A Specific Role for eNOS-Derived Reactive Oxygen Species in Atherosclerosis Progression

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Objective—When the availability of tetrahydrobiopterin (BH4) is deficient, endothelial nitric oxide synthase (eNOS) produces superoxide rather than NO (uncoupled eNOS). We have shown that the atherosclerotic lesion size was augmented in apolipoprotein E–deficient (ApoE-KO) mice overexpressing eNOS because of the enhanced superoxide production. In this study, we addressed the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus nonspecific antioxidant strategies in restoring eNOS coupling.

Methods and Results—We crossed mice overexpressing eNOS in the endothelium (eNOS-Tg) with mice overexpressing GTP-cyclohydrolase I (GCH), the rate-limiting enzyme in BH4 synthesis, to generate ApoE-KO/eNOS-Tg/GCH-Tg mice. As a comparison, ApoE-KO/eNOS-Tg mice were treated with vitamin C. Atherosclerotic lesion formation was increased in ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice. GCH overexpression in ApoE-KO/eNOS-Tg/GCH-Tg mice increased vascular BH4 levels and reduced plaque area. This reduction was associated with decreased superoxide production from uncoupled eNOS. Vitamin C treatment failed to reduce atherosclerotic lesion size in ApoE-KO/eNOS-Tg mice, despite reducing overall vascular superoxide production.

Conclusion—In contrast to vitamin C treatment, augmenting BH4 levels in the endothelium by GCH overexpression reduced the accelerated atherosclerotic lesion formation in ApoE-KO/eNOS-Tg mice, associated with a reduction of superoxide production from uncoupled eNOS. (Arterioscler Thromb Vasc Biol. 2007;27:1632-1637.)

Key Words: eNOS uncoupling ■ tetrahydrobiopterin ■ vitamin C ■ atherosclerosis ■ apolipoprotein E–deficient mice

Nitric oxide (NO) derived from endothelial NO synthase (eNOS) is a critical signaling molecule in the vasculature and has a range of antiatherogenic effects. In eNOS/apolipoprotein E (ApoE) double-knockout mice, atherosclerosis is increased, suggesting a protective effect of eNOS-derived NO. However, certain vascular diseased states are associated with an increase rather than a decrease in the expression of eNOS. We have shown that endothelium-targeted overexpression of eNOS in ApoE-KO (ApoE-KO/eNOS-Tg) mice surprisingly resulted in decreased endothelial NO availability, increased vascular superoxide production, and accelerated atherosclerosis.

These observations could be explained by a relative deficiency of the cofactor tetrahydrobiopterin (BH4) causing eNOS uncoupling, where the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. In ApoE-KO/eNOS-Tg mice, dietary BH4 supplementation reduced superoxide production and increased NO availability, although it was unclear whether this was a general antioxidant effect of BH4 or a specific effect on eNOS coupling. Indeed, dietary supplementation with the antioxidant vitamin C can also reduce vascular oxidative stress, increase BH4 levels, and was sufficient to improve the depressed endothelium-dependent relaxation in ApoE-KO mice fed a high fat diet, although a specific effect on eNOS coupling was not investigated. These preclinical data suggest a potential role for vitamin C therapy in vascular disease, yet large scale clinical trials have failed to demonstrate an effect on major clinical end points in human atherosclerosis. It is possible that interventions targeted at specific redox mechanisms may be more effective than nonspecific antioxidant strategies for treatment of vascular disease states.
Substantial evidence points to an important role for BH4 in regulating eNOS coupling in atherosclerosis. Indeed, even in the absence of vascular disease, eNOS overexpression in the endothelium without a concomitant increase in BH4 levels can result in eNOS uncoupling, but augmenting endothelial BH4 levels, by overexpression of the rate-limiting enzyme in BH4 biosynthesis, GTP-cyclohydrolase I (GCH), is able to restore eNOS/BH4 stoichiometry and eNOS coupling.11

In this study, we sought to address the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus nonspecific antioxidant strategies in restoring eNOS coupling. We used the ApoE-KO/eNOS-Tg mouse as a model of eNOS uncoupling in atherosclerosis, and investigated the relationships between eNOS protein, BH4 availability, and atherosclerotic plaque progression. Furthermore, we compared the strategy of directly augmenting endothelial BH4 levels by transgenic GCH overexpression, versus vitamin C supplementation.

