In Vivo Bioluminescence Imaging Reveals Redox-Regulated Activator Protein-1 Activation in Paraventricular Nucleus of Mice With Renovascular Hypertension

Melissa A. Burmeister, Colin N. Young, Valdir A. Braga, Scott D. Butler, Ram V. Sharma, Robin L. Davisson

Abstract—Renovascular hypertension in mice is characterized by an elevation in hypothalamic angiotensin II levels. The paraventricular nucleus (PVN) is a major cardioregulatory site implicated in the neurogenic component of renovascular hypertension. Increased superoxide (O$_2^-$) production in the PVN is involved in angiotensin II–dependent neurocardiovascular diseases such as hypertension and heart failure. Here, we tested the hypothesis that excessive O$_2^-$ production and activation of the redox-regulated transcription factor activator protein-1 (AP-1) in PVN contributes to the development and maintenance of renovascular hypertension. Male C57BL/6 mice underwent implantation of radiotelemeters, bilateral PVN injections of an adenovirus (Ad) encoding superoxide dismutase (AdCuZnSOD) or a control gene (LacZ), and unilateral renal artery clipping (2-kidney, one-clip [2K1C]) or sham surgery. AP-1 activity was longitudinally monitored in vivo by bioluminescence imaging in 2K1C or sham mice that had undergone PVN-targeted microinjections of an Ad encoding the firefly luciferase (Luc) gene downstream of AP-1 response elements (AdAP-1Luc). 2K1C evoked chronic hypertension and an increase in O$_2^-$ production in the PVN. Viral delivery of CuZnSOD to the PVN not only prevented the elevation in O$_2^-$ but also abolished renovascular hypertension. 2K1C also caused a surge in AP-1 activity in the PVN, which paralleled the rise in O$_2^-$ production in this brain region, and this was prevented by treatment with AdCuZnSOD. Finally, Ad-mediated expression of a dominant-negative inhibitor of AP-1 activity in the PVN prevented 2K1C-evoked hypertension. These results implicate oxidant signaling and AP-1 transcriptional activity in the PVN as key mediators in the pathogenesis of renovascular hypertension. (Hypertension. 2011;57:00-00.) * Online Data Supplement

Key Words: 2-kidney, one-clip (2K1C) Goldblatt ■ CuZnSOD ■ superoxide ■ adenosine-virus-mediated gene transfer

The central nervous system (CNS) is strongly implicated in the pathogenesis of renovascular hypertension (RVH), a disease that is characterized by renal artery stenosis most commonly caused by atherosclerosis. The stenotic kidney responds to reduction in perfusion pressure by secreting renin from juxtaglomerular cells, which leads to an initial increase in circulating levels of angiotensin II (Ang II) and an elevation in blood pressure. However, as demonstrated in experimental animal models of RVH such as 2-kidney, one-clip (2K1C) Goldblatt hypertension, initial elevations in circulating Ang II levels subside, and renal homeostasis returns to normal in later phases. Nevertheless, pathological hypertension is maintained. Although several potential mechanisms may contribute (eg, altered renal reflexes), several laboratories, including ours, have identified the brain renin-angiotensin system (ie, Ang II that is produced and acts specifically in the brain) as a critical mediator of chronic hypertension in this model. Abundant evidence now suggests that a key mechanism in the neurogenic control of blood pressure is the production of nicotinamide adenine dinucleotide phosphate-oxidase (Nox)–derived reactive oxygen species (ROS) such as superoxide (O$_2^-$) in the CNS. Work from our laboratory and others has shown that Ang II, administered either directly into the CNS or systemically in subpressor doses over weeks (ie, "slow-pressor"), causes hypertension along with Ang II receptor 1– and Nox-mediated ROS production in central cardioregulatory nuclei. Both the ROS production and hypertension can be prevented by genetic or chemical ROS scavengers or Nox inhibitors administered in the brain, suggesting a causative role of brain ROS signaling in Ang II–dependent hypertension.

The paraventricular nucleus (PVN) of the hypothalamus is a key site of autonomic and neurohumoral regulation, and both Ang II and ROS are known to be potent mediators of these processes. Either local production of Ang II in the PVN...
or activation of angiotensinergic signaling in the PVN through blood-borne Ang II binding Ang II receptor type 1 (AT₁) in upstream circumventricular organs can cause alterations in sympathetic reflexes/activity, baroreflex sensitivity, or secretion of arginine vasopressin. Recently, oxidant signaling has been shown to be important in at least some of these Ang II–mediated effects in the PVN. For example, the cardiac sympathetic afferent reflex involves Ang II–mediated ROS formation in the PVN. In addition, both acute central and chronic systemic pressor effects of Ang II are linked to Nox-dependent ROS formation in the PVN. Recent work from our laboratory shows that Nox4-mediated O₂⁻ production in the PVN mediates sympathoexcitation and cardiac dysfunction in a mouse model of heart failure. Blood-borne Ang II signaling through the circumventricular subfornical organ is a likely upstream mediator of this response. In 2K1C RVH, aberrant Ang II signaling in the PVN has been implicated in the hypertension, increased sympathetic discharge, and attenuation of baroreflex function observed in this model. However, the underlying signaling mechanisms are largely unknown.

