Objective—Tetrahydrobiopterin (BH₄) is of fundamental importance for the normal function of endothelial NO synthase. The purpose of this study was to investigate the effects of hyperlipidemia on vascular BH₄ levels and the effect of supplementation with sepiapterin in the presence and absence of N-acetylcysteine (NAC).

Methods and Results—New Zealand White rabbits were fed normal chow (normocholesterolemic [NC] group) or hyperlipidemic chow (hyperlipidemic [HL] group) for 8 to 10 weeks. Mean cholesterol levels were 1465±333 and 53±17 mg/dL in the HL and NC group, respectively. Markedly diminished BH₄ levels were found in the HL group compared with the NC group, but these levels could be restored after 6 hours of incubation with sepiapterin. Peak relaxations to acetylcholine and A23187 were impaired in the HL group. Supplementation with sepiapterin resulted in a further diminution of relaxation in the HL but not NC group. Incubation with NAC for 6 hours failed to raise BH₄ levels, whereas NAC in conjunction with sepiapterin raised BH₄ levels ∼221-fold. However, this increase did not improve relaxations to A23187 and acetylcholine.

Conclusions—Prolonged exposure to sepiapterin impairs vasorelaxation in hyperlipidemia despite repletion of endogenous BH₄. Antioxidant thiols do not correct this impairment. These studies have implications for the use of sepiapterin in the correction of vasomotor tone in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:1655-1661.)

Key Words: sepiapterin ■ N-acetylcysteine ■ endothelium ■ hypercholesterolemia ■ nitric oxide ■ tetrahydrobiopterin

Depletion of vascular NO (NO) has been shown to play a fundamental role in the pathogenesis of atherosclerosis. A large body of evidence has corroborated heightened levels of superoxide (O₂⁻) in the inactivation of NO as a physiologically relevant mechanism in vivo.¹ The sources of O₂⁻ in the vasculature are numerous and include NAD(P)H-dependent oxidases, xanthine oxidase, and the mitochondrial respiratory chain. Recently, it has been demonstrated that under limiting concentrations of tetrahydrobiopterin (BH₄), endothelial NO synthase (eNOS) generates O₂⁻.²⁻⁴ In support of a critical role for BH₄ in mediating O₂⁻ formation from eNOS are the observations that reduction of BH₄ levels by inhibition of GTP cyclohydrolase I, the rate-limiting enzyme for BH₄ synthesis in cells and intact vessel segments, results in reduced NO generation, increased generation of O₂⁻ and hydrogen peroxide, and impairment of vascular relaxation.⁵⁻⁷ In addition, supplementation of cellular BH₄ increases the ability of NO synthase to generate NO.⁸⁻¹⁰ These findings have led to the hypothesis that correction of BH₄ levels by supplementation with BH₄ analogues, such as sepiapterin, may represent a therapeutic strategy to ameliorate vascular function. Sepiapterin is an oxidized BH₄ analogue that generates BH₄ on 2 sequential enzymatic reductions by sepiapterin reductase and dihydrofolate reductase (Figure 1). This compound has been extensively used to augment BH₄ in conditions associated with altered BH₄ metabolism, such as diabetes, atherosclerosis, ischemia/reperfusion, smoking, and hypertension. Studies in experimental animal models and humans in these conditions have demonstrated favorable effects on endothelial function with short-term (=60-minute) exposure to BH₄.¹¹⁻¹⁶ It has also been hypothesized that a considerable proportion of BH₄ undergoes oxidation in conditions associated with heightened oxidative stress, contributing to further BH₄ depletion.¹⁷ Consistent with this, it has been demonstrated in cultured endothelial cells that vitamin C, an antioxidant, stimulates NO synthase secondary to increases in BH₄ levels through its chemical stabilization.¹⁸⁻²⁰ The implication of these findings on the in vivo stability of BH₄ in the vessel wall and its effects on endothelial function are currently unknown. Accordingly, the purpose of the present study was to investigate the effects of experimental atherosclerosis on vascular BH₄ levels and the consequences...
Dihydrobiopterin (7,8-BH₂) is the stable intermediate formed by sepiapterin reductase and/or oxidation of BH₄.

