Sex Differences in Protection Against Angiotensin II–Induced Endothelial Dysfunction by Manganese Superoxide Dismutase in the Cerebral Circulation

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Sex Differences in Protection Against Angiotensin II–Induced Endothelial Dysfunction by Manganese Superoxide Dismutase in the Cerebral Circulation

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Abstract—Angiotensin II (Ang II) produces oxidative stress and endothelial dysfunction in blood vessels. The vasculature from females may be protected against deleterious effects of Ang II. We tested the hypothesis that manganese superoxide dismutase (MnSOD) protects against Ang II–induced endothelial dysfunction. Experiments were performed in C57BL/6, wild-type (MnSOD^+/+), and MnSOD-deficient (MnSOD^+/−) mice treated systemically with vehicle or Ang II. Basilar arteries were isolated from mice treated for 1 week with a nonpressor dose of Ang II (0.28 mg/kg per day). Ang II treatment produced superoxide-mediated impairment of responses to the endothelium-dependent vasodilator acetylcholine (P<0.05). In male but not female MnSOD^+/+ mice, Ang II modestly inhibited responses to acetylcholine (P<0.05). In contrast, Ang II selectively impaired these responses by up to 70% in male MnSOD^+/− mice (P<0.05), and this effect was reversed by Tempol (P<0.05). Ang II had no effect on acetylcholine responses in MnSOD^+/− female mice. Vascular superoxide levels after treatment with an inhibitor of CuZn and extracellular superoxide dismutase were higher in Ang II–treated versus vehicle-treated MnSOD^+/− mice. Thus, a nonpressor dose of Ang II produces endothelial dysfunction in male mice only, suggesting that the female vasculature is protected from Ang II. In male but not female mice, MnSOD deficiency enhanced endothelial dysfunction, suggesting that MnSOD normally protects the vasculature during disease states in which Ang II contributes to vascular dysfunction. (Hypertension. 2010;55:905-910.)

Key Words: genetically altered mice ■ cerebral arteries ■ mitochondria ■ oxidative stress

Chronic hypertension has many deleterious effects on blood vessels, particularly in the cerebral circulation, where hypertension is a major risk factor for stroke and a leading cause of cognitive decline. The renin-angiotensin system and its main effector angiotensin II (Ang II) underlie many of the changes in vascular structure and function that occur in several forms of hypertension.1–4 Pharmacological inhibitors of the renin-angiotensin system are very useful clinically in the treatment of hypertension.5 In addition to mediating many of the negative effects of hypertension, Ang II also contributes to vascular disease in other states, including atherosclerosis and aging.6,7

Ang II increases the production of reactive oxygen species (ROS) in vascular cells via several mechanisms,4,8–12 including increased formation in mitochondria.13–15 Mitochondria may be a particularly important source of superoxide in the cerebral vasculature, because the mitochondrial content in cerebral endothelium is relatively high.16 Steady-state levels of superoxide in mitochondria are dependent on both its rate of production and activity of manganese superoxide dismutase (MnSOD), which converts superoxide to hydrogen peroxide.17 MnSOD is more abundantly expressed in endothelial cells compared with other cell types,17,18 suggesting that it may be particularly important in protecting against endothelial dysfunction. Although expression and activity of MnSOD in blood vessels can change in disease states including hypertension,17 the functional importance of MnSOD in relation to Ang II and hypertension is unknown.

Thus, the goal of this study was to examine the hypothesis that MnSOD protects against Ang II–induced vascular dysfunction. For our approach, we used mice genetically deficient in MnSOD to examine the role of this form of SOD.18 These studies were performed using a nonpressor dose of Ang II administered systemically. Recent studies suggest that inhibitory effects of Ang II on vascular responses are much less in females,19,20 and this was confirmed in the present study. A second goal was to test the hypothesis that Ang II produces vascular dysfunction in females in the presence of MnSOD deficiency.

