Role of Vascular Extracellular Superoxide Dismutase in Hypertension

Heinrich E. Lob, Antony Vinh, Li Li, Yelena Blinder, Stefan Offermanns, David G. Harrison

Abstract—Previous studies indicate that superoxide is important in the modulation of blood pressure but have not specifically identified the cell types or organs involved. We created mice with loxP sites flanking the extracellular superoxide dismutase (SOD3) gene. These mice were crossed with mice expressing inducible Cre-recombinase driven by the smooth muscle myosin heavy chain promoter allowing tissue-specific deletion of SOD3. Deletion of SOD3 increased vascular superoxide and reduced vascular NO levels as detected by electron spin resonance. Despite these changes in NO and superoxide, we did not observe increases in vascular inflammation caused by angiotensin II. Moreover, deletion of vascular SOD3 did not augment hypertension in response to angiotensin II. In additional studies, we also deleted SOD3 from the circumventricular organs by intracerebroventricular injection of an adenovirus encoding Cre-recombinase. Although this raised blood pressure and augmented the hypertension caused by angiotensin II, these responses were not further increased by vascular deletion of SOD3. These data suggest that the extracellular superoxide dismutase in vascular smooth muscle is not involved in the genesis of angiotensin II–induced hypertension and further emphasize the role of central SOD3 in the modulation of blood pressure. (Hypertension. 2011;58:232-239.) ● Online Data Supplement

Key Words: superoxide dismutase ● blood pressure ● inflammation ● vasculature ● central nervous system

Reactive oxygen species, and in particular the superoxide anion (O$_2^-$), play a critical role in the genesis of hypertension via mechanisms that are incompletely understood. Experimental hypertension is ameliorated by treatment with membrane-targeted forms of superoxide dismutase (SOD) or by SOD mimetics, and mice lacking the NADPH oxidase subunit p47phox have reduced hypertensive responses to either angiotensin II or deoxycorticosterone acetate-salt challenge. The precise cause of chronic angiotensin II infusion, renal clipping, and deoxycorticosterone acetate-salt challenge. In keeping with a role of O$_2^-$ in hypertension, we and others have found that mice lacking the extracellular SOD (SOD3) have augmented hypertension in response to chronic angiotensin II infusion, renal clipping, and deoxycorticosterone acetate-salt challenge. The precise cause of excessive hypertension in these animals remains unclear. Superoxide produced in various sites of the central nervous system (CNS) modulates sympathetic outflow, and intracerebroventricular injection of an adenovirus that expresses SOD blunts the hypertensive response to either local or systemically administered angiotensin II. In the kidney, O$_2^-$ enhances tubular sodium reabsorption. Finally, O$_2^-$ is a major determinant of vasmotor tone. Superoxide reacts rapidly with endothelium-derived NO and directly promotes vasoconstriction. Superoxide has also been implicated in vascular smooth muscle hypertrophy and remodeling in response to angiotensin II. Thus, deletion of SOD3 in the vascular smooth muscle could increase systemic vascular resistance and, thus, enhance experimental hypertension.

To understand the mechanisms by which O$_2^-$ promotes hypertension, we have produced mice that allow targeted deletion of SOD3 using Cre-lox technology. In a recent study, we used these animals to show that SOD3 in the circumventricular organs (CVOs) modulates baseline blood pressure and the hypertensive response to angiotensin II, likely by augmenting sympathetic outflow. We also found that this central intervention markedly enhances the vascular inflammation, as characterized by macrophage and T-cell accumulation, during induction of experimental hypertension. Of interest, deletion of SOD3 in the CVO caused a striking increase in vascular O$_2^-$ production. This study emphasized that central oxidant injury can promote vascular oxidant stress and inflammation. It remained possible, however, that the augmented hypertension caused by angiotensin II after CVO SOD3 deletion might have been in part because of increased vascular O$_2^-$.

