Mechanism of Endothelial Dysfunction in Apolipoprotein E–Deficient Mice
Livius V. d’Uscio, Timothy A. Baker, Carlos B. Mantilla, Leslie Smith, Deborah Weiler, Gary C. Sieck, Zvonimir S. Katusic

Abstract—Endothelium-dependent relaxations mediated by NO are impaired in a mouse model of human atherosclerosis. Our objective was to characterize the mechanisms underlying endothelial dysfunction in aortas of apolipoprotein E (apoE)-deficient mice, treated for 26 to 29 weeks with a lipid-rich Western-type diet. Aortic rings from apoE-deficient mice showed impaired endothelium-dependent relaxations to acetylcholine (10⁻⁹ to 10⁻⁵ mol/L) and Ca²⁺ ionophore (10⁻⁹ to 10⁻⁶ mol/L) and endothelium-independent relaxations to diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate (DEA-NONOate, 10⁻¹⁰ to 10⁻⁵ mol/L) compared with aortic rings from C57BL/6J mice (P<0.05). By use of confocal microscopy of an oxidative fluorescent probe (dihydroethidium), increased superoxide anion (O₂⁻) production was demonstrated throughout the aortic wall but mainly in smooth muscle cells of apoE-deficient mice. CuZn–superoxide dismutase (SOD) and Mn-SOD protein expressions were unaltered in the aorta exposed to hypercholesterolemia. A cell-permeable SOD mimetic, Mn(III) tetra(4-benzoic acid) porphyrin chloride (10⁻⁵ mol/L), reduced O₂⁻ production and partially normalized relaxations to acetylcholine and DEA-NONOate in apoE-deficient mice (P<0.05). [¹⁴CJ]-Citrulline assay showed a decrease of Ca²⁺-dependent NOS activity in aortas from apoE-deficient mice compared with C57BL/6J mice (P<0.05), whereas NO synthase protein expression was unchanged. In addition, cGMP levels were significantly reduced in the aortas of apoE-deficient mice (P<0.05). Our results demonstrate that in apoE-deficient mice on a Western-type fat diet, impairment of endothelial function is caused by increased production of O₂⁻ and reduced endothelial NO synthase enzyme activity. Thus, chemical inactivation of NO with O₂⁻ and reduced biosynthesis of NO are key mechanisms responsible for endothelial dysfunction in aortas of atherosclerotic apoE-deficient mice. (Arterioscler Thromb Vasc Biol. 2001;21:1017-1022.)

Key Words: endothelium n nitric oxide n superoxide anion n apolipoprotein E n atherosclerosis

Atherosclerosis is a chronic process, which can be triggered by cardiovascular risk factors such as hypercholesterolemia, aging, hypertension, and diabetes mellitus. Endothelium-derived vasoactive factors play an important regulatory role in vascular homeostasis and pathogenesis of atherosclerosis because of the strategic position of the endothelium between the vascular smooth muscle cells (VSMCs) and the circulating blood.

NO is a potent vasodilator that is formed in endothelial cells from L-arginine by endothelial NO synthase (eNOS), which is constitutively expressed.⁴–⁶ NO production is activated by the stimulation of cell surface receptors or by mechanical forces such as shear stress.⁷,⁸ Accumulating evidence suggests that alterations in the NO pathway play a central role in endothelial dysfunction induced by hypercholesterolemia. This may be of major importance inasmuch as NO can substantially inhibit several components of the atherogenic process, such as VSMC contraction and proliferation, platelet aggregation, and monocyte adhesion.⁹,¹⁰ Previous studies identified 3 mechanisms responsible for reduced bioavailability of NO in arteries exposed to hypercholesterolemia: (1) enhanced degradation of NO by superoxide anions (O₂⁻), (2) functional abnormalities of NO synthase (NOS) due to deficiency of substrate or cofactor,¹²,¹³ and (3) alteration in eNOS activity and/or protein expression.¹⁴–¹⁶

Mice homozygous for the inactivated apoE gene provide a new model of human atherosclerosis. These mice develop spontaneous hypercholesterolemia and aortic atherosclerosis, which can be accelerated by a lipid-rich Western-type diet.¹⁷–¹⁹ Indeed, impaired endothelium-dependent relaxation in response to acetylcholine (ACh) has been observed in the aortas of apoE-deficient mice on a Western-type diet but not on a normal diet.²⁰,²¹ However, the exact mechanisms of altered endothelial function (ie, role of O₂⁻, eNOS expression, and eNOS activity) have not been determined in this animal model of human atherosclerosis.

