Inactivation of Extracellular Superoxide Dismutase Contributes to the Development of High-Volume Hypertension

Oliver Jung, Stefan L. Marklund, Ning Xia, Rudi Busse, Ralf P. Brandes

Objectives—Extracellular superoxide dismutase (ecSOD) lowers superoxide anions and maintains vascular nitric oxide level. We studied the function of ecSOD in high-volume hypertension induced by the 1-kidney-1-clip model in wild-type, ecSOD−/− mice, and endothelial nitric oxide synthase (eNOS)−/− mice.

Methods and Results—The 1-kidney–1-clip model resulted in impaired endothelium-dependent relaxation and hypertension and vascular oxidative stress in wild-type and ecSOD−/− mice. Recombinant ecSOD lowered the blood pressure and improved aortic nitric oxide bioavailability in wild-type and ecSOD−/− but not eNOS−/− mice. ecSOD had no effect on blood pressure in eNOS−/− or wild-type mice treated with a nitric oxide synthase inhibitor. The 1-kidney–1-clip model markedly induced ecSOD protein expression, whereas activity was increased by only 25%, suggesting a partial inactivation of ecSOD in high-volume hypertension. Incubation of aortic segments with peroxynitrite or hydrogen peroxide attenuated ecSOD activity, but peroxynitrite did not induce tyrosine nitration of ecSOD, suggesting oxidative inactivation of the enzyme. Administration of polyethyleneglycol-catalase for 3 days selectively lowered the blood pressure in ecSOD−/− but not ecSOD−/− mice and improved nitric oxide bioavailability. In contrast, acute application of catalase had no effect.

Conclusions—Nitric oxide mediates the vascular effects of ecSOD. Vascular dysfunction in 1-kidney–1-clip model hypertension is partially a consequence of inactivation of ecSOD by reactive oxygen species. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

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

Oxidative stress is a feature of all cardiovascular diseases and it is thought that reactive oxygen species (ROS) contribute to the initiation and progression of the disease process.

Among ROS, superoxide anions (O2−), have a prominent position. Most other ROS are formed from reactions of O2− and O2. Also limits the bioavailability of nitric oxide (NO), thereby promoting endothelial dysfunction. Three superoxide dismutases (SOD) are involved in the detoxification of O2. MnSOD is localized in mitochondria, CuZnSOD is expressed in the cytosol and extracellular SOD (ecSOD) is expressed in the cytosol and extracellular SOD (ecSOD) is synthesized in the endoplasmic reticulum and is secreted into the interstitial space. There, the enzyme binds to matrix components such as heparin sulfate proteoglycans and fibulin-5. The matrix content of most organs is relatively low and thus in most organs CuZnSOD activity is several orders of magnitudes higher than that of ecSOD. Vascular structures, however, contain large amounts of matrix rendering them the tissue with the highest ecSOD activity in the body. Consequently, a role of endogenous ecSOD for controlling NO bioavailability has been demonstrated in animal experiments and a mutation of ecSOD which attenuates binding of the enzyme to matrix, is associated with an increased risk of cardiovascular fatalities.

It is unclear whether pharmacological application of ec-SOD confers protection against oxidative stress in the vasculature. Most studies reporting a beneficial effect were performed in rats. ecSOD in rats has a low affinity for heparan sulfate, and shows very low levels in the arterial wall. This is because the enzyme is a dimer in rats, whereas it is a tetramer in other mammals. Matrix binding is absolutely required for the positive effects of ecSOD, and thus rats are almost a physiological knockout model for this enzyme. In mice with renovascular hypertension elicited by the 2-kidney–1-clip (2K1C) model, we previously demonstrated that application of ecSOD failed to improve vascular function in wild-type (WT) mice but not in knockout mice. However, it has been demonstrated that in rabbit models of balloon angioplasty ecSOD application prevents inward remodel-
The effects in the 2K1C model are mediated by the renal release of rennin, which leads to the generation of angiotensin II. We have previously demonstrated that the endothelial dysfunction in 2K1C renovascular hypertension in mice is exclusively mediated by the endothelial production of O$_2^·$. In the balloon injury model, O$_2^·$ production is accompanied by a concomitant increase in NO production, resulting in oxidative as well as nitrosative stress and the formation of peroxynitrite. We therefore hypothesize that NO has a central role in determining the effect of SOD application. To test this hypothesis, we studied the vascular function in the 1-kidney–1-clip (1K1C) model of renovascular hypertension in WT, ecSOD$^{-/-}$, and endothelial nitric oxide synthase (eNOS)$^{-/-}$ mice. The 1-kidney–1-clip model induces nonre- nin, low-angiotensin II hypertension, which is mediated by salt and water retention and thus resembles a situation of high-voltage hypertension with increased cardiac output and NO formation.

