Extracellular Superoxide Dismutase Is a Major Determinant of Nitric Oxide Bioavailability

In Vivo and Ex Vivo Evidence From ecSOD-Deficient Mice

Oliver Jung, Stefan L. Marklund, Helmut Geiger, Thierry Pedrazzini, Rudi Busse, Ralf P. Brandes

Abstract—The bioavailability of nitric oxide (NO) within the vascular wall is limited by superoxide anions (O$_2^-$). The relevance of extracellular superoxide dismutase (ecSOD) for the detoxification of vascular O$_2^-$ is unknown. We determined the involvement of ecSOD in the control of blood pressure and endothelium-dependent responses in angiotensin II–induced hypertension and renovascular hypertension induced by the two-kidney, one-clip model in wild-type mice and mice lacking the ecSOD gene. Blood pressure was identical in sham-operated ecSOD$^{+/+}$ and ecSOD$^{-/-}$ mice. After 6 days of angiotensin II–treatment and 2 and 4 weeks after renal artery clipping, blood pressure was significantly higher in ecSOD$^{-/-}$ than ecSOD$^{+/+}$ mice. Recombinant ecSOD selectively decreased blood pressure in hypertensive ecSOD$^{-/-}$ mice, whereas ecSOD had no effect in normotensive and hypertensive ecSOD$^{+/+}$ mice. Compared with sham-operated ecSOD$^{+/+}$ mice, sham-operated ecSOD$^{-/-}$ mice exhibited attenuated acetylcholine-induced relaxations. These responses were further depressed in vessels from clipped animals. Vascular O$_2^-$, as measured by lucigenin chemiluminescence, was higher in ecSOD$^{-/-}$ compared with ecSOD$^{+/+}$ mice and was increased by clipping. The antioxidant tiron normalized relaxations in vessels from sham-operated and clipped ecSOD$^{-/-}$, as well as from clipped ecSOD$^{+/+}$ mice. In contrast, in vivo application of ecSOD selectively enhanced endothelium-dependent relaxations in vessels from ecSOD$^{+/+}$ mice. These data reveal that endogenous ecSOD is a major antagonistic principle to vascular O$_2^-$, controlling blood pressure and vascular function in angiotensin II–dependent models of hypertension. ecSOD is expressed in such an abundance that even in situations of high oxidative stress no relative lack of enzyme activity occurs. (Circ Res. 2003;93:622-629.)

Key Words: superoxide dismutase ■ oxidative stress ■ angiotensin ■ endothelium ■ hypertension

Nitric oxide (NO) is the predominant antiatherosclerotic principle in the vascular wall. The amount of free, bioactive NO in the vascular wall is determined by the activity of the endothelial NO synthase (eNOS) and by NO-scavenging mechanisms, such as the reaction of NO with superoxide anions (O$_2^-$).1 Numerous studies have demonstrated that indeed the local O$_2^-$ concentration is the main limiting factor for the availability of bioactive NO in healthy and diseased vessels.1

Multiple enzymatic systems have been identified that contribute to vascular O$_2^-$ formation including NADPH oxidases, xanthine oxidase, and cytochrome P450 monoxygenases. Also NOS under certain conditions can switch from an NO- to an O$_2^-$-generating enzyme. O$_2^-$ is relatively stable and detoxification of this type of radical is catalyzed by superoxide dismutase (SOD), yielding hydrogen peroxide (H$_2$O$_2$).2

Three different isoforms of SOD are found in the vasculature, a manganese containing SOD in the mitochondria and copper-zinc containing SOD (CuZnSOD) in the cytoplasm of all vascular cells and an extracellular copper-zinc SOD (ecSOD). The latter enzyme is secreted by smooth muscle cells and macrophages and binds to glycosaminoglycans in the vascular extracellular matrix.3,4

Nonselective pharmacological inhibition of SODs increases vascular oxidative stress and attenuates endothelium-dependent relaxation.5 Recently, it has been suggested using knockout mice that this observation can be attributed to the inhibition of the cytoplasmic CuZnSOD.6 Nevertheless, the role of ecSOD in the maintenance of vascular function is completely unknown and appears questionable as after secretion from smooth muscle cells into the interstitial space, the extracellular localization of this enzyme precludes any effect on intracellular O$_2^-$? Moreover, a large part of the diffusion...
distance nitric oxide has to pass through to reach the effector enzyme guanylyl cyclase is within the cell.

