Endothelial Regulation of Vasomotion in ApoE-Deficient Mice
Implications for Interactions Between Peroxynitrite and Tetrahydrobiopterin

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Background—Altered endothelial cell nitric oxide (NO ·) production in atherosclerosis may be due to a reduction of intracellular tetrahydrobiopterin, which is a critical cofactor for NO synthase (NOS). In addition, previous literature suggests that inactivation of NO by increased vascular production superoxide (O 2 ·) also reduces NO bioactivity in several disease states. We sought to determine whether these 2 seemingly disparate mechanisms were related.

Methods and Results—Endothelium-dependent vasodilation was abnormal in aortas of apoE-deficient (apoE<sup>−/−</sup>) mice, whereas vascular superoxide production (assessed by 5 μmol/L lucigenin) was markedly increased. Treatment with either liposome-entrapped superoxide dismutase or sepiapterin, a precursor to tetrahydrobiopterin, improved endothelium-dependent vasodilation in aortas from apoE<sup>−/−</sup> mice. Hydrogen peroxide had no effect on the decay of tetrahydrobiopterin, as monitored spectrophotometrically. In contrast, superoxide modestly and peroxynitrite strikingly increased the decay of tetrahydrobiopterin over 500 seconds. Luminol chemiluminescence, inhibitable by the peroxynitrite scavengers ebselen and uric acid, was markedly increased in apoE<sup>−/−</sup> aortic rings. In vessels from apoE<sup>−/−</sup> mice, uric acid improved endothelium-dependent relaxation while having no effect in vessels from control mice. Treatment of normal aortas with exogenous peroxynitrite dramatically increased vascular O 2 · production, seemingly from eNOS, because this effect was absent in vessels lacking endothelium, was blocked by NOS inhibition, and did not occur in vessels from mice lacking eNOS.

Conclusions—Reactive oxygen species may alter endothelium-dependent vascular relaxation not only by the interaction of O 2 · with NO but also through interactions between peroxynitrite and tetrahydrobiopterin. Peroxynitrite oxidation of tetrahydrobiopterin may represent a pathogenic cause of “uncoupling” of NO synthase. (Circulation. 2001;103:1282-1288.)

Key Words: endothelium ■ genes ■ vessels

There has been interest in mechanisms underlying abnormal endothelium-dependent vascular relaxation in hypercholesterolemia and atherosclerosis. Superoxide (O 2 ·) production is increased in atherosclerotic vessels<sup>1,2</sup> and reacts rapidly with nitric oxide (NO) to form the peroxynitrite anion. Increased nitrotyrosine immunostaining has provided indirect evidence for this interaction in atherosclerotic vessels.<sup>3</sup> Membrane-targeted forms of superoxide dismutase (SOD) or antioxidant vitamins can enhance endothelium-dependent vascular relaxation in diverse pathological conditions, such as hypercholesterolemia, diabetes, hypertension, and cigarette smoking.<sup>4</sup>

Recently, there has been interest in the possibility that tetrahydrobiopterin, a critical cofactor for NO synthase (NOS), may be deficient in various conditions associated with altered endothelial function. For example, treatment with tetrahydrobiopterin has been shown to augment endothelium-dependent vasodilation in humans with hypercholesterolemia.<sup>5</sup> In studies of purified NOS, removal of tetrahydrobiopterin results in endothelial NOS (eNOS) becoming “uncoupled,” such that electrons flowing from the reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine.<sup>6-7</sup> In this uncoupled state, O 2 · is produced rather than NO. There is indirect evidence to suggest that this might also occur in intact endothelial cells<sup>8</sup> and vessels.<sup>9</sup>

The present experiments were performed to examine mechanisms that might be responsible for tetrahydrobiopterin depletion in endothelial cells leading to eNOS uncoupling. Our findings suggest that reactive oxygen species produced in...
endothelial cells not only enhance degradation of NO but also alter eNOS function by oxidation of tetrahydrobiopterin.

