iments utilizing a conditional transgenesis approach may provide important new insights regarding eNOS overexpression and MI/R injury.

The primary mechanism(s) responsible for the observed cardioprotective effects in the two strains of eNOS TG mice remains unknown. Future studies in our laboratory will focus on the elucidation of the precise cellular mechanisms responsible for cytoprotection in the eNOS TG mice. NO derived from eNOS has a highly diverse biological profile that includes antiplatelet (21) and antileukocyte actions (16), vasodilator effects (7, 26), and direct cytoprotective actions (11). It is likely that the cardioprotective actions observed in eNOS TG mice are the result of a number of these actions of NO. Recently, experimental evidence indicated that NO can modulate mitochondrial K_{ATP} channels in the setting of myocardial preconditioning and thereby induce myocardial protection (23). We treated both NTG and eNOS TG mice with a selective inhibitor of mitochondrial K_{ATP} channels before the onset of myocardial ischemia and observed no attenuation of myocardial infarct size. These data suggest that the reduction in myocardial infarct size observed in the eNOS TG mouse is not dependent on activation of mitochondrial K_{ATP} channels in the myocardium.

In summary, overexpression of the eNOS enzyme significantly attenuates the extent of myocardial infarct size after coronary artery ischemia and reperfusion. These data provide clear evidence of the beneficial role of enhanced eNOS-derived NO production during myocardial reperfusion injury. Future studies should be directed toward identifying the mechanism of this protective effect. In the future, therapies might be developed to improve vascular eNOS function as a means to improve outcomes in patients suffering from acute myocardial infarction.

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Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis

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Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis

Seinosuke Kawashima, Mitsuhiro Yokoyama

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 NOS, 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.[1–4] 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.[5] 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.[6–8] Although the underlining 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.[8] 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.[9] 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.[10,11]

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.[12–14] Hypercholesterolemic serum and LDL upregulate caveolin abundance, augments caveolin–eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells.[15,16] Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) and N-monometylarginine (NMA) are also revealed to be involved in the mechanisms of reduced EDR in atherosclerosis.[17,18]

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From the Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kusunoki-cho Chuo-ku Kobe, 0017, Japan.

Correspondence to Dr Mitsuhiro Yokoyama, Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho Chuo-ku Kobe, 650-0017, Japan. E-mail yokoyama@med.kobe-u.ac.jp

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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.[8] Superoxide production from atherosclerotic vessels is augmented in human and animal models with atherosclerosis.[19–22] 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.[19] 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.[20,23,24] 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.[20] Antioxidants improve EDR in human and animal models with atherosclerosis.[25–27] 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.[28–30]

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.[31,32] In normal vessels, NADPH oxidase is present in adventitial fibroblasts. In atherosclerotic vessels, increased expression of subcomponents of NADPH oxidase has been found.[33–36] 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.[37]

However, in vitro biochemical studies demonstrated that NOS can independently produce superoxide under certain conditions.[38–41] The catalytic mechanisms of NOS 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 NOS leads to "uncoupling of NOS" and subsequent production of superoxide.[42–45] In "uncoupled NOS," 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 NOS to produce superoxide was first demonstrated in neuronal NOS (nNOS) and then extended to eNOS.[46,47] In the recombinant bovine eNOS, the heme moiety was identified as the main source for superoxide production.[45] 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.[48,49] It is also demonstrated that addition of exogenous BH4 increases NO production and decreases superoxide production from endothelial cells.[40] 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.[50–53] 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.[54] 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.[55–57] 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.[58] 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).[49] 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.[38,56] Endothelial cells from diabetic BioBreeding (BB) rats have a marked reduction in BH4 contents.[59] 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.[60] 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.[61] 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.[62]

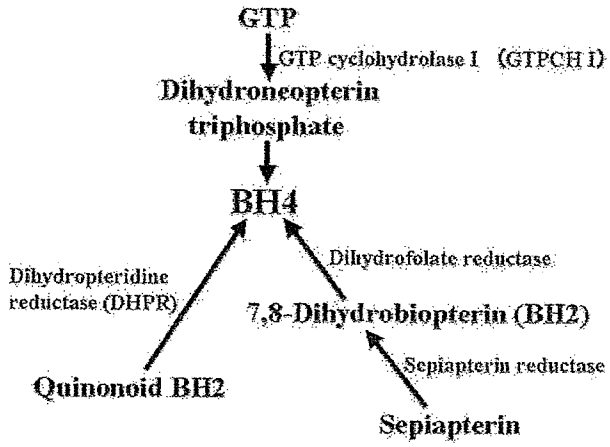

Figure 1. Scheme of BH4 biosynthesis.

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.[58] We have also demonstrated the BH4 levels in the aortas were decreased ≈50% in apoE-KO mice with marked hypercholesterolemia compared with normocholesterolemic wild-type mice.[63] In contrast, d'Uscio et al reported that in the aortas of apoE-KO mice with moderate hypercholesterolemia, BH4 levels were increased by ≈1.8-fold compared with those in control mice.[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.[65] 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-α and IL-1β, which are activated in atherosclerotic vessels, GTPCH I gene expression is reduced by oxidized LDL.[66–68] 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.[58] Studies in vitro showed that BH4 can be rapidly oxidized by reactive oxygen species such as peroxynitrite.[62,69] 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.[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.[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.[58] Therefore, oxidative stress causes "uncoupling" of eNOS not only by decreasing BH_4 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.[54]

The mechanism of the improvement of endothelial dysfunction by vitamin C includes its effects on BH4.[71,72,73] Vitamin C not only scavenges superoxide but also enhances NO synthase activity. Vitamin C increases the Kmax 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.[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.[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.[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.[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.[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. L-NAME, the NOS 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 crossbred 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

Atherosclerotic Lesion Formation in eNOS Gene-Engineered Mice

Model of Atherosclerosis	Lesion Size	Reference
eNOS-KO Mice cross-breeding with apo E-KO mice (caused by hypertension or sex hormones?)	Augmented	84, 85
eNOS-KO Mice cross-breeding with apo E-KO mice (unrelated to hypertension)	Augmented	86, 87
eNOS-KO Mice, diet-induced atherosclerosis	Reduced	88
eNOS-Tg Mice, cross-breeding with apo E-KO mice	Augmented	63
eNOS-Tg Mice, cross-breeding with apo E-KO mice	Reduced	91