Methods

Experimental Animals
Male C57BL/6J (control) mice and homozygous apoE-deficient mice (4 to 5 weeks old) were obtained from The Jackson Laborato-
Experimental Setup

Isolated aortic rings from C57BL/6J and apoE-deficient mice were studied in parallel. Rings were connected to a force transducer for recording of isometric force and placed in organ baths filled with 25 mL Krebs’ solution (37°C, 94% O2/6% CO2, pH 7.4). After an equilibration period of 30 minutes, the rings were progressively stretched to their optimal passive tension as assessed by the response to 100 mmol/L KCl. Concentration-dependent response curves to ACh (10⁻⁹ to 10⁻³ mol/L), Ca²⁺ ionophore (A23187, 10⁻⁹ to 10⁻⁵ mol/L), and diethylammonium (Z)-1-(N,N-diethy lamino) diazen-1-ium-1,2-diolate (DEA-NONOate, 10⁻¹⁰ to 10⁻⁴ mol/L) were obtained. In a separate protocol, aortic rings were preincubated with or without the cyclooxygenase inhibitor indomethacin (10⁻⁷ mol/L) and the cell-permeable SOD mimetic Krebs-Ringer bicarbonate solution (mmol/L: NaCl 118.6, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.1, EDTA 0.026, and glucose 10.1). The aorta was cut into 4-mm rings (proximal, distal thoracic, and first part of abdominal aorta).

Oxidative Fluorescence Microscopy

The oxidative fluorescent dye dihydroethidium (Molecular Probes) was used as described previously. Unfixed frozen rings of distal thoracic aortic segments were cut into 30-μm-thick sections and placed on a glass slide. Samples were incubated with dihydroethidium (2×10⁻⁴ mol/L) in a light-protected humidified chamber at 37°C for 30 minutes. Tissue sections were imaged by us of an Olympus Fluoview laser scanning confocal microscope.

Quantification of Vascular O₂⁻ Production

Lucigenin (5 μmol/L, Molecular Probes) was used to measure O₂⁻ levels in the aorta as described previously. The results were expressed as counts per minute per microgram dry weight.

Measurement of Ca²⁺-Dependent NOS Enzyme Activity

[³⁵S] Citrulline formation was measured by using a liquid scintillation counter (Beckman Instruments) as described. Briefly, 4 whole aortas (n=1 experiment) were homogenized on ice in lysis buffer (Sigma Chemical Co). After centrifugation, equal amounts of total protein were added to the enzymatic reactions.

Western Blot Analysis

Mouse monoclonal anti-eNOS (Transduction), sheep polyclonal anti-von Willebrand factor (vWF, Cedarlane), and rabbit polyclonal anti-Cu/Zn-SOD and anti-Mn-SOD (StressGen) were used. For actin, blots were rehybridized with monoclonal anti-actin (Sigma). Densitometry was carried out by using NIH Image, and the results were expressed as optical density (OD) per square millimeter aortic surface (eNOS and vWF) or relative to the respective intensity of the actin blot (Cu/Zn-SOD and Mn-SOD).

Measurements of cGMP and cAMP

After homogenization, cGMP and cAMP radioimmunoassay kits (Amersham) were used as described.

Calculations and Statistical Analysis

Results are given as mean±SEM. The concentration-response curves of the different groups were compared by ANOVA for repeated measurements, followed by the Bonferroni correction. For simple comparison between 2 values, a paired or unpaired Student t test was used, where appropriate. A value of P<0.05 was considered significant.

