Critical Role for CuZn–Superoxide Dismutase in Preventing Angiotensin II–Induced Endothelial Dysfunction

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Abstract—The goal of the present study was to test the hypothesis that the CuZn isoform of superoxide dismutase (CuZnSOD) protects against angiotensin II (Ang II)–induced endothelial dysfunction. Vascular responses of carotid arteries from control, CuZnSOD-deficient (CuZnSOD<sup>+/−</sup>), and CuZnSOD transgenic mice were examined in vitro after overnight incubation with either vehicle or Ang II (1 or 10 nmol/L). In control mice, acetylcholine produced concentration-dependent relaxation that was not affected by 1 nmol/L Ang II. In contrast, relaxation to acetylcholine in arteries from CuZnSOD<sup>+/−</sup> mice was markedly and selectively attenuated after incubation with 1 nmol/L Ang II (eg, 100 μmol/L acetylcholine produced 93±6% and 44±15% relaxation in vehicle- and Ang II–treated arteries, respectively). A higher concentration of Ang II (10 nmol/L) selectively impaired relaxation to acetylcholine in arteries from control mice (eg, 100 μmol/L acetylcholine produced 96±4% and 45±7% relaxation in vehicle- and Ang II–treated vessels, respectively). In contrast, 10 nmol/L Ang II had no effect on responses to acetylcholine in carotid arteries from CuZnSOD transgenic mice (or in control mice treated with the superoxide scavenger Tiron [1 mmol/L]). Superoxide levels in control mice were higher in aorta treated with Ang II than with vehicle and were markedly reduced in CuZnSOD transgenic mice. These findings provide the first direct evidence that CuZnSOD limits Ang II–mediated impairment of endothelial function and that loss of 1 copy of the CuZnSOD gene is sufficient to enhance Ang II–induced vascular dysfunction. (Hypertension. 2005;46:1147-1153.)

Key Words: mice ■ oxidative stress ■ endothelium ■ vessels

Angiotensin II (Ang II) increases arterial pressure, produces oxidative stress, and impairs endothelial function.<sup>1–10</sup> For example, levels of superoxide within the vessel wall are increased, and endothelial dysfunction is present in animals made hypertensive by infusion of Ang II as well as in mice that chronically overexpress human renin and human angiotensinogen.<sup>2,5,6,8,10</sup> The effect of Ang II on endothelial function appears to be related in large part to a superoxide–angiotensinogen.2,5,6,8,10 The effect of Ang II on superoxide levels and responses of carotid artery from CuZnSOD<sup>+/−</sup> mice is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000187532.80697.15

Experimental Animals

Because the direct effect of Ang II on endothelial function has not been determined previously in mouse carotid artery, C57BL/6J (male) mice were used in initial experiments designed to determine the concentration of Ang II necessary to produce vascular dysfunction in these vessels. C57BL/6 mice were also used to eliminate the possibility that any effect of Ang II in wild-type mice is attributable to genetic background.<sup>19</sup> CuZnSOD-deficient (male and female) mice were derived from breeding pairs of heterozygous CuZnSOD-deficient (B6;129S-SODtm1Leb) mice. We studied heterozygous CuZnSOD-deficient (CuZnSOD<sup>+/−</sup>) mice and their wild-type (CuZnSOD<sup>+/+</sup>) littermates. CuZnSOD-Tg mice (male and female) used for this study were derived from breeding male hemizygous CuZnSOD (human) trans-
effective in reducing superoxide levels. We have shown previously that this concentration of Tiron is very effective in reducing superoxide levels.

Experimental Protocols

Relaxation of carotid arteries in response to acetylcholine (an endothelium-dependent agonist) and nitroprusside (an endothelium-independent agonist) was measured after submaximal precontraction using the thromboxane analog U46619 (9,11-dideoxy-11a,9a-epoxy-methanoprostoglandin-F2a). Using pharmacological approaches and measurement of chemiluminescence in aorta from wild-type and CuZnSOD-Tg mice treatment with vehicle (control; ) or 1 mmol/L Ang II ( ). Right, Relaxation of carotid arteries from control mice ( ) to acetylcholine after treatment with vehicle (control; ) or 1 mmol/L Ang II ( ), vehicle plus 1 mmol/L Tiron ( ) or 10 mmol/L Ang II plus 1 mmol/L Tiron ( ), *P<0.05 vs control.

