Increased Endothelial Tetrahydrobiopterin Synthesis by Targeted Transgenic GTP-Cyclohydrolase I Overexpression Reduces Endothelial Dysfunction and Atherosclerosis in ApoE-Knockout Mice

Objective—Increased production of reactive oxygen species and loss of endothelial nitric oxide (NO) bioactivity are key features of vascular disease states such as atherosclerosis. Tetrahydrobiopterin (BH4) is a required cofactor for NO synthesis by endothelial nitric oxide synthase (eNOS); pharmacologic studies suggest that reduced BH4 availability may be an important mediator of endothelial dysfunction in atherosclerosis. We aimed to investigate the importance of endothelial BH4 availability in atherosclerosis using a transgenic mouse model with endothelial-targeted overexpression of the rate-limiting enzyme in BH4 synthesis, GTP-cyclohydrolase I (GTPCH).

Methods and Results—Transgenic mice were crossed into an ApoE knockout (ApoE-KO) background and fed a high-fat diet for 16 weeks. Compared with ApoE-KO controls, transgenic mice (ApoE-KO/GCH-Tg) had higher aortic BH4 levels, reduced endothelial superoxide production and eNOS uncoupling, increased cGMP levels, and preserved NO-mediated endothelium dependent vasorelaxations. Furthermore, aortic root atherosclerotic plaque was significantly reduced in ApoE-KO/GCH-Tg mice compared with ApoE-KO controls.

Conclusions—These findings indicate that BH4 availability is a critical determinant of eNOS regulation in atherosclerosis and is a rational therapeutic target to restore NO-mediated endothelial function and reduce disease progression.

Keywords: nitric oxide synthase ■ endothelium ■ atherosclerosis ■ hypercholesterolemia

Nitric oxide (NO) produced in the endothelium by endothelial nitric oxide synthase (eNOS) is a key mediator of vascular homeostasis. NO bioavailability is reduced early in vascular disease states such as hypercholesterolemia and atherosclerosis because of reduced NO synthesis and increased NO consumption by reactive oxygen species. A critical determinant of eNOS activity is the availability of the NOS cofactor tetrahydrobiopterin (BH4). When BH4 levels are inadequate, the enzymatic reduction of molecular oxygen by BH4 is no longer coupled to l-arginine oxidation, resulting in generation of superoxide rather than NO, thus contributing to vascular oxidative stress and endothelial dysfunction. BH4 bioavailability in the vasculature appears to be regulated at the level of biosynthesis by the rate-limiting enzyme GTP-cyclohydrolase I (GTPCH) and by oxidative degradation of BH4 to dihydrobiopterin (BH2) that is inactive for eNOS cofactor function.

Several pharmacologic studies suggest a possible role for BH4 availability in regulating NO-mediated endothelial function. Acute administration of BH4 improves some features of endothelial dysfunction in smokers, and in patients with type II diabetes, hypercholesterolemia, or coronary atherosclerosis. In hypercholesterolemic ApoE-knockout (ApoE-KO) mice, endothelium-dependent vascular relaxations are impaired, NO synthesis is reduced, and vascular superoxide production is increased. However, endothelial dysfunction in ApoE-KO mice can be reduced by incubation of vessels in the BH4 precursor sepiapterin. Transgenic overexpression of eNOS in ApoE-KO mice surprisingly leads to enhanced vascular superoxide production, reduced NO bioavailability, and accelerated atherosclerosis. BH4 levels are reduced in the aortas of these mice compared with wild-type controls, but dietary BH4 supplementation with sapropterin reduces superoxide production and increases NO synthesis. These results suggest that increased eNOS protein alone is insufficient to maintain NO synthesis in hypercholesterolemia, and that adequate BH4 levels are essential to prevent eNOS uncoupling in endothelial dysfunction states. However, the effects of topical or systemic pharmacologic BH4 supplementation in these studies may be mediated in part by nonspecific antioxidant effects of acute high-dose BH4, which can...
increase apparent NO bioavailability by nonspecific ROS scavenging. Furthermore, the long-term effects of endothelial BH4 augmentation in the pathogenesis of vascular disease states are uncertain. Indeed, the effects of pharmacologic supplementation with BH4 or other biotin analogues on NO bioactivity are unpredictable in vascular disease states in which oxidative stress is increased and in which oxidation of BH4 to BH2 by reactive oxygen species such as peroxynitrite may be an important mechanism underlying BH4 loss.

