

# Altered Tetrahydrobiopterin Metabolism in Atherosclerosis Implications for Use of Oxidized Tetrahydrobiopterin Analogues and Thiol Antioxidants

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**Objective**—Tetrahydrobiopterin (BH<sub>4</sub>) 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 BH<sub>4</sub> 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 BH<sub>4</sub> 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 BH<sub>4</sub> levels, whereas NAC in conjunction with sepiapterin raised BH<sub>4</sub> 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 BH<sub>4</sub>. 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 (O<sub>2</sub><sup>-</sup>) in the inactivation of NO as a pathophysiologically relevant mechanism *in vivo*.<sup>1</sup> The sources of O<sub>2</sub><sup>-</sup> 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 (BH<sub>4</sub>), endothelial NO synthase (eNOS) generates O<sub>2</sub><sup>-</sup>.<sup>2-4</sup> In support of a critical role for BH<sub>4</sub> in mediating O<sub>2</sub><sup>-</sup> formation

sepiapterin, may represent a therapeutic strategy to ameliorate vascular function. Sepiapterin is an oxidized BH<sub>4</sub> analogue that generates BH<sub>4</sub> on 2 sequential enzymatic reductions by sepiapterin reductase and dihydrofolate reductase (Figure 1). This compound has been extensively used to augment BH<sub>4</sub> in conditions associated with altered BH<sub>4</sub> 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 BH<sub>4</sub>.<sup>7,11-16</sup> It has also been hypothesized that a considerable proportion of BH<sub>4</sub> undergoes oxidation in conditions associated with heightened oxidative stress, contributing to further BH<sub>4</sub> depletion.<sup>17</sup> Consistent with this, it has been demonstrated in cultured endothelial cells that vitamin C, an antioxidant, stimulates NO synthase secondary to increases in BH<sub>4</sub> levels through its chemical stabilization.<sup>18-20</sup> The implication of these findings on the *in vivo* stability of BH<sub>4</sub> 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 BH<sub>4</sub> levels and the consequences

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from eNOS are the observations that reduction of BH<sub>4</sub> levels by inhibition of GTP cyclohydrolase I, the rate-limiting enzyme for BH<sub>4</sub> synthesis in cells and intact vessel segments, results in reduced NO generation, increased generation of O<sub>2</sub><sup>-</sup> and hydrogen peroxide, and impairment of vascular relaxation.<sup>5-7</sup> In addition, supplementation of cellular BH<sub>4</sub> increases the ability of NO synthase to generate NO.<sup>8-10</sup> These findings have led to the hypothesis that correction of BH<sub>4</sub> levels by supplementation with BH<sub>4</sub> analogues, such as

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Received May 3, 2002; revision accepted June 17, 2002.

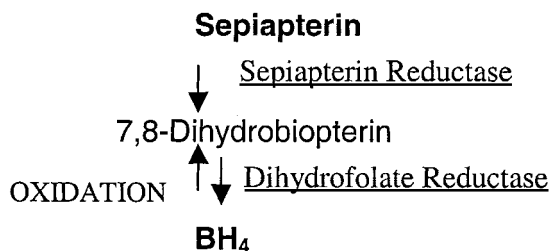
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*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000029122.79665.D9



**Figure 1.** Scheme I:  $\text{BH}_4$  production from sepiapterin. 7,8-dihydrobiopterin is the stable intermediate formed by sepiapterin reductase and/or oxidation of  $\text{BH}_4$ .

of modulating the latter with sepiapterin in the presence and absence of *N*-acetylcysteine (NAC), a thiol antioxidant compound.

Scheme I involves  $\text{BH}_4$  production from sepiapterin. 7,8-Dihydrobiopterin (7,8- $\text{BH}_2$ ) is the stable intermediate formed by sepiapterin reductase and/or oxidation of  $\text{BH}_4$ .

## 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 (normocholesterolemic [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 (hyperlipidemic [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 0.1 mmol/L and was incubated for another 6 hours in a humidified incubator under an atmosphere of 5%  $\text{CO}_2$ /95% air at 37°C. At the end of this period, a segment from each well was removed for organ chamber studies as detailed below. The other segment was snap-frozen in liquid nitrogen for determination of  $\text{BH}_4$  levels.

