Superoxide Dismutase 1 Limits Renal Microvascular Remodeling and Attenuates Arteriole and Blood Pressure Responses to Angiotensin II via Modulation of Nitric Oxide Bioavailability

Mattias Carlström, En Yin Lai, Zufu Ma, Andreas Steege, Andreas Patzak, Ulf J. Eriksson, Jon O. Lundberg, Christopher S. Wilcox, A. Erik G. Persson

Abstract—Oxidative stress is associated with vascular remodeling and increased pregglomerular resistance that are both implicated in the pathogenesis of renal and cardiovascular disease. Angiotensin II induces superoxide production, which is metabolized by superoxide dismutase (SOD) or scavenged by NO. We investigated the hypothesis that SOD1 regulates renal microvascular remodeling, blood pressure, and arteriolar responsiveness and sensitivity to angiotensin II using SOD1-transgenic (SOD1-tg) and SOD1-knockout (SOD1-ko) mice. Blood pressure, measured telemetrically, rose more abruptly during prolonged angiotensin II infusion in SOD1-ko mice. The afferent arteriole media:lumen ratios were reduced in SOD1-tg and increased in SOD1-ko mice. Afferent arterioles from nontreated wild types had graded contraction to angiotensin II (sensitivity: 10^{-9} mol/L; responsiveness: 40%). Angiotensin II contractions were less sensitive (10^{-8} mol/L) and responsive (14%) in SOD1-tg but more sensitive (10^{-13} mol/L) and responsive (89%) in SOD1-ko mice. Afferent arterioles from SOD1-ko had 4-fold increased superoxide formation with angiotensin II at 10^{-9} mol/L. N^{G}-nitro-L-arginine methyl ester reduced arteriole diameter of SOD1-tg and enhanced angiotensin II sensitivity and responsiveness of wild-type and SOD1-tg mice to the level of SOD1-ko mice. SOD mimetic treatment with Tempol increased arteriole diameter and normalized the enhanced sensitivity and responsiveness to angiotensin II of SOD1-ko mice but did not affect wild-type or SOD1-tg mice. Neither SOD1 deficiency nor overexpression was associated with changes in nitrate/nitrite excretion or renal mRNA expression of NO synthase, NADPH oxidase, or SOD2/SOD3 isoforms and angiotensin II receptors. In conclusion, SOD1 limits afferent arteriole remodeling and reduces sensitivity and responsiveness to angiotensin II by reducing superoxide and maintaining NO bioavailability. This may prevent an early and exaggerated blood pressure response to angiotensin II. (Hypertension. 2010;56:00-00.)

Key Words: afferent arterioles ▪ CuZnSOD ▪ hypertension ▪ ICSOD ▪ oxidative stress ▪ superoxide ▪ SOD1 ▪ kidney

Oxidative stress implies a shift in the balance between the production of reactive oxygen species (ROS) and the action of antioxidant systems and has been implicated in the pathogenesis of renal and cardiovascular disease.1,2 NADPH oxidase is an important source of superoxide (O_2^-) in the vasculature. O_2^- that escapes metabolism by superoxide dismutase (SOD) can inactivate NO,3 and growing evidence demonstrates that oxidative stress and NO deficiency in the kidney contribute to vascular dysfunction and hypertension.3 Furthermore, a potential crosstalk between NADPH oxidase and SOD activities has been suggested.4,5

SOD exists as 3 different isoforms: copper-zinc SOD (SOD1), predominately located in the cytoplasm; manganese SOD (SOD2) in the mitochondria; and extracellular SOD (SOD3) in the extracellular space. SOD1 accounts for 60% to 80% of SOD activity in the kidneys and also has an important role in blood vessels, where it preserves NO release from the endothelium.6,7 The renal afferent arterioles are the major resistance vessels to the kidneys and play an important role in blood pressure regulation.8 The afferent arteriolar tone and resistance are determined by the balance between constrictor agents, such as angiotensin II (Ang II) and vasodilator pathways, notably NO.9

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Infusions of Ang II induce NO release in afferent arterioles, but the response to this is limited by a concomitant increase in $O_2^-$ produced by NADPH oxidase. Indeed, an infusion of Ang II may produce sufficient ROS to overwhelm the antioxidant systems, with subsequent NO deficiency, hyperreactivity, and remodeling of afferent arterioles. Increased arteriolar reactivity may reduce renal perfusion and filtration, increase extracellular volume, and, thus, contribute to the development of Ang II–induced hypertension.

These properties of ROS underscore the importance of systems that limit $O_2^-$ in adjusting renal arteriolar tone. However, there is less information regarding the functional significance of selected SOD isoforms in the regulation of renal microcirculation and blood pressure. We tested the hypothesis that SOD1 regulates renal microvascular remodeling and limits afferent arteriolar responsiveness and hypertension in response to Ang II.

**Materials and Methods**

**Experimental Design**

The experiments were conducted on homozygous littermates from heterozygous breeding pairs of SOD1-transgenic (SOD1-tg; C57BL/6-Tg[SOD1]3CjeJ [stock No. 002629]) or SOD1-knockout (SOD1-ko; B6;129S7-Sod1tm1Leb/J [stock No. 002972]) mice from the Jackson Laboratory (Bar Harbor, ME). Wild-type littermates from the breeding colonies served as controls. Genotyping of the offspring was performed as described previously (please see the online Data Supplement). Both sexes were used, with equal distribution and similar age (10 to 16 weeks) in every set of experiments. The animals were fed standardized mouse chow (0.7% NaCl, R36, batch SD389, Lactamin) and tap water ad libitum. Telemetric blood pressure measurements were performed in wild-type and SOD1-ko mice before and after NADPH oxidase-mediated infusions of a slowpressor dose of Ang II for 14 days. Renal excretion of nitrate/nitrite was analyzed to determine total NO production. All of the afferent arteriole experiments (ie, isometric contractions and changes in $O_2^-$ levels) and gene expression studies were performed in nontreated mice (ie, without prolonged Ang II infusion).