The molecular pathways by which ROS mediate long-term changes in central neural circuits involved in diseases such as hypertension and heart failure are yet to be elucidated. Emerging evidence suggests that redox-regulated transcription factor (TF) activation and ensuing alterations in gene profiles in these circuits may be involved. The redox-regulated activator protein 1 (AP-1) transcription complex may have particular relevance in this regard because 2 of its members, c-Fos and c-Jun, have been used extensively as marker proteins for activated CNS neurons in models of chronic cardiovascular disease. Furthermore, abundant evidence shows that Ang II increases AP-1 family member levels and activity in the PVN and other central cardiovascular regions in vivo. Considering the importance of ROS signaling in the PVN in several neurocardiovascular diseases, we hypothesized that oxidant signaling in the PVN plays an important role in the hypertension evoked by renal artery stenosis. Further, given recent evidence that redox-regulated TFs mediate Ang II–induced changes in CNS circuits involved in chronic diseases such as hypertension, we hypothesized that AP-1 activation in the PVN would be associated with redox signaling and elevations in blood pressure induced by renal artery clipping. We used the 2K1C model of RVH in mice and modulated O₂⁻ levels selectively in the PVN by targeted adenoviral (Ad) delivery of cytoplasmic superoxide dismutase (AdCuZnSOD) to this brain region. In addition, we used an Ad encoding a luciferase reporter downstream of AP-1 response elements (AdAP-1Luc) in conjunction with bioluminescence imaging (BLI) to longitudinally track AP-1 activity in the PVN in vivo during the development and progression of RVH. Our data show that hypertension in this model is associated with increased O₂⁻ production and AP-1 activation in the PVN. PVN-targeted overexpression of CuZnSOD abolished these increases in O₂⁻ and AP-1 activation. Further, expression of either CuZnSOD or a dominant-negative inhibitor of AP-1 activity in the PVN protected against RVH in this model.

### Methods

An expanded Methods section can be found in an online supplement available at http://hypertension.ahajournals.org.

### Animals

Adult C57BL/6 mice (8 to 12 weeks of age) were used. All procedures were approved by the institutional animal care and use committee at Cornell University.

### Adenoviral Vectors

Ad vectors encoding for human cytoplasmic superoxide dismutase (AdCuZnSOD) and bacterial β-galactosidase (AdLacZ) were obtained from the University of Iowa Gene Transfer Vector Core. Ad vectors encoding an AP-1–responsive luciferase reporter construct (AdAP-1Luc) and a dominant-negative c-Jun NH₂-terminal kinase 1 JNK1 (Ad-dnJNK1) were kindly provided by Dr John F. Engelhardt (University of Iowa, Iowa City, IA).

### Radiotelemeter Implantation and Gene Transfer to the PVN

Mice were instrumented with radiotelemeters as described, followed by stereotaxic bilateral PVN microinjections of AdLacZ, AdCuZnSOD, or Ad-dnJNK1. In vivo BLI studies, nontelemetered mice underwent bilateral PVN microinjection of a 1:1 mixture of AdAP-1Luc/AdLacZ or AdAP-1Luc/AdCuZnSOD as described.

### 2K1C RVH Model

One week after telemeter implantation and PVN microinjection, mice were anesthetized with isofluorane. The right renal artery was exposed through a midline abdominal incision, and a silver clip was placed over the vessel as described. A sham procedure served as control.

### In Vivo BLI

To longitudinally track AP-1 activation in the PVN in vivo, AdAP-1Luc was used in conjunction with in vivo BLI as described. Animals underwent sham or 2K1C surgery 2 weeks after AdAP-1Luc gene transfer, after which daily bioluminescence images were acquired with the IVIS200 daily until 28 days after surgery. At the end of the study, systemic endotoxin-induced activation of AP-1 was assessed by injection of lipopolysaccharide.

### ROS Measurements in Brain Sections

Dihydroethidium (DHE) staining was performed as described. On days 5 and 14 after surgery, brains were removed, cryosectioned, and treated with DHE. DHE fluorescence was visualized by confocal microscopy and quantified using ImageJ as described.