### 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,  $\text{CaCl}_2$  1.87,  $\text{MgSO}_4$  1.20,  $\text{K}_2\text{HPO}_4$  1.03,  $\text{NaHCO}_3$  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,  $\text{CaCl}_2$  1.87,  $\text{MgSO}_4$  1.20,  $\text{K}_2\text{HPO}_4$  1.03,  $\text{NaHCO}_3$  25.0, and glucose 11.1, pH 7.40. The solution was aerated continuously with a 95%  $\text{O}_2$ /5%  $\text{CO}_2$  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  $\mu\text{mol/L}$ ) to prevent prostaglandin synthesis. The vessels were then precon-

stricted with gradual doses of L-phenylephrine (0.15  $\mu\text{mol/L}$ ). 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 nmol/L to 1  $\mu\text{mol/L}$ ), the endothelium-independent vasorelaxant nitroglycerin (1 nmol/L to 10  $\mu\text{mol/L}$ ), and the calcium ionophore A23187 (1 nmol/L to 1  $\mu\text{mol/L}$ ). 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 nmol/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 $\text{BH}_4$ in Aortic Segments

Measurement of  $\text{BH}_4$  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  $\text{BH}_4$  analogue. Oxidation of  $\text{BH}_4$  to biopterin under acidic conditions is quantitative. Under basic conditions, however,  $\text{BH}_4$  is further oxidized to nonfluorescent compounds. Thus,  $\text{BH}_4$  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%  $\text{I}_2$ /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 a water/ice bath for 1 minute by use of 25% sonicator full-power potency to break open the cells. After a 90-minute incubation at room temperature, 50  $\mu\text{L}$  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  $\mu\text{m}$ , 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  $\mu\text{L}$ . 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  $\mu\text{g/mL}$ ),  $\text{BH}_4$  (1  $\mu\text{mol/L}$ ), L-arginine (40  $\mu\text{mol/L}$ ), and 5-ethoxycarbonyl-5-methyl-pyrroline *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.<sup>2</sup>

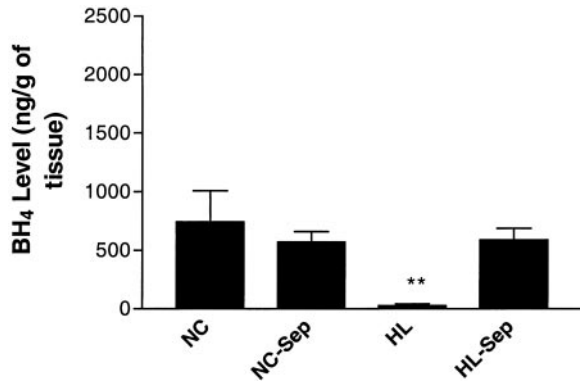
### Statistical Analysis

All data are expressed as mean  $\pm$  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



**Figure 2.** BH<sub>4</sub> 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% CO<sub>2</sub>/95% air at 37°C. \*\**P*<0.001 vs NC; *P*=NS for HL-Sep vs NC.