Methods

Experimental Design

The animals used in these experiments were offspring from breeding between ApoE-KO/eNOS-Tg mice (ApoE-KO mice overexpressing a bovine eNOS transgene in the endothelium) and ApoE-KO/GCH-Tg mice (ApoE-KO mice overexpressing a human GCH transgene in the endothelium), as previously reported in detail.6,12 The background strain was C57BL/6 mouse. ApoE-KO, ApoE-KO/eNOS-Tg, and ApoE-KO/eNOS-Tg/GCH-Tg mice were generated and fed standard mouse chow from weaning. Experimental mice were euthanized at 16 weeks of age and organs used for histological and biochemical analysis. In parallel experiments, another group of ApoE-KO/eNOS-Tg mice were treated with 500 mg/kg body weight/d vitamin C dissolved in drinking water starting from 4 weeks of age until 16 weeks of age.

All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine. All commercial drugs besides those mentioned specifically were purchased from Sigma Chemical Co.

Plasma Lipid Analyses, Vitamin C Levels, and Hemodynamic Analyses

After overnight fasting, blood was collected by the cardiac puncture under anesthetic using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation of the blood for 10 minutes at 5500g at 4°C and stored at −80°C. Concentrations of plasma total cholesterol and triglyceride were determined by use of an automated chemistry analyzer. High density lipoprotein cholesterol levels were quantified by enzymatic reaction using a commercially-available kit (Wako). Plasma concentrations of vitamin C were measured with reverse-phase high-performance liquid chromatography (HPLC).

Heart rate and systolic blood pressure were measured at 16 weeks of age using a tail cuff photoelectric device. All measurements were repeated 6 times for each mouse.

Measurement of Vascular Biopterin Concentrations

Mice were anesthetized and the aorta sample was dissected from the ascending aorta to the iliac bifurcation. The dissected aorta was placed in a mixture of 0.5 mol/L perchloric acid containing 0.1 mmol/L disodium EDTA, 0.1 mmol/L Na2S2O3 for protein separation, and 0.1 mmol/L ascorbic acid to prevent oxidation. Then the aorta was homogenized on ice in 200 μL homogenate buffer. After centrifugation (15 000g for 10 minutes) and filtration (0.45 μm pore size; Millex-HV Filter Unit, Millipore), we measured vascular BH4 and dihydrobiopterin (BH2) concentrations by HPLC developed by Tani et al.13 Briefly, by post-column NaNO2 oxidation with a reversed-phase ion-pair LC system, BH4 and BH2 were detected fluorometrically at wave lengths of 350 nm for excitation and 440 nm for emission (LC-10 series, Shimadzu). Protein concentrations of aortic homogenates were measured by the Bradford method, and BH4 or BH2 concentration was corrected for protein concentration.

Analysis of eNOS Protein

The levels of total eNOS protein in aorta homogenates were analyzed by Western blotting, and in addition, to investigate the ratio of eNOS dimer to monomer, Western blotting was performed by use of nonboiled lung homogenates and low-temperature SDS-page as previously described.14,15

Superoxide Production in the Aortic Endothelium

First, we measured overall superoxide production from aortas. The aorta was cut into 4 pieces (5 mm length per each piece), which were incubated with the Cu-Zn superoxide dismutase inhibitor for 30 minutes at 37°C. Then vascular superoxide production levels were measured by chemiluminescence with 10 μmol/L lucigenin (bis-N-methylacridinium nitrate).

By this method, however, we cannot differentiate endothelium-derived superoxide from superoxide produced in other vascular cell components. Therefore we measured in situ superoxide production from vessel wall by use of dihydroethidium (DHE; Molecular Probe).16 Briefly, the unfixed tissues embedded in OCT were cut into 10-μm-thick sections, and incubated with or without 5 mmol/L Nω-nitro-arginine methyl ester (L-NAME) for 5 minutes at 37°C, and then incubated with 2 μmol/L DHE at 37°C for 10 minutes in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope (Carl ZEISS). DHE fluorescence from high-power (>×200) images was quantified by automated image analysis using the Image J software (National Institutes of Health). For quantification of endothelial cell ethidium fluorescence, fluorescence (intensity×area) was measured only on the luminal side of the internal elastic lamina. For each vessel, mean fluorescence was calculated from 3 separate high-power fields taken in each sections of the vessel to produce n=1.

Atherosclerotic Lesion Assessment at the Aortic Sinus

At 16 weeks of age, mice were anesthetized and the aorta was perfused as above. The aorta was dissected from the middle of the left ventricle to the aortic arch, and fixed with 4% paraformaldehyde for 16 hours. The samples were cut in the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compounds (Tissue-Tek). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Sudan III. For quantitative analysis of atherosclerosis, the total lesion area of 5 separate sections from each mouse was obtained with the use of the Image J.5

Statistical Analysis

Data were expressed as means±SEM. One-way ANOVA or two-way ANOVA were used to compare the differences among the 4 experimental groups, and then appropriate post hoc analysis with Bonferroni correction for each result was performed. Values of P<0.05 were considered statistically significant.