Materials and Methods

Animals and 1-Kidney–1-Clip Model
C57/b6-ecSOD$^{-/-}$ and C57/b6-eNOS$^{-/-}$ mice were obtained from the breeding facility in Umeå, Sweden, and C57/b6-ecSOD$^{-/-}$ mice were kindly provided by Axel Gödeke, Heinrich-Heine-Universität Düsseldorf. The animals were bred at the local animal facility at Hannover Medical School. Only male mice were used for this study. At an age of 8 weeks, animals were subjected to sham operation or renal artery clip application of the left kidney as described previously. A sham procedure, which included the entire surgery with the application of the left kidney as described previously.

Organ Chamber Experiments
Organ chamber experiments were performed as described. Relaxations to cumulatively increasing concentrations of acetylcholine or deta-NONOate were recorded in vessels preconstricted to 80% of the maximal KCl (80 mmol/L)-induced contraction using phenylephrine in the presence of diclofenac (10 μmol/L). Relaxations are denoted as percent of the initial constriction obtained by phenylephrine. NO bioavailability was estimated from the constrictor response to the NO synthase inhibitor N$\text{-}$nitro-L-arginine (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.

SOD Activity
For activity determination, 3 whole mouse aorta were pooled per data point and total SOD activity, ConA-sepharose–binding SOD activity (ecSOD), and cyanide-sensitive SOD activity (CuZn-SOD) were determined by the potassium superoxide assay as described previously.

Immunoblotting
Western blot analysis from Triton x-100 (1%) soluble aortic protein was performed as described previously. 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 relaxations were calculated from individual dose-response curves. Statistical analysis was performed using 1-way analysis of variance for repeated measurements followed by Fisher least significant difference test, or paired t test, if appropriate. Values of P<0.05 were considered statistically significant.

Results

Vascular Dysfunction in 1K1C Hypertension Is Independent of Endogenous ecSOD
The 1K1C model resulted in a marked increase in systolic blood pressure as determined by tail-cuff technique in WT mice (Figure 1A). Moreover, endothelium-dependent relaxation was blunted in isolated aortic rings obtained from 1K1C hypertensive WT mice as compared with segments from control animals (Figure 1B), and this was associated with >3-fold increase in vascular O$_2^·$ concentrations as determined by lucigenin chemiluminescence (Figure 1C). This was accompanied by formation of peroxynitrite as aortic protein tyrosine nitration was increased in 1K1C hypertensive mice (data not shown).

O$_2^·$ concentrations in aortic segments from sham-operated ecSOD$^{-/-}$ mice were significantly higher even than those determined in segments from 1K1C-operated ecSOD$^{-/-}$ mice. In comparison to aortic rings from sham-operated ecSOD$^{-/-}$ mice, O$_2^·$ concentrations in segments from 1K1C were further enhanced (Figure 1C). Interestingly, despite this difference in O$_2^·$ concentrations endogenous ecSOD appears to be less important for hypertension development and endothelial dysfunction in the 1K1C model. No difference in the hypertensive response to the clip application was observed between ecSOD$^{-/-}$ and ecSOD$^{-/-}$ mice and the extent of the procedure-induced attenuation of endothelium-dependent relaxation was identical in both strains. However,
acetylcholine-induced relaxation in aortic segments from sham-operated ecSOD+/+ mice was slightly smaller than that observed in vessels from ecSOD+/+ mice and this difference was maintained in the 1K1C group despite a marked attenuation of the endothelium-dependent relaxation (Figure 1B). These observations are in striking contrast to the data previously obtained by our group in the 2K1C model of renovascular hypertension. In this model blood pressure was significantly higher in clipped ecSOD+/+ than ecSOD−/− mice and aortic endothelial dysfunction was more pronounced in clip-operated ecSOD+/+ than ecSOD−/− mice.4