Methods

Experimental Animals

Animals for study were derived from breeding MnSOD^+/+ and MnSOD^−/− mice, thus providing both genotypes and littermate controls as described.21 For some protocols, additional C57BL/6 male mice were studied (see below). Mice had access to regular diet and water ad libitum. Animals were permitted to acclimatize for at least 1 week before experiments began. A nonpressor dose of Ang II (0.28 mg/kg per day) or vehicle was administered systemically. Basilar arteries were isolated from mice treated for 1 week with a nonpressor dose of Ang II (0.28 mg/kg per day) or vehicle. Arteries were precontracted with U46619 (0.1 μM) and acetylcholine (5 nM to 10 μM) was added. Acetylcholine responses were determined as a percentage of the maximum difference between baseline and acetylcholine responses. "Female" refers to the sex of the offspring of the breeding pair, not the sex of the person who bred the mice.

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chow and water ad libitum. All of the protocols and procedures conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee at the University of Iowa.

**Administration of Ang II and Measurement of Blood Pressure**

After anesthesia with ketamine/xylazine (87.5 and 12.5 mg/kg SC, respectively), an osmotic minipump (Alzet, model 1002) was placed subcutaneously in the midscapular region to administer vehicle (isotonic saline) or Ang II (0.28 mg/kg per day for 7 days). Systolic blood pressure (BP) was measured using an automated tail-cuff device (BP-2000, Visitech Systems). Before surgery, mice were trained for 5 days and baseline BP was recorded, followed by implantation of minipumps and measurements of BP.

**Studies of Basilar Arteries**

Isolation and preparation of basilar arteries were described previously. In most experiments, we recorded changes in the diameter of the basilar artery in response to KCl (50 mmol/L). To examine responses to acetylcholine and nitroprusside, arteries were constricted submaximally (~40% of the response to KCl) using U46619. After the development of a stable baseline diameter, dose-response curves were obtained. In some experiments, vessels were treated with Tempol (100 μmol/L, 30 minutes) before the addition of acetylcholine. Papaverine (100 μmol/L) was added at the end of each experiment to produce maximal vasodilation.

**Measurement of ROS**

Vascular superoxide levels were measured in aorta using 5 μmol/L lucigenin-enhanced chemiluminescence. After measurements under basal conditions, NADPH (100 μmol/L) was added to stimulate NADPH oxidase–dependent superoxide formation. Tiron (10 mmol/L, a superoxide scavenger) inhibited the lucigenin signal by ~80% to 85% (data not shown). To minimize confounding effects of other superoxide dismutases, some experiments were performed in the presence of an inhibitor of CuZn superoxide dismutase and extracellular superoxide dismutase (diethyldithiocarbamate [DETC], 10 mmol/L).17

**Drugs**

Acetylcholine, Ang II, nitroprusside, papaverine, and Tempol were dissolved in saline. Tiron, lucigenin, NADPH, and DETC were dissolved in Dulbecco PBS. Most drugs were obtained from Sigma. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilutions being made with Krebs buffer.

**Statistical Analysis**

All of the data are expressed as mean±SE. Vasodilator responses are expressed as the percentage of dilation (percentage of induced tone), with 100% representing the difference between the resting value under basal conditions and the constricted value with U46619. Vasoconstriction to KCl is expressed as the percentage of change in diameter over baseline. For experiments using lucigenin-enhanced chemiluminescence, data were normalized to tissue dry weight and expressed as relative light units (RLUs) per second per milligram. Changes in BP were calculated by subtracting the baseline BP from the average BP over days 5 to 7 of treatment. Comparisons of vasodilation and vasoconstriction, superoxide levels, and BP were made using ANOVA with Student-Newman-Keuls post hoc test or Student t test, as appropriate. Statistical significance was accepted at P<0.05.

**Results**

**Effects of Ang II in Male C57Bl/6 Mice**

To establish the protocol in this model, we first studied male C57Bl/6 mice. Resting BP was similar in vehicle (118±7 mm Hg; n=10) and Ang II–treated (112±5 mm Hg; n=10) mice. Vascular and Ang II treatment had no significant effect on BP (ΔBP in vehicle-treated mice: 1±4 mm Hg, n=10; ΔBP in Ang II–treated mice: 5±6 mm Hg, n=10; P>0.05). These results are consistent with previous work.23 Body weight was unaffected by vehicle (weight before treatment: 25±1 g; weight after treatment: 26±1 g; n=10) or Ang II (weight before treatment: 27±1 g; weight after treatment: 28±1 g; n=10) treatment.