**Methods**

**Animals Studied**
ApoE-deficient (apoE−/−) mice were obtained from Breslow and colleagues (Rockefeller University, New York, NY).10 Offspring from these animals of either sex, 6 to 18 months old, were studied. Mice lacking eNOS (eNOS−/− mice) were obtained from Jackson Laboratories (Bar Harbor, Maine). C57Blk/6 mice were used as controls.

**Materials Used**
Liposome-entrapped Cu/Zn-SOD was prepared as described previously.11 Sepiapterin was obtained from B. Schrick or from RBI. Ebselen was obtained from Cayman Biochemicals and dissolved in ethanol (final concentration 0.2%). Peroxynitrite was synthesized as previously described12 or purchased from Alexis. Luminol was dissolved in DMSO (final concentration 0.1%). All other agents were obtained from Sigma in the highest grade available. All drugs were prepared from stock solutions and dissolved in distilled water immediately before use, unless otherwise stated.

**Studies of Vascular Reactivity**
Thoracic aortas were rapidly removed and cut into ring segments ≈3 mm long and studied as previously described.13 In preliminary studies, the optimum resting tension for both control and apoE−/− mouse aortas (n = 5 for each) for tone development to 80 mmol/L KCl was found to be 1.15 g. Vessels were gradually stretched to this resting tension over 1 hour. In general, 3 to 4 ring segments were studied from each animal. Relaxation responses were performed on 2 separate rings, and the responses were averaged and used to represent an “n” of 1.

**Western Analysis**
Western blots were performed as previously described14 with a polyclonal anti-eNOS antibody (1:1000 dilution) obtained from Transduction Laboratories, and a mouse anti-rabbit IgG secondary antibody (1:1000 dilution) obtained from BioRad.

**Spectrophotometric Analysis of Tetrahydrobiopterin After Exposure to Reactive Oxygen Species**
Tetrahydrobiopterin (1 mmol/L stock) was dissolved in a 200-mmol/L Tris-HCl buffer (pH 7.4) previously purged extensively with argon gas. Tetrahydrobiopterin (0.1 mmol/L final concentration) was maintained anaerobically at 5°C until used (<1 hour). In control experiments in which the spontaneous oxidation of tetrahydrobiop-

**Chemiluminescence Estimates of Reactive Oxygen Species Produced by Vascular Segments**
To estimate vascular O2·− production, we used lucigenin-enhanced chemiluminescence. Two 2.5-mm ring segments of mouse aorta were placed in scintillation vials containing Krebs-HEPES buffer with 5 μmol/L lucigenin. This concentration of lucigenin has been shown to accurately reflect levels of ambient O2·− and is not subject to the redox cycling and artifactual production of superoxide observed with higher concentrations of the agent.15,16 In other studies, luminol (100 μmol/L) chemiluminescence was examined by similar methods. Light emission was detected with a scintillation counter programmed to an out-of-coincidence mode. Mean chemiluminescence yields observed during the period of 15 to 20 minutes after addition of the vessel segments were used to estimate rates of production of the respective reactive oxygen species.

**Statistical Analysis**
Data are presented as mean±SEM. Peak relaxations and EC50s are compared by ANOVA. When significance was indicated, a Duncan’s multiple range post hoc test was used. The oxidation of tetrahydrobiop-

**Results**
**Responses to Acetylcholine, the Calcium Ionophore A23187, and Nitroglycerin**
For studies of vascular relaxations, vessels were preconstricted with 1.0 μmol/L phenylephrine. This resulted in 1.01±0.07 and 0.97±0.11 g tone in the control and apoE−/− mouse aortas, respectively. These values were similar between untreated vessels and vessels pretreated with liposome-entrapped SOD and uric acid. In control mice, acetylcholine and the calcium ionophore A23187 produced relaxations of 83±3% (n = 20) and 89±2% (n = 15), respectively. These responses were significantly reduced in the aortas of apoE−/− mice, 52±4% (n = 10) and 52±10% (n = 6) (Figure 1). To determine whether O2·− reduces endothelium-dependent vascular relaxation in apoE−/− mouse aortas, ring segments were incubated in solutions of Krebs/HEPES buffer containing liposome-entrapped SOD (500 U in 1:1 dilution) for 20 minutes and then mounted in the organ chamber for study. Liposome-entrapped SOD had no effect on endothelium-dependent responses of normal aortas (Figure 1). In contrast, in vessels from apoE−/− mice, relaxations to acetylcholine and A23187 were markedly enhanced by preincubation with liposome-entrapped SOD (Figure 1). Responses to nitroglycerin were shifted rightward in vessels from apoE−/− mice. Surprisingly, liposome-entrapped SOD reduced relaxations to nitroglycerin in control mice, while having no effect on responses to nitroglycerin in apoE−/− mice (Figure 1).