An expanded Materials and Methods section can be found in an online data supplement, which can be accessed at http://atvb.ahajournals.org.

Results

Vascular Relaxations

In the aortas of apoE-deficient and C57BL/6J mice, endothelium-dependent relaxation in response to ACh was completely blocked by the NOS inhibitor L-NAME (0±1% and 1±1% for maximal relaxations, respectively; n=5). NO-mediated endothelium-dependent relaxations were reduced in apoE-deficient mice (P<0.0001 versus C57BL/6J for maximal relaxation, Figure 1A). The sensitivity (pD₂) to ACh was significantly shifted to the right in apoE mice (P<0.05) compared with C57BL/6J mice (pD₂ 7.0±0.3 for C57BL/6J, pD₂ 7.4±0.1 for apoE, Figure 1B). Preincubation with SOD (75 U/mL) had no effect on relaxations to ACh in apoE-deficient and control mice (61±6% versus 59±3% and 91±2% versus 88±1% with and without SOD, respectively). In addition, indomethacin (10⁻⁵ mol/L) did not affect ACh-induced relaxations in either group (64±4% for apoE-deficient mice and 93±2% for control mice, n=5 or 6).

A23187 also caused endothelium-dependent relaxations, which were impaired in the aortas of apoE-deficient mice compared with C57BL/6J mice (P<0.05, Figure 1B).

Endothelium-independent relaxations to the NO donor DEA-NONOate were reduced, and the concentration-response curve was shifted 3-fold to the right in apoE-deficient mice (P<0.05 versus C57BL/6J mice, Figure 1C; n=5). Maximal relaxations were unaltered.

Constrictions of VSMCs

Constrictions to 100 mmol/L KCl did not differ among control mice (1.4±0.05 mN/mm) and apoE-deficient mice (1.35±0.03 mN/mm).
Concentration-dependent contractions to phenylephrine were unchanged in apoE-deficient mice compared with C57BL/6J mice. Maximal contraction was 90±3% in apoE-deficient mice and 82±8% in control mice, and pD₂ was 6.9±0.1 and 7.0±0.1, respectively (n=6 to 8, P=NS).

**Effect of SOD Mimetic**

A novel cell-permeable SOD mimetic MnTBAP (10⁻⁵ mol/L) significantly improved endothelium-dependent relaxation to Ach in aortas from apoE-deficient mice compared with untreated aortic rings from apoE-deficient mice (P<0.05, ANOVA+Bonferroni). Note that relaxations were still impaired compared with C57BL/6J mice (P<0.05, ANOVA+Bonferroni). MnTBAP did not affect relaxations to DEA-NONOate in aortas of apoE-deficient mice compared with control C57BL/6J mice (P=0.2, Figure 4A). Treatment of aortas with MnTBAP (10⁻⁵ mol/L) reduced O₂⁻ levels in apoE-deficient mice (P<0.05). MnTBAP had no effect on sensitivity or maximal relaxations to Ach in C57BL/6J mice.

MnTBAP also significantly improved endothelium-independent relaxations to DEA-NONOate in aortas of apoE-deficient mice (pD₂ 7.7±0.1 versus 7.2±0.1, P<0.05 for aorta with MnTBAP versus aorta without MnTBAP; Figure 2B). MnTBAP did not affect relaxations to DEA-NONOate in C57BL/6J mice (pD₂ 8.1±0.1, P=NS).

**Vascular O₂⁻ Production**

After loading with the oxidation-sensitive dye dihydroethidium, a marked increase in ethidium bromide (EtBr) fluorescence was found throughout the vascular wall of apoE-deficient mouse aorta, which reflected an increase in O₂⁻ (Figure 3B) compared with O₂⁻ in C57BL/6J mice (Figure 3A). The increase in EtBr fluorescence was observed mainly in VSMCs but also in endothelial cells and atheromatous plaques (Figure 3B). MnTBAP reduced EtBr fluorescence (Figure 3D) in apoE-deficient mice compared with C57BL/6J mice (Figure 3C). Interestingly, MnTBAP reduced the increase in EtBr fluorescence, not only in endothelial cells but also in VSMCs and atheromatous plaques of aortas from apoE-deficient mice (Figure 3D).