Baseline diameter of the basilar artery in C57Bl/6 mice treated with vehicle and Ang II was 137±4 μm (n=8) and 137±5 μm (n=10), respectively (P>0.05). Ang II treatment impaired vasodilator responses to acetylcholine compared with vehicle treatment (Figure 1A). Responses to nitroprusside (Figure 1B), KCl, and papaverine (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org) were not affected by Ang II. Ang II–induced impairment of vasodilation to acetylcholine was reversed by Tempol, suggesting that the endothelial dysfunction was superoxide mediated (Figure S1).

Ang II Had No Significant Effect on Arterial Pressure in Male MnSOD+/+ or MnSOD−/− Mice

Resting BP was similar in male MnSOD+/+ (117±5 mm Hg; n=14) and MnSOD−/− (114±2 mm Hg; n=16) mice. Vascular and Ang II treatment had no significant effect on BP in MnSOD+/+ mice (ΔBP in vehicle-treated mice: 3±6 mm Hg, n=7; ΔBP in Ang II–treated mice: 14±7 mm Hg, n=7;

**Figure 1.** Vasodilation to acetylcholine (A: vehicle, n=8; Ang II, n=10), and nitroprusside (B: vehicle, n=9; Ang II, n=10) in C57Bl/6 mice. All of the data are mean±SE.
P>0.05), consistent with results in C57Bl/6 mice. Similarly, treatment with vehicle and Ang II had no significant effect on BP in MnSOD+/+ mice (ΔBP in vehicle-treated mice: 9 ± 3 mm Hg, n = 6; ΔBP in Ang II–treated mice: 7 ± 6 mm Hg, n = 10; P>0.05), suggesting that MnSOD deficiency does not augment changes in BP in response to a low dose of Ang II. Thus, differences in vascular function observed in MnSOD−/− mice are related to MnSOD deficiency and not to differences in BP. Body weight in MnSOD+/+ and MnSOD−/− mice was unaffected by vehicle or Ang II treatment (data not shown).

Endothelial Dysfunction in Response to Ang II Was Enhanced in Male MnSOD−/− Mice
Baseline diameter of the basilar artery in female MnSOD+/+ mice treated with vehicle and Ang II was 138 ± 4 μm (n = 9) and 140 ± 7 μm (n = 10), respectively (P>0.05). Baseline vessel diameter in MnSOD+/+ mice was 132 ± 6 μm (n = 11; P>0.05 versus vehicle-treated MnSOD+/+ in animals treated with vehicle and 149 ± 5 μm (n = 14) in mice treated with Ang II (P<0.05 versus vehicle-treated MnSOD+/+). Acetylcholine caused similar dilation in arteries from vehicle-treated male MnSOD+/+ and MnSOD−/− mice (Figure 2).

Vasodilation to acetylcholine was modestly impaired in male MnSOD+/+ mice treated with Ang II compared with vehicle (Figure 2A). In contrast, Ang II treatment impaired vasodilator responses to acetylcholine in MnSOD−/− mice by as much as 70% (P<0.05; Figure 2B). These findings suggest that partial MnSOD deficiency markedly enhances Ang II–induced endothelial dysfunction.

Vasodilation to nitroprusside (Figure 2C and 2D) and papaverine (Table S2) were similar after vehicle and Ang II treatment in MnSOD+/+ and MnSOD−/− mice. Vasoconstriction to KCl was also unaffected by genotype and Ang II treatment (Table S2). These data suggest that the inhibitory effects of Ang II were selective for endothelium-dependent responses.

Ang II–Induced Endothelial Dysfunction in MnSOD−/− Mice Was Reversed by Tempol
In MnSOD+/+ mice, Tempol restored acetylcholine-induced vasodilator responses in Ang II–treated mice to normal (Figure 3), whereas Tempol had no significant effect on responses to acetylcholine in vehicle-treated mice (data not shown). These data suggest that increases in superoxide mediate endothelial dysfunction in response to Ang II in MnSOD−/− mice.

Ang II Did Not Cause Endothelial Dysfunction in Female Mice
Baseline diameter of the basilar artery in female MnSOD+/+ mice treated with vehicle and Ang II was 143 ± 12 μm (n = 7) and 157 ± 5 μm (n = 6), respectively (P>0.05). Baseline vessel diameter in female MnSOD+/+ mice was 149 ± 4 μm (n = 6; P>0.05 versus vehicle-treated MnSOD+/+) in animals treated with vehicle and 157 ± 11 μm (n = 6) in mice treated with Ang II (P>0.05 versus vehicle-treated MnSOD+/+). Body weight in MnSOD+/+ and MnSOD−/− mice was unaffected by vehicle or Ang II treatment (data not shown).