Accordingly, we sought to investigate the importance of BH4 availability in experimental atherosclerosis using a novel transgenic mouse model with endothelial-targeted overexpression of GTPCH, the rate-limiting enzyme in BH4 synthesis. In this model, endothelial cell BH4 levels are specifically increased 3- to 4-fold, without elevation of plasma BH4 levels. We crossed this transgenic mouse line into an ApoE-KO background to investigate the effects of sustained targeted increases in endothelial BH4 synthesis on NO-mediated endothelial function and on progression of atherosclerosis.

**Methods**

**Animals**

All studies involving laboratory animals were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Mice were housed in temperature-controlled cages (20°C to 22°C) with a 12-hour light–dark cycle and were given free access to water and formulated diets. GTPCH transgenic (GCH-Tg) mice, in which human GTPCH transgene overexpression is targeted to the vascular endothelium under the control of the mouse Tie-2 promoter, were generated in a C57B/6 background as previously described. GCH-Tg mice have increased endothelial GTPCH mRNA expression and protein production, resulting in a 3- to 4-fold augmentation of vascular BH4 levels. These mice were cross-bred through two generations with ApoE-KO mice, also in a C57BL/6 background (purchased from Jackson Laboratory, Bar Harbor, ME), to produce ApoE-KO/GCH-Tg heterozygote breeders. Breeders were mated with pure ApoE-KO mice to produce equal numbers of GCH-Tg (ApoE-KO/GCH-Tg) and nontransgenic (ApoE-KO) littermates in an ApoE-KO background, which were used in all experiments. Mice were weaned at age 3 weeks onto a high-fat diet, to 60 mmol/L KCl, followed by cumulative half-log concentrations of isoprotanol (isoprotanol) at 37°C, and gassed with 95% O2/5% CO2. All experiments were performed in the presence of 10 μmol/L indo-1 to inhibit vascular prostaglandin synthesis. Serial responses to 60 mmol/L KCl, followed by cumulative half-log concentrations of phenylephrine (PE) (1 × 10-10 to 1 × 10-8 mol/L) and acetylcholine (Ach) (1 × 10-7 to 1 × 10-5 mol/L) after preconstriction with PE (3 × 10-6 mol/L), and the NO donor sodium nitroprusside (SNP) (1 × 10-10 to 1 × 10-6 mol/L) were determined as previously described.

**Histologic Analysis of Aortic Root Plaque**

Immediately after being euthanized, mice were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline via the left ventricle. Hearts were dissected and immersed in fixative for a further 24 hours. Each heart was transected at the level of the atria, and gassed with 95% O2/5% CO2. All experiments were performed in the presence of 10 μmol/L indo-1 to inhibit vascular prostaglandin synthesis. Serial responses to 60 mmol/L KCl, followed by cumulative half-log concentrations of phenylephrine (PE) (1 × 10-10 to 1 × 10-8 mol/L) and acetylcholine (Ach) (1 × 10-7 to 1 × 10-5 mol/L) after preconstriction with PE (3 × 10-6 mol/L), and the NO donor sodium nitroprusside (SNP) (1 × 10-10 to 1 × 10-6 mol/L) were determined as previously described.
Statistical Analysis

For isometric tension studies, mean responses of two rings from each animal were combined to produce \( n = 1 \). Dose response curves from groups were compared using a general linear model ANOVA test for repeated measures (SPSS v10.0). For other comparisons, one-way ANOVA or \( t \) tests were used. \( P < 0.05 \) was considered significant.

Data are expressed as means and SEM.

Results

Plasma Lipid Profiles in Mice Fed a High-Fat Diet

Both ApoE-KO and ApoE-KO/GCH-Tg mice had markedly increased plasma total cholesterol and triglyceride levels when fed a high-fat diet for 16 weeks, but there were no significant differences between the two groups (Table).

