Brain Angiotensin

Genetic Silencing of Nox2 and Nox4 Reveals Differential Roles of These NADPH Oxidase Homologues in the Vasopressor and Dipsogenic Effects of Brain Angiotensin II

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Abstract—The renin-angiotensin system exerts a tremendous influence over fluid balance and arterial pressure. Angiotensin II (Ang-II), the effector peptide of the renin-angiotensin system, acts in the central nervous system to regulate neurohumoral outflow and thirst. Dysregulation of Ang-II signaling in the central nervous system is implicated in cardiovascular diseases; however, the mechanisms remain poorly understood. Recently we established that NADPH oxidase (Nox)–derived superoxide acting in the forebrain subfornical organ is critical in the physiological responses to central Ang-II. In addition, we have found that Nox2 and Nox4 are the most abundantly expressed Nox homologues within Ang-II–sensitive sites in the forebrain. To dissect out the functional importance and unique roles of these Nox enzymes in the pressor and dipsogenic effects of central Ang-II, we developed adenoviral vectors expressing small interfering RNA to selectively silence Nox2 or Nox4 expression in the subfornical organ. Our results demonstrate that both Nox2 and Nox4 are required for the full vasopressor effects of brain Ang-II but that only Nox2 is coupled to the Ang-II–induced water intake response. These studies establish the importance of both Nox2- and Nox4-containing NADPH oxidases in the actions of Ang-II in the central nervous system and are the first to reveal differential involvement of these Nox enzymes in the various physiological effects of central Ang-II. (Hypertension. 2009;54:1106-1114.)

Key Words: hypertension ■ blood pressure ■ water intake ■ subfornical organ ■ adenovirus ■ siRNA

Highly conserved throughout evolution, the renin-angiotensin system (RAS) is vital for maintaining fluid and arterial pressure homeostasis. Osmoregulation in the leech, water swallowing in euryhaline fish, and sodium appetite in pigeons all are mediated by the RAS.1,2 As cardiovascular systems have evolved, the RAS has kept pace; in higher-order animals, the RAS is intricately involved with nearly every aspect of cardiovascular function. Angiotensin II (Ang-II), the primary effector peptide of the RAS, acts in the central nervous system (CNS) to modulate autonomic tone, increase thirst, and initiate the release of neurohormones.3 Circulating Ang-II is detected by circumventricular organs (CVOs), specialized brain regions that lie outside of the blood-brain barrier and connect to key cardio-regulatory regions within the CNS.4 In addition, because most of these CNS cardiovascular nuclei possess the enzymatic machinery to generate Ang-II locally,5,6 this source of the peptide is also important in modulating neurotransmission. The tremendous impact of RAS-CNS interactions on normal cardiovascular physiology has prompted many investigations of their role in cardiovascular disease. Indeed, mounting evidence has implicated dysregulation of central Ang-II signaling in hypertension and heart failure both in the clinic and in experimental models.7 However, despite a century’s worth of studies aimed at uncovering the physiology and pathophysiology of the RAS,8 the precise neural pathways and molecular mechanisms involved in Ang-II signaling in the CNS remain unresolved.

Pioneering studies by Mangiapane and Simpson9 >30 years ago implicated the subfornical organ (SFO), a prominent forebrain CVO, as a key interface between circulating Ang-II and cardio regulatory centers of the brain. Recently, we demonstrated a critical role for superoxide (O2•-) production in this brain region in mediating the classical pressor, bradydyic, and dipsogenic responses elicited by intracerebroventricular (ICV) delivery of Ang-II.10 In addition, we have identified a Rac1-dependent NADPH oxidase (Nox), a multisubunit enzyme that catalyzes the 1 electron transfer from NADPH to molecular oxygen,11 as a primary source of Ang-II–dependent O2•- production in the SFO.12 Genetic inhibition of this enzyme complex in the SFO attenuated the cardiovascular and dipsogenic profiles to ICV administration of Ang-II.12 Recent studies using pharmacological or peptide inhibitors (eg, gp91dsat13) have also confirmed NADPH...
oxidase as a key player in the actions of Ang-II in other CNS cardiovascular nuclei.\textsuperscript{14,15} An entire family of Nox enzymes has now been described, each containing a unique homologue of the catalytic subunit of the oxidase.\textsuperscript{16} Peripheral cardiovascular tissues express distinct complements of Nox1, Nox2, and Nox4, and these different Nox enzymes have been shown to subserve diverse roles in the pathogenesis of numerous cardiovascular diseases.\textsuperscript{17} Although the studies described above clearly demonstrate the general importance of NADPH oxidase in central Ang-II signaling, such broad inhibition of the enzyme through targeting either assembly or activation does not provide information about the molecular identity of the oxidase(s) involved. Recently we reported that the Nox homologues exhibit a differential expression pattern across key cardioregulatory regions in the CNS, with Nox2 and Nox4 being the most abundantly expressed homologues in forebrain CVOs.\textsuperscript{17} Although little information exists regarding the functional roles of the Nox homologues in the CNS, a few recent studies implicate Nox2 in Ang-II signaling within brainstem cardiovascular nuclei. In the rostral ventrolateral medulla, Gao et al\textsuperscript{18} reported upregulation of Nox2 expression in response to central Ang-II treatment. In addition, Wang et al\textsuperscript{19} showed that Nox2 colocalizes with Ang-II receptors (Ang-II type 1 [AT1] subtype) in the nucleus tractus solitarius and further demonstrated that Ang-II–dependent responses are significantly impaired in neurons cultured from the nucleus tractus solitarius of animals lacking Nox2.\textsuperscript{20} Much less is known regarding the role of Nox4 in central Ang-II or other signaling pathways of the brain.