In the present study, we sought to specifically examine the role of vascular O$_2^-$ in hypertension by deleting SOD3 in vascular smooth muscle cells. Using a vascular smooth muscle–targeted Cre-recombinase that could be induced in adult animals, we were able to enhance O$_2^-$ levels specifically in the vascular smooth muscle. We compared the effects
of vascular versus CVO SOD3 deletion and further examined the effect of simultaneous deletion of SOD3 in these 2 sites. We found that, whereas vascular smooth muscle SOD3 deletion increases vascular O$_2^-$ production, it does not affect blood pressure either at baseline or during angiotensin II infusion and has only modest effects on vascular inflammation.

**Materials and Methods**

**Mice Studied and Protocols**

Mice with loxP sites flanking the SOD3 coding region (SOD3$^{loxP/loxP}$ mice), created previously in our laboratory, were used in these studies. These animals have been backcrossed for >11 generations with C57Bl/6 background. To delete SOD3 in vascular smooth muscle cells, we crossed SOD3$^{loxP/loxP}$ mice with mice transgenic for Cre-recombinase driven by a tamoxifen-inducible smooth muscle cell myosin heavy chain promoter (tgcre/SMMHC). The resultant male SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice were studied at 3 months of age. To delete vascular SOD3 in these animals, tamoxifen (3 mg/20 g of body weight) was injected daily for 5 days beginning at 3 months of age. As controls, we treated tamoxifen-injected SOD3$^{loxP/loxP}$ mice that were negative for Cre-recombinase with tamoxifen in an identical fashion. In other experiments, we deleted SOD3 in the CVO of SOD3$^{loxP/loxP}$ mice by intraperitoneal injection of adenoviral Cre-recombinase, as described previously. In yet other SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice, we initially performed intraperitoneal Cre-recombinase injections of adenoviral Cre-recombinase and then administered intraperitoneal injections of tamoxifen to permit the deletion of both vascular smooth muscle and CVO SOD3. Two weeks after the final tamoxifen injection, osmotic minipumps (ALZET, model 2002) were implanted for subcutaneous infusion of either angiotensin II (140 ng/kg per minute) or vehicle. This low dose of angiotensin II was chosen because it causes minimal elevation of blood pressure in control mice. In additional mice, we infused a higher dose of angiotensin II (500 ng/kg per minute) or vehicle. This low dose of angiotensin II was chosen because it causes minimal elevation of blood pressure in control mice. In additional mice, we infused a higher dose of angiotensin II (500 ng/kg per minute), which induces severe hypertension in C57Bl/6 mice.

Blood pressure was measured using either radiotelemetry, as described previously, or in some cases noninvasively by using the tail cuff method. When tail cuff recordings were made, mice were accustomed to this procedure for 3 days before measurements were obtained. For telemetry studies, mice were allowed to recover for 10 days before recording blood pressure, and osmotic minipumps containing either angiotensin II or vehicle for angiotensin II were inserted 13 days after telemetry implantation. All of the studies were performed according to a protocol approved by the Emory University Institutional Animal Care and Use Committee.

**Measurement of Vascular Reactivity, Superoxide, Hydrogen Peroxide Levels, and NO**

After euthanasia, the aortas were immediately removed. Isometric tension studies were performed using 2-mm aortic segments as described previously. Aortic O$_2^-$ levels were measured using electron spin resonance (ESR) spectroscopy with CAT-1H as a spin probe. The SOD-inhibited amplitude of the ESR signal was used to quantify extracellular O$_2^-$ production. In separate animals, we also measured aortic O$_2^-$ levels by monitoring the oxidation of dihydroethidium to dihydroethidium by monitoring the oxidation of dihydroethidium to dihydroethidium. Values were normalized to 2-mm aortic segments or tissue wet weight of the mesenteric arteries.