Preincubation with the tetrahydrobiopterin precursor sepi-

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increased endothelium-dependent vascular relaxations to A23187, while having no effect on responses to acetylcholine (Figure 2).

Expression of eNOS in Control C57Blk/6 and apoE\(^{-/-}\) Mouse Aortas
As shown in Figure 1, liposome-entrapped SOD increased endothelium-dependent vascular relaxations to levels exceeding that of normal vessels, suggesting that eNOS protein might be increased in apoE\(^{-/-}\) mouse aortas. Indeed, levels of eNOS protein, as assessed by Western analysis, were significantly greater in apoE\(^{-/-}\) than in C57Blk/6 aortic homogenates (n=3, Figure 3).

Superoxide Production by Control C57Blk/6 and apoE\(^{-/-}\) Mouse Aortas
The finding that liposome-entrapped SOD enhanced endothelium-dependent vascular relaxation in aortas of apoE\(^{-/-}\) mice strongly suggested that production of O\(_2^-.\) may be increased in these vessels. Estimates of O\(_2^-.\) production by use of lucigenin-enhanced chemiluminescence confirmed this. As shown in Figure 4, O\(_2^-.\) production was markedly increased in apoE\(^{-/-}\) mouse aortas compared with controls. Removal of the endothelium dramatically reduced O\(_2^-.\) production from apoE\(^{-/-}\) mouse aortas, while having little effect in control vessels. Exposure of aortic segments from apoE\(^{-/-}\) mice to sepiapterin (10 \(\mu\)mol/L) for 1 hour before the lucigenin assay reduced O\(_2^-.\) production in aortas of apoE\(^{-/-}\) mice, while having no effect in vessels from control C57Blk/6 mice (Figure 4). NG-Nitro-L-arginine methyl ester

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**Figure 1.** Vascular relaxations to acetylcholine, calcium ionophore A23187, and nitroglycerin in aortas from control and apoE\(^{-/-}\) mice. Vessels were studied as ring segments in organ chambers. After preconstriction with phenylephrine (1.0 \(\mu\)mol/L), respective agents were added in cumulative fashion. In other experiments, ring segments were incubated with liposome-entrapped SOD (500 U/ring) for 20 minutes before mounting in organ chamber.

**Figure 2.** Effect of sepiapterin on endothelium-dependent vasodilation of aortas from apoE\(^{-/-}\) mice. Vessels were studied in organ chambers as described in Figure 1. Sepiapterin (10 \(\mu\)mol/L) was added 60 minutes before responses to acetylcholine or calcium ionophore were examined.

**Figure 3.** A, Representative Western blot showing eNOS expression in aortas from control (left 2 lanes) and apoE\(^{-/-}\) (right 2 lanes) mice. B, Densitometric analysis of eNOS expression in control and apoE\(^{-/-}\) mice (n=3 for both).

**Figure 4.** Vascular O\(_2^-.\) production estimated by lucigenin chemiluminescence in aortic segments from control and apoE\(^{-/-}\) mice. Two 2.5-mm segments of aortic rings were placed in scintillation vials containing 2 mL of Krebs buffer and 5 \(\mu\)mol/L lucigenin. Vessels were incubated with sepiapterin (10 \(\mu\)mol/L) in some experiments for 30 minutes before assay. Counts were obtained from 15 to 20 minutes after addition of vessel and averaged. Numbers of experiments are given above each bar. Endo indicates endothelium.

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(L-NAME) (1 mmol/L) dramatically increased $O_2^-$ production in control vessels, while having no effect in the apoE$^{-/-}$ vessels. Taken together, these data suggest that the endothelium produces large amounts of $O_2^-$ in the atherosclerotic aortas of apoE$^{-/-}$ mice.