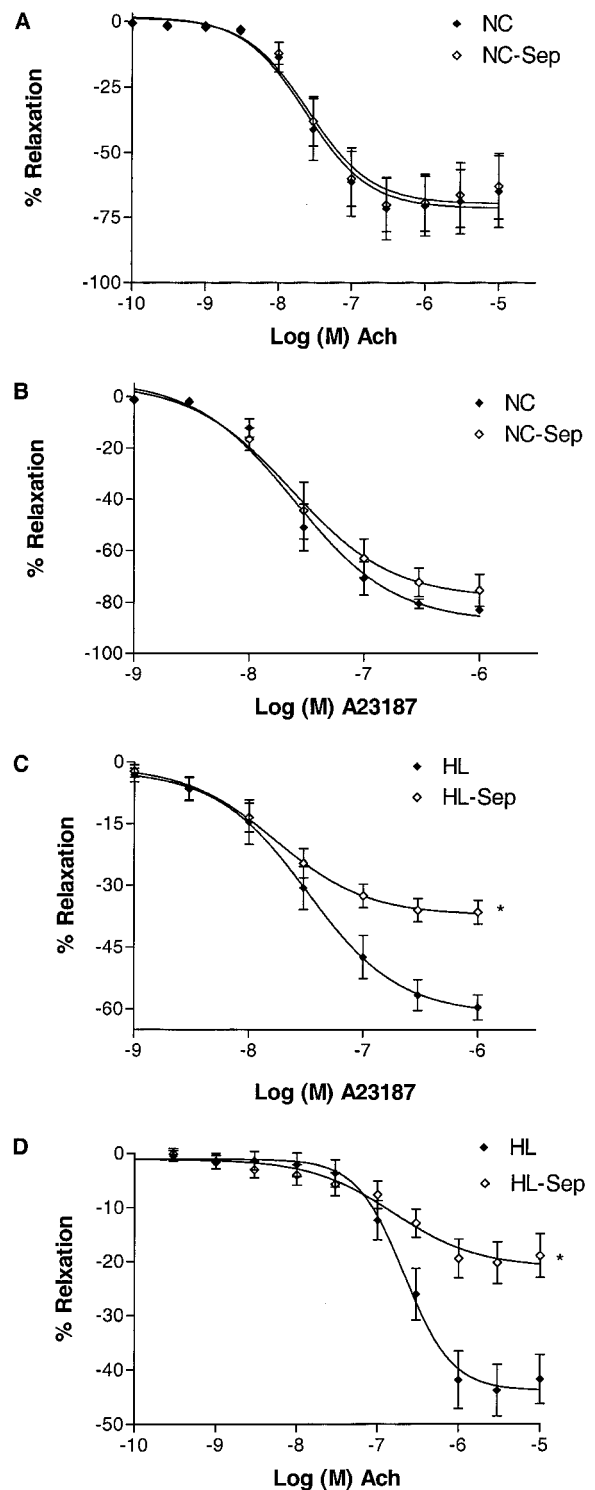
1465 ± 333 mg/dL. The total cholesterol level in the control group was 52 ± 17 mg/dL.

### BH<sub>4</sub> Levels in Aortas From HL Rabbits

Figure 2 depicts BH<sub>4</sub> 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 BH<sub>4</sub> per gram tissue, whereas aortic segments from HL rabbits contained 28 ± 14 ng BH<sub>4</sub> per gram tissue (*P*<0.001 versus NC by ANOVA), a 27-fold reduction in levels. Oxidized pteridine (BH<sub>2</sub>) concentration (298.1 ± 105.8 ng per gram tissue) was found in NC tissue, which represents ≈30% of the total oxidized and reduced BH<sub>4</sub> content. In HL tissue, the BH<sub>2</sub> 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 BH<sub>4</sub> content in HL aortas increased 21-fold (to 589 ± 97 ng BH<sub>4</sub> 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 BH<sub>4</sub> content (570 ± 91 ng BH<sub>4</sub> 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. BH<sub>4</sub> 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 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% CO<sub>2</sub>/95% air at 37°C. Segments were precontracted with L-phenylephrine (PE), and relaxation to cumulative doses of ACh (10<sup>-10</sup> to 10<sup>-5</sup>) and A23187 (10<sup>-10</sup> to 10<sup>-5</sup>) was examined in NC aortas (A and B) and HL aortas (C and D). \**P*<0.05 for HL vs HL-Sep.

**Endothelial-Dependent (Top) and Endothelial-Independent (Bottom) Vascular Responses in Normal Cholesterol-Fed Aorta Rabbits (NC) and Hyperlipidemic-Fed Aorta Rabbits With and Without Sepsiapterin Supplementation**

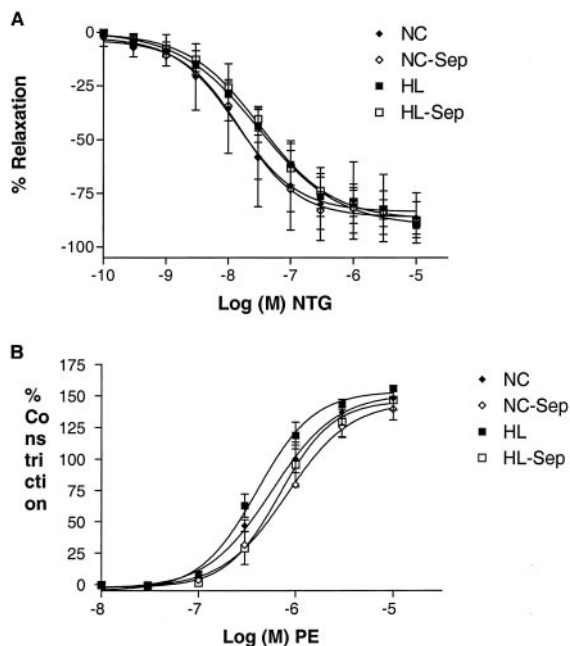
	Peak Relaxation to Ach	ED <sub>50</sub>	Peak Relaxation to A23187	ED <sub>50</sub>
NC	-65±14	7.62±0.18	-84±2	-7.31±0.31
NC-Sep	-63±13	7.59±0.17	-65±19	-7.42±0.41
HL	-42±5†	6.67±0.09†	-60±3‡	-7.56±0.17
HL-Sep	-17±4*†	6.79±0.27†	-37±3*†	-7.84±0.16