Given the importance of NADPH oxidase–derived O$_{2}^{ullet-}$ in the actions of Ang-II in the brain, our knowledge that the various Nox enzymes serve distinctive physiological roles and our findings that both Nox2 and Nox4 are expressed in forebrain CVOs at high levels, here we sought to identify the functional significance of Nox2- and Nox4-containing NADPH oxidases in brain Ang-II–elicited physiological responses. To dissect out the relative contributions of these Nox homologues, we used adenoviral-mediated delivery of small interfering RNA (siRNA) to the SFO of mouse brain to induce stable and localized knockdown of Nox2 or Nox4. We then examined the impact of selectively silencing these Nox homologues on central Ang-II–induced vasopressor and dipsogenic responses, as well as on Ang-II–induced reactive oxygen species (ROS) formation in cultured neurons.

Materials and Methods

What follows is a brief summary of the experimental protocols. A detailed description of all of the methods can be found in the expanded Materials and Methods section in the online Data Supplement (available at http://hyper.ahajournals.org).

Adenoviral Vectors

Adenoviral vectors expressing siRNA targeted against Nox2 (Adsi-Nox2), Nox4 (AdsiNox4), or the control message enhanced green fluorescent protein (GFP; AdsiGFP) were constructed, purified, and provided by the University of Iowa Gene Vector Core, as described previously.\textsuperscript{21,22} In brief, 21-bp short hairpin RNAs representing sequences directed against Nox2, Nox4, or enhanced green fluorescent protein were placed under the control of the mouse U6 promoter. A separate CMV promoter drives expression of a reporter gene (GFP for siNox2- and siNox4-expressing constructs and LacZ for the small interfering GFP–expressing construct).

Measurement of Nox Homologue Expression

In Vitro

Primary neonatal rat cardiomyocytes were treated with vehicle, AdsiGFP (100 plaque-forming units per cell), AdsiNox2 (100 plaque-forming units per cell), or AdsiNox4 (100 plaque-forming units per cell). In separate experiments, M-1 kidney cortical duct cells were also treated with vehicle, AdsiGFP, or AdsiNox4 at the same concentrations as above. After 48 hours, total RNA was isolated, and relative expression levels of Nox mRNAs were analyzed using real-time RT-PCR with Sequence Detection Software version 1.4 (Applied Biosystems) and expressed relative to vehicle-treated controls using the ΔΔCt method.\textsuperscript{22}

Detection of O$_{2}^{ullet-}$ Production in Primary Cell Culture

Primary neuronal cultures were treated with vehicle (saline) or infected with AdsiGFP, AdsiNox2 alone, AdsiNox4 alone, or Adsi-Nox2 and AdsiNox4 together 24 hours before loading with dihydroethidium (DHE; 5 μmol/L). DHE fluorescence was imaged using confocal microscopy, as described previously.\textsuperscript{10,12} After obtaining baseline images (0 minutes), cells were stimulated with Ang-II (1 μmol/L) and reimaged after 30 minutes. DHE fluorescence was quantified using ImageJ analysis software (version 1.33; National Institutes of Health) and expressed relative to baseline fluorescence in individual cells, as described previously.\textsuperscript{10}

Physiological Studies

Adult C57Bl/6 mice (Harlan, Indianapolis, IN) underwent ICV microinjection of saline, AdsiGFP, AdsiNox2, AdsiNox4, or Adsi- Nox2 plus AdsiNox4 for targeted gene delivery to the SFO, as described previously.\textsuperscript{21,23} Mice were then instrumented with ICV canulae for central delivery of Ang-II, and radiotelemeters were implanted for conscious recordings of mean arterial pressure (MAP) and heart rate (HR), as described previously.\textsuperscript{10,12} After 7 days of recovery, MAP, HR, and dipsogenic responses were recorded after ICV Ang-II (200 ng, 200 nL), as described previously.\textsuperscript{10,12} All of the procedures conformed to the guidelines set forth by the National Institutes of Health and were approved by the University of Iowa and Cornell University Animal Care and Use Committees.

Viral Transduction Efficiency Assessed by Reporter Gene Expression

One week after virus injections, mice were euthanized and perfused with 4% paraformaldehyde. Coronal sections from AdsiGFP(LacZ)-treated mice were processed for ß-galactosidase activity and analyzed using real-time RT-PCR with Sequence Detection Software version 1.4 (Applied Biosystems) and expressed relative to vehicle-treated controls using the ΔΔCt method.\textsuperscript{22}

Immunoblot Analysis for Nox Homologue Expression in the SFO

Brains were removed from animals 7 days after SFO-targeted delivery of saline, AdsiGFP, AdsiNox2, or AdsiNox4, and micropunches from the SFO and immediately surrounding tissue were pooled from 3 animals in each group. Western analysis was performed using anti-gp91phox antibody (BD Biosciences; 1:1000) or anti-Nox4 antibody (kind gift of Dr David Lambeth, Emory University, Atlanta, GA).