**Interaction of Tetrahydrobiopterin With Reactive Oxygen Species**

Because both liposome-entrapped SOD and sepiapterin corrected endothelium-dependent vascular relaxations in atherosclerotic vessels of the apoE$^{-/-}$ mice and these vessels seemed to produce large quantities of reactive oxygen species, we considered the hypothesis that either $O_2^-$ or a reactive oxygen species derived from $O_2^-$ might oxidize tetrahydrobiopterin. Tetrahydrobiopterin and dihydrobiopterin could easily be differentiated by their respective absorbances at 282 and 297 nm (Figure 5A, top). Simple exposure to air resulted in loss of 17% of tetrahydrobiopterin over the 500 seconds of observation. Exposure of tetrahydrobiopterin to hydrogen peroxide did not alter this rate of degradation (Figure 5B). In contrast, exposure to superoxide, derived from KO$_2$, modestly increased degradation of tetrahydrobiopterin ($P=0.04$). Exposure to even lower concentrations of peroxynitrite resulted in a marked decline of tetrahydrobiopterin such that only $\approx 40\%$ remained after 500 seconds ($P=0.0004$). The spectra after exposure to peroxynitrite resembled that of dihydrobiopterin (Figure 5A). Addition of sodium borohydride (1 mmol/L) restored the tetrahydrobiopterin absorbance after exposure to peroxynitrite (Figure 5B).

**Evidence for Increased Production of Peroxynitrite in ApoE$^{-/-}$ Mouse Aortas**

Using luminol chemiluminescence, we next sought to determine whether apoE$^{-/-}$ mouse vessels produced excessive quantities of peroxynitrite. Luminol chemiluminescence was dramatically enhanced in apoE$^{-/-}$ mouse aortas. Both eb-selen, a scavenger of both H$_2$O$_2$ and peroxynitrite, and uric acid, a scavenger of peroxynitrite, reduced luminol chemiluminescence in control and apoE$^{-/-}$ mouse aortas, although the degree of inhibition in apoE$^{-/-}$ aortas was much greater than in control C57Blk/6 mouse aortas (Figure 6). These data indicate that atherosclerotic vessels from apoE$^{-/-}$ mice pro-

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**Figure 5.** A, Representative spectra for tetrahydrobiopterin (top), tetrahydrobiopterin after exposure to 250 μmol/L peroxynitrite (middle), and dihydrobiopterin (bottom). B, Effect of exposure to various reactive oxygen species on absorbance of tetrahydrobiopterin. Values are given as percentage of absorbance immediately on exposure to air and before addition of reactive oxygen species.

**Figure 6.** Luminol chemiluminescence in aortas of C57Blk/6 and apoE$^{-/-}$ mice. Studies were performed as described in Figure 4, except that luminol (100 μmol/L) was used rather lucigenin. Numbers of experiments are given above each bar.
Evidence That Peroxynitrite Can Increase \( \text{O}_2^- \) Production From eNOS in Intact Vascular Segments

The data above suggest that in atherosclerotic aortas, peroxynitrite could readily oxidize tetrahydrobiopterin in vitro and that peroxynitrite is made in large quantities in atherosclerotic vessels. To determine whether peroxynitrite might impair endothelium-dependent vascular relaxation in intact vessels, 12 additional mice were studied. These included 6 C57Blk/6 and 6 apoE-/- mice, 18 months old. Studies of vascular relaxation were performed as described above in 4 rings from each animal. In these studies, half of the ring segments were exposed to the peroxynitrite scavenger uric acid (100 \( \mu \text{mol/L} \)) for 20 minutes before and during vasodilation studies. As shown in Figure 7, uric acid significantly increased the sensitivity of vessels from apoE-/- mice to both acetylcholine and A23187, but not to nitroglycerin. In contrast, uric acid had no effect on responses in aortas of C57Blk/6 mice (Figure 7).