In the present study, we set out to elucidate the role of ecSOD in the control of blood pressure, NO bioavailability, and vascular O$_2^-$ level under physiological conditions, in renovascular hypertension and after angiotensin II infusion in mice.

Materials and Methods

Study Design and Animal Procedures

Animals and Clip Application
ecSOD$^{-/-}$ and ecSOD$^{+/+}$ mice were obtained from the breeding facility in Umeå, Sweden, and bred at the local animal facility at Frankfurt Medical School. Only male mice were used for this study. At an age of 8 weeks, animals were subjected to sham operation or clip application as described previously. Briefly, mice were anesthetized by isoflurane inhalation (1% to 2% in room air). The kidney was exposed through a small flank incision, externalized, and carefully held with an ophthalmic chalazion forceps. The renal artery of the left kidney was isolated over a short segment by blunt dissection, and a clip (U-shaped, stainless steal, 3×2×1 mm with a 2-mm-long, 0.12-mm-wide slit, Exelix SA) was placed close to the aorta. The kidney was then gently pushed back into the retroperitoneal cavity. The muscle layer was sutured, and the skin incision was closed with surgical staples. A sham procedure, which included the entire surgery with the exception of artery clipping, was performed in control mice. Blood pressure was measured 14 and 28 days after the operation and organ chamber experiments were performed 29 days after the operation. Successful induction of renovascular hypertension, defined by a blood pressure over 120 mm Hg 2 weeks after clip application was observed in ~70% of the animals. Animals that did not meet this criterion were excluded from the study. Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23) and were approved by the local government (II25.3-19c2015-F61/16).

Blood Pressure Measurements and ecSOD Application

Blood pressure was measured at the same time every day using tail cuff technique with the aid of a computerized system (Visitech Systems). Measurements were performed after 5 days of training. For intravenous application of human recombinant ecSOD$^+$ supplied by Dr Marklund, mice were anesthetized by isoflurane and the right external jugular vein was exposed through a small incision. The vessels was cannulated using a Teflon catheter and 30 000 U/kg ecSOD were applied in 100 μL of normal saline solution. Subsequently, the catheter was removed, the vessel ligated, and the skin sutured. Animals were allowed to recover for 16 hours before the next blood pressure measurements. The dose of 30 000 U/kg was chosen as in a pilot study, it restored vascular ecSOD content and normalized aortic superoxide production in ecSOD$^{-/-}$ mice to the level of ecSOD$^{+/+}$ mice (see expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org)

Effect of N"-Nitro-l-Arginine Methyl Ester (L-NAME) on Blood Pressure

L-NAME (2 mg/mL) was added to the drinking water, which was provided ad libitum, and blood pressure measurements were continued for another 6 days.

Effect of [\text{\textbf{5\text{\texthextext{v}}\text{\texttextit{a}}}]}\text{-Angiotensin II on Blood Pressure}

In subgroups ecSOD$^{-/-}$ and ecSOD$^{+/+}$ mice were implanted with osmotic minipumps (Alzet, purchased via Charles Rivers) as described$^{[10]}$ and [5\text{\text{\text{v}}}]-angiotensin II (1 mg/kg per day) was infused over a period of 6 days. Subsequently, mice were anesthetized using isoflurane and catheters were placed in the right common carotid artery and the left external jugular vein. Blood pressure was measured continuously using a Statham pressure transducer. ecSOD was applied as a bolus (30 000 and 100 000 U/kg dissolved in 100 μL of normal saline) via the vein catheter.

Organ Chamber Experiments

Organ chamber experiments were performed as described$^{[11]}$ using mouse aortic rings from ecSOD$^{-/-}$ and ecSOD$^{+/+}$ mice preconstricted with phenylephrine to 80% of the contraction elicited by KCl (80 mmol/L). Endothelium-dependent relaxation to acetylcholine (ACh) were recorded in the presence or absence of the antioxidant tiron (1 mmol/L) and in the presence or absence of catalase (1200 U/mL, Sigma C40 from bovine liver, applied 30 minutes before the application of ACh). Endothelium-independent relaxations were studied in response to sodium nitroprusside (SNP). NO bioavailability was estimated from the constrictor response to the NOS inhibitor N$^\text{\text{\text{\textomega}}}\text{-nitro-l-arginine (L-NA, 300 μmol/L)}$ in aortic rings preconstricted to 10% of the maximal KCl constriction using phenylephrine.

