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Mitochondrial-localized NADPH oxidase 4 is a source of superoxide in angiotensin II-stimulated neurons

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Mitochondrial-localized NADPH oxidase 4 is a source of superoxide in angiotensin II-stimulated neurons. Am J Physiol Heart Circ Physiol 305: H19–H28, 2013. First published April 26, 2013; doi:10.1152/ajpheart.00974.2012.—Angiotensin II (ANG II) plays an important role in the central regulation of systemic cardiovascular function. ANG II-mediated intraneuronal signaling has been shown to be mediated by an increase in mitochondrial superoxide (O$_2^{\cdot-}$), yet the source of this reactive oxygen species (ROS) production remains unclear. NADPH oxidase 4 (Nox4), a member of the NADPH oxidase family, has been reported to be localized in mitochondria of various cell types and has been implicated in brain angiotensinergic signaling. However, the subcellular localization and function of Nox4 in neurons has not been fully elucidated. In this study, we hypothesized that Nox4 is expressed in neuron mitochondria and is involved in ANG II-dependent O$_2^{\cdot-}$-mediated intraneuronal signaling. To query this, Nox4 immunofluorescent staining and mitochondrial enrichment were performed in a mouse catecholaminergic neuronal cell model (CATH.a). Nox4 was shown to be present in neuron mitochondria as evidenced by colocalization with both the mitochondrial-localized protein manganese superoxide dismutase (MnSOD) and dye MitoTracker Red. Moreover, Nox4 expression was significantly increased in enriched mitochondrial fractions compared with whole cell lysates. Additionally, adenoviral-encoded small interfering RNA for Nox4 (AdsiNox4) caused a robust knockdown in Nox4 mRNA and protein levels, which led to the attenuation of ANG II-mediated mitochondrial O$_2^{\cdot-}$ production. Finally, in the subfornical organ (SFO) of the brain, Nox4 not only demonstrated mitochondrial localization but was induced by chronic peripheral infusion of ANG II. Collectively, these data suggest that Nox4 is a source of O$_2^{\cdot-}$ in neuron mitochondria that contributes to ANG II intraneuronal signaling.

Nox4; mitochondria; angiotensin II; siRNA; CATH.a neurons; subfornical organ

Over the past decade, the importance of superoxide (O$_2^{\cdot-}$) in mediating angiotensin II (ANG II) intraneuronal signaling and contributing to the pathogenesis of neurocardiovascular diseases, including hypertension and heart failure, has been well established (19, 20, 38, 39, 41). Initial in vivo studies revealed that overexpression of superoxide dismutase (SOD) in various cardiovascular control brain regions improves systemic cardiovascular function in models of hypertension and heart failure (6, 19–21, 41). Considering SOD is an antioxidant enzyme that specifically scavenges O$_2^{\cdot-}$, these studies indicated that O$_2^{\cdot-}$ plays a critical role in cardiovascular diseases associated with enhanced brain angiotensinergic signaling. Subsequently, investigation turned to understanding ANG II intraneuronal signaling pathways modulated by O$_2^{\cdot-}$. We and others reported that the O$_2^{\cdot-}$ produced by ANG II stimulation of neurons leads to a variety of downstream sequelae including modulation of ion channel activity and increases in neuronal firing (31, 37, 42). Increasing evidence implicates that certain members of the NADPH oxidase (Nox) family are the primary source of O$_2^{\cdot-}$ in ANG II-stimulated neurons (24, 31, 40). However, other studies strongly suggest mitochondria as a primary source of O$_2^{\cdot-}$ as overexpression of manganese SOD (MnSOD), the SOD isoform specifically targeted to mitochondria, in the brain virtually abolished the acute, central ANG II-induced pressor response and decreased blood pressure in spontaneously hypertensive rats (SHR) (6, 41). Further, we recently reported that O$_2^{\cdot-}$ produced specifically in neuron mitochondria also contributes to the ANG II-mediated signaling cascade (37), but the cellular and molecular mechanisms underlying this mitochondrial-localized O$_2^{\cdot-}$ generation remain to be fully elucidated.