**SOD Activity Assay**

SOD activity was measured using a commercially available kit (Cayman Chemicals, Inc.). To distinguish between the different SOD isoforms, homogenized aortic tissue was centrifuged at 10,000 g to separate mitochondrial and extramitochondrial cell lysates. The mitochondrial fraction was treated with NaCN to inactivate possible contamination of copper/zinc containing SODs. The mitochondrial fraction was used to measure SOD2 activity. To differentiate between SOD1 and SOD3, Concanavalin A Sepharose beads (Amersham) were added to the extramitochondrial fraction in batch and agitated for 10 minutes. Low speed centrifugation (500 g) was performed to remove SOD3, and the remaining SOD1 activity was measured in the supernatant. We have used this method previously.

**Immunohistochemistry and Flow Cytometry**

After euthanasia, a stainless-steel cannula was placed in the right ventricle, and the mice were initially perfused at 100 mm Hg with saline and then with 10% formaldehyde. Aortas were harvested and embedded in paraffin. Primary antibodies used were a polyclonal anti-SOD3, as described previously. Primary antibodies were visualized using the DAKO LSAB+ System-HRP kit (DAKO, Carpinteria, CA) according to the manufacturer’s protocol. Fluorescent cell sorting was performed on peripheral blood mononuclear cells and single cell aortic suspensions as described previously.

**Statistical Analysis**

Data are presented as mean±SEM. Comparisons between groups were performed using ANOVA and a Bonferroni post hoc test for comparison of selected groups. When only 2 comparisons were made, we used a Student t test, and the reported P value represents a Bonferroni correction if >1 comparison was made. Blood pressure was analyzed using 2-way ANOVA with repeated measures. Data from fluorescence-activated cell sorters were analyzed with FlowJo software (Tree Star, Inc.). For data from aortic fluorescence-activated cell sorters we used 2-way ANOVA to detect an interaction between the angiotensin II treatment and SOD3 deletion.

**Results**

**Expression of SOD3 in Aortic Tissue**

In initial experiments, we confirmed that 5 days of tamoxifen administration successfully deleted SOD3 in SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice. Using Western blots we found that vessels from mice treated with the vehicle for tamoxifen (corn oil) expressed all 3 of the SOD isotypes at the predicted molecular sizes (Figure 1A). Two days after the final tamoxifen injection, SOD3 was undetectable by Western blot in both the aorta and mesenteric arteries of SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice, whereas SOD1 and SOD2 levels were unchanged (Figure 1A through 1D). Western blots of the lung, heart, and kidney showed that tamoxifen injection had no effect on SOD3 in these organs (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Activity assays for each of the respective SOD isoforms further confirmed that tamoxifen injection markedly reduced SOD3 activity in vessels of SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice (Figure 1E) but not in SOD3$^{loxP/loxP \times tgcre/SMMHC}$ mice lacking Cre-recombinase.

**Effect of VSMC SOD3 Deletion on Reactive Oxygen Species, Endothelial Function, and NO Production**

As expected, deletion of SOD3 in vascular smooth muscle increased vascular O$_2^-$ production by 2-fold at baseline as.
detected by ESR (Figure 2A) and by dihydroethidium and high-pressure liquid chromatography (Figure 2B). Infusion of low-dose angiotensin II did not further increase vascular $O_2^{-}$ production in either Cre-negative mice or SOD3loxP/loxP x tgcre/SMMHC mice (Figure 2A and 2B). The vascular production of hydrogen peroxide was not affected by SOD3 deletion in vascular smooth muscle (Figure 2C).

An increase in vascular $O_2^{-}$ can decrease vascular NO bioavailability and impair endothelium-dependent vasodilation. In keeping with this, deletion of SOD3 caused a modest reduction in endothelium-dependent vasodilation in animals treated with low-dose angiotensin II compared with controls. In contrast, endothelium-independent vasodilation to sodium nitroprusside was not altered (Figure 2D and 2E).
In mice lacking SOD3 we observed a dramatic reduction of NO bioavailability at baseline in aortic and mesenteric tissue (Figure 2F and 2G). After high-dose angiotensin II infusion, NO levels were not further decreased in aortic tissue (Figure 2F). In mesenteric arteries, however, angiotensin II tended to decrease NO in mice lacking SOD3, but this difference did not achieve statistical significance (Figure 2G).

