechanisms of reduced EDR in atherosclerosis (15).

On the other hand, the accelerated degradation of NO by superoxide is another important mechanism of the impaired EDR (16). Because of an extremely rapid reaction between NO* and O₂**, there is always some O₂** reacting with NO* within cells and in the extracellular space. Under physiological conditions, there is a minute balance between O₂** and NO*, whereas under pathological conditions such as hyperlipidemia and atherosclerosis, this minute balance shifts toward the state where superoxide production becomes predominant (5). Animal models of hyperlipidemia and atherosclerosis demonstrate an excess vascular superoxide production that is linked to reduced NO bioactivity. The augmented superoxide production from atherosclerotic vessels has been demonstrated in both

![Fig. 1. Augmented superoxide production from the aorta of a WHHL rabbit assessed at the aortic sinus levels. Superoxide production in situ was assessed by the MCLA method. JW: Japanese white rabbit. WHHL: WHHL rabbit.](image)
human and animal models with atherosclerosis (Fig. 1). Also in patients with hyperlipidemia and atherosclerosis, the restoration of EDR by antioxidants such as vitamin C and superoxide dismutase has been shown as evidence for the involvement of superoxide in the impaired EDR (17 – 19).

As mentioned previously in this manuscript, NO from eNOS inhibits leukocyte-endothelial adhesion, vascular smooth muscle migration and proliferation, and platelet aggregation, all of which are important steps of atherogenesis. In vivo studies directed to modify the production of NO support the concept that eNOS-derived NO acts as an anti-atherogenic molecule. Chronic treatment with L-arginine, a substrate for NO, inhibited atherosclerotic lesion formation in animal models of atherosclerosis such as the diet-induced atherosclerosis model of rabbits (20). On the contrary, NOS inhibitors like L-NAME were demonstrated to accelerate atherosclerotic lesion development, suggesting that inhibition of endogenous NO synthesis facilitated the progression of atherosclerosis (21). Also, local adenovirus-mediated nNOS gene transfer to atherosclerotic carotid arteries resulted in rapid reduction of adhesion molecule expression and inflammatory cell infiltration in cholesterol-fed rabbits, which likely served to inhibit atherogenesis (22).

In humans, there has been limited information that proves the anti-atherogenic action of eNOS-derived NO. Recently it was reported in angiographic studies that coronary vascular endothelial dysfunction predicted a worse long-term outcome from coronary events such as acute myocardial infarction. Coronary events were shown to be more frequent in patients with depressed coronary vasodilatory responses to acetylcholine and/or adenosine (23). These clinical studies demonstrated that the reduced bioactivity of eNOS-derived NO is closely linked to prognosis of atherosclerotic diseases. Although the endothelium secretes multiple factors other than NO, these studies strongly suggest that eNOS-derived NO serves to protect vessels from progression of atherosclerosis.

**The role of eNOS in atherogenesis: studies in eNOS gene-deficient mice**

Recently eNOS-gene-deficient mice were used to clarify directly the role of NO produced by eNOS in atherogenesis. Knowles et al. first demonstrated that genetic lack of eNOS resulted in enhanced atherosclerosis in association with hypertension in apoE/eNOS double-knockout mice, which were produced by crossing apoE-KO mice with eNOS knockout (eNOS-KO) mice (7). They suggested that the elevation of blood pressure was responsible for the augmented lesion size in these mice because there was a positive correlation between blood pressure and size of atherosclerotic lesions in aortas. Kuhlencordt et al. also reported that eNOS deficiency promotes atherosclerosis in apoE/eNOS double knockout mice (8). Under feeding with “western-type” diet, apoE/eNOS double knockout mice showed a significant increase in aortic lesion area, which was associated with peripheral coronary atherosclerosis and aortic aneurysm formation. Different from the report by Knowles et al., Kuhlencordt et al. concluded that the acceleration of atherogenesis was not due to the elevation of blood pressure because this acceleration was not inhibited by hydralazine treatment, which reduced blood pressure to the level comparable to that of apoE-KO mice (24). Therefore, although the participation of elevated blood pressure remains to be further clarified, these reports indicated that the absence of endogenous eNOS-derived NO due to the lack of eNOS gene accelerated atherosclerosis.

On the other hand, Shi et al. demonstrated the paradoxical reduction of atherosclerotic lesion area in eNOS-KO mice compared with wild type mice in the model of high cholesterol diet-induced atherosclerosis (25). L-NAME, the NOS inhibitor, reduced LDL oxidation by endothelial cells from wild type mice but not from eNOS-KO mice. Based on this finding, they speculated that eNOS might contribute to the oxidation of LDL under the circumstance of hypercholesterolemia and that the absence of eNOS-mediated LDL oxidation might lead to the reduction of atherosclerotic lesion formation in eNOS-KO mice. I will discuss the possible mechanisms of this eNOS-mediated LDL oxidation later in this manuscript.

