Role of the NADPH Oxidases in the Subfornical Organ in Angiotensin II–Induced Hypertension

Heinrich E. Lob, David Schultz, Paul J. Marvar, Robin L. Davisson, David G. Harrison

Abstract—Reactive oxygen species and the NADPH oxidases contribute to hypertension via mechanisms that remain undefined. Reactive oxygen species produced in the central nervous system have been proposed to promote sympathetic outflow, inflammation, and hypertension, but the contribution of the NADPH oxidases to these processes in chronic hypertension is uncertain. We therefore sought to identify how NADPH oxidases in the subfornical organ (SFO) of the brain regulate blood pressure and vascular inflammation during sustained hypertension. We produced mice withloxP sites flanking the coding region of the NADPH oxidase docking subunit p22phox. SFO-targeted injections of an adenovirus encoding cre-recombinase markedly diminished p22phox, Nox2, and Nox4 mRNA in the SFO, as compared with a control adenovirus encoding red-fluorescent protein injection. Increased superoxide production in the SFO by chronic angiotensin II infusion (490 ng/kg min−1 x 2 weeks) was blunted in adenovirus encoding cre-recombinase–treated mice, as detected by dihydroethidium fluorescence. Deletion of p22phox in the SFO eliminated the hypertensive response observed at 2 weeks of angiotensin II infusion compared with control adenovirus encoding red-fluorescent protein–treated mice (mean arterial pressures=97±15 versus 154±6 mm Hg, respectively; P=0.0001). Angiotensin II infusion also promoted marked vascular inflammation, as characterized by accumulation of activated T-cells and other leukocytes, and this was prevented by deletion of the SFO p22phox. These experiments definitively identify the NADPH oxidases in the SFO as a critical determinant of the blood pressure and vascular inflammatory responses to chronic angiotensin II, and further support a role of reactive oxygen species in central nervous system signaling in hypertension. *(Hypertension. 2013;61:382-387.)* ● Online Data Supplement

Key Words: blood pressure ■ central nervous system ■ inflammation ■ NADPH oxidase ■ vasculature

It is now clear that reactive oxygen species (ROS) are involved in the genesis of experimental hypertension. Superoxide (O2−) production is increased in the vasculature, kidney, and central nervous system in various models of hypertension,1,2 and scavenging O2− using membrane-targeted forms of superoxide dismutase (SOD) or SOD mimetics blunts hypertension in these models.3,4 The NADPH oxidases are major sources of ROS in hypertension. The catalytic subunits of these enzymes are the Nox proteins, which facilitate transfer of electrons from NADPH to molecular oxygen to form O2−. There are 5 Nox proteins, which vary in their means of activation, subunit binding partners, and output of O2−. Genetic deletion of the NADPH oxidase subunits Nox1, Nox2, and p47phox prevents many of the consequences of angiotensin II and renal artery stenosis, including elevation of blood pressure, increased renal vascular resistance, and endothelial dysfunction.5−7 All Nox proteins, except Nox 5, which does not exist in rodents, require the small membrane subunit p22phox. Angiotensin II increases vascular p22phox expression in vivo, and deletion of p22phox in vascular smooth muscle cells prevents ROS formation and the growth response to angiotensin II.8,9 Conversely, transgenic overexpression of vascular smooth muscle p22phox enhances hypertension and vascular smooth muscle hypertrophy, in response to angiotensin II.10 Recently, it has been recognized that ROS production is increased in several regions of the brain in hypertension, including the circumventricular organs,11 hypothalamic centers, and the brainstem.12 ROS increase neuronal firing in these regions, and ultimately increases sympathetic outflow. The subfornical organ (SFO) is particularly important in modulating these responses. This region is rich in angiotensin type 1 receptors, and is a major cardiovascular regulatory and dipsogenic center in the brain.13,14 Intracerebroventricular (ICV) administration of adenoviruses overexpressing SOD blunts the acute pressor effects of angiotensin II in the SFO, and in the long-term, prevents the hypertension caused by chronic angiotensin II infusion.11,15 Recent studies have shown that both Nox2 and Nox4 are involved in the acute pressor response to angiotensin II, whereas the dipsogenic response is dependent on Nox2.16 Likewise, ICV administration of an adenovirus expressing a dominant-negative form of the small G-protein, Rac1, prevents the acute pressor and water-drinking.
responses to angiotensin II.17 The roles of Nox2, Nox4, and Rac1 in chronic hypertension have not been defined in these acute studies.