Figure 2. Levels of reactive oxygen species, vascular function, and NO after vascular extracellular superoxide dismutase (SOD3) deletion. SOD3loxP/loxP with and without the vascular smooth muscle inducible Cre-recombinase were treated with tamoxifen and 1 week later received osmotic minipumps for infusion of either vehicle or angiotensin II (140 or 490 ng/kg per minute). A shows estimates of extracellular superoxide production as determined by the spin probe CAT1-H and electron spin resonance. B shows estimates of vascular superoxide production as detected by formation of 2-hydroxyethidium from dihydroethidium, determined using high-pressure liquid chromatography. C shows vascular production of hydrogen peroxide as determined using Amplex Red. Aortic rings were studied in organ chambers. Vessels were contracted by phenylephrine, and the relaxations evoked by either acetylcholine (D) or sodium nitroprusside (E) were studied. Estimates of NO production in different tissue as determined by the spin trap Fe[DETC]2 and electron spin resonance is shown in F and G. F reflects aortic NO, whereas G shows NO production in mesenteric tissue. Two-way ANOVA was used to compare groups in A through C and F and G, whereas 2-way ANOVA with repeated measures was used in D and E. In D, * = 0.04 between Cre-negative sham and Cre-positive angiotensin II.

Effect of Vascular SOD3 Deletion on Blood Pressure

Previous studies from our laboratory and others have shown that global embryonic deletion of SOD3 predisposes to hypertension and that this is recapitulated by deletion of SOD3 in the CNS. In the present study, we sought to determine the role of SOD3 in the vasculature in modulation...
of blood pressure. Using telemetry recordings of blood pressure, we found that low-dose angiotensin II had only a modest effect on blood pressure in both the Cre-negative SOD3loxP/loxP mice and in the SOD3loxp/loxP/H11003tgcre/SMMHC mice and that these responses were not different between the 2 groups of animals (Figure 3A and 3B). In additional experiments, we infused a higher dose of angiotensin II (490 ng/kg per minute) for 2 weeks. This dose, which is known to cause severe hypertension in control mice, increased blood pressure to a similar extent in SOD3loxP/loxP/H11003tgsmmhc/cre mice and in SOD3loxP/loxP mice without Cre-recombinase (Figure 3C). Additional experiments were performed in Cre-positive mice without loxP sites flanking SOD3. These animals demonstrated similar blood pressures at baseline and in response to angiotensin II as the Cre-negative mice, indicating that the presence of the Cre-transgene alone had no effects on blood pressure modulation (data not shown).

The above findings are in contrast to our previous findings with deletion of SOD3 in the CVO, which caused an elevation of blood pressure at baseline and a striking increase in the hypertensive response to this identical dose of angiotensin II. We considered the possibility that central and vascular oxidative stresses might synergistically increase blood pressure. To test this possibility, we performed an additional group of experiments in which we deleted SOD3 either in the vascular smooth muscle or in both the CVO and the vascular smooth muscle simultaneously and then examined the hypertensive response to angiotensin II. The results of these experiments are shown in Figure 3D. These studies confirmed that deletion of vascular smooth muscle SOD3 had no effect on blood pressure at baseline and did not alter the hypertensive response to angiotensin II. In addition, the simultaneous deletion of SOD3 in the CNS and vasculature led to a hypertensive phenotype that was identical to what we observed previously with deletion of SOD3 in the CVO alone (Figure S2).