**eNOS as the superoxide-producing enzyme**

Increased superoxide production is an important feature of atherosclerotic vessels and contributes to the pathophysiology of atherosclerosis. Reaction with superoxide reduces the bioavailability of NO, impairs vasomotor function, increases platelet aggregation, and monocyte and leukocyte adhesion to the endothelium. Superoxide and other reactive oxygen species are involved in modification of lipids, induce proinflammatory genes, and increase cellular proliferation. Superoxide can also activate matrix metalloproteinases and produce apoptosis, which may contribute to instability of atherosclerotic lesions. Therefore superoxide can be regarded in general as a pro-atherogenic molecule.

Superoxide is produced by a variety of enzymes including mitochondrial respiration, lipoxigenase,
cyclooxygenase, cytochrome p450s, NO synthase, xanthine oxidase, and NAD(P)H oxidase. Among them, NAD(P)H oxidase is the major superoxide producing enzyme in vascular cells (26), and increased expressions of subcomponents of NAD(P)H oxidase have been revealed in atherosclerotic vessels (27, 28). In the early stage of atherosclerosis, superoxide seems to be produced from NAD(P)H oxidase localized in the endothelium, and in the advanced atherosclerosis, vascular smooth muscle cells serve as the major source of NAD(P)H oxidase-derived superoxide.

NOS is also an important enzyme capable of producing superoxide. In the presence of suboptimal concentrations of L-arginine and/or BH$_4$, activation of NOS leads to “uncoupling of NOS” and subsequent production of superoxide (5, 6, 29). The precise role of BH$_4$ in the formation of NO is not completely understood, but it is postulated that BH$_4$ donates electrons from the reductase domain to the heme in the oxygenase domain. In the absence of BH$_4$, electrons are diverted to molecular oxygen rather than to L-arginine, and thereby production of superoxide occurs. In endothelial cells, a close link between cellular BH$_4$ levels and NO synthesis was demonstrated, suggesting that an optimal concentration of BH$_4$ is essential for NO production. It was also demonstrated that addition of exogenous BH$_4$ increased NO production and decreased superoxide production from endothelial cells.

In the last decade, it was revealed that superoxide is produced in vivo from dysfunctional “uncoupled” eNOS under certain pathological conditions. Clinical as well as experimental studies demonstrated that acute administration of BH$_4$ improved endothelial dysfunction associated with hypercholesterolemia, atherosclerosis, hypertension, and cigarette smoking (30, 31). These data have been used as an evidence for the presence of “uncoupled eNOS”, which produces superoxide leading to impaired EDR, although the effect of exogenous BH$_4$ may be simply due to its potent direct antioxidant action. Regarding atherosclerotic vessels, Laursen et al. demonstrated that in apoE-KO mice, there was increased production of superoxide from the endothelium of atherosclerotic aortas, which was reduced by treatment of vessels with sepiapterin, a precursor for BH$_4$. Sepiapterin had no effects on superoxide production in vessels from control mice (32). This study showed the superoxide production from “uncoupled” eNOS in atherosclerotic vessels. Therefore it is conceivable that there is an abnormality of pteridine metabolisms, particularly a reduction of BH$_4$ contents, in the vascular tissue of atherosclerotic vessels, which causes dysfunctional eNOS leading to superoxide production.

Only limited information is available on BH$_4$ contents and the pteridine metabolism in the vessel wall of the diseased states. A reduction of BH$_4$ contents and increased levels of its oxidative form 7,8-dihydrobiopterin (7,8-BH$_2$) and biopterin have been demonstrated in vessel wall of animal models of diabetes mellitus, such as diabetic BioBreeding (BB) rats and high fructose diet-induced insulin resistance rats, and vessel wall of DOCA-salt hypertensive mice (33, 34).