**Blood Pressure Response to Prolonged Ang II Infusion**

Telemetric devices (PA-C10, DSI) were implanted in wild-type and SOD1-ko mice. After surgery, the animals were allowed to recover for ≥10 days. Thereafter, the blood pressure was measured continuously for 96 hours to determine basal levels (please see the online Data Supplement). The mice were anesthetized by spontaneous inhalation of isoflurane (Forene, Abbot Scandinavia AB) in air (~2.2%), and an osmotic minipump (ALZET, Durect) was implanted subcutaneously, delivering Ang II (Sigma-Aldrich) at 400 μg/kg per 24 hours for 14 days. After implantation, blood pressure was recorded continuously throughout the Ang II delivery period.

**Renal Excretion of Nitrate/Nitrite**

Mice were placed in metabolic cages for 24 hours, with food and water given ad libitum. Water consumption and urine production were measured gravimetrically. Samples of fresh urine were stored at −70°C until analysis. Nitrate and nitrite in urine were measured with a dedicated high-performance liquid chromatography system (ENO-20, EiCom) described previously (please see the online Data Supplement).

**Afferent Arteriole Measurements**

**Dissection and Perfusion of Arterioles**

Dissection and perfusion were performed as described previously (please see the online Data Supplement).

**Figure 1.** Setup for the renal afferent arteriole experiments. The microphotograph shows a glomerulus and its afferent arteriole held by 2 holding pipettes. The perfusion pipette (5-μm diameter), inserted into the holding pipette on the right side, was connected to a reservoir containing the perfusion solution to provide a pressure of 100 mm Hg in the pressure head, which produced a flow of ~50 nL/min. The inner luminal diameter (Ø) of the arteriole was measured at the most active site to estimate the effect of vasoactive substances.

**Measurements of Arteriolar Diameter**

The technique for the renal afferent arterioles is demonstrated in Figure 1. The experiments were recorded by a video system, digitized offline, and analyzed as described previously. The inner luminal diameters of the arterioles were measured at the most active site to estimate the effect of vasoactive substances. Responsiveness (ie, maximal change in arteriolar diameter) and sensitivity (ie, threshold concentration of Ang II mediating a significant change in arteriolar diameter) were calculated to investigate the roles of NO and $O_2^-$ for the Ang II–induced contraction. In all of the series, the last 10 seconds of a control or treatment period were used for statistical analysis of steady state responses. Each experiment used a separate dissected afferent arteriole; only 1 arteriole was used per animal. The inner luminal diameter and media thickness were measured during baseline (before application of any substances), and the areas were calculated to compute the media:lumen ratios to assess vascular remodeling.

**$O_2^-$ Measurements in Afferent Arterioles**

$O_2^-$ generation was assessed by fluorescence microscopy of perfused afferent arterioles with dihydroethidium or temp–9AC, as described previously (please see the online Data Supplement).

**Analysis of mRNA Expression**

Infusion of cold PBS was started once the vena cava was cut to remove the blood. The heart and kidneys were explanted, blotted, and weighed. The renal cortex was separated and homogenized, and quantitative PCR analysis was performed (please see the online Data Supplement).

**Drugs and Reagents**

All of the drugs were applied to the bath solution in the arteriolar contraction experiments (please see the online Data Supplement).

**Statistical Analysis**

Values are presented as mean±SEM. Repeated-measures ANOVA was used to test time- or concentration-dependent changes in the arteriolar diameter and to assess differences between the groups. Post hoc comparisons were performed with a Fisher test. ANOVA, followed by the Fisher post hoc test, when appropriate, was used for analysis of blood pressure, changes in $O_2^-$ levels, and vascular...
remodeling. Wilcoxon tests were applied for gene expression data. Differences were considered to be statistically different if $P<0.05$.

**Ethics**

The experiments were approved by the Uppsala Ethical Committee for Animal Experiments and Georgetown University Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Results**

All of the animals were in good condition, and at the time of euthanasia, there were no differences in body weights among the groups. The heterozygous SOD1-ko breeding generated a lower number of homozygous offspring ($+/+$, 29%; $+/-$, 52%; $-/-$, 19%), but no differences in mendelian distribution were found for the heterozygous SOD1-tg breeding. SOD1-ko mice displayed higher kidney and heart masses, adjusted for body weights (Table).

**Blood Pressure Response to Prolonged Ang II Infusion**

There was no difference in basal blood pressure (ie, 4 days mean) between the wild-type (107±1 mm Hg) and SOD1-ko (110±5 mm Hg) mice. SOD1-ko mice displayed a dramatic initial blood pressure response to Ang II but reached a similar blood pressure level as wild-type mice after 10 days of treatment. During the first 4 days of Ang II infusion, blood pressure increased with 37±11 mm Hg in SOD1-ko mice ($P<0.05$), whereas no significant change was observed in wild-type mice (3±1 mm Hg). The wild-type mice displayed a typical slowpressor response to Ang II with a significant blood pressure response after 10 days of treatment. Averaged blood pressure data for baseline period and for the whole Ang II treatment period are presented in Figure 2.