Effect of GTPCH Overexpression on Aortic Biopterin Levels

We first determined whether increased GTPCH expression within the endothelium of ApoE-KO/GCH-Tg mice would lead to increased BH4 levels by measuring biopterins in homogenates of snap-frozen aorta. Total biopterin levels were approximately 2-fold higher and BH4 levels were 3-fold higher in ApoE-KO/GCH-Tg compared with ApoE-KO aorta (Figure 1A) as a result of GTPCH over-expression. In ApoE-KO/GCH-Tg mice, BH4 levels represented 71% of total biopterins, compared with 38% in ApoE-KO mice, suggesting increased oxidative degradation of BH4 in ApoE-KO mice (Figure 1B). This finding suggests that increased endothelial BH4 levels in ApoE-KO/GCH-Tg mice are associated with reduced aortic oxidative stress in atherosclerosis.

Effect of Increased BH4 Levels on Endothelial Superoxide Production and eNOS Coupling

To investigate the source of oxidative stress in mouse aorta, we measured superoxide production using dihydroethidium oxidative fluorescent microtopography. Ethidium fluorescence was observed throughout all layers of the vessel wall that could be inhibited by preincubation with PEG-SOD (data not shown). We next focused on the contribution of eNOS to vascular superoxide production in endothelial cells by measuring ethidium fluorescence specifically on the luminal side of the internal elastic lamina only. Endothelial ethidium fluorescence in ApoE-KO mice (black bars, expressed in arbitrary units) was increased 2-fold compared with ApoE-KO/GCH-Tg mice (white bars; \( * P < 0.05 \)). Incubation of sections with 1 mmol/L L-NAME resulted in decreased endothelial fluorescence in ApoE-KO mice, but increased fluorescence in ApoE-KO/GCH-Tg mice \( (P < 0.05) \). White bar represents 20 \( \mu \)m; elastic laminae exhibit green autofluorescence.

Cyclic GMP Levels in Aortas

To evaluate whether maintenance of eNOS coupling in ApoE-KO/GCH-Tg mice increased NO bioavailability, we

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cholesterol (mmol/L)</th>
<th>HDL Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-KO/GCH-Tg</td>
<td>33.1±4.2</td>
<td>3.6±0.8</td>
<td>1.7±0.8</td>
</tr>
<tr>
<td>ApoE-KO</td>
<td>37.1±2.6</td>
<td>4.0±0.4</td>
<td>1.9±0.8</td>
</tr>
</tbody>
</table>

Data shown are means±SEM (n=7 to 9 per group).

Figure 1. A, Total biopterin (white bars) and BH4 content (hatched bars) in aortas of ApoE-KO/GCH-Tg and ApoE-KO mice. Total aortic biopterin concentration was 2-fold higher and BH4 concentration was 3-fold higher in ApoE-KO/GCH-Tg compared with ApoE-KO mice \( (** P < 0.01 ; n=5 \) to 6 per group). B, Ratio of BH4 to total biopterin in aortas of ApoE-KO/GCH-Tg (white bars) and ApoE-KO mice (black bars). The aortic BH4 to total biopterin ratio was lower in ApoE-KO mice \( (P < 0.05 , n=5 \) to 6), suggesting increased BH4 oxidation in these animals.

Figure 2. Dihydroethidium (DHE) staining of aortic sections for endothelial cell superoxide production. Representative sections (60x) are shown, with specific endothelial cell ethidium fluorescence (white arrows) measured on the luminal side of the internal elastic lamina only. Endothelial ethidium fluorescence in ApoE-KO mice (black bars, expressed in arbitrary units) was increased 2-fold compared with ApoE-KO/GCH-Tg mice (white bars; \( * P < 0.05 \)). Incubation of sections with 1 mmol/L L-NAME resulted in decreased endothelial fluorescence in ApoE-KO mice, but increased fluorescence in ApoE-KO/GCH-Tg mice \( (P < 0.05) \). White bar represents 20 \( \mu \)m; elastic laminae exhibit green autofluorescence.
measured aortic cGMP levels in ApoE-KO/GCH-Tg and ApoE-KO mice. cGMP levels were increased 2-fold in ApoE-KO/GCH-Tg mice compared with ApoE-KO mice (Figure 3). These results indicate that maintenance of endothelial BH4 levels by GTPCH overexpression in ApoE-KO mice augments NO bioavailability and signaling in the vascular wall.

