Role of Extracellular Superoxide Dismutase in Hypertension

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Abstract—We previously found that angiotensin II–induced hypertension increases vascular extracellular superoxide dismutase (ecSOD), and proposed that this is a compensatory mechanism that blunts the hypertensive response and preserves endothelium-dependent vasodilatation. To test this hypothesis, we studied ecSOD-deficient mice. ecSOD−/− and C57Blk/6 mice had similar blood pressure at baseline; however, the hypertension caused by angiotensin II was greater in ecSOD−/− compared with wild-type mice (168 versus 147 mm Hg, respectively; P<0.01). In keeping with this, angiotensin II increased superoxide and reduced endothelium-dependent vasodilatation in small mesenteric arterioles to a greater extent in ecSOD−/− than in wild-type mice. In contrast to these findings in resistance vessels, angiotensin II paradoxically improved endothelium-dependent vasodilatation, reduced intracellular and extracellular superoxide, and increased NO production in aortas of ecSOD−/− mice. Whereas aortic expression of endothelial NO synthase, Cu/ZnSOD, and MnSOD were not altered in ecSOD−/− mice, the activity of Cu/ZnSOD was increased by 80% after angiotensin II infusion. This was associated with a concomitant increase in expression of the copper chaperone for Cu/ZnSOD in the aorta but not in the mesenteric arteries. Moreover, the angiotensin II–induced increase in aortic reduced nicotinamide-adenine dinucleotide phosphate oxidase activity was diminished in ecSOD−/− mice as compared with controls. Thus, during angiotensin II infusion, ecSOD reduces hypertension, minimizes vascular superoxide production, and preserves endothelial function in resistance arterioles. We also identified novel compensatory mechanisms involving upregulation of copper chaperone for Cu/ZnSOD, increased Cu/ZnSOD activity, and decreased reduced nicotinamide-adenine dinucleotide phosphate oxidase activity in larger vessels. These compensatory mechanisms preserve large vessel function when ecSOD is absent in hypertension. (Hypertension. 2006;48:473-481.)

Key Words: angiotensin II ■ endothelium ■ hypertension, experimental ■ nitric oxide

Hypertension increases vascular production of superoxide (O2−), which, in turn, leads to inactivation of NO-altering endothelium-dependent vasodilatation.1 In some models, such as angiotensin II–induced hypertension and in the spontaneously hypertensive rat, membrane-targeted forms of superoxide dismutase (SOD) and SOD mimetics lower blood pressure suggesting that the oxidative inactivation of NO contributes to blood pressure elevation.2–4 Increased production of O2− in kidneys and circumventricular organs of the central nervous system also contributes to the hypertension caused by angiotensin II.5,6 A major antioxidant mechanism against O2− is the SODs, including the cytosolic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular superoxide dismutase (ecSOD or SOD3).7 In most tissues, ecSOD is present in small amounts, however, in vascular tissues ecSOD represents 30% to 50% of total SOD.8 We have shown previously that angiotensin II increases ecSOD expression in mice and human vascular smooth muscle cells.9 One consequence of this increase in ecSOD might be a blunting of hypertension and prevention of endothelial dysfunction caused by angiotensin II.

To test this hypothesis, we have examined alterations in blood pressure and vascular reactivity caused by low-dose angiotensin II in wild-type and ecSOD-deficient (ecSOD−/−) mice. Our data indicate that ecSOD plays a major role in blood pressure modulation and vascular function in resistance vessels. In contrast, we observed a paradoxical improvement in NO production and endothelium-dependent vasodilatation in aortas of ecSOD−/− mice during angiotensin II infusion and defined a potential mechanism for this adaptive response.

Methods

Animals and Angiotensin II Infusion
ecSOD-deficient mice backcrossed to the C57Blk/6 background for 10 generations were weaned at 4 weeks of age and maintained on regular chow for 3 months. C57Blk/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were fed regular chow. Diet and water were provided ad libitum. The Emory University Animal Care and Use Committee approved the protocol for animal use.