### CuZnSOD and Luciferase Immunohistochemistry

Mice were perfused, and brains were removed and cryosectioned. Free-floating sections were processed for immunofluorescence with antibodies to luciferase, CuZnSOD, neuronal nuclei, or glial fibrillary acidic protein as described. Sections were analyzed by confocal microscopy.

### Body and Kidney Weights

Mice were weighed and euthanized at the end of the telemetry experiments (day 28), and kidneys were removed and weighed to confirm atrophy of the clipped kidney and hypertrophy of the contralateral unclipped kidney.

### Statistical Analyses

Results are expressed as mean±SEM. All data were analyzed by 1-way analysis of variance (ANOVA) and Newman–Keuls post tests, except changes in mean arterial pressure (MAP) and heart rate (HR) from baseline, which were assessed by repeated-measures ANOVA with the Tukey multiple comparison post test. Significance was defined as P<0.05.
treatment groups, suggesting that $O_2^{-}$ scavenging in the PVN does not alter basal cardiovascular function. Together, these results suggest that $O_2^{-}$ signaling in the PVN is critical in mediating 2K1C hypertension, both during the early development and chronic phases.

**Renal Artery Clipping Induces CuZnSOD-Sensitive AP-1 Transcriptional Activity in the PVN**

Given that AP-1 activation is redox sensitive and may be important in mediating long-term changes in CNS cardiovascular networks, we hypothesized that RVH involves ROS-dependent AP-1 activation in the PVN. To test this, the AP-1 reporter virus Ad-AP-1Luc was microinjected selectively into PVN. This was then coupled with in vivo BLI for noninvasive real-time monitoring of AP-1 activation after renal artery occlusion. Figure 2 shows representative BLI images (Figure 2A) and a summary of AP-1–dependent photon flux over time (grouped in 2-day bins; Figure 2B). Baseline photon flux, averaged over 4 consecutive days before 2K1C or sham surgery, was at low levels and not different between the groups. This level of background flux is consistent with what we have shown previously using this technology. Starting 3 to 4 days after clipping, the AP-1–dependent luminescent signal began to rise significantly compared with sham animals (Figure 2A and 2B). AP-1–dependent photon flux in clipped mice remained significantly elevated compared with sham animals until 13 to 14 days postclip (2K1C + AdLacZ versus Sham + AdLacZ; $P < 0.05$). By 5 to 6 days after surgery, AP-1 activation in the PVN of the 2K1C group surged to ~5-fold greater levels compared with sham controls (Figure 2A and 2B). AP-1–dependent photon flux in clipped mice remained significantly elevated compared with sham animals until 13 to 14 days postclip (2K1C + AdLacZ versus Sham + AdLacZ; $P < 0.05$), albeit at lower levels compared with the surge at 5 to 6 days (Figure 2B). To examine the role of redox signaling in this 2K1C-induced AP-1 activation, photon flux was investigated in mice that had undergone PVN-selective injections of Ad-AP-1Luc and AdCuZnSOD. In these mice, the 2K1C-induced increases in AP-1 activity observed in AdLacZ-treated mice on days 3 through 14 were significantly attenuated (Figure 2B; 2K1C + AdCuZnSOD versus 2K1C + AdLacZ; $P < 0.05$).

After the 2-week time point and for the duration of the study, AP-1 activity was not significantly different between the groups. In addition, photon flux was not altered at any time point in sham animals compared with baseline. On the final day of the study (day 28; ~6 weeks after initial gene transfer), we verified that AP-1Luc had retained the functional capacity for activation in vivo using systemic challenge with lipopolysaccharide (4 μg/g IP). AP-1 is known to be activated in the PVN on systemic lipopolysaccharide administration. Lipopolysaccharide-induced profound increases in AP-1–dependent luminescence in both sham and 2K1C groups, confirming functional expression of AP-1Luc until the end of the study (Figure 2B, far right panel).

It should be noted that in addition to temporal resolution, spatial localization of AP-1 activation in PVN is verified by virtue of the fact that the Ad-AP-1 reporter was injected site selectively. This is an important point because the scattering of visible light through tissue prevents the surface-weighted signal itself from strictly informing of the 3D position of the luminescence source. However, because the luciferase reporter was delivered selectively to PVN, we have independent confirmation of spatial localization of AP-1 activation.
To provide further evidence of this, immunohistochemical staining of luciferase was performed at day 28. As seen in Figure 2C, luciferase was expressed at high levels bilaterally in the PVN. Together, these results suggest that renal artery clipping induces O$_2^-$/H$_2$O$_2$-dependent AP-1 transcriptional activity in the PVN during the initiation and early phase of hypertension in this model.