Figure 1. Scheme I: BH₄ production from sepiapterin. 7,8-Dihydrobiopterin is the stable intermediate formed by sepiapterin reductase and/or oxidation of BH₄.

Methods

Animal Model
Male New Zealand White rabbits (n=18) were used in the present study. A total of 9 rabbits were fed a standard diet of rabbit chow (normcholesterolemic [NC] rabbits), and the remaining rabbits were fed an atherogenic diet consisting of standard rabbit chow supplemented with 1.0% cholesterol (Purina Chow) for 8 to 10 weeks (hypercholesterolemic [HL] rabbits). At the end of this period, blood samples for lipid profiles were determined for all rabbits. Rabbits were then euthanized with an intravenous injection of sodium pentobarbital, and tissues were harvested for investigation.

Organoid Cultures of Rabbit Aorta
After dissection of adventitial tissue, 2 aortic segments (3 mm) from each animal (NC rabbits, n=5; HL rabbits, n=5) were incubated in a 6-well plate that contained DMEM (GIBCO-BRL), antibiotics (100 U/mL penicillin and 100 mg/L streptomycin), and 0.1% calf serum. Sepiapterin was added to 1 of the segments at a final concentration of 1.03 mmol/L and was incubated for another 6 hours in a humified incubator under an atmosphere of 5% CO₂/95% air at 37°C.

Organ Chamber Studies
Aortas harvested from rabbits were placed in chilled modified Krebs-HEPES buffer (composition in mmol/L: NaCl 99.01, KCl 4.69, CaCl₂ 1.87, MgSO₄ 1.20, K₂HPO₄ 1.03, NaHCO₃ 25.0, HEPES 20.0, and glucose 11.1, pH 7.4). Eight 3- to 5-mm ring segments of the thoracic aorta were suspended in individual organ chambers filled with Krebs’ buffer (25 mL) of the following composition (mmol/L): NaCl 118.3, KCl 4.69, CaCl₂ 1.87, MgSO₄ 1.20, K₂HPO₄ 1.03, NaHCO₃ 25.0, and glucose 11.1, pH 7.4. The solution was aerated continuously with a 95% O₂/5% CO₂ mixture and maintained at 37°C. Care was taken not to injure the endothelium during preparation of the rings. Tension was recorded with a linear force transducer. Over a period of 1 hour, the resting tension was gradually increased, and the ring segment was frequently exposed to 80 mmol/L KCl, until the optimal tension for generating force during isometric contraction was reached. In preliminary experiments, this proved to be 3.0 g in all subsets of animals. The vessels were left at this resting tension throughout the remainder of the study. Experiments were performed in the presence of indomethacin (10 μmol/L) to prevent prostaglandin synthesis. The vessels were then preconstricted with gradual doses of L-phenylephrine (0.15 μmol/L). After a stable contraction plateau that approximated 40% to 50% of peak tension generated with the maximal dose of KCl was reached, the rings were exposed to the endothelium-dependent agonist acetylcholine (ACh, 1 mmol/L to 1 μmol/L), the endothelium-independent vasorelaxant nitroglycerin (1 mmol/L to 10 μmol/L), and the calcium ionophore A23187 (1 mmol/L to 1 μmol/L). The vessels were then washed thoroughly and allowed to equilibrate for another hour before being subjected to vasoconstrictors. Vessels were allowed to equilibrate for at least 2 hours at a resting tension of 3 g before being subjected to graded doses of phenylephrine (1 mmol/L to 0.1 mmol/L). Responses were then expressed as a percentage of the peak response to 80 mmol/L KCl.