Mice were studied between 8 and 12 weeks of age. Angiotensin II was delivered at a rate of 500 ng/kg per minute for 14 days using osmotic minipumps as described previously.10 Sham-operated ani-
mals underwent an identical surgical procedure, except that no pump was implanted. Blood pressure was measured before and during infusion of angiotensin II (on days 7 and 14) using the tail-cuff method (BP2000 Visitech System Inc).

Studies of Vascular Reactivity
For measurements of vascular reactivity in resistance vessels, third-order mesenteric vessels were dissected and mounted in a small vessel myograph for isometric tension recording (JP Trading), as described previously. Concentration-response curves to acetylcholine were performed after precontraction of the vessel with \(3 \times 10^{-8}\) M prostaglandin E\(_2\). Isometric tension studies of aortic rings were performed as described previously.12

Measurements of Vascular O\(_2^-\) Production and SOD Activity
To measure intracellular O\(_2^-\), we quantified formation of hydroxyethidium from dihydroethidium using high-performance liquid chromatography as described previously. To detect aortic extracellular O\(_2^-\) production, we used electron spin resonance (ESR) spectroscopy with CAT1H spin probe as described in the supplemental Methods section (available online at http://hyper.ahajournals.org). The SOD-inhibited amplitude of the low field component of the ESR spectra of oxidized CAT1H was used to quantify extracellular O\(_2^-\) production and was normalized to 2-mm ring segments. In mesenteric arteries, extracellular O\(_2^-\) production was estimated using SOD-inhibitable cytochrome c reduction. O\(_2^-\) production was quantified in picomoles per 2-mm segment of vessel by calculating the difference between absorbance with or without SOD as described previously.12 SOD isoform activity was determined spectrophotometrically by monitoring the inhibition of the rate of xanthine oxidase-mediated reduction of cytochrome c (pH 7.4), as described previously.9 Activity of the NADPH oxidase in aortic membranes was performed as described in the supplemental Methods section.

Determination of Aortic NO Production
Five 2-mm aortic rings were incubated for 60 minutes in 1.5 mL of Krebs/Hepes buffer containing 200 \(\mu\)mol/L iron diethyldithiocarbamate (Fe [DETCCl]) and 10 \(\mu\)mol/L A23187 at 37°C. The NO-Fe [DETCCl] complex was detected using ESR as described previously.14

Western Blotting
Protein expression was examined using Western analysis as described previously. Antibodies used were the following: endothelial NO synthase (eNOS) monoclonal antibody (BD Transduction), MnSOD (Stressgen), rabbit polyclonal antibody against ecSOD,4 copper chaperone for SOD ([CCS] Santa Cruz Biotechnology), Cu/ZnSOD (Biodesign International), and Nox1 (a gift from Harald Schmidt, Monash University, Victoria, Australia). Equal gel loading was determined by Ponceau staining of Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Bio- tech) after transfer and also by blotting for \(\beta\)-actin.

Statistical Analysis
Data are presented as mean±SEM. Comparisons of dose-response curves were performed using 1-way ANOVA for repeated measures. Other comparisons were made using 1-way ANOVA and Student–Newman–Keuls post hoc test when significance was indicated. \(p\) values <0.05 were considered significant.

Results
Effect of Angiotensin II on Blood Pressure in ecSOD-Deficient Mice
Baseline blood pressure was similar in both groups (102±2 versus 95±5 mm Hg in control versus ecSOD\(^{-/-}\) mice, respectively). Angiotensin II increased blood pressure to a greater extent in ecSOD\(^{-/-}\) than in C57Blk/6 mice (168±3 versus 144±2 mm Hg respectively; Figure 1). Sham surgery had no effect on blood pressure.

