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# Hypertension Caused by Angiotensin II Infusion Involves Increased Superoxide Production in the Central Nervous System

Matthew C. Zimmerman, Eric Lazartigues, Ram V. Sharma, Robin L. Davisson

**Abstract**—Hypertension caused by angiotensin II (Ang II) infusion is associated with oxidative stress in the peripheral vasculature and kidney. The role of redox mechanisms in the central nervous system (CNS), a tissue known to be pivotal in Ang II-dependent hypertension, has not been investigated. We recently identified superoxide ( $O_2^{\cdot-}$ ) in the brain as a key signaling intermediate in the transient pressor response elicited by acute injection of Ang II directly into the CNS. Here we tested the hypothesis that hypertension caused by chronic systemic infusion of Ang II is mediated by a central neurogenic mechanism involving  $O_2^{\cdot-}$ . Infusion of Ang II ( $600 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) over a 2-week period in mice caused a gradually developing hypertension that was correlated with marked elevations in  $O_2^{\cdot-}$  production specifically in the subfornical organ (SFO), a brain region lying outside the blood-brain barrier and known to be a primary sensor for blood-borne Ang II. Adenoviral-mediated delivery of cytoplasmically targeted superoxide dismutase (SOD) selectively to this site prevented the hypertension and the increased  $O_2^{\cdot-}$  production, whereas gene transfer of SOD targeted to the extracellular matrix had no effect. These data suggest that increased intracellular  $O_2^{\cdot-}$  production in the SFO is critical in the development of Ang II-induced hypertension. (*Circ Res.* 2004;95:210-216.)

**Key Words:** reactive oxygen species ■ brain ■ subfornical organ ■ neurons ■ blood pressure

Various forms of hypertension are characterized by an elevation in angiotensin II (Ang II) levels.<sup>1,2</sup> Abundant evidence now suggests that a key mechanism by which Ang II influences blood pressure is via its ability to produce reactive oxygen species (ROS). A decade ago, Griendling et al first discovered that Ang II activates the vascular smooth muscle NAD(P)H oxidase, an important cellular source of ROS.<sup>3</sup> Subsequently, it was shown that hypertension caused by Ang II infusion, but not norepinephrine infusion, increased vascular superoxide ( $O_2^{\cdot-}$ ) production in vivo<sup>4</sup> and that treatment with liposome-encapsulated superoxide dismutase (SOD) was effective in preventing this form of hypertension.<sup>5</sup> More recent studies demonstrating that genetic disabling of the NADPH oxidase complex attenuates Ang II-induced increases in vascular  $O_2^{\cdot-}$  production and hypertension further implicates ROS, particularly  $O_2^{\cdot-}$ , in the pathogenesis of Ang II-dependent hypertension.<sup>6</sup>

Most investigations of Ang II hypertension and oxidant stress have focused on the vasculature as a key player, and in particular the notion that increased levels of  $O_2^{\cdot-}$  lead to diminished bioactivity of nitric oxide and thus vasoconstriction.<sup>4,7</sup> An important role for ROS-mediated vascular smooth muscle hypertrophy and remodeling in Ang II-dependent hypertension has also received considerable attention.<sup>8,9</sup>

While these mechanisms are likely to be important, there are a number of other potential sites where increased ROS production could contribute to hypertension caused by Ang II infusion. For example, it is well known that circulating Ang II has potent effects on blood pressure and cardiovascular function through its activation of neurons located in specialized brain regions called circumventricular organs (CVOs).<sup>10</sup> These unique areas are unprotected by the blood-brain barrier and are thought to couple circulating signals such as Ang II with neural networks that mobilize various effector systems (eg, sympathetic outflow, vasopressin release, thirst, and salt appetite) involved in maintaining blood pressure and body fluid homeostasis.<sup>11,12</sup> The CVOs and, in particular, the subfornical organ (SFO), are among regions of the brain most densely populated with Ang II receptors.<sup>13,14</sup> Interestingly, studies have shown that lesioning the CVOs markedly attenuates hypertension caused by increased levels of circulating Ang II.<sup>15-19</sup> Although this suggests that the CVOs are critically involved in Ang II-induced hypertension, the underlying mechanisms remain unknown.

Recent evidence from our laboratory suggests that ROS are important signaling intermediates in the cardiovascular effects elicited by administration of Ang II directly in the central nervous system (CNS). Intracerebroventricular injec-

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From the Department of Anatomy and Cell Biology (M.C.Z., E.L., R.V.S., R.L.D.), Free Radical and Radiation Biology Program, Department of Radiation Oncology (M.C.Z., R.L.D.), and The Cardiovascular Center (R.V.S., R.L.D.), The University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City.