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 1 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 endothelium.[94] They reported that in the rat model of streptozotocin-induced diabetes, overexpression of GTPCH I augmented endothelial BH4 levels, improved the impaired vascular function, and decreased superoxide production from vessels. They suggested that a small increase in BH4 levels in the tissue was sufficient to maintain normal eNOS function. The beneficial effects of GTPCH I gene transfer was also

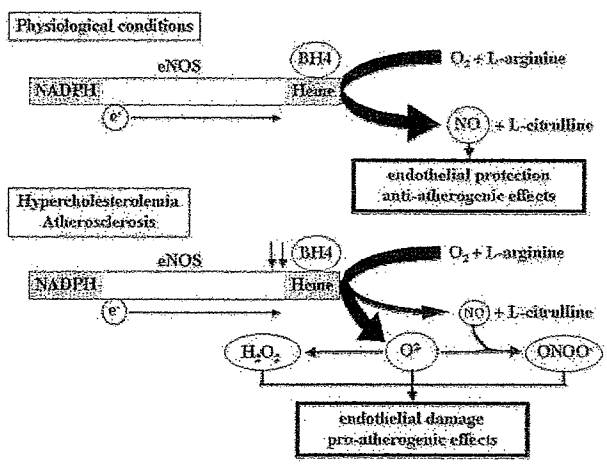

Figure 2. Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherogenesis. Under physiological conditions, tissue levels of BH4 are optimal for eNOS catalytic activity, and activation of eNOS generates NO and L-citrulline. NO generated by eNOS serves as an anti-atherogenic molecule. With hypercholesterolemia and atherosclerosis, when oxidative stress is increased, tissue levels of BH4 are reduced. In the presence of suboptimal levels of BH4, activation of eNOS leads to "uncoupling of NOS" with subsequent generation of superoxide rather than NO. Superoxide and, subsequently, peroxinitrite and hydrogen peroxide serve to damage endothelial cells and thus may promote atherosclerosis.

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 metabolisms 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.

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EPR quantification of vascular nitric oxide production in genetically modified mouse models

Jeffrey P. Khoo,[a] Nicholas J. Alp,[a] Jennifer K. Bendall,[a] Seinosuke Kawashima,[b] Mitsuhiro Yokoyama,[b] Yin-Hua Zhang,[a] Barbara Casadei,[a] and Keith M. Channon[a,*]

[a] *Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK*
[b] *The First Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan*

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Abstract

With increasing use of genetically modified mice to study endothelial nitric oxide (NO) biology, methods for reliable quantification of vascular NO production by mouse tissues are crucial. We describe a technique based on electron paramagnetic resonance (EPR) spectroscopy, using colloid iron (II) diethyldithiocarbamate [$Fe(DETC)_2$], to trap NO. A signal was seen from *C57BL/6* mice aortas incubated with $Fe(DETC)_2$, that increased 4.7-fold on stimulation with calcium ionophore A23187 [3.45 ± 0.13 vs 0.73 ± 0.13 au (arbitrary units)]. The signal increased linearly with incubation time ($r^2 = 0.93$), but was abolished by addition of N^G-nitro-L-arginine methyl ester (L-NAME) or endothelial removal. Stimulated aortas from eNOS knockout mice had virtually undetectable signals (0.14 ± 0.06 vs 3.17 ± 0.21 au in littermate controls). However, the signal was doubled from mice with transgenic eNOS overexpression (7.17 ± 0.76 vs 3.37 ± 0.43 au in littermate controls). We conclude that EPR is a useful tool for direct NO quantification in mouse vessels.

Keywords: Nitric oxide; Measurement; EPR; Colloid iron (II) diethyldithiocarbamate; eNOS knockout; eNOS-Tg; Transgenic; Aorta; Mouse

Nitric oxide (NO), produced by endothelial NO synthase (eNOS), regulates numerous pivotal processes in vascular homeostasis. Reduced NO bioavailability is implicated in several vascular disorders such as hypertension, diabetes, and atherosclerosis [1]. Loss of NO bioavailability results in endothelial dysfunction and directly contributes to vascular disease pathogenesis. The development of genetically modified mouse models has provided invaluable insights into NO biology and mechanisms of oxidative stress in vascular disease. However, direct and accurate measurement of NO in small murine vessels remains technically challenging. Existing methods such as those using electrochemical electrodes or chemiluminescence may not be sufficiently specific or sensitive [2]. In vitro enzymatic measurements, for example radioisotopic L-arginine to L-citrulline conversion, require addition of non-physiological concentrations of co-factors and reflect arginine consumption by eNOS rather than tissue NO bioactivity. Other widely used techniques rely on downstream NO signalling pathways, such as cGMP levels, or endothelium-dependent vasorelaxation bioassays which provide an indirect measure of NO action that may also be influenced by other signalling pathways. The recently recognized biological importance of discordance between eNOS enzymatic activity, NO production, and NO bioactivity further highlights the need for a range of complementary approaches to NO measurement in animal models.

Electron paramagnetic resonance (EPR) spin trapping is one of the most specific methods for NO detection in biological tissues [3,4]. Previous spin traps were limited by inadequate sensitivity to detect low-level endothelial NO production. For example, one commonly used spin trapping method using non-colloidal iron diethyldithiocarbamate (Fe/DETC) requires

* Corresponding author. Fax: + 44-1865-222077.
E-mail address: keith.channon@cardiov.ox.ac.uk (K.M. Channon).