**Renal Nitrate/Nitrite Excretion**

There were no differences in renal nitrate/nitrite excretion (in nanomoles per 24 hours per gram of body weight) among SOD1-tg (37±4; $n=7$), wild-type (50±15; $n=7$), and SOD1-ko mice (53±20; $n=7$), indicating that the systemic NO production was similar in all 3 of the genotypes.

**Afferent Arteriole Measurements**

To investigate the hypothesis that increased arteriolar responsiveness/sensitivity contributes to the early and rapid blood pressure response observed in SOD1-ko mice, all of the afferent arteriole experiments were performed in nontreated mice (ie, without prolonged Ang II infusion).

**Basal Arteriolar Areas and Vascular Remodeling**

The basal luminal area of afferent arterioles was significantly smaller in SOD1-ko mice than in wild-type and SOD1-tg mice (Table). No difference was found between the SOD1-tg and wild-type mice. Compared with wild-type mice, the arteriolar media thickness and media:lumen ratios were in-

### Table. Renal and Cardiovascular Characteristics of SOD1 Mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild-Type</th>
<th>SOD1-ko</th>
<th>SOD1-tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney and heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>30.1±1.3</td>
<td>27.2±1.8</td>
<td>26.5±1.1</td>
</tr>
<tr>
<td>TKW, g</td>
<td>0.328±0.004</td>
<td>0.329±0.017</td>
<td>0.279±0.010†</td>
</tr>
<tr>
<td>TKW/BW×10³</td>
<td>10.9±0.1</td>
<td>12.2±0.5*</td>
<td>10.6±0.2†</td>
</tr>
<tr>
<td>HW, g</td>
<td>0.155±0.502</td>
<td>0.155±0.004</td>
<td>…</td>
</tr>
<tr>
<td>HW/BW×10³</td>
<td>5.1±0.0</td>
<td>5.8±0.3*</td>
<td>…</td>
</tr>
<tr>
<td>Afferent arterioles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media area, μm²</td>
<td>181±10</td>
<td>202±8*</td>
<td>138±10†</td>
</tr>
<tr>
<td>Lumen area, μm²</td>
<td>108±8</td>
<td>77±5*</td>
<td>113±9†</td>
</tr>
<tr>
<td>Media:lumen ratio</td>
<td>1.83±0.14</td>
<td>2.85±0.18*</td>
<td>1.35±0.12†</td>
</tr>
<tr>
<td>Afferent arteriole O₂⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change with Ang II, %</td>
<td>6.6±1.9</td>
<td>28.9±6.8*</td>
<td>…</td>
</tr>
</tbody>
</table>

BW indicates body weight; TKW, total kidney weight; and HW, heart weight in wild-type ($n=7$), SOD1-ko ($n=7$), and SOD1-tg ($n=7$) mice. Afferent arterioles include evaluation of vascular properties, as a measure for vascular remodeling, in wild-type ($n=22$), SOD1-ko ($n=23$), and SOD1-tg ($n=26$) mice. Afferent arteriole $O_2^-$ shows changes in $O_2^-$ levels in response to extraluminal application of Ang II at $10^{-9}$ mol/L in wild-type ($n=7$) and SOD1-ko ($n=5$) mice. Values are presented as mean±SEM.

* $P<0.05$ compared with wild-type mice.
† $P<0.05$ compared with SOD1-ko mice.

Figure 2. SOD1 prevents a rapid and exaggerated blood pressure response to angiotensin II. Top, Mean arterial pressure in conscious wild-type ($n=5$) and SOD1-ko mice ($n=5$). The telemetry measurements were conducted continuously under control conditions for 4 days (Baseline) and then with chronic Ang II infusion at a slow-pressor rate (400 μg/kg per 24 hours) for 14 days. Bottom, Mean arterial pressure during baseline period (4 days average) and the whole Ang II infusion period (14 days average). Basal blood pressures were similar in both genotypes; however, Ang II elevated blood pressure more in SOD1-ko mice than in wild-type mice. Values are presented as mean±SEM.

$P<0.05$.
 increased in SOD1-ko mice and reduced in SOD1-tg mice (Table). To control for these differences in baseline luminal area, results are presented for absolute and fractional changes of the vessels. Please see the online Data Supplement for absolute changes in arteriole diameters.

**Effect of Ang II**

Concentration-response curves were obtained by cumulative application of Ang II (10^{-14} to 10^{-6} mol/L; each for 2 minutes). Ang II constricted afferent arterioles from wild-type mice in a concentration-dependent manner (threshold response: 10^{-9} mol/L), with a maximum response of 40%. Afferent arterioles of SOD1-ko mice were more sensitive (threshold response: 10^{-13} mol/L) and more responsive (89%) to Ang II, but arterioles from SOD1-tg mice were less sensitive (threshold response: 10^{-8} mol/L) and less responsive (14%) to Ang II (Figure 3).

**Effect of NO Synthase Inhibition With N^G-Nitro-L-Arginine Methyl Ester**

N^G-nitro-L-arginine methyl ester (L-NAME; 10^{-4} mol/L), applied for 15 minutes, constricted afferent arterioles of wild-type mice by 8%, but no contractile response was observed in the SOD1-ko mice. Arterioles from the SOD1-tg mice had a stronger contraction (-38%) after 15 minutes of treatment (Figure 4).

**Effect of L-NAME Treatment on Ang II–Induced Contractions**

To investigate the role of NO in offsetting Ang II–induced arteriolar contraction, vessels were treated with L-NAME (10^{-4} mol/L) for 15 minutes before and during treatment with Ang II (10^{-14} to 10^{-6} mol/L; 2 minutes each dose). L-NAME did not affect the responsiveness to Ang II in SOD1-ko mice but enhanced the response of wild-type and SOD1-tg mice to the level of SOD1-ko mice (Figure 5). L-NAME treatment increased the sensitivity to Ang II in SOD1-ko (10^{-13} to 10^{-14} mol/L) but to a much higher degree in wild-type mice (10^{-9} to 10^{-14} mol/L) and in SOD1-tg mice (10^{-8} to 10^{-13} mol/L).