Correspondence to Robin L. Davisson, PhD, Department of Anatomy and Cell Biology, 1-251 Bowen Science Building, The University of Iowa College of Medicine, Iowa City, IA 52242. E-mail robin-davisson@uiowa.edu

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tion of Ang II causes a well-known transient pressor and bradycardic response,<sup>20,21</sup> and our results demonstrated that these effects were abolished by genetic overexpression of SOD in the SFO.<sup>22</sup> Moreover, we showed that Ang II stimulates O<sub>2</sub><sup>-</sup> production in cultured neurons isolated from the CVOs, and this was prevented by the Ang II type 1 receptor (AT<sub>1</sub>) antagonist losartan or SOD. Taken together with a recent report by Oury et al showing particularly high endogenous levels of SOD in the CVOs,<sup>23</sup> these data suggest an important functional role for ROS in these unique brain regions.

Given the importance of the CNS in hypertension caused by Ang II infusion, along with recent evidence showing that ROS play a role in Ang II signaling in the brain, we hypothesized that oxidative stress in the CVOs play a pivotal role in hypertension caused by chronic peripheral Ang II infusion. To address this, we used the Ang II "slow-pressor" model of experimental hypertension in mice because of its similarities to some forms of Ang II-dependent hypertension in humans.<sup>24,25</sup> In this model, we modulated O<sub>2</sub><sup>-</sup> levels in the CVOs *in vivo* by delivering SOD via adenoviral vectors. SOD was targeted either to the cytoplasm (CuZnSOD) or to the extracellular matrix (ECSOD). Our results show that infusion of Ang II at doses that are initially subpressor causes a gradually developing hypertension that is paralleled by an increase in O<sub>2</sub><sup>-</sup> production in the SFO. Both the O<sub>2</sub><sup>-</sup> production and the hypertension were prevented by overexpression of CuZnSOD in the SFO, whereas ECSOD was ineffective.

## Methods

### Animals

Adult C57BL/6 mice (20 to 25 g; Harlan, Indianapolis, Ind) were used for all experiments in this study. Mice were fed standard chow (Harlan) and water *ad libitum*. All procedures were approved by the University of Iowa Animal Care and Use Committee.

### Adenoviral Vectors

Recombinant E1-deleted adenoviral vectors encoding human cytoplasmic superoxide dismutase (AdCuZnSOD), extracellular superoxide dismutase (AdECSOD), or bacterial  $\beta$ -galactosidase genes (AdLacZ) were obtained from the University of Iowa Gene Therapy Center (J. F. Engelhardt, Director). Construction and characterization of each of these viral vectors have been detailed previously.<sup>26,27</sup>

### Blood Pressure Studies

Mice were instrumented with intracerebroventricular (ICV) cannulae for central injection of adenoviruses and radiotelemeters for chronic recording of mean arterial pressure (MAP) as described in detail previously.<sup>28,29</sup> After 1-week recovery from surgery and 4 days of recording baseline MAP, separate groups of mice received one of the adenoviral vectors ( $5 \times 10^8$  particles, 500 nL) or vehicle in the brain via the ICV cannula. We have established previously that central injection of this adenovirus concentration results in robust transgene expression in the brain by 3 days and for up to 4 weeks without inflammatory effects.<sup>22,30,31</sup> After 3 additional days of MAP recording, mice were implanted subcutaneously with 14-day Ang II osmotic minipumps (Alzet; Durect Corporation). In initial studies, 3 different doses of Ang II (1500, 600, or 60 ng  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>) were used to establish the slow-pressor model of hypertension in mice. All subsequent studies were performed using the 600-ng  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> dose. At the time of Ang II osmotic minipump implantation, an additional group of mice were instrumented with ICV minipumps for

central infusion of the AT<sub>1</sub> receptor specific antagonist, losartan (2  $\mu$ g/h; Alzet). It should be noted that although 14-day osmotic minipumps were used, the actual calculated infusion times for these pumps was 16 days (0.25  $\mu$ L/h, fill volume of  $98.6 \pm 3$  mL). MAP was recorded daily for 3 weeks to evaluate the effects of Ang II throughout the entire infusion period and several days after infusion. Additionally, a separate group of mice was instrumented, injected with ICV with AdCuZnSOD, and MAP was recorded as described, but they did not receive the Ang II infusion.