Results

Plasma Analyses and Hemodynamics

Plasma total cholesterol, triglyceride, and high density lipoprotein cholesterol levels were not different among the 4 groups (Table). Body weight was similar among the 4 groups in both gender mice. As previously reported,14 eNOS overexpression in the endothelium significantly decreased systolic blood pressure, but neither concomitant GCH overexpression nor vitamin C treatment had any further effect on blood pressure. There were no differences in heart rate among the 4 groups (data not shown). In either ApoE-KO mice or ApoE-KO/eNOS-Tg mice, plasma vitamin C concentrations were not different from those in
control C57BL/6 mice (55.2±5.4 μmol/L, n=5). Vitamin C treatment increased its plasma concentrations approximately 1.5-fold in ApoE-KO/eNOS-Tg mice (Table).

**Vascular Biopterin Concentrations**

We next measured aortic BH4 concentrations and the oxidative state of biopterins. In ApoE-KO mice, aortic BH4 concentrations were approximately 4 times higher compared with BH2 concentrations. Aortic BH4 concentrations in ApoE-KO/eNOS-Tg mice were similar compared with ApoE-KO mice (Figure 1A). However, overexpression of GCH increased vascular BH4 concentrations by more than 2-fold in ApoE-KO/eNOS-Tg mice. In contrast, vitamin C treatment did not induce significant changes in vascular BH4 concentrations. Overexpression of eNOS reduced the BH4/BH2 ratio in all groups, reflecting increased oxidative loss of BH4 (Figure 1B).

**eNOS Dimerization in Lung Homogenates**

As expected, transgenic overexpression of eNOS in the endothelium significantly increased eNOS protein levels in the aorta. However, neither GCH overexpression in the endothelium nor vitamin C treatment affected eNOS protein levels in the aorta of ApoE-KO/eNOS-Tg mice (data not shown).

To investigate the effect of GCH overexpression versus vitamin C supplementation on the stability of eNOS dimer, we used low-temperature SDS-PAGE and Western blotting to determine the ratio of eNOS dimer to monomer. We used lung homogenates for this assay, because much higher level of eNOS protein could be obtained in the lung compared with in aortas. In ApoE-KO/eNOS-Tg mice, eNOS dimer to monomer ratio was significantly reduced compared with ApoE-KO mice in lung homogenates (Figure 2A and 2B), reflecting an imbalance between eNOS protein and BH4 concentrations. GCH overexpression partially restored the eNOS dimer to monomer ratio in ApoE-KO/eNOS-Tg mice, but vitamin C treatment had no effect.

**Superoxide Production from the Endothelium**

In the lucigenin method, eNOS overexpression augmented chemiluminescence signals from aortas compared with ApoE-KO mice. Both concomitant overexpression of GCH and vitamin C treatment reduced the augmented chemiluminescence signals in ApoE-KO/eNOS-Tg mice (Figure 3).

Then, superoxide production in the endothelium, and in particular the contribution of uncoupled eNOS to endothelial superoxide production, was evaluated using DHE oxidative fluorescent microtopography. Ethidium fluorescence was detected throughout all layers of the vessel wall (Figure 4A). We focused on the vascular superoxide production in endothelial cells by measuring ethidium fluorescence specifically on the luminal side of the internal elastic lamina. Endothelial ethidium fluorescence was increased in ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice, and both GCH overexpression and vitamin C treatment decreased its fluorescence (Figure 4A and 4B). In particular, vitamin C treatment suppressed fluorescence signals in all layers of the vessel wall. We now probed the contribution of eNOS to net superoxide levels in the endothelium using the NOS inhibitor L-NAME. Although L-NAME had no effect on ethidium fluorescence in ApoE-KO mice, it decreased DHE fluorescence in the ApoE-KO/eNOS-Tg mice.

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**Table 1.** Body Weight, Plasma Lipid Contents, Vitamin C Levels, and Blood Pressure