Vascular Radical Generation

Measurements were performed using a lucigenin (5 μmol/L)-enhanced chemiluminescence assay in intact mouse aortic rings as described previously.$^{[11]}$

Immunoblotting

Western blot analysis from Triton X-100 (1%) soluble aortic protein was performed as described previously.$^{[11]}$ The rabbit anti-MnSOD antibody was from Dr W. Gwinner (Medizinische Hochschule Hannover, Germany). The following commercially available antibodies were used: mouse anti-eNOS (BD Transduction), sheep anti-CuZnSOD (Calbiochem), and rabbit anti-catalase (Calbiochem).

Statistics

All values are mean±SEM. Maximal relaxation were calculated from individual dose-response curves. Statistical analysis was performed using one-way analysis of variance for repeated measures followed by Fisher LSD test, or paired t test, if appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Body weight, heart weight, and kidney weight were similar between ecSOD$^{-/-}$ and ecSOD$^{+/+}$ mice. Also, no differences in the degree of cardiac and renal weight changes after clipping were observed between the two strains (Table 1).

ecSOD$^{-/-}$ Mice Exhibit Endothelial Dysfunction Under Basal Conditions

Endothelium-dependent relaxations in aortic rings of sham-operated ecSOD$^{-/-}$ were significantly smaller than those observed in vessels from sham-operated ecSOD$^{+/+}$ mice. Compared with the sham-operated group, clip application resulted in a significant attenuation of endothelium-dependent relaxation in vessels from ecSOD$^{+/+}$, as well as ecSOD$^{-/-}$ mice. The extent of attenuation of vascular function by clipping was similar in both strains (Figure 1A, Table 2). Endothelium-independent relaxation to the vasodilator SNP was identical in the different study groups (Figure 1B).

ecSOD$^{-/-}$ Mice Have Enhanced O$_2^-$ Levels

Vascular O$_2^-$ levels, as determined by lucigenin-chemiluminescence were markedly increased in aortic segments from ecSOD$^{-/-}$ as compared with ecSOD$^{+/+}$ mice. Clipping enhanced O$_2^-$ levels in vessels from both knockout as well as control animals. Although the absolute increase in lucigenin-chemiluminescence was more pronounced in
clipped ecSOD^{−/−} mice compared with ecSOD^{+/+} mice, the relative increase in O_2^− level induced by clipping was similar in both strains (Figure 2A). In order to determine the relevance of increased O_2^− level for endothelium-dependent relaxation, organ chamber studies were performed in the presence of the antioxidant tiron (1 mmol/L). Tiron significantly enhanced relaxations in all groups. In the presence of tiron, relaxations of aortic rings from sham-operated ecSOD^{−/−} and clipped ecSOD^{−/−} mice did not significantly differ from those observed in sham-operated ecSOD^{+/+} mice. Tiron also enhanced ACh-induced relaxations in vessels from clipped ecSOD^{−/−} mice, but the response was not completely normalized (Figure 2B).

Antioxidant Protein Expression Is Not Altered in ecSOD^{−/−} Mice

In order to study whether the lack of ecSOD is compensated by other antioxidative enzymes, Western blot analysis of relevant proteins was performed in aortic segments of all groups. Protein expression of eNOS, catalase, MnSOD, and cytoplasmic CuZnSOD was not different between the four study groups. As expected, ecSOD expression was undetectable in vessels from ecSOD^{−/−} mice. Interestingly, ecSOD protein levels in vessels from clipped wild-type mice were also not different to those obtained in vessels from sham-operated wild-type mice (Figure 3).

ecSOD^{−/−} Mice Exhibit More Pronounced Angiotsin II–Induced Hypertension and Renovascular Hypertension Than ecSOD^{+/+} Mice

In order to determine the role of ecSOD for renovascular hypertension in vivo, blood pressure was measured using tail-cuff technique. Basal blood pressure was not different between the two strains and also sham operation had no effect on blood pressure. Renal artery clipping resulted in a dramatic rise in blood pressure in both mouse strains. Nevertheless, blood pressure was significantly higher in clipped ecSOD^{−/−} than ecSOD^{+/+} mice (Figure 4A). In order to demonstrate that the lack of ecSOD is responsible for this difference, recombinant ecSOD was administered in some mice. Whereas this approach had no effect in sham-operated mice as well as clipped ecSOD^{+/+} mice, a significant decrease in blood pressure was observed in clipped ecSOD^{−/−} mice (Figure 4B).