In contrast to males, Ang II had no significant effect on responses to acetylcholine in female mice of either genotype (Figure 4A and B). Responses to nitroprusside (Figure 4C) were increased in female MnSOD+/+ mice treated with Ang II compared with vehicle, suggesting that responses to NO were increased. There was no effect of Ang II treatment on nitroprusside responses in MnSOD−/− mice (Figure 4D). In MnSOD−/− and MnSOD−/− mice, vasoconstriction to KCl (Table S2) was similar after vehicle and Ang II treatment.
Superoxide Levels
In both strains of male mice, there were no significant differences in vascular superoxide levels under basal conditions, in response to this low dose of Ang II (MnSOD+/+; vehicle-treated, 16±4 RLU/s per milligram, n=11; Ang II-treated, 21±7 RLU/s per milligram, n=11; MnSOD+/-: vehicle-treated, 17±8 RLU/s per milligram, n=10; Ang II–treated, 27±8 RLU/s per milligram, n=10) or in the presence of NADPH (data not shown). Superoxide levels were higher in the presence of DETC and NADPH compared with DETC alone (Figure 5). In the presence of DETC, Ang II tended to increase superoxide levels in MnSOD+/- (0.05<P<0.1) but not MnSOD+/- mice (P=0.31; power: 18%; Figure 5). Superoxide levels in the presence of NADPH (and DETC) were greater in MnSOD+/- than in MnSOD+/+ mice treated with Ang II (Figure 5B), suggesting that MnSOD deficiency increases vascular superoxide production after Ang II treatment.

Discussion
The major new finding of the present study was that Ang II–induced endothelial dysfunction in a cerebral artery is markedly enhanced in male heterozygous MnSOD-deficient mice, suggesting that MnSOD is a critical component of mechanisms that protect the vasculature against Ang II.

Cerebral arteries like the basilar artery are important resistance vessels in the brain.24 To our knowledge, this is the first data of its kind for any blood vessel. The inhibitory effect of Ang II was reversed by a superoxide scavenger, and vascular superoxide levels after Ang II treatment were increased in MnSOD+/- mice. The effects on vascular function were seen with a low dose of Ang II that had little effect on arterial pressure. Interestingly, Ang II had no effect on vascular function in MnSOD+/+ or MnSOD+/- female mice, suggesting the concept that the female cerebral vasculature is protected from deleterious effects of Ang II,19,20 even in the presence of MnSOD deficiency. Protection against the deleterious effects of Ang II in the cerebral vasculature in females may occur at the level of sources of superoxide (NADPH oxidase).25 Suppression of ROS formation in the cerebral vascular wall may be estrogen dependent.19

Ang II–Induced Oxidative Stress and Endothelial Dysfunction
Scavengers of superoxide and other ROS protect against endothelial dysfunction in carotid arteries and cerebral blood vessels, as well as impairment of functional hyperemia in the brain in response to acute and chronic treatment with Ang II, and in a genetic model of Ang II–dependent hypertension.4,9,10,26,27 One mechanism by which superoxide impairs
endothelium-dependent relaxation is by decreasing the bioavailability of NO. Because Ang II is a major stimulus for the production of superoxide in the vasculature, impaired endothelium-dependent vasodilation in response to Ang II may be attributed in large part to increased superoxide levels. In the present study, we found that Ang II produced endothelial dysfunction that was reversed by a superoxide scavenger, suggesting that superoxide was the mediator of the dysfunction. We observed no significant changes in arterial scavenger, suggesting that superoxide was the mediator of the endothelial dysfunction that was reversed by a superoxide levels.