Effect of Angiotensin II on Mesenteric Vascular Function and O\(_2^-\) Production in ecSOD\(^{-/-}\) Mice and Wild-Type Mice
Because systemic vascular resistance is largely regulated at the arteriolar level, we studied mesenteric arteries 200 \(\mu\)m in diameter using the wire myograph approach. At baseline, endothelium-dependent vasodilatation to acetylcholine was similar between segments of C57Blk/6 and ecSOD\(^{-/-}\) mice. In contrast, angiotensin II caused a marked impairment in endothelium-dependent vasodilatation in mesenteric arteries of the ecSOD\(^{-/-}\) compared with C57Blk/6 mice (37±4% versus 78±6%, respectively; Figure 2A).

At baseline, O\(_2^-\) production measured using cytochrome c reduction was similar in mesenteric vessels from ecSOD\(^{-/-}\) compared with C57Blk/6 mice. The increase in O\(_2^-\) production caused by angiotensin II was significantly greater in ecSOD\(^{-/-}\) as compared with wild-type animals (Figure 2B).

Effect of Angiotensin II on Aortic Vascular Reactivity and NO Production
Under basal conditions, acetylcholine-evoked relaxations were impaired in ecSOD\(^{-/-}\) mouse aortic segments compared with those from C57Blk/6 (69% versus 96%, respectively, Figure 3A and 3B). Angiotensin II impaired relaxations to acetylcholine in vessels from C57Blk/6 mice (79% after Angiotensin II versus 96% at baseline). In striking contrast, endothelium-dependent vasodilatation improved in aortic segments of ecSOD\(^{-/-}\) mice after angiotensin II (69±3% at baseline versus 83±3% after angiotensin II). As in the case with acetylcholine, relaxations to the calcium ionophore A23187 were improved in ecSOD\(^{-/-}\) mice after treatment with angiotensin II (Figure 3C). Relaxations to the endothelium-independent vasodilator nitroglycerin were similar in C57Blk/6 and ecSOD\(^{-/-}\) mice at baseline and were not altered by angiotensin II (Figure 3D).
These data indicate that aortic endothelial NO bioavailability is paradoxically increased by angiotensin II in ecSOD−/− mice. To directly measure NO, we used Fe[DETC]2 and ESR. In C57Blk/6, angiotensin II markedly decreased aortic NO production in response to A23187. In ecSOD−/−, NO production was approximately one-third that observed in the wild-type mice in the absence of angiotensin II and was paradoxically increased by 50% after angiotensin II infusion (Figure 4A and 4B).

Influence of Angiotensin II–Induced Hypertension on Aortic O2− Levels

We used 2 independent approaches to detect O2− production by aortic segments. In aortas from C57Blk/6 mice, extracellular O2− production, as estimated by the SOD-inhibitable oxidation of CAT1H to CAT1, was increased 2-fold by angiotensin II. At baseline, the aortic O2− production was markedly increased in ecSOD−/− mice. Angiotensin II, however, decreased aortic O2− production in these mice (Figure 5A). We also used high-performance liquid chromatography to monitor the oxidation of dihydroethidium to hydroxyethidium, a reaction specifically mediated by O2−. This assay revealed qualitatively similar results to those obtained with ESR (Figure 5B). Thus, using 2 independent approaches, we found that whereas angiotensin II increases aortic O2− levels in wild-type mice, it paradoxically reduces O2− levels in mice lacking ecSOD.
O$_2^-$ Production by Reduced NADPH Oxidase and Nox1 Protein Expression

Angiotensin II acutely stimulates NADPH oxidase activity and chronically increases Nox1 expression. Because we observed improvement in vascular function and decrease in O$_2^-$ production in ecSOD−/− mice after angiotensin II, we considered the possibility that NADPH oxidase activity might not be affected by angiotensin II in these animals. At baseline, NADPH-dependent O$_2^-$ production was similar in both groups. Angiotensin II increased NADPH oxidase activity in both C57Blk/6 and ecSOD−/− mice, although this effect was significantly greater in the C57Blk/6 mice (Figure 6). In keeping with this, the increase in Nox1 caused by angiotensin II was greater in wild-type than in ecSOD−/− mice (Figure 7).