The Nox family of enzymes consists of seven isoforms (Nox1–5, and DUOX1–2), with varied tissue- and subcellular-specific expression of each enzyme (2). The Nox4 isoform has been shown to be expressed in an array of cell types including cardiomyocytes, mesangial cells, and neurons (12, 16, 18, 22, 33). In the brain, Nox4 has been demonstrated to play an important role in regulating neural control of cardiovascular function, as silencing of Nox4 in the paraventricular nucleus (PVN) attenuated sympathetic nerve activity and cardiac dysfunction in mice with myocardial infarction (15). Furthermore, knockdown of Nox4 in the subfornical organ (SFO), a cardiovascular control brain region that lacks a blood-brain barrier, significantly attenuated the acute pressor response to centrally administered ANG II (24). In cardiomyocytes and mesangial cells of the kidney, Nox4 was found to be expressed in mitochondria (3, 18). However, whether Nox4 is localized to the mitochondria in neurons, and if a relationship exists between Nox4, mitochondria-produced O$_2^{\cdot-}$, and ANG II intraneuronal signaling, remains unclear.

In the present study, we tested the hypothesis that Nox4 is localized to mitochondria in neurons and is a primary source of mitochondrial O$_2^{\cdot-}$ in ANG II-stimulated neurons. We show Nox4 protein is indeed localized in mitochondria of the catecholaminergic neuronal cell model (CATH.a neurons) as well as...
as the SFO. Furthermore, we demonstrate a causal relationship between Nox4 expression and mitochondrial $\text{O}_2^{-}$ production after ANG II stimulation. Overall, this report supports mitochondrial-localized Nox4 as a mechanistic player in mitochondrial-derived $\text{O}_2^{-}$ in ANG II-stimulated neurons.

**MATERIALS AND METHODS**

**Cell culture.** Mouse catecholaminergic CATH.a neuronal cells were purchased from American Tissue Cell Collection (ATCC, stock no. CRL-11179) and cultured in RPMI 1640 medium supplemented with 8% normal horse serum, 4% fetal bovine serum, and 1% 

![Image](image_url)
To examine mitochondrial localization of Nox4, neurons were differentiated for 6–8 days prior to experimentation by adding N2-2-O-dibutyryladenosine 3,5'-cyclic monophosphate sodium salt (1 mM, Sigma, St. Louis, MO) to the culture medium every other day, as we previously described (37). CATH.a neurons have been established to demonstrate ANG II-dependent intraneuronal signaling mechanisms through activation of AT1 and AT2 receptors, thus indicating these neurons are a viable model for these studies (30, 35, 37).

**Adenoviral vectors encoding siRNA.** Replication-deficient recombinant adenovirus encoding small interfering RNA (siRNA) for Nox4 (AdsiNox4) or the control vector encoding siRNA for the green fluorescent protein (AdsiGFP) were generous gifts from Dr. Robin Davison (Cornell University). Adenoviral vectors were constructed and purified by the University of Iowa Gene Vector Core, as described previously (24). Briefly, short hairpin RNAs (21 base pair) directed against Nox4 or GFP were placed under control of the U6 mouse promoter. Notably, the siNox4 adenosine also encodes for a GFP reporter gene, which is under the control of the CMV promoter. To determine the optimal silencing of Nox4, differentiated CATH.a neurons were infected with a range of viral multiplicity of infection (MOI) units ranging from 0 to 100. Additionally, a time-course experiment was performed on CATH.a neurons with 100 MOI of adenovirus for 0, 6, 12, 24, 48, or 72 h. Conventional PCR was used to validate transduction of either virus into CATH.a neurons utilizing the following primers: siVector forward, 5'-GCT GCA ATG ATA CCG CGA GAC CCA C-3'; siVector reverse, 5'-CAA GAG CAA CTC GGT CGC CGC ATA C-3'; GAPDH forward, 5'-GCC TTG CCC TTT GAG CTT GCT GAT G-3'; GAPDH reverse, 5'-GAC TGT GGC GTC TAA TTC GGC TTG-3'.