Regarding hyperlipidemia and atherosclerosis, Vasquez-Vivar et al. reported that that BH$_4$ levels in aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits (35). We also demonstrated that BH$_4$ levels in the aortas were approximately 50% decreased in apoE-KO mice with marked hypercholesterolemia compared with normocholesterolemic wild-type mice (9). Therefore, although there still exists some controversy, tissue levels of BH$_4$ seem to be reduced in atherosclerotic vessels in the presence of hypercholesterolemia. The tissue levels of BH$_4$ are determined by a balance between its production and degradation. BH$_4$ is a molecular target for oxidative stress and can rapidly be oxidized by reactive oxygen species such as peroxynitrite (36, 37). It is shown that oxidation of BH$_4$ is enhanced and vascular tissue levels of 7,8-BH$_2$ increase under the elevated oxidative stress. In hyperlipidemia and atherosclerosis, it is very likely that oxidation of BH$_4$ to 7,8-BH$_2$ is enhanced, because these conditions are associated with increased oxidative stress. The reduced synthesis may also be involved in the reduced BH$_4$ contents. BH$_4$ is synthesized from GTP via a de novo pathway by the rate-limiting enzyme GTP cyclo-hydrolase-I (GTPCH-I) and alternatively via a so-called salvage pathway, which uses BH$_2$ as a substrate (37). Therefore, the reduced activity or expression of GTPCH-I results in the decreased BH$_4$ levels in tissue. Reduced GTPCH-I activity has been reported 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 (33), but limited information is available on GTPCH-I activity in atherosclerotic vessels.

**Superoxide production from dysfunctional eNOS and atherogenesis**

We have examined the effects of eNOS overexpression on atherosclerotic lesion formation with the use of eNOS-Tg that overexpress bovine eNOS mainly in the endothelium (38, 39). We crossed eNOS-Tg with apoE-KO mice and developed apoE-KO overexpressing eNOS, which were backcrossed to C57BL/6 background (apoE-KO/eNOS-Tg) (9). Then we fed these
mice a “high cholesterol diet”. After 8 weeks on a high-cholesterol diet, plasma cholesterol levels increased to 1850 – 2050 mg/dl, but there were no differences in plasma cholesterol levels between apoE-KO mice and apoE-KO/eNOS Tg mice. Blood pressure was approximately 15 mmHg lower in apoE-KO/eNOS-Tg mice compared with apoE-KO mice. The atherosclerotic lesion areas in the aortic sinus were unexpectedly increased by more than twofold in apoE-KO/eNOS-Tg mice compared with apoE-KO mice both in males and females (Fig. 2). Also, aortic tree lesion areas were approximately 50% larger in apoE-KO/eNOS-Tg mice after 12 weeks on a high-cholesterol diet.

We measured production of NO from aortas in situ by fluorescence indicator DAF-2 DA. The emitted fluorescent image caused by the reaction of DAF-2 DA with NO was obtained by the luminograph (LB981, NightOWL imaging system; EG&G Berthold, Bad Wildbad, Germany) as described previously (40). We also assessed spatial distribution and quantitative determination of superoxide production by chemiluminescent detection with a sensitive and specific probe, MCLA, which is a Cypiridina luciferin analogue (41). The chemiluminescent light emission due to the in situ MCLA reaction with superoxide anion was measured by the NightOWL luminograph. Quantification of both NO and superoxide production was evaluated separately in approximately ten randomly selected sites (0.0005 – 0.002 cm²) of either the plaque or non-plaque area in aortas that were discriminated by Sudan III staining. Expression of eNOS assessed by immuno-blotting and NO production by DAF-2 DA in aortas from apoE-KO/eNOS-Tg mice were higher than those in apoE-KO mice. However, NO production was low relative to eNOS expression. Superoxide production in non-plaque areas was increased 3.3-fold in apoE-KO/eNOS-Tg mice and 2.1-fold in apoE-KO mice compared with that in normal aortic vessels of wild type mice (Fig. 3). In plaque areas, superoxide generation was further increased to more than tenfold in both apoE-KO and apoE-KO/eNOS-Tg mice compared with wild type mice. Superoxide production was more significantly augmented in apoE-KO/eNOS-Tg mice in both plaque and non-plaque areas compared with apoE-KO mice. In apoE-KO/eNOS-Tg, endothelial denudation dramatically decreased superoxide production in the non-plaque area, showing that the endothelium was the main source of superoxide production. Furthermore, we measured vascular tissue levels of pteridine by HPLC and found the decreased vascular BH₄ levels and increased 7,8-BH₂ levels in apoE-KO/eNOS-Tg mice. These findings suggested that under hypercholesterolemia, eNOS became dysfunctional and produced superoxide rather than NO, which resulted in acceleration of atherosclerotic lesion formation in apoE-KO/eNOS-Tg mice. The mechanisms of the acceleration of atherosclerotic lesion formation by eNOS-derived superoxide were not clarified, but it was conceivable that oxidation of LDL
by superoxide as demonstrated by the study of Shi et al. (25) was at least partly involved.

