MnSOD Deficiency Increases Endothelial Dysfunction in ApoE-Deficient Mice

Masuo Ohashi, Marshall S. Runge, Frank M. Faraci, Donald D. Heistad

Objective—In mice that are heterozygous for mitochondrial superoxide dismutase (SOD2+/−) with apoE deficiency (apoE−/−), mitochondrial DNA damage increases formation of atherosclerotic lesions. The purpose of this study was to determine whether SOD2 provides protection against increased vascular superoxide and endothelial dysfunction in apoE-deficient mice.

Methods and Results—Four groups of mice [apoE−/−/SOD2+/− (apoelsod2), apoE−/−/SOD2+/+ (apoelsod2), apoE−/−+/SOD2+/− (apoelsod2), and apoE−/−+/SOD2+/+ (apoelsod2)] were fed normal chow diet, and studied at 15 to 17 months of age. Serum cholesterol levels were similar in apoelsod2 and apoelsod2 mice, and also were similar in apoelsod2 and apoelsod2 mice. Intimal area was increased in aorta, but not carotid artery, of apoelsod2 and apoelsod2 mice. In carotid artery, superoxide was increased (67±5.2 relative fluorescence intensity/vessel area [RI] in apoelsod2 mice, 31±3.1 RI in apoelsod2 mice, P<0.05), and relaxation to acetylcholine was impaired in apoelsod2 mice versus apoelsod2, apoelsod2, apoelsod2 mice. Tiron improved relaxation to acetylcholine. In aorta, superoxide levels were increased and relaxation to acetylcholine was impaired in apoelsod2 and apoelsod2 mice, but responses were similar in apoelsod2 and apoelsod2 mice.

Conclusion—SOD2 protects against oxidative stress and endothelial dysfunction in carotid artery of apoE-deficient mice. (Arterioscler Thromb Vasc Biol. 2006;26:2331-2336.)

Key Words: MnSOD ■ apoE ■ endothelial ■ superoxide ■ carotid artery

Mitochondria are biologically important sources and targets for reactive oxygen species.1-2 The mitochondrial isoform of superoxide dismutase (SOD), MnSOD (SOD2), is essential for life, because homozygous mice that are deficient for SOD2 (SOD2−/−) die within days of birth.3 Mice that are heterozygous for SOD2 (SOD2+/−, sod2) have mitochondrial dysfunction and exhibit diminished enzymatic activity of SOD2 that is indicative of oxidative stress.3,4 Consequently, SOD2−/− mice are more susceptible than wild-type mice to oxidative stress, including cerebral5,6 and myocardial ischemia/reperfusion.7 Vasomotor function in aorta of young SOD2−/− mice, however, is not impaired under normal conditions or during some forms of oxidative stress.8

ApoE-deficient mice (apoE−/−, apoe) develop spontaneous hypercholesterolemia and atherosclerosis. Endothelium-dependent relaxation in response to acetylcholine is impaired in the aorta of apoe-deficient mice.9,10

There are 3 isoforms of SOD: SOD2, CuZn-SOD (SOD1, cytosolic), and extracellular SOD (EC-SOD, SOD3).11 Several studies have examined effects of SODs on atherogenesis and endothelial function. In general, the studies have failed to observe protective effects with CuZn-SOD or MnSOD12,13 under pathological conditions, such as reperfusion injury. In addition, overexpression of CuZn-SOD does not affect atherogenesis in mice fed a high fat diet14 or in apoE−−/− mice.15 Arterial EC-SOD also might be expected to protect arteries against atherosclerotic vascular disease.11,16–18 In apoE−−/− mice, however, EC-SOD deficiency had no effect on atherogenesis.19 Furthermore, gene transfer of CuZn-SOD or EC-SOD failed to improve endothelial dysfunction in hypercholesterolemic rabbits.20

ApoE−−/− mice that are also deficient in SOD2 (SOD2−−/−) exhibit early increases in mitochondrial DNA damage and accelerated atherogenesis at arterial branch points.21 In this study, we tested the hypothesis that SOD2 may protect against oxidative stress and vasomotor function in apoE−−/− mice. We examined vasomotor function and superoxide levels in carotid artery and aorta of apoE−−/−/SOD2−−/− mice at 15 to 17 months of age, when they had developed moderately severe atherosclerosis in the aorta.
Methods