**Nox4 immunofluorescence, mitochondrial Co-localization, and confocal microscopy.** Differentiated CATH.a neurons were fixed with 4% paraformaldehyde and subjected to a standard immunofluorescence protocol as previously described (37). Briefly, neurons were incubated with blocking buffer [10% normal horse serum (NHS) and 0.3% Triton X-100 in 0.1 mol/l phosphate buffer (PB)]. After 1 h of incubation, neurons were incubated with Nox4 primary antibody (1:1,000 dilution, rabbit anti-human Nox4, Novus Biologicals, Littleton, CO) and then incubated with Alexa Fluoro 488 or 594 secondary antibody (1:500, anti-rabbit, Invitrogen, Carlsbad, CA) at room temperature for 2 h. After washout of secondary antibody, fluorescent images were captured by confocal laser scanning microscopy (Zeiss LSM 510 Meta). To examine mitochondrial localization of Nox4, neurons were coinubated with Nox4 antibody and an antibody directed against the mitochondria matrix protein, manganese superoxide dismutase (MnSOD; 1:500 dilution; sheep anti-human MnSOD, The Binding Site, Birmingham UK). Co-localization of Nox4 and MnSOD was determined by using an anti-rabbit Alexa Fluor 594 (red) and anti-sheep FITC-conjugated (green) secondary antibodies to detect Nox4 and MnSOD, respectively. In separate colocalization experiments, CATH.a neurons were loaded with MitoTracker Red (250 nmol/l, 20 min at 37°C), a red fluorescent mitochondrial marker, and Nox4 expression was observed using an anti-rabbit Alexa Fluor 488 (green) secondary antibody. For both colocalization experiments, expression of Nox4 in mitochondria was detected as yellow fluorescence in merged images.

**Mitochondria isolation.** Mitochondria were isolated from differentiated CATH.a neurons as previously described (37). Briefly, neurons were homogenized in ice-cold buffer A (225 mmol/l mannitol, 65 mmol/l sucrose, 10 mmol/l HEPES, and 1 mmol/l EGTA) using a glass Dounce homogenizer. The homogenates were centrifuged at 500 g for 6 min at 4°C and supernatants were collected and centrifuged at 10,000 g for 10 min at 4°C to obtain mitochondria-enriched fractions.

**Western blot analysis.** Standard Western blot analysis was utilized to examine Nox4 protein expression in whole neuronal cell lysates and isolated neuron mitochondria. Briefly, whole cell lysate or mitochondrial protein (5–20 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. To confirm the purity and enrichment of isolated mitochondrial fractions, membranes were incubated with antibodies directed against various subcellular proteins, including 1) mitochondria: MnSOD (1:2,000 dilution, Millipore, Billerica, MA); 2) endoplasmic reticulum (ER): calnexin (1:1,000 dilution, Abcam, Cambridge, MA); 3) cell membrane: pan-cadherin (1:2,000 dilution, Abcam); and 4) cytosol: lactate dehydrogenase (LDH, 1:1,000 dilution, Abcam). Nox4 expression in whole cell lysates and mitochondrial fraction was detected by incubating membranes with rabbit anti-human Nox4 (1:1,000 dilution, Novus Biologicals, Littleton, CO) at 4°C overnight. Protein levels were determined by the Pierce enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL) after membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Densitometric analysis of band intensity was determined using ImageJ analysis software.

**RNA extraction, cDNA, and quantitative real-time RT-PCR.** Total RNA was extracted from CATH.a neurons using the RNAeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Concentration of RNA was determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). Taqman reverse transcription kit (Applied Biosystems) was used to obtain
cDNA from total RNA. Generated cDNA was then subjected to SYBR green quantitative real-time PCR with primers specific to the coding sequence of Nox4 and GAPDH. Primer sequences were as follows (14): Nox4 forward, 5′-GGA TCA CAG AAG GTC CCT AGC AG-3′; Nox4 reverse, 5′-GCA GCT ACA TGC ACA CCT GAG AA-3′; GAPDH forward, 5′-CGT CCC GTA GAC AAA ATG-3′; GAPDH reverse, 5′-TAG TGG GGT CTC GCT CC-3′. PCR product specificity was determined by thermal dissociation. A threshold in the linear range of PCR amplification was selected and the cycle threshold (Ct) determined. Levels of transcripts were then normalized to the GAPDH loading control and compared relative to the control sample (noninfected neurons) using the ΔΔCt method. Experiments were run in triplicate, and data are expressed as percent change of control noninfected neurons.

