Superoxide Mediates the Actions of Angiotensin II in the Central Nervous System

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Abstract—Angiotensin II (Ang II) has profound effects in the central nervous system (CNS), including promotion of thirst, regulation of vasopressin secretion, and modulation of sympathetic outflow. Despite its importance in cardiovascular and volume homeostasis, angiotensinergic mechanisms are incompletely understood in the CNS. Recently, a novel signaling mechanism for Ang II involving reactive oxygen species (ROS) has been identified in a variety of peripheral tissues, but the involvement of ROS as second messengers in Ang II–mediated signaling in the CNS has not been reported. The hypothesis that superoxide is a key mediator of the actions of Ang II in the CNS was tested in mice using adenoviral vector–mediated expression of superoxide dismutase (AdSOD). Changes in blood pressure, heart rate, and drinking elicited by injection of Ang II in the CNS were abolished by prior treatment with AdSOD in the brain, whereas the cardiovascular responses to carbachol, another central vasopressor agent, were unaffected. In addition, Ang II stimulated superoxide generation in primary CNS cell cultures, and this was prevented by the Ang II receptor (Ang II type 1 subtype) antagonist losartan or AdSOD. These results identify a novel signaling mechanism mediating the actions of Ang II in the CNS. Dysregulation of this signaling cascade may be important in hypertension and heart failure triggered by Ang II acting in the CNS. (Circ Res. 2002;91:1038-1045.)

Key Words: blood pressure ■ thirst ■ reactive oxygen species ■ renin-angiotensin system ■ brain

Angiotensin II (Ang II) is a primitive peptide to which the survival of most species is tightly linked. Highly conserved across evolution, it participates in myriad functions to maintain homeostasis of the organism. Cardiorenal, neural, endocrine, and behavioral activities are all regulated by Ang II. Key aspects of Ang II–mediated effects on hemodynamic function and fluid balance occur in the central nervous system (CNS). Through stimulation of the Ang II type 1 (AT₁) receptor subtype, Ang II has profound actions in the CNS to promote vasopressin secretion, thirst and salt appetite, sympathetic outflow, and modulation of a variety of cardiovascular reflexes, including the baroreflex. Dysregulation of brain angiotensinergic systems is implicated in a number of cardiovascular diseases, including hypertension and heart failure.

Despite its importance in central cardiovascular and volume homeostasis, the precise pathways and signaling mechanisms used by Ang II in the brain are incompletely understood. Recently, a novel signaling mechanism for Ang II involving superoxide and other reactive oxygen species (ROS) has been identified in peripheral tissues. Ang II stimulates the production of ROS and activates molecules associated with redox regulation in the vasculature, heart, and kidney. In these peripheral tissues, ROS have come to be recognized as important intracellular second messengers in a number of Ang II–regulated cellular processes, including growth, contraction, and inflammation. Overproduction of ROS in the vasculature is implicated in the pathogenesis of hypertension caused by systemic Ang II infusion.

In the brain, ROS are best known for their role in the pathogenesis of primary neurodegenerative diseases, such as amyotrophic lateral sclerosis and Alzheimer’s disease. It has been postulated that neuronal death in these diseases may be mediated by oxidative and/or nitrosative stress caused by the aberrant metabolism of superoxide via superoxide dismutase (SOD). Although neurons in the brain appear to contain predominantly mitochondrial SOD (MnSOD), both MnSOD and cytoplasmic SOD (CuZnSOD) are thought to play key antioxidant and neuroprotective roles in the brain.

Despite their well-known role in neurodegeneration, very little is understood about ROS as second messengers in normal neural processes in the brain, and even less is known about the role of redox mechanisms in CNS-mediated regulation of cardiovascular function. Given the evidence for superoxide-generating and -scavenging systems throughout...
the brain\textsuperscript{13,16} and the importance of ROS signaling in a wide range of Ang II–regulated cellular processes, we sought to establish a causal link between Ang II–mediated signaling in the CNS, alterations in blood pressure/fluid regulation, and superoxide radicals. Using integrative physiological analyses, selective genetic tools, and primary cell culture, we suggest that Ang II–mediated signaling in the CNS impacts systemic cardiovascular function via superoxide-dependent mechanisms.