### Immunohistochemistry

On the final day of MAP recording, a subset of mice from each treatment group was anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, as previously described.<sup>22</sup> Brains were removed, postfixed for 2 hours, and then transferred to 20% sucrose in phosphate buffer overnight. Cryostat sections (30  $\mu$ m, coronal) were incubated with human CuZnSOD antibody (sheep anti-human IgG; The Binding Site Limited) or human ECSOD antibody (rabbit anti-human IgG, kind gift from D. D. Heistad, University of Iowa, Iowa City) diluted 1:500 and 1:1000, respectively, in 2% NHS and 0.3% Triton for 24 hours at 4°C. Sections were washed with phosphate buffer, incubated with secondary antibodies (donkey antisheep FITC conjugated, 1:200 for anti-CuZnSOD; goat anti-rabbit 488 Alexa Fluor, 1:200 for anti-ECSOD), and washed before being mounted onto microscope slides and imaged using a Zeiss LSM 510 confocal microscope.

### Measurement of Cardiac Mass

At the conclusion of the blood pressure studies, another subset of mice from each group was euthanized and hearts were removed and weighed. In addition, tibia lengths were measured 24 hours after proteinase K digestion of the lower leg. Cardiac mass was expressed as the ratio of heart weight (mg) to tibia length (mm), as previously reported.<sup>32</sup>

### Measurement of Superoxide in the Brain

Separate groups of animals underwent ICV injection of the adenoviruses and subcutaneous Ang II infusion as described, except radiotelemeters were not implanted. Mice that were not infused with Ang II served as a control. On day 8 or day 16 of Ang II infusion, brains were removed, quickly frozen, embedded into OCT, and cryostat sectioned (30  $\mu$ m, coronal) directly onto chilled microscope slides. Sections were thawed at room temperature, rehydrated with 1X phosphate-buffered saline, and incubated for 5 minutes in the dark with the O<sub>2</sub><sup>-</sup>-specific fluorogenic probe dihydroethidium (DHE; 1  $\mu$ mol/L). After washing with phosphate-buffered saline, DHE fluorescence was visualized by confocal microscopy (Zeiss LSM 510) using an excitation wavelength of 543 nm and a rhodamine emission filter. Detector and laser settings were kept constant across all samples within individual experiments, and control and experimental samples were always processed in parallel. DHE fluorescence was quantified using ImageJ version 1.31, NIH analysis software. The RGB confocal images were loaded into the program and converted to 8-bit gray-scale before subtracting background fluorescence equivalently for all images (setting the threshold to 50% maximum intensity). The mean fluorescence was quantified and expressed relative to values obtained for control mice that were not infused with Ang II.

### Statistical Analysis

All data are expressed as mean  $\pm$  SEM and were analyzed by ANOVA (after Bartlett test of homogeneity of variance), followed by the Dunnett posttest for multiple comparisons. Statistical analyses were performed using Prism (GraphPad Software, Inc).

## Results

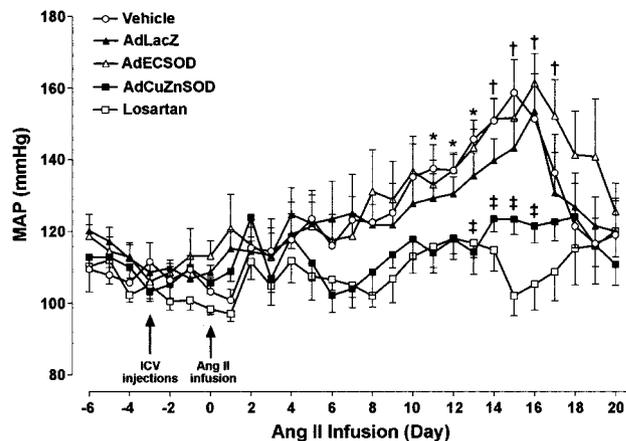
### Slow-Pressor Effects of Ang II in Mice