Mice
CS7BL/J6 control and apoE<sup>+/−</sup> (apoE, CS7BL/J6 background) mice were purchased from Jackson Laboratories (Bar Harbor, Me). We bred pairs of apoE or CS7BL/J6 mice to obtain 3-week-old apoE<sup>+/−</sup> or control pups. SOD2<sup>+/−</sup> mice (sod2), which have been described previously, were backcrossed at least 8 times into the CS7BL/J6 background. apoE<sup>+/−</sup>/SOD2<sup>+/−</sup> (apoE/sod2) animals were generated by crossing SOD2<sup>+/−</sup> and apoE<sup>+/−</sup> mice (F0). F1 double heterozygotes were backcrossed with apoE<sup>+/−</sup> mice to generate appropriate F2 breeders (apoE/sod2). Genotypes were determined through polymerase chain reaction (PCR) analysis of tail clips. SOD2<sup>+/−</sup> mice were used as breeders because SOD2<sup>−/−</sup> animals die within a few weeks after birth.

We studied 4 groups of mice: (1) apoE<sup>+/−</sup>/SOD2<sup>+/−</sup> (apoE/sod2), (2) apoE<sup>−/−</sup>/SOD2<sup>−/−</sup> (apoE/sod2), (3) apoE<sup>+/−</sup>/SOD2<sup>−/−</sup> (apoE/sod2), and (4) apoE<sup>−/−</sup>/SOD2<sup>−/−</sup> (apoE/sod2). Male and female mice were fed normal chow diet and were studied at 15 to 17 months of age. Experiments were conducted in accordance with guidelines of the Animal Care and Use Committee of the University of Iowa.

Mice were killed with an intraperitoneal injection of pentobarbital sodium (150 mg/kg). Common carotid arteries, aortic arch, and descending thoracic aorta were removed. Loosely adhering adventitia was removed, and vessels were kept in Krebs buffer (4°C) containing (in mmol/L) NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 23, KH<sub>2</sub>PO<sub>4</sub> 1.2, and D-glucose 11. Blood samples were taken for measurement of serum cholesterol. Serum total cholesterol was measured enzymatically (Cholesterol Liquid Stable Reagent, Infinity).

Histology and Morphometric Analysis
Sections of carotid artery, aortic arch, and descending aorta were fixed in formalin, embedded in paraffin, and stained with Verhoeff-van Gieson stain. Intimal area was measured as described previously. Morphometric analysis was performed in vascular segments that avoided arterial branches.

Vascular Function
Rings of carotid artery, aortic arch, and descending thoracic aorta were studied in organ chambers. Vascular rings were obtained from segments without arterial branches. Vascular rings (3 to 4 mm length) were mounted on a pair of triangular hooks and suspended in individual organ chambers containing 20 mL Krebs solution maintained at 37°C and bubbled continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The rings were connected to a force transducer to measure isometric tension (contraction and relaxation). Resting tension was increased stepwise to reach final resting tension of 0.25g (carotid artery) and 0.5g (aortic arch or descending thoracic).

After equilibration, we measured responses to the thromboxane analogue 9,11-dideoxy-11a,9α-epoxy-methanoprostaglandin F<sub>2</sub> (U46619, 1 nmol/L to 300 nmol/L) to determine the maximal contractile response of each vessel. Vessels were then contracted submaximally (50% to 60% of maximum) with U46619. After reaching a stable contraction plateau, we measured responses to acetylcholine (0.1 nmol/L to 300 nmol/L) and chemiluminescence was normalized to the cross-sectional area of the vessel wall. Fresh unfixed vessel segments were then incubated in OCT compound until detection of O<sub>2</sub>−. Transverse sections (30 μm thick) were cut in a cryostat and placed on glass slides. Samples were then incubated at room temperature for 30 minutes with DHE (2 μmol/L) and protected from light. Images were obtained using a Bio-Rad MRC-1024 laser (krypton/argon) scanning confocal microscope. The fluorescence excitation/emission spectrum for ethidium bromide was used during the imaging process (488 and 610 nm, respectively). Fluorescence was detected with a 585-nm long-pass filter. Relative increases in ethidium bromide fluorescence were determined using ImageJ software for PC (version 1.32) as previously described.

Dihydroethidine (DHE), an oxidative fluorescent dye, was used to localize O<sub>2</sub>−, as described previously, in layers of carotid artery, aortic arch, descending thoracic aorta in situ. Ethidium bromide fluorescence was normalized to the cross-sectional area of the vessel wall for each section. Fresh unfixed vessel segments were then incubated at room temperature for 30 minutes with DHE (2 μmol/L) and protected from light. Images were obtained using a Bio-Rad MRC-1024 laser (krypton/argon) scanning confocal microscope. The fluorescence excitation/emission spectrum for ethidium bromide was used during the imaging process (488 and 610 nm, respectively). Fluorescence was detected with a 585-nm long-pass filter. Relative increases in ethidium bromide fluorescence were determined using ImageJ software for PC (version 1.32) as previously described.