**Effect of Increased BH4 Levels on Endothelial Function**

We next determined the functional relationships between aortic BH4 levels and eNOS-dependent vasomotor function in ApoE-KO and ApoE-KO/GCH-Tg mice. Isometric tension studies revealed no difference in vascular contractions to phenylephrine among the two groups of mice (Figure 4A). However, endothelium-dependent relaxations to the receptor-mediated eNOS agonist acetylcholine (ACh) were significantly impaired in ApoE-KO mice compared with relaxations in ApoE-KO/GCH-Tg mice (Figure 4B). Indeed, endothelium-dependent relaxations in ApoE-KO/GCH-Tg mice were not significantly impaired in comparison with control C57Bl/6J mice fed a high-fat diet (data not shown). Endothelium independent relaxations to the NO donor sodium nitro-

**Development of Aortic Root Plaque**

To investigate the effect of preserved endothelial function in ApoE-KO/GCH-Tg mice on the progression of atherosclerosis, we quantified aortic root plaque area after 16 weeks of high-fat feeding. Mean aortic root plaque area after 16 weeks of high-fat diet was 28% lower in ApoE-KO/GCH-Tg mice (0.39 mm²) compared with ApoE-KO mice (0.54 mm²) (n=9 animals per group; *P<0.05). Black bars on photomicrographs represent 200 μm.

**Discussion**

In this study we describe a new mouse model in which endothelial-targeted overexpression of GTPCH leads to a persistent increase in endothelial BH4 levels in an ApoE-KO background. We used this model to investigate the role of BH4 in hypercholesterolemic endothelial dysfunction and report the following major findings. First, endothelial-specific overexpression of GTPCH is sufficient to increase vascular BH4 levels in atherosclerosis. Second, uncoupled eNOS contributes to endothelial superoxide production in ApoE-KO aortas, but eNOS coupling is preserved by increased vascular BH4 levels in ApoE-KO/GCH-Tg aortas. Third, increased BH4 synthesis in ApoE-KO mice is associ-
ated with increased NO bioavailability, as demonstrated by increased aortic cGMP levels and preserved endothelial-dependent vascular relaxations. Fourth, preserved endothelial function by GTPCH overexpression in ApoE-KO mice reduces atherosclerosis progression.

Previous studies have reported increased superoxide production, at least partly derived from uncoupled eNOS, in the aorta of ApoE-KO mice, diabetic rats, and hyperlipidemic rabbits. In agreement with these studies, we also found that uncoupled eNOS contributes to superoxide production specifically within endothelial cells and is associated with increased oxidation of BH4, forming BH2 and biopterin. The mechanisms underlying increased oxidative stress in vascular disease states include enzyme systems such as the NADPH oxidases, in addition to eNOS uncoupling in the endothelium. Reducing NADPH oxidase-mediated ROS production has salutary effects on atherosclerotic progression in ApoE-KO mice and on NO-mediated vascular function and eNOS coupling in DOCA-salt hypertension, suggesting that NADPH oxidase-mediated ROS can initiate eNOS uncoupling through BH4 oxidation. However, initial eNOS uncoupling may progressively increase oxidative degradation of BH4, resulting in a positive feed-forward spiral of reduced NO production and increasing eNOS-mediated superoxide generation. In support of this model, some studies have suggested that agents such as ascorbate and folate, known to improve endothelial dysfunction, may act through stabilization or regeneration of BH4. We now add further direct evidence suggesting a central role for BH4-mediated eNOS uncoupling in the progressive endothelial dysfunction of atherosclerosis. By specifically augmenting vascular BH4 levels in ApoE-KO mice, using endothelial-targeted transgenic overexpression of GTPCH, we found that eNOS coupling was maintained, leading to reduced endothelial superoxide production and increased NO bioavailability. Our observations suggest that maintenance of BH4 levels in atherosclerosis is alone sufficient to rescue the deficit in eNOS function, despite the overall increase in vascular oxidative stress. However, it should be noted that direct evidence of eNOS uncoupling in vascular disease has not been demonstrated in vivo, because of the technical limitations involved in the measurement of vascular superoxide production. In addition, our experiments cannot exclude a possible effect of increased endothelial BH4 levels on the activity of the inducible NOS isoform in other cells of the vascular wall, which likely contributes to vascular pathophysiology in ApoE-KO mice.