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addition of strong reducing agents to successfully detect NO, and the large amounts of DETC added can also inhibit superoxide dismutase [5]. These limitations make this approach less useful for quantification of endothelial NO production in vascular disease models where biologically important alterations in NO bioactivity are mediated through the effects of reactive oxygen species. However, recent studies have identified colloid iron (II) diethyldithiocarbamate [$Fe(DETC)_2$] as a promising approach for vascular NO spin trapping [6,7]. Colloidal $Fe(DETC)_2$, being lipophilic, appears to localize predominantly in cell membranes, where eNOS also resides and to where neutrally charged NO partitions [8]. Thus, the spin trap can efficiently capture NO and form a stable spin adduct NO–$Fe(DETC)_2$. Previous studies using this method found no requirement for reducing agents, and the concentration of DETC used was much lower. Furthermore, vascular SOD activity remained unaffected by colloid $Fe(DETC)_2$, and the addition of nitrite or superoxide-generating xanthine/xanthine oxidase did not interfere with NO–$Fe(DETC)_2$ signal [7].

We investigated whether EPR spin trapping, using colloid $Fe(DETC)_2$, could reliably measure NO production from murine aortas, and validated its application in comparing vascular NO production in wild-type animals with that in genetic mouse models of absent or increased eNOS expression.

Materials and methods

Chemicals

NaDETC was obtained from Alexis. L-NAME, NaCl, and Hepes were obtained from Sigma–Aldrich. All other chemicals were obtained from BDH Laboratory Sciences, UK.

Animals

C57BL/6 wild-type mice, aged between 14 and 20 weeks, were used for initial validation of the method. Mice with targeted deletion of the *eNOS* gene (eNOS-KO) mice were obtained from Jackson Laboratories, USA, and their wild-type (wt) littermates were used as controls [9]. Mice with targeted endothelial eNOS overexpression (eNOS-Tg), as described previously [10], were used to assess the effects of elevated vascular eNOS levels, in comparison with their wild-type (wt) littermates. Mice were genotyped by polymerase chain reaction (PCR) methods. All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986. Mice were kept in cages with 12 h light–dark cycle and controlled temperature (20–22 °C), and fed normal chow and water ad libitum.

Animals were killed by an overdose of inhaled isofluorane. Freshly harvested thoracic aortas were cleaned of adherent fat, opened longitudinally, and placed in ice-cold Krebs–Hepes buffer (consisting of, in mM: NaCl 99; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.0; $CaCl_2$ 1.9; $NaHCO_3$ 25; glucose 11.1; and Na-Hepes 20; pH 7.4).

Colloid $Fe(DETC)_2$ spin trapping

NaDETC (3.6 mg) and $FeSO_4 \cdot 7H_2O$ (2.25 mg) were separately dissolved under argon gas bubbling in two 10 ml volumes of ice-cold Krebs–Hepes buffer. These were rapidly mixed to obtain a pale yellow-brown opalescent colloid $Fe(DETC)_2$ solution (0.4 mM), which was used immediately.

Aortas were placed separately in 24-well plates each filled with 100 µl Krebs–Hepes buffer, with or without the addition of calcium ionophore A23187 (final concentration 1 µM), to stimulate NOS. Colloid $Fe(DETC)_2$ was then added to each well (250 µl, final concentration 285 µM) and incubated at 37 °C for 90 min. To study the time course of NO accumulation in stimulated aortas, incubation times of 30, 60, 90, and 120 min were also performed. Separate experiments were done to examine the effects of endothelial removal, or NOS inhibition by addition of N^G-nitro-L-arginine methyl ester (L-NAME) to the incubation medium (final concentration 1 mM). Incubations were also performed with colloid $Fe(DETC)_2$ alone (without aortas) to correct for any background signals.

EPR spectroscopy

After incubation, the aorta was removed and frozen, in liquid nitrogen, into the middle of a column of Krebs–Hepes buffer. The needle-end was cut from a 1 ml syringe barrel, the plunger retracted 1.5 cm from the cut end, and one aorta was placed into the syringe barrel, which was then filled with Krebs–Hepes buffer and frozen in liquid nitrogen. After freezing, the syringe was removed from the liquid nitrogen and warmed for 5 s; the plunger was then removed, and the remaining barrel was filled with Krebs–Hepes buffer and frozen in liquid nitrogen. The syringe was then re-warmed for 5 s, and the syringe plunger was used to push the frozen column out of the syringe barrel directly into a finger Dewar vacuum flask (Magnettech, Germany) containing liquid nitrogen. EPR spectra were obtained using a benchtop X-band EPR spectrometer (Miniscope MS 200, Magnettech, Germany), equipped with a rectangular resonator cavity H102. Instrument settings were: centre-field (B_0) 3276G, sweep 115G, microwave power 10 mW, modulation frequency 100 kHz, amplitude modulation 8000 mG, sweep time 60 s, and number of scans 4. Signals were quantified by measuring the total amplitude, after correction of baseline as done previously [7,11],

and after subtracting background signals from incubation with colloid $Fe(DETC)_2$ alone.

Statistical analysis

One-way ANOVA was used to compare data sets.

Results

A characteristic NO–$Fe(DETC)_2$ signal with three peaks (g value approximately 2.035) was detected in wild-type C57BL/6 mice aortas incubated with $Fe(DETC)_2$ for 30 min in buffer, without agonist stimulation (Fig. 1). The magnitude of this signal was greatly increased by stimulation with the calcium ionophore A23187 (Fig. 1). However, the EPR signal from A23187-stimulated aortas was largely suppressed after addition of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), or after removal of the endothelium (Fig. 2). The NO–$Fe(DETC)_2$ signal was not detected in the incubation medium after removal of the aortic tissue segment (data not shown).

Systematic quantification of the NO–$Fe(DETC)_2$ signal from the EPR spectra revealed a 4.7-fold increase on stimulation with the calcium ionophore A23187 compared with basal conditions (Fig. 3; 3.45 ± 0.13 vs 0.73 ± 0.13 au [arbitrary units], $n = 3$). Signals from stimulated aortas were inhibited after addition of L-NAME (0.03 ± 0.08 au, $n = 3$), or after endothelial denudation (0.23 ± 0.08, $n = 3$). The NO–$Fe(DETC)_2$ signal increased linearly with incubation time ($r^2 = 0.93$), up to 2 h (Fig. 4).