In the present study, we sought to definitively define the role of NADPH oxidases in the SFO in hypertension caused by prolonged angiotensin II infusion. To accomplish this, we created mice with loxP sites flanking the coding region of p22phox, and used cre/lox technology to delete this molecule in the SFO. Our findings show that p22phox in the SFO is essential for hypertension and vascular inflammation caused by angiotensin II.

Materials and Methods

Creation of p22phox/flox Mice

A targeting vector was created in which a neomycin cassette flanked by loxP sites was cloned into an Apo1 site 7.1-kb upstream of the p22phox transcription start site. An additional loxP site was placed in intron 1. This was microinjected into embryonic stem cells, and proper homologous recombination was confirmed using Southern blots. Mice containing this targeted mutation were produced using standard techniques and backcrossed to C57Bl/6 mice for >11 generations. These mice were viable, developed normally, and had normal growth. All studies were performed according to a protocol approved by Emory and Cornell Universities Institutional Animal Care and Use Committees. For detailed Material and Methods, please see the online-only Data Supplement.

Results

Effect of Adenovirus Encoding Cre-Recombinase on p22phox mRNA and O2− Production in the SFO

In previous studies, we and others found that uptake of adenovirus after SFO-targeted ICV injection is greatest in the SFO, with uptake in other circumventricular organs to be limited to ependymal cells.18 We therefore focused on the SFO for measurements of p22phox mRNA and O2− production in the present studies. Quantitative real-time PCR on punch biopsies of the SFO showed markedly reduced p22phox mRNA after injection of adenovirus encoding cre-recombinase (AdCre), as compared with adenovirus encoding red-fluorescent protein (AdRFP) control injection, confirming successful targeting (Figure 1A). Interestingly, this was accompanied by a marked downregulation of Nox2 and Nox4 mRNA at baseline, and after 14 days of angiotensin II infusion (Figure 1A and Figure S1 in the online-only Data Supplement). Real-time PCR also revealed low levels of Nox1 in the SFO after 14 days angiotensin II in mice injected with AdRFP, and this was unchanged by AdCre injection (data not shown).

To investigate whether deletion of p22phox reduces O2− production in the SFO, we performed dihydroethidium staining using confocal microscopy and measured fluorescence at 405 nm. In keeping with previous studies,11 angiotensin II increased dihydroethidium fluorescence in the SFO of AdRFP-injected mice, and this was markedly reduced in mice with SFO deletion of p22phox by AdCre (Figure 2B). Heart rate was diminished by SFO p22phox deletion before and during angiotensin II infusion. Power spectral analysis

Effect of SFO p22phox Deletion on Hemodynamics

In mice treated with AdRFP, angiotensin II infusion caused a progressive increase in blood pressure to a value of 154±6 mm Hg. In striking contrast, mice with SFO deletion of p22phox had blood pressure that rose transiently at days 1 to 4 of angiotensin II infusion and then returned to normal values (Figure 2A).

To investigate whether deletion of p22phox in the SFO affects sympathetic activity, we performed power spectral analysis of mean arterial pressure. Blood pressure oscillations are influenced by sympathetic and parasympathetic neurons, the renin–angiotensin system, and vasoactive factors like nitric oxide. Power spectral analysis was used to detect which factors are influencing the blood pressure. Sympathetic outflow modulates low-frequency oscillations (0.015–0.6 Hz), thus absolute values divided by the total power of low-frequency provides an indirect measurement of sympathetic outflow.19 The power of low-frequency blood pressure oscillations was significantly increased after 14 days of angiotensin II infusion in AdRFP-injected mice, but not in mice with SFO deletion of p22phox by AdCre (Figure 2B).
of heart rate variability showed that angiotensin II increased the low-frequency/high-frequency ratio in AdRFP-treated, but not AdCre-treated, mice.