**Effect of Tempol**

Tempol (10^{-7} mol/L) was applied for 15 minutes to reduce basal levels of O2^-. Tempol dilated afferent arterioles of SOD1-ko mice by 11% but did not change the diameter of arterioles from wild-type or SOD1-tg mice (Figure 6).

**Effect of Tempol Treatment on Ang II–Induced Contractions**

To investigate the role of O2^- on Ang II–induced arteriolar contraction, vessels were treated with Tempol (10^{-4} mol/L) for 15 minutes before and during treatment with Ang II (10^{-14} to 10^{-6} mol/L; 2 minutes each dose). The addition of Tempol did not influence the responsiveness to Ang II in SOD1-tg (18%) or wild-type (43%) mice (Figure 7) but strongly attenuated the contractile response to Ang II in the SOD1-ko mice, with a reduction in the maximal response from 89% to 47%. Tempol treatment did not change the sensitivity to Ang II in wild-type or SOD1-tg mice.
SOD1-tg mice but normalized the enhanced sensitivity in SOD1-ko mice (10⁻¹⁳ to 10⁻⁹ mol/L).

**Afferent Arteriolar O₂⁻ Levels**

**Effect of Ang II**

To investigate whether the enhanced contractile response to Ang II in SOD1-tg mice was related to compromised antioxidant defense with increased oxidative stress, O₂⁻ levels were measured in response to Ang II (10⁻⁹ mol/L). As shown in the Table, Ang II–induced changes in O₂⁻ levels were significantly higher in SOD1-ko than in wild-type mice.

**Gene Expression Studies**

Real-time PCR revealed no differences in renal cortical mRNA expression for NO synthase (NOS), NADPH oxidase, or SOD2/SOD3 isoforms or in Ang II receptors among the genotypes (please see the online Data Supplement).

**Figure 6.** SOD mimic treatment dilates renal microvessels that lack SOD1. Effect of Tempol (10⁻⁴ mol/L) on diameters of isolated and perfused afferent arterioles from nontreated SOD1-tg (n=6), wild-type (n=7), and SOD1-ko (n=7) mice. Values are presented as mean±SEM. c, *P*<0.05 compared with control period in SOD1-ko mice. *P*<0.05 compared with wild-type and SOD1-tg mice.

**Figure 7.** SOD mimic treatment normalizes the exaggerated Ang II response of renal microvessels that lack SOD1. Effect of Tempol (10⁻⁴ mol/L) on Ang II concentration response in isolated and perfused afferent arterioles of nontreated SOD1-tg (n=6), wild-type (n=7), and SOD1-ko (n=7) mice. Control period represents the arteriolar diameter after Tempol pretreatment for 15 minutes. Values are presented as mean±SEM. a, *P*<0.05 compared with control period in SOD1-tg mice. b, *P*<0.05 compared with control period in wild-type mice. c, *P*<0.05 compared with control period in SOD1-ko mice. *P*<0.05 compared with wild-type and SOD1-ko mice.

**Discussion**

The main new findings are that the SOD1 attenuates responsiveness and sensitivity of renal afferent arterioles to Ang II and moderates the early increase in blood pressure to prolonged Ang II. Furthermore, the study suggests that SOD1 deficiency is associated with renal and cardiac hypertrophy and hypertrophic remodeling of renal microvessels.

SOD1 is the predominant isoenzyme in the vascular wall and accounts for the majority of SOD activity in the kidney.⁶,²⁰ Didion et al²¹ have demonstrated that SOD1 deficiency is associated with increased O₂⁻ levels in carotid arteries and endothelial dysfunction,²⁰ which can be prevented by a SOD mimic. Furthermore, increased SOD1 expression markedly reduced Ang II–induced vascular O₂⁻ levels in the aorta.²² SOD1-ko mice are normotensive but display renal oxidative stress and increased blood pressure during high sodium treatment, which can be reversed by treatment with a SOD mimic.²³

