Tetrahydrobiopterin Deficiency and Nitric Oxide Synthase Uncoupling Contribute to Atherosclerosis Induced by Disturbed Flow

Li Li, Wei Chen, Amir Rezvan, Hanjoong Jo, David G. Harrison

Objective—Tetrahydrobiopterin (BH₄) is a critical cofactor for nitric oxide (NO) synthesis by NO synthase (NOS). Recently, we demonstrated that disturbed flow produced by partial carotid ligation decreases BH₄ levels in vivo. We therefore aimed to determine whether atherosclerosis induced by disturbed flow is due to BH₄ deficiency and NOS uncoupling and whether increasing BH₄ would prevent endothelial dysfunction, plaque inflammation, and atherosclerosis.

Methods and Results—We produced a region of disturbed flow in apolipoprotein E⁻/⁻ mice using partial carotid ligation and fed these animals a high-fat diet. This caused endothelial NOS uncoupling as characterized by increased vascular superoxide production, altered vascular reactivity, and a change in endothelial NOS migration on low-temperature gel. These perturbations were accompanied by severe atherosclerosis, infiltration of T cells and macrophages, and an increase in cytokine production. Treatment with BH₄ recoupled NOS, decreased superoxide production, improved endothelium-dependent vasodilatation, and virtually eliminated atherosclerosis. BH₄ treatment also markedly reduced vascular inflammation and improved the cytokine milieu induced by disturbed flow.

Conclusion—Our results highlight a key role of BH₄ deficiency and NOS uncoupling in atherosclerosis induced by disturbed flow and provide insight into the effect of modulating vascular BH₄ levels on atherosclerosis and inflammation at these sites of the circulation. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis • immune system • nitric oxide synthase • reactive oxygen species • superoxide
The purposes of this study were therefore to determine whether atherosclerosis induced by disturbed flow is due to low levels of BH₄ and NOS uncoupling and to determine whether increasing BH₄ prevents endothelial dysfunction, plaque inflammation, and atherosclerosis. To perform these studies, we used an in vivo model of disturbed flow by inducing partial carotid ligation in hyperlipidemic mice. This model is associated with rapid atherosclerotic lesion formation and facilitates the examination of therapeutic interventions.

**Methods**

**Reagents and Materials**

BH₄ and 5,6,7,8-tetrahydropterin (NH₄) were from Schircks Laboratories. All other biochemicals were purchased in the highest available grade from Sigma-Aldrich (St Louis, MO). Live/Dead Fixable Near-IR stain and PE-TR rat anti-mouse CD45 antibody were from Invitrogen (Carlsbad, CA). PE Cy7 rat anti-mouse F4/80 antibody was from eBiosciences (San Diego, CA). All other antibodies for flow cytometry were from BD Biosciences (San Jose, CA). The eNOS antibody used for Western blot was from BD Biosciences (San Jose, CA).

**Animals Studied**

The Institutional Animal Care and Use Committee at Emory University approved all experimental protocols. C57Bl/6 and apolipoprotein E (Apoe)⁻⁻ mice were obtained from the Jackson Laboratory. Partial ligation of left common carotid artery (LCA) was carried out at 8 weeks of age as previously described to create low and oscillatory shear stress in the LCA. The right common carotid artery (RCA) was not ligated and was used as a control.

To augment vascular levels of BH₄, this pterin was added to the drinking water to provide an approximate dose of 10 mg/kg per day. To minimize oxidation of BH₄ in the drinking water, 0.04% vitamin C was also added. In preliminary experiments, we found that this prevented BH₄ oxidation for up to 24 hours. As a vehicle control, mice not receiving BH₄ received only vitamin C in their drinking water to provide an approximate dose of 10 mg/kg per day.

**Plasma Lipid Analysis**

Blood was aspirated directly into syringes containing heparin via cardiac puncture. Plasma was obtained through centrifugation of blood for 15 minutes at 5500g and 4°C and then stored at −20°C until each assay was performed. Lipid analysis was performed commercially (Cardiovascular Specialty Laboratories, Atlanta, GA). All lipid determinations were performed using a Beckman CX7 chemistry analyzer and reagents from Beckman Diagnostics (Fullerton, CA) for total cholesterol and triglycerides.