Materials and Methods

Animals

Adult C57BL/6 mice (18 to 24 g, 2 to 6 months old; Harlan, Indianapolis, Ind) were used for physiological and immunohistochemical studies. Mice were fed standard chow (Harlan) and water ad libitum. Preweanling (14-day) Sprague-Dawley rat pups were used for primary cell cultures. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Adenoviral Vectors

Construction of recombinant E1-deleted adenoviral vectors encoding human mitochondrial superoxide dismutase (AdMnSOD), human cytoplasmic superoxide dismutase (AdCuZnSOD), or bacterial \(\beta\)-galactosidase genes (AdLacZ) (kind gift of J.F. Engelhardt, University of Iowa, Iowa City) has been detailed previously.\textsuperscript{17,18} Purified high-titer stocks of each of the viral vectors were generated by double CsCl banding and gel-filtration as described.\textsuperscript{17} All viral titers were evaluated by plaque assays on 293 cells.\textsuperscript{17} Titer-matched stocks of AdMnSOD, AdCuZnSOD, and AdLacZ (\(2\times10^8\) particles in PBS) were used for CNS injections. We have shown previously that at this concentration, adenoviruses do not cause significant in PBS) were used for CNS injections. We have shown previously that adenoviruses do not cause a 50% reduction in product formation using purified CuZn-SOD, or bacterial \(\beta\)-galactosidase genes (AdLacZ) (kind gift of J.F. Engelhardt, University of Iowa, Iowa City) has been detailed previously.\textsuperscript{17,18} Purified high-titer stocks of each of the viral vectors were generated by double CsCl banding and gel-filtration as described.\textsuperscript{17} All viral titers were evaluated by plaque assays on 293 cells.\textsuperscript{17} Titer-matched stocks of AdMnSOD, AdCuZnSOD, and AdLacZ (\(2\times10^8\) particles in PBS) were used for CNS injections. We have shown previously that at this concentration, adenoviruses do not cause significant inflammatory responses in the mouse brain.\textsuperscript{19}

Surgical Procedures and Experimental Protocol for Physiological Studies

Mice were instrumented with intracerebroventricular cannulas and left carotid arterial catheters for central administration of drugs or viruses and for direct measurement of mean arterial pressure (MAP) and heart rate (HR), respectively, as described previously.\textsuperscript{20} At the end of surgery, mice were injected intracerebroventricularly with either saline, AdLacZ, or AdMnSOD (500 nL) before being placed on a heating pad for recovery. Three days later, physiological experiments were performed in conscious, freely moving mice in the home cage. This amount of time has been shown to be sufficient for both recovery from surgery\textsuperscript{20} and adenovirus-mediated expression of transgenes in the brain.\textsuperscript{19} In one set of experiments, MAP and HR were recorded continuously before and up to 30 minutes after intracerebroventricular administration of Ang II (200 ng, 200 nL) in saline-treated (n=5), AdLacZ-treated (n=5), or AdMnSOD-treated (n=7) mice. We have established previously that this dose of Ang II in the brain produces robust pressor and bradycardic responses in mice.\textsuperscript{20,21} In separate saline-treated (n=4), AdLacZ-treated (n=4), and AdMnSOD-treated (n=4) mice (without arterial catheters), the effects of intracerebroventricular Ang II (200 ng, 200 nL) on drinking behavior was recorded as described in detail previously.\textsuperscript{21} The total amount of time spent drinking was recorded for up to 30 minutes after Ang II injection.

In a separate series of studies, these cardiovascular and drinking experiments were repeated in mice that were pretreated intracerebroventricularly with AdCuZnSOD 3 days earlier. The surgical procedures and experimental protocols for recording central Ang II–induced cardiovascular effects (saline, n=4; AdLacZ, n=4; and AdCuZnSOD, n=5) and dipsogenic effects (saline, n=4; AdLacZ, n=3; and AdCuZnSOD, n=4) in these animals were identical to those described above. For both series of experiments, the selectivity of AdMnSOD and AdCuZnSOD on Ang II–mediated responses was established by determining the cardiovascular effects of another central pressor agent, the cholinergic agonist carbachol\textsuperscript{22} (50 ng, 200 nL ICV) in separate mice (saline, n=7; AdLacZ, n=4; AdMnSOD, n=4; and AdCuZnSOD, n=3).