Results

Adenoviral-Mediated Delivery of siRNA Effectively Silences Nox Homologue Expression

Viraically mediated delivery of RNA interference has emerged as a valuable tool for stable and localized knockdown of specific genes in isolated brain regions.\textsuperscript{24} We demonstrated...
Recently, AdsiGFP targeted to the SFO of GFP transgenic mice silenced GFP fluorescence selectively in this brain region, demonstrating the feasibility of using virally mediated delivery of siRNA to study targeted molecules within isolated cardioventricular regions of the CNS. In this study, we sought to use this strategy to evaluate the functional significance of individual Nox homologues in the SFO. To achieve this, we developed adenoviral vectors expressing siRNA targeted against either Nox2 (AdsiNox2) or Nox4 (AdsiNox4) and tested their ability to efficiently and selectively silence Nox2 or Nox4 expression, respectively. For these studies, we used primary rat neonatal cardiomyocytes, because both Nox2 and Nox4 are expressed endogenously at high levels in these cells. Cardiomyocytes were treated with vehicle, AdsiNox2, AdsiNox4, or the control vector AdsiGFP, and Nox1, Nox2, and Nox4 homologue transcript levels were assessed by real-time RT-PCR. As shown in Figure 1A, AdsiNox2 and AdsiNox4 markedly diminished each of their respective targeted Nox homologues but had no effect on the other homologues. For AdsiNox2, this further verifies our earlier data showing that this construct is selective and efficient in silencing Nox2. To further confirm the effectiveness of AdsiNox4 in another cell type in which endogenous Nox4 is expressed at high levels, M-1 kidney cortical duct cells were transduced with AdsiNox4 or AdsiGFP. AdsiNox4 caused an ~90% decrease in Nox4 levels compared with AdsiGFP (8.7±0.4×10⁻³ AdsiGFP versus 0.9±0.1×10⁻³ AdsiNox4 4-fold β-actin; n=3; *P<0.05).

Next we tested the ability of AdsiNox2 and AdsiNox4 to effectively silence Nox homologue expression within the SFO in vivo. We first verified effective viral delivery of siRNA constructs to the SFO by visualizing reporter gene expression in this brain region after ICV delivery of AdsiNox2 or AdsiNox4. These constructs harbor CMV-driven GFP as a reporter gene, whereas AdsiGFP contains CMV-driven LacZ. As shown in Figure 1B, reporter gene expression was robust and localized to the SFO 1 week after stereotaxic gene transfer of AdsiNox2, AdsiNox4, or AdsiGFP, verifying efficient viral transduction of this region. To confirm effective silencing of Nox2 or Nox4 in the SFO with these viruses, we performed Western blot analysis on microinjections taken from the SFO and the immediate surrounding tissue 1 week after injection. As shown in Figure 1C, Nox2 and Nox4 protein levels were significantly diminished in mice treated with AdsiNox2 or AdsiNox4, respectively. Together, these results confirm that Ad-mediated delivery of siNox2 or siNox4 results in effective transduction and inhibition of Nox homologue expression in targeted brain regions.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Adenovirus-mediated delivery of RNA interference effectively silences Nox homologue expression in the SFO. A, Real-time RT-PCR data showing the effects of AdsiGFP, AdsiNox2, or AdsiNox4 treatment on endogenous Nox1, Nox2, and Nox4 transcript levels in primary neonatal cardiomyocytes. Levels of Nox1, Nox2, and Nox4 mRNA were first normalized to β-actin and expressed relative to levels in vehicle-treated cells. Experiments were performed in triplicate. B, ICV viral delivery results in robust and widespread transgene expression in the SFO. One week after adenoviral delivery, coronal brain sections through the SFO were visualized for reporter gene expression. The SFO is depicted in the top left panel. Representative light and fluorescence microscopy images of sections from AdsiGFP(LacZ)-(top right panel), AdsiNox2(GFP)-(bottom left panel), or AdsiNox4(GFP)-(bottom right panel) treated mice demonstrate robust LacZ or GFP expression in the SFO. C, SFO-targeted delivery of AdsiNox2 or AdsiNox4 significantly silences Nox2 and Nox4 expression, respectively. Representative Western blot and summary data of Nox2 (left) and Nox4 (right) expression in the lamina terminals of mice microinjected with saline, AdsiGFP, or AdsiRNA targeted against Nox2 or Nox4 1 week earlier. Brain tissue was pooled from 3 animals per group. Experiment was performed in triplicate. Data are expressed as mean±SEM. *P<0.05 vs saline and AdsiGFP. †P<0.05 vs AdsiNox4. ‡P<0.05 vs AdsiNox2.
Previous studies in our laboratory have established that ICV delivery of adenoviruses is a highly effective and reliable method for targeting transgene expression to the SFO.6,10,12,21,23,26 To confirm these earlier findings and to further explore the brain regions affected by ICV delivery of our AdsiRNAs, we examined various regions throughout the CNS for GFP expression in 4 C57Bl/6 mice 1 week after ICV delivery of AdsiNox2 and AdsiNox4 concomitantly. As shown in Figure 2, ICV injection of AdsiNox2 and AdsiNox4 resulted in highly robust GFP expression throughout the SFO. Similar to our previous studies,27 GFP expression was also found along the ependymal layer of the lateral ventricles. There was sparse GFP expression in the organum vasculosum of the lamina terminalis (OVLT) in 1 animal (see Figure 2), but no expression was detected in the median preoptic nucleus (MnPO), paraventricular nucleus (PVN), or area postrema (AP; right). LV indicates lateral ventricle; 3V, third ventricle.

