Oral Administration of Tetrahydrobiopterin Slows the Progression of Atherosclerosis in Apolipoprotein E-Knockout Mice

Yoshiyuki Hattori, Sachiko Hattori, Xi Wang, Hiroko Satoh, Nobuo Nakanishi, Kikuo Kasai

Objective—Although it has been reported that oral administration of tetrahydrobiopterin (BH4) prevents endothelial dysfunction and vascular oxidative stress in various rat models, the effect of treatment with BH4 on atherogenesis remains unclear.

Methods and Results—In this study, we investigated whether oral BH4 treatment might slow the progression of atherosclerosis using hypercholesterolemic apolipoprotein E-knockout mice. We report that ingesting BH4 in drinking water is sufficient to inhibit atherogenesis in mice. Furthermore, we report that BH4 treatment improves endothelial dysfunction and attenuates increased mRNA expression of NADPH oxidase components, as well as a number of inflammatory factors, such as LOX-1 and MCP-1, in the aortas of apolipoprotein E-knockout mice.

Conclusion—Strategies such as oral administration of BH4 to ensure continuous BH4 availability may be effective in restoring nitric oxide-mediated endothelial function and limiting vascular disease and the progression of atherosclerosis.

Key Words: apolipoprotein E-knockout mouse ▪ atherosclerosis ▪ endothelial function ▪ tetrahydrobiopterin

Nitric oxide (NO) inhibits atherogenesis through a number of mechanisms, including anti-inflammatory, anti-thrombotic, antiproliferative, and antioxidant effects.1 Loss of NO bioavailability is a cardinal feature of endothelial dysfunction2 preceding the development of atherosclerosis, and is an independent risk factor for cardiovascular disease.3,4 A critical determinant of endothelial nitric oxide synthase (eNOS) activity is the availability of the nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH4). When BH4 levels are inadequate, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in superoxide, rather than NO, production, thereby inducing vascular oxidative stress and endothelial dysfunction.

In hypercholesterolemic apolipoprotein E-knockout (ApoE-KO) mice, endothelium-dependent vascular relaxation is impaired, NO synthesis is reduced, and vascular superoxide production is increased.5,6 Transgenic overexpression of eNOS in ApoE-KO mice surprisingly results in enhanced vascular superoxide production, reduced NO bioavailability, and accelerated atherosclerosis.7 BH4 levels are reduced in the aortas of these mice compared with wild-type (WT) controls, but dietary BH4 supplementation reduces superoxide production and increases NO synthesis. Furthermore, transgenic overexpression of GTP-cyclohydrolase I, the rate-limiting enzyme of de novo BH4 synthesis, in ApoE-KO mice attenuates endothelial dysfunction and atherosclerosis.8 In this model, endothelial cell BH4 levels are specifically increased without elevation of plasma BH4 levels. These results suggest that increased eNOS protein alone is insufficient to maintain NO synthesis in the setting of hypercholesterolemia, and that adequate BH4 levels are essential to prevent eNOS uncoupling where endothelial dysfunction exists. In contrast, van Haperen et al9 also crossedbred ApoE-KO mice with another line of eNOS transgenic mice that they created and reported that atherosclerotic lesion size was reduced by eNOS overexpression. Regarding the mechanisms, they cited the reductions of blood pressure and plasma cholesterol levels.

Several pharmacological 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,10 and in patients with type II diabetes,11 hypercholesterolemia,12,13 and coronary atherosclerosis.14 Shinozaki et al15 have shown that oral administration of BH4 prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. In addition, we have shown that supplementation with BH4 prevents the cardiovascular effects of angiotensin II-induced oxidative and nitrosative stress in rats.16 Thus, BH4 not only may improve NO-mediated endothelial function but also may reduce vascular oxidative and nitrosative stress, thereby...
potentially reducing the development of atherosclerosis. Because the long-term effects of BH4 supplementation on the development of vascular disease remain uncertain, we sought to investigate the effects of oral administration of BH4 on the development of atherosclerosis in ApoE-KO mice.