Immunohistochemistry

Three or 4 days after the physiological experiments, mice were anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB). Brains were removed, postfixed for 2 hours, and then transferred to 20% sucrose in PB overnight. Cryostat sections (30 \(\mu\)m, coronal) were prepared and free-floated in PB. Sections were incubated in 10% normal horse serum (NHS, 1 hour at room temperature [RT]), followed by 30 minutes with blocking salts (0.5% blocking powder, 0.1 mol/L Tris-HCl, and 0.15 mol/L NaCl, pH 7.6, NEN Life Science Products) before incubation with a rabbit polyclonal antibody targeted against the carboxy terminus of the rat AT\(_1\) receptor (generous gift from M. McKinley, Howard Florey Institute, Victoria, Australia) diluted in PB (1:10 000) containing 2% NHS and 0.3% Triton for 48 hours at RT. AT\(_1\) immunoreactivity was detected by the tyramide signal amplification technique, described in detail previously.\textsuperscript{23} After the amplification technique, the human MnSOD antibody (sheep anti-human IgG, The Binding Site Limited), diluted 1:500 in 2% NHS and 0.3% Triton, was applied to the free-floating sections for 24 hours at 4\(^\circ\)C in the dark. Sections were washed with PB, incubated with the secondary antibody (donkey anti-sheep FITC-conjugated, 1:200) for 2 hours at RT in the dark, and washed before being mounted and coverslipped.

SOD Activity Assay

MnSOD activity was measured in brain tissue dissected from a subset of saline-injected (n=3), AdLacZ-injected (n=3), or AdMnSOD-injected (n=3) mice (see above) as described previously.\textsuperscript{24} Briefly, brains were rapidly removed, flash-frozen in dry ice, and sectioned to the anterior border of the lateral ventricles. Tissue surrounding the ventricles, including the lamina terminals and subfornical organ (SFO), was dissected using a micropunch (1.24-mm diameter) and homogenized on ice with a polytetrafluoroethylene (Teflon) pestle Dounce homogenizer in 0.05 mol/L PB (pH 7.8) with 1 mmol/L diethylenetriamine-pentacetic acid. Tissue protein content was measured by the method of Lowry et al,\textsuperscript{25} and increasing quantities (0 to 500 \(\mu\)g) were placed in a solution containing 1 mmol/L diethylenetriamine-pentacetic acid, 1 U catalase, 5.6\(\times10^{-3}\) mol/L nitro blue tetrazolium (NBT), 10\(^{-4}\) mol/L xanthine, 5 mmol/L NaCN, and 50 \(\mu\)mol/L bathocuproinedisulfonic acid. Xanthine oxidase was added to initiate superoxide-mediated NBT reduction to blue formazan, and the rate of formation of colored product was monitored spectrophotometrically at 560 nm.\textsuperscript{26} The rate of NBT reduction in the absence of tissue was used as the reference rate. Data are expressed in units of SOD activity per milligram protein, where 1 U is defined as the amount of enzyme needed to cause a 50% reduction in product formation using purified CuZn-SOD in the absence of CN\(^{–}\) as the standard.\textsuperscript{24}

Primary Cell Culture and Fluorogenic Monitoring of Superoxide Production

The lamina terminalis of Sprague-Dawley rat pups (14 days old, 8 to 10 pups per culture) was dissected with the aid of a dissecting scope and cultured as described in detail previously.\textsuperscript{26} Cultured cells were plated on coated (poly-L-lysine, Sigma Chemical Co) chamber slides containing prewarmed serum-containing media and allowed to recover overnight. Cultures were then washed and grown in serum-free media for 24 hours. Primary cultures were treated for 45 minutes with either vehicle, Ang II alone (100 nmol/L), or Ang II (100 nmol/L) subsequent to pretreatment (30 minutes) with the AT\(_1\) receptor antagonist losartan (1 \(\mu\)mol/L). This was followed by loading with the oxidant-sensitive fluorogenic probe dihydroethidine (DHE, 2 \(\mu\)mol/L) for 30 minutes. In a separate study, cultures were infected with AdMnSOD or AdLacZ for 24 hours before vehicle or Ang II stimulation and DHE loading. Each condition was run in triplicate within experiments, and each set of experiments was
performed three separate times. Cultures were washed with PBS, coverslipped, and examined by confocal microscopy (Zeiss LSM510). Eight-bit gray-scale images (in a fluorescent configuration) were captured digitally using constant pinhole, detector gain, and laser power settings. The percentage of DHE-positive cells was determined by counting the number of cells in 4 or 5 randomly selected visual fields (per condition) showing unequivocally bright red fluorescence, defined as <170 gray-scale units, relative to all cells counted. All nonpositive cells exhibited very low-level background fluorescence, ranging from ~200 to 230 gray-scale units.