Interestingly, as compared with the level previously measured in ecSOD+/+ mice subjected to the 2K1C protocol, the 1K1C procedure resulted in markedly higher vascular O2− concentrations in the same strain (ecSOD+/+; sham, 39±12; 2K1C: 79±24 cpm; 1K1C, 167±9 cpm; P<0.05, 2K1C versus 1K1C, n=8). This difference, however, was not observed in the absence of ecSOD, because both models increased the radical production to the same level in ecSOD−/− mice (ecSOD−/−: sham, 249±66; 2K1C, 442±78; 1K1C, 404±126 cpm; P=not significant, 2K1C versus 1K1C, n=8) (Figure 1C).

**Figure 2.** Effect of human recombinant ecSOD on mice with 1-kidney-1-clip hypertension. (A) Mean blood pressure as determined by carotid artery catheter before and after intravenous application of 100,000 U/kg ecSOD in mice 4 weeks after 1K1C operation (n=8): Acetylcholine (ACh)-induced relaxation (B) and nitro-L-arginine (L-NA)-induced contraction (C) in aortic rings from mice 4 weeks after 1K1C with (+) and without (−) in vivo application of ecSOD 16 hours before euthanization. n=8, *P<0.05.

**Human Recombinant ecSOD Improves Vascular Function in 1K1C Hypertension**

Because no difference between ecSOD+/+ and ecSOD−/− mice was observed after the 1K1C procedure, it was tested whether in this form of hypertension ecSOD plays any role for vascular function. When human recombinant ecSOD was administered intravenously the mean blood pressure recorded in the carotid artery declined by >15 mm Hg. The blood pressure lowering effect of ecSOD, however, was identical between ecSOD+/+ and ecSOD−/− mice (Figure 2A). Similar observations were made in isolated aortic segments: In vivo application of human recombinant ecSOD improved acetylcholine-induced relaxation as well as basal NO formation in aortic segments from animals subjected to the 1K1C protocol.
mice. Importantly, application of human ecSOD had no effect on blood pressure in eNOS−/− mice, suggesting that either the hypotensive effect of ecSOD requires endothelial NO or that the presence of eNOS alters endogenous ecSOD activity to render the mice sensitive to ecSOD application (please see http://atvb.ahajournals.org).

In organ chamber experiments, the relaxations to deta-NONOate of aortic rings from eNOS−/− mice were found to be unaffected by in vivo ecSOD treatment. In contrast, relaxations to deta-NONOate in rings from ecSOD−/− and ecSOD+/− mice were improved by in vivo ecSOD application. As the effect of ecSOD on the deta-NONO−induced relaxation involves scavenging of O2−, it could to be concluded that O2− levels are lower in aortas of eNOS−/− mice compared with control mice. A possible explanation could be that eNOS uncoupling largely contributes to the oxidative stress in the 1K1C model. Alternatively, the concomitant generation of O2− and NO will yield peroxynitrite (ONOO−), which may inactivate ecSOD in WT animals. Interestingly, ecSOD expression was also much higher in eNOS−/− mice than in WT mice (Figure ID, http://atvb.ahajournals.org), which could be another explanation for the failure of exogenous ecSOD to affect relaxations.