**Western Blot Analysis**

Expressions of cytosolic CuZn-SOD and Mn-SOD proteins were not different between apoE-deficient and C57BL/6J mice (n=3, Figure 4B and 4C). In addition, eNOS expression was not altered in apoE-deficient mice (n=3, Figure 5A). Interestingly, vWF protein expression was increased in aortas of apoE-deficient mice (3.9±0.2 versus 2.6±0.2 OD/mm² [P<0.05] for apoE-deficient mice versus control mice, respectively; n=3).

**Ca²⁺-Dependent NOS Activity**

We measured Ca²⁺-dependent NOS activity in the aortas of apoE-deficient and C57BL/6J mice by assaying the conversion of [¹⁴C]L-arginine to [¹⁴C]L-citrulline in tissue homogenates. Aortas from apoE-deficient mice showed 2.3-fold less NOS enzyme activity than aortas from C57BL/6J mice (P<0.05, Figure 5B).

**cGMP and cAMP Levels**

Basal cGMP level was reduced in aortas from apoE-deficient mice compared with C57BL/6J mice (P<0.05, Figure 6A), but reduced fluorescence in apoE-deficient mice (D).
whereas basal cAMP levels were not different (n=10, P=NS; Figure 6B).

Discussion

There are several novel findings in the present study. The cell-permeable SOD mimetic MnTBAP reduced O$_2^-$ formation and augmented endothelium-dependent and endothelium-independent relaxations mediated by NO. Increased O$_2^-$ production was found in endothelial cells, VSMCs, and atheromatous plaques of apoE-deficient aorta. Increased breakdown of NO by O$_2^-$ appears to be the key mechanism responsible for the decrease in biological activity of NO. This was reflected in selective impairment of cGMP production in aortic walls of apoE-deficient mice.

One of the hallmarks of atherosclerosis is impairment of endothelial function, which is present even before vascular structural changes occur. Indeed, endothelial dysfunction is a common feature in subjects with cardiovascular risk factors, suggesting a role for the initiation of pathological changes in atherosclerosis. Treatment with the SOD mimic MnTBAP, but not SOD, normalized in part endothelium-dependent relaxations in the aorta of apoE-deficient mice, suggesting that excess production of free radicals throughout the aortic wall is responsible for the breakdown of NO.

It is known that superoxide radicals react with NO more rapidly to form peroxynitrite [k = 6.7 x 10$^9$ (mol/L)$^{-1}$ · s$^{-1}$] than with SOD [k ≈ 2 x 10$^7$ (mol/L)$^{-1}$ · s$^{-1}$], which converts O$_2^-$ to hydrogen peroxide. MnTBAP is a cell-permeable Mn$^{2+}$ (III)–containing metalloporphyrin that is able to catalyze the dismutation of O$_2^-$ with a rate constant of ≈ 10$^7$ (mol/L)$^{-1}$ · s$^{-1}$, which converts O$_2^-$ to hydrogen peroxide. Moreover, Mn$^{2+}$ (III)–containing SOD mimetics have been reported to exhibit a catalase-like activity, converting hydrogen peroxide to water and oxygen, and they are also reported to be scavengers of peroxynitrite. Importantly, in contrast to other metalloporphyrins, MnTBAP did not inhibit vascular relaxations in response to NO, deficienct mice on a Western-type diet. This is in line with the study in humans, which demonstrated a reduction of the initial rate of NO release in advanced atherosclerosis. Treatment with the SOD mimetic MnTBAP, but not SOD, normalized in part endothelium-dependent relaxations in the aorta of apoE-deficient mice, suggesting that excess production of free radicals throughout the aortic wall is responsible for the breakdown of NO.

