Modulation of Dilator Responses of Cerebral Arterioles by Extracellular Superoxide Dismutase

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Background and Purpose—Extracellular superoxide dismutase (ECSOD) is highly expressed in the wall of blood vessels and plays an important role in modulation of vascular function in extracranial arteries. Expression of ECSOD appears to affect cerebral vascular responses during disease states. Effects of ECSOD on dilator function in cerebral arterioles, however, have not been fully elucidated. In the present study, we examined effects of ECSOD deficiency on cerebrovascular reactivity under control conditions and during oxidative stress.

Methods—Dilator responses of cerebral arterioles were examined in cranial windows in vivo in anesthetized ECSOD-deficient (−/−) and wild-type (+/+ ) mice under normal conditions and during oxidative stress induced by angiotensin II.

Results—Total SOD activity in the aorta in ECSOD−/− mice (176±24 [mean±SEM] U/mg) was approximately 30% lower than in ECSOD+/+ mice (270±38, P=0.051). Dilator responses to acetylcholine (10 μmol/L) in cerebral arterioles were similar under control conditions in ECSOD+/+ (34±5% changes in diameter) and −/− mice (32±4%). Angiotensin II (500 nmol/L for 30 minutes) tended to reduce responses to acetylcholine in ECSOD+/+ mice (not significant) and significantly impaired responses in ECSOD−/− mice (42% reduction, P<0.05). Tempol (1 mmol/L), a scavenger of superoxide, restored the impaired dilator responses in ECSOD−/− mice. Responses to nitroprusside in cerebral arterioles were similar in ECSOD+/+ and −/− mice and were not affected by angiotensin II nor by tempol.

Conclusions—ECSOD deficiency has little effect on cerebrovascular reactivity in control conditions but plays an important role in the regulation of vascular tone during oxidative stress produced by angiotensin II. (Stroke. 2006;37:2802-2806.)

Key Words: angiotensin II  ■  cerebral circulation  ■  endothelium  ■  nitric oxide  ■  oxidative stress

Under physiological conditions, small amounts of reactive oxygen species are generated in the wall of blood vessels and are important in signaling and regulation of vascular tone.1-3 Overproduction of reactive oxygen species, mainly superoxide, is associated with several cardiovascular risk factors, including hypertension,4 hyperlipidemia,5 diabetes,6 cigarette smoking,7 and aging8 and may play an important role in vascular dysfunction. Superoxide reacts with nitric oxide (NO) released from endothelial cells limits bioavailability of NO and thus impairs endothelium-dependent dilator responses.

To protect against oxidative damage, there are several endogenous antioxidant mechanisms, including a group of superoxide dismutase (SODs).9 Extracellular SOD (ECSOD) is the only isoform of SOD that is distributed in the extracellular matrix and plasma.9,10 ECSOD is highly expressed in blood vessels, particularly in the arterial wall, and its activity constitutes almost half of the total SOD activity in aorta of humans.11,12 Thus, expression and activity of ECSOD in the arterial wall may be important in protection of vascular function during oxidative stress.

A study13 in transgenic mice with overexpression of ECSOD reported that baseline regional cerebral blood flow and responses to acetylcholine were significantly greater than in ECSOD-deficient mice, but responses to acetylcholine were similar in ECSOD-deficient mice and in wild-type mice. Thus, under baseline conditions, endogenous ECSOD does not appear to regulate basal regional cerebral blood flow or vasodilator responses. Vasomotor responses were not examined during oxidative stress.

In this study, we tested the hypothesis that ECSOD may modulate endothelium-dependent dilator responses of cerebral arterioles during oxidative stress. We examined effects of angiotensin II, which generates oxidative stress,14,15 on dilator responses of cerebral arterioles in vivo and in ECSOD-deficient mice. Responses also were examined in the presence of tempol,16 a scavenger of superoxide.