High-Performance Liquid Chromatographic Measurements of BH₄ in Aortic Segments
Measurement of BH₄ by high-performance liquid chromatography (Hewlett Packard Series 1100, Agilent Technologies) with fluorescence detection is indirect and is based on the quantification of biopterin, a highly fluorescent BH₄ analogue. Oxidation of BH₄ to biopterin under acidic conditions is quantitative. Under basic conditions, however, BH₄ is further oxidized to nonfluorescent compounds. Thus, BH₄ concentrations are calculated from the difference of biopterin measured in these conditions. Frozen aortic segments from normal and hypercholesterolemic rabbits isolated as described above were cryopulverized and divided into 2 fractions of known weight. One fraction was suspended in HCl (0.25 mL, 0.1N), and the other was suspended in NaOH (0.3 mL, 0.1N). A solution of 4% I₂/8% KI (0.25 mL) was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice on an ice bath for 1 minute by use of 25% sonicator full-power power to break open the cells. After a 90-minute incubation at room temperature, 50 μL of a 50% ascorbate solution was added to remove excess iodine solution and then centrifuged at 14,000 rpm for 10 minutes to remove tissue debris. After adjustment of pH to 4.0 with HCl, supernatants were injected onto a Kromasil C-18 column (5 μm, Alltech) equilibrated with phosphate buffer (0.15 mmol/L, pH 6.4), and biopterin was analyzed by authentic standards.

Electron Spin Resonance Measurements
Electron spin resonance spectra were recorded at room temperature on a Varian E-109 spectrometer operating at 9.5 GHz and with a 100-kHz field modulation equipped with a loop gap resonator. This device allows electron paramagnetic resonance (EPR) measurements of small sample volumes, typically <20 μL. Reactions were initiated by the addition of eNOS to the incubation mixtures containing NADPH (0.1 mmol/L), calcium (0.2 mmol/L), calmodulin (20 μg/mL), BH₄ (1 μmol/L), l-arginine (40 μmol/L), and 5-ethoxycarbonyl-5-methyl-pyrrrole N-oxide (EMPO, 50 mmol/L), DTPA (0.1 mmol/L), and HEPES buffer (50 mmol/L, pH 7.4). The EPR spectra were recorded at room temperature as previously described.

Statistical Analysis
All data are expressed as mean±SE. Comparisons across groups were made by 1-way ANOVA. For differences between paired observations, a t test was used when appropriate. When significance was detected, a post hoc Newman-Keuls multiple comparison test was performed. All statistical analyses were performed with the use of GraphPad software (version 3.02).

Results

Rabbit Plasma Cholesterol Levels
At the end of the 8- to 10-week period of 1% cholesterol administration, the average total plasma cholesterol level was
1465±333 mg/dL. The total cholesterol level in the control group was 52±17 mg/dL.

BH₄ Levels in Aortas From HL Rabbits

Figure 2 depicts BH₄ levels in aortic segments cultured in DMEM culture media for 6 hours in the presence and absence of sepiapterin. Aortic segments from control NC rabbits contained 743±264 ng BH₄ per gram tissue, whereas aortic segments from HL rabbits contained 28±14 ng BH₄ per gram tissue (P<0.001 versus NC by ANOVA), a 27-fold reduction in levels. Oxidized pteridine (BH₂) concentration (298.1±105.8 ng per gram tissue) was found in NC tissue, which represents ~30% of the total oxidized and reduced BH₄ content. In HL tissue, the BH₂ concentration was 52.5±23.6 ng per gram tissue, representing ~63% of the total. In the analysis of HL tissue, the presence of other fluorescent products was evident. However, their concentration could not be determined and was not included in our calculations because their identity remains to be established. On 6 hours of incubation with sepiapterin, the BH₄ content in HL aortas increased 21-fold (to 589±97 ng BH₄ per gram tissue, P=NS for HL group with sepiapterin [HL-Sep] versus the NC group and the NC group with sepiapterin [NC-Sep]), whereas incubation of the aortic segments from NC animals demonstrated no significant change in BH₄ content (570±91 ng BH₄ per gram tissue) compared with untreated NC segments. We also performed experiments in freshly isolated segments of aorta derived from animals fed control chow (NC group) and from animals fed hyperlipidemic chow (HL group) for a duration of 8 to 10 weeks. BH₄ levels were similarly diminished in the HL group (0.69±0.92 ng per gram tissue, n=4), with a 17-fold reduction compared with control aortas (11.73±4.8 ng per gram tissue, n=4). Endothelial denudation reduced the levels in control rabbits to those seen with hyperlipidemia (1.66 ng/mg tissue, n=1).