MnSOD Haploinsufficiency Enhances Ang II–Induced Oxidative Stress and Endothelial Dysfunction

Consistent with previous findings, deletion of a single copy of the gene for MnSOD did not alter responses in male or female cerebral arteries under baseline conditions. Although Ang II is known to increase ROS in mitochondria in endothelial cells, the functional importance of this effect in relation to the regulation of vascular tone has been unknown. MnSOD is more abundantly expressed in endothelial cells relative to other cell types, and the mitochondrial content in the cerebral endothelium is greater than that in other cells. Mitochondria may be a particularly important source of superoxide and, thus, MnSOD may play an important role in the cerebral circulation during oxidative stress. Perhaps the most important finding of this study is the observation that a dose of Ang II that produced only modest impairment of endothelial function in male wild-type mice produced marked impairment of endothelial function in male MnSOD-deficient mice. Thus, MnSOD is an important mediator of vascular protection in response to Ang II.

Our data with Tempol suggest that increased superoxide mediates the deleterious effects of Ang II under control conditions and in the presence of MnSOD deficiency. Ang II increased vascular superoxide levels in MnSOD-deficient mice under conditions where CuZn superoxide dismutase and extracellular superoxide dismutase were inhibited, suggesting that Ang II increased superoxide production. Furthermore, superoxide levels in the presence of NADPH (and DETC) were greater in MnSOD-deficient versus MnSOD mice treated with Ang II. This finding is consistent with the concept that Ang II–induced activation of NADPH oxidase leads to mitochondrial ROS production. There are several isoforms of the catalytic subunit of NADPH oxidase (Nox1, Nox2, Nox4, and Nox5), and the subcellular distribution of these proteins varies with cell type and tissue. There has not been much evidence for NADPH oxidase localization in mitochondria to our knowledge. However, a recent study has suggested that Nox4 may be present in mitochondria.

Mitochondrial-derived ROS may activate NADPH oxidase, resulting in increased superoxide production and reduced NO bioavailability. Such a sequence would be consistent with our result showing enhanced NADPH-induced superoxide production in the presence of Ang II under conditions of MnSOD deficiency.

MnSOD Deficiency Does Not Enhance Vascular Dysfunction After Treatment With Ang II in Females

It is well established that premenopausal women have a lower incidence of cardiovascular disease compared with age-matched men. Female sex is associated with vascular protection. Pressor doses of Ang II impair cerebral vascular responses to endothelium-dependent agonists in male but not in female mice. Our finding that Ang II had little effect on endothelial function in female mice is consistent with these previous reports. Our finding that, even in the presence of MnSOD deficiency, endothelial function is not impaired after Ang II treatment in females is novel and further emphasizes the marked sex-dependent effects of Ang II on blood vessels.

Perspectives

ROS are thought to play a major role in vascular disease. The present study supports that concept and provides the first evidence that MnSOD deficiency promotes endothelial dysfunction in response to Ang II in males. Thus, MnSOD may be an important mediator of vascular protection during hypertension but may also play a role in other states where Ang II is thought to contribute to vascular disease, such as atherosclerosis and aging. Because we observed significant vascular effects after deletion of only 1 copy of the MnSOD gene, our findings have implications for disease states or genetic polymorphisms that cause decreased expression or activity of MnSOD. We also demonstrated for the first time that, even in the face of MnSOD deficiency, female mice are protected against vascular effects of Ang II.

Acknowledgments

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Disclosures

None.

References


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Running Title: MnSOD Protects Against Vascular Dysfunction

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**Supplemental Table 1.** Vasodilator responses to papaverine, and vasoconstrictor responses to KCl in vehicle and Ang II-treated C57Bl/6 mice. Data are expressed as % change in diameter over baseline. All data are mean±SE. Numbers in brackets indicate n value.

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<tr>
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<td>Papaverine (100 µmol/L)</td>
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<td>74±9 (10)</td>
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<td>KCl (50 mmol/L)</td>
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<td>-50±2 (10)</td>
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**Supplemental Table 2.** Vasodilator responses to papaverine, and vasoconstrictor responses to KCl in vehicle and Ang II-treated MnSOD\(^{+/+}\) and MnSOD\(^{+/−}\) male and female mice. Data are expressed as % change in diameter over baseline. All data are mean±SE. Numbers in brackets indicate n value.

*P<0.05 vs vehicle-treated MnSOD\(^{+/+}\)

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<td>81±3 (10)</td>
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<table>
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<tr>
<td>KCl (50 mmol/L)</td>
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S1. Effect of tempol (100 µmol/L) on vasodilation to acetylcholine in Ang II-treated (0.28 mg/kg x d for 14 days) male C57Bl/6 mice (n=7). All data are mean±SE.