Methods

Experimental Animals

Breeding pairs of ECSOD-deficient (ECSOD−/−) and wild-type (ECSOD+/+) mice were kindly provided by Dr Ralf Brandes. The mice were originally obtained from Dr Stefan Marklund, who inbred the mice for more than 10 generations. Male F2 ECSOD−/− mice, derived from breeding pairs of heterozygous F1 ECSOD-deficient mice, were used for this study. Animals were studied at 4 to 7 months...
of age. Sex- and age-matched litter ECSOD+/+ mice were used as controls. Genotype was determined by polymerase chain reaction (PCR) analysis of DNA extracted from tail snips. Two primers (5' -CCA CGA AGT TGC CAA AGT C-3' and 5' -CCG ACA CGC ATG CCA AAG-3') were used for PCR. After a hot start at 94°C for 3 minutes, PCR was performed for 35 cycles at 94°C for 45 seconds, 59°C for 45 seconds, and 72°C for 4 minutes. Bands for wild-type and mutant ECSOD genes ran at approximately 300 bp and approximately 1.5 kbp, respectively. All procedures followed institutional guidelines approved by the Animal Care and Use Committee at the University of Iowa.

**Cranial Window**

Mice were anesthetized with pentobarbital sodium (75 to 90 mg/kg intraperitoneal). A catheter in the femoral artery was used to measure arterial pressure and to obtain blood. Mice were ventilated mechanically with supplemental oxygen. Depth of anesthesia was evaluated by applying pressure to a paw or tail and by observing changes in heart rate and blood pressure. Additional anesthetic (approximately 20 mg/kg per hour) was administered when such changes occurred. Arterial blood gases were maintained within normal limits throughout each experiment (pH = 7.35 ±0.01 [mean ±SEM], PCO2 = 39±1 mm Hg, and PO2 =119±4 mm Hg). Body temperature was maintained at approximately 37°C with a heating pad.

A cranial window was made over the left parietal cortex, and a segment of pial arteriole (27±1 μm diameter) was exposed. After part of the dura was opened, the cranial window was suffused with artificial cerebrospinal fluid at approximately 3 mL/minute beginning at least 30 minutes before measurement. In cerebrospinal fluid sampled from the cranial window, pH was 7.39±0.02, PCO2 was 36±1 mm Hg, and PO2 was 131±2 mm Hg.

Diameter of cerebral arterioles was recorded and measured using a microscope, video recorder, and a dimension analyzer. The diameter of one arteriole per animal was measured under control conditions, but endogenous ECSOD appears to play a protective role against oxidative stress. The diameter of the pial arteriole was measured. Topical application of these agents did not produce any changes in systemic arterial pressure or prolonged change in arteriolar diameter after superfusion of the cranial window with 500 nmol/L of angiotensin II for 30 minutes did not alter diameter of cerebral arterioles significantly (not shown). Topical application of angiotensin II did not also change systemic blood pressure. In the presence of angiotensin II, dilator responses to acetylcholine (10^−5 mol/L) tended to be reduced in ECSOD+/+ mice compared with controls (34±5 and 28±6%, NS, Figure 1A). In ECSOD−/− mice, responses to acetylcholine (10^−5 mol/L) were significantly reduced by angiotensin II (32±4 and 18±4%, P<0.05 versus control, Figure 1C). Nitroprusside-induced dilator responses of cerebral arterioles in ECSOD+/+ and ECSOD−/− mice were similar to those of ECSOD+/+ mice (Figure 1).

**Superoxide Dismutase Assay**

Total SOD activity of aortic homogenates from ECSOD+/+ and ECSOD−/− mice was determined as previously described.18,19 Measurement of enzyme activity is based on competition of SOD and nitroblue-tetrazolium (NBT) for superoxide; thus, the percent inhibition of NBT reduction is a measure of the amount of SOD present. The rate of NBT reduction to formazan, the photoabsorbant product, was measured spectrophotometrically for 5 minutes at 560 nm. One unit of SOD activity is defined as the amount of protein that results in 50% inhibition of NBT reduction. SOD activity is expressed as units per milligram of protein. Total protein content was determined using a Bio-Rad protein assay kit.