To further determine the specificity of the NO–$Fe(DETC)_2$ signal for endothelial NO production, we next incubated aortas from both eNOS knockout (eNOS-KO) mice and endothelial-targeted eNOS transgenic (eNOS-Tg) mice with $Fe(DETC)_2$. Despite stimulation with calcium ionophore, the NO–$Fe(DETC)_2$ signal from aortas from eNOS-KO mice was essentially undetectable (Fig. 5; 0.14 ± 0.06 vs 3.17 ± 0.21 au in littermate controls, $n = 3$). In contrast, stimulated aortas from eNOS-Tg mice showed a striking increase in NO–$Fe(DETC)_2$ signal (7.17 ± 0.76 vs 3.37 ± 0.43 au in littermate controls, $n = 5$).

Discussion

In this paper, we describe and validate an EPR based method capable of quantifying NO production in mouse aortas using colloid $Fe(DETC)_2$. A characteristic NO–$Fe(DETC)_2$ signal was detected from aortas under basal conditions that increased with calcium ionophore

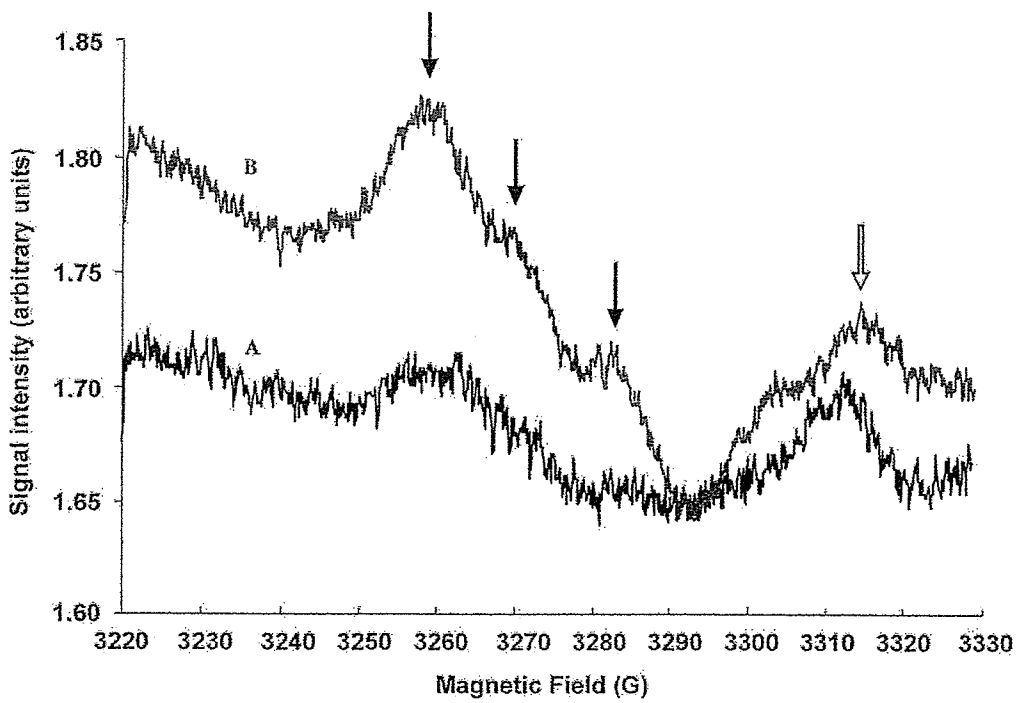

Fig. 1. EPR spectra of mouse aortas incubated for 90 min at 37 °C with 285 μM colloid $Fe(DETC)_2$. (A) Aorta alone and (B) aorta stimulated with 1 μM calcium ionophore A23187 (g values approximately 2.04). EPR spectra were recorded at 77 K and instrument settings are indicated in Materials and methods. The vertical axis represents signal intensity in arbitrary units. The characteristic triplet peaks associated with NO–$Fe(DETC)_2$ signal are shown by the three small arrows. The $Cu(DETC)_2$ signal to the right of the spectra is indicated by the open arrow. Spectra are representative of three separate experiments.

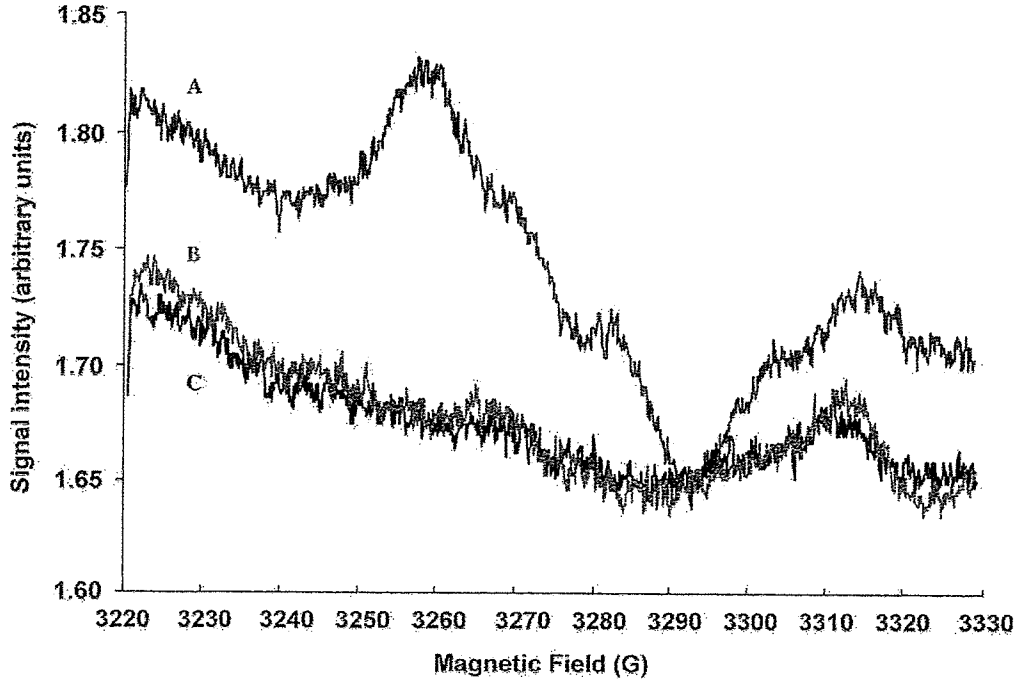

Fig. 2. EPR spectra of mouse aortas incubated with 1 μM calcium ionophore A23187 for 90 min at 37 °C with 285 μM colloid $Fe(DETC)_2$. (A) Stimulated aorta alone (g value = 2.04), (B) stimulated aorta treated with 1 mM L-NAME, and (C) stimulated aorta with endothelium removed. EPR spectra were recorded at 77 K and instrument settings are indicated in Materials and methods. The vertical axis represents signal intensity in arbitrary units. Spectra are representative of three separate experiments.