Effect of Sepiapterin on Responses to ACh and A23187

Figure 3 demonstrates responses to the endothelium-dependent agonists ACh and A23187 in NC rabbits after constriction with

Figure 2. BH₄ levels in NC and HL rabbit aortas. Tissues were incubated in the presence or absence of sepiapterin in DMEM culture media containing antibiotics and 0.1% calf serum. Sepiapterin was added to 1 of the segments at a final concentration of 0.1 mmol/L and was incubated for 6 hours under an atmosphere of 5% CO₂/95% air at 37°C. **P<0.001 vs NC; P=NS for HL-Sep vs NC.

Figure 3. Vascular relaxation in NC and HL aortas with and without sepiapterin supplementation. Tissues were incubated in the presence or absence of sepiapterin in DMEM culture media containing antibiotics and 0.1% calf serum. Sepiapterin was added to 1 of the segments at a final concentration of 0.1 mmol/L and was incubated for 6 hours under an atmosphere of 5% CO₂/95% air at 37°C. Segments were preconstricted with L-phenylephrine (PE), and relaxation to cumulative doses of ACh (10⁻¹⁰ to 10⁻⁵) and A23187 (10⁻¹⁰ to 10⁻⁵) was examined in NC aortas (A and B) and HL aortas (C and D). *P<0.05 for HL vs HL-Sep.
l-phenylephrine. Incubation with sepiapterin for 6 hours failed to improve peak relaxations and ED$_{50}$ to both agonists (see Table). In contrast to the lack of an effect of BH$_4$ in NC aortas, incubation of HL aortic segments with sepiapterin resulted in a pronounced impairment in responses to both agonists, as shown in Figure 3 and the Table.

### Effects of Sepiapterin on Smooth Muscle Function

Nitroglycerin induced dose-dependent relaxations in preconstricted NC and HL ring segments, which did not differ with supplementation to BH$_4$ (see Figure 4 and Table). Ring segments from HL aortas demonstrated a trend toward heightened peak constriction to the vasoconstrictor phenylephrine (156±2% versus 148±10% for HL and NC groups, respectively; P<0.05 by ANOVA; see Table). Responses to phenylephrine were unchanged in NC and HL aortas subjected to sepiapterin (NC-Sep and HL-Sep groups, respectively; Table).

### Effect of NAC on Endothelial Function and BH$_4$ Levels

In a separate set of experiments, we examined the effects of the thiol antioxidant compound NAC (1 mmol/L) in preserv-
ing BH₄ levels. NAC by itself did not augment BH₄ levels (7±3 ng BH₄ per gram tissue) in HL aortic segments. However, in the presence of sepiapterin, there was a 221-fold increase in BH₄ (1570±683 versus 7±3 ng BH₄ per gram tissue, P<0.0001 by paired t test). Figure 5 depicts peak responses to ACh and A23187. NAC did not improve responses to ACh or A23187. Interestingly, although NAC, when used in conjunction with sepiapterin, markedly increased BH₄ levels, this failed to translate into improvements in responses to ACh and A23187 (see Figure 5).