Methods

Animals
ApoE-KO mice (n=40) were weaned at 5 weeks of age onto a high-cholesterol diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, 0.5% sodium cholate; Oriental Yeast Co., Tokyo, Japan) and maintained on the diet for 10 weeks. Half of the ApoE-KO mice (ApoE-KO/BH4 mice, n=20) were maintained on the high-cholesterol diet supplemented with 10 mg/kg per day BH4 in drinking water as described previously.16 WT (C57B/6j) mice were used to provide a control group in some experiments (n=12). Animals were provided their diet and water ad libitum and maintained on a 12-hour light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Dokkyo University School of Medicine.

Assessment of Atherosclerosis
The extent of atherosclerosis was determined macroscopically on opening the aortas and by examination of serial sections from the aortic roots. Each aorta was cut open longitudinally and pinned flat onto a silicone-coated dissecting dish. Each aorta was fixed with 10% neutral buffered formalin for 24 hours. After fixation, each aorta was washed with phosphate-buffered saline for 1 hour and stained with oil red O solution (0.3% in isopropyl alcohol diluted with water, 3:2 vol/vol) for 15 minutes. Excess stain was washed off with 60% isopropyl alcohol. Images were captured with an Olympus digital camera mounted on an Olympus BX417 microscope and an Olympus digital camera. The lipid-stained areas were analyzed with Adobe Photoshop 7 software and MacSCOPE software. The lipid-stained area of the aortic root was reported as the mean area of 5 sections from each mouse. Those image analyses were performed with help from Fumie Yokotsuka.

Immunohistochemical Analysis of eNOS, iNOS, and gp91phox Expression
Paraffin sections of 2 μm were prepared for analysis using the avidin-biotin-horseradish peroxidase complex method. This experiment was performed by Noriko Suzuki.

Organ Chamber Experiments
Organ chamber experiments were performed as previously described.16 The experimental procedure was instructed by Dr. Kohsuke Uchida. The animals were anesthetized with pentobarbital and euthanized by exsanguinations. The thoracic aortas were carefully dissected and any perivascular tissue removed under microscopy in physiological salt solution. Each aorta was homogenized with 60% isopropyl alcohol diluted with water, 3:2 vol/vol) for 50 minutes. Excess stain was washed off with 60% isopropyl alcohol. Images were captured with an Olympus BX417 microscope and an Olympus digital camera. The lipid-stained areas were analyzed with Adobe Photoshop 7 software and MacSCOPE software. The lipid-stained area of the aortic root is reported as the mean area of 5 sections from each mouse. Those image analyses were performed with help from Fumie Yokotsuka.

Measurement of Vascular Superoxide Anion Production
Superoxide anion production was measured using lucigenin chemiluminescence, as previously described.16 Briefly, each thoracic aorta was carefully dissected and the perivascular tissue and blood contaminants removed under microscopy in physiological salt solution. Before placement in HEPES-buffered physiological salt solution, scintillation vials containing HEPES-buffered physiological salt solution with 5 μmol/L lucigenin (bis-N-methylacridinium nitrate) were placed into a scintillation counter (Luminescence Reader BLR 301; ALOKA). Scintillation counts were recorded every minute for 10 minutes, and the respective background counts subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts per minute per milligram of dry weight. Measurements were performed in the presence of L-NAME (10 μmol/L), which inhibits NOS activity. Measurements were also performed in the presence of apocynin (100 μmol/L), a NADPH oxidase inhibitor, which inhibits the assembly of NADPH oxidase.12

Plasma Lipid Analysis
After mice were deprived of food for at least 12 hours, blood was collected by cardiac puncture into heparin-coated tubes. Plasma was obtained through centrifugation of blood for 15 minutes at 5500 g and 4°C, then stored at −80°C until each assay was performed. Total cholesterol and triglyceride levels were determined using an automated clinical chemistry analyzer.