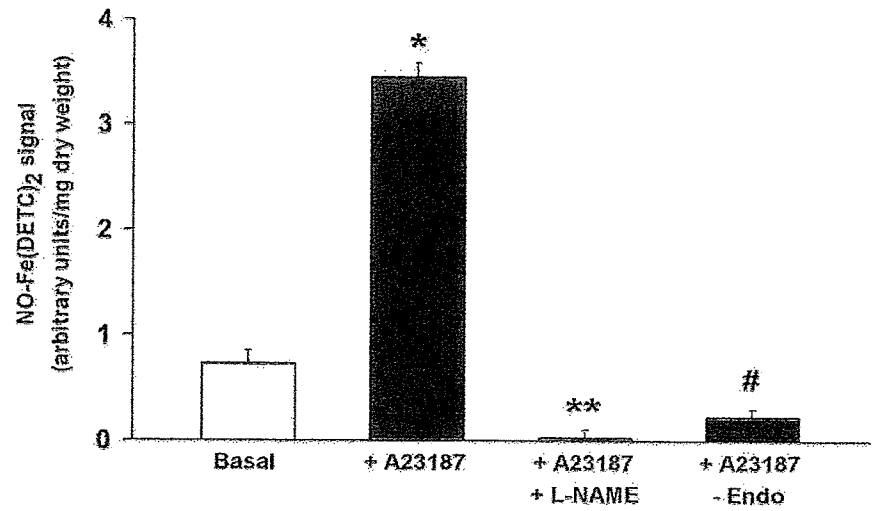

Fig. 3. Quantification of NO–$Fe(DETC)_2$ signal from mouse aortas incubated for 90 min at 37 °C, under basal condition with 285 μM colloid $Fe(DETC)_2$, stimulated condition with the addition of 1 μM calcium ionophore A23187, and also in stimulated conditions with 1 mM L-NAME, or removal of endothelium (-Endo). Data are expressed as signal intensity in arbitrary units, normalized to dry weight of aorta in milligrams, and corrected for background signals. Shown are mean values ± SEM from $n = 3$ experiments in each group. $^{*}P < 0.001$ vs basal, $^{**}P < 0.0001$ vs stimulated, and $^{\#}P < 0.0001$ vs stimulated.

stimulation, and increased in a linear relation with time of incubation, at least up to 2 h. The stimulated NO signal was abolished by L-NAME incubation or by endothelial removal, indicating that the source of NO production in the murine aorta is from endothelial NOS. This was further confirmed by undetectable signals from the eNOS-KO murine aortas, despite stimulation with calcium ionophore. Lack of detectable NO production is consistent with previous studies showing absent acetylcholine-induced relaxation in these mice [9].

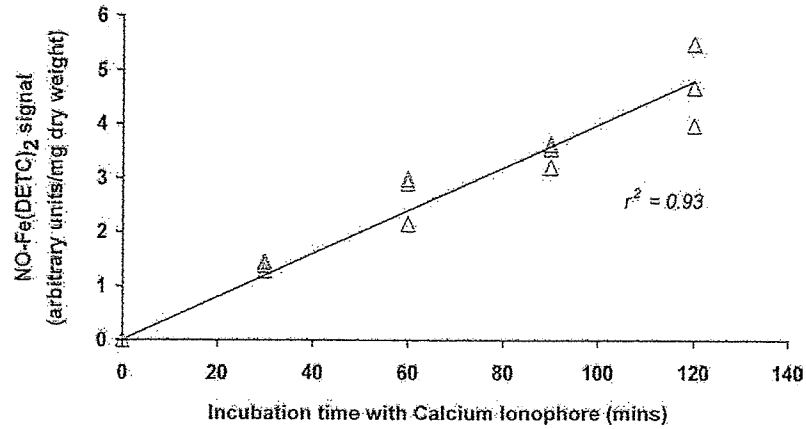

Fig. 4. Time course showing accumulation of NO–$Fe(DETC)_2$ signal from mouse aortas incubated at 37 °C with 285 μM colloid $Fe(DETC)_2$ and 1 μM calcium ionophore A23187. Data points represent three separate experiments performed at time points of 0, 30, 60, 90, and 120 min.