Electron paramagnetic resonance (EPR) spectroscopy. EPR spectroscopy was used to measure intracellular $\text{O}_2^\cdot$ levels in noninfected, AdsiNox4-, or AdsiGFP-infected CATH.a neurons with or without ANG II as previously described (27). Briefly, neurons were incubated for 1 h at 37°C with the cell-permeable $\text{O}_2^\cdot$-sensitive spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 200 μM/l, Noxygen Science Transfer and Diagnostics, Elzach, Germany) in a Krebs-HEPES buffer (pH 7.4) containing (in mmol/l) 99 NaCl, 4.69 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.03 KH₂PO₄, 5.6 d-glucose, 20 HEPES, and supplemented with the metal chelators DETC (5 μM) and deferoxamine (25 μM). After this preloading period, cells were stimulated with ANG II (100 mmol/l) or saline for 5 min, then 50 μl of neuronal cell suspension was analyzed using a Bruker e-scan EPR spectrometer. EPR-CMH spectra were normalized to the number of cells in each sample, which were counted using a standard Coulter Counter (Beckman Coulter). The following EPR settings were used: field sweep width, 60.0 gauss; microwave frequency, 9.75 kHz; microwave power, 21.90 mW; modulation amplitude, 2.37 gauss; conversion time, 10.24 ms; time constant, 40.96 ms.

MitoSOX Red fluorescence. To measure mitochondrial-localized $\text{O}_2^\cdot$ levels, CATH.a neurons were loaded with MitoSOX Red (1 μmol/l for 20 min; Invitrogen, Carlsbad, CA), a mitochondria-targeted, $\text{O}_2^\cdot$-sensitive fluorogenic dye, as previously described (37). After the preloading period, fluorescence images were captured with confocal laser scanning microscopy (Zeiss LSM 510 Meta) before (baseline) and after ANG II (100 mmol/l) for 20 min) stimulation. MitoSOX Red fluorescence was detected using 405 nm excitation wavelength, which selectively detects the $\text{O}_2^\cdot$ specific 2-hydroxyethidium product of MitoSOX Red, as previously described (25, 26). Fluorescence intensity was quantified by establishing each neuron as a region of interest (ROI) using the Zeiss LSM 510 analysis software. Fluorescence was normalized in each cell by dividing the post-ANG II fluorescence intensity value by the baseline value of that particular cell.

Mouse studies. Adult C57Bl/6 mice (20–25 g; Harlan Sprague Dawley, Indianapolis, IN) were used for all in vivo experiments in this study. Mice were fed standard chow and water ad libitum. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Mice were implanted subcutaneously with osmotic mini-pumps (Alzet, Durect) delivering either ANG II (400 ng·kg⁻¹·min⁻¹) or saline for ~3 wk. At day 5 or 15 after the start of ANG II infusion, mice were euthanized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/l phosphate buffer (PB). Brains were harvested and transferred to 30% sucrose in PB prior to sectioning. Immunofluorescence was carried out as previously described for CATH.a neurons. For blood pressure recordings, mice were instrumented with radiotelemeters as previously described (4). Mice were allowed 1 wk for recovery before implantation of osmotic mini-pumps with measurements of mean arterial pressure (MAP) recorded daily until completion of the study.

Statistical analysis. Data are presented as means ± standard error of the mean (SE) and were analyzed by Student’s $t$-test for two-group comparisons or by ANOVA followed by Newman-Keuls correction for multiple comparisons. GraphPad Prism 5.0 statistical and graphing software was used. Differences were considered significant at $P < 0.05$.