To further analyze the role of ecSOD for hypertensive response, the effect of L-NAME on blood pressure was determined in ecSOD^{−/−} and ecSOD^{+/+} mice. In this model, we assume that NO production is inhibited and that NO-scavenging by O_2^− cannot underlie the hypertensive response. Indeed, under steady-state conditions (3 or more days of L-NAME treatment), the blood pressure was elevated to a similar level in both mouse strains. In contrast, during the initial phase of L-NAME treatment (days 1 and 2), blood pressure in ecSOD^{+/+} mice was even higher than that measured in ecSOD^{−/−} mice (Figure 4C).

In order to exclude that counter-regulatory mechanisms occurring in renovascular hypertension attenuate the difference in the hypertensive response between the two strains, the effect of angiotensin II on blood pressure was tested. In these experiments, measurements were performed via a carotid artery catheter in anesthetized mice to exclude inaccuracies potentially occurring with the tail-cuff technique. Blood pressure in ecSOD^{−/−} mice 6 days after osmotic mini pump implantation was significantly higher than that in ecSOD^{+/+} mice. Administration of human recombinant ecSOD (30 000 U/kg) reduced within seconds blood pressure in ecSOD^{−/−} mice to the level

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**TABLE 1. Basal Characteristics of the Animals**

<table>
<thead>
<tr>
<th></th>
<th>ecSOD^{+/+} Mice</th>
<th>ecSOD^{−/−} Mice</th>
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<tbody>
<tr>
<td></td>
<td>Sham Clip</td>
<td>Sham Clip</td>
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<tr>
<td>Body weight, g</td>
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<td>29.9±1.0</td>
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<td>Heart, mg</td>
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<td>133±6</td>
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<tr>
<td>Kidney, mg</td>
<td>162±4</td>
<td>74±15*</td>
</tr>
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<td>Kidney left relative, mg/g BW</td>
<td>5.22±0.12</td>
<td>2.48±0.51*</td>
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<tr>
<td>Kidney right, mg</td>
<td>170±6</td>
<td>203±8*</td>
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<tr>
<td>Kidney right relative, mg/g BW</td>
<td>5.49±0.28</td>
<td>6.62±0.28*</td>
</tr>
<tr>
<td>Left kidney/relative kidney ratio</td>
<td>0.95±0.04</td>
<td>0.37±0.06*</td>
</tr>
</tbody>
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BW indicates body weight. *P<0.05 clipped vs sham-operated group of identical strain.

**Figure 1.** Effect of renal artery clip application on vascular function. Concentration-response curves to the endothelium-dependent vasodilator acetylcholine (ACh, A) and the endothelium-independent vasodilator sodium nitroprusside (SNP, B) were obtained in phenylephrine-preconstricted mouse aortic rings from clipped and sham-operated ecSOD^{+/+} (+/+) and ecSOD^{−/−} (−/−) mice. ns indicates not significant; ¢P<0.05 clipped vs sham-operated group of identical strain; #P<0.05 ecSOD^{−/−} vs ecSOD^{+/+} mice with identical treatment.
Observed in ecSOD+/+ mice. ecSOD had no effect in ecSOD−/− mice (Figure 5) and additional administration of 100 000 U/kg in mice treated with 30 000 U/kg ecSOD had no effect on blood pressure (data not shown).

**Recombinant Human ecSOD Restores Endothelium-Dependent Responses in Aortic Rings From ecSOD−/− Mice**

The different hypertensive responses between the two strains could be interpreted as differences in basal NO bioavailability resulting from higher amount of NO scavenging in ecSOD−/− mice. To address this aspect, the constrictor response to L-NA was studied in isolated aortic ring preparations precontracted to 10% of the maximal force developed in response to depolarizing KCl solution (80 mmol/L). Maximal force development to KCl was identical in all groups (data not shown). In line with the in vivo data, L-NA-induced constriction was significantly lower in aortic rings from sham-operated ecSOD−/− mice as compared with those obtained from sham-operated ecSOD+/+ mice. Comparing responses in vessels from sham-operated and clipped mice revealed a significantly attenuated constrictor response in vessels from clipped animals of both groups. In vivo application of ecSOD had no effect on the L-NA-evoked constrictor response observed in ecSOD+/+ mice, but significantly increased that observed in rings from clipped and sham-operated ecSOD−/− mice (Figure 6A). In vivo ecSOD application had no effect on the ACh-induced endothelium-dependent relaxation of aortic rings from clipped and sham-operated ecSOD−/− mice. In contrast, in vessels from ecSOD−/− mice an increase in relaxation was observed, which was much more pronounced in vessels from clipped than from sham-operated animals (Figures 6B and 6C, Table 2).