Drugs
Acetylcholine, nitroprusside, and Tiron were obtained from Sigma. Acetylcholine and nitroprusside were dissolved in saline, and Tiron directly in Krebs solution. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilution being made with saline. Final concentration of ethanol was <0.01%. Hydroethidine was obtained from Molecular Probes and dissolved in DMSO at a concentration of 0.1 mol/L.

Statistical Analysis
All data are expressed as means±SE. Relaxation to acetylcholine and nitroprusside is expressed as percent relaxation to U46619-induced contraction. Comparison of relaxation was made using ANOVA followed by Bonferroni multiple comparison test. Statistical significance was accepted at P<0.05.

Results

Serum Cholesterol and Atherosclerotic Lesions
Serum cholesterol level was higher in apoE<sup>+/−</sup> mice than in apoE<sup>−/−</sup> mice (Figure 1a). There was no difference in serum cholesterol level between SOD2<sup>+/−</sup> and SOD2<sup>−/−</sup> mice.

Intimal area was increased in apoE<sup>+/−</sup> mice in aortic arch and descending thoracic aorta (Figure 1b). There was, however, no significant difference in intimal area of the aorta between apoE/sod2 and apoE/SOD2 mice (Figure 1b). In carotid artery, intimal area was increased in only a few mice (2 of 9 in apoE/sod2 and 1 of 7 in apoE/SOD2 mice), and there were no significant differences in intimal area of carotid artery between the four groups of mice. No apoE<sup>+/−</sup> mice (either SOD2<sup>−/−</sup> or SOD2<sup>+/−</sup>) had a detectable intimal area in aortic arch, descending thoracic aorta, or carotid artery.

Vasomotor Function
Contraction to U46619 (10<sup>−9</sup> to 10<sup>−7</sup> mol/L) was similar in carotid artery of apoE/sod2, apoE/SOD2, apoE/sod2, and wild-type mice (Figure 2a). In aortic arch and descending aorta, contractile responses to U46619 were greater in apoE<sup>+/−</sup> mice than in apoE<sup>−/−</sup> mice (Figure 2b and 2e). Contraction to U46619 was not different between SOD2<sup>−/−</sup> and SOD2<sup>+/−</sup> mice in carotid artery and aorta.

In carotid artery, relaxation to acetylcholine was impaired only in apoE/sod2 mice (Figure 3a). In aortic arch (Figure 3b) and descending thoracic aorta (Figure 3c), responses to acetylcholine were impaired in apoE/sod2 and apoE/SOD2 mice, and there were no differences between apoE/sod2 and apoE/SOD2 mice. In apoE/sod2 and apoE/SOD2 mice, re-
laxation to acetylcholine was similar in carotid artery, aortic arch, and descending thoracic aorta.

Tiron (10 mmol/L) improved relaxation to acetylcholine in apoe/sod2 and apoe/SOD2 mice in carotid artery (Figure 4a). Tiron, however, did not improve relaxation to acetylcholine in aorta of apoe/sod2 and apoe/SOD2 mice (Figure 4b and 4c). Tiron did not affect relaxation to acetylcholine in carotid artery, aortic arch, or descending aorta of apoE/sod2 and apoE/SOD2 mice (data not shown).

There were no significant differences in relaxation to nitroprusside in carotid artery, aortic arch, and descending thoracic aorta in the 4 groups of mice although responses tended to be impaired in apoE/sod2 and apoE/SOD2 mice (Figure 5).

**Superoxide Levels in Vessels**

Basal levels of superoxide, as measured using lucigenin-enhanced chemiluminescence, were approximately 2-fold higher ($P<0.05$) in aorta of apoE/sod2 mice compared with levels in aorta of apoE/SOD2, and apoE mice (supplemental Figure I, available online at http://atvb.ahajournals.org).

In carotid artery and aorta, superoxide levels (hydroethidine) tended to be higher in apoE/sod2 and apoE/SOD2 mice than in apoE/sod2 and apoE/SOD2 (Figure 6). In carotid artery, superoxide levels were higher in apoE/sod2 mice compared with apoE/SOD2, apoE/sod2, and wild-type mice by quantification of dihydroethidine bromide fluorescence (based on relative difference in fluorescent intensity) ($P<0.05$, supplemental Figure I). There were no significant differences in aorta among these groups.