RESULTS

Nox4 is localized to CATH.a neuron mitochondria. In the present study, we sought to determine if Nox4 is localized to neuron mitochondria. Using immunofluorescence staining and

Fig. 3. AdsiNox4 and AdsiGFP are effectively transduced and functional. A: conventional PCR analysis showing the presence of either adenoviral constructs within transduced CATH.a neurons, but absent in nontransduced cells. GAPDH shown for loading of equal DNA template. B: representative confocal microscopy images of CATH.a neurons 24 h after transduction with adenoviral constructs. Strong green fluorescence is noted in AdsiNox4 + AdsiEmpty transduced cells due to GFP coexpression in the AdsiNox4 construct. In contrast, GFP fluorescence from the AdsiNox4 is knocked down when AdsiNOX4 + AdsiGFP are cotransduced, thus demonstrating functionality of either adenoviral construct. Scale bar = 50 μm.
high-magnification confocal microscopy, we found Nox4 to be expressed in CATH.a neuron subcellular compartments with punctate morphology (Fig. 1A), which is characteristic of mitochondrial localization. Additionally, Nox4 immunofluorescence colocalized with a mitochondrial matrix protein, MnSOD (Fig. 1B). To rule out cross-reactivity of the Nox4 and MnSOD antibodies, Nox4 immunofluorescence was also colocalized with the mitochondrial dye, MitoTracker Red (Fig. 1C). Taken together, these data are strong evidence that Nox4 is found in the mitochondria of CATH.a neurons. To further evaluate Nox4 localization in neuron mitochondria, cellular fractionation was performed to isolate the mitochondrial subcellular compartment. Enrichment of the mitochondrial fraction was successful as indicated by strong immunoreactivity to MnSOD (mitochondrial protein), with little to no reactivity with calnexin (ER protein), pan-cadherin (cell membrane protein), or LDH (cytosolic protein; Fig. 2A). Furthermore, Nox4 immunoreactivity was enhanced in the mitochondrial fraction compared with the whole cell lysate in a similar manner to that observed with MnSOD (Fig. 2, B and C). Overall, these data strongly indicate Nox4 localization to the mitochondria in CATH.a neurons.

Knockdown of Nox4 in CATH.a neurons infected with AdsiNox4. To determine the causal relationship between Nox4 activation and ANG II-mediated mitochondrial \( \text{O}_2^- \) production in CATH.a neurons, we sought to decrease the expression of Nox4 using siRNA. To first characterize this knockdown, CATH.a neurons were transduced with adenoviruses encoding either Nox4 siRNA (AdsiNox4) or control siRNA (AdsiGFP). Transduction of the adenoviral vectors was validated by PCR (Fig. 3A). The AdsiNox4 construct coexpresses GFP and siRNA to Nox4 for further validation of transduction efficiency. Confocal microscopy images clearly show efficient viral transduction as strong GFP fluorescence was observed in CATH.a neurons infected with AdsiNox4 (Fig. 3B). The AdsiGFP vector does not possess a reporter construct, so to demonstrate functionality of the AdsiGFP construct we performed a cotransduction with the GFP-expressing AdsiNox4. Cotransduction of the AdsiNOX4 along with AdsiGFP resulted in decreased GFP fluorescence (Fig. 3B), thus confirming the functionality of the control siRNA for a non-Nox4 target. Next, we found Nox4 mRNA levels were reduced in a dose-dependent manner 24 h after AdsiNox4 infection (Fig. 4A). Compared with nontransduced and AdsiGFP-transduced neurons, 50
Mitochondrial Nox4 is a source of superoxide in ANG II-stimulated neurons. Using CATH.a neurons transduced with AdsiNox4 and EPR spectroscopy, we investigated mitochondrial Nox4 as a source of $\cdot O_2^-$ following ANG II stimulation. In noninfected and AdsiGFP-transduced neurons, ANG II significantly increased the amplitude of the EPR-CMH spectrum compared with nontransduced neurons without ANG II stimulation, indicating an increase in $\cdot O_2^-$ levels (Fig. 5, A and B). In contrast, the ANG II-induced increase in $\cdot O_2^-$ was markedly attenuated in AdsiNox4-transduced neurons. It was anticipated that knockdown of Nox4 in neurons would reduce basal levels of $\cdot O_2^-$; however, the baseline (i.e., without ANG II stimulation) EPR-CMH amplitude in AdsiNox4-transduced and 100 MOI of AdsiNox4 significantly decreased Nox4 mRNA levels in CATH.a neurons. Using 100 MOI of AdsiNox4, we assessed the time course of Nox4 mRNA levels and protein levels in CATH.a neurons transduced with AdsiNox4 for 24 h compared with nontransduced or AdsiGFP-transduced neurons (Fig. 4 C). Similar to the mRNA levels, AdsiNox4 decreased Nox4 protein only at the 24 h posttransduction time point (Fig. 4 D). These data indicate that AdsiNox4 (100 MOI) for 24 h significantly and specifically knocks down Nox4 mRNA and protein levels.