**AdCuZnSOD Prevents 2K1C-Induced ROS Formation in the PVN**

Data in Figure 1 suggest that 2K1C-induced increases in O$_2^-$ levels in the PVN are associated with increased MAP in this model. BLI results in Figure 2 show that AP-1 activation in the PVN in response to renal artery clipping is CuZnSOD sensitive and with a time course that parallels the rise in MAP. These results implicate 2K1C-induced increases in O$_2^-$ in the PVN, and here we sought to confirm this directly by measuring ROS levels in this brain region at 5 and 14 days postclip using DHE confocal microscopy. As shown in representative images of DHE fluorescence (day 14; Figure 3A) and summary data at both time points (Figure 3B), renal artery clipping increased ROS levels in the PVN of mice treated with the control virus. This occurred during the early-phase postclip, coinciding with the start of the rise in MAP and the peak AP-1 activation response (day 5). Elevated ROS levels in the PVN were maintained at day 14, a time when MAP was maximum in the AdLacZ group but back to baseline in the AdCuZnSOD-treated animals (Figure 1). 2K1C-induced increases in PVN ROS levels were significantly blunted at both time points by AdCuZnSOD (Figure 3A and 3B), implicating O$_2^-$ as the radical species involved in both the initiation and maintenance of hypertension in this model.

**Localization of CuZnSOD in PVN**

We have shown previously that AdCuZnSOD induces robust, localized, and stable CuZnSOD expression and SOD activity in cardiovascular nuclei of the CNS.$^{9,18,19}$ As shown in Figure
4A, we confirmed this here and demonstrate that the stereotaxic coordinates used resulted in viral injections in the parvocellular region of PVN. In addition, to determine the subcellular targeting of CuZnSOD in the PVN of mice injected with AdCuZnSOD, double immunohistochemistry was performed for CuZnSOD with either the neuronal cell marker neuronal nuclei or the glial cell marker glial fibrillary acidic protein. As shown in Figure 4B, double immunolabeling revealed CuZnSOD expression in both neurons and glia in this region. This confirms what we and others have shown previously that Ad vectors transduce both cell types in the hypothalamus and other central cardioregulatory nuclei.28,29

**PVN-Targeted Expression of dnJNK1 Ameliorates 2K1C Hypertension**

Data presented in Figures 1 and 2 demonstrate that 2K1C-induced hypertension is associated with AP-1 activation in the PVN. To determine whether there is a causal link between these 2 responses, we injected an adenovirus encoding a dominant-negative inhibitor of AP-1 transcriptional activation (Ad-dnJNK1) into the PVN before renal artery clipping. Summary data in Figure 5 demonstrate that PVN-targeted dnJNK1 expression prevented the 2K1C-induced rise in MAP in both the early and chronic phases. Similar to Figure 1, blood pressure was not significantly altered in sham mice treated with AdLacZ, nor was HR different between the 3
groups at any time point. Baseline preclip MAP (Sham + AdLacZ = 102 ± 4, n = 3; 2K1C + AdLacZ = 98 ± 2, n = 4; 2K1C + Ad-dnJNK1 = 98 ± 2, n = 5; P > 0.05) and HR (Sham + AdLacZ = 485 ± 17, n = 4; 2K1C + AdLacZ = 487 ± 21, n = 4; 2K1C + Ad-dnJNK1 = 485 ± 5, n = 5; P > 0.05) were also not different between the treatment groups.

Renal Atrophy Induced by Renal Artery Clipping
Kidneys were weighed at the end of the 4-week period in a subset of sham, 2K1C + AdLacZ, and 2K1C + Ad-dnJNK1 animals to confirm atrophy of the clipped kidney and hypertrophy of the contralateral unclipped kidney. As shown in the Table, there were significant decreases and increases in renal mass of right (clipped) and left (nonclipped) kidneys, respectively, compared with shams. This was unaffected by PVN-targeted AdLacZ, AdCuZnSOD, or Ad-dnJNK1 treatment. Finally, body weights were not different between the groups.