**Effect of Sepiapterin on NO/O₂⁻ Production From eNOS**

The effects of sepiapterin on NO and O₂⁻ formation from eNOS were examined by measuring l-citrulline formation and by EPR spin trapping with EMPO, respectively. Incubations of eNOS with BH₄ (1 μmol/L) supported l-citrulline formation at a rate of 148.3±1.2 mmol/min per milligram protein. Inclusion of sepiapterin (500 μmol/L) to the eNOS incubation mixture diminished the rate of NO formation to 38.7±0.4 mmol/min per milligram protein. EPR experiments showed that inhibition of NO formation was paralleled by an increase in O₂⁻ formation. Sepiapterin augmented O₂⁻ release from eNOS in a concentration-dependent fashion. Together, these results demonstrate that sepiapterin at higher doses may uncouple NADPH from l-arginine oxidation, enhancing O₂⁻ formation from eNOS.

**Discussion**

The key findings of the present study are as follows: (1) Hypercholesterolemia diminishes vascular BH₄. (2) Supplementation with sepiapterin, an oxidized BH₄ analogue, for 6 hours paradoxically worsens responses to endothelium-dependent agonists ACh and A23187. (3) Incubation with NAC, a thiol antioxidant, does not restore depleted BH₄ levels in hyperlipidemia. (4) Sepiapterin in high concentrations uncouples purified eNOS and leads to the generation of O₂⁻.

**BH₄ Levels and Atherosclerosis**

Although a number of studies have inferred alterations in BH₄ levels in atherosclerosis, none has provided direct measurements in the vessel wall. The present study demonstrates marked decreases in BH₄ in the HL model within 10 weeks of lipid feeding. The levels of reduction (>95%) were profound and were associated with marked abnormalities in agonist responsiveness to ACh and A23187. These results are consistent with prior studies involving in vitro manipulation of BH₄ levels in the aortic wall and in cultured cells with 2,4-diamino-6-hydroxypyrimidine, an inhibitor of BH₄ biosynthesis, demonstrating that substantial depletion of BH₄ is required before there are reductions in NO production.²⁻³¹ Recently, a genetic model of GTP cyclohydrolase I deficiency has been described, characterized by ∼60% reduction in vascular BH₄ levels. Interestingly, the animals did not exhibit differences in baseline agonist responsiveness compared with their wild counterparts. However, they demonstrated decreases in eNOS activity with a corresponding increase in reactive oxygen species that was attribut-

able to uncoupled NO synthase, which was corrected by short-term exposure to BH₄. At first glance, these results could be attributed to the in vitro culture system used in the study, because it is certainly possible that culturing diseased vessel segments from animals that have alterations in free radical defense systems may confer a selective vulnerability to oxidant stress that is not seen in control vessels. Therefore, we performed additional experiments in which we measured BH₄ levels in freshly isolated segments. The results confirmed the fact that HL vessels had BH₄ levels that were 15- to 30-fold lower than the levels in control animals. These results reiterate prior observations that cell culture conditions do not have an impact on BH₄ levels over the short term (<24 hours) in intact preparations.²²

In the present study, supplementation of aortas with sepiapterin in NC animals for 6 hours did not increase BH₄ levels compared with the levels in nonsupplemented aortas in NC animals, whereas it restored levels to near normal in atherosclerotic vessels and, in combination with NAC, led to further increases beyond those in NC aortas. There are a variety of potential explanations for these findings. BH₄ depletion in HL aortas could occur secondary to impairment in BH₄ synthesis, increased BH₄ oxidation, and/or diminished BH₄ recycling (see scheme I). The finding that levels increased only under conditions of BH₄ depletion but not under control conditions suggests that there are indeed mechanisms regulating optimal BH₄ concentrations in the vessel wall. The observation that one is able to further potentiate levels with NAC in the presence of sepiapterin in hyperlipidemia suggests disruption in the mechanisms maintaining optimal intracellular BH₄ concentrations.