Fig. 5. Nox4 silencing attenuates ANG II-induced increase in intracellular superoxide levels. A: representative electron paramagnetic resonance (EPR) spectra obtained from nontransduced CATH.a neurons or neurons infected with AdsiGFP or AdsiNox4 (100 MOI, 24 h) and stimulated with ANG II (100 nmol/l, 5 min). Neurons were incubated with the superoxide-sensitive EPR spin probe CMH (200 µmol/l) for 1 h prior to ANG II administration and experimentation. B: summary data (n = 5 biological replicates in all experimental groups) of EPR amplitude obtained from CMH-treated CATH.a neurons either nontransduced or transduced with AdsiGFP or AdsiNox4 (100 MOI, 24 h) with and without ANG II stimulation (100 nmol/l, 5 min). AU, arbitrary units. *P < 0.05 vs. respective group without ANG II; #P < 0.05 vs. nontransduced or AdsiGFP + ANG II.

Fig. 6. Nox4 knockdown inhibits ANG II-induced increase in mitochondrial superoxide levels. A: representative confocal microscopy images of CATH.a neurons 24 h after transduction with either AdsiGFP or AdsiNox4. MitoSOX Red was allowed to preincubate with cells for 20 min and baseline images were recorded (left panels). ANG II (100 nmol/l) was added and images acquired after a 20-min incubation (right panels). Scale bar = 10 µm. B: summary data of ANG II-induced (100 nmol/l, 20 min) increase in MitoSOX Red fluorescence (mitochondrial superoxide sensitive dye) in CATH.a neurons infected with AdsiGFP or AdsiNox4 (n = 3 biological replicates, with >40 cells in all experimental groups quantified). Data expressed as fold increase in MitoSOX Red fluorescence from baseline (pre-ANG II; indicated by dotted line). Fluorescence intensity quantified using Zeiss LSM 510 confocal analysis software. *P < 0.05 vs. baseline; #P < 0.05 vs. AdsiGFP.
neurons was similar to that obtained from nontransduced and AdsI-GFP-transduced neurons (Fig. 5B). These data suggest that in CATH.a neurons Nox4 is not a constitutively active NADPH oxidase, but directly contributes to ANG II-mediated \( \text{O}_2^- \) production.

Knockdown of Nox4 inhibits ANG II-induced increase in mitochondrial \( \text{O}_2^- \) levels. We recently reported that ANG II stimulates an increase in mitochondrial-localized \( \text{O}_2^- \) in CATH.a neurons (37). To more closely investigate the contribution of Nox4 to generate mitochondrial \( \text{O}_2^- \), we queried mitochondrial \( \text{O}_2^- \) using MitoSOX Red fluorescence in neurons transduced with AdsI-Nox4 or AdsI-GFP. ANG II significantly increased MitoSOX Red fluorescence in control AdsI-GFP-transduced neurons indicating an increase in mitochondrial-localized \( \text{O}_2^- \) (Fig. 6, A and B). However, knockdown of Nox4 in AdsI-Nox4-transduced neurons significantly attenuated the ANG II-induced increase in mitochondrial-localized \( \text{O}_2^- \). Together, the data presented here strongly indicate that Nox4 in mitochondria is a primary source of mitochondrial-localized \( \text{O}_2^- \) in ANG II-stimulated neurons.

Nox4 is mitochondrial-localized and ANG II-regulated in the SFO of mice. To extend our findings outside of an in vitro neuronal cell culture model, we examined Nox4 expression and subcellular localization in the SFO, a known cardiovascular regulatory brain region. Nox4 demonstrated robust immunofluorescent staining in the SFO, and this immunoreactivity highly colocalized with the mitochondrial dye MitoTracker Green (Fig. 7). Interestingly, while the expression level of Nox4 appeared to increase with subcutaneous infusion of ANG II, the amount of colocalization remained static suggesting Nox4 mitochondrial localization at baseline conditions. To understand if Nox4 upregulation was a sustained event with ANG II infusion, SFO regions were examined in brains of mice infused with ANG II or saline for 5 or 15 days. Similar to Fig. 7, the SFO of ANG II-infused animals possessed higher levels of Nox4 immunofluorescence and this increase was sustained at both 5 and 15 days of infusion (Fig. 8A). Validating this model of ANG II-dependent hypertension, systemic blood pressure was increased in these animals throughout the infusion period and returned to near baseline after the minipumps emptied (Fig. 8B). Overall, these in vivo studies confirm and extend our findings in the CATH.a neurons, and create a link among mitochondrial \( \text{O}_2^- \), Nox4, and ANG II-mediated signaling in neurons.