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suggesting that MnTBAP is not a scavenger of NO. In the present study with apoE-deficient mouse aortas, MnTBAP was able to reduce the increased O$_2^-$ production in endothelial cells, VSMCs, and atheromatous plaques as detected by lucigenin-enhanced chemiluminescence and EtBr fluorescence. Thus, the ability of MnTBAP to scavenge O$_2^-$ may explain the improvement of endothelial function in apoE-deficient mice. Accordingly, treatment of hypercholesterolemic rabbits with polyethylene-glycolated SOD, but not native SOD, has been shown to restore, in part, endothelium-dependent relaxations in rabbit atherosclerotic aorta. In addition, ex vivo gene transfer of CuZn-SOD and extracellular SOD reduced production of O$_2^-$ in endothelium but not in VSMCs and failed to improve impaired relaxations to ACh in rabbit aortas.

Cytosolic CuZn-SOD and Mn-SOD protein levels were unaltered in the aortas of apoE-deficient mice compared with control mice despite increased O$_2^-$ production. These findings are consistent with results reported by Fukai et al (1998). They showed an increase of extracellular SOD activity in aortic macrophages, whereas the activities of cytosolic CuZn-SOD and Mn-SOD were unaltered in aortas of apoE-deficient mice. These findings suggest that reduced SOD expression is not responsible for the increased formation of O$_2^-$, and they are in agreement with the most recent study. In addition, the cyclooxygenase pathway is unlikely to be a source of O$_2^-$ production because indomethacin did not affect endothelium-dependent relaxations in the aorta of apoE-deficient mice. The exact source of O$_2^-$ in apoE-deficient mouse aortas remains to be determined.

Because endothelial function in the presence of MnTBAP was not completely normalized, we investigated whether other major mechanisms in the alteration of the NO pathway may be involved. Indeed, we found a reduction of Ca$^{2+}$-dependent NOS enzyme activity in the aortas of apoE-deficient mice compared with aortas of control mice (see below), whereas Western blot analysis showed no change in eNOS protein expression. These findings were in contrast to studies on human arteries in which a reduction of immunoreactive eNOS in luminal endothelial cells was found. On the other hand, an increase of eNOS protein expression and mRNA in the atherosclerotic aortas of rabbits was found despite enhanced O$_2^-$ production and impaired endothelium-dependent relaxations. The discrepancy may be related to the differential duration of the high cholesterol diet or to species differences or duration of the atherosclerotic process, ie, months in experimental animals versus decades in patients.

Deficiency of a substrate L-arginine may also lead to the reduced eNOS enzyme activity. However, chronic treatment with the NO substrate L-arginine had no effect on impaired endothelial function in apoE-deficient mice, suggesting that the endothelial dysfunction found in apoE-deficient mice is not due to the deficiency of the substrate L-arginine. Endothelium-dependent relaxations to ACh and A23187 were impaired, indicating that reduced Ca$^{2+}$-dependent NOS activity rather than impairment of receptor-mediated signal transduction mechanisms is an important component responsible for endothelial dysfunction in apoE aortas. In addition, under in vivo conditions, several mechanisms may contribute to decreased eNOS activity. Chronic inhibition of the enzyme by free radicals or endogenous antagonists of NO, such as asymmetric dimethyl arginine, may reduce the activity of eNOS in atherosclerotic apoE-deficient mouse aortas.

The observed alteration in vascular responses may be dependent not only on the reduced availability of endothelium-derived relaxing factor(s) but also on the altered responsiveness of VSMCs to NO. Indeed, we found that basal cGMP levels were reduced in the aortas of apoE-deficient mice, although tissue cAMP levels were not different, indicating a selective loss of cGMP-dependent vascular function. Most importantly, relaxation of VSMCs to NO was impaired; the exact mechanism of VSMC dysfunction is unknown. This phenomenon may contribute to abnormal endothelium-dependent relaxation mediated by NO in atherosclerosis and is consistent with results of the previous studies.

In summary, an increased production of O$_2^-$ throughout the aortic wall selectively impaired NO-mediated relaxations in apoE-deficient mice. Treatment with a SOD mimetic only in part improved endothelium-dependent relaxations. Increase of O$_2^-$ production and the reduced activity of eNOS enzyme appear to be major mechanisms responsible for impaired endothelial function in the aortas of apoE-deficient mice.