**Figure 2.** ICV delivery of virus results in robust and localized transgene expression in the SFO. Serial coronal sections (30 µm) throughout the CNS from 4 mice injected ICV with AdsiNox2 plus AdsiNox4 7 days earlier were processed for GFP expression using confocal microscopy. Both AdsiNox2 and AdsiNox4 harbor GFP as a reporter gene. Robust GFP expression was seen in the SFO (top left) and along the lining of the lateral ventricle (left middle). Although sparse expression was found in the OVLT of 1 animal (bottom left), expression could not be detected in the median preoptic nucleus (MnPO), paraventricular nucleus (PVN), or area postrema (AP; right). LV indicates lateral ventricle; 3V, third ventricle.

Nox2 and Nox4 Mediate Ang-II–Induced ROS Production in SFO Neurons

We have previously established a Rac1-dependent NADPH oxidase as the primary enzymatic source of Ang-II–induced ROS in neurons12 and have found that Nox2 and Nox4 are the most abundantly expressed Nox homologues within forebrain CVOs.17 As a first step in identifying the importance of these Nox homologues in the actions of Ang-II in the CNS, we examined the impact of silencing Nox2 or Nox4 on Ang-II–dependent ROS production in neurons cultured from the lamina terminalis, a forebrain region that includes the SFO and is critical for the physiological actions of Ang-II in the CNS.7,17 In primary cultures treated with either vehicle or the control AdsiGFP vector, Ang-II induced an ∼3-fold increase in fluorescence from the ROS indicator DHE by 30 minutes (Figure 3). However, treatment with either AdsiNox2 or AdsiNox4 alone significantly inhibited this response (Figure 3). Furthermore, concomitant treatment with both AdsiNox2 and AdsiNox4 abolished Ang-II–induced increases in DHE fluorescence (Figure 3), suggesting that both Nox2- and Nox4-containing NADPH oxidases are important sources of Ang-II–induced ROS in neurons from this brain region.

Both Nox2 and Nox4 Are Required for the Full Vasopressor Effects of Central Ang-II

Having established the use of adenovirus-mediated delivery of siRNA for selective silencing of Nox homologues in vivo, we next examined the impact of inhibiting Nox2 and/or Nox4 expression in the SFO on the physiological profile elicited by central Ang-II. MAP and HR responses to ICV Ang-II were recorded in conscious mice 7 days after implantation of radiotelemeters and gene transfer of AdsiNox2, AdsiNox4, AdsiNox2 and AdsiNox4 concomitantly, AdsiGFP, or saline injection. As shown in the representative tracings (Figure 4A), mice treated previously with either saline or AdsiGFP showed the classic pressor and bradycardic responses to ICV injection of Ang-II. It should be noted that ICV treatment with vehicle had no effect on blood pressure or HR (ΔMAP: −0.4±1.0 mm Hg; ΔHR: −2±7 bpm; n=3), confirming that changes in these responses are specific to Ang-II treatment.
Silencing Nox2 or Nox4 prevents Ang-II-induced ROS production in neurons cultured from the lamina terminalis. A, Representative confocal images of DHE-loaded (5 μmol/L; 30 minutes) cells cultured from the lamina terminalis showing the effects of Ang-II (1 μmol/L; 30 minutes) on production of ROS in noninfected cells and cells treated with AdsiGFP, AdsiNox2 alone, AdsiNox4 alone, or AdsiNox2 and AdsiNox4 together. Effective transduction of AdsiNox2 and/or AdsiNox4 was verified by visualization of expression of the reporter gene GFP. B, Summary of relative DHE fluorescence in individual cells before Ang-II stimulation (0 minutes) and 30 minutes after Ang-II stimulation. Cells were stimulated with Ang-II 24 hours after treatment with AdsiGFP, AdsiNox2, AdsiNox4, or AdsiNox2 plus AdsiNox4 together. Vehicle-treated cells served as a control. Data are expressed as mean±SEM (n=10 to 14 cells per group over 3 separate experiments) and expressed relative to DHE fluorescence before Ang-II treatment (0 minutes). *P<0.05 vs 0 minutes. †P<0.05 vs saline and AdsiGFP.

Figure 3. Silencing Nox2 or Nox4 prevents Ang-II–induced ROS production in neurons cultured from the lamina terminalis. A, Representative confocal images of DHE-loaded (5 μmol/L; 30 minutes) cells cultured from the lamina terminalis showing the effects of Ang-II (1 μmol/L; 30 minutes) on production of ROS in noninfected cells and cells treated with AdsiGFP, AdsiNox2 alone, AdsiNox4 alone, or AdsiNox2 and AdsiNox4 together. Effective transduction of AdsiNox2 and/or AdsiNox4 was verified by visualization of expression of the reporter gene GFP. B, Summary of relative DHE fluorescence in individual cells before Ang-II stimulation (0 minutes) and 30 minutes after Ang-II stimulation. Cells were stimulated with Ang-II 24 hours after treatment with AdsiGFP, AdsiNox2, AdsiNox4, or AdsiNox2 plus AdsiNox4 together. Vehicle-treated cells served as a control. Data are expressed as mean±SEM (n=10 to 14 cells per group over 3 separate experiments) and expressed relative to DHE fluorescence before Ang-II treatment (0 minutes). *P<0.05 vs 0 minutes. †P<0.05 vs saline and AdsiGFP.