SOD Activity

Measurement of Superoxide

Total SOD activity of aortic homogenates from wild-type and CuZnSOD+/− mice as well as from nontransgenic and CuZnSOD-Tg mice was determined as described previously.

Measurement of Superoxide

Vascular superoxide levels were measured using lucigenin-enhanced chemiluminescence in aorta from wild-type and CuZnSOD+/− mice as well as nontransgenic and CuZnSOD-Tg mice treated with vehicle and Ang II (10 nmol/L) as described previously.

Drugs

Acetylcholine, Ang II, lucigenin, nitroprusside, and Tiron were obtained from Sigma and all were dissolved in saline. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilution being made with saline.

Statistical Analysis

All data are expressed as means±SE. Relaxation to acetylcholine and nitroprusside is expressed as a percent relaxation to U46619-induced contraction. Contractile responses to U46619 are expressed in grams of tension. Comparisons of relaxation and contraction were made using ANOVA followed by Student-Newman–Keuls post hoc test. Comparison of total SOD activity and superoxide levels was made using paired and unpaired t tests where appropriate. Statistical significance was accepted at P<0.05.

Results

Concentration-Dependent Effects of Ang II on Vascular Responses in C57BL/6J Mice

In carotid arteries incubated with vehicle, acetylcholine produced relaxation in C57BL/6J (control) mice that was not affected (P>0.05) by 1 nmol/L Ang II treatment (Figure 1). In contrast, 10 nmol/L Ang II produced marked impairment (P<0.05) of acetylcholine-induced relaxation in arteries from control mice (Figure 1). The effect of Ang II appears to be selective for endothelium because relaxation in response to nitroprusside was similar in carotid arteries from all groups treated with vehicle or Ang II (1 and 10 nmol/L; data not shown).

To determine whether endothelial dysfunction produced by Ang II in mouse carotid artery was mediated by superoxide, vascular responses were examined in the presence of vehicle...
or Tiron (1 mmol/L) after treatment with either vehicle or 10 nmol/L Ang II. Tiron had no effect on relaxation to acetylcholine (Figure 1) or nitroprusside (data not shown) in control mice incubated with vehicle. Acute incubation with Tiron produced almost complete restoration of responses to acetylcholine in vessels incubated with 10 nmol/L Ang II (Figure 1). These data suggest that Ang II produces concentration-dependent endothelial dysfunction in carotid artery of mice, which is mediated by superoxide and can be reversed acutely.

**SOD Activity in CuZnSOD+/− and CuZnSOD-Tg Mice**

Total SOD activity was reduced by ≈30% in vehicle-treated aorta from CuZnSOD+/− mice compared with aorta from wild-type mice (Figure 2). Incubation with Ang II (1 nmol/L) had no effect (P>0.05) on total SOD activity in aorta from either wild-type or CuZnSOD+/− mice (Figure 2).

Total SOD activity was increased (P<0.05) in aorta from CuZnSOD-Tg mice (283±52 and 534±113 U/mg protein in nontransgenic [n=6] and CuZnSOD-Tg mice [n=6], respectively). The effect of Ang II on total SOD activity in CuZnSOD-Tg mice was not determined, but Ang II (10 nmol/L) had no effect on total SOD activity in nontransgenic mice (229±46 and 211±12 U/mg protein in vehicle-treated [n=6] and Ang II–treated [n=4] vessels, respectively).