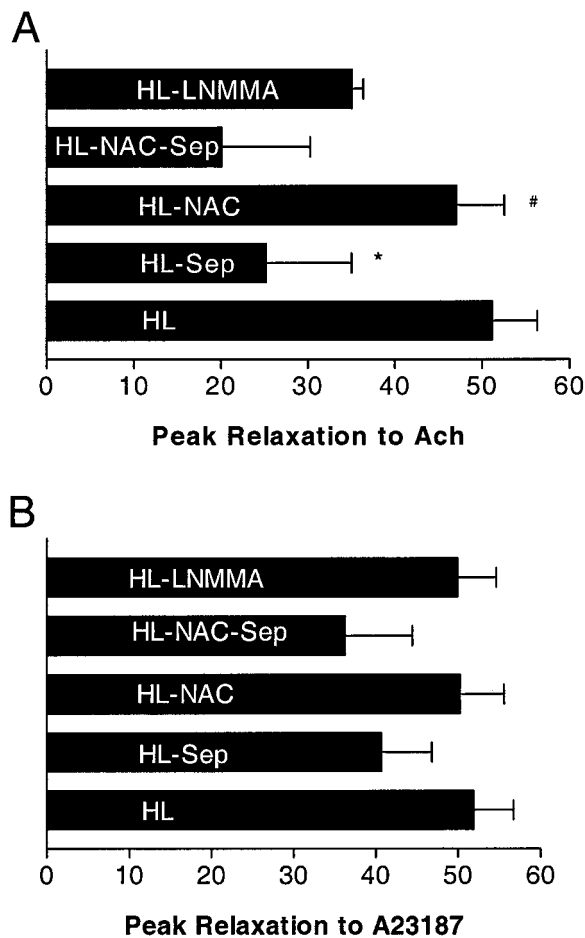
	Peak Relaxation to NTG	ED <sub>50</sub>	Peak Constriction to PE	EC <sub>50</sub>
NC	93.2±4.0	-7.72±0.12	148.0±9.6	-6.23±0.03
NC-Sep	94.1±3.6	-7.66±0.09	139.4±8.5	-6.10±0.05
HL	90±4.0	-7.56±0.14	155.8±2.4	-6.37±0.09‡
HL-Sep	88.3±2.3	-7.53±0.10	146.8±7.7	-6.13±0.13

\*P<0.05 vs HL. †P<0.05 vs NC and NC-Sep. ‡P<0.05 vs NC.

L-phenylephrine. Incubation with sepsiapterin for 6 hours failed to improve peak relaxations and ED<sub>50</sub> to both agonists (see Table). In contrast to the lack of an effect of BH<sub>4</sub> in NC aortas, incubation of HL aortic segments with sepsiapterin resulted in a pronounced impairment in responses to both agonists, as shown in Figure 3 and the Table.



**Figure 4.** Vascular relaxation in NC and HL rabbit aortas with and without sepsiapterin supplementation to nitroglycerin (NTG, A) and PE (B). Tissues were incubated in the presence or absence of sepsiapterin in DMEM culture media containing antibiotics and 0.1% calf serum. Sepsiapterin 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% CO<sub>2</sub>/95% air at 37°C. Segments were precontracted with PE, and vascular response to cumulative doses of NTG (10<sup>-10</sup> to 10<sup>-5</sup>) and PE (10<sup>-10</sup> to 10<sup>-5</sup>) was examined.