Discussion

Isometric tension studies of isolated vascular rings showed that both \( \text{O}_2^- \) scavenging and treatment with a tetrahydrobiopterin precursor improved endothelium-dependent vasodilation in apoE-/- mouse aortas. Although there may be completely independent explanations for these findings, studies of interactions between reactive oxygen species and tetrahydrobiopterin suggested a common mechanism. Peroxynitrite potently oxidized tetrahydrobiopterin to dihydrobiopterin. Of note, peroxynitrite had no effect on \( \text{O}_2^- \) production in vessels from eNOS+/- mice (Figure 8).

Effect of Uric Acid on Endothelium-Dependent Vascular Relaxations

The above experiments indicated that peroxynitrite could readily oxidize tetrahydrobiopterin in vitro and that peroxynitrite is made in large quantities in atherosclerotic vessels. To determine whether peroxynitrite might impair endothelium-dependent vascular relaxation in intact vessels, 12 additional mice were studied. These included 6 C57Blk/6 and 6 apoE-/- mice, 18 months old. Studies of vascular relaxation were performed as described above in 4 rings from each animal. In these studies, half of the ring segments were exposed to the peroxynitrite scavenger uric acid (100 \( \mu \text{mol/L} \)) for 20 minutes before and during vasodilation studies. As shown in Figure 7, uric acid significantly increased the sensitivity of vessels from apoE-/- mice to both acetylcholine and A23187, but not to nitroglycerin. In contrast, uric acid had no effect on responses in aortas of C57Blk/6 mice (Figure 7).

Evidence That Peroxynitrite Can Increase \( \text{O}_2^- \) Production From eNOS in Intact Vascular Segments

The data above suggest that in atherosclerotic aortas, peroxynitrite is formed and may interfere with eNOS function by oxidizing tetrahydrobiopterin. We therefore reasoned that exogenously administered peroxynitrite might uncouple eNOS and thus increase vascular \( \text{O}_2^- \) production in normal mouse aortas. Aortic segments of C57Blk/6 mice were exposed to peroxynitrite (100 \( \mu \text{mol/L} \)). Thirty minutes later, the vascular rings were washed 2 times in Krebs/HEPES buffer and then examined for lucigenin-enhanced chemiluminescence. In vessels with intact endothelium, treatment with peroxynitrite increased lucigenin-enhanced chemiluminescence almost 3-fold (1124±189 versus 2965±377 counts \( \text{min}^{-1} \cdot \text{mg}^{-1} \) in control versus peroxynitrite-treated aortas, respectively, Figure 8). Because peroxynitrite (which has a half-life of only several seconds) was added >0.5 hour before the lucigenin estimates of \( \text{O}_2^- \) production, these findings strongly suggest that peroxynitrite produces a long-lasting effect on vascular \( \text{O}_2^- \) production. In vessels in which the endothelium had been removed, peroxynitrite did not increase, and in fact paradoxically decreased, lucigenin-enhanced chemiluminescence. The NO synthase inhibitor L-NAME completely prevented the increase in \( \text{O}_2^- \) production caused by peroxynitrite, as did treatment of the vessels with sepiapterin. Importantly, peroxynitrite had no effect on \( \text{O}_2^- \) production in vessels from eNOS+/- mice (Figure 8).
In keeping with the concept that peroxynitrite may alter endothelium-dependent vascular relaxation, we found that uric acid, a peroxynitrite scavenger, significantly enhanced responses to acetylcholine and the calcium ionophore. The effect of uric acid was not as great as that of liposome-entrapped SOD. This is compatible with the notion that scavenging of O$_3^-$ would improve endothelium-dependent vascular relaxation via 2 mechanisms, preventing direct reactions with NO and secondarily preventing the formation of peroxynitrite and consequent oxidation of tetrahydrobiopterin. In contrast, treatment with uric acid would be expected to prevent the only latter of these. Importantly, the reduction potentials of uric acid and superoxide do not favor reactions between the two. Uric acid could react with other strong oxidants that might also oxidize tetrahydrobiopterin, including hydroxyl and hypohalous acid. Our studies do not exclude a role for such oxidants, although these would not likely be eliminated by liposome-entrapped SOD.