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Expanded Materials and Methods for On-line Publication

**Experimental Animals:** Male C57BL/6J (control) mice and homozygous apoE-deficient mice (C57BL/6J-ApoEM1Unc) were obtained at the age of 4-5 weeks from Jackson Laboratory (Bar Harbor, ME). The Institutional Animal Care and Use Committee of Mayo Clinic approved housing facilities and all experimental protocols. In our preliminary study we found no endothelial dysfunction in age-matched apoE-deficient mice on normal diet as previously reported.1 To accelerate the development of spontaneous atherosclerotic lesions in apoE-deficient mice, control C57BL/6J and apoE-deficient mice were fed a lipid rich Western-type diet for 26-29 weeks (0.15% cholesterol and 42% milk fat by weight, TD88137, Harlan Teklad, Madison, WI).2,3

The mice were anesthetized (pentobarbital, 60 mg/kg body weight, i.p.) and sacrificed. The whole aorta was carefully removed and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; CaCl2 2.5; MgSO4 1.2; KH2PO4 1.2; NaHCO3 25.1; EDTA 0.026; glucose 10.1). The aorta was carefully dissected free from connective tissue in cold Krebs solution under a microscope (Carl Zeiss) and cut into rings from the proximal (closed to descending) and distal (closed to abdominal) thoracic aorta of 4 mm in length. For cGMP and cAMP analysis (see below), 10-15 mm of the abdominal aorta per analysis was immediately frozen in liquid nitrogen and stored at -80°C until assayed.

For plasma cholesterol measurements, blood samples were obtained through puncture of the right ventricle. The blood was immediately transferred to a tube containing heparin and centrifuged at 4°C for 10 minutes. Plasma was separated immediately at 4°C and kept at -80°C until assayed. Cholesterol was determined using a colorimetric-based assay on a Cobas Mira®
Total plasma cholesterol levels were 22.2±1.6 mmol/L in apoE-deficient mice and 6.4±0.7 mmol/L in C57BL/6J mice (P<0.05; n=10).

**Experimental Set-up:** Isolated aorta rings from C57BL/6J and apoE-deficient mice were studied in parallel. Rings were connected to a force transducer (Gould Instrument Systems, Valley View, OH) for recording of isometric force and placed in organ baths filled with 25 mL Krebs solution and maintained at 37°C and aerated continuously with 94% O₂/6% CO₂. After an equilibration period of 30 min, rings were progressively stretched to their optimal passive tension (2.0±0.1 g) as assessed by the response to 100 mmol/L KCl in modified Krebs solution and maintained for 30 min before the experiments.

In a preliminary study, endothelium-dependent relaxations to Ach was unaltered in plaque-free segments of medial thoracic aorta in apoE-deficient mice as compared with C57BL/6J (n=6-7, data not shown). Thus, segments from proximal, distal thoracic and/or first part of abdominal aortas were randomly used. Concentration-dependent response curves to Ach (10⁻⁹-10⁻⁵ mol/L), Ca²⁺-ionophore (A23187; 10⁻⁹-10⁻⁶ mol/L) and diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate; 10⁻¹⁰-10⁻⁵ mol/L) were cumulatively obtained during submaximal contractions to norepinephrine. Concentrations of norepinephrine (1-5 x10⁻⁸ mol/L) were selected in order to obtain the same submaximal contraction in both apoE-deficient and C57BL/6J mice. In a separate protocol, aortic rings were preincubated with or without cyclooxygenase-inhibitor indomethacin (10⁻⁵ mol/L; 15 min) or superoxide dismutase (SOD; 75 U/mL; 5 min) or NO-synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 3x10⁻⁴ mol/L; 20 min) and endothelium-dependent relaxations to Ach were obtained.
A novel cell permeable SOD mimetic Mn(III) tetra (4-benzoic acid) porphyrin chloride (MnTBAP; 10^{-5} mol/L for 15 minutes) was also used. This concentration was found to be optimal in control mice. In our preliminary study, we found that higher concentration of MnTBAP inhibited endothelium-dependent relaxations to Ach in control mouse (data not shown). Rings were contracted with submaximal concentrations of phenylephrine (1-6 x 10^{-7} mol/L) and endothelium-dependent relaxation was tested using Ach (10^{-9}-10^{-5} mol/L). Endothelium-independent relaxation was assessed using DEA-NONOate (10^{-10}-10^{-5} mol/L) in untreated vessels and in those incubated with MnTBAP (10^{-5} mol/L).