**Figure 5.** Vascular relaxation in NC and HL rabbit aortas with and without sepsiapterin supplementation to ACh (A) and A23187 (B). Tissues were incubated in the presence or absence of sepsiapterin in DMEM culture media containing antibiotics and 0.1% calf serum. Sepsiapterin was added to 1 of the segments at a final concentration of 0.1 mmol/L and incubated for 6 hours under an atmosphere of 5% CO<sub>2</sub>/95% air at 37°C. Segments were precontracted with PE, and vascular response to ACh (10 μmol/L) and A23187 (10 μmol/L) was recorded. N<sup>G</sup>-Monomethyl-L-arginine (L-NMMA, 10 μmol/L) was preincubated for 30 minutes before stimulation with the agonists. \*P<0.05 by ANOVA vs HL; #P=NS vs HL-Sep.

**Effects of Sepsiapterin on Smooth Muscle Function**

Nitroglycerin induced dose-dependent relaxations in precontracted NC and HL ring segments, which did not differ with supplementation with BH<sub>4</sub> (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=NS) and a shift in sensitivity (EC<sub>50</sub> 6.37±0.1 and 6.23±0.03 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 sepsiapterin (NC-Sep and HL-Sep groups, respectively; Table).

**Effect of NAC on Endothelial Function and BH<sub>4</sub> Levels**

In a separate set of experiments, we examined the effects of the thiol antioxidant compound NAC (1 mmol/L) in preserv-

ing BH<sub>4</sub> levels. NAC by itself did not augment BH<sub>4</sub> levels (7±3 ng BH<sub>4</sub> per gram tissue) in HL aortic segments. However, in the presence of sepiapterin, there was a 221-fold increase in BH<sub>4</sub> (1570±683 versus 7±3 ng BH<sub>4</sub> 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 BH<sub>4</sub> levels, this failed to translate into improvements in responses to ACh and A23187 (see Figure 5).

### Effect of Sepiapterin on NO/O<sub>2</sub><sup>-</sup> Production From eNOS

The effects of sepiapterin on NO and O<sub>2</sub><sup>-</sup> formation from eNOS were examined by measuring L-citrulline formation and by EPR spin trapping with EMPO, respectively. Incubations of eNOS with BH<sub>4</sub> (1 μmol/L) supported L-citrulline formation at a rate of 148.3±1.2 nmol/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 nmol/min per milligram protein. EPR experiments showed that inhibition of NO formation was paralleled by an increase in O<sub>2</sub><sup>-</sup> formation. Sepiapterin augmented O<sub>2</sub><sup>-</sup> 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 O<sub>2</sub><sup>-</sup> formation from eNOS.

### Discussion

The key findings of the present study are as follows: (1) Hypercholesterolemia diminishes vascular BH<sub>4</sub>. (2) Supplementation with sepiapterin, an oxidized BH<sub>4</sub> 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 BH<sub>4</sub> levels in hyperlipidemia. (4) Sepiapterin in high concentrations uncouples purified eNOS and leads to the generation of O<sub>2</sub><sup>-</sup>.

### BH<sub>4</sub> Levels and Atherosclerosis

Although a number of studies have inferred alterations in BH<sub>4</sub> levels in atherosclerosis, none has provided direct measurements in the vessel wall. The present study demonstrates marked decreases in BH<sub>4</sub> 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 BH<sub>4</sub> levels in the aortic wall and in cultured cells with 2,4-diamino-6-hydroxypyrimidine, an inhibitor of BH<sub>4</sub> biosynthesis, demonstrating that substantial depletion of BH<sub>4</sub> is required before there are reductions in NO production.<sup>7,21</sup> Recently, a genetic model of GTP cyclohydrolase I deficiency has been described, characterized by ≈60% reduction in vascular BH<sub>4</sub> 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 BH<sub>4</sub>. 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 BH<sub>4</sub> levels in freshly isolated segments. The results confirmed the fact that HL vessels had BH<sub>4</sub> 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 BH<sub>4</sub> levels over the short term (<24 hours) in intact preparations.<sup>22</sup>

In the present study, supplementation of aortas with sepiapterin in NC animals for 6 hours did not increase BH<sub>4</sub> 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. BH<sub>4</sub> depletion in HL aortas could occur secondary to impairment in BH<sub>4</sub> synthesis, increased BH<sub>4</sub> oxidation, and/or diminished BH<sub>4</sub> recycling (see scheme I). The finding that levels increased only under conditions of BH<sub>4</sub> depletion but not under control conditions suggests that there are indeed mechanisms regulating optimal BH<sub>4</sub> 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 BH<sub>4</sub> concentrations.