In the present study, sepiapterin only partially decreased O$_3^-$ production in apoE$^{-/-}$ mice, suggesting that sources other than eNOS may produce O$_3^-$ in these vessels. Studies using L-NAME to inhibit O$_3^-$ production were difficult to interpret, because L-NAME dramatically increased O$_3^-$ production in normal vessels, while having no effect in the apoE$^{-/-}$ aortas. The fact that L-NAME can increase lucigenin-enhanced chemiluminescence production in normal vessels is in keeping with the concept that a portion of NO produced in these vessels is tonically inactivated by O$_3^-$.

The fact that L-NAME had minimal effect in the atherosclerotic vessels indicates that sources other than NO synthase almost certainly contribute. One such source is the NADH/NADPH oxidase, which represents a major source of reactive oxygen species in vascular cells and in atherosclerotic vessels. Our data are compatible with the concept that the simultaneous production of both O$_3^-$ (regardless of the source) and NO, leading to peroxynitrite formation and oxidation of tetrahydrobiopterin, could result in uncoupling of eNOS such that O$_3^-$ was formed rather than NO. This would result in a self-propagating condition whereby peroxynitrite formation could promote production of O$_3^-$.

Indeed, this seemed to be the case in our studies of normal vessels exposed briefly to exogenous peroxynitrite. This led to a sustained production of O$_3^-$ that was almost certainly from eNOS, because it could be prevented by either L-NAME or sepiapterin and did not occur when aortic segments from eNOS$^{-/-}$ mice were exposed to peroxynitrite.

Recently, the heme domain of eNOS has been crystallized and the tetrahydrobiopterin-binding site characterized. This region contains paired cysteine residues that coordinate binding of a zinc sulfate, which in turn seems to be critical in maintaining the integrity of tetrahydrobiopterin binding. The authors proposed that ZnS$_5$ release may be controlled by redox status of the cell. Thus, exposure of eNOS to a strong oxidant, such as peroxynitrite, may not only oxidize intracellular tetrahydrobiopterin but also affect tetrahydrobiopterin binding by eNOS. It is also possible that oxidation of cytosolic tetrahydrobiopterin could affect eNOS function by reducing pterin availability for the enzyme. Addition of tetrahydrobiopterin to endothelial cell homogenates is essential to demonstrate optimum activity of eNOS, suggesting that the pterin may be readily lost from the intact enzyme.

By Western analysis, eNOS expression was not reduced but rather was increased in the descending thoracic aortas of apoE$^{-/-}$ mice compared with control aortas. This is in accordance with the finding that liposome-entrapped SOD increased endothelium-dependent vascular relaxation to supernormal levels in these vessels. Recently, we have shown that H$_2$O$_2$, the product of O$_3^-$ dismutation, is also a potent stimulus for eNOS expression. Thus, H$_2$O$_2$ and other factors may increase eNOS expression in endothelial cells overlying atherosclerotic lesions.

Previous work has suggested that inflammatory cytokines, like those found in atherosclerotic lesions, can stimulate expression of cyclohydrolase-1 in a variety of cell types, including endothelial cells. This would lead to higher rather than lower levels of pterins in atherosclerotic vessels. Our studies do not refute this finding but suggest that when the production of reactive oxygen species is increased, excessive oxidation of the pterins may be produced by the induced cyclohydrolase.

Taken together, these findings provide a new mechanism whereby reactive oxygen species can affect endothelial production of NO. In addition to the well-recognized reaction between NO and O$_3^-$, it appears that the radical-radical termination product of the reaction between these 2 species, OONO$^-$, can oxidize a critical cofactor for the NO synthase enzyme tetrahydrobiopterin, leading to uncoupling of the enzyme. In either case, endothelial control of vasomotion would be impaired and would be improved by scavenging of O$_3^-$. It should be noted that tetrahydrobiopterin is also a cofactor for aromatic amino acid hydroxylases, such as phenylalanine hydroxylase, which catalyzes formation of L-tyrosine from L-phenylalanine. Thus, these results may have implications for other aspects of cellular metabolism in conditions in which OONO$^-$ is produced in excess. Finally, oxidation of tetrahydrobiopterin by peroxynitrite may result in uncoupling of eNOS in numerous conditions other than atherosclerosis in which this oxidant is formed.

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