**Statistical Analysis**

All values are expressed as mean ±SEM. Dilatation of pial arterioles to acetylcholine and sodium nitroprusside is expressed as percent change from baseline diameter. Single comparisons were made with unpaired t test, and multiple comparisons were made with repeated measures one-way ANOVA. P<0.05 was considered to be statistically significant.

**Results**

**Extracellular Superoxide Dismutase in Tissue**

In the aorta, total SOD activity was 270±38 U/mg in ECSOD+/+ mice (n=7) and 176±24 U/mg in ECSOD−/− mice (n=8), respectively (P=0.051 versus ECSOD+/+ mice).

**Dilator Responses of Cerebral Arterioles**

Baseline diameter of cerebral arterioles was similar in both groups of mice and averaged 27±1 μm. In control conditions, dilator responses of cerebral arterioles to acetylcholine and nitroprusside in ECSOD−/− mice were similar to those of ECSOD+/+ mice (Figure 1). Superfusion of the cranial window with 500 nmol/L of angiotensin II for 30 minutes did not alter diameter of cerebral arterioles in vivo, deficiency of ECSOD does not affect basal diameter or dilatation of cerebral arterioles to acetylcholine in control mice (data not shown). In the presence of angiotensin II, responses to acetylcholine (10^−5 mol/L) tended to be reduced in ECSOD+/+ mice compared with controls (34±5 and 28±6%, NS, Figure 1A). In ECSOD−/− mice, responses to acetylcholine (10^−5 mol/L) were significantly reduced by angiotensin II (32±4 and 18±4%, P<0.05 versus control, Figure 1C). Nitroprusside-induced dilator responses of cerebral arterioles in ECSOD+/+ and ECSOD−/− mice were not affected by angiotensin II with or without tempol (Figures 1B, 1D and 2B and 2D).

Application of 1 mmol/L of tempol alone did not affect baseline diameter or dilatation of cerebral arterioles to acetylcholine in control mice (data not shown). In the presence of tempol, dilator responses to acetylcholine were not altered by angiotensin II in ECSOD+/+ mice (Figure 2A). Impaired dilator responses to acetylcholine in ECSOD−/− mice in the presence of angiotensin II were restored to normal by tempol (Figure 2C).

**Discussion**

The major new findings in this study are that in cerebral arterioles in vivo, deficiency of ECSOD does not affect acetylcholine-induced, NO-mediated dilator responses under control conditions, but endogenous ECSOD appears to play an important role in modulation of dilator responses during oxidative stress produced by angiotensin II.

**Superoxide Dismutases in Vascularulature**

SODs dismutate superoxide and play an important role in protecting normal vascular function against oxidative stress. There are 3 different isoforms of SODs with different localization: cytosolic copper zinc SOD (CuZnSOD), mitochondrial manganese SOD (MnSOD), and ECSOD. These isoforms are encoded by separate genes, and deficiency or reduction of each SOD isoform has been implicated in vascular dysfunction using genetically altered mice.17,21,22 In aorta from CuZnSOD-deficient mice, total SOD activity is reduced by approximately 60% and superoxide levels are 2-fold greater compared with levels in wild-type mice.17

Statistical Analysis

All values are expressed as mean±SEM. Dilatation of pial arterioles to acetylcholine and sodium nitroprusside is expressed as percent change from baseline diameter. Single comparisons were made with unpaired t test, and multiple comparisons were made with repeated measures one-way ANOVA. P<0.05 was considered to be statistically significant.
Maximal relaxation of the carotid artery to acetylcholine is reduced by approximately 30% in CuZnSOD-deficient mice compared with that in wild-type mice. MnSOD comprises only 10% or less of total SOD activity in the mouse aorta. Although MnSOD is the only isoform that is essential for life and its deficiency induces death soon after birth, vascular function and superoxide levels in the aorta appear to be normal in mice heterozygous for MnSOD (MnSOD+/−) under control conditions. In addition, acute oxidative stress, induced by hypoxia, does not affect endothelial function in the aorta from MnSOD+/− mice.