Statistical Analysis
All data are expressed as mean±SEM and were analyzed by the Student t test or ANOVA (after Bartlett’s test of homogeneity of variance), followed by the Newman-Keuls correction for multiple comparisons. Statistical analyses were performed using Prism (GraphPad Software, Inc).

Results
Effects of AdMnSOD on Cardiovascular and Dipsogenic Responses
Given that MnSOD is thought to be the predominant form of SOD in brain neurons,13 we focused our initial studies on genetic modulation of this isozyme. Three days after the administration of AdMnSOD in the brain, the acute effects of intracerebroventricular Ang II on MAP and HR were determined in conscious mice. Brain Ang II is a potent regulator of arterial blood pressure, and when administered acutely intracerebroventricularly, it elicits a transient systemic vaspressor and bradycardic response in a number of species, including mice.21 As seen in representative recordings in Figure 1A, saline- and AdLacZ-treated mice showed the classic intracerebroventricular Ang II–induced pressor and bradycardic response4: increases in MAP and decreases in HR occurred rapidly and were relatively short-lived. In contrast, this classic Ang II–elicited response was virtually abolished in AdMnSOD-infected mice (Figure 1A). The peak effects of Ang II are summarized in Figure 1B, which demonstrates that modulation of the redox state of the brain by increasing superoxide scavenging prevented the acute cardiovascular effects of central Ang II. The adenovirus itself did not affect Ang II–elicited responses because the changes in blood pressure and HR were not different in AdLacZ- and saline-treated mice. It should also be noted that basal MAP and HR were not affected by the viruses (for MAP, 122±3 mm Hg for saline, 120±3 mm Hg for AdLacZ, and 121±3 mm Hg for AdMnSOD [P>0.05]; for HR, 652±44 bpm for saline, 635±70 bpm for AdLacZ, and 690±27 bpm for AdMnSOD [P>0.05]).

Along with its role in blood pressure regulation, the brain angiotensinergic system has evolved to meet the fluid homeostatic needs of the organism. Ang II promotes thirst in states of volume depletion, and when given acutely in the brain, it has potent dipsogenic actions.2 To determine whether superoxide is involved in this classic Ang II response, drinking behavior was recorded before, during, and after intracerebroventricular administration of Ang II in separate groups of saline-, AdLacZ-, and AdMnSOD-treated mice. Similar to the cardiovascular responses, overexpression of MnSOD in the brain attenuated Ang II–stimulated drinking behavior (Figure 1C). Measured as the total amount of time spent drinking, the
robust dipsogenesis produced by intracerebroventricular Ang II in saline- and AdLacZ-treated mice was absent in the AdMnSOD-treated mice. It should be noted that the adenoviruses did not alter basal water intakes and that spontaneous water drinking in mice before Ang II administration and in non-Ang II–infused mice did not occur during the experimental period (data not shown).

MnSOD Transgene Expression and Activity in the Brain
To confirm MnSOD transgene expression and activity in the brain and to determine potential sites where intracerebroventricularly administered AdMnSOD could be acting to block Ang II–mediated responses, immunohistochemical analyses and analyses of enzyme activity of brains from saline-, AdLacZ-, and AdMnSOD-treated mice were performed. Human MnSOD was widespread to periventricular tissue of the third and fourth ventricles in AdMnSOD-infected mice, suggesting that the virus was transported throughout the ventricular system and that the tissue surrounding the ventricles was transduced. One structure was particularly prominent and intense regarding MnSOD immunofluorescence staining—the SFO. Bulging into the lumen of the third ventricle, the SFO is a primary sensor for blood-borne and ventricular Ang II and is dense with AT1 receptors. To ventricle, the SFO is a primary sensor for blood-borne and saline- and AdLacZ-treated mice, AT1 staining was similar in human SOD immunoreactivity was detected in brains of the outer annular and lateral horn portions of the SFO (Figure 2, bottom left panel). Similarly, intense MnSOD staining was observed in SFO of AdMnSOD-infected mice. It was particularly prominent in the lateral horns (Figure 2A, bottom middle panel) and outer perimeter of the organ (top middle panel), whereas immunoreactivity in the core region of the SFO was more sparse (top middle panel). Thus, coexpression of the MnSOD transgene with AT1 receptors was observed in the outer annular and lateral horn portions of the SFO (Figure 2A, top and bottom right panels). It should be noted that no human SOD immunoreactivity was detected in brains of saline- and AdLacZ-treated mice, AT1 staining was similar in each of the three groups, and colocalization of MnSOD and AT1 was not detected in any other periventricular sites besides SFO (data not shown).