**Effect of p22phox Deletion in the SFO on Vascular Inflammation**

Flow cytometric analysis indicated that angiotensin II infusion promoted vascular inflammation, as reflected by infiltration CD45+ (Figure S2A) and CD3+ (Figure 3A) cells detected in single-cell suspensions of vascular homogenates from AdRFP-treated mice. Moreover, the infiltrating T-cells expressed the early activation marker CD69 (Figure 3B) and CD44high (Figure S2B), characteristic of effector memory T-cells. Deletion of p22phox in the SFO virtually eliminated the increase in vascular leukocytes, including CD69+ and CD44high T-cells.

**Effect of p22phox Deletion in the SFO on Vascular NADPH Oxidase Expression**

To determine whether deletion of p22phox or angiotensin II in the SFO affects vascular NADPH oxidase subunits, we measured levels of p22phox and Nox2 mRNA in the aorta. There was no change in p22phox or Nox2 expression in vehicle-infused mice (Figure 4A and Figure S3A). Vascular p22phox and Nox2 mRNA expression was upregulated in the aortas of both AdCre- and AdRFP-injected mice after 14 days of angiotensin II infusion. As in other studies,20,21 we observed a trend of reduced aortic Nox4 mRNA expression in response to angiotensin II; however, this did not reach statistical significance (Figure S3B).

Previous studies from our group and others have shown that angiotensin II stimulates vascular preproendothelin-1 expression.22 We confirmed this in the present study, and found that this was unaffected by deletion of SFO p22phox (Figure 4D). These data suggest that angiotensin II directly affects vascular p22phox, Nox2, and endothelin-1 expression, independent of sympathetic outflow or pressure increases.

**Discussion**

In the present study, we used cre–lox methodology to specifically delete p22phox in the SFO and showed that this reduced O2·− in this region. Moreover, deletion of p22phox in this brain region attenuated the hypertensive response and eliminated the vascular inflammation caused by angiotensin II. This study definitively identifies a critical role of the NADPH oxidases in the brain SFO, in this form of experimental hypertension.

Our findings are in agreement with previous reports showing that augmenting SOD or inhibiting Rac-1 activity in the SFO reduces the acute pressor and dipsogenic responses to ICV injections of angiotensin II.17,18 Previous studies by Peterson et al showed that silencing either Nox2 or Nox4 blocks the acute pressor response to angiotensin II. Silencing Nox2 also prevented the dipsogenic response to angiotensin II, but silencing Nox4 did not.16 Although it has been shown that augmenting SOD in the SFO inhibits chronic angiotensin II hypertension, our current study extends this previous observation by specifically identifying a critical role of the NADPH oxidases in the SFO in the long-term effect of angiotensin II.

In these experiments, we specifically targeted p22phox for selective deletion in the SFO. Although this subunit of the NADPH oxidase is not catalytically active, it is critical for functioning of all Nox isoforms. In the case of Nox2 activation, the proline-rich area in the C-terminal region of p22phox serves as a docking unit for activated p47phox.23 This region also binds p40phox, which activates Nox2 and competes for p47phox binding.24 Moreover, p22phox associates with and stabilizes the
Nox2 during processing and transport to the cell membrane. A similar role for Nox1 likely exists, in that we previously found overexpression of p22phox in transgenic mice markedly increased Nox1 expression. Nox1 associates with p22phox and point mutations in the proline-rich region of p22phox inhibit Nox1 function. Likewise, p22phox binds Poldip2, a newly recognized activator of Nox4. Thus, our deletion of p22phox in the SFO should have functionally disabled all Nox isoforms.