Effect of Angiotensin II–Induced Hypertension on SOD Isoforms and eNOS

To gain insight into other factors that modulate O$_2^-$ production and endothelium-dependent vasodilatation, we examined protein levels of SOD isoforms and eNOS at baseline and after angiotensin II. Aortic protein levels of MnSOD, Cu/ZnSOD, and eNOS were similar between C57Blk/6 and ecSOD−/− mice and were not altered by angiotensin II treatment. As expected, ecSOD was absent in vessels of ecSOD−/− mice and, as reported previously, angiotensin II increased ecSOD protein levels in C57Blk/6 mice (Figure 8).

In resistance vessels, levels of eNOS were similar at baseline and during angiotensin II infusion in wild-type and ecSOD−/− mice. Levels of Cu/ZnSOD were slightly higher in ecSOD−/− mice mesenteric arteries compared with wild-type. In wild-type mice, angiotensin II markedly increased mesenteric artery in Cu/ZnSOD, while having no effect on Cu/ZnSOD in ecSOD−/− mice mesenteric arteries. Finally, MnSOD levels were similar in wild-type and ecSOD−/− mice at baseline; however, angiotensin II infusion caused a significant decline in MnSOD in ecSOD−/− mice (Figure 9).

In keeping with the effects on aortic ecSOD protein levels, aortic ecSOD enzymatic activity was increased by angiotensin II in C57Blk/6 and was absent in ecSOD−/− mice (Figure 10A). The activity of MnSOD was similar between C57Blk/6 and ecSOD−/− aortas and was not altered by angiotensin II treatment (data not shown). At baseline, aortic Cu/ZnSOD activity was similar in these 2 groups of mice; however, the response to angiotensin II was very different. In C57Blk/6 mice, angiotensin II had no effect on Cu/ZnSOD activity, whereas infusion of this octapeptide almost doubled it in ecSOD−/− mice (Figure 10B).

An important determinant of Cu/ZnSOD activity is its copper content, which is regulated by delivery from the CCS. To ascertain if CCS could be altered in ecSOD−/− mice, we performed Western blots at baseline and in vessels after angiotensin II infusion. At baseline, aortic CCS expres-
sion was similar between C57Blk/6 and ecSOD⁻/⁻ mice; however, the response to angiotensin II–induced hypertension was different. Angiotensin II had no effect in vessels of C57Blk/6 but doubled CCS expression in the aortas of ecSOD⁻/⁻/⁻ mice (Figure 11A). These changes in CCS expression were not observed in mesenteric arteries (Figure 11B).

**Discussion**

In the present study, we found that angiotensin II increases blood pressure and O$_2^-$ production in mesenteric arteries of C57Blk/6 mice and that these effects were augmented in mice lacking ecSOD. Also, endothelium-dependent relaxation was altered in the mesenteric arteries of ecSOD⁻/⁻ mice when treated with angiotensin II. In contrast to the effects on resistance vessels in ecSOD⁻/⁻ mice, angiotensin II paradoxically improved endothelium-dependent vasodilatation in aortic segments. This was associated with an increase in NO as detected by ESR and with a reduction in aortic O$_2^-$ production. NADPH oxidase activity was increased by angiotensin II in C57Blk/6 and ecSOD⁻/⁻/⁻ mice but to a lesser extent in the latter group. Our data also show that the CCS plays a potentially important role in modulation of O$_2^-$ production in large vessels during angiotensin II–induced hypertension.

The exaggerated hypertensive response to angiotensin II in ecSOD⁻/⁻/⁻ mice is in keeping with the marked alteration in endothelium-dependent vasodilatation and the increase in O$_2^-$ observed in the resistance vessels in these animals. Previous studies have shown that O$_2^-$ clearly alters endothelium-dependent vasodilatation in resistance vessels, such as the mesenteric arterioles, hamster cheek pouch arterioles, and the afferent arteriole of the kidney.18–20 This impairment in arteriolar vasodilatation likely increases systemic vascular resistance in ecSOD⁻/⁻ mice during angiotensin II infusion and, thus, augments the hypertensive response observed in these animals.