Discussion
Using a combination of brain site–directed viral gene transfer, longitudinal monitoring of TF activation in vivo by BLI, and radiotelemetry in the 2K1C model of hypertension in mice, this study reveals that redox-regulated AP-1 activation in the PVN of the hypothalamus is causally linked to RVH. The time course and magnitude of hypertension observed in this study are consistent with what we and others have reported for this model in a variety of species, and that is, blood pressure rose gradually over the first week (ie, early phase) and was sustained at this level over the following weeks after clipping (ie, chronic phase). Also consistent with what others have reported, HR was not significantly altered at any time postclip, likely because of impaired baroreflex sensitivity. The initial rise in MAP coincided with increases in ROS production and a dramatic surge in AP-1 transactivation in the PVN. Each of these PVN responses was sustained through 2 weeks postclip, coinciding with the chronic phase of hypertension. AdCuZnSOD targeted to the PVN abolished both the ROS and AP-1 responses. This was associated with an attenuation of both the early and chronic phases of hypertension. Similarly, dominant-negative inhibition of AP-1 translocation in PVN with Ad-dnJNK1 prevented both phases of 2K1C-evoked hypertension. These results implicate O$_2$$^\cdot$ signaling and AP-1 transactivation activity in the PVN as key mediators of RVH.

The upstream signaling events that lead to increased O$_2$$^\cdot$ production in the PVN after renal artery stenosis remain unclear, although locally generated Ang II is a leading candidate given its well-established role in several cardiovascular diseases including RVH, as well as previous data showing that Ang II is elevated in the hypothalamus during both the early and chronic phases of 2K1C hypertension, with AT$_1$ receptor mRNA levels also being upregulated in the PVN. Given that a major mechanism through which Ang II exerts its powerful effects on central control of blood pressure is redox signaling, we first tested the hypothesis that 2K1C hypertension involves oxidant stress in the PVN. Indeed, renal artery clipping induced CuZnSOD-sensitive ROS increases in the PVN during the initial rise in MAP (day 5), suggesting that O$_2$$^\cdot$ signaling in this brain region may be involved in the development of RVH. To test this directly, PVN-targeted AdCuZnSOD treatment was used before placement of the clip. This led to attenuation of 2K1C-induced hypertension during the early phase, suggesting that the development of hypertension in this model does involve cytoplasmic O$_2$$^\cdot$ signaling in the PVN. Because AdCuZnSOD targeted to this brain region also ameliorated hypertension during the chronic phase, in addition to normalizing ROS levels in the PVN at 2 weeks postclip, these data suggest that O$_2$$^\cdot$ signaling in the PVN is also critical in the maintenance phase of RVH. Importantly, this occurred despite sustained 2K1C-induced renal atrophy. These results are consistent

Table. Body and Kidney Weights 4 Weeks After Surgery

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham + AdLacZ (n = 9)</th>
<th>2K1C + AdLacZ (n = 8)</th>
<th>2K1C + AdCuZnSOD (n = 6)</th>
<th>2K1C + Ad-dnJNK1 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>23.3 ± 0.9</td>
<td>25.4 ± 0.7</td>
<td>23.5 ± 0.8</td>
<td>25.3 ± 1.0</td>
</tr>
<tr>
<td>Left kidney weight, mg</td>
<td>135.0 ± 5.3</td>
<td>211.2 ± 7.9*</td>
<td>202.5 ± 11.5*</td>
<td>202.1 ± 12.6*</td>
</tr>
<tr>
<td>Right kidney weight, mg</td>
<td>154.3 ± 5.8</td>
<td>64.0 ± 5.8*</td>
<td>66.5 ± 6.9</td>
<td>74.0 ± 7.5*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs Sham + AdLacZ.
with those of Oliveira-Sales et al, whose findings support a role for ROS in the PVN in RVH; however, their studies used acute microinjection of Tempol unilaterally into PVN of anesthetized rats and showed that established hypertension (after 6 weeks) could be transiently reversed by this treatment.21 Our experimental design involving long-term gene transfer of CuZnSOD to PVN before renal artery clipping leads us to conclude that cytoplasmic O$_2^-$ signaling in the PVN plays a causal role in RVH. Studies using PVN-specific ablation of AT$_1$ receptors through Cre-loxP technology35 will be important to verify whether Ang II signaling in the PVN is indeed functionally linked to 2K1C-induced oxidant stress and RVH. In addition, because Ad-mediated CuZnSOD transduction occurred in both neurons and glia of the PVN, additional studies will be required to determine the relative role of O$_2^-$ signaling in these 2 cell types.

Additional future studies should aim to determine the enzymatic source of 2K1C-induced ROS formation in the PVN. It is certainly well established that Nox is a primary source of ROS, mediating many cardiovascular responses, including central neural control of blood pressure and related parameters.6,9–12,36 Recently, Nox-derived ROS have been implicated in RVH. Several studies have shown that systemic treatment with the antioxidants Tempol or apocynin relieve several peripheral pathologies associated with RVH in rats, including endothelial dysfunction, impairment in renal hemodynamics, and increased renal sympathetic nerve activity.31–33,38 In addition, Wang et al demonstrated that Ang II–stimulated Nox contributes to the development of 2K1C hypertension and cardiac remodeling in rats.39 Interestingly, Nox has also been implicated recently in the CNS component of RVH. Oliveira-Sales et al showed that both Nox2 and p47phox were markedly upregulated in PVN of rats with 2K1C hypertension.21 Using viruses encoding small interfering RNAs targeted against Nox1, Nox2, or Nox4,12,18,40 it should be possible to dissect the relative functional role of each of these homologues in ROS formation in the PVN and 2K1C RVH.