In a previous study, we showed that IVC injection of AdCre in mice with floxed extracellular SOD (SOD3flox mice) specifically deleted SOD3 in the SFO, but had no effect on SOD3 in peripheral tissues, suggesting that the adenosivirus does not gain access to peripheral sites, when injected ICV. We further showed that even intraperitoneal injection of the small amount of AdCre used for ICV injection had no effect on vascular SOD3 levels in this study. It is therefore unlikely that a recent study in which anteroventral third ventricle lesions prevented T-cell activation and vascular inflammation. The precise reason for this is unclear. It is possible that mRNA transcription or stability is redox regulated in neuronal cells. Although previously uninvestigated, it is also possible that p22phox has functions other than simply acting as a docking subunit for the Nox subunits that could modulate expression of other genes. Additional studies of examining transcriptional and posttranscriptional regulation of Nox2 and Nox4 mRNA would be necessary to address this issue.

There is ample evidence that oxidative signaling in central cardiovascular control centers modulates sympathetic outflow. Increased oxidative stress in the SFO increases sympathetic outflow and moderately elevates blood pressure. In elegant studies, Chan et al showed that angiotensin II in the rostral ventrolateral medulla increases O2·− via NADPH oxidase activation and causes increased neural firing. In spontaneous hypertensive rats, a model of neurogenic hypertension characterized by increased sympathetic nervous activity, reduction of O2·− in the rostral ventrolateral medulla blunts hypertension. In keeping with these data, power spectral analysis indicated that sympathetic outflow in response to angiotensin II is significantly diminished by deletion of p22phox in the SFO, supporting the concept that NADPH oxidase-derived O2·− enhances neuronal firing in these centers.

Recent studies from our laboratory have shown that T-cells are essential for the development of various forms of experimental hypertension. In these studies, we found that hypertension promotes accumulation of activated T-cells and macrophages in the perivascular fat of both large and small arteries. Our current findings are also compatible with a recent study in which anteroventral third ventricle lesions prevent T-cell activation and vascular inflammation. The

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**Figure 3.** The role of p22phox in the circumventricular organ on vascular inflammation in p22phox mice. A, CD3+ cell infiltration in aortic tissue in p22phox mice that received intracerebroventricular (ICV) injection of either adenosvirues encoding cre-recombinase (AdCre) or adenoviruses encoding red-fluorescent protein (AdRFP). Osmotic minipumps to deliver angiotensin II (490 ng/kg min−1) or vehicle were inserted after 10 days. B, the T-cell activation marker CD69 in aortic tissue. n=6; *P<0.05.

**Figure 4.** The effects of p22phox in the subfornical organ (SFO) on vascular NADPH oxidase and preproendothelin-1 expression. A, mRNA expression of p22phox in aortas of p22phox mice treated with either adenosvirues encoding cre-recombinase (AdCre) or adenoviruses encoding red-fluorescent protein (AdRFP), and 14 days of angiotensin II or vehicle infusion (n=4–5). B, mRNA expression levels of preproendothelin-1 in aortas of the same mice. n=5; *P<0.05.
virtual elimination of vascular inflammation after central deletion of p22phox might be a consequence of simply preventing hypertension. In this regard, we have previously shown that lowering blood pressure with hydralazine also prevents vascular inflammation in response to either angiotensin II or norepinephrine infusion. It is also possible that disruption of sympathetic stimulation reduces T-cell activation and vascular inflammation. In keeping with this concept, Ganta et al have shown that angiotensin II stimulates cytokine production by splenocytes via sympathetic activation. Innate and adaptive immune cells possess both α- and β-adrenergic receptors, and modulate immune functions such as antigen presentation by dendritic cells, clonal expansion of lymphocytes, migration, and cell trafficking.

Interestingly, the increase in vascular p22phox, Nox2, and preproendothelin-1 caused by angiotensin II infusion was not affected by p22phox deletion in the SFO. These results indicate that p22phox, Nox2, and preproendothelin-1 are regulated by direct actions of angiotensin II on vascular cells, independent of blood pressure and sympathetic outflow, and are in keeping with studies in which angiotensin II induces expression of these genes in cultured vascular cells. These findings also suggest that blockade of angiotensin II production and its receptors could have additional benefit beyond blockade of sympathetic outflow.