Silencing Nox2 Expression Alone in the SFO Is Sufficient to Prevent the Dipsogenic Effect of Central Ang-II

The cardiovascular responses to central Ang-II are accompanied by a brisk and pronounced water drinking response that is prevented by genetic inhibition of NADPH oxidase–dependent O_2•^- generation in the SFO. To examine the roles of Nox2 and Nox4 in the dipsogenic response elicited by central Ang-II, time spent licking the water bottle for 15 minutes after ICV Ang-II (saline: 5.8±1.1×10^5 mm Hg*s, AdsiGFP: 7.7±0.8×10^5 mm Hg*s versus AdsiNox2: 1.3±0.7×10^5 mm Hg*s, AdsiNox4: 2.8±0.8×10^5 mm Hg*s; P<0.05). Even more importantly, treatment with both AdsiNox2 and AdsiNox4 together abolished thepressor and bradycardic responses with respect to both the peak (AdsiNox2 + AdsiNox4: 1±1 mm Hg, −18±8 bpm, P<0.05 versus all other groups) and duration (AdsiNox2 + AdsiNox4: −0.6±0.6×10^3 mm Hg*s, P<0.05 versus all other groups). Together, these results demonstrate that both Nox2- and Nox4-containing NADPH oxidases are required for the full cardiovascular response profile to central Ang-II.

A role for ROS in the regulation of baseline blood pressure and HR remains controversial. Because both cytosolic and membrane-bound Nox enzyme subunits, including Nox2 and Nox4, are expressed at baseline conditions in central cardiovascular control regions, we examined the effects of silencing Nox2 and Nox4 in the SFO on resting cardiovascular function 6 days after brain gene transfer. As summarized in the Table, average baseline MAP and HR were comparable in all of the groups, indicating that neither Nox2 nor Nox4 in the SFO is involved in the maintenance of basal cardiovascular parameters.

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drinking response to a similar extent as did treatment with AdsINox2 alone (AdsINox2 plus AdsINox4: 20±3 seconds, *P<0.05 versus saline, AdsIGFP, or AdsINox4). These results demonstrate a crucial role for Nox2 in the central effects of Ang-II on water intake and suggest that Nox4-containing NADPH oxidase is not involved in mediating this response.

**Discussion**

We and others have previously established the importance of NADPH oxidase–derived ROS in the brain as a key feature of Ang-II–mediated physiological responses and in the pathogenesis of neurogenic hypertension.10,12,14,15,26 Although a wealth of evidence has demonstrated the importance and unique roles of the various Nox enzymes in the actions of Ang-II in the periphery, the function of the Nox homologues involved in brain Ang-II signaling is not clear. Here, through the use of adenovirus-mediated delivery of siRNA to the SFO of mouse brain, we have identified Nox2 and Nox4 as key elements of Ang-II–mediated ROS production and actions in the CNS. Our data demonstrate that Nox2 and Nox4 each contribute to the vasopressor effects of central Ang-II; however, neither one alone can account for the entire response. In contrast, Nox2 alone is selectively linked to the water intake effects of ICV Ang-II. These results demonstrate for the first time that Nox2 and Nox4 are differentially linked to brain Ang-II effects.

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<thead>
<tr>
<th>Variable</th>
<th>Saline</th>
<th>AdsIGFP</th>
<th>AdsINox2</th>
<th>AdsINox4</th>
<th>AdsINox2/4</th>
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Baseline blood pressure and HR were recorded in mice 6 days after SFO-targeted delivery of saline (n=12), AdsIGFP (n=15), AdsINox2 (n=14), AdsINox4 (n=8), and AdsINox2/4 (n=7). Data are expressed as mean±SEM. *P>0.05 between all of the groups.
time a functional divergence between Nox2- and Nox4-containing NADPH oxidases in central Ang-II signaling.

A key feature of our studies involved the use of adenovirus-mediated delivery of siRNA for selective silencing of Nox homologues within targeted brain regions. Although this approach has just recently emerged,^{24,25} it has already proven to be a powerful strategy for unraveling complex molecular mechanisms in the CNS, including central Ang-II signaling.^{26} Although previous studies of NADPH oxidase in the CNS established the importance of this enzyme within discrete brain regions,^{12,14,15} these experiments were not able to address the contributions of specific Nox homologues. Furthermore, recent studies have questioned the specificity of currently available pharmacological inhibitors of NADPH oxidase.^{30} Genetic ablation of specific Nox homologues in knockout animals overcomes such limitations, and these models have served as valuable tools for studying the pathophysiological roles of specific Nox homologues in cardiovascular disease.^{7,17} However, global deletions of specific Nox enzymes make it difficult to interpret their role(s) within distinct brain regions.

The use of adenovirus-mediated delivery of siNox2 or siNox4 in this study enabled the selective silencing of distinct Nox homologues in discrete CNS nuclei. It should be noted that, whereas our cell culture studies show ≈80% to 90% silencing of Nox2^{22} and Nox4 using these reagents in vitro, Western blot analysis shows significant but incomplete silencing of Nox2 or Nox4 within brain tissue. We believe that this result is most likely related to the technical challenge of selectively harvesting the virally transduced SFO, which is only 0.1×0.2 mm in the mouse. As such, the Western data probably reflect Nox2 and Nox4 levels in areas immediately surrounding the SFO that were not transduced by our targeted delivery of AdsiNox2 or AdsiNox4. Related to this, we confirmed that ICV delivery of viral vectors is an efficient and reproducible method for targeting the SFO. However, as demonstrated in previous studies,^{27} this strategy also results in transduction of the ventricle lining, as well as occasional sparse transgene expression in the OVLT. Although it is highly unlikely that silencing Nox2 and/or Nox4 in the ependymal layer could impact the physiological responses to central Ang-II, we cannot entirely rule out the possibility that occasional targeting of a few cells of the OVLT might play some role in changes in cardiovascular parameters seen in our studies, especially given the pivotal role of this region in regulating water intake.^{31,32}