The downstream molecular mechanisms by which increased O$_2^-$ in the PVN leads to chronic hypertension in this model was the next focus of this study. Effectors of O$_2^-$ in CNS cardioregulatory nuclei have been the subject of several recent studies, with evidence that kinase cascades, calcium transients, and delayed rectifier potassium channels may be involved.6,11,41–43 However, many of these studies focused on the effects of relatively short-term stimulation of ROS production. Because O$_2^-$ signaling in the context of our study was involved in chronic regulation of blood pressure, we focused on ROS-sensitive TFs because of their well-known role in long-term modulation of a wide variety of CNS parameters44 and an emerging role in CNS cardiovascular circuits. For example, Liu et al have shown that Ang II induces AT$_1$ receptor upregulation in the rostral ventrolateral medulla by an increase in ROS-dependent activation of AP-1 in brains of chronic heart failure rabbits.46 In addition, a recent report by Kang et al shows that chronic systemic infusion of Ang II in rats causes ROS-dependent activation of nuclear factor κB in the PVN.17 Both of these studies used gel mobility shift assays to examine TF activation in brain nuclei at a few select time points during these chronic conditions. However, because we were interested in the full time course of TF activation in the PVN during the development and progression of RVH, we turned to in vivo BLI technology for longitudinal real-time monitoring of AP-1 activation. Our results demonstrate that renal artery clipping caused a small but significant increase in AP-1 transcriptional activation in the PVN as early as 3 to 4 days postclip. This transitioned to a marked surge in activity over the following 2 days, followed by sustained elevations compared with shams for another week, albeit at lower levels compared with the early surge. AP-1 activity in the PVN then returned to basal levels for the remaining 2 weeks of the experiment. The sensitivity of these increases in AP-1–dependent photon flux to AdCuZnSOD suggests that activity of this TF in PVN is O$_2^-$ regulated.

It is interesting to speculate about the time course of AP-1 activation in the context of the different phases of 2K1C-induced RVH in this study. Genes encoding Fos and Jun family proteins, which form the AP-1 complex that binds AP-1 sites in gene promoters, are termed immediate early genes because of their classic quick and transient induction (ie, hours) in response to a variety of stimuli.45 However, more recently, some family members such as isoforms of FosB, which are important in CNS adaptation, were shown to be induced only by more chronic stimuli and persist for longer periods of time (ie, days to weeks to months).46 Although our strategy for measuring AP-1 activation in the current study does not allow for resolution of the various family members involved, we speculate that the early robust increases in AP-1 transcriptional activity leads to changes in PVN neural networks that play a key role in initiating the long-term changes in blood pressure in this model. This was supported by the finding that AdCuZnSOD injection into PVN blocked both the AP-1 transactivation and chronic hypertension in this model. Further, because dominant-negative inhibition of AP-1 in the PVN prevented 2K1C-evoked rises in MAP, this suggests that its transcriptional activity is functionally linked to hypertension in this model.

The physiological effectors in the PVN that lead to 2K1C hypertension have been the subject of many investigations. On the one hand, increased arginine vasopressin release has been implicated in this model over the years,47,48 and there are strong links between Ang II, ROS, and AP-1, with increased activation of vasopressinergic neurons in the PVN.49–51 However, there are other reports that arginine vasopressin is not involved in 2K1C hypertension.52 More recently, it is thought that PVN involvement in RVH is through activation of rostral ventrolateral medulla–projecting parvocellular neurons in this region, leading to increased sympathoexcitation.53 Interestingly, ROS are now also implicated in this pathway.21 Further studies will be required to determine the physiological effectors that are downstream of ROS and AP-1 activation in PVN during 2K1C hypertension.