**BH₄ Measurements by High-Performance Liquid Chromatography**

Tissue bipterins (BH₄ and more oxidized species) were measured by high-performance liquid chromatography (HPLC) as previously described. Carotids were homogenized with lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA) and oxidized by exposure to 1% I₂ and 2% KI at room temperature for 1 hour under dark conditions. Ascorbic acid was added to stop the reaction, and the mixture was then centrifuged for 10 minutes at 12,000g. Bipterins in the supernatant were quantified by HPLC on a C18 column with fluorescence detection.

**Measurement of Vascular Superoxide Production**

Carotid superoxide production was quantified by measuring formation of 2-hydroxyethidium from dihydroethidium (DHE) by HPLC. This product specifically reflects the reaction of superoxide with DHE as previously validated. To localize superoxide within the vascular wall, we used DHE staining. As previously described, frozen sections (30 µm) obtained from LCA and RCA of partially ligated mice were stained in 2 µmol/L DHE for 30 minutes at 37°C. Slides were mounted with Dako mounting medium and immediately imaged with a Zeiss LSM 510 META confocal microscope.

**Assessment of Atherosclerosis by Oil Red O Staining**

Mice were euthanized and perfused at physiological pressure with saline containing heparin. The left and right carotid arteries were removed en bloc with the trachea and esophagus. For frozen sections, tissue was embedded in Tissue-Tek optimum cutting temperature medium, frozen in liquid nitrogen, and stored at −80°C until stained. Oil Red O staining was carried out using frozen sections as previously described, and nuclei were stained with hematoxylin.

**Vascular Isometric Tension Studies**

Endothelium-dependent and -independent vasodilatation was examined using carotid rings obtained from LCA and RCA of partially ligated ApoE⁻⁻ mice, as previously described. Isometric tension was measured using MultiWire Myograph System Model 610 (DMT). After being equilibrated for at least 30 minutes, the rings were preconstricted using phenylephrine. After a stable contraction was achieved, the rings were exposed to acetylcholine to assess endothelium-dependent vasodilatation. Endothelium-independent relaxation to sodium nitroprusside was also examined.

**Flow Cytometry for Measurement of Vascular Inflammatory Cells**

Mouse carotids were minced with fine scissors and digested using collagenase type IX (125 U/mL), collagenase type I (450 U/mL), and hyaluronidase I (60 U/mL) dissolved in PBS containing calcium and magnesium for 1 hour at 37°C, with agitation every 20 minutes. Digested samples were then homogenized using an 18-gauge needle yielding single cell suspensions. Cells were then centrifuged at 1200 rpm and resuspended in fluorescence-activated cell sorting buffer. Cells were stained for 30 minutes at 4°C with antibodies and then washed and resuspended in fluorescence-activated cell sorting buffer. Antibodies used for staining were fluorescein isothiocyanate rat anti-mouse CD4, PE-TR rat anti-mouse CD45, PerCP-Cy5.5 rat anti-mouse CD3, V450 rat anti-mouse CD8, APC rat anti-mouse CD44, APC Cy7 rat anti-mouse CD62L, fluorescein isothiocyanate rat anti-mouse CD45, PerCP-Cy5.5 rat anti-mouse cd11c, APC rat anti-mouse cd11b, PE rat anti-mouse CD86, PE Cy7 rat anti-mouse F4/80, and Live/Dead Fixable Near-IR stain. Absolute cell counting was performed by adding a known quantity of calibration beads to a known sample volume. After staining, cells were analyzed immedi-
Cytokine Measurements

Mouse carotids were placed into each well of a 96-well plate containing 60 μL of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C under 5% CO2 for 16 hours. During this time, a combination of phorbol myristate acetate (10 μmol/L) was added to stimulate vascular cytokine production. Following this, levels of cytokines tumor necrosis factor-α, interferon-γ (IFN-γ), and interleukin-2 (IL-2) in the media were measured using a cytokine bead array kit (BD Biosciences).