**Discussion**

There are two major novel findings in this study. First, in carotid artery, relaxation to acetylcholine was impaired more in apoe/sod2 mice than apoe/SOD2 mice, although serum cholesterol level and intimal area were not different. SOD2 deficiency did not affect acetylcholine-induced relaxation in apoE$^{+/+}$ mice. Tiron improved relaxation to acetylcholine in carotid artery of apoE$^{-/-}$ mice. Second, superoxide levels were higher in carotid artery of apoE$^{-/-}$ mice than apoE/SOD2 or apoE$^{+/+}$ mice. Taken together, the findings suggest that SOD2 deficiency contributes to elevated superoxide levels and endothelial dysfunction in carotid artery of apoE$^{-/-}$ mice, but not in apoE mice.
In contrast to findings in carotid artery, relaxation to acetylcholine and contraction to U46619 were impaired in aortic arch and descending aorta of \textit{apoe} mice, but there were no differences in impairment of relaxations to acetylcholine between \textit{apoe/sod2} and \textit{apoe/SOD2} mice, and Tiron did not improve relaxation to acetylcholine, although superoxide levels detected by lucigenin were increased in \textit{apoe/sod2} mice.

We studied mice that were 15 to 17 months old, which were fed normal chow. Aging produces oxidative stress to mitochondria, and SOD2 appears to protect against organ damage during aging.\textsuperscript{29} Most previous studies used mice that were much younger (typically 4 to 6 months old), and the mice were fed a high fat diet. Thus, the conditions in this study are closer to those observed in older humans with hypercholesterolemia.

**Endothelial Dysfunction in Apoe/Sod2 Mice**

The present findings suggest that deficiency of SOD2 affects endothelial function when oxidative stress is augmented by apoE deficiency, in carotid artery, even without detectable atherosclerosis.

Aorta develops mitochondrial dysfunction and atherosclerosis (especially at the branch points) earlier in \textit{apoe/sod2} mice than in \textit{apoe/SOD2} mice.\textsuperscript{21} In the present study of 15-month-old mice, there were, however, no significant differences in intimal area of aorta between \textit{apoe/sod2} and \textit{apoe/SOD2} mice. In aorta of apoE\textsuperscript{-/-} mice, there were no significant differences in impairment of relaxation to acetylcholine between \textit{apoe/sod2} and \textit{apoe/SOD2} mice. A possible explanation for differences in morphometric findings in the previous\textsuperscript{21} and present study is that, in this study, vascular branches were avoided in morphometric analysis.

In mice, atherosclerosis in aortic arch and thoracic aorta is more severe, especially at branch points, than atherosclerosis in carotid artery.\textsuperscript{30} In the present study, contractile responses to U46619 were also decreased in aorta of apoE\textsuperscript{-/-} mice, although contraction was not impaired in carotid artery of these mice. Severe atherosclerosis in apoE\textsuperscript{-/-} mice has been observed previously to impair contractile responses to U46619.\textsuperscript{10} Responses to acetylcholine reflect severity of atherosclerosis in monkey\textsuperscript{31} and carotid arteries of mice.\textsuperscript{24} In the present study, there is a weak, but significant, correlation between intimal area and maximum relaxations to acetylcholine (10 \textmu mol/L) in aortic
arch and descending aorta of apoe/sod2, apoe/SOD2, apoE/sod2, and apoE/SOD2 mice (supplemental Figure II). There was no significant correlation in carotid artery, perhaps because intimal proliferation was present in only a few mice. These data suggest that there is a modest relation between severity of atherosclerosis and abnormal vasomotor responses to acetylcholine, as in previous reports.24,31 We speculate that differences in responses to acetylcholine in carotid artery and aorta of apoe/sod2 mice may reflect differences in severity of atherosclerosis.

Vascular Superoxide in Apoe/Sod2 Mice
There were no significant increases in superoxide in carotid artery and aorta of SOD2-deficient apoE\(^{+/+}\) mice. This finding is consistent with previous findings in aorta of SOD2-deficient mice.8 In the present study, however, we observe in apoe/sod2 mice an increase in levels of superoxide in carotid artery (detected by hydroethidine) and in aorta (detected by lucigenin chemiluminescence). Thus, SOD2 appears to be an important determinant of superoxide levels in vessels of apoE-deficient mice.