O$_2^-$ rapidly reacts with NO forming peroxynitrite,21 which, in turn, can alter eNOS function.12,22 It is, therefore, not surprising that the vascular O$_2^-$ was inversely related to levels of NO production as measured by ESR. Our studies indicate that, under baseline conditions, ecSOD has an important role in modulating NO bioavailability as it traverses between the endothelium and vascular smooth muscle in large vessels. In wild-type C57Blk/6 mice, angiotensin II dramatically reduced NO production in response to A23187. In contrast, in ecSOD⁻/⁻ mice, angiotensin II caused a paradoxical increase in aortic NO production. These changes in NO were paralleled by alterations in endothelium-dependent vasodilatation in that angiotensin II worsened these
responses in C57Blk/6 mice and improved them in ecSOD<sup>−/−</sup> mice.

Our findings indicate during angiotensin II–induced hypertension that the absence of ecSOD has opposite effects on conduit arteries and resistance vessels. In thoracic aorta, the baseline production of O<sub>2</sub><sup>−</sup> was increased in the ecSOD<sup>−/−</sup> as compared with the wild-type mice. Paradoxically, during angiotensin II infusion, aortic O<sub>2</sub><sup>−</sup> production was markedly increased in wild-type animals but declined in the ecSOD<sup>−/−</sup> mice. It is highly unlikely that these results represent a measurement artifact, because they were confirmed using 2 independent methods. Moreover, in preliminary studies we obtained similar results using cytochrome c reduction.

Our experiments also provide some insight into the paradoxical improvement in endothelium-dependent vasodilatation in the large vessels of ecSOD<sup>−/−</sup> mice caused by angiotensin II. In ecSOD<sup>−/−</sup> mice, although there was no change in the expression of eNOS or the other SOD isoforms after angiotensin II, the enzymatic activity of the cytosolic Cu/ZnSOD (SOD1) was almost doubled. Although the explanation for this remains unclear, we found that expression of the CCS was also increased in ecSOD<sup>−/−</sup> mice after angiotensin II. This metalloprotein can insert copper into preformed apolipoprotein SOD1 and increase its activity without the need for new protein synthesis.Interestingly, the dismutation product of O<sub>2</sub><sup>−</sup>, hydrogen peroxide, particularly in the presence of bicarbonate, can react with the copper center of both the ecSOD and Cu/ZnSOD, leading to formation of the Cu–OH radical and, ultimately, loss of copper leading to enzyme inactivation. This can be prevented by scavenging the Cu–OH radical with small anionic antioxidants, such as urate or nitrite. In apolipoprotein E<sup>−/−</sup>, we have shown previously that the activity of both Cu/ZnSOD and ecSOD is depressed via this mechanism. It is possible that a similar mechanism is operative in the setting of angiotensin II–induced hypertension and that CCS helps preserve full activ-
It is also of note that, in neural tissues, the Cu/ZnSOD is in 12- to 30-fold excess of CCS. If true in vascular cells, this might also predispose to some of the dismutase being copper deficient. It is conceivable that the increase in CCS observed in the ecSOD mice during angiotensin II infusion helps to maintain Cu/ZnSOD activity without changing Cu/ZnSOD protein levels. The precise mechanism underlying the increase in CCS remains undefined; however, it is possible that extracellular O$_2$ or a derivative of O$_2$ reenters the cells to stimulate CCS expression.