Despite the lack of effect of ecSOD in eNOS−/− mice, the aortic responses to deta-NONOate were largely blunted in hypertensive animals of this strain (Figure 4B). It is possible that this difference is a consequence of a dysfunction of the smooth muscle cells in hypertensive eNOS−/− mice. Indeed, in preliminary experiments lower aortic expression of soluble guanylyl cyclase was observed in eNOS−/− mice subjected to the 1K1C procedure as compared with sham operated animals (Brandes RP, unpublished observation, 2006).

Hydrogen Peroxide and Peroxynitrite Inactivate ecSOD

To determine whether ecSOD is inactivated by ROS, isolated aortic segments were exposed to peroxynitrite (ONOO−) and hydrogen peroxide (H2O2). Both ROS decreased ecSOD activity, although the effect of ONOO− was more pronounced than that of H2O2. In contrast, CuZnSOD activity was similarly impaired by ONOO− and H2O2, whereas MnSOD activity remained unaffected (Figure 4A). To determine whether the inhibitory effect of ONOO− results from tyrosine, nitration of ecSOD immunoprecipitates was performed. These experiments, however, revealed that several proteins but not ecSOD are tyrosine-nitrated on exposure to ONOO− (Figure 4B), suggesting that rather thiol oxidation or the formation of amino acid radicals mediate inactivation of ecSOD.

Prolonged Treatment With Catalase Selectively Improves Vascular Function in ecSOD+/+ Mice

To determine whether H2O2 contributes to the inactivation of ecSOD in vivo, mice were treated with daily injections of PEG-catalase for 3 days and studied subsequently. This prolonged treatment selectively lowered the blood pressure in ecSOD+/+ mice but was without effect in ecSOD−/− mice. Importantly, injection of ecSOD in PEG-catalase–pretreated mice only lowered the blood pressure in animals of the
Acute Blood Pressure-Lowering Effect of ecSOD

It has been suggested that H₂O₂ through the peroxidase activity of SODs leads to the generation of hydroxyl radicals, which subsequently inactivate SOD. Therefore, the plasma concentrations of the hydroxyl radical scavenger uric acid were increased by treating mice with oxonic acid for 7 days, as reported previously by others. Indeed, similar to PEG-catalase, in vivo treatment of 1K1C hypertensive ecSOD+/+ mice with oxonic acid increased the endothelium-dependent relaxation of the isolated aorta (Figure 5C).

Figure 5. Effects of in vivo treatment with PEG-catalase, L-NAME, and oxonic acid in 1K1C hypertension. A, Mean blood pressure as determined by carotid artery catheter before and after intravenous application of 100 000 U/kg ecSOD in ecSOD+/+ and ecSOD−/− mice 4 weeks after 1K10 operation. Subgroups were treated with daily injections of PEG-catalase (catalase, 40 000 U/kg) for 3 days before the catheterization (n=6). *P<0.05 vs vs ecSOD, #P<0.06 with vs without PEG-catalase. Acetylcholine (ACH)-induced relaxation in aortic rings from ecSOD−/− mice 4 weeks after 1K1C operation without (control) and with in vivo pretreatment with PEG-catalase (B) or oxonic acid (C) (applied by osmotic minipump at a rate of 2.8 mg/kg per day over 7 days; n=8). D, Mean blood pressure before and 15 minutes after acute intravenous application of PEG-catalase (40 000 U/kg, n=4) or N^nitro-l-arginine methyl ester (L-NAME) (30 mg/kg, n=3). Subsequently, ecSOD (100 000 U/kg) were injected and the blood pressure was measured again after 15 minutes. P<0.05 vs “before.”

NO and Not Hydrogen Peroxide Mediates the Acute Blood Pressure-Lowering Effect of ecSOD

It is unclear whether the blood pressure-lowering effect of ecSOD is mediated by an increased generation of H₂O₂ or an impaired scavenging of NO. Therefore, the effects of acute application of PEG-catalase and the NO synthase inhibitor L-NAME were compared. PEG-catalase had no effect on blood pressure, whereas L-NAME resulted in a marked increase in pressure, which was fatal for the mice within 60 minutes. ecSOD had no effect on blood pressure in L-
NAME–treated mice. In contrast, in PEG-catalase–pretreated animals, the blood pressure-lowering effect was similar to that obtained in untreated 1K1C hypertensive animals (Figure ●●). These data clearly demonstrate that NO, but not H$_2$O$_2$, mediates the vascular effects of ecSOD.