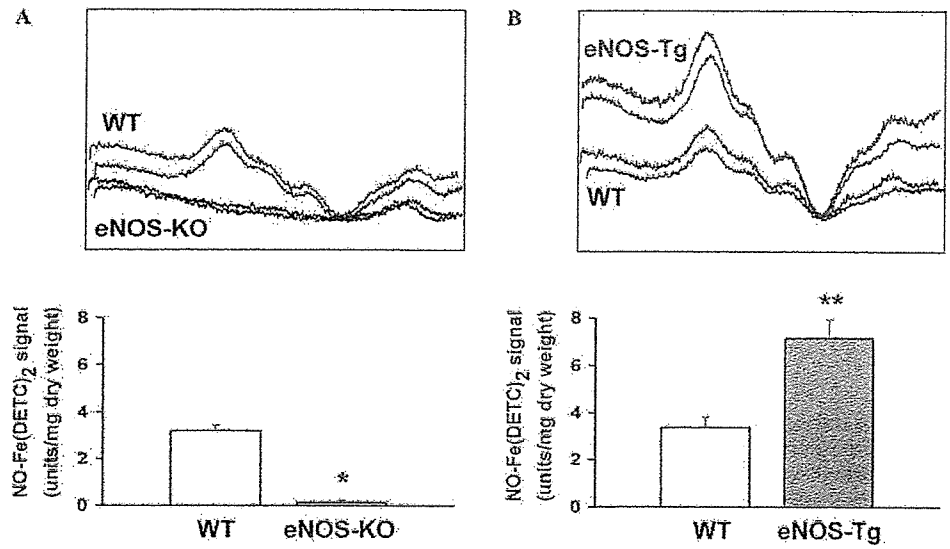

Fig. 5. Comparison of NO production from aortas of (A) *eNOS-KO* and (B) *eNOS-Tg* mice. Aortas were incubated with 285 μM colloid $Fe(DETC)_2$ and 1 μM calcium ionophore A23187 for 90 min at 37 °C. Representative EPR spectra from the genetically modified mice and their wild-type littermates are shown in the top panels (*g* values approximately 2.035). Comparative analyses of the NO–$Fe(DETC)_2$ signals are shown in the bottom panels. Data are means ± SEM. $^*P < 0.001$ vs wt littermate ($n = 3$) and $^{**}P < 0.01$ vs wt littermate ($n = 5$).

In contrast, eNOS-Tg mice showed a 2-fold increase in NO production compared with their wild-type littermates. It is important to also note that both the eNOS-KO and eNOS-Tg mice were bred on a C57BL/6 background, and as expected, the level of stimulated NO production in the wild-type offspring of both colonies was similar to each other and to the separate C57BL/6 colony used for initial method validation.

Previous studies showed that eNOS protein levels in eNOS-Tg mice are substantially elevated, and enzymatic L-citrulline conversion assay (with full supplementation of co-factors) suggests a dramatic 8-fold increase in total NOS enzymatic activity [10]. However, we have shown here that direct measurement of vascular NO production with EPR spin trapping revealed a more modest 2-fold increase in eNOS activity, suggesting that eNOS may be limited in vivo by insufficient levels of co-factor(s). Hence, by allowing direct measurement of NO in fresh vascular tissue, EPR spin trapping complements existing methods of NO detection, and can offer further insights into eNOS regulation in mouse models of vascular physiology and vascular disease [12,13].

The high efficiency of spin trapping with colloid $Fe(DETC)_2$ is probably due to partitioning of the lipophilic spin trap into the cell membranes, where it is able to form a stable spin adduct with NO. Indeed, no signal was obtained from the incubation medium after removal of the aorta. The method of spin trapping NO

using non-colloidal Fe/diethyldithiocarbamate (Fe/DETC) required the addition of strong reducing agents to detect a signal [3]. No reducing agent was required to detect NO in our experiments using colloid $Fe(DETC)_2$.

The $Cu(DETC)_2$ signal results from ligand exchange of Fe in the $Fe(DETC)_2$ complex with endogenous Cu present in the tissues, which may vary depending on the amount of tissue present [8]. However, this does not affect the quantification of the Fe(DETC)NO signal as it is small and separate. The $Cu(DETC)_2$ signal, when analysed per milligram of tissue, decreases as the NO–$Fe(DETC)_2$ signal increases, consistent with previous observations in rabbit vessels, suggesting that the FeNO group may compete more efficiently with Cu for DETC than Fe [8].

In conclusion, EPR spectroscopy using colloid $Fe(DETC)_2$ is a sensitive and specific approach to directly measure endothelial NO production from isolated mouse aortas. This approach has broad applications for comparative studies in genetically modified murine models of vascular disease.

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Functional Expression of Sodium-Dependent Vitamin C Transporter 2 in Human Endothelial Cells

Tadashi Seno Nobutaka Inoue Kiyoko Matsui Junya Ejiri Ken-ichi Hirata
Seinosuke Kawashima Mitsuhiro Yokoyama

Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Key Words
Antioxidants · Endothelium · Free radicals

Abstract
Since oxidative stress plays an important role in dysregulation of the microcirculation as well as the pathogenesis of atherosclersosis, therapeutic intervention with antioxidants has been speculated to prevent cardiovascular diseases. Ascorbic acid (AA) has been reported to improve endothelial function; however, its intracellular metabolic pathway has not been fully determined. Sodium-dependent vitamin C transporter (SVCT) types 1 and 2 were recently cloned. In the present study, we investigated whether SVCT-2 is functionally expressed in vascular endothelial cells and, if so, what factors modulate its activity. The uptake of AA into human umbilical vein endothelial cells (HUVECs) was examined by incubation with radiolabeled AA (^{14}C-AA). AA was transported into HUVECs in a dose- and time-dependent manner. Replacement of sodium chloride with choline chloride in the medium suppressed the uptake of AA. RT-PCR revealed that HUVECs expressed SVCT-2 mRNA, but not SVCT-1. Transfection of HUVECs with the antisense oligonucleotide of SVCT-2 significantly suppressed the uptake of AA. Furthermore, tumor necrosis factor-α and interleukin-1β inhibited the transport activity of AA. Thus, SVCT-2 is functionally expressed in human endothelial cells, and its activity is negatively regulated by inflammatory cytokines. Our findings might provide a new insight into understanding the treatment of cardiovascular diseases with AA.