<table>
<thead>
<tr>
<th></th>
<th>ApoE-KO</th>
<th>ApoE-KO eNOS-Tg</th>
<th>ApoE-KO eNOS-Tg GCH-Tg</th>
<th>ApoE-KO eNOS-Tg + Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female body wt, g</td>
<td>21.8±0.5</td>
<td>22.1±1.3</td>
<td>22.5±0.4</td>
<td>21.9±0.9</td>
</tr>
<tr>
<td>Male body wt, g</td>
<td>28.7±0.6</td>
<td>27.5±0.7</td>
<td>29.2±0.7</td>
<td>27.1±0.7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>12.4±0.7</td>
<td>12.3±0.7</td>
<td>12.2±0.9</td>
<td>12.7±1.4</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>0.45±0.04</td>
<td>0.44±0.08</td>
<td>0.46±0.07</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>0.26±0.03</td>
<td>0.25±0.02</td>
<td>0.28±0.03</td>
<td>0.30±0.04</td>
</tr>
<tr>
<td>Vitamin C, μmol/L</td>
<td>56.3±4.0</td>
<td>54.5±8.0</td>
<td>52.8±6.8</td>
<td>80.7±6.3*</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>108.8±1.3</td>
<td>102.0±1.4†</td>
<td>103.5±1.6‡</td>
<td>103.1±1.5§</td>
</tr>
</tbody>
</table>

Data were shown as means ± SEM (*P<0.05 vs ApoE-KO/eNOS-Tg mice, †P<0.01 vs ApoE-KO mice, ‡P<0.05 vs ApoE-KO mice, n=10 per each group).
mice, indicating the presence of NOS-mediated superoxide production in the endothelium. In contrast, endothelial ethidium fluorescence was increased by L-NAME in ApoE-KO/eNOS-Tg/GCH-Tg mice, indicating that NOS was a net producer of NO rather than superoxide in this group. Importantly, although basal endothelial ethidium fluorescence was lower in vitamin C–treated ApoE-KO/eNOS-Tg mice than in control ApoE-KO mice or ApoE-KO/eNOS-Tg mice, there was no change after L-NAME incubation, suggesting that the reduction in superoxide levels was attributable to an antioxidative effect of vitamin C rather than a direct effect on eNOS coupling.

Atherosclerotic Lesion Formation

To evaluate the net effect of these different vascular biochemical changes in the 4 groups on atherosclerosis progression, we measured atherosclerotic lesion formation at the aortic sinus. In accordance with our previous study, performed in mice with more severe hypercholesterolemia than that in the present study, histological examination staining revealed that eNOS overexpression markedly accelerated the atherosclerotic lesion formation in both genders (Figure 5). Augmented endothelial BH4 levels by GCH overexpression significantly reduced atherosclerotic lesion formation compared with ApoE-KO/eNOS-Tg mice, and indeed restored plaque progression to control ApoE-KO mice levels. In contrast, vitamin C treatment had no effects on accelerated atherosclerotic lesion formation in ApoE-KO/eNOS-Tg mice.
These results indicate that overexpression of eNOS in ApoE-KO mice leads to eNOS uncoupling attributable to a stoichiometric imbalance between levels of eNOS and BH4. The reduction in vascular NO bioavailability and increased superoxide production are associated with accelerated atherosclerosis. A general reduction in vascular superoxide levels alone, by vitamin C treatment, is insufficient to retard atherosclerosis. In contrast, restoration of eNOS/BH4 stoichiometry and eNOS coupling, by augmented endothelial BH4 biosynthesis, is able to restore disease progression to control ApoE-KO mice levels.

**Discussion**

In this article we investigate the mechanisms by which BH4 and vitamin C regulate production of NO or superoxide from eNOS, and how this relates to atherosclerosis progression. To focus on and accentuate the effect of uncoupled eNOS in atherosclerosis progression, we chose to evaluate an ApoE-KO mouse model with eNOS overexpression in the vascular endothelium—previously shown to have accelerated atherosclerosis and eNOS uncoupling. By crossing this mouse with an endothelium-targeted GCH transgenic mouse, we compared the effect of specific augmentation of endothelial BH4 synthesis with vitamin C treatment, and report the following novel findings: First, GCH overexpression, but not vitamin C treatment, was able to increase aortic BH4 levels and increase eNOS dimer stability. Second, endothelial superoxide production was higher in eNOS overexpressing ApoE-KO mice compared with controls, and this was at least in part attributable to superoxide derived from uncoupled eNOS. Compared with vitamin C treatment, GCH overexpression led to a marked reduction in eNOS-derived superoxide production. Third, only a specific increase in endothelial BH4 synthesis by GCH overexpression was able to inhibit the accelerated atherosclerosis progression seen in eNOS overexpressing mice; vitamin C treatment had no effect on accelerated atherosclerosis in ApoE-KO/eNOS-Tg mice, despite a general reduction in vascular oxidative stress.

We previously demonstrated that eNOS overexpression in the endothelium of high-fat–fed ApoE-KO mice caused greater atherosclerotic lesion formation, in association with increased endothelial superoxide production mediated by uncoupled eNOS. In the present study, we now show that the acceleration of atherogenesis attributable to uncoupled eNOS also occurs in ApoE-KO mice with less severe hypercholesterolemia. These and other reports suggest that the relationship between eNOS and BH4 availability is a critical determinant of eNOS enzymatic function in both health and disease states.