Increases in superoxide levels produce endothelial dysfunction in apoE-deficient mice.8 Tiron improved relaxation to acetylcholine in apoe/sod2 and apoe/SOD2 mice in the present study. Thus, it is likely that increased levels of superoxide in apoe/sod2 contribute to impaired relaxation to acetylcholine in carotid artery. Even after Tiron, however, responses to acetylcholine were less in apoe/sod2 mice than in apoe/SOD2 mice. One possible interpretation of the failure of Tiron to restore normal responses is that chronic mitochondrial damage by deficiency of SOD2 may produce irreversible changes. We used 10 mmol/L Tiron to scavenge superoxide in present study, and considered the possibility that this dose of Tiron may not be effective. The same dose of Tiron, however, improves endothelial dysfunction in cerebral arteries in mice treated with angiotensin II,32 in hyperhomocysteinemic mice,33 and in aorta in hypertensive mice.34 A lower concentration of Tiron (1 mmol/L) also reduced vascular superoxide in mouse aorta, and restored endothelial function in mouse carotid artery.35 Thus, SOD2 deficiency appears to be responsible for endothelial dysfunction by increasing superoxide and causing chronic mitochondrial damage in apoE\(^{+/+}\) mice.

Superoxide levels detected by hydroethidine in aorta, in contrast to carotid artery, did not change significantly in the present study, although superoxide levels in aorta detected by lucigenin chemiluminescence were increased in apoe/sod2 mice. Reasons for the discrepancy between findings with lucigenin chemiluminescence and hydroethidine fluorescence are not clear. There are several limitations in both methods. Lucigenin chemiluminescence detects superoxide in endothelium, but is not as sensitive in detection of superoxide in deeper layers of the vessel wall. There is some quenching of photons emitted within the media, before they reach the photomultiplier tube.20 Perhaps quenching of photons by the vessel wall is greater in the thickened atherosclerotic vessel. There are, however, also important limitations in estimation of vascular superoxide with dihydroethidine. The method allows detection of superoxide in the vessel wall in situ, but

Figure 5. Relaxation to sodium nitroprusside of carotid artery (a), aortic arch (b), and descending aorta (c) from apoe/sod2 (n=8), apoe/SOD2 (n=5), apoE/sod2 (n=7), and wild-type (n=6) mice. Values are mean±SE.

Figure 6. Confocal fluorescent sections of carotid arteries (upper row), and thoracic aorta (lower row) from apoe/sod2, apoe/SOD2, apoE/sod2, and wild-type mice. These sections are representative of vessels from 7 to 10 mice in each group. Vessels were incubated with hydroethidine for detection of superoxide in situ.
should be viewed as an estimate of superoxide, rather than a precise measurement.

In conclusion, SOD2 protects against oxidative stress and endothelial dysfunction in apoE-deficient mice. We conclude that a genetic determinant of levels of reactive oxygen species, in addition to hypercholesterolemia, may be a risk factor for endothelial dysfunction during atherosclerosis.

Acknowledgments
We thank Drs Yoshimasa Watanabe and Jon J. Andresen for assistance with confocal microscopy, and Arlinda LaRose for typing the manuscript.

Tompkins for technical assistance with confocal microscopy, and

Sources of Funding
This work was supported by National Institutes of Health grants HL 62984, HL 16066, NS 24621, HL14388, HL 38901, DK 54759, DK 15843, DK 52617, HL 55006, funds provided by the Veterans Affairs Medical Service, and a Carver Trust Research Program of Excellence.

Disclosures
None.

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*Arterioscler Thromb Vasc Biol.* 2006;26:2331-2336; originally published online July 27, 2006; doi: 10.1161/01.ATV.0000238347.77590.c9

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure I

A. aorta

B. a. carotid artery

b. aortic arch

c. thoracic aorta

<table>
<thead>
<tr>
<th>apoE</th>
<th>SOD2</th>
<th>+/+</th>
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*RLU/mg*
a. carotid artery

b. aortic arch

c. descending aorta

Figure II
Figure I
Vascular superoxide levels by lucigenin chemiluminescence (A) in aortas of \textit{apoel/sod2} (n=9), \textit{apoel/SOD2} (n=6), \textit{apoE/sod2} (n=8) and wild type (n=7) mice, and by hydroethidine-based confocal microscopy (B) in carotid artery (Ba), aortic arch (Bb), and thoracic aorta (Bc) from \textit{apoel/sod2} (n=10), \textit{apoel/SOD2} (n=8), \textit{apoE/sod2} (n=9) and wild type (n=7) mice. Values are mean ± SE; *P<0.05 vs \textit{apoE/SOD2}. †P<0.05 vs wild type.

Figure II
Correlations between maximum relaxations to acetylcholine and intimal area in carotid (a), aortic arch (b), and thoracic aorta (c) from \textit{apoel/sod2} (n=8), \textit{apoel/SOD2} (n=6), \textit{apoE/sod2} (n=9) and wild type (n=6) mice.