Extracellular Superoxide Dismutase ECSOD and Vascular Function

ECSOD is a major SOD isoform in the arterial wall, and it is the only SOD in extracellular space. It has been suggested that ECSOD is the major determinant of NO bioavailability in blood vessels. Like other isoforms of SODs, ECSOD appears to play an important role in modulation of reactivity of systemic blood vessels.

A previous study using ECSOD-deficient mice indicated that arterial blood pressure is similar under control conditions in ECSOD-deficient and wild-type mice, although basal

\[\text{Figure 1. Dilator responses of cerebral arterioles to acetylcholine and nitroprusside in ECSOD+/+ mice (A and B) and ECSOD−/− mice (C and D) under control conditions and in the presence of angiotensin II. Under control conditions, dilator responses to acetylcholine in ECSOD−/− mice were similar to those in ECSOD+/+ mice. A and C. Dilator responses to acetylcholine in ECSOD+/+ mice were not significantly impaired by angiotensin II (A); however, responses to acetylcholine (10^{-5} \text{ mol/L}) were significantly impaired in ECSOD−/− mice (B) during angiotensin II. Responses to nitroprusside were normal in the presence of angiotensin II in both ECSOD+/+ and −/− mice (C and D).}\]

\[\text{Figure 2. Effects of tempol on responses to acetylcholine and nitroprusside in ECSOD+/+ mice (A and B) and in ECSOD−/− mice (C and D) under control conditions and in the presence of angiotensin II. In ECSOD+/+ mice, dilator responses to acetylcholine (A) and nitroprusside (B) were normal in the presence of tempol and similar to responses without tempol (Figure 1A and 1B). Impaired dilator responses to acetylcholine in ECSOD−/− mice during angiotensin II (Figure 1C) were restored to normal by tempol (C). Responses to nitroprusside were normal in the presence of angiotensin II and tempol in ECSOD−/− mice (D).}\]
superoxide levels in the aorta are higher in ECSOD-deficient mice than in wild-type mice. Clipping of a renal artery, which produces hypertension and increases superoxide levels in blood vessels, induces greater endothelial dysfunction and elevation of arterial pressure in ECSOD-deficient mice. Our present data are consistent with previous results\textsuperscript{22} that blood pressure under control conditions is similar in ECSOD\textsuperscript{+/+} and /−/− mice.

In a previous study,\textsuperscript{22} absence of ECSOD activity was associated with normal to very mildly impaired endothelium-dependent dilator responses in the aorta under control conditions. We demonstrated here that vasodilator responses of cerebral arterioles were normal during control conditions in ECSOD−/− mice. We speculate that increased antioxidant mechanisms such as other SODs may compensate under normal conditions for loss of ECSOD activity in cerebral arterioles.

**Extracellular Superoxide Dismutase and Cerebral Blood Vessels**

Total SOD activity in the aorta observed here was approximately 30\% lower in ECSOD−/− mice than in ECSOD+/+ mice. This result is compatible with previous reports.\textsuperscript{12,22} The reduction of total SOD activity induced by selective loss of ECSOD is not as great as that in CuZnSOD-deficient mice (approximately 60\% of reduction) in which acetylcholine-induced dilator responses in cerebral arterioles were reduced by approximately 50\% compared with responses in wild-type mice under control conditions.\textsuperscript{17} It is possible that the reduction in total SOD activity in ECSOD−/− mice is not sufficient to affect endothelial function in small arterioles. It is of interest that endothelial function is not impaired in ECSOD-deficient mice under normal conditions in small pulmonary arteries\textsuperscript{28} or, using a hydrogen electrode to measure responses, in the cerebral microcirculation.\textsuperscript{13}