Our previous studies have demonstrated that the pressor, bradycardic, and dipsogenic responses to central Ang-II are almost entirely attenuated either by scavenging O$_2^-$ or by treatment with a dominant-negative mutant of Rac1, a major regulatory component of NADPH oxidase.^{10,12} Placed in the context of these findings, the present study suggests that both Nox2- and Nox4-containing NADPH oxidases are key sources of Ang-II--generated O$_2^-$ production in the SFO. Furthermore, our data suggest that Nox4, like Nox2, may be regulated either directly or indirectly by Rac1. Although the mechanisms of Nox2 activation have been studied extensively,^{16,33} the molecular events involved in the activation of Nox4 remain unresolved, including the specific role of Rac-GTPase. In studies involving heterologous Nox4 expression, activation of Nox4 may be independent of cytosolic subunits required for Nox2 activation, including p47, p67, and Rac.^{33,34} In addition, Nox4 lacks the specific residues present in Nox2 that are required for Rac-dependent regulation.^{35} It has also been suggested that Nox4 activity is regulated primarily at the transcriptional level.^{36} However, the kinetics of Nox4 activation observed in systems that express this homologue endogenously have suggested a requirement for regulatory subunits,^{33,34} and a recent study has provided indirect evidence for Nox4 regulation by the Rho GTPases Rac1 and RhoA.^{37}

A separate controversy exists regarding the mechanisms involved in the generation of ROS by Nox4. Although initial characterization studies demonstrated that Nox4 constitutively generates hydrogen peroxide,^{38} a more recent study using nitro blue tetrazolium staining suggests that Nox4, like Nox2, primarily generates O$_2^-$.^{36} The use of nitro blue tetrazolium in this study also underscores discrepancies regarding the measurement of Nox4-dependent ROS production using DHE. In the study by Serrander et al.,^{36} nitro blue tetrazolium was used because of the suggestion that Nox4-dependent ROS production occurs within an intracellular compartment that is accessible to nitro blue tetrazolium but not DHE. However, in the present study, we were indeed successful in using DHE to monitor Nox4-dependent ROS production, with our data showing that Ang-II--induced increases in DHE fluorescence in primary neurons were significantly blunted by inhibiting Nox4 expression. Our findings are supported by a study by Peshavariya et al.^{39} demonstrating that siRNA-mediated silencing of Nox4 expression inhibits DHE fluorescence in an endothelial cell line. The discrepancies in studies of Nox4 regulation, as well as inconsistencies in Nox4-dependent ROS measurements, are likely because of differences in cell type and systems used, as well as in variations in experimental procedure. Additional studies of Nox enzyme regulation and activation patterns in neurons are needed, because a full understanding of the biology of Nox4 and other Nox homologues will yield important clues regarding their unique functional roles in central Ang-II and other types of signaling.

At the present time we can only speculate as to the precise mechanisms by which ROS production by different Nox enzymes mediates specific physiological responses to central Ang-II. The SFO consists of an extremely diverse and uncharted population of cells,^{40} and it has been suggested that separate populations of neurons in the SFO differentially regulate the blood pressure and drinking effects of Ang-II. In rats, Ang-II infusion at low doses selectively activates neurons at the core of the SFO (as indicated by c-Fos staining) and raises pressure but does not induce a drinking response.^{31} Higher doses of Ang-II induce dipsogenesis and activate neurons both in the core as well as on the periphery of the SFO.^{31} In addition, other dipsogenic stimuli, including relaxin and hypertonic saline, activate neurons exclusively at the outer boundary of the SFO.^{31} In addition, patterns of known effluent projections from the SFO seem to support a separation of function. Projections to the bed nucleus of the stria terminalis, a region known to be important in regulating autonomic tone,^{41,42} arise only from the core of the SFO.^{41}
whereas projections to the paraventricular nucleus originate from the outer regions. In addition, it is thought that projections to the median preoptic nucleus originating in the periphery of the SFO are involved in dipsogenesis. Additional intrigue is added by the fact that the vasopressor and dipsogenic responses to central Ang-II are mediated by distinct isoforms of the AT1 receptor in the mouse; the AT1α receptor is linked to the pressor response, whereas the AT1β receptor is linked to the drinking response. Interestingly, the AT1α receptor is expressed primarily in the core of the SFO, supporting the notion that the location of this receptor is linked to its function. Although a lack of appropriate antibodies has prevented comprehensive immunohistochemical localization of these receptor subtypes, as well as the Nox homologues, in the SFO, it is tantalizing to consider the possibilities that the Ang-II receptor isoforms are differentially expressed in the core and peripheral regions of the SFO and that Nox2 and Nox4 are uniquely coupled to these different AT1 receptor isoforms, whereby both Nox2 and Nox4 are linked to the AT1α receptor, whereas only Nox2 is linked to the AT1β receptor.