**Effect of Vascular SOD3 Deletion on Peripheral Inflammation**

We have found previously that deletion of SOD3 in the CVO causes a striking increase in vascular inflammation, characterized by infiltration of T cells and total leukocytes.12 In...
Vascular smooth muscle deletion of SOD3 had no effect on vascular T-cell infiltration in mice lacking SOD3 in the CVO. In the present study, we also showed that CVO deletion of SOD3 increases blood pressure and vascular inflammation when combined with vascular smooth muscle deletion of SOD3 but to the same extent that we observed previously for deletion of SOD3 in the CNS alone. Of interest, we showed previously that CVO SOD3 deletion also increases vascular smooth muscle O$_2^-$ production, in a fashion similar to vascular smooth muscle deletion of this antioxidant.

In the present study, we infused angiotensin II at rates of 140 and 490 ng/kg per day. In normal mice, the 140 ng/kg per day dose of angiotensin II has minimal effect on blood pressure at baseline or in response to angiotensin II, and does not augment the inflammatory response to angiotensin II. Moreover, deletion of SOD3 in both the CVO and the vascular smooth muscle had no greater effect than deletion in the CVO alone. These findings indicate that SOD3 in the CNS likely plays a more important role in modulation of blood pressure than SOD3 in the vasculature.

There are several reasons to suspect that an increase in vascular O$_2^-$ might promote hypertension. Superoxide inactivates endothelium-derived NO and, therefore, could indirectly enhance vasoconstriction. Indeed, in the present study, we found that SOD3 deletion reduced ambient levels of NO, as detected by ESR, and that angiotensin II modestly impairs endothelium-dependent vasodilatation in mice lacking vascular smooth muscle SOD3. In addition, O$_2^-$ has been implicated as an endothelium-derived vasoconstrictor agent and could enhance formation of vasoconstrictor isoprostanes. Superoxide can alter vascular smooth muscle calcium handling, predisposing to vasoconstriction. These factors could increase systemic vascular resistance. Despite these potential roles of vascular O$_2^-$, we observed no change in blood pressure at baseline or in response to angiotensin II in animals lacking vascular smooth muscle SOD3. In keeping with our findings, vascular O$_2^-$ is increased in experimental models of type 1 diabetes mellitus, obesity, and atherosclerosis, and in these conditions blood pressure is not consistently increased.

Our present findings also emphasize the importance of oxidative events in the CNS and, in particular, the CVO in the regulation of blood pressure. In our previous study, we showed that deletion of SOD3 in the CVO caused an elevation of baseline blood pressure and markedly enhanced the hypertension caused by low-dose angiotensin II. In the present study, we also showed that CVO deletion of SOD3 increases blood pressure and vascular inflammation when combined with vascular smooth muscle deletion of SOD3 but to the same extent that we observed previously for deletion of SOD3 in the CNS alone. Of interest, we showed previously that CVO SOD3 deletion also increases vascular smooth muscle O$_2^-$ production, in a fashion similar to vascular smooth muscle deletion of this antioxidant.

In the present studies, we infused angiotensin II at rates of 140 and 490 ng/kg per day. In normal mice, the 140 ng/kg per day dose of angiotensin II had minimal effect on blood pressure. It is possible that vascular smooth muscle deletion of SOD3 could have augmented the hypertensive response to a higher dose of angiotensin II; however, we found that the
higher dose caused an increase in blood pressure that is identical with vascular smooth muscle deletion of SOD3 versus control animals. Moreover, the low-dose angiotensin II used in the present studies caused severe hypertension in mice lacking CVO SOD3. Thus, it is reasonable to conclude that the vascular smooth muscle deletion of SOD3 does not augment hypertension in response to either dose of angiotensin II.

Our present findings do not exclude a role of intracellular \( \text{O}_2^- \) in the vascular smooth muscle in the modulation of blood pressure. Deletion of SOD3 would be expected to predominantly increase extracellular \( \text{O}_2^- \); however, extracellular \( \text{O}_2^- \) can affect the activity of the cytoplasmic SOD (SOD1) in other models. Moreover, changes in the extracellular redox state can induce striking signaling events within the cell. In keeping with these considerations, we found that SOD3 deletion increased \( \text{O}_2^- \) measured by high-pressure liquid chromatography detection of 2-hydroxyethylidium, which predominantly reflects intracellular \( \text{O}_2^- \). Thus, although we cannot exclude a role of intracellular \( \text{O}_2^- \), our data suggest that vascular smooth muscle \( \text{O}_2^- \) in general has minimal effects on blood pressure.