An attenuated blood pressure and vascular response to l-NAME has been reported in SOD3-ko mice²⁴ and was ascribed to reduced NO bioactivity. The responsiveness of afferent arterioles to Ang II was reduced in SOD1-tg mice but strongly augmented in SOD1-ko mice. NOS inhibition did not change arteriolar diameter in the SOD1-ko mice but reduced the diameter in SOD1-tg mice. This suggests that the basal NO bioavailability in afferent arterioles was low in SOD1-ko mice but high in SOD1-tg mice. This was confirmed by the finding that, after l-NAME treatment, the contractile response to Ang II became similar among all 3 of the genotypes. Furthermore, l-NAME enhanced the sensitivity to Ang II in wild-type and SOD1-tg mice (10⁻⁷ times) to the level of SOD1-ko mice. This suggests that NO bioavailability was a critical determinant for the differences in responsiveness and sensitivity to Ang II among the groups. SOD1-ko mice have enhanced bioactivation of NO and increased formation of peroxynitrite²⁵ and lipid peroxidation.²⁶ The proposal that increased microvascular O₂⁻ levels were responsible for the enhanced responses to Ang II in SOD1-ko mice was supported by measurements of Ang II–induced changes in O₂⁻ and experiments with Tempol in afferent arterioles. Administration of Ang II (10⁻⁹ mol/L) caused a 4-fold greater arteriolar contraction in SOD1-ko mice than in wild-types, accompanied by a 4-fold greater increase in microvascular O₂⁻. Tempol is a redox-cycling nitroxide that effectively dismutates O₂⁻.²⁶ Tempol dilated afferent arterioles in SOD1-ko mice and prevented their exaggerated contractile response to Ang II. Furthermore, Tempol normalized the enhanced sensitivity to Ang II in SOD1-ko mice (reduced by 10⁴ times) but had no effects in wild-type or SOD1-tg mice. This finding is consistent with previous studies that the renal vascular resistance of normal mice is little affected by Tempol but is reduced in mice with oxidative stress because of prolonged Ang II infusion.²⁷ Taken together, this demonstrates that SOD1-ko mice have increased microvascular oxidative stress and reduced NO bioavailability that enhances their response to Ang II. However, the diminished contraction to Ang II in SOD1-tg mice persisted after Tempol, indicating the operation of a mechanism other than reduced O₂⁻ that limited responsiveness in the group.
Oxidative stress and NO deficiency have been implicated in microvascular remodeling and endothelial dysfunction in cardiovascular disease.28 Scavenging of ROS or overexpression of SOD suppresses Ang II–induced hypertrophic remodeling in aortic smooth muscle cells.29 Furthermore, endothelial NOS–deficient mice and rats with prolonged NOS blockade displayed vascular remodeling that is independent of blood pressure,28 demonstrating that basal NO regulates vascular growth. Thus, renal microvascular hypertrophic remodeling in SOD1-ko mice and reduced wall thickness of SOD1-tg mice may relate to O₂⁻–dependent changes in NO bioavailability. We have demonstrated previously that kidneys from SOD1-ko mice displayed hypertrophy and fibrotic and inflammatory changes.23 The present study confirms renal hypertrophy and shows further that SOD1 deficiency caused cardiac hypertrophy.

Our findings that SOD1 limits vascular remodeling of afferent arterioles might have contributed to the differences in contractile behavior. However, our results dissociate the effects of vascular remodeling from arteriole contractile responses to Ang II, because the responses became indistinguishable by short-term inhibition of NOS. This indicates that the effects of SOD1 on the response to Ang II are attributed to modulation of the offsetting effect of bioactive NO. Furthermore, the finding that Ang II caused a 4-fold greater increase in afferent arteriolar O₂⁻ in SOD1-ko mice and that Tempol normalized the enhanced contractile responses demonstrates an important role of SOD1 in limiting the vascular accumulation of O₂⁻ during challenge with Ang II.

Evaluation of mRNA expression in the renal cortex did not reveal any differences between SOD1-tg and SOD1-ko mice for gene expression of NOS, NADPH oxidase, or SOD2/SOD3 isoforms or Ang II receptors. Although expression studies in afferent arterioles would have been more conclusive, our renal cortical measurements indicate that differences among the genotypes are mainly attributed to the ability of SOD1 to metabolize O₂⁻ and modulate NO bioavailability rather than changes in other key enzymes/receptors. Studies in SOD3-knockout mice also have determined that a lack of this SOD isoform is not compensated for by other antioxidative enzymes.30 Moreover, unchanged expression of NOS isoforms in the kidney and similar rates of nitrate/nitrite excretion among the genotypes are consistent with similar overall NO production and suggest that the major effects of SOD1 expression are to increase NO bioavailability. A recent study demonstrated that glutathione S-transferase α4 is compensatory induced in young SOD1-ko mice and may have a protective role against oxidative stress.30 However, the present study suggests that changes in glutathione S-transferase α4 do not, or only partly, compensate for SOD1 deficiency in adult mice.

Ang II treatment can activate NADPH oxidases and increase O₂⁻ production with subsequent renal NO deficiency. The slow-pressor response to Ang II is suggested to be of renal origin,31 because the blood pressure is salt sensitive22 and is associated with elevated renal microvascular resistance,33 enhanced Ang II induced responses of afferent arterioles,12,34 and salt retention.35 In the present study, a slow-pressor infusion of Ang II was converted into a pressor infusion in SOD1-ko mice in which it elevated blood pressure within the first day. Interestingly, SOD3-ko mice also displayed an exaggerated hypertensive response to Ang II at a pressor rate, which was attributed to reduced NO bioavailability.8,24,36 In contrast, Ang II infused at a slow-pressor rate led to similar increases in blood pressure in SOD3-ko and wild-type mice,8 which were attributed to down-regulation of SOD3 and upregulation of SOD1 in the kidney.8 Thus, SOD1 appears to provide a major defense in the kidney against Ang II–induced ROS accumulation. A limitation of the present study is that blood pressure responses to Ang II were not investigated in SOD1-tg mice. We have demonstrated previously that Tempol treatment in wild-type mice reduced the slow-pressor response to Ang II.27 Based on this finding we would expect SOD1-tg mice to have an attenuated response compared with wild-type mice given Ang II. The mechanisms responsible for returning blood pressure to SOD1-ko mice to levels of wild-type mice during prolonged infusion of Ang II remain to be investigated.

Our findings provide evidence that reduced antioxidant capacity, because of lack of SOD1, rendered the renal microvascular system sensitive to changes in O₂⁻ production. Increased O₂⁻ availability may contribute to the exaggerated blood pressure response to Ang II in SOD1-ko mice by preventing buffering by NO in blood vessels or in the kidney.37 Both effects can increase the sensitivity of the tubuloglomerular feedback response, which may reduce the glomerular filtration rate and cause volume retention, which has been demonstrated in the development of hypertension in several experimental models.16,38–40 In conclusion, the present study provides evidence that SOD1 attenuates renal microvascular responses to Ang II by ROS scavenging and maintaining NO bioavailability. This feature of SOD1 may prevent an early and exaggerated blood pressure response to Ang II. Furthermore, SOD1 plays an important role in regulating renal microvascular hypertrophic remodeling.