Improving eNOS coupling by constitutive augmentation of endothelial BH4 levels in the ApoE-KO mouse model of atherosclerosis also allowed us to investigate the long-term effects of this intervention on NO-mediated endothelial function and on atherosclerotic plaque progression in vivo. Reduced endothelial superoxide production and increased NO bioactivity, as evidenced by cGMP levels, significantly improved vascular relaxations to acetylcholine in GCH-Tg atherosclerotic mice. Indeed, in these animals, vaso relaxations were maintained at levels indistinguishable from levels in control C57Bl/6J mice fed a high-fat diet. Importantly, these findings demonstrate that targeted preservation of NO-mediated endothelial function is sufficient to reduce plaque progression in atherosclerosis, adding further weight to the concept that endothelial dysfunction is indeed a direct contributor in the pathogenesis of atherosclerosis, rather than an indirect marker of disease progression.

Our study suggests that BH4 is a rational therapeutic target to correct endothelial dysfunction in atherosclerosis. Strategies to persistently improve BH4 availability may be effective in restoring NO-mediated endothelial function and limiting vascular disease progression in several conditions, such as atherosclerosis, diabetes, and hypertension. Paradoxically, targeted eNOS overexpression alone as a strategy to restore or increase vascular NO bioactivity in atherosclerosis neither restores NO-mediated endothelial function nor reduces atherosclerosis. Rather, eNOS overexpression in atherosclerosis has detrimental effects because of superoxide generated by eNOS uncoupling, which is corrected by high-dose BH4 supplementation. However, high pharmacological doses of sepiapterin or BH4 (often >100-fold in excess of physiological concentrations) may increase NO bioactivity via nonspecific antioxidant effects. The present study addresses this potential limitation by using GTPCH overexpression in transgenic mice to only modestly increase endothelial BH4 synthesis and vascular BH4 levels. Taken together with previous studies, our observations suggest that BH4 availability, rather than total eNOS enzymatic activity, is a more critical regulator of NO production in atherosclerosis and has direct effects on NO-mediated endothelial function and on atherosclerotic progression.

The mechanisms underlying reduced BH4 availability in vascular diseases remain incompletely understood and may involve several pathways. BH4 levels in inflammatory cells are regulated principally by transcriptional upregulation of GTPCH in response to cytokine stimulation. Although similar observations have been described in cultured endothelial cells, there is little evidence for major changes in endothelial GTPCH expression and BH4 synthesis in vivo in the setting of vascular diseases. For example, GTPCH expression does not appear upregulated in the vasculature in a mouse model of diabetes, and there are conflicting comparisons of aortic BH4 levels in ApoE-KO mice compared with controls. Reduced endothelial BH4 levels in vascular diseases could be caused by inhibition of GTPCH enzymatic activity secondary to either phosphorylation or feedback inhibition through GTPCH feedback regulatory protein. However, previous data suggest that oxidative degradation of BH4 by peroxynitrite (forming BH2 and biopterin via the nonprotonated BH3 radical) is a more likely explanation for reduced BH4 levels in atherosclerosis and in other vascular disease states. Indeed, in the present study we observed that the ratio of BH4 to oxidized biopterins was reduced in aortas of ApoE-KO mice compared with ApoE-KO/GCH-Tg mice, adding further support to this conclusion. We found, in addition, that maintenance of endothelial BH4 levels appears sufficient to limit BH4 oxidation by preservation of eNOS coupling. Thus, eNOS-dependent superoxide production mediated by BH4 insufficiency further reduces BH4 availability. Although reduced biosynthesis of BH4 may not be the principal mechanism of BH4 loss in vascular disease, the
GCH-Tg mouse model clearly shows that increasing endothelial BH4 biosynthesis is nevertheless effective in restoring BH4 availability. Strategies aimed at increasing BH4 biosynthesis, reducing BH4 oxidation, or enhancing BH4 regeneration may be equally valid as therapeutic approaches in vascular disease states.

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References

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