We found, however, that endothelium-dependent dilator responses of cerebral arterioles are significantly impaired in ECSOD−/− mice during increased oxidative stress produced by angiotensin II. Angiotensin II generates superoxide through activation of NADPH oxidase in the vascular wall.\textsuperscript{15,20,30} A recent report indicated that systemic administration of angiotensin II attenuates endothelium-dependent responses in the cerebral microcirculation through AT1 receptor-mediated, Nox2 (gp91phox)-mediated oxidative stress.\textsuperscript{15} In mouse pial arterioles, AT1 receptors and Nox2 are present in endothelium and adventitia but not in smooth muscle cells.\textsuperscript{31} Superoxide from activation of NADPH oxidase reacts with NO immediately after generation within the endothelial cell, i.e., an intracellular reaction. In vessels from ECSOD−/− mice, superoxide levels are higher than in wild-type mice during renovascular hypertension,\textsuperscript{22} suggesting that a substantial amount of superoxide may be dissipated by ECSOD (i.e., extracellularly) under conditions with oxidative stress.

In our study, topical application of angiotensin II to the cranial window did not affect systemic arterial pressure, but nevertheless attenuated endothelium-dependent dilator responses of cerebral arterioles in ECSOD−/− mice. A previous study demonstrated\textsuperscript{31} that superfusion of the cerebral cortex with 100 nmol/L of angiotensin II for 1 hour increased superoxide by approximately 50\% in cerebral microvessels in vivo. It is likely that similar or greater levels of superoxide were produced in cerebral arterioles in the present study during superfusion of high doses of angiotensin II. Superoxide inactivates endothelium-derived NO, contributing to impairment of endothelial function. Impairment of dilator responses to acetylcholine in ECSOD−/− mice were restored by tempol, a superoxide scavenger, which supports the conclusion that deficiency of ECSOD augments oxidative stress during angiotensin II.

Like many previous articles that studied endothelial functions of vessels in vivo and in vitro,\textsuperscript{15,32} we did not find impairment of SNP-induced vasodilatation by oxidative stress even when responses to acetylcholine were impaired. The explanation is not entirely clear, but SNP apparently generates NO within smooth muscle and thus may be relatively inaccessible to extracellular oxidative stress. Thus, bioavailability of NO may be relatively preserved during application of nitroprusside.

Several cerebrovascular diseases are associated with oxidative stress. In pathologic conditions, vasodilator responses of cerebral arterioles are impaired by several mechanisms associated with oxidative stress. SODs play an important role in protection against oxidative stress.\textsuperscript{9,20} ECSOD activity has been reported to affect outcome of cerebrovascular diseases. ECSOD deficiency worsens the outcome after focal cerebral ischemia in mice.\textsuperscript{33} In contrast, transgenic mice overexpressing ECSOD have increased resistance to focal and global cerebral ischemia.\textsuperscript{34,35} After subarachnoid hemorrhage, overexpression of ECSOD by gene transfer reduces the risk of vasospasm in rabbits\textsuperscript{56} but not in dogs.\textsuperscript{37} This study is the first to demonstrate that ECSOD deficiency modulates cerebrovascular function during oxidative stress. Thus, we conclude that ECSOD may play an important role in cerebral vasculature in protection against oxidative stress.

In contrast to our findings, that dilator responses of cerebral arterioles are normal in ECSOD-deficient mice under control conditions, responses of aorta to acetylcholine are impaired in ECSOD-deficient mice.\textsuperscript{22} It is possible that in ECSOD−/− mice, other antioxidant mechanisms may upregulate so that responses of cerebral arterioles are normal under control conditions. We measured total SOD activity in aorta, and it was reduced approximately 35\% in ECSOD−/− mice compared with ECSOD+/+ mice. It would be very difficult to measure ECSOD activity in cerebral arterioles of mice.

**Summary**

We conclude that ECSOD deficiency may not affect cerebrovascular reactivity under control conditions but plays an important role in regulation of vascular tone under conditions with increased oxidative stress. Angiotensin II induces oxidative stress by activation of NADPH oxidase in wild-type mice, and we speculate that a similar mechanism may generate oxidative stress in ECSOD-deficient mice. We did not, however, measure activity of NADPH oxidase or the levels of superoxide radical in cerebral arterioles in ECSOD-deficient mice. Thus, this hypothesis has not yet been tested directly.
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Disclosures
None.

References
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