In mesenteric arteries, the levels of CCS were similar between wild-type and ecSOD mice and were not changed by angiotensin II infusion. Thus, the compensatory response observed in the aorta of ecSOD mice during angiotensin II infusion does not occur in the resistance vessels. In addition, in the resistance vessels of ecSOD mice, angiotensin II infusion induced a significant decrease in MnSOD protein. It has been shown previously that MnSOD can be oxidatively inactivated by oxidants such as hypochlorous acid and peroxynitrite. Oxidatively modified proteins are often targeted for degradation, and this might explain the lower levels of MnSOD in the ecSOD mice treated with angiotensin II. Finally, in mesenteric arteries, the protein levels of Cu/ZnSOD were low in wild-type animals at baseline and increased significantly during angiotensin II infusion. This might explain the lack of effect of angiotensin II on endothelium-dependent vasodilation in these vessels and the relatively modest increase in blood pressure. In contrast, in the ecSOD mouse mesenteric vessels, Cu/ZnSOD levels were relatively high at baseline and were not changed by angiotensin II infusion. The decline in MnSOD and the lack of response of CCS and Cu/ZnSOD in resistance vessels of ecSOD mice likely contribute to the alteration in vascular function and hypertension caused by angiotensin II.

**Figure 11.** Aortic (A) and mesenteric arteries (B) expression of CCS protein. Protein levels of CCS were measured using Western analysis. Top, representative blot; Bottom, mean data of densitometry for 3 experiments normalized to actin.

**Figure 12.** Proposed effect of angiotensin II in ecSOD knockout mice in aorta and mesenteric vessels. See Discussion for details.
The net vascular level of $\text{O}_2^{-}$ depends on the rate of its production and scavenging. To understand the capacity to generate $\text{O}_2^{-}$, we measured NADPH oxidase activity. At baseline, aortic oxidase activity was identical between the wild-type and ecSOD$^{-/-}$ mice, suggesting that the increase in $\text{O}_2^{-}$ in ecSOD$^{-/-}$ mice was largely because of reduced scavenging. After angiotensin II, NADPH oxidase activity increased in both groups of animals, albeit to a lesser extent in the ecSOD$^{-/-}$ mice. The predominant long-term effect of angiotensin II on the aortic NADPH oxidase is induction of Nox1.16 In keeping with this, we observed a greater increase in Nox1 protein during angiotensin II infusion in wild-type as compared with the ecSOD$^{-/-}$ mouse aortas. It has been shown recently that $\text{O}_2^{-}$ can stimulate Nox1 expression in breast and ovarian tumor cells.30 It is possible that the increase in Cu/ZnSOD activity blunted the rise in $\text{O}_2^{-}$ in the ecSOD$^{-/-}$ vessels and that this reduced the stimulus for Nox1 expression.

Previously, Jung et al31 have also examined the role of the ecSOD in modulation of blood pressure in the setting of both angiotensin II–induced hypertension and 2-kidney/1-clip hypertension. In this previous study, the authors also showed that lack of ecSOD augmented the hypertensive response to angiotensin II–induced hypertension and 2-kidney/1-clip hypertension. The biochemical events that occur in mesenteric vessels of ecSOD$^{-/-}$ mice, including a decline in MnSOD and a lack of increase in the CCS and Cu/ZnSOD protein predistore to an increase in $\text{O}_2^{-}$, a marked impairment in endothelium-dependent vasodilation and augmented hyper- tension caused by angiotensin II.

**Perspectives**

This study clearly demonstrates that not only intracellular $\text{O}_2^{-}$ but also extracellular $\text{O}_2^{-}$ is responsible for the bioavailability of NO. The extracellular isoform of the SODs is indispensable for allowing transfer of NO through the extracellular matrix, crossing from the endothelium to the smooth muscle. These properties of ecSOD suggest that it might be a therapeutic target and that increasing its levels or activity could blunt the harmful effects of oxidative stress associated with hypertension. Our data also indicate that a rather complex interplay exists between the ecSOD and copper delivery enzymes that modulate activity of other SOD isoforms and likely influence vascular $\text{O}_2^{-}$ and NO levels (Figure 12).

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**Disclosures**

None.

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