In order to determine whether H2O2 formed by SOD contributes the endothelium-dependent relaxation in the present study, the effect of catalase was studied. Catalase uneffectively attenuated endothelium-dependent relaxation in all groups (data not shown). This may indicate that H2O2, derived

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Involvement of oxidative stress in vascular dysfunction. A, Lucigenin (5 μmol/L)-enhanced chemiluminescence in mouse aortic segments from sham-operated (gray bars) and clipped (black bars) animals of both strains. n=8 in each group. *P<0.05 clipped vs sham-operated group of identical strain; †P<0.05 ecSOD−/− vs ecSOD+/+ with identical treatment. B, Effect of the antioxidant tiron (1 mM) on the acetylcholine (Ach)-induced endothelium-dependent relaxation of aortic rings from sham-operated and clipped ecSOD+/+ and ecSOD−/− mice. n=8, *P<0.05 clipped ecSOD−/− vs sham-operated ecSOD+/+ mice.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Aortic protein expression in sham-operated and clipped ecSOD+/+ and ecSOD−/− mice. Aortic protein of vessels from the different treatment groups were subjected to SDS-PAGE and proteins were detected by Western blot analysis. Provided are representative blots. Numbers below the lanes indicate the results of the relative densitometry. Numbers on the left-hand side indicate the staining of the molecular weight markers. Expression of endothelial NO synthase (eNOS), catalase, ecSOD, manganese SOD, as well as cytoplasmic copper-zinc SOD was not different between the 4 groups. n=5.
from $\cdot$O$_2^{-}$ by spontaneous dismutation or by the action of any SOD contributes to endothelium-dependent relaxation in mouse vessels. As this effect of catalase was however not different between vessels containing ecSOD and those lacking the enzyme, it is unlikely that H$_2$O$_2$ is a main effector for the permissive effects of ecSOD on endothelium-dependent relaxation.

**Discussion**

In the present study, we demonstrate for the first time that ecSOD substantially contributes to the modulation of vascular O$_2^{-}$ levels. Lack of ecSOD was associated with attenuated endothelium-dependent relaxation and reduced basal vascular NO levels in vivo and in vitro. Moreover, ecSOD-/- mice exhibited enhanced hypertensive responses during angiotensin II–induced as well as high-renin–induced hypertension (two-kidney, one-clip), which was ameliorated by in vivo treatment with ecSOD.

A multitude of studies have demonstrated that unspecific inhibition of SOD is deleterious for endothelium-dependent relaxation. The role however of ecSOD in controlling vascular level of bioactive NO until now was completely unknown. Although ecSOD is localized in that area of the vascular wall through which NO must pass on its way from the endothelium to the smooth muscle cells, only extracellular superoxide anions can be detoxified by the enzyme. Certainly, some extracellular sources of superoxide anions, such as xanthine oxidase, are found in the arterial wall, but many enzymes including vascular NADPH oxidase and endothelial NO synthase apparently generate $\cdot$O$_2^{-}$ primarily within cells, where it reacts very rapidly with intracellular SODs (rate constant $2\times10^{9}$ mol/L$^{-1} \cdot$ sec$^{-1}$) that are expressed in large
dependent responses in ecSOD
ecSOD also resulted in normalization of endothelium-
tiron. Moreover, in vivo treatment with human recombinant
bars) and clipped (hatched bars) ecSOD
Depicted response in segments from sham-operated (open
constricted with phenylephrine to 10% of the constrictor
rings from sham-operated (B) and clipped (C) ecSOD
and C, Effect of in vivo ecSOD treatment (& ecSOD) on endo-

These effects were a consequence of an increased vascular
level of O2
oxidative stress but are rather a direct consequence of the

In the present study, we now provide clear evidence that
the absence of vascular ecSOD activity by limiting availability
ability of bioactive NO results in attenuated endothelium-
dependent relaxation and increased hypertensive responses.
These effects were a consequence of an increased vascular
level of O2
, as determined by lucigenin chemiluminescence.
Indeed, normal endothelium-dependent relaxation in vessels
from ecSOD
mice could be restored by the antioxidant
tiron. Moreover, in vivo treatment with human recombinant
cSOD also resulted in normalization of endothelium-
dependent responses in ecSOD
mice. Therefore, the attenu-
ated endothelium-dependent responses cannot be attributed
to secondary phenomena such as strain differences or chronic
irreversible changes resulting from continuous enhanced
oxidative stress but are rather a direct consequence of the
scavenging of endothelium-derived NO in ecSOD
mice.