### Sepiapterin and Endothelium-Dependent Responses in Atherosclerosis

In spite of restoration of BH<sub>4</sub> levels, sepiapterin paradoxically worsened responses to ACh and A23187. Recent *in vitro* studies demonstrated that 7,8-BH<sub>2</sub> enhances O<sub>2</sub><sup>-</sup> generation by uncoupling NADPH from L-arginine oxidation by eNOS.<sup>22</sup> This effect is also observed with BH<sub>4</sub>-replete eNOS, demonstrating that sepiapterin in high concentrations enhances O<sub>2</sub><sup>-</sup> generation from eNOS. In aortas derived from HL animals, it is likely that sepiapterin itself and/or the accumulation of 7,8-BH<sub>2</sub> produced from sepiapterin reduction (see scheme I) enhances O<sub>2</sub><sup>-</sup> 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.<sup>22,23</sup>

There are several important differences between this and other studies that have demonstrated an improvement in endothelium-dependent relaxation with BH<sub>4</sub> 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).<sup>11–13,24,25</sup> Second, most studies that have demonstrated an improvement in agonist

responses have been short-term incubations or infusions with BH<sub>4</sub> rather than its precursor sepiapterin.<sup>13,24,25</sup> The possibility of BH<sub>4</sub> acting as an antioxidant in these studies cannot be ruled out. In this regard, we have recently determined the rate constant for the reaction between BH<sub>4</sub> and O<sub>2</sub><sup>•-</sup> to be ≈10<sup>5</sup> mol/L per second, which is close to the reported rate constant for ascorbate with O<sub>2</sub><sup>•-</sup>.<sup>26,27</sup> However, in view of the lower prevailing concentrations of BH<sub>4</sub> compared with the 30 to 50 μmol/L concentrations of ascorbate *in vivo*, it is likely that BH<sub>4</sub> acts through mechanisms other than O<sub>2</sub><sup>•-</sup> scavenging. In studies that have used sepiapterin and that have demonstrated an improvement in vasomotor responsiveness, the exposures times to the drug have been brief,<sup>28,29</sup> and these studies did not provide measurements of intracellular BH<sub>4</sub> levels to firmly conclude that the effects were secondary to increases in intracellular BH<sub>4</sub> levels.

### Thiol Antioxidants as a Strategy to Rescue Cellular BH<sub>4</sub> Levels

Incubations with NAC, a thiol antioxidant, failed to raise BH<sub>4</sub> levels, whereas in the presence of sepiapterin, they resulted in marked elevations in atherosclerotic vessels. The lack of effect of NAC on BH<sub>4</sub> is in contrast to previous studies in endothelial cells, which have suggested that antioxidant therapy (such as with ascorbate) increases BH<sub>4</sub> levels.<sup>18–20</sup> The lack of increase in atherosclerotic vessels with NAC alone but a marked increase in the presence of sepiapterin suggest that oxidative modification of BH<sub>4</sub> is not the sole mechanism involved in the lack of BH<sub>4</sub>. Recently, it has been suggested that depletion of GTP plays a role in BH<sub>4</sub> deficiency, although this possibility is unlikely.<sup>30</sup> Alternatively, there is experimental evidence that oxidized LDL downregulates the expression of GTP cyclohydrolase I.<sup>31</sup> Of note, the increase in cellular BH<sub>4</sub> 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 BH<sub>4</sub> alone is not the only variable controlling NO and O<sub>2</sub><sup>•-</sup> formation from eNOS. Even though BH<sub>4</sub> levels are augmented, it is the ratio between BH<sub>4</sub> and oxidized BH<sub>4</sub> metabolites such as 7,8-BH<sub>2</sub> that controls eNOS activity. This suggests the existence of a BH<sub>4</sub> 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 O<sub>2</sub><sup>•-</sup> formation from eNOS as a consequence of the lack of BH<sub>4</sub> may cause oxidative damage to eNOS, perpetuating a “dysfunctional” eNOS.

In summary, these data provide novel insights into the magnitude and mechanisms underlying BH<sub>4</sub> depletion in atherosclerosis, reemphasizing the critical role of the cofactor in the vasculature. Prolonged exposure to oxidized BH<sub>4</sub> analogues may potentially worsen endothelial function. Our observations reemphasize the need to understand pathways regulating BH<sub>4</sub> metabolism in hypercholesterolemia to provide a rationale for its therapeutic application.

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