**Perspectives**

This study definitively identifies a critical role of the NADPH oxidases, and by inference, a role for ROS derived from the NADPH oxidases, in the SFO in modulation of hypertension and the systemic inflammation caused by angiotensin II. Furthermore, our study suggests that therapeutic interventions that target this central site could prevent the untoward effects of this common disease.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- This study confirms that NADPH oxidases in the subfornical organ are major sources of oxidative stress during angiotensin II–induced hypertension. This study is the first to make use of a mouse model in which p22<sup>lox/lox</sup>, necessary for function of all rodent Nox subunits, is selectively deleted using cre–lox technology. We also provide the first evidence that angiotensin II–induced changes in the vasculature, such as expression of preproendothelin and Nox2 are independent of blood pressure or sympathetic outflow.

**What Is Relevant?**

- This study is relevant because it confirms that NADPH oxidases in the circumventricular organs of the brain are the major reactive oxygen species–producing enzymes involved in angiotensin II–induced hypertension. These studies aid in understanding the mechanisms by which angiotensin II activates the subfornical organ.

**Summary**

In this study, we definitively identified a critical role of the NADPH oxidases in the subfornical organ in modulation of hypertension, and the systemic inflammation caused by angiotensin II. Furthermore, our study suggests that vascular effects of angiotensin II are independent of blood pressure. Therapeutic interventions that target the subfornical organ could prevent the untoward effects of this common disease.
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Heinrich E. Lob
David Schultz
Paul J Marvar
Robin L Davisson
David G. Harrison

Department of Biomedical Sciences, Cornell University, Ithaca, NY
Division of Cardiology, Department of Medicine Emory University School of Medicine, Atlanta, GA
Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY
Divisions of Clinical Pharmacology and Cardiology, Department of Internal Medicine, Vanderbilt University, Nashville, TN

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Corresponding Author:
David G. Harrison, MD,
Director of Clinical Pharmacology,
536 Robinson Research Building,
Vanderbilt University School of Medicine,
Nashville, TN, 37232-6602,
Phone: (615)-875-3049,
Fax: (615)-875-3297,
e-mail: david.g.harrison@vanderbilt.edu
Material and Methods

Deletion of p22\textsuperscript{phox}: Male p22\textsuperscript{phox/flox} mice at 3 months of age underwent a surgical procedure during which either an adenovirus expressing Cre-recombinase (AdCre) or red fluorescent protein (AdRFP) was injected ICV to target the subfornical organ as described (Coordinates: -0.3 mm bregma, ±1.0 mm midline, 3.2 mm dorsal).\textsuperscript{1} A radiotelemeter transmitter was also implanted for blood pressure measurements.\textsuperscript{2} Ten days later osmotic mini-pumps (Alzet Model 2002) were inserted for infusion of angiotensin II (490 ng/kg/min x 2 weeks) or its vehicle. All of the studies were performed according to protocols approved by the Emory and Cornell University Institutional Animal Care and Use Committee.

Quantitative real-time polymerase chain reaction (qrtPCR): Brains were removed immediately after euthanasia and frozen on dry ice. Micropunches of the subfornical organ were taken and homogenized. RNA was extracted using Trizol reagent and the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s protocol. RNA with an A260/280-ratio between 1.8 and 2.0 was used for reverse transcription using the qScript cDNA kit (Quanta BioSciences, USA). Quantitative real-time PCR was performed in an ABI 7500. Fast Thermocycler (Applied Bioscience, USA) using SYBR Green (SuperArray Bioscience). Primers for p22\textsuperscript{phox}, Nox2, Nox4, Preproendothelin-1 and 18S RNA were obtained from Integrated DNA Technologies. Total mRNA copy numbers were normalized to 18S RNA.