**Oxidative Fluorescent Microscopy:** The oxidative fluorescent dye hydroethidine (Molecular Probes) was used to evaluate the in situ concentration of O_2^-, as described previously.\(^5\) Hydroethidine is freely permeable to cells and, in the presence of O_2^-, is oxidized to ethidium bromide (EtBr), where it is trapped by intercalation with DNA. EtBr fluorescence is excited at 488 nm with an emission wavelength of 610 nm. In cell-free assays, hydrogen peroxide, NO, and peroxynitrite do not react with hydroethidine. Unfixed frozen rings of distal thoracic aortic segments were cut into 30 µm thick sections and placed on a glass slide and hydroethidine (2 x 10^{-6} mol/L) was added. Samples were cover-slipped and incubated in a light-protected, humidified chamber at 37°C for 30 min. Tissue sections were imaged using an Olympus Fluoview laser scanning confocal microscope mounted on an Olympus BW50WI upright microscope, equipped with Ar and Kr lasers. The 488-nm Ar laser line was used to excite EtBr fluorescence, which was then detected with a 585-nm long-pass filter. Unlabeled sections were used to obtain background images of aorta from both C57BL/6J and apoE-deficient mice. Identical photomultiplier settings were then used for the image acquisition.
from all samples. Images were analyzed using a comprehensive image processing software (MetaMorph 3.7). Sections were subsequently stained with nuclear fast red. For MnTBAP experiments, aortic rings of C57BL/6J and apoE-deficient mice were incubated with MnTBAP (10^{-5} \text{ mol/L}) for 30 min prior to staining.

**Quantification of Vascular Superoxide Anion:** Superoxide anion production was measured using lucigenin-enhanced chemiluminescence. Low concentration of lucigenin (5 \mu mol/L) was used to avoid the autoxidation of lucigenin. Briefly, thoracic aorta was cut in 4 mm segments. Rings were placed in a modified Krebs-Hepes buffer (pH 7.4) containing sodium diethyldithiocarbamate (10 mmol/L), in order to inhibit vascular CuZn-SOD activity, and equilibrated for 30 minutes at 37°C. Scintillation vials containing 2 mL Krebs-Hepes buffer with 5 \mu mol/L lucigenin were placed into a scintillation counter (LS 5000, Beckman Instruments Inc., Fullerton, CA) switched to the out-of-coincidence mode. After dark adaptation, background signals were recorded and vascular rings were then added to the vial. For MnTBAP experiments, aortic rings of C57BL/6J and apoE-deficient mice were incubated with MnTBAP (10^{-5} \text{ mol/L}) for 15 min prior to recording. Photon counts were recorded every 2 minutes for 8 minutes, and the background was subtracted. The vessels were then dried for 24 hrs at 90°C and weighed. The results were expressed as counts per min per \mu g dry weight.