Introduction

Oxidative stress plays a crucial role in dysregulation of the microcirculation as well as the pathogenesis of atherosclerotic cardiovascular diseases. Therefore, some antioxidant vitamins such as α-tocopherol (vitamin E), β-carotene and ascorbic acid (AA) are speculated to function as therapeutic drugs against such diseases. AA is an indispensable vitamin as the cofactor of several enzymes such as proline and lysine hydroxylase in collagen synthesis, dopamine β-hydroxylase in epinephrine synthesis and 7α-hydroxylase in bile acid formation. On the other hand, since AA has the property of a potent radical scavenger in human blood [1], it protects against lipid peroxidation by reactive oxygen species (ROS) [2]. Its antioxidative properties might exert beneficial effects on the cardiovascular system. Indeed, several clinical investigations have demonstrated that AA restores the impaired endothelium-dependent vasodilation in essential hypertension, diabetes mellitus, chronic heart disease and coronary artery disease. Previous investigations have also indicated an

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Dr. Nobutaka Inoue
Division of Cardiovascular and Respiratory Medicine
Department of Internal Medicine, Kobe University Graduate School of Medicine
7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017 (Japan)
Tel. +81 78 382 5846, Fax +81 78 382 5859, E-Mail nobutaka@med.kobe-u.ac.jp

inverse association between antioxidant intake and cardiovascular disease [3]. Furthermore, the Third National Health and Nutritional Examination Survey III has suggested that ascorbate supplementation has a protective effect against ischemic cardiovascular diseases [4]. However, whether AA can decrease the risk of cardiovascular events is still a subject of controversy.

Vitamin C is ubiquitously distributed in blood plasma, tissue interstitial fluid and the cytoplasm, and highly accumulated in several tissues, such as the central nervous system, retina, adrenal cortex, immune cells and platelets. The precise mechanism and regulation of the uptake of vitamin C into such tissues are unclear. Vitamin C exists as two forms, i.e. a reduced form, AA, and an oxidized form, dehydroascorbic acid (DHAA). Since the latter is structurally similar to glucose, its transport to the cytosol has been proposed to be mediated by glucose transporter (GLUT) [5–7]. On the other hand, another transport system of AA, the sodium-dependent vitamin C transporter (SVCT), has been identified [8]. To date, two subtypes of SVCT have been cloned, i.e. rat and human SVCT-1 and SVCT-2 [9, 10]. The expression of SVCT-1 is detected mainly in the intestinal epithelium and the liver, whereas SVCT-2 is ubiquitously expressed in various organs, including the placenta, liver, brain and heart [9, 10]. SVCT-2-deficient mice show a phenotype of respiratory distress and intracerebral hemorrhage, indicating the requirement of AA in the perinatal period [11]. In the present study, we investigated whether SVCT-2 is functionally expressed in human umbilical vein endothelial cells (HUVECs) and, if so, what kinds of mediators modify its activity in vascular endothelial cells.

Materials and Methods

Materials

L-AA, phloretin, dithiothreitol, choline chloride and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). *L*-[Carboxyl-^{14}C]-AA (16.7 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, N.J., USA). Recombinant human tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were purchased from R&D Systems Inc.

Cell Cultures

HUVECs, HepG2 cells (human hepatoma cell line) and CoCa-2 cells were obtained from the American Type Culture Collection. HUVECs were cultured to confluency in RPMI-1640 medium supplemented with 20% fetal calf serum, 100 IU/ml heparin, 100 IU/ml endothelial cell growth supplement, 100 U/ml penicillin and 100 μg/ml streptomycin. For the uptake study, 4th–6th passage HUVECs were seeded onto 24-well plates. Since a previous report indicates that there is considerable variability in AA metabolism between cell cultures, three independent batches were used [12].

Uptake Study of AA

To examine the activity of ascorbate transport, the uptake of radiolabeled AA (^{14}C-AA) was measured as previously described [10]. The transport buffer was composed of 25 m*M* N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (pH 7.5), Tris (pH 7.5), 140 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgSO_4$ and 5.0 m*M* glucose. To prevent the auto-oxidation of AA, 1.0 m*M* dithiothreitol was added to the transport buffer.

HUVECs were washed twice with prewarmed phosphate-buffered saline, and then incubated with the transport buffer adjusted to each concentration of ^{14}C-AA (0–200 μ*M*) for 15 min at 37 °C. In the time course study, the cells were incubated for each period with the transport buffer containing 50 μ*M* ^{14}C-AA. The buffer was then removed, and the cells were washed twice with ice-cold transport buffer and solubilized in 1.0 ml of lysis buffer composed of 0.5% SDS and 0.2 *N* NaOH. The cell lysate of 500 μl was transferred into vials, and intracellular radioactivity was quantified by liquid scintillation spectrometry. The sodium-dependent uptake of AA was defined as the uptake in the presence of Na^+ in media minus the uptake in the absence of Na^+. To estimate the competitive inhibition of ^{14}C-AA uptake by nonlabeled AA, the cells were incubated with the transport buffer containing 5–200 μ*M* ^{14}C-AA and nonlabeled AA (200 μ*M*). To examine the effects of TPA and phloretin, HUVECs were preincubated with these drugs for 30 min before the uptake study. In the case of examination of inflammatory cytokines, HUVECs were preincubated with TNF-α or IL-1β for the indicated periods, and then the uptake studies were performed.

RT-PCR

The RT reaction was carried out with 4 μg of total RNA isolated from HUVECs, HepG2 cells, and CoCa-2 cells. The RT product of 1 μl was used as a template for PCR amplification. PCR amplification was conducted for 1 min at 94 °C, 3 min at 61 °C and 3 min at 72 °C. Amplified DNA was separated on 1% agarose gel with ethidium bromide and visualized under UV. The two different sets of specific primers used to detect mRNA of SVCT-2 [13] were as follows: 5′-ACGTTTGGATGCAGGTTACCC-3′ and 5′-TGAAGCAGAGCAGCCAGGATAC-3′, and 5′-TTCTATGCTCGCACAGATGCC-3′ and 5′-TAAAAGCCACACAGCCCCCTAC-3′. The SVCT-1-specific primers were as follows: 5′-ACTCTCCTCCGCATCCAGAT-3′ and 5′-CCAGGCGGGCACAGGCGTAG-3′ [14].