Vitamin C may be important in maintaining BH4 levels in the setting of vascular oxidative stress. Vitamin C supplementation improves endothelial dysfunction in smokers, in subjects with hypercholesterolemia, and in patients with diabetes mellitus or coronary artery disease. In addition, chronic dietary supplementation with vitamin C increased the BH4/BH2 ratio and NO bioavailability in the aortas of ApoE-KO mice; and there is a dose-dependent effect of vitamin C on BH4 levels and NO synthesis in cultured endothelial cells. Although the mechanism for this effect is not fully resolved, recent data indicate that vitamin C may act by reducing the BH3 radical to regenerate BH4.

The reported effects of long-term vitamin C treatment on atherosclerotic lesion formation in mice have been variable, likely because of differences in the mouse models and experimental conditions used. d’Uscio et al observed that vitamin C improved endothelial function and suppressed atherosclerotic lesion formation in ApoE-KO mice fed a high-fat Western-type diet, whereas Nakata et al showed that vitamin C did not affect atherosclerotic plaque area but reduced plaque collagen content in ApoE-KO mice that were further genetically modified to be unable to synthesize vitamin C. We now report findings in the ApoE-KO/eNOS-Tg mice fed a standard (non–high fat) chow. We used the ApoE-KO/eNOS-Tg mouse model to specifically evaluate aspects of eNOS uncoupling, because we know from previous studies that eNOS overexpression leads to eNOS uncoupling in both normal animals and in the ApoE-KO mouse. Furthermore, we used 500 mg/kg body weight/d vitamin C supplementation, a dose based on previous studies, whereas d’Uscio et al used 1%/kg chow/d, implying that the administered dose of vitamin C in that protocol was approximately 3 to 5 times higher. In the study of d’Uscio et al, vitamin C treatment resulted in the reduction of vascular BH2 concentrations without changing BH4 concentrations, suggesting that vitamin C inhibited BH4 oxidation. It is possible that, in the setting of marked hypercholesterolemia, their model was associated with more oxidation of vascular BH4 than we observed in our study, and the higher dose of vitamin C inhibited BH4 oxidation and increased BH4/BH2 ratio in vessels.

It remains uncertain whether the ratio of BH4 to the oxidized bioppterin BH2 is as important as the absolute BH4.
concentrations in determining eNOS enzymatic activity in vivo. Biochemical studies indicate that BH2, which has no eNOS cofactor activity, can compete with BH4 for eNOS binding, and experiments in the DOCA-salt model of mouse hypertension suggest that the ratio of BH4/BH2 may be a determinant of NOS uncoupling. In contrast, in the streptozotocin mouse model of diabetes, the absolute concentration of endothelial BH4 appeared to be more important than the BH4/BH2 ratio in NOS regulation. In the present study, the BH4/BH2 ratio did not change either in mice with GCH overexpression or in those treated by vitamin C, suggesting that in our model absolute BH4 levels rather than the BH4/BH2 ratio determine eNOS coupling.

As the limitation of our study, we used DHE staining to evaluate superoxide production from the endothelium. Ethidium, the oxidation product of DHE, intercalates with DNA. The ethidium signals may, therefore, be influenced by cellular chromatin density, and the HPLC-based technique is recommended to omit such a problem in the use of DHE. In the present study, however, it was important to differentiate the signals only from the endothelium, which is the site of eNOS uncoupling, from those from other vascular cells. We also measured superoxide from whole aortic tissue by the lucigenin method and confirmed that the increased superoxide production in BH4-overexpressed mice, which was decreased by BH4 treatment. In addition, as we mentioned already, we cannot rule out the possibility that, in the presence of high oxidative stress, higher dose of vitamin C might reduce atherosclerotic lesion formation by inhibiting BH4 oxidation.

In conclusion, our findings demonstrate that increased synthesis of BH4 by GCH overexpression in the endothelium was able to specifically restore eNOS uncoupling, and resulted in the inhibition of atherosclerotic lesion formation. In contrast, a general reduction in vascular superoxide levels by oral vitamin C treatment had no effect on eNOS coupling and did not inhibit atherosclerosis. These data support the concept that strategies to increase eNOS expression without a concomitant augmentation of endothelial BH4 concentrations may be detrimental in the setting of atherosclerotic disease, and may offer further mechanistic data to explain the failure of nonspecific antioxidant therapies to reduce vascular disease progression in large clinical trials.

Disclosures

None.

References

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