Measurement of superoxide: To measure O\textsubscript{2}\textsuperscript{−} in the subfornical organ we used dihydroethidium staining as described previously.\textsuperscript{3} Brains were frozen on dry ice and covered in Optimal-Cutting-Temperature (OCT). Slides were thawed at room temperature and warmed to 37°C for incubation with dihydroethidium for 30 minutes. Images were obtained using a confocal microscope at an excitation wavelength of 405 nm. Fluorescence at 405 nm excites 2-OH-E\textsuperscript{+} selectively, with minimal interference from other oxidation products, allowing the selective detection of O\textsubscript{2}\textsuperscript{−}.\textsuperscript{4}

Power Spectral Analysis of Heart rate and blood pressure: Data were extracted with a sampling rate of 2000 Hz using the Hemolab Software Suite Version 14.8 (http://www.haraldstauss.com/HemoLab). Artifact free heart rate and mean arterial pressure beat-to-beat data were resampled at a frequency of 25 Hz and converted from non-equidistant to equidistant time series. From this equidistant data a spectral analysis was performed using the Fast Fourier Transformation technique as previously described.\textsuperscript{5,6,7}

Flow cytometric analysis of inflammatory cells: Aortas were cleared of blood by perfusion with phosphate-buffered saline (PBS), excised, and digested using collagenase type IX (125 U/ml), collagenase type IV (450 U/ml), and hyaluronidase IS (60 U/ml) dissolved in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-PBS buffer containing calcium for 45 min. The digested tissue was then passed through a 70 mm sterile cell strainer (Falcon, BD), yielding single cell suspensions. Cells were then centrifuged (400 g), washed twice with PBS, resuspended in the fixable Near-IR dead cell staining solution (Invitrogen). After 15 min on ice 1% bovine serum albumin/PBS
buffer (FACS buffer) was added to the cells. Following another centrifugation step, cells were stained for 25 min at 4°C with antibodies and washed twice with FACS buffer. Cell labeling was performed using the following antibodies (all from BD Pharmingen): FITC anti-CD69 (H1.2F3); FITC CD44 (IM7); PerCP anti-CD45 (30-F11); APC anti-TCR-β (145-2C11). After immunostaining, cells were resuspended in FACS buffer containing 50 mL fluorescent counting beads (CountBright™, Invitrogen) and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). An initial gate was applied to exclude cell debris from further analysis, and only live cells positive for CD45 staining were used to identify leukocytes within the aortic cell suspension. All data were analyzed as total cell number.

**Statistical analysis:** Data are presented as mean ± SEM. Data from flow cytometry were analyzed with FlowJo software (Tree Star, Inc.) and two-way ANOVA was used to detect an interaction between the angiotensin II treatment and p22phox deletion. Blood pressure was analyzed using two-way ANOVA with repeated measures. Repeated measures ANOVA was employed to compare measurements of blood pressure and heart rate over time. Two-tailed Student’s t-test was used when two variables were compared.

**Literature:**

Figure S1: Selective deletion of p22^{phox} in the SFO. Real-time PCR confirmed diminished p22^{phox} mRNA after ICV injection targeting the SFO in mice that were injected with an adenovirus encoding for Cre-recombinase (AdCre). Levels of p22^{phox} mRNA were normalized to 18S RNA (n = 3, 2 brains per sample, *: P < 0.05).
Figure S2: The role of p22phox in the circumventricular organ on vascular inflammation in p22phox/flox mice. Panel A shows CD45+ cell infiltration in aortic tissue in p22phox/flox mice that received intracerebroventricular injection of adenoviruses encoding either cre-recombinase or red-fluorescent protein. Osmotic minipumps to deliver angiotensin II (490 ng/kg/min) or vehicle were inserted 10 days later. Panel B shows the T cell activation and homing marker CD44^{high} in aortic tissue. n = 6, *: P < 0.05.
Figure S3: The effects of p22^{phox} in the SFO on vascular NADPH oxidase subunit expression. Panels A shows mRNA expression of Nox2 in aortas of p22^{phox/flox} mice treated with AdCre or AdRFP and 14 days of angiotensin II or vehicle infusion. Panel B shows Nox4 mRNA expression in aortas of the same mice. n = 5-6, *: P<0.05.