Statistical Analysis

Data are expressed as mean ± standard error of the mean. When 2 groups were compared, unpaired t tests were used. ANOVA was used for multiple comparisons and when significance was indicated, a 2-tailed Bonferroni post hoc test was used to make selected comparisons. When 1 group served as a control, the Dunnett post hoc test was used. A value of P<0.05 was considered significant.

Results

Disturbed Flow Induced by Partial Carotid Ligation Causes NOS Uncoupling

We have previously shown that partial carotid ligation reduces total biopterin production and increases BH4 oxidation to BH2 in the LCA of the C57bl/6 mice.11 In the present study, we confirmed that this also occurred in ApoE−/− mice under partial carotid ligation and were fed a high-fat diet for 1 week. Mice were further randomized to receive either oral BH4 or vehicle in their drinking water. A, During incubation with DHE ex vivo, vessels were coincubated with and without NG-L-nitro-arginine methyl ester (L-NAME), and formation of 2-hydroxyethidium was subsequently measured using HPLC (n=6). Values were compared using ANOVA, and selected comparisons were made using a Bonferroni post hoc test. B, Representative Western blot for eNOS dimer/monomer in the carotids using a low-temperature gel. D, Densitometry ratios for eNOS dimers and monomers (n=3). Values were compared using ANOVA, and selected comparisons were made using a Bonferroni post hoc test.

Effect of BH4 Treatment on NOS Uncoupling and Vascular Superoxide Production at Areas of Disturbed Flow

We treated mice with BH4 in their drinking water to examine whether increasing BH4 levels can reverse NOS uncoupling and decrease superoxide levels at areas of disturbed flow. BH4 treatment for 1 week normalized the total biopterin and BH4 levels in the LCA of the ApoE−/− mice (Supplemental Figure IA and IC). This confirmed that oral BH4 supplementation increased vascular BH4 levels. BH4 treatment also reduced superoxide production in the LCA but did not affect RCA superoxide levels. Moreover, in BH4-treated mice, NG-L-nitro-arginine methyl ester increased superoxide production, suggesting that BH4 treatment was able to recouple NOS in the setting of disturbed flow (Figure 1A). Using DHE staining, we found that BH4 treatment markedly reduced superoxide production in all 3 layers of the vessel wall (Figure 1B). In keeping with this, BH4 treatment also normalized the ratio of eNOS dimers to monomers observed on low-temperature Western blots (Figure 1C and 1D). Together, these results indicate that NOS uncoupling enhances vascular superoxide production at areas of disturbed flow and that this can be ameliorated by BH4 treatment.
Effect of NOS Uncoupling on Atherosclerosis at Areas of Disturbed Flow

We next sought to determine the role of NOS uncoupling on the severity of atherosclerosis at sites of disturbed flow. Nam et al have previously shown that partial carotid ligation in ApoE<sup>−/−</sup> mice fed a high-fat diet causes accelerated atherosclerosis within 3 weeks. In keeping with this, Oil Red O staining showed that the LCA of vehicle-treated mice developed severe atherosclerosis. In striking contrast, BH<sub>4</sub> treatment for 3 weeks virtually eliminated atherosclerotic lesion formation in the LCA (Figure 2A and 2B). The unligated RCA did not develop atherosclerotic lesions in this time frame. BH<sub>4</sub> treatment for 3 weeks did not affect plasma cholesterol levels of the ApoE<sup>−/−</sup> mice fed a high-fat diet for 3 weeks (Supplemental Figure II). To exclude the possibility that the benefit of BH<sub>4</sub> was due to a nonspecific antioxidant effect, we also treated mice with NH<sub>4</sub>. NH<sub>4</sub> has a redox potential similar to that of BH<sub>4</sub> but does not sustain NOS catalysis. In contrast to BH<sub>4</sub> treatment, oral treatment with NH<sub>4</sub> did not affect atherosclerosis development (Figure 2A) (Supplemental Figure III).

Prior studies of ApoE<sup>−/−</sup> mice have shown that atherosclerosis preferentially develops at the origin of the brachiocephalic and intercostal arteries, areas where shear stresses are likely reduced. In keeping with these prior observations, we found that high-fat feeding induced small lesions in the brachiocephalic artery and in some of the intercostal artery branches of the aorta. These were reduced by BH<sub>4</sub> treatment (Supplemental Figure III).