**Endothelial Dysfunction in Response to Ang II Is Enhanced in CuZnSOD+/− Mice**

In wild-type and CuZnSOD+/− mice, acetylcholine produced relaxation that was similar (P>0.05) in arteries incubated with vehicle (Figure 3). These findings suggest that loss of a single copy of the CuZnSOD gene is not sufficient to alter endothelial function under basal conditions.

Consistent with that observed in C57BL/6 mice, acetylcholine produced relaxation that was similar in carotid arteries from wild-type mice incubated with 1 nmol/L Ang II compared with that in vehicle-treated arteries (Figure 3). In contrast, 1 nmol/L Ang II produced marked impairment of relaxation in response to acetylcholine in carotid artery from CuZnSOD+/− mice (Figure 3). These findings suggest that deficiency in a single copy of the CuZnSOD gene markedly enhances Ang II–induced endothelial dysfunction at a concentration of Ang II that has no effect in vessels from wild-type mice. Responses to nitroprusside (Figure 3) and U46619 (data not shown) were similar (P>0.05) in vehicle-treated and Ang II (1 nmol/L)–treated vessels from wild-type mice.

**Figure 2.** Total SOD activity is reduced in CuZnSOD+/− mice (n=11) compared with wild type (n=13). Ang II treatment had no effect (P>0.05) on total SOD activity in either wild-type (n=5) or CuZnSOD+/− (n=5) mice or vehicle (control)-treated vessels. *P<0.05 vs respective vehicle (control)-treated and Ang II–treated wild type.

**Figure 3.** Ang II (1 nmol/L) produces impairment of endothelial function in CuZnSOD+/− but not wild-type mice. A, Left, Relaxation of carotid arteries to acetylcholine from wild-type mice (n=6) treated with vehicle (control; □) or 1 nmol/L Ang II (●); Right, relaxation of carotid arteries to acetylcholine from CuZnSOD+/− mice (n=7) treated with vehicle (control; ○) or 1 nmol/L Ang II (●). B, Left, Relaxation of carotid arteries to nitroprusside from wild-type mice (n=6) treated with vehicle (control; □) or 1 nmol/L Ang II (●); Right, relaxation of carotid arteries to nitroprusside from CuZnSOD+/− mice (n=7) treated with vehicle (control; ○) or 1 nmol/L Ang II (●). *P<0.05 vs vehicle.
Ang II–Induced Endothelial Dysfunction Is Prevented in CuZnSOD-Tg Mice

In CuZnSOD-Tg and nontransgenic mice, carotid arteries relaxed in a similar manner in response to acetylcholine (Figure 4). These findings suggest that overexpression of CuZnSOD per se does not alter endothelial function and is consistent with previous findings.\(^23,24\)

In nontransgenic mice, 10 nmol/L Ang II markedly impaired responses of carotid arteries to acetylcholine but not nitroprusside (Figure 4). In contrast, overexpression of CuZnSOD completely prevented 10 nmol/L Ang II–induced alterations in responses to acetylcholine (Figure 4). Responses to nitroprusside (Figure 4) and U46619 (data not shown) were similar (\(P>0.05\)) in vehicle-treated and Ang II (10 nmol/L)–treated vessels from nontransgenic and CuZnSOD-Tg mice. These findings suggest that increases in CuZnSOD expression and activity are sufficient to prevent Ang II–induced endothelial dysfunction.

Superoxide levels were higher (\(P<0.05\)) in aorta from nontransgenic mice treated with 10 nmol/L Ang II compared with vehicle-treated vessels (Figure 5). Basal superoxide levels as well as the increase in superoxide in response to 10 nmol/L Ang II were inhibited (\(P<0.05\)) by Tiron (\(-4 \pm 9\) and \(1 \pm 15\) RLU/s per mg tissue in vehicle- and Ang II–treated vessels, respectively). Additionally, the increase in superoxide in response to Ang II (10 nmol/L) was prevented in vessels from CuZnSOD-Tg mice (Figure 5).