Prolonged infusion of low doses of Ang II leads to a gradually developing pressor response that is thought to be a good

model of Ang II–dependent hypertension in humans.<sup>24,25</sup> Kawada et al recently described a mouse model of the Ang II slow-pressor response<sup>33</sup> in which blood pressure was measured via tail-cuff plethysmography. However, because of recent evidence associated with tail-cuff measurements exaggerating the pressor effects of Ang II,<sup>34</sup> we used radiotelemetry in this study. Therefore, our first goal was to establish the optimal dose of subcutaneous Ang II infusion to elicit slow-pressor hypertension as measured by radiotelemetry. At the high dose of Ang II ( $1500 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $n=6$ ), MAP increased rapidly within 24 hours ( $105 \pm 5 \text{ mm Hg}$  to  $128 \pm 4 \text{ mm Hg}$ ,  $P < 0.05$ ) and peaked between days 5 and 6 of infusion ( $145 \pm 5 \text{ mm Hg}$ ,  $P < 0.05$  versus baseline). The low dose ( $60 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $n=3$ ) had no effect on MAP throughout the 2-week infusion period ( $100 \pm 7$  to  $107 \pm 3$ ,  $P > 0.05$ ). However, the  $600\text{-ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  dose ( $n=4$ ) elicited a slow-pressor response with a time course and peak values that were similar to what has been reported in other species.<sup>33,35,36</sup> MAP increased gradually and did not increase significantly over baseline ( $111 \pm 4 \text{ mm Hg}$ ) until day 11 of infusion ( $126 \pm 3 \text{ mm Hg}$ ,  $P < 0.05$  versus baseline). The peak elevation in MAP occurred between 15 and 16 days after the start of Ang II treatment ( $144 \pm 6 \text{ mm Hg}$ ,  $P < 0.05$  versus baseline), and blood pressure resolved to basal levels quickly thereafter on minipump emptying ( $115 \pm 9 \text{ mm Hg}$  on day 20,  $P > 0.05$  versus baseline). Thus, all subsequent studies were performed using the  $600\text{-ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  dose of Ang II.

### Increased Scavenging of Cytoplasmic $\text{O}_2^{\cdot-}$ in the Brain Prevents Ang II–Induced Hypertension

To investigate whether increased  $\text{O}_2^{\cdot-}$  production in the CNS is involved in the development of Ang II–dependent hypertension, MAP was recorded by radiotelemetry before, during, and after Ang II infusion in mice that received ICV injections of vehicle ( $n=6$ ), a control adenovirus (AdLacZ,  $n=5$ ), or adenoviruses encoding SOD targeted either to the cytoplasm (AdCuZnSOD,  $n=6$ ) or to the extracellular matrix (AdECSOD,  $n=6$ ). Baseline MAP was not affected by any of the viruses (AdLacZ,  $109 \pm 2 \text{ mm Hg}$ ; AdCuZnSOD,  $106 \pm 3 \text{ mm Hg}$ ; AdECSOD,  $107 \pm 3 \text{ mm Hg}$ ) as compared with vehicle-treated mice ( $109 \pm 4 \text{ mm Hg}$ ,  $P > 0.05$ ). Furthermore, the adenoviral vector itself had no effect on the gradually developing Ang II hypertension as indicated by nearly identical patterns of blood pressure change in vehicle-treated and AdLacZ-treated mice (Figure 1). However, virally mediated expression of CuZnSOD in the brain prevented the Ang II–induced elevation in MAP. In fact, treatment with AdCuZnSOD was as effective in ameliorating the hypertension as chronic ICV infusion of the  $\text{AT}_1$  receptor antagonist losartan (Figure 1). In contrast, CNS overexpression of ECSOD had no effect on the Ang II pressor response, because the blood pressure changes in these mice were indistinguishable from those observed in the control groups. As in the preliminary dose–response studies, there was a rapid return of MAP to baseline after the Ang II osmotic minipumps emptied, verifying that the pressor response is Ang II–dependent (Figure 1). It should be noted that overexpression of CuZnSOD in the brain in otherwise untreated animals



**Figure 1.** Increased scavenging of cytoplasmic  $\text{O}_2^{\cdot-}$  in the brain prevents Ang II–induced hypertension. Summary of mean arterial pressure (MAP) recorded by radiotelemetry before, during, and after subcutaneous infusion of Ang II ( $600 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). Mice received intracerebroventricular (ICV) injections of vehicle ( $n=6$ ), a control adenovirus (AdLacZ,  $n=5$ ), or adenoviruses encoding SOD targeted either to the cytoplasm (AdCuZnSOD,  $n=6$ ) or to the extracellular matrix (AdECSOD,  $n=6$ ) 3 days before the start of Ang II infusion. An additional group of mice was infused subcutaneously with Ang II and ICV with losartan. \* $P < 0.05$  versus day 0 for vehicle; † $P < 0.05$  versus day 0 for vehicle, AdLacZ, AdECSOD. ‡ $P < 0.05$  AdCuZnSOD and losartan versus vehicle, AdLacZ, and AdECSOD.