An equivalent attenuation of endothelium-dependent relax-
lation was observed in sham-operated ecSOD
mice and in
wild-type mice after renal artery clipping, despite a higher
lucigenin signal in the former group. This discrepancy might
suggest that lucigenin chemiluminescence occurs in a differ-
ent compartment than the activation of the soluble guanylyl
cyclase by NO. Alternatively, the extracellular oxidative
stress observed in ecSOD
mice elicits distinct signaling
events to those activated by renal artery clipping.

Although in vivo administration of ecSOD improved en-
dothelium-dependent relaxation of isolated aortic rings from
sham-operated ecSOD
mice, this treatment had no effect
on blood pressure in sham-operated ecSOD
mice. Several
explanations may account for this observation. Firstly, the
antioxidant milieu in vivo is very different to the situation in
the organ bath set-up. In vivo, numerous small molecules
such as glutathione and vitamins are present in abundance,
increasing the antioxidative defense. Secondly, the oxygen
tension in the organ chamber is 5 to 10 times higher than in
vivo, resulting in a high spontaneous formation of O2
in
aqueous solutions. Finally and most importantly, ecSOD
mice are chronically adapted to oxidative stress and only a
conditional knockout can ultimately solve the question
whether blood pressure under normal conditions is modulated
by ecSOD. That such an adaptation is present in ecSOD
mice can be derived from the different blood pressure
responses to an NO synthase inhibitor. During the initial
phase of L-NAME treatment, blood pressure increased sig-
ificantly more in ecSOD
than ecSOD
mice, despite
identical weight and fluid intake of the two groups (data not
shown). This observation can only be interpreted in such a
way that ecSOD
mice are chronically exposed to lower NO
concentrations than ecSOD
mice, and thus, a slight with-
drawal of NO has less effect on peripheral resistance than in
wild-type mice. Of note, after 3 days of L-NAME treatment
and therefore presumably almost complete inhibition of NO
release, blood pressure was identical in both strains, demon-
strating that differences in the myogenic tone or the central
blood pressure regulation do not underlie the different hyper-
tensive response in ecSOD
and ecSOD
mice. This
interpretation is further supported by the finding that inhibi-
tion of NOS leads to a significantly greater constrictor
response in vessels isolated from ecSOD
than from ec-
SOD
mice. This observation is a consequence of the fact
that NO produces a rightward shift in the concentration-
response curve to phenylephrine. Such an approach previ-
ously revealed low basal NO bioavailability in stroke-prone
spontaneously hypertensive rats, that also experience sub-
stantial oxidative stress.

To induce oxidative stress in the present study, the two-

kidney, one-clip model of renovascular hypertension was
used. By increasing the release of renin from the clipped
kidney, this model leads to hypertension via angiotensin II
formation, and facilitates the angiotensin II–mediated induc-
tion and activation of the vascular NADPH oxidase, resulting
in oxidative stress and NO scavenging. Recently, it has been
demonstrated that such a mechanisms is also functional in
 renovascular hypertension in humans. In the present study,
renal artery clipping resulted in a substantial rise in blood
pressure as well as an significant increase in vascular O2
generation. This was associated with an attenuation of the

abundance. Consequently, examples for a role of ecSOD for
oxidant protection have been restricted to hypoxia, a
situation where O2
 is primarily found in the extracellular
space due to high oxygen tension.14–16

In the present study, we now provide clear evidence that
the absence of vascular ecSOD activity by limiting availability
of bioactive NO results in attenuated endothelium-
dependent relaxation and increased hypertensive responses.
These effects were a consequence of an increased vascular
level of O2
, as determined by lucigenin chemiluminescence.

