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 (O₂·⁻) 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 (apoE²/²) 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 apoE²/² 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 apoE²/² aortic rings. In vessels from apoE²/² 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 O₂·⁻ 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 O₂·⁻ 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
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. Luminal 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 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) 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 tetrahydrobiopterin was examined, 100 μL of the tetrahydrobiopterin solution was added to 900 μL of deoxygenated Tris buffer in a 1-mL sample cuvette. Buffer without tetrahydrobiopterin was used in the reference cuvette. Absorbance of tetrahydrobiopterin at 297 nm15 was followed for 500 seconds at room temperature. Under these conditions, loss of absorbance represents the tetrahydrobiopterin oxidation after solvation of atmospheric oxygen into the sample and was considered a background tetrahydrobiopterin oxidation. The effect of 1 mmol/L hydrogen peroxide on tetrahydrobiopterin oxidation was examined with catalase (1000 U/mL), Cu/Zn-SOD (100 U/mL), and hydrogen peroxide (1 mmol/L) added to the reference cuvette and hydrogen peroxide with SOD (100 U/mL) to the sample cuvette. To examine the reaction of O2− with tetrahydrobiopterin, KO2 was dissolved in 50 mmol/L NaOH (8 mmol/L stock) and diluted to a final concentration of 800 μmol/L in sample cuvettes containing catalase (1000 U/mL, to scavenge H2O2 formed by dismutation of O2−). Decayed KO2 and catalase (1000 U/mL) were added to the reference cuvette. Peroxynitrite was added for a final concentration of 250 μmol/L in sample cuvettes containing catalase (1000 U/mL) and SOD (100 U/mL). Reference cuvettes for these experiments contained decayed peroxynitrite, SOD, and catalase. Decayed KO2 and OONO− controls were made by adjusting the pH of stock solutions to 7 at 5 minutes before addition to tetrahydrobiopterin. Representative absorbance spectra were obtained before and after 500 seconds of exposure to the various reaction conditions.

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.16,17 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 were compared by ANOVA. When significance was indicated, a Duncan’s multiple range post hoc test was used. The oxidation of tetrahydrobiopterin by various reactive oxygen species was compared by unpaired t tests with a Bonferroni correction for multiple comparisons. Significance was considered present when probability values were <0.05.

Results

Responses to Acetylcholine, the Calcium Ionophore A23187, and Nitroglycerin
For studies of vascular relaxations, vessels were precontracted 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 sepiapterin (10 μmol/L) for 1 hour also improved peak endothelium-dependent vascular relaxation to acetylcholine and A23187 in vessels from apoE−/− mice (Figure 2). In normal vessels, sepiapterin slightly, but not significantly,
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<sup>−/−</sup> Mouse Aortas

As shown in Figure 1, liposome-entrapped SOD increased endothelium-dependent vascular relaxation to levels exceeding that of normal vessels, suggesting that eNOS protein might be increased in apoe<sup>−/−</sup> mouse aortas. Indeed, levels of eNOS protein, as assessed by Western analysis, were significantly greater in apoe<sup>−/−</sup> than in C57Blk/6 aortic homogenates (n=3, Figure 3).

Superoxide Production by Control C57Blk/6 and apoe<sup>−/−</sup> Mouse Aortas

The finding that liposome-entrapped SOD enhanced endothelium-dependent vascular relaxation in aortas of apoe<sup>−/−</sup> mice strongly suggested that production of O<sub>2</sub>·<sup>−</sup> may be increased in these vessels. Estimates of O<sub>2</sub>·<sup>−</sup> production by use of lucigenin-enhanced chemiluminescence confirmed this. As shown in Figure 4, O<sub>2</sub>·<sup>−</sup> production was markedly increased in apoe<sup>−/−</sup> mouse aortas compared with controls. Removal of the endothelium dramatically reduced O<sub>2</sub>·<sup>−</sup> production from apoe<sup>−/−</sup> mouse aortas, while having little effect in control vessels. Exposure of aortic segments from apoe<sup>−/−</sup> mice to sepiapterin (10 μmol/L) for 1 hour before the lucigenin assay reduced O<sub>2</sub>·<sup>−</sup> production in aortas of apoe<sup>−/−</sup> mice, while having no effect in vessels from control C57Blk/6 mice (Figure 4). NG-Nitro-L-arginine methyl ester...
(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 ebselen, 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-
exposed to peroxynitrite (100 μmol/L) 189 versus 2965 ± 377 counts per minute 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 C57B/6 mice (Figure 7).

**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 μmol/L) 21 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 C57B/6 mice (Figure 7).

**Evidence That Peroxynitrite Can Increase O₂⁻• 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 O₂⁻• production in normal mouse aortas. Aortic segments of C57B/6 mice were exposed to peroxynitrite (100 μmol/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 · min⁻¹ · 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 O₂⁻• production, these findings strongly suggest that peroxynitrite produces a long-lasting effect on vascular O₂⁻• 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 O₂⁻• production caused by peroxynitrite, as did treatment of the vessels with sepiapterin. Importantly, peroxynitrite had no effect on O₂⁻• production in vessels from eNOS–/– mice (Figure 8).

**Discussion**

Isometric tension studies of isolated vascular rings showed that both O₂⁻• scavenging and treatment with 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 was more potent than larger concentrations of O₂⁻• or H₂O₂. The relative effectiveness of these various reactive species in oxidizing tetrahydrobiopterin is in keeping with their known redox properties. In the absence of reductants and metals that would catalyze the reduction of H₂O₂ to ·OH, H₂O₂ is relatively stable and is a mild oxidant (E°’ = +320). Likewise, although O₂⁻• has both reducing and oxidizing capacity, in most instances, donation of electrons (reduction) from superoxide rather than oxidation is favored. Our findings are also compatible with a recent report showing that peroxynitrite may oxidize tetrahydrobiopterin.
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$_2^-$ 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 hypochlorous 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$_2^-$ production in apoE$^{-/-}$ mice, suggesting that sources other than eNOS may produce O$_2^-$ in these vessels. Studies using L-NAME to inhibit O$_2^-$ production were difficult to interpret, because L-NAME dramatically increased O$_2^-$ 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$_2^-$. 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$_2^-$ (regardless of the source) and NO, leading to peroxynitrite formation and oxidation of tetrahydrobiopterin, could result in uncoupling of eNOS such that O$_2^-$ was formed rather than NO. This would result in a self-propagating condition whereby peroxynitrite formation could promote production of O$_2^-$. 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$_2^-$ 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 in the presence of a putative substrate, 

\[ \text{Cu}^{2+}_{\text{apo}} + \text{Fe}^{2+} \rightarrow \text{Cu}^{3+}_{\text{apo}} + \text{Fe}^{3+} \]

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References
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