Within this framework, one possibility is that the Nox homologues are differentially expressed in the SFO, whereby both Nox2 and Nox4 are expressed at the core of the SFO, in circuitry that gives rise to the pressor response, whereas only Nox2 would be expressed in outer regions of the SFO, in circuitry that underlies the dipsogenic response. However, it is just as possible that Nox2 and Nox4 are coexpressed in cells throughout the SFO but that Nox4-dependent ROS production is not a key feature of neurons involved in dipsogenesis. Such a scenario would involve compartmentalization of Nox-dependent redox signaling, whereby Nox2 or Nox4 may be used differentially by Ang-II within separate neuronal populations in the SFO. Interestingly, numerous examples of specialized roles for different Nox homologues within the same cell have been discovered in peripheral cardiovascular cells. Work from Hilenksi et al demonstrated that Nox1 and Nox4 to unique subcellular compartments in vascular smooth muscle cells, where these different homologues are thought to regulate unique temporal phases of Ang-II signaling. In this same cell type, Miller et al demonstrated that Nox1-dependent ROS production and subsequent transcription factor activation after cytokine stimulation occur within tightly regulated and compartmentalized signaling endosomes. In addition, a recent study by Wu et al demonstrated that Nox2 and Nox4 are independently linked to the activation of separate mitogen-activated protein kinase pathways in endothelial cells by HIV Tat. In support of this, Anilkumar et al reported that overexpression of Nox2 or Nox4 in HEK293 cells triggers unique profiles of protein kinase activation. Interestingly, in this same study, whereas both Nox2 and Nox4 were involved in intracellular responses to insulin, only Nox2 was found to be linked to Ang-II signaling, suggesting that Nox4 is not a player in the actions of Ang-II in this cell type. However, a recent study by Block et al demonstrated that, in glomerular mesangial cells, silencing of Nox4 prevents ROS formation, activation of protein kinases Src and 3-phosphoinositide-dependent protein kinase-1, and hypertrophy after Ang-II stimulation. These findings, along with our studies demonstrating that Nox4 mediates Ang-II-dependent ROS generation in neurons, indicate that the extent of Nox4 involvement in Ang-II signaling may vary by cell type and the stimulus used. Although subcellular expression patterns of Nox homologues in neurons have yet to be established, accumulating evidence in peripheral cell types strongly suggests that compartmentalization of redox signaling is a prevailing feature of Nox enzyme biology. Importantly, in the present study we found that both Nox2 and Nox4 are key sources of Ang-II-induced ROS formation in primary lamina terminalis cultures from mouse pups. Although it is possible that Nox expression patterns change during development and maturation, this finding suggests that at least a subset of neurons in the lamina terminalis does indeed coexpress Nox2 and Nox4. Thus, it is likely that Nox2 and Nox4 are coexpressed in cells throughout the SFO and that compartmentalization of Nox-dependent redox signaling exists such that Nox2 or Nox4 may be used differentially by Ang-II within separate neuronal populations in the SFO. As the molecular and biochemical tools become available, careful and meticulous localization of the Nox homologues at the regional, cellular, and subcellular levels will be a critical next step in determining the mechanisms linking Nox2 and Nox4 with central Ang-II–induced physiological responses.

**Perspectives**

Our results demonstrate that both Nox2- and Nox4-containing NADPH oxidases are required for the pressor effects of Ang-II, whereas only Nox2 is coupled with Ang-II–induced drinking. This study is the first to demonstrate that these Nox enzymes are differentially involved in mediating the volume regulatory and blood pressure effects of brain Ang-II. We speculate that the dysregulation of these enzymes is a major contributor to the pathogenesis of neurocardiovascular disease and that further characterization of the mechanisms underlying the differential roles of Nox homologues will lead to novel treatment strategies for a variety of cardiovascular disorders.

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**Disclosures**

None.

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Genetic silencing of Nox2 and Nox4 reveals differential roles of these NADPH oxidase homologues in the vasopressor and dipsogenic effects of brain angiotensin-II

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Short Title: Nox2 and Nox4 differentially linked to brain AngII
Material and Methods

Adenoviral Vectors

To achieve stable knockdown of specific Nox enzymes in targeted brain regions, we developed adenoviral vectors expressing siRNA targeted against Nox2, Nox4, or a control message (eGFP). All viral vectors were constructed, purified and provided by the University of Iowa Gene Vector Core as previously described ¹. In brief, 21 base pair short hairpin RNAs representing sequences directed against Nox2, Nox4, or eGFP were placed under the control of the mouse U6 promoter. The hairpin was juxtaposed almost immediately to the U6 transcription start site (within 6 base pairs) and was followed by a synthetic, minimal polyA cassette. After cloning into expression plasmids, in which a separate CMV promoter drives expression of a reporter gene (GFP for siNox2 and siNox4 expressing constructs, LacZ for the siGFP expressing construct), recombinant adenoviruses were generated from these siNox2 (AdsiNox2), siNox4 (AdsiNox4), and siGFP (AdsiGFP) constructs. Generation of siRNA targeted against Nox2 and GFP have been described previously ², ³. In designing siRNA targeted against Nox4, we initially tested 5 different sequences spaced through the mRNA of Nox4 using general rules for siRNA selection as described previously ⁴. In initial screening, the most effective siRNA directed against Nox4 (5' GGAACAAGTGCAATTTCTAAG-3') was located 1821 - 1841 bases from the start codon.