Figure 6. Effect of in vivo ecSOD treatment on basal and
agonist-induced NO bioavailability. A, Basal NO bioavailability
as determined by the constrictor response to the NO synthase
inhibitor N-nitro-L-arginine (300 μmol/L) in aortic segments pre-
constriicted with phenylephrine to 10% of the constrictor
response evoked with depolarizing KCl solution (80 mmol/L).
Depicted response in segments from sham-operated (open
bars) and clipped (hatched bars) ecSOD
and ecSOD
mice
with and without prior treatment with ecSOD.
B and C, Effect of in vivo ecSOD treatment (& ecSOD) on endo-
thelium-dependent relaxation to acetylcholine (ACh) in aortic
rings from sham-operated (B) and clipped (C) ecSOD
and
ecSOD
mice.
bioavailability of endothelium-derived NO, determined in isolated aortic segments. Incubation of vascular segments with the antioxidant tiron improved endothelium-dependent relaxation, indicating that the reduction of NO bioavailability is a consequence of enhanced O$_2^-$ formation, as previously suggested in rat models of angiotensin II–induced endothelial dysfunction. Vascular dysfunction and O$_2^-$ levels were more pronounced in vessels from clipped ecSOD$^{-/-}$ mice compared to clipped ecSOD$^{+/+}$ mice, suggesting that even during renovascular hypertension a substantial amount of O$_2^-$ is detoxified by ecSOD.

Although the constrictor response to nitro-L-arginine, taken as an index of NO bioavailability was normalized by in vivo application of ecSOD in clipped ecSOD$^{-/-}$ mice, this treatment failed to completely normalize endothelium-dependent relaxation in this group of animals. As similar observations were obtained by in vitro treatment with tiron, these data may suggest that after 4 weeks of hypertension in ecSOD$^{-/-}$ mice already some degree of fixed vascular dysfunction is present.

Blood pressure in sham-operated ecSOD$^{+/+}$ and ecSOD$^{-/-}$ mice was identical, despite higher O$_2^-$ level in the latter strain. This might suggest that in healthy mice substantial alterations in NO bioavailability are required to overcome the regulatory mechanisms involved in blood pressure control. In this context, it is noteworthy that also the opposing beneficial effects of genetic deletion of NADPH oxidase subunits on this context, it is noteworthy that also the opposing beneficial effects of genetic deletion of NADPH oxidase subunits on NO bioavailability are only evident under pathophysiological situations.

Blood pressure in clipped ecSOD$^{-/-}$ mice was significantly higher than in clipped ecSOD$^{+/+}$ mice and in vivo application of ecSOD selectively lowered blood pressure in clipped knockout mice. Similar results were obtained in mice subjected to angiotensin II–treatment. It is likely that the blood pressure lowering effect of ecSOD is a consequence of an attenuated scavenging of NO by O$_2^-$ in vivo. It should however also be considered that H$_2$O$_2$ formed from O$_2^-$ in the presence of SOD can act as a direct vasodilator in some vessels. In any case, the present observation indicate that the higher blood pressure in ecSOD$^{-/-}$ mice is indeed a consequence of the lack of ecSOD. Moreover the angiotensin II– or clip-induced increase of blood pressure in ecSOD$^{+/+}$ mice is obviously not due to insufficient extracellular SOD activity. Indeed, in wild-type mice, ecSOD appears to be expressed in such an abundance that even the high activities of ecSOD exogenously administered, have no effect on NO bioavailability. These data are in contrast to observations in rats where heparin-binding SOD and ecSOD, respectively, were able to lower blood pressure$^{23}$ and to improve endothelium-dependent relaxation.$^{24}$ via a mechanism requiring the heparin-binding domain of ecSOD.$^{25}$ It is however important to note that rats exhibit a very low vascular ecSOD activity, as a mutation of the gene in these animals prevents binding of the enzyme to glycosaminoglycans.$^{26}$

Certainly, the difference in the extent of hypertension between hypertensive ecSOD$^{+/+}$ and ecSOD$^{-/-}$ mice was small, an effect that might in part be a consequence of inaccuracy of the tail cuff method. However, considering that a complete inhibition of NOS increased blood pressure only by 30 mm Hg, the 10 mm Hg difference observed due to the lack of ecSOD illustrates that this enzyme has an important impact on NO bioavailability. Given the fact that angiotensin II exerts its vasoconstrictor function by modulating sympathetic tone, by increasing the production of endothelin and 20-HETE and through aldosterone-induced sodium and volume retention, it is erroneous to assume that O$_2^-$ is the predominant vasoconstrictor principle in the two-kidney, one-clip model of hypertension. This conclusion is supported by observations made in animals lacking vascular NADPH oxidase activity. In these animals, angiotensin II fails to increase vascular radical generation, yet blood pressure was only slightly lower than in wild-type animals treated with angiotensin II (0 to 25 mm Hg depending on the model)$^{22,27,28}$