Our studies also do not exclude a role of vascular hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) on blood pressure regulation. Deletion of SOD3 would be expected to increase vascular \( \text{O}_2^- \) but not \( \text{H}_2\text{O}_2 \). In keeping with this, we found no change in vascular \( \text{H}_2\text{O}_2 \) as measured by the Amplex red assay. \( \text{H}_2\text{O}_2 \) can promote vascular smooth muscle hypertrophy and, thus, could promote vascular remodeling characteristic of hypertension. Indeed, mice overexpressing thioredoxin, which eliminates \( \text{H}_2\text{O}_2 \), are protected against hypertension. In contrast, mice lacking the \( \text{H}_2\text{O}_2 \) scavenging enzyme glutathione peroxidase do not develop excessive hypertension when given angiotensin II. Nevertheless, we cannot exclude a role for vascular \( \text{H}_2\text{O}_2 \) in the modulation of blood pressure based on our present findings.

We and others have also shown an important role for inflammatory cells in hypertension and have shown that hypertension causes infiltration of both macrophages and activated T cells into the perivascular fat. A potential signal for this could be oxidative stress, which could increase vascular expression of adhesion molecules and chemokines that signal inflammatory cell accumulation. Surprisingly, we did not observe an increase in vascular inflammation after deletion in vascular smooth muscle SOD3, despite an increase in vascular \( \text{O}_2^- \) levels. In contrast, deletion of SOD3 in the CVO enhanced vascular inflammation in response to angiotensin II, as reflected by total vascular T cells and CD44(+) T cells. These findings again reflect the importance of CNS signaling in vascular inflammation.

**Significance and Perspectives**

This study extends previous research devoted to understanding how the extracellular SOD and, by inference, the superoxide anion contribute to hypertension. In previous studies, it has been shown that mice in with global deletion of SOD3 have augmented hypertension in response to either angiotensin II or deoxy corticalosterone acetate-salt challenge. More recently, we found that deletion of SOD3 in the CVOs caused an increase in baseline blood pressure and markedly enhanced the hypertension caused by low-dose angiotensin II infusion. The present research now demonstrates that, surprisingly, deletion of SOD3 in the vasculature has no effect on the hypertension caused by angiotensin II and does not augment the blood pressure elevation caused by the elimination of SOD3 in the CNS. These data do not exclude a role of other vascular reactive oxygen species in the genesis of hypertension but provide new insight into the relative roles of \( \text{O}_2^- \) in the vasculature and the CNS in the regulation of blood pressure. Therapeutic interventions directed toward the CVOs rather than the vasculature might be more effective in the treatment of hypertension.

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**Disclosures**

None.

**References**


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ROLE OF VASCULAR EXTRACELLULAR SUPEROXIDE DISMUTASE IN HYPERTENSION

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Supplemental Figure 1: Effect of tamoxifen injection on SOD3 expression in different organs in SOD3^{loxP/loxP} mice with or without Cre-recombinase. Panel A shows SOD3 and β-actin expression in lungs, heart and kidney tissue of SOD3^{loxP/loxP} mice with or without Cre-recombinase, the densitometric quantification is in Panel B (n = 4).
Supplemental Figure 2: The role of vascular SOD3 in regulation of blood pressure. Blood pressure was measured non-invasively using the tail cuff method. Mice underwent ICV injection of an adenovirus encoding Cre-recombinase (AdCre) and then received tamoxifen for 5 consecutive days. Osmotic minipumps for infusion angiotensin II (140 ng/kg/min) were then inserted three weeks after ICV injection. In some SOD3^loxP/loxP mice only ICV AdCre was delivered (black dotted line). For statistical analysis two-way ANOVA with Bonferroni post-hoc test was employed.