Discussion

There are several major findings of the present study. First, Ang II impairs endothelial function in mouse carotid artery in a concentration-dependent manner. This effect was selective for endothelium because Ang II had no effect on responses to nitroprusside. Because these studies were performed in vitro, these changes reflect direct effects of Ang II on the vessel wall independent of changes in arterial pressure. Second, despite a reduction in total SOD activity of \(\approx 30\%\), loss of a single gene for CuZnSOD does not appear to alter endothelial
function under basal conditions. Importantly, Ang II produced marked endothelial dysfunction in CuZnSOD−/− mice at a concentration (1 nmol/L) that had no effect in wild-type mice. These results suggest that deficiency in a single copy of the CuZnSOD gene increases vascular sensitivity to Ang II–induced endothelial dysfunction. Third, overexpression of CuZnSOD is very effective in protecting against increases in superoxide and endothelial dysfunction in response to a higher concentration of Ang II (10 nmol/L). Ang II–induced endothelial dysfunction could also be reversed by acute treatment with the superoxide scavenger Tiron, providing pharmacological and genetic evidence that the effects of Ang II are mediated by superoxide. Together, the results of the present study provide direct evidence that CuZnSOD plays a critical role in protecting the vessel wall against Ang II–induced increases in superoxide and endothelial function.

**Ang II–Induced Endothelial Dysfunction**

It is well documented that systemic administration of Ang II can increase vascular superoxide levels and arterial blood pressure as well as produce endothelial dysfunction.1–15 For example, previous studies have shown that mice that overexpress the human renin and human angiotensinogen genes exhibit increased plasma Ang II levels, hypertension, and increases in vascular superoxide and vascular dysfunction.2,8,28,29 Infusion of Ang II (via osmotic minipump) also produces hypertension as well as increased vascular superoxide and endothelial dysfunction.3,5,6,10–13 Several lines of evidence suggest that these effects are mediated in large part by activation of a vascular NAD(P)H oxidase.3,5,6,12–15 For example, expression of components of the NAD(P)H oxidase (eg, p67phox and gp91phox) as well as oxidase activity is increased in aortic homogenates from Ang II–infused mice.5 Consistent with this concept, the pressor response and increase in vascular superoxide, as well as vascular dysfunction induced by Ang II, are attenuated in mice deficient in the expression of NAD(P)H oxidase components (ie, p47phox- and gp91phox-deficient mice).11,13–15,30

In the present study, Ang II produced impairment of endothelial function in carotid arteries in a concentration-dependent manner. A very low concentration of Ang II (1 nmol/L) had no effect on vascular responses, whereas a higher concentration of Ang II (10 nmol/L) produced marked impairment of endothelial function in control mice. These results are consistent with those described previously, in which incubation with 30 and 100 nmol/L Ang II for 24 hours produced impairment of endothelial function in mouse aorta.14 In addition to the effect of Ang II on endothelial function, we found that incubation of aorta with Ang II (10 nmol/L) increased superoxide levels in control mice. This result is consistent with a previous study, in which Ang II treatment increased superoxide levels in human internal mammary artery.4

Although relatively few previous studies have examined the direct effects of Ang II on vascular function and superoxide levels,4,14 we feel that in vitro incubation of vessels with Ang II is useful experimentally for ≥2 reasons. First, this approach allows examination of effects of Ang II on intact vascular segments. Thus, the model may be more physiological than simply studying single vascular cells in culture. This is potentially important because the synergy between the various vascular layers is becoming increasingly apparent.31 Second, in vitro incubation of blood vessels with Ang II allows for assessment of direct effects of Ang II within the vessel wall independent of systemic effects (eg, central and renal) of in vivo Ang II administration.