( $n=4$ ) caused no change in blood pressure. MAP in this control group ranged between  $100 \pm 3$  and  $108 \pm 4 \text{ mm Hg}$  over the 3-week recording period. In addition, control mice given saline-filled osmotic minipumps did not exhibit any significant changes in MAP over the 2-week infusion period, with blood pressure ranging between  $98 \pm 2$  and  $108 \pm 3 \text{ mm Hg}$ . These data suggest that intracellular, but not extracellular, scavenging of  $\text{O}_2^{\cdot-}$  in the CNS protects against hypertension caused by Ang II infusion.

### Ang II–Induced Cardiac Hypertrophy Is Unaffected by CNS Overexpression of SOD

To determine whether the protective effect of  $\text{O}_2^{\cdot-}$  scavenging in the brain is specific to the Ang II–induced increase in blood pressure or more broadly impacts other pathophysiological responses elicited by Ang II infusion, cardiac mass was measured in mice from each of the ICV treatment groups (vehicle,  $n=7$ ; AdLacZ,  $n=4$ ; AdCuZnSOD,  $n=5$ ; AdECSOD,  $n=5$ ) at the end of the 2-week Ang II (or saline) infusion period. There is now ample evidence that Ang II acts as a growth factor in cardiomyocytes,<sup>37</sup> and the link between hypertension caused by Ang II infusion and cardiac hypertrophy is well-established.<sup>35,38,39</sup> As seen in the Table, Ang II infusion caused a significant increase in cardiac mass as indicated by an increase in heart weight/tibia length ratio. However, in contrast to what was seen with the hypertensive response, Ang II–induced cardiac hypertrophy was not affected by CNS overexpression of either SOD isozyme. In all groups of animals, regardless of ICV treatment, Ang II infusion elicited equivalent increases in cardiac mass compared with saline-infused mice (Table). These data not only provide further validation of the Ang II infusion model used in this study but also

**Effect of Ang II Infusion (600 ng · kg<sup>-1</sup> · min<sup>-1</sup>) on Cardiac Mass**

Treatment		n	Heart Weight (mg)	Tibia Length (mm)	Heart Weight/Tibia Ratio (mg/mm)
ICV Injection	SC Infusion				
Vehicle	Saline	4	121±7	17.4±0.1	6.9±0.9
Vehicle	Ang II	7	181±14*	17.3±0.2	10.4±0.7*
AdLacZ	Ang II	4	169±11*	16.8±0.2	10.1±0.7*
AdECSOD	Ang II	5	174±6*	17.6±0.2	9.9±0.2*
AdCuZnSOD	Ang II	5	185±16*	17.9±0.6	10.6±0.8*

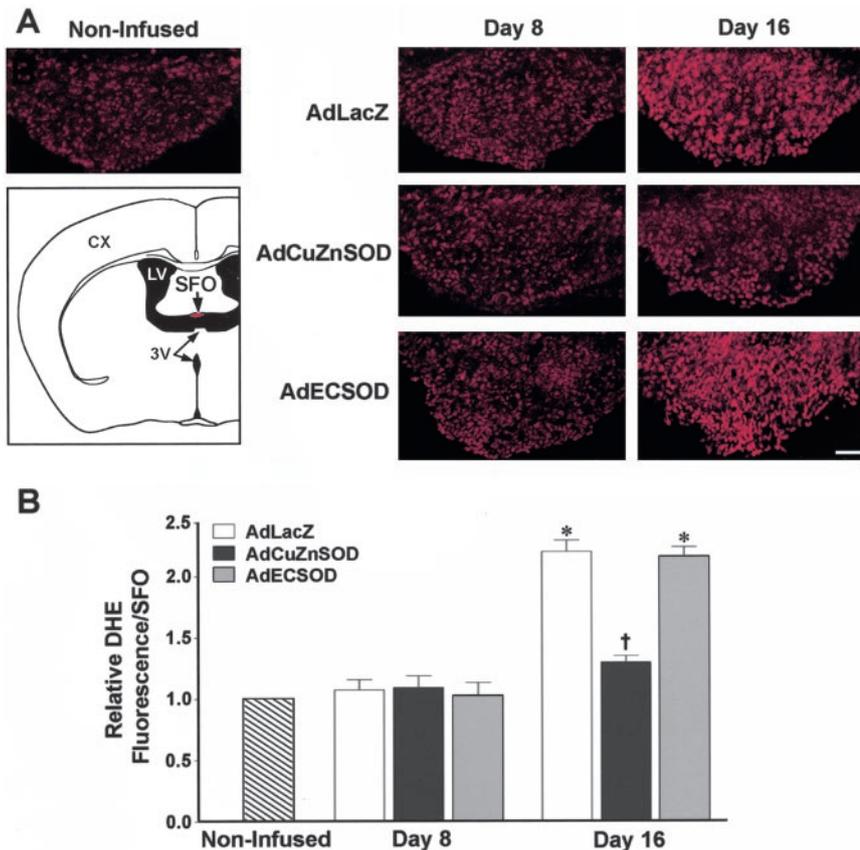
ICV indicates intracerebroventricular; SC, subcutaneous.  
\**P*<0.05 vs saline.