Assay of Biopterin
Biopterin (BH4 and more oxidized species) was measured as described by Fukushima and Nixon.19 Each aorta was homogenized with 0.2 mol/L perchloric acid and oxidized by exposure to 0.2 mol/L perchloric acid containing 0.2% I2 and 0.4% KI at room temperature for 1 hour under dark conditions. Ascorbate (2%) was added to remove residual free I2, and the mixture was then centrifuged for 10 minutes at 10,000 g. Biopterin in the supernatant was quantified by reversed-phase high-performance liquid chromatography on a C18 column with fluorescence detection.

Real-Time Polymerase Chain Reaction
For quantitative measurement of mRNA, 2 μg of total RNA were treated with DNase I for 15 minutes and subsequently used for cDNA synthesis. Polymerase chain reaction reactions with cDNA were conducted in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95°C for 5 minutes, and 40 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec. eNOS, iNOS, gp91phox, p47, p67, Cz-SOD, ICAM, vascular cell adhesion molecule, L-0X-1, and MCP-1 mRNA levels were expressed as a ratio of the glyceraldehyde-3-phosphate dehydrogenase mRNA level. The sequences of the primers used for real-time polymerase chain reaction were as follows: eNOS, 5'-GATGCGAGAGATGGCACTCC-3' and 5'-CTTGCAAGTCTGCAGTCTCAT-3'; iNOS, 5'-GCCACACCAGCT-CTTCTG-3' and 5'-GGTGGTTGGAGGACA-3'; glyceraldehyde-3-phosphate dehydrogenase, 5'-GCCACCCAACGCTTTCGTG-3' and 5'-GGTGGTTGGAGGACA-3'; however, the remaining primers were based on the report by Furukawa et al.20

Statistical Analysis
Data were expressed as mean±SD. An unpaired Student t test was used to detect significant differences whenever 2 groups were compared. One-way ANOVA was used to compare differences among 3 or 4 groups, with Bonferroni test for post hoc analysis. P<0.05 was considered statistically significant.
Results

Plasma Lipid Levels and Hemodynamics
Plasma cholesterol levels were markedly elevated by ingestion of a high-cholesterol diet by ApoE-KO and ApoE-KO/BH4 mice and significant differences between the 2 groups were not observed (Table). In contrast, triglyceride levels were not affected by the high cholesterol diet in either group (Table).

Aortic Biopterin Levels
We first determined whether BH4 treatment might lead to increased BH4 levels by measuring biopterin levels in homogenates of snap-frozen aorta. In ApoE-KO mice, total biopterin contents in aortas were 50% decreased compared with WT mice. Total biopterin levels were significantly greater in the ApoE-KO/BH4 mice compared with the ApoE-KO mice as a result of oral ingestion of BH4. In ApoE-KO/BH4 mice, the biopterin level was 142% that observed in ApoE-KO mice (Figure 1).

Effect of BH4 on Endothelial Function
Endothelium-dependent relaxation caused by acetylcholine, a receptor-mediated eNOS agonist, was significantly impaired in ApoE-KO mice, compared with ApoE-KO/BH4 mice (Figure 2A). In fact, endothelium-dependent relaxation was not significantly impaired in ApoE-KO/BH4 mice, compared with control WT or WT/BH4 mice fed a high-fat diet (Figure 2A). Endothelium-independent relaxation in response to the NO donor sodium nitroprusside was identical in 4 groups, demonstrating no differences in vascular smooth muscle responses to NO (Figure 2B). These observations indicate that increased endothelial BH4 levels preserve NO-mediated endothelial function in the setting of atherosclerosis.