Blockade of SVCT-2 by Antisense Oligonucleotides

To assess the contribution of SVCT-2 in AA transport, the effects of antisense oligonucleotides against SVCT-2 were examined with a Morpholino Oligomers kit [15]. This system avoids concerns regarding the degradation of oligonucleotides, because of its resistance to a wide range of nucleases, including DNase I, DNase H and RNase A [16]. Antisense oligonucleotide was generated against nucleotides 387–411 of human SVCT-2 cDNA, and its sequence was 5′-CTTACCAATACCCATCATTAAGAGA-3′. The invert of the antisense oligonucleotide, 5′-AGAGAATT CTACCCATAACCATTC-3′, was used as negative control. To transfect these oligonucleotides, the Special Delivery System (Gene Tools, LCC, Philomath, Oreg., USA) was used, because of its high efficiency in oligonucleotide delivery [15]. After transfection, HUVECs were incubated for 48 h, and then the uptake of AA was determined.

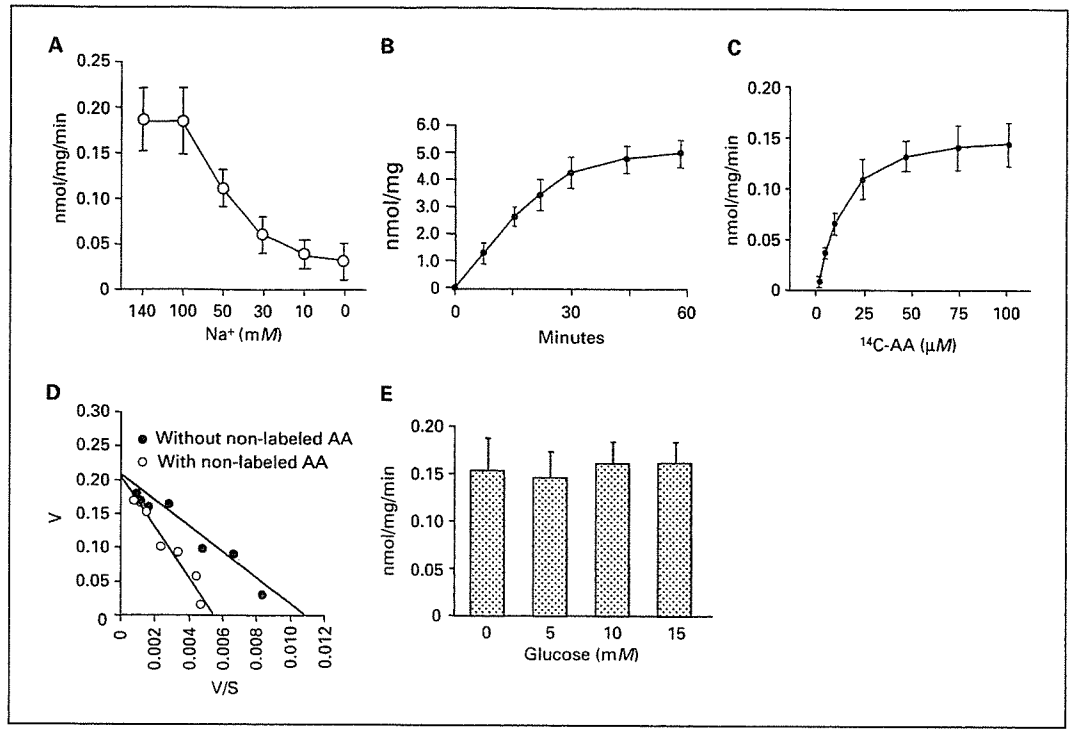

Fig. 1. A Effect of extracellular Na^+ on AA uptake into HUVECs. The replacement of Na^+ with choline chloride suppressed the uptake of AA in a dose-dependent manner. The results were obtained from twelve wells of three different cell cultures. **B** Sodium-dependent uptake of AA. HUVECs were incubated for each period with transport buffer containing 50 μ*M* ^{14}C-AA in the presence or absence of extracellular Na^+. The sodium-dependent uptake of AA increased in a time-dependent manner and was linear until 15 min. **C** Dose response of sodium-dependent uptake of AA. The cells were incubated with the transport buffer adjusted to each concentration of ^{14}C-AA (0–200 μ*M*) for 15 min. The ^{14}C-AA count increased in a dose-dependent manner, and reached a plateau level at 100 μ*M*. The results were obtained from twelve wells of three different cell cultures. The kinetic analysis revealed that K_m and V_{max} values were 13.2 μmol and 0.13 nmol/min/mg, respectively. **D** An Eadie-Hofstee plot showing the inhibition of transport of radiolabeled AA by nonlabeled AA. The cells were incubated with the transport buffer containing ^{14}C-AA (5–200 μ*M*) with or without nonlabeled AA (200 μ*M*). V = Rate of AA uptake (nmol/mg/min); S = concentration of AA (μ*M*). **E** Effect of extracellular glucose on sodium-dependent AA transport into HUVECs. To examine the involvement of GLUT in AA transport into HUVECs, the effect of glucose in the transport buffer on the sodium-dependent uptake of ^{14}C-AA was examined. Glucose in the transport buffer up to a concentration of 15 m*M* had no effect on the uptake of ^{14}C-AA. The results were obtained from nine wells of three different cell cultures.

Statistical Analysis

All values are presented as the mean ± standard error. Comparisons between different groups were performed using one-way analysis of variance. When significance was indicated, a Student-Newman-Keuls post hoc analysis was employed. The accepted level of significance was $p < 0.05$.

Results

Transport of AA into HUVECs

Firstly, we examined whether the transport of ^{14}C-AA into HUVECs was dependent upon the presence of extracellular Na^+. As shown in figure 1A, the replacement of Na^+ with choline chloride suppressed the uptake of AA in a dose-dependent manner, and the uptake was strongly suppressed by 81% in the absence of extracellular Na^+. In the following experiments, the sodium-dependent uptake of AA was defined as the uptake in the presence of Na^+ minus the uptake in the absence of Na^+.

Next, we examined the kinetics of sodium-dependent uptake of AA. The sodium-dependent uptake of AA was clearly increased when HUVECs were incubated with the transport buffer containing 50 μ*M* ^{14}C-AA. The transport to the cells was linear until 15 min (fig. 1B); therefore, the rate of uptake at this time point was used in the following assay. The transport of ^{14}C-AA increased in a dose-depen-