**DISCUSSION**

Nox4, a member of the NADPH oxidase family, was first identified as a renal-specific protein and was originally named renal NADPH oxidase (Renox) (9, 12). Since then, numerous studies have demonstrated Nox4 expression in a variety of cell types including cardiomyocytes and neurons (1, 33). Moreover, utilizing in vivo models of neurocardiovascular diseases, Nox4 has been shown to play a distinct role in neural control of cardiovascular function. For example, silencing of Nox4 in the paraventricular nucleus (PVN) of the brain greatly improved cardiac function, attenuated sympathoexcitation, and decreased myocardial apoptosis in a mouse model of myocardial infarction (15). Furthermore, Nox4 expression in the PVN of mice has been shown to contribute to sympathoexcitation and hypertension induced by mineralocorticoid excess (34). Additionally, silencing Nox4 in the blood-brain barrier-deficient SFO has been shown to inhibit the central ANG II-induced pressor response in mice (24). While these previous studies clearly elucidated an in vivo functional role for neuronal Nox4 in the pathogenesis of neurocardiovascular diseases, our current study focused on the subcellular location of Nox4 in neurons and examined the contribution of Nox4 to the elevated levels of mitochondrial-localized \( \text{O}_2^- \) observed in ANG II-stimulated neurons (36).

The mitochondrion is a major source of \( \text{O}_2^- \) generation during both normal and pathological cellular processes. The
first study implicating a contribution of mitochondrial-produced $\text{O}_2^{\cdot\cdot}$ in mediating central ANG II-induced cardiovascular dysfunction showed that overexpression of MnSOD in the SFO of mice prevents the pressor response to centrally-administered ANG II (41). Subsequent studies have provided abundant and clear evidence that mitochondrial-produced $\text{O}_2^{\cdot\cdot}$ in the rostral ventrolateral medulla (RVLM) of rats contributes to neurogenic hypertension (5, 6, 17, 23). More recently, we have shown that ANG II stimulation of neurons increases levels of mitochondrial-derivative $\text{O}_2^{\cdot\cdot}$, which mediates, at least in part, ANG II-induced inhibition of outward $\text{K}^{\cdot\cdot}$ current (37). This increase in $\text{O}_2^{\cdot\cdot}$ and modulation of $\text{K}^{\cdot\cdot}$ current is attenuated by the overexpression of MnSOD (37). Collectively, these studies demonstrate that mitochondrial-produced $\text{O}_2^{\cdot\cdot}$ in central neurons contributes to ANG II pathophysiological responses; however, the precise source of the mitochondrial-produced $\text{O}_2^{\cdot\cdot}$ has remained unclear.

Nox4 has been reported to be localized to the nucleus and endoplasmic reticulum (8), but recently has been demonstrated as a mitochondrial-targeted enzyme possessing a mitochondrial localization sequence (3, 13, 18). The first description of Nox4 in mitochondria was in the renal cortex of diabetic rats (3). Later, it was reported that cardiomyocytes also display mitochondrial localized Nox4 that is upregulated by hypertrophic stimuli (1). In this pivotal study, Nox4 was implicated in oxidative stress-mediated apoptosis and mitochondrial dysfunction. Furthermore, deletion of Nox4 was shown to attenuate mitochondrial $\text{O}_2^{\cdot\cdot}$ production, which suggested a causal relationship between mitochondrial $\text{O}_2^{\cdot\cdot}$ generation and pathogenesis (1, 18). It must be noted that in these studies the electron transport chain (ETC) was also implicated in mitochondrial $\text{O}_2^{\cdot\cdot}$ generation, but that Nox4 augmented this production in all experimental conditions. The data presented herein clearly show Nox4 colocalization with mitochondrial markers in both mouse CATH.a neurons and in the SFO of mouse brains. Moreover, Nox4 detection was significantly enhanced in mitochondria-enriched protein fractions. These data support the notion that Nox4 is highly expressed in mitochondria of ANG II-sensitive neurons.