Measurement of Nox homologue expression in vitro

Primary neonatal mouse cardiomyocytes were dissociated from the hearts of 1-2-day old mouse pups and cultured using the Neonatal Cardiomyocyte Isolation System (Worthington, Lakewood, NJ) according to manufacturer’s instructions. After 24 hr of incubation under serum-free conditions, cardiomyocytes were treated with vehicle, AdsiGFP, AdsiNox2, or AdsiNox4 at 100 pfu/cell. M-1 kidney cortical duct cells (ATCC) were treated with vehicle, AdsiGFP or AdsiNox4 at the same concentrations. After 24 hours, effective viral transduction was assessed by monitoring for the presence of the reporter gene GFP using fluorescence microscopy. Cells were harvested 48 hours after transduction, and total RNA was isolated and reverse-transcribed. Quantification of Nox1, Nox2 and Nox4 transcript levels was performed by amplification of cDNA prepared from total RNA with an ABI 7500, using SYBR Green and primers specific for each of the Nox homologues and β-actin mRNA. Primer sequences used for Nox1, Nox2 and Nox4 have been described previously ². Primers used for β-actin are as follows: sense, 5' CATCCTCTTCCCTGGAGAAGA-3'; anti-sense, 5' ACAGGATTCCATACCAAGGAAGG-3'. Standard amplification conditions were utilized according to the manufacturer's specifications (Applied Biosystems). Relative expression levels of Nox mRNAs were analyzed using Sequence Detection Software v1.4 (Applied Biosystems) and the ΔΔCt method. Experiments were run in triplicate.

Detection of superoxide production in primary cell culture
Primary neuronal cultures were established from the lamina terminalis of C57Bl/6 pre-weanling pups (2-3 days old, 8-10 pups per culture) as previously described \(^5, 6\). Cells were cultured for 4 days in Dulbecco’s Modified Eagles Medium (DMEM):Ham’s F12 medium (1:1) supplemented with 10% FBS and 1% L-glutamine-penicillin-streptomycin. Cultures were treated with vehicle (saline) or infected with AdsiGFP, AdsiNox2, AdsiNox4, or AdsiNox2 and AdsiNox4 concomitantly 24 hours prior to loading with dihydroethidium (DHE, 5 \(\mu\)M) for 30 minutes. DHE fluorescence was imaged using confocal microscopy as described \(^6, 7\). After obtaining baseline images (0 minutes), cells were stimulated with Ang-II (1 \(\mu\)M) and reimaged after 30 minutes. DHE fluorescence was quantified using ImageJ analysis software (version 1.33, NIH) and expressed relative to baseline fluorescence in individual cells as described \(^7\). Experiments were run in triplicate.

**Physiological Studies**

Adult C57Bl/6 mice (Harlan, Indianapolis, Ind) (25-27g) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Mice then underwent microinjection of saline, AdsiGFP, AdsiNox2, AdsiNox4, or AdsiNox2 + AdsiNox4 for targeted gene delivery to the SFO as described previously \(^1, 8\). We have previously confirmed that our gene transfer protocols result in robust and stable transgene expression in the SFO \(^1, 6-9\). Following brain microinjections, mice were instrumented with ICV cannulae for central delivery of Ang-II, and radiotelemeters were implanted for conscious recordings of MAP and HR as described \(^7, 9, 10\). After 7 days of recovery from surgery, MAP and HR were recorded following ICV Ang-II (200ng, 200nl) as described \(^7, 9\). Central Ang-II-induced dipsogenic responses were measured by recording the total time spent drinking for 15 minutes following ICV Ang-II by an investigator blinded to treatment. All procedures conformed to the guidelines set forth by the NIH and were approved by the University of Iowa and Cornell University Animal Care and Use Committees.

**Viral transduction efficiency assessed by reporter gene expression**

One week after virus injections, mice were sacrificed and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in PBS. Brains were removed and saturated in 20% sucrose overnight at 4°C for cryoprotection. Serial coronal sections (30 \(\mu\)m) were cut on a cryostat. Sections from AdsiGFP(LacZ)-treated mice were processed for \(\beta\)-galactosidase activity via 2-hr incubation in 5-bromo-4-chloro-3-indoyl-\(\beta\)-D-galactopyranoside (X-gal, Boehringer Mannheim), counterstained with eosin, and analyzed by light microscopy as described \(^1\). Sections from AdsiNox2(GFP)- and/or AdsiNox4(GFP)-treated mice were processed for GFP expression by confocal microscopy (Zeiss 510).

**Immunoblot analysis for Nox homologue expression in the lamina terminalis of mouse brain**

Brains were removed from animals 7 days after ICV injection of saline, AdsiGFP, AdsiNox2, or AdsiNox4, and micropunches from the lamina terminalis, a brain region
encompassing the SFO, were pooled from 3 animals in each group and analyses were performed in triplicate. For analysis of Nox2 protein levels, membranes were probed with an anti-gp91phox antibody (BD Biosciences, 1:1000) and bands were visualized using IRDye 680 goat anti-mouse IgG (LI-COR Biotechnology, 1:20,000) with a LI-COR infrared imager (Odyssey). For analysis of Nox4 protein levels, membranes were probed with anti-Nox4 antibody (kind gift of Dr. David Lambeth, Emory University), and bands were visualized using HRP-conjugated goat anti-rabbit IgG (Santa Cruz, 1:10,000) with standard chemiluminescence detection. To ensure equal loading, actin levels were analyzed simultaneously.

Data and statistical analyses

Results are expressed as mean±SEM. Data were analyzed by Student’s t test for comparisons between groups, or ANOVA followed by the Tukey test for multiple comparisons. Statistical analyses were performed using Prism (GraphPad Software, Inc).

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