suggest that alteration of central redox mechanisms impacts hemodynamic events independently of other cardiovascular pathophysiological changes. Furthermore, the data demonstrate a direct effect of Ang II on cardiomyocytes rather than an influence of blood pressure in mediating cardiac hypertrophy.

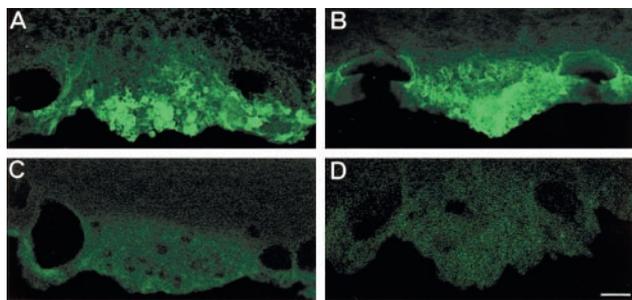
**Ang II Infusion Causes Increased O<sub>2</sub><sup>-</sup> Production in the SFO**

To provide direct evidence that peripheral Ang II infusion causes an increase in intracellular O<sub>2</sub><sup>-</sup> levels in the CNS, and to determine which sites are involved, confocal analysis of DHE fluorescence was used to estimate O<sub>2</sub><sup>-</sup> levels in brain sections of mice from each adenoviral treatment group after 8 or 16 days of Ang II infusion (n=3 to 6 in each group at each time point). Mice that were not infused with Ang II served as a control (n=4). These time points were selected based on the

slow-pressor Ang II profile that was established indicating no significant change in MAP at day 8, but peak Ang II-induced increases in blood pressure at day 16 (see Figure 1). Sections from throughout the brain were examined; however, only the SFO, which lacks a blood-brain barrier,<sup>40</sup> showed a significant increase in DHE fluorescence with Ang II infusion. As such, quantitative analyses focused on this region. As seen in Figure 2, only low-level DHE staining was observed in the SFO of control vector (AdLacZ)-treated mice after 8 days of Ang II treatment, and this was not different relative to basal fluorescence observed in the noninfused mice. Further, neither AdECSOD nor AdCuZnSOD had any effect on DHE staining at this early time point. However, by 16 days of Ang II infusion, the time when Ang II exerts its maximum effects on blood pressure, the AdLacZ-treated mice exhibited more than a doubling in relative DHE fluorescence in the SFO. This response was significantly attenuated in AdCuZnSOD-treated mice,



**Figure 2.** Ang II infusion causes increased intracellular O<sub>2</sub><sup>-</sup> production in the SFO. A, Representative confocal images showing DHE fluorescence in the SFO at days 8 or 16 of Ang II infusion in mice that had received ICV injections of AdLacZ, AdECSOD, or AdCuZnSOD 3 days before the start of Ang II infusion. Mice that were not infused with Ang II served as controls (upper left panel). The SFO is depicted in a schematic in the lower left panel. B, Summary of DHE fluorescence intensity in the SFO at days 8 or 16 of Ang II infusion in AdLacZ-treated, AdCuZnSOD-treated, and AdECSOD-treated mice (n=3 to 6 in each group at each time point). Data are expressed as mean±SEM for each group relative to mice not infused with Ang II (n=4). \**P*<0.05 versus noninfused and day 8; †*P*<0.05 versus AdLacZ and AdECSOD. CX indicates cortex; LV, lateral ventricle; 3V, third ventricle; SFO, subfornical organ. Scale bar=50 μm.



**Figure 3.** Adenoviral-mediated SOD expression is robust and localized to the SFO. Representative confocal images of coronal sections of the brain showing human CuZnSOD (A) and human ECSOD (B) immunoreactivity in the SFO of mice 3 weeks after ICV injection of AdCuZnSOD or AdECSOD, respectively. C and D, No staining of human CuZnSOD or human ECSOD, respectively, in mice injected with ICV with vehicle. Scale bar=50  $\mu\text{m}$ .

whereas DHE fluorescence was unaffected in mice that had received AdECSOD in the brain. These data demonstrate that the development of Ang II–dependent hypertension is paralleled by robust increases in intracellular  $\text{O}_2^{\cdot-}$  levels in the SFO.