In addition to confirming transgene expression and distribution, MnSOD activity was measured in periventricular brain tissue by the method of Spitz and Oberley. AdMnSOD-treated mice exhibited an ~3-fold increase in MnSOD activity compared with saline- and AdLacZ-treated mice (Figure 2B). The adeno virus itself did not alter endogenous MnSOD activity, inasmuch as basal levels were similar in saline- and AdLacZ-treated mice (Figure 2B).

Fluorogenic Detection of Superoxide Generation
Although our physiological studies implicate superoxide radicals in central Ang II actions, we wanted to provide direct evidence that Ang II increased intracellular superoxide production in CNS cells accessed by intracerebroventricular Ang II. Fluorogenic confocal analyses using DHE were performed in primary cultures derived from the lamina terminalis, a region that encompasses tissue surrounding the third ventricle and includes the SFO and a number of other AT1-rich sites. Because of the small size of this region and relatively limited number of cells that can be isolated compared with other areas, we used rat rather than mouse brain for these studies. Importantly, the culture is rich with cells critical in intracerebroventricular Ang II–mediated effects. Commonly used for monitoring intracellular superoxide levels, the fluorogenic probe DHE is oxidized to fluorescent ethidium by superoxide radicals; however, it should be noted that some fluorogenic probes can be oxidized by other reactive molecules, including NO, peroxynitrite, H2O2, and hydroxyl radical. Compared with cells that were treated with vehicle control, cells in primary cultures incubated with Ang II showed marked increases in fluorescence (Figures 3A and 3B). The response was limited to cells with typical neuronal morphology, ie, phase-bright domed somata ranging in size from 10 to 30 μm, and occurred only in a subset of neurons. Of 147 neurons counted over two series of experiments (each run in triplicate), 40.1±3.6% were DHE positive when they were incubated with Ang II, whereas no vehicle-treated neurons...
(137 neurons counted) exhibited fluorescence above background levels. Pretreatment of the cultures with the selective AT1 receptor antagonist losartan abolished Ang II–stimulated increases in DHE fluorescence (Figure 3C) (3.5 ± 0.7% DHE positive, 93 neurons counted; P < 0.001 versus Ang II alone), implicating the AT1 receptor in Ang II–stimulated superoxide production in lamina terminalis neurons. Infection with AdMnSOD 24 hours before Ang II stimulation also prevented the increase in fluorescence (2.1 ± 0.5% DHE positive, 73 neurons counted; P < 0.001 versus no infection), corroborating the fidelity of the assay. AdLacZ-treated cultures exhibited Ang II–stimulated changes in fluorescence (35.7 ± 6.2% DHE positive, 64 neurons counted) that were similar to those in noninfected cells (P > 0.05).

Effects of AdCuZnSOD on Cardiovascular and Dipsogenic Responses

To extend the studies of MnSOD, physiological studies similar to those described above were performed with the adenoviral vector encoding the cytoplasmic form of the SOD enzyme (AdCuZnSOD). Interestingly, AdCuZnSOD was just as effective as AdMnSOD at inhibiting the pressor, bradycardic (Figure 4A), and drinking responses (Figure 4B) elicited by CNS infusion of Ang II (Figure 1). Also similar to the AdMnSOD study, basal MAP and HR were not affected by the vectors (for MAP, 111 ± 9 mm Hg for saline, 112 ± 11 mm Hg for AdLacZ, and 117 ± 5 mm Hg for AdCuZnSOD [P > 0.05]; for HR, 628 ± 43 bpm for saline, 554 ± 81 bpm for AdLacZ, and 657 ± 71 bpm for AdCuZnSOD [P > 0.05]), nor were basal water intakes (data not shown).