**CuZnSOD Deficiency Enhances Ang II–Induced Vascular Dysfunction**

We and others have previously shown that complete CuZnSOD deficiency (CuZnSOD−/− mice) increases vascular superoxide as well as peroxynitrite levels.22,32,33 CuZnSOD−/− mice also display enhanced responsiveness to vasoconstrictors as well as impaired endothelial responses.22,32,33 Thus, selective loss of both genes for CuZnSOD produces a dramatic vascular phenotype under basal conditions. Because CuZnSOD−/− mice display impaired vascular responses under basal conditions and because it would be difficult to examine the effects of Ang II–mediated endothelial dysfunction in CuZnSOD−/− mice, we elected to examine in the present study whether Ang II–induced endothelial dysfunction is greater in CuZnSOD−/− mice. Heterozygous deficient mice are important in relation to discovery of vascular phenotypes associated with loss of a single gene copy.34 Studies involving heterozygous mice are potentially relevant to genetic polymorphisms in humans as well as disease conditions associated with reduced activity of CuZnSOD.

The effect of heterozygous CuZnSOD deficiency on endothelial function is more difficult to predict than with homozygous CuZnSOD deficiency and, to our knowledge, has not been examined previously. We found that loss of a single copy of the CuZnSOD gene was associated with an ∼30% reduction in total vascular SOD activity. However, despite a significant reduction in SOD activity, endothelial responses to acetylcholine and nitroprusside were similar in carotid artery in CuZnSOD+/− and wild-type mice. These findings suggest that the loss of a single gene for CuZnSOD is not sufficient to alter endothelial function under basal conditions.

Perhaps the most important finding of the present study was that 1 nmol/L Ang II, which had no effect on endothelial responses in control mice, produced marked endothelial dysfunction in CuZnSOD−/− mice. Thus, these data provide direct evidence that both copies of the CuZnSOD gene are required to protect blood vessels from Ang II–induced endothelial dysfunction. The data also provide an example of the importance of studies involving heterozygote gene deficiency in relation to vascular biology in disease models (ie, vascular phenotypes not evident in control mice may be unmasked in mice lacking 1 gene copy).

**Overexpression of CuZnSOD Prevents Ang II–Induced Endothelial Dysfunction**

Previously, we have shown that CuZnSOD protein expression and total SOD activity are increased (several-fold) in the vasculature of CuZnSOD-Tg mice.23,24 Thus, our present findings are consistent with these previous studies because total SOD activity was higher in aortic homogenates from
CuZnSOD-Tg compared with nontransgenic mice. More important, overexpression of CuZnSOD was very effective in preventing Ang II–induced increases in vascular superoxide levels and endothelial dysfunction. The higher concentration of Ang II (10 nmol/L), which produced >50% inhibition of acetylcholine–induced relaxation in nontransgenic mice, was completely prevented in CuZnSOD-Tg mice. These findings provide additional evidence that overexpression of CuZnSOD is very effective in limiting increases in superoxide and preventing endothelial dysfunction in response to stimuli that produce oxidative stress (eg, amyloid precursor protein, ceramide, or lipopolysaccharide).23,24,35 In relation to Ang II, a previous study showed that overexpression of CuZnSOD attenuates increases in vascular superoxide (consistent with the present findings) in response to Ang II infusion and that the pressor response to Ang II infusion could be blunted in CuZnSOD-Tg mice.18 However, this previous study did not examine the effect of Ang II on vascular responses.18 Thus, our findings are the first to demonstrate the effectiveness of CuZnSOD in preventing Ang II–induced endothelial dysfunction.

**Perspectives**

It is well recognized that both experimental models of hypertension as well as certain forms of human hypertension are associated with increases in Ang II.37,39 More important, increases in Ang II have been associated with increased levels of oxidative stress and endothelial dysfunction.36–39 The present findings suggest that reductions in CuZnSOD activity are sufficient to sensitize blood vessels to Ang II–mediated endothelial dysfunction, whereas increases in CuZnSOD activity are very effective at limiting endothelial function produced by Ang II. Together, the present study provides another example of the CuZnSOD in protecting the vasculature.22–24,32,33,35 Because superoxide has been shown to be a mediator of changes in vascular structure and end-organ damage in response to Ang II,40 the present findings have broader implications. Alterations in SOD activity would be predicted to have important consequences on vascular responses to Ang II, including Ang II–dependent hypertension.

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**References**


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