### Adenoviral-Mediated Transgene Expression Is Robust and Localized to the SFO

To verify adenoviral-mediated transgene expression and to determine distribution in the CNS, immunohistochemical analyses were performed on brains of mice from the ICV treatment groups (vehicle,  $n=3$ ; AdCuZnSOD,  $n=3$ ; AdECSOD,  $n=3$ ). In ICV AdCuZnSOD-injected or AdECSOD-injected mice, human CuZnSOD and ECSOD staining was detected in the ependymal layer of the lateral and third ventricles (data not shown). In addition, very high levels of CuZnSOD and ECSOD protein expression were observed in the SFO (Figure 3A and 3B, respectively). These findings are consistent with our previous studies showing localization of another SOD isozyme, MnSOD, specifically to the SFO in ICV AdMnSOD-injected animals.<sup>22</sup> It should be noted that brains from control mice injected with vehicle ICV showed no CuZnSOD or ECSOD immunoreactivity (Figure 3C and 3D, respectively), demonstrating the specificity of the human antibodies used to detect human CuZnSOD or ECSOD transgene expression. In addition, transgene expression was not detected outside of the brain in any other peripheral organs (data not shown).

### Discussion

Chronic infusion of subpressor doses of Ang II in experimental animals, the so-called slow-pressor Ang II model, induces a gradual increase in blood pressure that mimics human essential hypertension.<sup>1,24,25</sup> Oxidative stress in the vasculature and kidney has begun to emerge as an important underlying mechanism in this and other Ang II infusion models,<sup>6,33</sup> although the role of ROS in alternative sites known to be pivotal in Ang II–mediated regulation of blood pressure has not been investigated. We report here for the first time that redox mechanisms in the CNS play a key role in the hypertension caused by peripheral Ang II infusion. Our data demonstrate that infusion of Ang II ( $600 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) over a 2-week period in mice causes a gradually developing

hypertension that is correlated with marked elevations in  $\text{O}_2^{\cdot-}$  production specifically in the SFO, a brain region lying outside the blood–brain barrier and known to be a primary sensor for blood-borne Ang II.<sup>10</sup> Gene transfer of cytoplasmically targeted SOD selectively to this site prevented the development of hypertension and the increased  $\text{O}_2^{\cdot-}$  production, whereas adenoviral-mediated delivery of ECSOD to this region was without effect. These data support the notion that intracellular, but not extracellular,  $\text{O}_2^{\cdot-}$  in the SFO is critical in the signaling mechanisms underlying the development of Ang II–induced hypertension.

A central neurogenic mechanism has long been implicated in the hypertensive effects of Ang II, especially that elicited by slow-pressor doses. In the original studies 40 years ago showing that prolonged infusion of nonpressor amounts of Ang II elicited a delayed but profound hypertension, investigators suggested that Ang II acting in the brain to increase sympathetic nerve activity was a key mechanism.<sup>41,42</sup> More recent studies demonstrating that central sympathoinhibitors,<sup>43</sup> ganglionic blockade,<sup>44</sup> or sympathetic denervation<sup>45</sup> attenuate slow-pressor Ang II hypertension further support these early ideas. Subsequent studies showing that Ang II–dependent hypertension could be attenuated or reversed by ablation of CVOs<sup>14–17</sup> has led to the concept that blood-borne Ang II increases blood pressure through its activation of these unique brain regions. More recent work by Hendel and Collister suggests that SFO ablation inhibits the increase in blood pressure after Ang II infusion in rats.<sup>19</sup> The current results not only lend additional support to this idea but also further suggest that ROS function as important signaling molecules in these sites.