Atherosclerotic Lesion Formation
The development of atherosclerosis was assessed by measuring lipid-stained areas within the aortas and aortic root sections. After ingestion of a Western-type diet for 10 weeks, atherosclerotic lesions were evident in the aortas of ApoE-KO mice and ApoE-KO/BH4 mice, whereas control C57Bl/6J mice were almost free of detectable lesions. Representative photographs of oil red O aortic staining are shown in Figure 3A. Lipid-stained areas were primarily observed at the aortic curvature and branching points of other vessels. Significantly fewer lipid-stained areas were observed in the aortic sinuses of ApoE-KO/BH4 mice, compared with ApoE-KO mice (41.2 ± 1.4 versus 52.3 ± 2.6%; Figure 3A). Five consecutive sections surrounding the root of the aortic valve were taken from each mouse and stained with oil red O to assess lesion formation in that region. Representative sections are shown in Figure 3B. After 10 weeks of a high-cholesterol diet, a significant reduction in plaque area was observed within aortic root sections from ApoE-KO/BH4 mice, compared with ApoE-KO mice. The mean aortic root plaque area was reduced by 25% in ApoE-KO/BH4 mice, compared with ApoE-KO mice (Figure 3B).

Thus, ApoE-KO/BH4 mice demonstrated reduced lesion size and thickness, compared with ApoE-KO mice. Thus, BH4 administration slows the progression of atherosclerosis in the setting of hypercholesterolemia.

Superoxide Production in the Aorta
A small amount of superoxide production was noted in the aortas of WT mice. Markedly increased aortic superoxide production was observed in ApoE-KO mice (Figure 4). This increased superoxide production was ~50% inhibited by L-NAME, suggesting that half of superoxide from the aorta of ApoE-KO mice be derived from uncoupled eNOS. Importantly, BH4 treatment significantly suppressed superoxide production within the aorta (Figure 4). Endothelial denudation normalized superoxide production in

Plasma Lipids in ApoE-KO Mice and ApoE-KO/BH4 Mice

<table>
<thead>
<tr>
<th></th>
<th>WT(C57BL/6)</th>
<th>ApoE-KO</th>
<th>ApoE-KO/BH4</th>
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<tbody>
<tr>
<td>N</td>
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<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
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<td>2512±278*</td>
<td>2537±128*</td>
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<tr>
<td>Triglyceride, mg/dL</td>
<td>60±12.5</td>
<td>8.3±2.08</td>
<td>22±10.5</td>
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Data shown are means±SD.  *P<0.01 vs. WT mice.

Figure 1. Total biopterin in the aortas of WT, ApoE-KO, and ApoE-KO/BH4 mice. *P<0.05; n=5, ApoE-KO vs. ApoE-KO/BH4 mice.

Figure 2. A, ApoE-KO aortic rings exhibited impaired endothelium-dependent relaxation in response to acetylcholine compared with ApoE-KO/BH4 mice. B, Endothelium-independent vessel relaxation in response to the NO donor sodium nitroprusside was identical in both groups of mice. WT mice: black squares, WT/BH4 mice: white squares, ApoE-KO mice: black circles, ApoE-KO/BH4 mice: white circles. n=5 to 6 animals per group; *P=0.03 vs. ApoE-KO/BH4 mice.

Figure 3. Representative photographs of oil red O aortic staining are shown in A. Lipid-stained areas were primarily observed at the aortic curvature and branching points of other vessels. Significantly fewer lipid-stained areas were observed in the aortic sinuses of ApoE-KO/BH4 mice, compared with ApoE-KO mice (41.2±1.4 versus 52.3±2.6%; Figure 3A). Five consecutive sections surrounding the root of the aortic valve were taken from each mouse and stained with oil red O to assess lesion formation in that region. Representative sections are shown in B. After 10 weeks of a high-cholesterol diet, a significant reduction in plaque area was observed within aortic root sections from ApoE-KO/BH4 mice, compared with ApoE-KO mice. The mean aortic root plaque area was reduced by 25% in ApoE-KO/BH4 mice, compared with ApoE-KO mice (Figure 3B).