**Sepiapterin and Endothelium-Dependent Responses in Atherosclerosis**

In spite of restoration of BH₄ levels, sepiapterin paradoxically worsened responses to ACh and A23187. Recent in vitro studies demonstrated that 7.8-BH₄ enhances O₂⁻ generation by uncoupling NADPH from l-arginine oxidation by eNOS.²² This effect is also observed with BH₄-replete eNOS, demonstrating that sepiapterin in high concentrations enhances O₂⁻ generation from eNOS. In aortas derived from HL animals, it is likely that sepiapterin itself and/or the accumulation of 7.8-BH₄ produced from sepiapterin reduction (see scheme I) enhances O₂⁻ production from eNOS, thereby further impairing vasorelaxation. Smooth muscle function was unimpaired, as evidenced by preserved responses to the NO donor nitroglycerin and the vasoconstrictor phenylephrine, ruling out direct toxic effects on the smooth muscle or on the guanylate cyclase–cGMP pathway, mediated by sepiapterin. In agreement with the present study, previous experiments in canine and human internal mammary artery segments have demonstrated worsening of responses to the endothelium-dependent agonist A23187 after 24 hours of exposure to sepiapterin in organoid cultures.²²⁻²³

There are several important differences between this and other studies that have demonstrated an improvement in endothelium-dependent relaxation with BH₄ or its analogue sepiapterin. First, the duration of exposure in the present study was much longer than that in studies that have demonstrated an improvement (6 hours versus ≤60 minutes in the present study).¹¹⁻¹³,²⁴,²⁵ Second, most studies that have demonstrated an improvement in agonist
Thiol Antioxidants as a Strategy to Rescue Cellular BH₄ Levels

Incubations with NAC, a thiol antioxidant, failed to raise BH₄ levels, whereas in the presence of sepiapterin, they resulted in marked elevations in atherosclerotic vessels. The lack of effect of NAC on BH₄ is in contrast to previous studies in endothelial cells, which have suggested that antioxidant therapy (such as with ascorbate) increases BH₄ levels.¹⁸⁻²⁰ The lack of increase in atherosclerotic vessels with NAC alone but a marked increase in the presence of sepiapterin suggest that oxidative modification of BH₄ is not the sole mechanism involved in the lack of BH₄. Recently, it has been suggested that depletion of GTP plays a role in BH₄ deficiency, although this possibility is unlikely.³⁰ Alternatively, there is experimental evidence that oxidized LDL downregulates the expression of GTP cyclohydrolase I.³¹ Of note, the increase in cellular BH₄ levels with NAC in combination with sepiapterin was not paralleled by improvements in endothelial function to ACh and A23187. This result further supports the idea that BH₄ alone is not the only variable controlling NO and O₂⁻ formation from eNOS. Even though BH₄ levels are augmented, it is the ratio between BH₄ and oxidized BH₄ metabolites such as 7,8-BH₄ that controls eNOS activity. This suggests the existence of a BH₄ concentration threshold in the control of eNOS function in vivo. Interestingly, NAC by itself did not improve responses to either ACh or A23187 in the present study. There is a variety of explanations for this finding. First, it is possible that the concentration of thiols used was insufficient to counteract ongoing oxidative stress over the 6-hour experiment. Second, it is possible that nonoxidative mechanisms control responsiveness such that antioxidants may be ineffective. Finally, the ongoing O₂⁻ formation from eNOS as a consequence of the lack of BH₄ may cause oxidative damage to eNOS, perpetuating a “dysfunctional” eNOS.

In summary, these data provide novel insights into the magnitude and mechanisms underlying BH₄ depletion in atherosclerosis, reemphasizing the critical role of the cofactor in the vasculature. Prolonged exposure to oxidized BH₄ analogues may potentially worsen endothelial function. Our observations emphasize the need to understand pathways regulating BH₄ metabolism in hypercholesterolemia to provide a rationale for its therapeutic application.

References


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