**Discussion**

In this study we observed that application of human recombinant ecSOD improves endothelial function and lowers blood pressure in a mouse model of high-volume hypertension induced by the 1K1C procedure. Therefore, direct evidence is provided that in this type of hypertension endogenous ecSOD does not provide sufficient activity to scavenge extracellular superoxide. The effects of ecSOD application were dependent on eNOS and evidence is presented that endogenous ecSOD is partially inactivated by hydroxyl radicals formed from H$_2$O$_2$.

In arteries, ecSOD is present in large abundance and is involved in the maintenance of vascular function in high-renin hypertension. Indeed, we previously observed that endogenous ecSOD attenuates the hypertensive response and endothelial dysfunction in ecSOD$^{+/−}$ mice with 2K1C-induced hypertension, whereas application of recombinant ecSOD failed to elicit any effects in WT mice. These former observations are in line with several studies performed in rats, which lack significant vascular ecSOD activity. In contrast to this, it was observed that in rabbits, a species with high-endogenous ecSOD activity, application of ecSOD either as recombinant enzyme or by gene therapy can have positive effects: This difference suggests that the pathophysiological situation determines whether ecSOD application elicits positive vascular effects and whether endogenous ecSOD is sufficient to dismute the majority of extracellular O$_2^−$. Indeed, different to the 2K1C model, the studies performed in rabbits utilized disease models with concomitant oxidative stress as indicated in studies using the aortic banding model. In these studies, arteries distal of the stenosis had low vascular O$_2^−$ production, whereas O$_2^−$ production was increased in the vascular areas exposed to hypertension and this is associated with the induction of NADPH oxidase subunits and protein kinase C. Also endothelial NO formation is increased in response to enhanced circumferential tension, and peroxynitrite formation has uniformly been observed in models of high-volume hypertension. The data obtained with eNOS$^{+/−}$ mice in this study suggest a critical role of endothelial NO for the action of recombinant ecSOD. eNOS-derived ONOO$^−$ could potentially inactivate abundantly expressed endogenous ecSOD, rendering the system sensitive to the exogenously applied enzyme. ENOS could also be a major source of O$_2^−$ in the present study and finally, the vasodilator effects of ecSOD could be mediated by increasing basal NO systemically, leading to direct vasodilatation. Our observations suggest that all these mechanisms are functional in high-volume hypertension. Acute inhibition of NOS completely blocks the effects of recombinant ecSOD on blood pressure, proving that the effect on blood pressure is mediated by increasing the NO bioavailability rather than that of H$_2$O$_2$. However, in 1K1C hypertensive eNOS$^{−/−}$ mice, in vivo treatment with recombinant ecSOD had no effect on the deta-NONOate–induced relaxation of isolated aortic segments, whereas the same approach improved dilator responses in ecSOD$^{+/−}$ as well as ecSOD$^{−/−}$ mice. This observation is indicative toward an inactivation of endogenous ecSOD by a mechanism involving eNOS. Indeed, evidence has been presented to suggest that ecSOD inactivation occurs under certain disease conditions. In a rabbit balloon injury model, a significant decrease in ecSOD activity was observed despite an increase in protein levels. Moreover, in apoE$^{−/−}$ mice, inactivation of ecSOD was detected. Increasing the plasma concentration of urate, a scavenger of hydroxyl radicals and ONOO$^−$, restored vascular ecSOD activities. The physiological relevance of ecSOD...
inactivation and the effect of different ROS on aortic ecSOD activity were not determined so far. The present observation that prolonged in vivo treatment with catalase selectively lowers the blood pressure in hypertensive ecSOD+/− mice and improves ex vivo aortic endothelial function clearly demonstrate that H2O2 is central for the inactivation of endogenous ecSOD. The effects of H2O2 appear to be mediated by hydroxyl radicals: When hypertensive mice were treated with oxonic acid for 7 days, the ex vivo aortic endothelial vasomotor function improved. In pilot experiments we also observed that oxonic acid lowered the blood pressure in hypertensive mice (Xia, unpublished observations, 2006). Oxonic acid inhibits uricase, and thus increases urate level in mice,17,33 and urate is known to scavenge hydroxyl radicals as well as ONOO−.34 Despite the lack of selectivity of uric acid, data to suggest an involvement of ONOO− for the present disease mechanisms could not be obtained. The mechanism of H2O2-mediated inactivation of Cu-containing SODs is relatively well understood and involves the peroxidase activity of the enzyme itself.17 ONOO−, in contrast could act via tyrosine nitration or oxidation of proteins. In the present study we found no evidence for tyrosine nitration of ecSOD using immunoprecipitation. It has been demonstrated that the inactivation of CuZnSOD by ONOO− involves histidyl radicals rather than tyrosine nitration.35 In addition to this aspect it is important to realize that under physiological conditions, ONOO− will preferentially react with abundantly expressed antioxidants such as glutathione or will mediate its effect through the formation of secondary radicals.36 Consequently, based on the present data it is impossible to role out but also to prove some contribution of ONOO− in the present model.