Figure 4. Total superoxide production in the aortas of WT, ApoE-KO, and ApoE-KO/BH4 mice. *P<0.05; n=5, ApoE-KO vs. ApoE-KO/BH4 mice.
Figure 3. A, Atherosclerotic lesions in aortas stained with oil red O solution, longitudinally opened aortas from control, ApoE-KO, and ApoE-KO/BH4 mice. Lipid stained area in the aortic sinus of ApoE-KO and ApoE-KO/BH4 mice was 52.3±2.6 vs. 41.2±1.4% (n=5; *P<0.05). B, Aortic root plaque area in ApoE-KO (white squares) and ApoE-KO/BH4 mice (black squares), with representative aortic root sections (5× objective) from each group, stained stained with oil red O solution. Mean plaque area (indicated by horizontal black lines) was 25% lower in ApoE-KO/BH4 mice (0.66 mm²) compared with ApoE-KO mice (0.88 mm²) (n=5 animals per group; *P<0.05). Black bars on photomicrographs represent 200 μm.

ApoE-KO and ApoE-KO/BH4 mice with a remaining production of superoxide ions in vascular smooth muscle cells.

mRNA Levels of Inflammatory Mediators in Aortic Tissue

The eNOS mRNA levels were greater in ApoE-KO mice, compared with WT or ApoE-KO/BH4 mice. The iNOS mRNA levels were barely detectable in WT mice, whereas they were clearly increased in ApoE-KO mice, and decreased by treatment with BH4 (ApoE-KO/BH4) (supplemental Figure I, available online at http://atvb.ahajournals.org). The mRNA levels of various components of the NADPH oxidase system, including gp91, p47, and p67, were markedly increased in ApoE-KO mice, compared with WT mice, which was significantly attenuated by treatment with BH4 (ApoE-KO/BH4). mRNA levels of Cu-SOD were increased in ApoE-KO and ApoE-KO/BH4 mice; however, this was not a significant finding. mRNA levels of ICAM and vascular cell adhesion molecule were clearly increased in ApoE-KO mice, compared with WT mice, and decreased by treatment with BH4 (ApoE-KO/BH4). LOX-1 and MCP-1 mRNA levels were increased in ApoE-KO mice, compared with WT mice, and significantly reduced by treatment with BH4 (ApoE-KO/BH4) (supplemental Figure I).

Immunohistochemistry

Supplemental Figure II shows representative sections of aortic tissue immunostained for eNOS, iNOS, and gp91. eNOS expression was similar among control C57/6J mice and ApoE-KO/BH4 mice, but slightly increased in ApoE-KO mice. iNOS immunoreactivity was significantly greater in ApoE-KO mice, compared with ApoE-KO/BH4 mice. Similarly, a component of NADPH oxidase gp91 staining was clearly observed in ApoE-KO mice, but significantly reduced in ApoE-KO/BH4 mice.

Discussion

In this study, we investigated whether oral treatment with BH4 might reduce the progression of atherosclerosis in hypercholesterolemic ApoE-KO mice. Although oral administration of BH4 has been reported to prevent endothelial dysfunction and vascular oxidative stress in various rat models,15,16 it remains unclear whether oral BH4 treatment might actually inhibit atherogenesis. We report that ingesting BH4 in drinking water slows the progression of atherosclerosis in mice. Furthermore, we report that BH4 treatment improves endothelial dysfunction and attenuates increased mRNA expression of various components of the NADPH oxidase system, as well inflammatory mediators, such as LOX-1 and MCP-1, in the aortas of ApoE-KO mice. These results suggest that BH4 is a rational therapy to ameliorate endothelial dysfunction and prevent atherosclerosis in the setting of factors such as hypercholesterolemia.

Strategies to consistently improve BH4 availability may be effective in restoring NO-mediated endothelial function and limiting the progression of vascular disease in the setting of atherosclerosis.6,7 diabetes,21 and hypertension.22 Paradoxically, inducing overexpression of eNOS as a strategy to restore or increase vascular NO bioactivity in atherosclerosis neither restores NO-mediated endothelial function nor reduces atherosclerosis.7 Rather, eNOS overexpression has, as Ozaki et al7 reported, detrimental effects in the setting of atherosclerosis because of generation of superoxide ions by eNOS uncoupling, which is corrected by BH4 supplementation. Furthermore, the importance of BH4 availability in slowing the progression of atherosclerosis has also been
demonstrated in a transgenic mouse model with endothelial overexpression of GTP-cyclohydrolase I, a rate-limiting enzyme in BH4 synthesis, in which endothelial cell BH4 levels were specifically increased 3-fold to 4-fold without an elevation of plasma BH4 levels. 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. 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. The discrepancy between this study and Ozaki’s study could 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 greater compared with that Ozaki et al achieved by feeding a “high-cholesterol” diet. Therefore, it is speculated that oxidative stress in the hypercholesterolemic mice of van Haperen et al was not increased as much as that in Ozaki’s model, although they did not describe oxidative stress and eNOS function in their model. In the present study, we used the same diet as used in Ozaki’s model, and hypercholesterolemia as severe as used in the Ozaki’s was achieved as shown in the Table.