NADPH oxidases are generally regarded as $\text{O}_2^{\cdot\cdot}$-producing enzymes, but recent evidence of $\text{H}_2\text{O}_2$ production by Nox4 contradicts this unifying theory (32). Utilizing human embryonic kidney cells, Serrander et al. showed $\text{H}_2\text{O}_2$ production by Nox4 in an mRNA dose-dependent manner (29). In rat aortic smooth muscle cells, Nox4 activation induced by ANG II was shown to contribute to basal $\text{H}_2\text{O}_2$ production, while NOX1 determined $\text{O}_2^{\cdot\cdot}$ production (11). Furthermore, Nox4 production of $\text{H}_2\text{O}_2$ was shown to be protective to the vasculature of mice in response to ischemic or inflammatory stress (28). In the current study, we demonstrate that Nox4 contributes to mitochondrial-produced $\text{O}_2^{\cdot\cdot}$ in response to ANG II in neurons. The discrepancy in the specific ROS produced may be due in part to cell type specificity. Relative levels of Nox4 enzyme, NADPH substrate, antioxidant enzyme concentrations, metabolic activity, and other Nox family isoforms may all contribute to the steady-state levels of mitochondrial-ROS produced by these enzymes. Furthermore, it has been suggested that Nox isoforms may interact with elements of the electron transport chain within the mitochondria, thus indirectly initiating production of mitochondrial $\text{O}_2^{\cdot\cdot}$ (10, 43). We show that ANG II-mediated mitochondrial $\text{O}_2^{\cdot\cdot}$ production in neurons can be inhibited by downregulation of Nox4 specifically. Nevertheless, whether this $\text{O}_2^{\cdot\cdot}$ is a direct product of Nox4 activity or indirectly produced through disruption of mitochondrial metabolism remains unknown.

Similar to previously published in vivo studies (24), we found that knockdown of Nox4 in CATH.a neurons using AdsNox4 had no effect on Nox2 mRNA (data not shown), thus implying Nox4 is a major mitochondrial $\text{O}_2^{\cdot\cdot}$-producing Nox isofrom in this cell type following ANG II stimulation. This implication may be of particular importance considering previous work by Peterson et al. (24). In their study, silencing Nox2 or Nox4 independently in the SFO of mice modestly, but significantly, inhibited the central ANG II-induced pressor response, whereas knockdown of both Nox2 and Nox4 concurrently virtually abolished this ANG II response. In addition, knockdown of Nox2, but not Nox4, in the SFO attenuated the ANG II-induced dipsogenic response. This study suggests cooperation and differential contribution of these Nox enzymes in mediating central ANG II-induced cardiovascular responses.
Together with our data presented herein, we speculate the differential contribution of Nox2 vs. Nox4 may be due, at least in part, to Nox4 initiating redox signaling from mitochondrial-produced $O_2^-$, while Nox2 is thought to increase levels of cytoplasmic $O_2^-$. Certainly, additional studies are needed to address this hypothesis.

**Conclusions.** ANG II plays a fundamental role in neurogenic cardiovascular regulation in which $O_2^-$ are implicated as downstream modulators of intraneuronal redox signaling (39). Accumulating evidence has demonstrated that the NADPH oxidase family of enzymes is the major source of ANG II-induced increases in $O_2^-$ levels (7, 39). Furthermore, recent evidence suggests that mitochondria are a major subcellular site of $O_2^-$ generation in neurons after ANG II stimulation (6, 37). The data presented here strongly illuminate Nox4 localization to neuron mitochondria, and furthermore suggest a causal link to Nox4 expression and mitochondrial $O_2^-$ production in ANG II-stimulated neurons. Overall, we propose that Nox4 is a major mitochondrial source of $O_2^-$ in neurons and plays a mechanistic role in ANG II-dependent intraneuronal signaling.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**