**Perspectives**

Our data suggest that 2K1C-induced RVH in mice is mediated by oxidative stress–induced AP-1 activation in the PVN. This is supported by the following observations: (1) the development of 2K1C hypertension is associated with a
marked increase in O$_2^-$ production in the PVN; (2) PVN-targeted overexpression of the CuZnSOD prevents both the increase in PVN O$_2^-$ levels and hypertension in this model; (3) AP-1 is robustly activated in the PVN with a time course that is consistent with its involvement in the development of hypertension of this model, and its inhibition by O$_2^-$ scavenging parallels the inhibition of hypertension; (4) dominant-negative inhibition of AP-1 transcriptional activity in the PVN prevents RVH. The present findings have the potential to fundamentally advance our understanding of the molecular mechanisms underlying RVH and could have important implications in the design of novel and innovative therapeutic approaches targeting the neurogenic component of this disease.

Acknowledgments
We thank Dr Carlos Alberto Aguiar da Silva (University of São Paulo Ribeirão Preto) for providing the renal artery clips.

Sources of Funding
These studies were supported by grants from the National Institutes of Health to R.L.D. (HL063887 and HL084624) and an American Heart Association Established Investigator Award to R.L.D. (0540114N).

Disclosures
None.

References


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Hypertension. published online December 20, 2010;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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In Vivo Bioluminescence Imaging Reveals Redox-regulated AP-1 Activation in Paraventricular Nucleus of Mice with Renovascular Hypertension

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Short Title: O₂⁻ and AP-1 in PVN cause 2K1C hypertension

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METHODS

Animals

Adult C57Bl/6 mice weighing 20-25g (8-12 wks) were used in these studies. Animals were housed in a climate-controlled room set to a 12-hr light/dark cycle and fed a standard rodent chow (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan, IN). All procedures were approved by the Institutional Animal Care and Use Committee at Cornell University. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations and the AVMA Panel on Euthanasia.

Adenoviral vectors

Recombinant E1-deleted adenoviral (Ad) vectors encoding human cytoplasmic superoxide dismutase (AdCuZnSOD, 1.3 x 10^{12} pfu/mL) or bacterial β-galactosidase (AdLacZ, 1.0x10^{12} pfu/mL) were obtained from the University of Iowa Gene Transfer Vector Core. Ad vectors encoding an AP-1-responsive luciferase reporter construct (AdAP-1Luc, 0.9x10^{13} pfu/mL) and a dominant-negative c-Jun NH2-terminal kinase 1 (Ad-dnJNK1) was a kind gift from Dr. John F. Engelhardt (University of Iowa). Construction and characterization of each of these viral vectors have been described previously1-4.

Radiotelemeter implantation and gene transfer to the PVN

Mice were anesthetized (ketamine, 150 mg/kg/xylazine, 15 mg/kg i.p.) and instrumented with TA11PA-C10 radio telemetry probes (Data Sciences International, Arden Hills, MN) for continuous recording of arterial pressure and heart rate (HR) as previously described5, 6. Briefly, the catheter of the telemeter was implanted in the thoracic aorta via the left common carotid artery. The body of the probe was placed in a subcutaneous pocket created in the right flank, and the wound was closed and sutured. Immediately following telemeter implantation, mice were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and the skull was exposed by an incision and leveled between lambda and bregma. AdLacZ, AdCuZnSOD or Ad-dnJNK1 were slowly microinjected bilaterally (200 nl/side, 1.0x10^{12} pfu/mL) into the PVN. Brain coordinates were as follows (relative to bregma): 0.7 mm caudal, 0.3 mm from midline and 5.0 mm ventral as previously described7. In separate cohorts used for in vivo bioluminescence imaging studies, non-telemetered mice underwent bilateral PVN microinjection of a 1:1 mixture of AdAP-1Luc/AdLacZ or AdAP-1Luc/AdCuZnSOD (200 nl/side) as previously described1. Following telemeter implantation and/or brain microinjections, mice were returned to their home cages where they remained throughout the studies.

2K1C Renovascular hypertension (RVH) model
Mice were allowed 1 week recovery before baseline arterial pressure and heart rate measurements were taken over 3 days. Mice were then anesthetized with 2% inhaled isoflurane, and a midline abdominal incision was made. The right renal artery was exposed and isolated over a short segment by blunt dissection. A U-shaped silver clip (0.126 mm internal diameter) was placed over the vessel proximal to the abdominal aorta as previously described. It should be noted that this size clip is known to produce RVH without significant renal infarction. The sham surgery consisted of the entire protocol with the exception of renal artery clipping. Wounds were sutured and mice were returned to their home cages placed atop telemetry receivers (DSI, Model RPC-1). Cardiovascular parameters were recorded over 28 days as described.