**Measurement of Ca^{2+}-dependent NOS Enzyme Activity:** \[^{14}\text{C}]\text{-Citrulline formation was measured as previously described.}^{9} \text{Briefly, after collection and removal of connective tissue, four whole aortas (n=1 experiment) were homogenized on ice in lysis buffer (pH 7.5) containing 50 mmol/L Tris-HCL, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPAL, and a 1000-fold dilution of a mammalian protease inhibitor}
cocktail (all from Sigma). The homogenate was then sonicated 30 s, rotated for 1 hr at 4°C, and centrifuged to remove insoluble matter. Total protein was determined using the BioRad (Hercules, CA), DC Protein assay kit, and equal amounts of total protein (119±4 µg, n=7) were added to the enzymatic reactions. Tissue from C57BL/6J and apoE-deficient mice was analyzed in parallel. Ca\(^{2+}\)-dependent NOS activity was determined in duplicate in reactions containing 1 mmol/L NADPH, 100 nmol/L calmodulin, 30 µM BH\(_4\), 40 µM cold (4°C) L-[U\(^{14}\)C]-arginine (348 mCi/mmol, Amersham Life Science) and either 2.5 mmol/L CaCl\(_2\) or 2 mmol/L EDTA. The reactions were stopped after 20 min with 1 mL of stop buffer containing 20 mmol/L HEPES, 2 mmol/L EGTA, and 2 mmol/L EDTA, pH 5.5. Labeled L-Arg was removed by passing the reactions through a cation-exchange resin, DOWEX 50X8-200 (Sigma), and \([^{14}\text{C}]-\text{citrulline}\) content in the eluant was determined using a liquid scintillation counter (LS 5000TD; Beckman Instruments, Fullerton, CA). eNOS enzyme activity was expressed as fmol of \([^{14}\text{C}]-\text{citrulline}\) produced per mg of protein per min.

**Western Blot Analysis:** The surface area of the open aorta was measured planimetrically by using a microscope containing a calibrated eyepiece. Total protein was determined with the BioRad kit. Equal surface area of aorta (for eNOS and vWF) or equal amounts of protein (100 µg/ lane for CuZnSOD and MnSOD) from C57BL/6J and apoE group were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham) using a semi-dry electrophoretic transfer cell for Western analysis. For eNOS and vWF analysis, mouse monoclonal anti-eNOS (1:500; Transduction Labs) and polyclonal anti-rat vWF (1:100; Cedarlane), respectively, were used. For SOD analysis, proteins were detected using their respective rabbit polyclonal antibodies against CuZnSOD or MnSOD (1:5000; StressGen). For
actin, blots were rehybridized with monoclonal anti-actin (1:5000; Sigma). Bands were visualized by enhanced chemiluminescence using a commercially available kit (Amersham Life Science). Densitometry was carried out using NIH-Image® (Scion-Image, Scion-Corp., Frederick, MD) and the results were expressed as O.D. per mm² aortic surface (eNOS and vWF) or relative to the respective intensity of the actin blot (SOD).

**Measurements of cGMP and cAMP:** Frozen aortic tissue was homogenized with a mortar and pestle and suspended in 25 mmol/L Tris buffer pH 7.4. After homogenization, cGMP and cAMP radioimmunoassay kits (Amersham Life Science) were used to perform the measurements. Aortic rings from C57BL/6J and apoE-deficient mice were analyzed in parallel and the results were expressed as pmol/mg protein.

**Drugs:** Ach hydrochloride, A23187, phenylephrine, L-norepinephrine bitartrate, indomethacin, L-NAME and SOD (from bovine erythrocytes, 5100 U/mg protein) were from Sigma Chemical Co. DEA-NONOate and MnTBAP were from Cayman Chemical and Biomol Laboratories, respectively. All drugs were dissolved in distilled water, except DEA-NONOate and A23187, which were prepared as stock solutions in 1.5 mol/L Tris buffer pH 8.8 and DMSO, respectively. All drugs were then diluted in Krebs solution and concentrations are expressed as final molar concentration (mol/L) in the organ bath.

**Calculations and Statistical Analysis:** Contractions were expressed as percent of the response to a second KCl (100 mmol/L) induced contraction, which was obtained at the beginning of each experiment. Results are given as mean ± SEM. Sensitivity was expressed as negative logarithm of the concentration causing half-maximal relaxation or contraction (pD₂ value). Maximal relaxation (expressed as percentage of precontraction) or contraction were
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determined for each individual concentration-response curve by non-linear regression analysis. The concentration-response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni's correction. For simple comparison between two values a paired or unpaired Student's t-test was used, where appropriate. A value of P<0.05 was considered significant.

References