Perspectives
Oxidative stress has been implicated in the pathogenesis of many human diseases, including hypertension. Emerging evidence suggests that increased O₂⁻ levels and NO deficiency in the kidney play crucial roles in the development and persistence of hypertension. SOD1 deficiency leads to endothelial dysfunction, and in the present study we demonstrate that SOD1 deficiency enhances preglomerular and blood pressure responses to Ang II and is associated with renovascular remodeling and cardiac hypertrophy. From the present study one could speculate that increased O₂⁻ levels in the kidney, because of compromised antioxidant defense, can cause hypertension by increasing preglomerular vascular resistance. Studies have shown that patients with endothelial dysfunction, vascular remodeling, or cardiac hypertrophy are at increased risk for subsequent cardiovascular events. Therefore, antioxidant strategies specifically targeting SOD isoforms could have therapeutic benefit in preventing a wide spectrum of adverse cardiovascular outcomes.

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Disclosures

None.

References

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ONLINE SUPPLEMENT

SOD1 Limits Renal Microvascular Remodeling and Attenuates Arteriole and Blood Pressure Responses to Angiotensin II via Modulation of Nitric Oxide Bioavailability

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Supplemental Methods

Genotyping
From all SOD1-tg and SOD1-ko offspring a piece of the tail was collected for DNA preparation. Each sample tube contained Tris buffer (50mM TrisBase, 100mM ethylenediaminetetraacetic acid [EDTA], and 100mM NaCl, 20% sodium dodecyl sulfate, pH 7.5) with 10 mg/ml Proteinase K (Sigma-Aldrich Sweden AB) and was incubated at 55ºC overnight. Samples were then vortexed and centrifuged at 13,000 3 g for 10 min. To precipitate DNA, 300 µl of each supernatant was transferred to a new tube and mixed with 200 µl of 2-propanol (Kebo lab AB, Spånga, Sweden) and gently shaken. Next, the samples were centrifuged at 13,000 x g for 10 min. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellets. Samples were then centrifuged at 13,000 x g for 5 min. Subsequently, the supernatant was removed and the pellets were dried and resuspended in RNase-free water. Finally the DNA concentration was estimated by measuring the absorbance of the samples.

Transgenic: Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 5.80 µl of RNase-free water, 25 mM MgCl2, 10x PCR gold buffer, 2.5 mM deoxy nucleotidyl triphosphate (dNTP) (Ampli Taq Gold with Gene Amp, Applied Biosystems, Branchburg, NJ), 20 µM of each mouse CuZnSOD and human CuZnSOD sense and antisense primers (TIB, Molbiol, Berlin, Germany) were used in the PCR reaction. Wild-type genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 324 bp). Heterozygous genotype is denoted if similar amounts of both the hCuZnSOD and mouse endogenous CuZnSOD PCR product are amplified (product size of 324 and 236 bp, respectively). Homozygous genotype is denoted if a higher amount of the inserted hCuZnSOD PCR product is amplified.

Knockout: Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 15.3 µl of RNase-free water, 25 mM MgCl2, 10x PCR gold buffer, 5 mM dNTP (Ampli Taq Gold with Gene Amp), 20 µM of each HPRT (hypoxanthine guanine phosphoribosyl transferase), and CuZnSOD sense and antisense primers (TIB, Molbiol, Berlin, Germany). The HPRT primers amplify a DNA fragment, which includes the CuZnSOD promoter region and exon 2, whereas the primers for mouse endogenous CuZnSOD amplify a DNA fragment from the wt allele on exon 2. Wild-type genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 123 bp). Heterozygous genotype is denoted if PCR products positive for both HPRT and mouse CuZnSOD are amplified. Homozygous genotype is denoted if PCR products positive for only HPRT are amplified (product size of 240 bp).

Separate PCR cycling programs were used for tg and ko samples. The following parameters were used: Transgenic: (1) denaturation at 95ºC for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 95ºC for 30 s, annealing temperature at 60ºC for 30 s, elongation temperature at 72ºC for 2 min. Knock-out: (1) denaturation at 94ºC for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 94ºC for 30 s, annealing temperature at 61ºC for 1 min, elongation temperature at 72ºC for 2 min.
**Telemetric Blood Pressure Measurements**

Anaesthesia was induced by spontaneous inhalation of isoflurane (Forene®, Abbot Scandinavia AB, Kista, Sweden) and was continued throughout surgery by inhalation of 2.2% isoflurane in air. A midline incision was made between the lower mandible and sternum and the catheter of the telemetric blood pressure device (PA-C10 (DSI™, Transoma Medical, St Paul, MN, USA) was then inserted into the left carotid lumen and secured by 6/0 silk sutures (Silk®, Ethicon, Johnson & Johnson Intl, USA). The entry site was sealed by application of n-butyl-cyanoacrylate tissue adhesive (VetbondTM, 3M Animal Care Products, St Paul, MN, USA) and the body of the transmitter was placed subcutaneously in the right flank. Finally the skin incision was closed using 5/0 polyamide sutures (Ethilon®, Ethicon, Johnson & Johnson Intl). The animals were placed in new cages and were allowed to wake up under a heating lamp. All animals were allowed to recover for at least 10 days before the telemetric measurements were commenced. The implanted receiver was switched on, and the mouse cage was placed on a receiver plate which transferred the signals to a computer, where calibrated blood pressure and heart rate values were sampled. Data were collected for 5 seconds every second minute throughout the measurements.