Effect of NOS Uncoupling on Endothelial Dysfunction Induced by Partial Ligation

Endothelial dysfunction is an early marker for atherosclerosis. A defect in BH<sub>4</sub> results in NOS uncoupling and reduces production of NO, leading to impaired endothelium-dependent vasodilation. We therefore sought to determine whether the endothelial dysfunction observed at sites of disturbed flow was due to NOS uncoupling. Carotid rings were obtained from LCA and RCA of partially ligated ApoE<sup>−/−</sup> mice fed a high-fat diet for 1 week. Carotid rings were preconstricted with phenylephrine and the relaxation

![Table. EC<sub>50</sub> and Peak Relaxation Values of LCA and RCA of ApoE<sup>−/−</sup> Mice Treated With Vehicle or BH<sub>4</sub> in Response to Acetylcholine](image)

<table>
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<th>Experimental Group</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; Log [M(Ach)]</th>
<th>Peak Relaxation (%)</th>
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<tr>
<td>Vehicle-LCA</td>
<td>−5.82±0.64*</td>
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<tr>
<td>Vehicle-RCA</td>
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<td>BH&lt;sub&gt;4&lt;/sub&gt;-RCA</td>
<td>−8.09±0.34‡</td>
<td>93.1±4.8</td>
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*P<0.001 vs RCA. †P<0.001 vs RCA. ‡P<0.05 vs vehicle-LCA (n=6 to 8).

Figure 3. Role of NOS uncoupling on endothelial dysfunction induced by partial carotid ligation. ApoE<sup>−/−</sup> mice underwent partial carotid ligation and were fed a high-fat diet for 1 week. The animals were randomized to receive oral BH<sub>4</sub> or vehicle following carotid ligation. Arterial rings were obtained from LCA and RCA of these mice for vascular ring activity measurements. A. Rings were preconstricted with phenylephrine, and responses were evoked by increasing concentrations of acetylcholine for endothelium-dependent relaxation (n=6 to 8). B. Responses to increasing concentrations of sodium nitroprusside (SNP) were studied to assess endothelium-independent relaxation (n=6 to 8). Values were compared using repeated-measures ANOVA, and selected comparisons were made using a Bonferroni post hoc test.
evoked by increasing concentrations of acetylcholine was examined. In the unligated RCA, acetylcholine elicited potent relaxations reaching 90% of the preconstricted tension at EC50 of 10^{-8} \text{mol/L} (Table). LCA segments from vehicle-treated mice exhibited significantly blunted relaxations to acetylcholine both in terms of peak relaxation and the EC50. BH4 treatment normalized the peak relaxation to acetylcholine and improved but did not normalize EC50 (Figure 3A and Table). Endothelium-independent relaxation in response to the NO donor sodium nitroprusside was identical among all the groups (Figure 3B). Thus, the alteration of endothelium-dependent vasodilatation observed at areas of disturbed flow was largely related to BH4 deficiency and NOS uncoupling.

**Role of NOS Uncoupling on Infiltration of Activated Immune Cells at Sites of Disturbed Flow**

Inflammatory cells, including T cells, antigen-presenting dendritic cells, and macrophages, are present in atherosclerotic lesions and are thought to promote the progression of atherosclerosis and plaque instability by releasing inflammatory cytokines. We therefore examined the role of NOS uncoupling in this inflammatory response in regions of disturbed flow. Using flow cytometry, we found that partial ligation of the LCA for 1 week caused marked infiltration of total leukocytes and T cells, characterized by the surface markers CD45+ and CD3+, respectively. BH4 treatment reduced the number of leukocytes and T cells in the LCA (Figure 4A to 4D). Within the T-cell population, we found that BH4 treatment reduced both CD4+ and CD8+ cells in the LCA (Figure 4E and 4F). In addition to reducing the total number of T cells in the LCA, BH4 also decreased the number of activated T cells, as indicated by a reduced number of CCR5+ and CD44High cells (Figure 4G and 4H). Immune cells in the RCA were virtually undetectable. Of note, BH4 treatment had no effect on levels of total leukocytes, T cells, CD4+ cells, CD8+ cells, or activated T cells in the peripheral blood or spleen (Supplemental Figure IV).