We could confirm the beneficial effects of BH4 on endothelial dysfunction and atherosclerosis in ApoE-KO mice in this study. We observed oral administration of BH4 to have comparable effects to those observed in mice engineered to overexpress GTP-cyclohydrolase I. Significantly increased (by 25%) aortic bioprotein levels were observed after oral BH4 ingestion with significantly elevated plasma bioprotein levels (by 14%). The dose examined (10 mg/kg per day) should be safe in humans because long-term oral treatment of 5 to 20 mg/kg per day BH4 in humans with BH4-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene has shown no adverse effects and is easy to achieve. Oral BH4 treatment may have nonspecific antioxidant effects. However, these appear beneficial, and a number of antioxidant effects, including reduction of NADPH oxidase expression, may be partly responsible for attenuation of endothelial dysfunction and inflammatory gene expression. We previously reported that oral BH4 administration restores decreased adiponectin levels in angiotensin II-treated rats by exerting antioxidant effects on adipocytes. Adiponectin is an adipocyte-derived cytokine that has anti-diabetic and anti-atherogenic effects. Thus, BH4 has beneficial effects on NO-mediated functions, as well as functions not mediated by NO, within the vasculature and elsewhere in the body. In this study, mice ingested BH4 in drinking water, in which BH4 is remarkably stable. We found that 85% of BH4 remained intact in drinking water shaded from light after 24 hours, whereas 100% remained intact in the presence of ascorbate (0.04%). Because we changed the drinking water every day, we estimate ingestion of ≈85% of the total amount of BH4 added to drinking water and therefore adjusted the BH4 concentration to ensure ingestion of 10 mg/kg per day in accordance with the total water intake of the mice.

Vascular NADPH oxidase has an important role in vascular dysfunction. A consistent association between endothelial dysfunction in hypercholesterolemic rabbits and in vascular tissue from patients with diabetes mellitus and increased NADPH oxidase-mediated vascular O$_2^-_*$ production has been observed. The present study shows increased vascular O$_2^-_*$ production in aortas from ApoE-KO mice, which is markedly attenuated by apocynin, indicating a role of NADPH oxidase as a source of O$_2^-_*$ within the aortic vasculature. Indeed, increased mRNA levels of gp91, p67, and p47 were observed in the aortas of ApoE-KO mice. The increased O$_2^-_*$ production and mRNA expression of gp91, p67, and p47 were significantly attenuated by treatment with BH4. However, because incubation of aortic sections with 10 μmol/L L-NAME to inhibit eNOS reduced endothelial superoxide production in ApoE-KO aortas, eNOS uncoupling also likely contributes to superoxide production in these mice. Thus, eNOS-dependent superoxide production mediated by BH4 insufficiency further reduces BH4 availability. Although the precise mechanism of inhibition of NADPH oxidase expression by BH4 remains elusive, anti-oxidative effect of BH4 could be involved in this suppression of NADPH oxidase. This effect might also cause the reduction of MCP-1 expression by inhibiting oxidative stress-induced NF-κB activation.

In conclusion, the present study demonstrates that oral administration of BH4 reduces atherosclerosis in experimental ApoE-KO mice. Oral administration of BH4 appears to be safe in humans, thus further research is required to establish the potential of oral BH4 treatment for prevention of endothelial dysfunction and protection against the development of cardiovascular disease and atherosclerosis in humans.

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Disclosures

None.

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