**Renal excretion of nitrate/nitrite**

To ensure a low overall nitrate intake we first measured nitrate content in several standard rodent diets and found that it varied considerably (0.14-1.5 mM). The chow containing 0.14 mM nitrate was given to all animals. Mice were placed in metabolism cages for 24-hours, with food and water given ad libitum. Water consumption and urine production were measured gravimetrically. Samples of fresh urine were stored at -70ºC until analysis. Nitrate and nitrite in urine (diluted 50 times with carrier buffer) were measured with a dedicated high performance liquid chromatography (HPLC) system (ENO-20; EiCom, Kyoto, Japan).¹ The method is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by on-line reduction of nitrate to nitrite with cadmium and reduced copper. Reduced nitrite was then derivatised with Griess reagent and the level of diazo compounds measured at 540 nm. The retention time was 7.0 minutes for nitrate and 4.5 minutes for nitrite.

**Dissection and perfusion of arterioles**

The outer cortical afferent arterioles were dissected at 4°C in Dulbecco’s modified Eagle’s medium (DMEM, 0.1% albumin). Arterioles with their glomeruli were perfused in a thermo-regulated chamber (37°C) by a perfusion system, which allowed adjustment of outer holding and inner perfusion pipettes (Vestavia Scientific, Vestavia Hills, AL, USA). The chamber and the perfusion system were fixed to the stage of an inverted microscope (Nikon, Badhoevedorp, Netherlands). A 5-µm diameter perfusion pipette was connected to a reservoir containing the perfusion solution to provide a pressure of 100 mmHg in the pressure head, which produced a flow of about 50 nl/min. The criteria for the use of an arteriole were: a satisfactory, remaining basal tone and an intact myogenic response. Both criteria were tested by increasing perfusion pressure rapidly and assessing the change in the luminal diameter, which produced a constriction. A further criterion was a fast and complete constriction in response to KCl (100 mmol/l) solution. Results from the afferent arteriole measurements are shown in Figure S1-S5).

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¹ The method is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by on-line reduction of nitrate to nitrite with cadmium and reduced copper. Reduced nitrite was then derivatised with Griess reagent and the level of diazo compounds measured at 540 nm. The retention time was 7.0 minutes for nitrate and 4.5 minutes for nitrite.
**Superoxide measurements in afferent arterioles**

Superoxide generation was assessed by fluorescence microscopy of isolated and perfused afferent arterioles with dihydroethidium (DHE) or tempo-9-AC, as previously described. DHE is freely permeable to cells and oxidized by O$_2^-$ to the highly fluorescent compound ethidium, which is trapped intracellularly and intercalated into DNA. The conversion of DHE to ethidium was quantified by a dual wavelength determination using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for DHE and an excitation wavelength of 480 nm and an emission wavelength of 605 nm for ethidium. Single-agent signal capture was achieved by cycling at 3-s intervals between a 460- and 605-nm filter. Changes in O$_2^-$ were expressed as the ratio of ethidium:DHE fluorescence. The system used an Olympus IX70 fluorescence microscope equipped with dual photomultipliers (PMT, Photon Technology Int., Lawrenceville, NJ). Excitation was provided by a 75-W xenon arc lamp using a 380/460 nm wavelength combination isolated with a computer-controlled monochromator. Ethidium and DHE emit blue and red light, respectively, that were directed to a dual PMT assembly by a beam splitter that directed light to the two separate PMT using a 400-nm dichroic mirror and barrier filters centred at 460 and 605 nm, respectively. The ratio of ethidium:DHE was monitored in real time and recorded by software (Felix32; Photon Technology Int.). This ethidium fluorescence method has also been shown to detect a product that differs from ethidium by the presence of an additional oxygen atom in its molecular structure. Therefore additional studies were undertaken using tempo-9-AC fluorescence to detect O$_2^-$.

There was good agreement between the two methods to detect Ang II induced O$_2^-$ generation in afferent arterioles.

**Analysis of mRNA expression**

Infusion of cold PBS (Phosphate Buffered Saline) was started once the vena cava was cut to remove the blood. The heart and kidneys were explanted, dried and weighed. The renal cortex was separated and homogenised in lysis buffer (1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 80mM Tris, pH 7.5) containing enzyme inhibitors (Complete Mini; 1 tablet/1.5 ml; Roche Diagnostics, Mannheim, Germany). RNA was isolated with RNA-Bee-reagent (Biozol, Eching, Germany) and reverse transcribed with random hexamers (High Capacity cDNA RT-Kit, Applied Biosystems, Foster City, CA, USA, #4374966), according to the manufacturer’s protocols. Quantitative PCR analysis was performed with a StepOnePlus device (Applied Biosystems, Foster City, CA, USA). SYBR Green was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 12.5 μl with 0.4 pmol/μl of each primer (Table S1), and ImmoMix (Bioline, Luckenwalde, Germany): 2μl cDNA corresponding to 20 ng RNA was used as a template. Experiments were performed in triplicate with similar results. The expression levels of mRNA were normalised to β-actin by the ΔCt-method. Parallelism of amplification curves of the test and control was confirmed. Results from the mRNA analysis are shown in Table S2 and Figure S6.
**Drugs and Reagents**

DMEM/F12 with 10mmol/l HEPES (Invitrogen AB, Lidingö, Sweden) was used for dissection, bath, and perfusion. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA: SERVA Electrophoresis Heidelberg, Germany). The concentration of BSA was 0.1% in dissection and bath solutions, and 1% in the perfusion solution. The K+ solution had the composition in mmol/l, NaCl 20, KCl 95, NaHCO3 25, K2HPO4 2.5, CaCl2 1.3, MgSO4 1.2, glucose 5.5, and was equilibrated with 5% CO2 in air.