Our findings that Ang II–elicited increases in  $\text{O}_2^{\cdot-}$  production were restricted to the SFO and that scavenging of  $\text{O}_2^{\cdot-}$  selectively in this region blunted Ang II hypertension strongly implicate this region in central redox regulation of blood pressure. However, this does not rule out the possibility that other sites are involved, particularly other CVOs, which could be accessed by ICV administration of the viruses. In light of earlier evidence that the AP and OVLT are important in Ang II–dependent hypertension,<sup>15–17</sup> we paid particular attention to these regions in the DHE and SOD immunoreactivity analyses. Although SOD staining was detected in the periventricular ependymal layer and occasionally in the OVLT, the primary site of consistent and intense SOD immunoreactivity was the SFO. We failed to detect either CuZnSOD or ECSOD in the AP of any of the animals examined. Perhaps more importantly, the Ang II–stimulated increase in DHE staining was only observed in the SFO, despite careful analysis of these other regions. Although these findings do suggest that the SFO figure prominently in this central redox mechanism, it must be recognized that Ang II–induced increases in  $\text{O}_2^{\cdot-}$  and/or adenoviral-mediated expression of SOD in these other CVOs, or in other sites of the brain, could be beyond the limits of detection for these assays. Furthermore, it is possible that SOD-mediated alteration in the redox state of the SFO could indirectly impact downstream networks that receive inputs from this region.

There have been a number of recent reports suggesting that redox mechanisms in peripheral tissues may be involved in hypertension caused by Ang II infusion. Chronic infusion of

low doses of Ang II in rats has been correlated with increased markers of oxidant stress in the plasma<sup>46</sup> and kidney,<sup>33,47</sup> and peripheral administration of antioxidants such as vitamin E or the membrane-permeable SOD mimetic tempol have been shown to ameliorate the hypertension in these models.<sup>33,47</sup> However, given that both of these treatments have the potential to impact the CNS, not only via interactions with CVOs but also by crossing the blood-brain barrier, one cannot rule out the possibility that at least part of the mechanism by which these or other antioxidants attenuate Ang II-induced hypertension is related to abrogation of central redox signaling. In fact, liposome-encapsulated or heparin-binding SOD, the first reagents used to suggest a role for superoxide in Ang II-induced hypertension,<sup>5,48</sup> could very well exert their effects via CVOs or other central sites. Indeed, the difficulty in targeting such reagents tissue-selectively is what prompted our use of SOD gene transfer to dissect out the role of ROS in the CNS. Our data showing that the CuZnSOD transgene was not found outside the CNS and was highly localized to the SFO provide strong evidence that the antihypertensive effects of this reagent were caused by O<sub>2</sub><sup>-</sup> scavenging in this site. Further evidence in support of this comes from our finding that Ang II-induced cardiac hypertrophy, a response known to be mediated by direct actions of Ang II in the heart,<sup>37</sup> was completely intact in AdCuZnSOD-treated animals.

Another recent area of focus in studies of oxidant stress and Ang II hypertension is on ECSOD and vascular function. In most tissues, the amount of ECSOD is very small, ≈1% to 5% of total SOD amounts. However, in the vasculature, nearly 70% of total SOD activity comprises ECSOD.<sup>49</sup> It is postulated that by scavenging O<sub>2</sub><sup>-</sup> in the vascular extracellular matrix, ECSOD plays an important role in protecting nitric oxide as it diffuses from the endothelium to the vascular smooth muscle.<sup>50</sup> Fukui et al demonstrated that Ang II infusion leads to an increase in ECSOD activity in the vasculature,<sup>51</sup> and hypertension caused by Ang II infusion is exacerbated in mice lacking ECSOD.<sup>52</sup> Interestingly, a recent study by Oury et al demonstrated that although ECSOD activity is low in whole brain homogenates, it is expressed at high levels in brain regions lacking a blood-brain barrier.<sup>23</sup> This is what prompted us to investigate the role of ECSOD in Ang II-induced hypertension in this study. However, the results suggest that Ang II infusion caused an increase in intracellular, not extracellular, O<sub>2</sub><sup>-</sup> in the SFO. Consistent with this, targeting ECSOD to this site did not protect against hypertension caused by Ang II. Although this does not rule out the possibility that O<sub>2</sub><sup>-</sup> in the extracellular matrix is important in other central cardiovascular sites, it suggests that the high levels of ECSOD observed in the CVOs play a role other than that related to signaling mechanisms involved in Ang II-dependent hypertension. An alternative hypothesis is that the high levels of endogenous ECSOD in the CVOs prevent a further functional effect of overexpressing ECSOD in these regions.

In summary, our results demonstrate that increased intracellular O<sub>2</sub><sup>-</sup> production in the SFO plays a crucial role in Ang II-dependent hypertension. Although understanding the mechanisms of ROS-mediated activation of neurons in this

region and subsequent alterations in central cardiovascular outputs will require further investigation, these findings suggest new potential targets of therapy for Ang II-dependent hypertension and other diseases characterized by neurocardiovascular dysregulation.

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