**ROS measurements in brain sections**

Dihydroethidium (DHE) staining was performed as described previously. On days 5 and 14 post-clip, brains from a subset of AdLacZ or AdCuZnSOD-treated 2K1C and sham controls were removed, cryosectioned (30 µm, coronal) through the PVN and mounted directly onto chilled microscope slides. Sections (4-6 per animal) were thawed at room temperature, rehydrated with 1X PBS and incubated for 7 minutes in the dark with DHE (1 µM; Invitrogen, Carlsbad, CA). After washing with 1X PBS, DHE fluorescence was visualized by confocal microscopy (Zeiss LSM 510) using an excitation wavelength of 543 nm and a rhodamine emission filter. Sections from all groups were processed and analyzed in parallel, using identical microscope settings. Fluorescence intensity was quantified using ImageJ as described and normalized to fluorescence levels observed in sham controls.

**In vivo bioluminescence imaging (BLI)**

To longitudinally track AP-1 activation in PVN in vivo, an Ad vector containing a luciferase cassette downstream of the consensus AP-1 binding sequence (AdAP-1Luc) was utilized in conjunction with in vivo BLI as described. Mice treated with AdAP-1Luc in the PVN were imaged 24 hrs after infection to confirm expression of the AP-1 construct, and then given a two-week recovery period to allow surgery-induced elevation in AP-1 activity to subside. Following recovery and baseline imaging over 4 days, animals underwent sham or 2K1C surgery, after which the temporal pattern of AP-1 activation was assessed longitudinally for 28 days. Daily in vivo bioluminescence images were acquired with the IVIS®200 (Caliper Life Sciences, Alameda, CA) following D-luciferin injections (150 mg/kg, i.p.) and transfer of mice to the imaging cabinet. For uniformity, animals across each of the experimental groups were imaged within the same daily session, which occurred at the same time each day. At the end of the study, systemic endotoxin-induced activation of AP-1 was assessed in mice by injection of lipopolysaccharide (LPS, 4 µg/g, i.p.) to confirm functional expression of the AP-1-driven luciferase reporter in PVN. Data were analyzed with Xenogen Living Image software as described.

**CuZnSOD and AP-1Luc immunohistochemistry**
Immunohistochemical analysis of PVN brain sections was performed to verify proper localization of virally-delivered transgenes as described previously\textsuperscript{5, 7, 9}. Briefly, AdCuZnSOD- and AdAP-1Luc-injected mice were perfused at the end of the studies, post-fixed, and sectioned (30 µm, coronal) through the PVN. After 2-h incubation in 10\% normal rabbit serum, free-floating sections were incubated with a sheep polyclonal CuZnSOD antibody (1:100, Calbiochem, San Diego, CA) for 24 hr at room temperature. After washing, sections were incubated in a fluorescein-conjugated rabbit anti-sheep secondary antibody (1:200, Vector Labs) for 45-min. In order to determine the cell-specificity of the AdCuZnSOD expression, sections were subsequently processed for immunofluorescence with antibodies labeling either neuronal nuclei (Neu-N) or glial fibrillary acidic protein (GFAP). After 2-h incubation in mouse IgG blocking reagent (Vector Labs, Burlingame, CA), one set of serial sections was incubated with a mouse monoclonal anti-Neu-N antibody (1:100, Chemicon), while the other set was incubated with a monoclonal mouse anti-GFAP antibody (1:200, Chemicon/Millipore, Billerica, MA) for 1-h using a mouse-on-mouse immunodetection kit (Vector Labs). This was followed by incubation in a secondary antibody consisting of a biotinylated anti-mouse IgG reagent for 15-min and subsequent treatment with Streptavidin Texas Red (1:200, Vector Labs) for 15-min. For AP-1Luc expression, free-floating sections were processed for immunofluorescence with a goat polyclonal luciferase antibody (1:100, Promega, Madison, WI) for 24 hr at room temperature. Incubation of some sections in goat IgG was performed as a negative control. After washing, sections were incubated in secondary biotinylated rabbit anti-goat antibody for 60-min and subsequent treatment with Streptavidin AlexaFluor 498 (1:200, Vector Labs) for 15-min. Sections were mounted onto microscope slides, coverslipped with Vectashield (Vector Labs) and analyzed by confocal laser microscopy (Zeiss LSM 510).

**Body and renal mass**

Mice from each of the groups were weighed and euthanized at the end of the telemetry experiments (day 28), and kidneys were removed and weighed.

**Statistical analyses**

Results are expressed as mean±SEM. Differences in cardiovascular parameters, ROS production and photon flux at various time-points following renal artery clipping or sham surgery were compared with respective controls by one-way analysis of variance (ANOVA) and Newman-Keuls post-tests. Changes in MAP and HR from baseline were assessed by repeated measures ANOVA with subsequent Tukey’s multiple comparison post-test. Statistical significance was defined as p<0.05.
REFERENCES