Then, we examined the effects of chronic BH₄ supplementation on the lesion size and superoxide production from the aortas (9). For this purpose, mice were maintained on a high-cholesterol diet supplemented with 10 mg/kg per day BH₄. After 12 weeks of administration, BH₄ treatment significantly reduced the lesion size in the aortic tree by 26% in male and by 28% in female apoE-KO/eNOS-Tg mice (Fig. 4). Plasma lipid profiles were not affected by BH₄ treatment. Along with these histological changes, superoxide production was significantly decreased by chronic treatment with exogenous BH₄. As I described previously, exogenous BH₄ has a
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Direct antioxidant effect, and the reduced lesion formation by exogenous BH₄ might not be mediated by improved eNOS function. Therefore, we tried to increase endogenous BH₄. Recently Alp et al. developed transgenic mice that overexpress human GTPCH-1 at the endothelium (GTPCH-Tg) by use of murine Tie-2 promoter (42). They reported that tissue BH₄ levels in aortas were increased approximately threefold in the GTPCH-Tg mice compared with wild type mice. Then they crossed GTPCH-Tg mice with apoE-KO mice and developed apoE-KO mice overexpressing GTPCH-1 (apoE-KO/GTPCH-Tg) that are of C57BL/6 background. By crossing these apoE-KO/GTPCH-Tg mice with apoE-KO/eNOS-Tg mice, we created apoE-KO/eNOS-Tg mice overexpressing GTPCH-1 (apoE-KO/eNOS-Tg/GTPCH-Tg). Then we examined atherosclerotic lesion formation in these apoE-KO/eNOS-Tg/GTPCH-Tg mice. Plasma cholesterol levels were increased to approximately 450 – 500 mg/dl in all three groups of mice and there were no differences among them. As in mice fed a “high cholesterol diet”, atherosclerotic lesion area at the aortic sinus was significantly increased in apoE-KO/eNOS-Tg mice compared with apoE-KO mice. GTPCH-1 overexpression, however, reduced the atherosclerotic lesion area in apoE-KO/eNOS-Tg mice to the extent comparable to that in apoE-KO mice, and this histological improvement was associated with reduced superoxide production from the endothelium. Therefore, restoration of endogenous vascular BH₄ levels in atherosclerotic vessels could improve eNOS function, reduce superoxide production, and reduce atherosclerotic lesion formation. Along with the data on exogenous BH₄ treatment, data on GTPCH-Tg clearly demonstrated the importance of vascular tissue levels of BH₄ in atherogenesis.

Therefore, eNOS can be regarded as both an NO⁻ as well as a superoxide-producing enzyme, and thus eNOS may have dual effects on vascular function depending on the vascular pteridine metabolisms. Under physiological condition with preserved pteridine metabolism, eNOS produces mainly NO, which serves as an anti-atherogenic molecule. Under pathological conditions where tissue BH₄ levels decrease, eNOS becomes dysfunctional and may promote atherosclerosis by producing superoxide rather than NO (Fig. 5) (43). Although no studies are available, it may be possible that the dysfunctional eNOS is involved in vascular disorders other than atherosclerosis, such as vascular remodeling, in various pathological conditions.

Summary

It is now widely becoming recognized that eNOS becomes dysfunctional and produces superoxide rather than NO in hyperlipidmia and atherosclerosis. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in atherosclerotic vessels. In addition, although eNOS with normal function inhibits atherogenesis, recent reports on eNOS-gene-engineered mice raised the possibility that dysfunctional eNOS may serve to promote athero-

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**Physiological conditions**

- **BH₄** (reduced form)
- NADPH
- FAD
- FMN
- CaM
- Heme
- eNOS
- L-arginine + O₂
- L-citrulline + NO

**Hypercholesterolemia**

- **BH₂** (oxidized form)
- NADPH
- FAD
- FMN
- CaM
- Heme
- eNOS
- L-arginine + O₂
- L-citrulline + NO
- “eNOS-uncoupling”
- O₂⁻
- Endothelial damage
- Atherosclerosis

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Fig. 5. Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherosclerosis. FAD: flavin adenine dinucleotide, FMN: flavin mononucleotide, CaM: calmodulin.
sclerotic lesion formation under severe hypercholesterolemia. For development of eNOS dysfunction, 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. There needs to be improved understanding of tissue BH4 metabolisms in atherosclerotic vessels in relation to conditions where eNOS dysfunction develops. It is also intriguing to know whether dysfunctional eNOS participates in the pathogenesis of vascular disorders other than atherosclerosis.

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