Selectivity of AdSOD for Ang II–Mediated Cardiovascular Responses

Finally, to verify that overexpressing either SOD isozyme did not have a generalized inhibitory effect on CNS-elicited cardiovascular responses, the effects of another central pressor agent, the muscarinic agonist carbachol, were examined in separate groups of saline-, AdLacZ-, AdMnSOD-, and AdCuZnSOD-treated mice. It should be noted that the dose of carbachol used in these studies (50 ng, 200 nL) was selected because it produces a similar magnitude pressor response as the dose of Ang II used. Furthermore, this dose of carbachol is near the low end of the dose–pressor-response curve.

Carbachol caused equivalent increases in MAP
(Δ16±2 mm Hg for saline, Δ18±2 mm Hg for AdLacZ, Δ19±2 mm Hg for AdMnSOD, and Δ12±6 mm Hg for AdCuZnSOD [P>0.05]) and decreases in HR (Δ−65±11 bpm for saline, Δ−81±14 bpm for AdLacZ, Δ−93±17 bpm for AdMnSOD, and Δ−59±13 bpm for AdCuZnSOD [P>0.05]) in all groups of mice, suggesting that the systems necessary for CNS-derived cardiovascular responses were intact in AdSOD-transduced mice.

Discussion

Ang II has profound blood pressure and volume-regulatory actions in the CNS, and results in the present study identify a novel mechanism by which this occurs. We demonstrated that genetic modulation of SOD activity in brain tissue virtually abolished the pressor, bradycardic, and dipsogenic actions of intracerebroventricularly administered Ang II. We confirmed that this was not due to an indiscriminate effect of SOD overexpression on central cardiovascular mechanisms, inasmuch as responses to the pressor agent carbachol were completely intact in AdMnSOD- and AdCuZnSOD-infected mice. The results showing that Ad II stimulates AT$_1$-dependent DHE fluorescence in neuron-like cells of the lamina terminalis (tissue directly accessed by intracerebroventricular injections) lends further support to the notion that superoxide radicals are critical determinants of the central actions of Ang II.

In the present study, we used two different isoforms of SOD to uncover the role of superoxide radicals in central Ang II–mediated signaling. Initially, these studies focused on mitochondria-targeted modulation of superoxide levels, because it had been reported that CNS neurons preferentially express MnSOD and because Ang II–mediated effects in the brain are thought to be neurally mediated. Subsequently, we also examined the effects of cytoplasmic SOD because of the importance of extramitochondrial superoxide-generating systems, such as NAD(P)H oxidase, in peripheral Ang II–stimulated responses. The findings that AdMnSOD and AdCuZnSOD were equally effective in inhibiting central Ang II–induced cardiovascular and dipsogenic responses suggests that superoxide radicals derived from both mitochondrial and extramitochondrial sources may be important in modulating these physiological responses and/or that there is cross talk between mitochondrial and cytoplasmic compartments. Recent evidence suggesting important interplay between free radicals derived from different subcellular compartments, along with reports that superoxide radicals can exit mitochondria through an anion channel and enter the cytoplasm, support the latter hypothesis. Alternatively, we cannot rule out the possibility that both adenovirus-expressed enzymes were targeted to the same subcellular compartments when injected in the CNS. Although previous studies in other cell types have demonstrated that these particular adenoviral vectors target the SOD enzymes to the appropriate respective compartments, further studies are required to demonstrate proper subcellular localization of AdMnSOD and AdCuZnSOD in the CNS in vivo. In any event, our findings suggest a significant role for superoxide radicals in Ang II–mediated actions in the brain.