Blood pressure in the present study was determined using 2 different methods: tail cuff technique in conscious animals and carotid artery catheter in anesthetized mice. Using both approaches it was observed that the blood pressure between ecSOD−/− and ecSOD+/− mice was identical. Both techniques, however, have substantial limitations and thus it cannot be excluded that slight differences that could only be detected by telemetry in large groups were overseen. This aspect does not relate to the central finding of this study that ecSOD improves vascular function; because this effect was also observed in isolated vessels and therefore was independent of any confounders in the blood pressure methods.

Interestingly, aortic ecSOD expression increased in the 1K1C model. It has been demonstrated previously that endothelial NO77 as well as angiotensin II via a pathway not involving oxidative stress88 increase ecSOD expression. It is unlikely that these 2 mechanisms contribute to the induction observed in the present study, because angiotensin II level are low in 1K1C hypertension (Jung O, 2006, unpublished observation) and because NO bioavailability was low through O2•−-mediated scavenging. Moreover, particular in eNOS−/− mice, aortic ecSOD expression was high and further increased by the clip procedure. These data are in contrast to a previous observation22; still, the 1K1C-mediated increase in ecSOD expression in eNOS−/− mice excludes endothelial NO to contribute to the induction observed in the present study. In cultured smooth muscle cells, NO rather decreases than increases ecSOD expression.39 It has recently been reported that the increased expression of ecSOD observed in apoE−/− mice is attributable to high expression of Atox-1.40 Therefore, it is attractive to speculate that a similar mechanism might be function in eNOS−/− mice as well as in 1K1C hypertension.

In conclusion, the present study provides evidence that shortage of endogenous vascular ecSOD activity occurs in situations of combined oxidative and nitrosative stress, and that replenishment of the vascular ecSOD activity pool by application of ecSOD or SOD mimetic might lead to normalization of endothelial function in hypertensive patients.

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Disclosures
None.

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**Effect of the 1-kidney-1-clip model in endothelial NO synthase-/- mice**

(A) Mean blood pressure as determined by carotid artery catheter before and after i.v. application of 100,000 U/kg ecSOD in eNOS-/- mice 4 weeks after 1K1C-operation (clip) or sham-procedure. n=6. (B&C): Deta-NONOate-induced relaxation in aortic rings from eNOS-/- mice (B) and ecSOD+/+ and ecSOD-/- mice (C) 4 weeks after 1K1C with (+ecSOD) and without in vivo application of ecSOD 16 hours prior to sacrifice. n=8, *<0.05. (D) Western blot analysis for aortic ecSOD and β-actin expression in aortic segments from ecSOD+/+ and eNOS-/- mice 4 weeks after 1K1C-procedure (Clip) or sham operation (Sham).