In the present study, we did not observe an effect of renal artery clip application on vascular ecSOD expression. This result was unexpected because it has been reported that angiotensin II infusion in mice significantly increases ecSOD expression.$^{29}$ Certainly, in renovascular hypertension, angiotensin II levels were lower than in the before mentioned study, and it is not known whether some threshold concentration has to be reached to modulate ecSOD expression. In addition, under physiological conditions, ecSOD expression in mice is mainly controlled by NO.$^{30}$ As NO bioavailability is attenuated in renovascular hypertension, angiotensin II may substitute for NO as an inducer of ecSOD, leaving its overall expression unchanged. Also in humans, ecSOD expression decreases in settings of attenuated NO bioavailability such as arteriosclerosis, heart failure, and endothelial dysfunction.$^{31}$

In conclusion, we have demonstrated that ecSOD is an important antagonistic principle to vascular O$_2^-$ and that the enzyme is crucial for NO bioavailability and consequently for the maintenance of normal vasomotor function and blood pressure. ecSOD, however, is expressed in such an abundance that even in models of increased oxidative stress, excessive application of the enzyme does not alter O$_2^-$ levels. Therefore, the destruction of NO in renovascular hypertension appears to occur in a compartment inaccessible by ecSOD.

Acknowledgments

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References


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Determination of the amount of ecSOD for intravenous application.
Different concentrations of ecSOD (3,000, 30,000 and 300,000 Units / kg) were applied to ecSOD -/- mice according to the procedure reported in the methods section. After 8 hours animals were sacrificed. The aorta was removed for measurement of endothelium-dependent relaxation and lucigenin chemiluminescence. ecSOD content was determined in blood samples and aortic tissue using the KO₂ assay as reported previously.¹

Effect of ecSOD application on plasma ecSOD content:
ecSOD was below the detection limit of the assay in plasma of ecSOD -/- and ecSOD +/+ mice without intervention, whereas application of human recombinant ecSOD increased plasma activity, dose dependently. (Fig 1).

Fig. 1: Measurements of plasma ecSOD content in ecSOD -/- and ecSOD +/+ mice following application of various amounts of ecSOD. kunits/kg denotes 1000 units/kg. * indicates values below detection limit. Each bar represents individual animals.
Effect of ecSOD application on aortic ecSOD content:
ecSOD was below the detection limit in vessels from ecSOD -/- mice and ecSOD -/- mice treated with 3,000 units / kg recombinant ecSOD. Application of 30,000 units / kg ecSOD in ecSOD -/- mice resulted in aortic levels similar to those observed in ecSOD +/- mice, whereas 300,000 units / kg further increased aortic activity. (Fig 2).

![Graph showing aortic ecSOD content](image)

**Fig. 2**: Measurements of aortic ecSOD content in ecSOD -/- and ecSOD +/- mice following application of various amounts of ecSOD. kunits/kg denotes 1000 units/kg. * indicates values below detection limit. Each bar represents individual animals.
Effect of ecSOD application on aortic superoxide formation as measured using lucigenin chemiluminescence:
Application of in vivo ecSOD application to ecSOD -/- mice dose-dependently reduced the chemiluminescence signal in aortic segments. Using 30,000 units/kg resulted in chemiluminescence signals similar to those observed in vessels from untreated ecSOD +/+ mice (Fig 3).

![Figure 3](image)

**Fig 3:** Measurements of vascular superoxide anion formation using lucigenin chemiluminescence in aortic segments from ecSOD +/+ and ecSOD -/- mice following application of various amounts of ecSOD. kunits/kg denotes 1000 units/kg. Each bar represents individual animals.

Endothelium-dependent relaxation:
In ecSOD -/- mice endothelium-dependent relaxation was not affected by 3,000 Units / kg but was completely restored to relaxation observed in aortic segments of ecSOD +/+ mice by 30,000 Units /kg (n=2), increasing the dose of ecSOD to 300,000 Units /kg had no additional effect (n=1). (Data not shown).

Reference List