Immunohistochemical data showing coexpression of the MnSOD transgene with AT$_1$ receptors in the SFO suggest a possible scenario for how AdSOD may be exerting its potent inhibitory effect on the vasopressor and bradycardic and dipsogenic actions of intracerebroventricular Ang II. The SFO is dense with AT$_1$ receptors and is known to be pivotal in intracerebroventricular Ang II–elicited blood pressure and dipsogenic actions. The colocalization of human MnSOD with AT$_1$ receptors in this important site, taken together with the finding that Ang II–stimulated ROS production in lamina terminalis cell cultures is AT$_1$-dependent, suggests that the SFO figures prominently in the AdSOD-mediated loss of central Ang II effects. This is not to suggest that the SFO is the only site involved. It is possible that through an effect on AT$_1$-positive cells in the SFO, AdSOD indirectly impacts downstream networks that receive inputs from the SFO. For example, angiotensinergic pathways projecting from the SFO to hypothalamic nuclei (key sites in the cardiovascular and dipsogenic effects of central Ang II$^{39}$) could potentially be affected by modulation of the upstream redox state. In addition, because other periventricular regions are accessed by intraventricular administration of AdSOD, this raises the possibility that sites other than the SFO are involved in Ang II–stimulated superoxide production. Indeed, we observed SOD immunoreactivity in tissue surrounding the ventricles throughout the brain, although none was as intense or highly localized as that observed in the SFO. Furthermore, the SFO was the only site in which we detected colocalization of the MnSOD transgene with AT$_1$ receptors. However, we cannot rule out the possibility that AdSOD transduced AT$_1$-containing cells but at levels beyond the limits of detection by our immunohistochemical protocol. The relative role of the SFO versus other CNS sites in ROS-mediated modulation of blood pressure and drinking responses is the subject of ongoing investigations.

The pattern of double immunofluorescence observed in SFO is interesting in light of studies showing that this structure is not homogeneous regarding morphology, cytology, or topographical organization of its neural connections. Although very little is known about the functional significance of the various SFO “zones,” the data showing colocalization of AT$_1$ receptors with the MnSOD transgene in the outer perimeter and lateral horns are intriguing because the evidence suggests that these circumscribed regions may be critical in central Ang II regulation of cardiovascular function. Angiotensinergic fiber staining is localized to the annular region of the SFO, and cells projecting to important cardiovascular networks in the hypothalamic nuclei are also concentrated in the outer parts of the nucleus. This raises the possibility that genetic alteration of the redox state in these important outer zones of the SFO interferes with key cardiovascular signaling.

The heterogeneity of angiotensinergic systems in the SFO is mirrored in other lamina terminalis sites and may explain the finding that not all lamina terminalis neurons showed increases in DHE fluorescence on stimulation with Ang II. Although the culture is rich with cells from periventricular Ang II–sensitive sites, it is heterogeneous regarding AT$_1$-positive cells. We postulate that neuron-like cells that did not
exhibit the marked increase in fluorescence observed in other cells were Ang II insensitive.

Finally, the intracellular mechanisms by which ROS links the activation of central Ang II receptors with the stimulation of neural circuitry involved in blood pressure and dipsogenic responses remain to be determined. It is known that similar to peripheral tissues, activation of G-protein-coupled AT1 receptors in cultured CNS neurons leads to phosphoinositol hydrolysis, Ca2+ mobilization, and diacylglycerol-mediated increases in protein kinase C activity.4,37,38 Interestingly, ROS have been shown to have links to these signaling events in a variety of cell types. The ability of protein kinase C to induce ROS formation is well established,29 as is the modulation of Ca2+ release by ROS.40 The recent identification of an association between the NAD(P)H complex and phosphoinositides41 further underscores the variety of potential mechanisms by which ROS may be involved in brain angiotensinergic signaling.

In summary, we have identified superoxide radicals as key mediators in the acute actions of Ang II on blood pressure and drinking behavior originating from the CNS. We hypothesize that ROS play a critical role in long-term regulation of cardiovascular and volume homeostasis and that dysregulation of central redox mechanisms is involved in the pathogenesis of hypertension and heart failure. We further speculate that oxygen radicals in the brain may be important new targets for therapeutic treatment of these diseases.

Acknowledgments

This study was funded by grants from the NIH (HL-14388, HL-63887, and HL-51469 to R.L.D. and CA-66081 to D.R.S.). M.C. Zimmerman is supported in part by an NIH-funded Institutional National Research Service Award (5T32 CA78586). Dr. Lartzigues and Dr. Sinnayah are funded by Postdoctoral Fellowships from the American Heart Association (Nos. 20572Z and 0225723Z, respectively). The authors would like to thank Dr. J.F. Engelhardt for generously providing recombinant adenoviruses and Drs. Engeldhart and L.W. Oberley for invaluable discussions.

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Circ Res. 2002;91:1038-1045; originally published online October 24, 2002;
doi: 10.1161/01.RES.0000043501.47934.FA

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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