Vascular monocyte infiltration and differentiation into macrophages is one of the hallmarks of atherosclerosis. We therefore examined vascular levels of monocytes and macrophages, characterized by surface markers CD11b+ and F4/80+, respectively, at areas of disturbed flow. In the LCA of
vehicle-treated animals, both CD11b+ and F4/80+ cells were markedly increased compared with the unligated RCA. BH4 treatment reduced infiltration of these cells by approximately one half (Figure 5A to 5D). Taken together, these data suggest that vascular leukocyte and monocyte infiltration at sites of disturbed flow is in part due to NOS uncoupling and that this inflammatory response can be reduced by BH4 treatment.

**Role of NOS Uncoupling on Ex Vivo Cytokine Production of the Carotid Exposed to Disturbed Flow**

It is known that activated immune cells in the plaque produce cytokines (tumor necrosis factor-α [TNF-α], IFN-γ, etc.) that promote further inflammation, induce plaque progression, and stimulate matrix proteases and ROS production. To examine the role of NOS uncoupling on cytokine levels in the LCA, we measured carotid cytokine production by incubating isolated mouse carotids ex vivo and stimulating it with a combination of phorbol myristate acetate and ionomycin. The cytokines released into the medium were then measured. We found that BH4 treatment dramatically decreased levels of cytokines, including TNF-α, IFN-γ, and IL-2 in the LCA of ApoE−/− mice that were partially ligated for 1 week (Figure 6A to 6C). These data further demonstrate NOS uncoupling promotes the inflammatory cascade that contributes to atherosclerosis at areas of disturbed flow and that this is suppressed by BH4 treatment.

**Discussion**

Recently, our laboratory demonstrated that disturbed flow produced by partial carotid ligation reduces GTPCH-1 phosphorylation, decreases total bipterin production, and augments BH4 oxidation in vivo. In the present study, we showed that these perturbations of BH4 homeostasis lead to NOS uncoupling and contribute to the accelerated lesion development at regions of altered shear stress, including areas of disturbed flow induced by partial carotid ligation and naturally formed in the arterial tree. Our findings indicate that BH4 deficiency and NOS uncoupling, in addition to promoting lesion development, contribute to vascular superoxide production and endothelial dysfunction associated with ath-

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**Figure 5.** Role of NOS uncoupling on infiltration and activation of monocytes and macrophages induced by partial carotid ligation. ApoE−/− mice were fed a high-fat diet and underwent partial carotid ligation for 1 week. Animals also received BH4 or vehicle in their drinking water. Flow cytometry was performed to detect CD11b (A) and F4/80 (B) in the carotids. Absolute numbers of CD11b+ (C) and F4/80+ (D) cells in the carotids were shown (n=6 to 8). Values were compared using ANOVA, and selected comparisons were made using a Bonferroni post hoc test.

**Figure 6.** Role of NOS uncoupling on ex vivo cytokine production of the carotid induced by partial ligation. ApoE−/− mice that underwent partial carotid ligation were fed a high-fat diet and treated with BH4 or vehicle for 1 week. Isolated mouse carotids were incubated in Dulbecco’s modified Eagle’s medium containing 10 μmol/L phorbol myristate acetate and 2 μmol/L ionomycin for 16 hours. TNF-α (A), IFN-γ (B), and IL-2 (C) in the culture media were measured by cytokine bead array (n=7 to 8). Values were compared using ANOVA, and selected comparisons were made using a Bonferroni post hoc test.
erosclerosis at sites of disturbed flow. Finally, our data indicate that BH₄ deficiency and NOS uncoupling likely contribute to the vascular inflammation and abnormal cytokine milieu induced by disturbed flow without affecting systemic immune cell numbers.