The drugs used were: Angiotensin II, Nω-Nitro-L-arginine methyl ester hydrochloride (L NAME), and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempol) from Sigma-Aldrich. All drugs were applied to the bath solution in the arteriolar contraction experiments.

**Supplemental References**


Table S1. Primers used for PCR analysis

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<th>Primer Name</th>
<th>Primer Sequence</th>
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<td>SOD1-fw</td>
<td>5'- TCC CTT CGA GCA GAA GGC AAG C -3'</td>
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<td>NM_011435</td>
<td>SOD1-rev</td>
<td>5'- ACA TGC CTC TCT TCA TCC GCC G -3'</td>
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<tr>
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<td>SOD2-fw</td>
<td>5'- ACG CGG CCT ACG TGA ACA ATC TC -3'</td>
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<td>SOD2-rev</td>
<td>5'- CAA CTC TCC TTT GGG TTC TCC ACC A -3'</td>
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<td>SOD3-fw</td>
<td>5'- CTC TAG CTG GGT GCT GGC CTG AAC T -3'</td>
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<td>NM_177322</td>
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<td>p67Phox-rev</td>
<td>5'- TCG GAC TTC ATG TTG GTC GCC AA -3'</td>
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Table S2. Fold changes in mRNA expression compared with wild-type mice.

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<th>Wild-type</th>
<th>SOD1-tg</th>
<th>SOD1-ko</th>
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<tr>
<td>SOD1</td>
<td>1.00 ± 0.43</td>
<td>1.44 ± 0.42</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>SOD2</td>
<td>1.00 ± 0.47</td>
<td>1.43 ± 0.44</td>
<td>1.51 ± 0.41</td>
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<tr>
<td>SOD3</td>
<td>1.00 ± 0.26</td>
<td>1.03 ± 0.18</td>
<td>1.49 ± 0.24</td>
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<tr>
<td>AT1A</td>
<td>1.00 ± 0.20</td>
<td>0.80 ± 0.28</td>
<td>0.79 ± 0.26</td>
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<tr>
<td>AT1B</td>
<td>1.00 ± 0.61</td>
<td>2.24 ± 1.07</td>
<td>0.92 ± 0.49</td>
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<tr>
<td>AT2</td>
<td>1.00 ± 0.48</td>
<td>1.96 ± 0.78</td>
<td>0.60 ± 0.37</td>
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<td>eNOS</td>
<td>1.00 ± 0.10</td>
<td>1.65 ± 0.22</td>
<td>1.98 ± 0.52</td>
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<td>iNOS</td>
<td>1.00 ± 0.73</td>
<td>3.44 ± 1.76</td>
<td>4.20 ± 1.80</td>
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<td>nNOS</td>
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<td>NOX1</td>
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<td>3.58 ± 1.40</td>
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<td>p67phox</td>
<td>1.00 ± 0.37</td>
<td>0.98 ± 0.37</td>
<td>0.79 ± 0.25</td>
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</table>

Superoxide dismutase isoforms (SOD1, SOD2, SOD3); Angiotensin I receptors (AT1A, AT1B, AT2); nitric oxide synthase isoforms (eNOS, iNOS, nNOS); and NADPH-oxidase isoforms (NOX1, NOX2, NOX4) and subunits (p22Phox, p47Phox, p67Phox) in the renal cortex of non-treated wild-type, SOD1-transgenic (SOD1-tg), and SOD1-knockout (SOD1-ko) mice. Fold changes are expressed as mean ± standard error.
Ang II concentration response curves in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=7), wild-type (n=11), and SOD1-knockout (SOD1-ko, n=7) mice. Control period represents the arteriolar diameter before Ang II application.
Figure S2.

Effect of L-NAME (10^{-4} mol/l) on diameters of isolated and perfused afferent arteriolar from SOD1-transgenic (SOD1-tg; n=7), wild-type (n=8), and SOD1-knockout (SOD1-ko; n=8) mice.

Figure S3.

Effect of L-NAME (10^{-4} mol/l) on Ang II concentration response in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=10), wild-type (n=8), and SOD1-knockout (SOD1-ko; n=7) mice. Control period represents the arteriolar diameter after L-NAME pre-treatment for 15 min.
Figure S4.

Effect of tempol ($10^{-4}$ mol/l) on diameters of isolated and perfused afferent arteriolar from SOD1-transgenic (SOD1-tg; n=6), wild-type (n=7), and SOD1-knockout (SOD1-ko; n=7) mice.

Figure S5.

Effect of tempol ($10^{-4}$ mol/l) on Ang II concentration response in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=6), wild-type (n=7), and SOD1-knockout (SOD1-ko; n=7) mice. Control period represents the arteriolar diameter after tempol pre-treatment for 15 min.
Figure S6.

Relative mRNA expression of (A) superoxide dismutase isoforms (SOD1, SOD2, SOD3); (B) Angiotensin I receptors (AT\textsubscript{1A}, AT\textsubscript{1B}, AT\textsubscript{2}); (C) nitric oxide synthase isoforms (eNOS, iNOS, nNOS); and, (D) NADPH-oxidase isoforms (NOX1, NOX2, NOX4) and subunits (p22Phox, p47Phox, p67Phox) in the renal cortex of non-treated wild-type, SOD1-transgenic (SOD1-tg), and SOD1-knockout (SOD1-ko) mice.