Evidence from animal models and human studies suggests that atherosclerosis preferentially develops at areas where blood flow is known to be disturbed, including the lesser curvature of the aortic arch, the infrarenal aorta, the carotid bulb, and the proximal coronary arteries. Flow separations occur at these sites, reducing wall shear stress. In addition, flow reversal occurs at some of these sites, leading to oscillatory shear. Low shear stress and oscillatory shear stress promote atherosclerotic lesion development through a number of molecular and cellular mechanisms, including attenuation of NO-dependent atheroprotection, promotion of oxidative stress and inflammation, induction of vascular smooth muscle cell migration, and enhancement of low-density lipoprotein cholesterol uptake. Oscillatory shear stress has been shown to activate the NADPH oxidases and cause eNOS uncoupling. Our previous studies have shown that oscillatory shear can reduce BH₄ levels and cause eNOS uncoupling by at least 2 mechanisms. One is that oscillatory shear causes oxidation of BH₄; the other being that it reduces the synthesis of BH₄ by reducing GTPCH-1 phosphorylation and activation. Our current findings indicate that the reduction in NO and concomitant increase in superoxide caused by NOS uncoupling likely play a critical role in the accelerated lesion formation caused by disturbed flow in vivo.

In the present study, we used a model of rapid developing atherosclerosis caused by partial carotid ligation in ApoE⁻/⁻ mice fed a high-fat diet. This intervention leads to both low and oscillatory shear stress in the carotid proximal to the site of ligation. The resulting lesions share many characteristics of complex human atherosclerotic lesions, including lipid deposition, inflammation, and fibrosis. This model allows examination of interventions such as BH₄ administration over a short period, but it does not provide insight into any long-term benefits of BH₄ therapy. In keeping with our studies, Schmidt et al have shown that oral BH₄ reduces atherosclerosis in the aortic root over 12 weeks. Likewise, Hattori et al have shown a benefit of BH₄ treatment on plaque development in ApoE⁻/⁻ mice over 10 weeks of high-fat feeding. Of note, in the study of Hattori et al, BH₄ therapy seemed to have a striking effect on lesion development in the lesser curvature of aorta, where shear stress has been shown to be oscillatory.

BH₄ exhibits modest antioxidant effects, and therefore could have prevented atherosclerosis via its ability to scavenge ROS. We and others have previously shown that BH₄ reacts with strong oxidants, including peroxynitrite, thiol, and carbonate radicals. To exclude the possibility that the effect of BH₄ was mediated by oxidant scavenging, we performed additional experiments with NH₄, an antioxidant structurally similar to BH₄ but without cofactor activity for NOS. Our data demonstrate that NH₄ did not protect against disturbed flow induced atherosclerosis, suggesting that the effect of BH₄ was primarily mediated by its ability to sustain NOS catalysis.

We found that BH₄ treatment decreased superoxide production not only in the endothelium but also in the media and adventitia. This might have been due to its ability to sustain catalysis of NOS isoforms in all these cell layers. In addition to eNOS, neuronal NOS is expressed in rat and human vascular smooth muscle cells. Expression of inducible NOS in the vessel wall can also occur in the setting of oxidative stress and inflammation. Importantly, Wilcox et al showed that atherosclerosis is associated with expression of all forms of NOS in the intima and adventitia. It is also conceivable that ROS produced by uncoupled NOS could activate other sources of superoxide, such as the NADPH oxidase, in a feed-forward fashion and that these could contribute to ROS production throughout the vessel wall.

In keeping with improved NOS catalysis, endothelium-dependent vasodilatation of the ligated carotid arteries was partially enhanced by BH₄ treatment. It is of interest that in normal vessels, the dose response curve to acetylcholine seemed biphasic, rather than sigmoidal. This response is similar to that observed by others. Of interest, BH₄ treatment normalized the relaxation to higher doses of acetylcholine and thus the peak response to acetylcholine and had a partial beneficial effect on the responses to the lower doses, and it thus partially shifted EC₅₀ values. It is conceivable that this might reflect a differential effect of BH₄ on different mechanisms of carotid artery relaxation.

It is well known that inflammation plays a key role in the pathogenesis of atherosclerosis. Flow disturbances promote endothelial expression of leukocyte adhesion molecules and chemokines, such as vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. These could in turn induce monocyte rolling, adhesion, and transmigration into the subendothelial space, promoting early lesion development. Monocytes differentiate into macrophages and dendritic cells that present antigens and trigger T-cell activation within the lesion. These activated T cells produce Th1 cytokines, such as TNF-α and IFN-γ, which further lead to inflammation and lesion progression. Our data indicate that NOS uncoupling has a dramatic impact on these inflammatory events. We found that BH₄ treatment reduced vascular accumulation of T cells and macrophages. Of note, the T cells present in the lesions caused by disturbed flow seemed to have the phenotype of effector T cells, in that they express CCR5 and are CD44High. In addition to preventing the accumulation of these cells, BH₄ treatment also markedly reduced the ability of vascular segments to elaborate TNF-α, IFN-γ, and IL-2. Taken together, these data indicate that recoupling of NOS has dramatic effects in reducing vascular inflammation in the setting of rapid atherosclerosis development.

In summary, our findings demonstrate that NOS uncoupling is a key mechanism by which disturbed flow induces accelerated atherosclerosis. We found that oral BH₄ supplementation prevented NOS uncoupling and improved endothelial function in the carotid exposed to disturbed flow induced by partial carotid ligation. The reduction in oxidative stress elicited by BH₄ treatment was associated with diminished monocyte adhesion and T-cell activation, as well as blunted cytokine production from the vessel wall. Taken together,
these results indicate that BH₄ treatment, by correcting NOS uncoupling and rectifying the oxidative status at areas of disturbed flow, targets the underlying mechanism of atherosclerosis progression by inhibiting inflammation. These results highlight a pivotal role of BH₄ deficiency and NOS uncoupling in atherosclerosis progression, particularly under the patterns of low and oscillatory shear flow, and indicate that modulation of vascular BH₄ levels could be a therapeutic target for preventing atherosclerosis at branches and curvatures in the arterial tree.

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**Disclosures**

None.

**References**

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Supplemental Material

Tetrahydrobiopterin Deficiency and Nitric Oxide Synthase Uncoupling Contribute to
Atherosclerosis Induced by Disturbed Flow

Li Li\textsuperscript{1,2}, Wei Chen\textsuperscript{1}, Amir Rezvan\textsuperscript{1}, Hanjoong Jo\textsuperscript{1,3}, David G. Harrison\textsuperscript{1,2,4}
Supplemental Figure I: Pterin levels in the carotids that underwent partial ligation for 1 week. The left carotid arteries of ApoE⁻/⁻ mice were partially ligated and mice were treated with an approximate dose of 10 mg/kg per day BH₄ in the drinking water or vehicle for 1 week. Total biopterin (Panel A), BH₂ (Panel B), BH₄ (Panel C) levels and the ratio of BH₄/BH₂ (Panel D) in the carotids were shown (n=5). Values were compared using two-way ANOVA and selected comparisons were made using a Bonferroni post hoc test.
Supplemental Figure II: Effect of BH₄ supplementation on plasma lipid profile in ApoE⁻/⁻ mice. ApoE⁻/⁻ mice were treated with BH₄ or vehicle in the drinking water and fed a high fat diet for 3 weeks (n=4-5). Values were compared using ANOVA and selected comparisons were made using a Bonferroni post hoc test.
Supplemental Figure III: Effect of BH4 treatment on atherosclerosis in the brachiocephalic and intercostal arteries of ApoE<sup>−/−</sup> mice fed a high fat diet for 3 weeks. Animals also received BH4 or vehicle in the drinking water for 3 weeks. A. Representative images of Hematoxylin and Oil red O staining of the brachiocephalic artery frozen sections. B. Quantification of the intimal lesion areas using Image J (n=5-6). Values were compared using an unpaired t-test. C. Representative images of en face Oil Red O staining of the aorta. Intercostal arteries were indicated by the yellow arrows. D. Quantification of the intercostal lesion area presented as percentage of entire aorta (n=5). Values were compared using an unpaired t-test.
Supplemental Figure IV: Effect of BH₄ treatment on T cell numbers and activation in the peripheral blood and spleen after partial carotid ligation. ApoE⁻/⁻ mice were fed a high fat diet and underwent partial carotid ligation for 1 week. Animals also received BH₄ or vehicle in the drinking water. Percentages of CD45⁺ (Panel A), CD3⁺ (Panel B), CD4⁺ (Panel C), CD8⁺ (Panel D), CD4⁺CCR5⁺ (Panel E), and CD4⁺CD44High (Panel F) cells were shown (n=6).