Nox2, Ca\(^{2+}\), and Protein Kinase C Play a Role in Angiotensin II–Induced Free Radical Production in Nucleus Tractus Solitarius

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Abstract—The dorsomedial portion of the nucleus tractus solitarius (dmNTS) is the site of termination of baroreceptor and cardiorespiratory afferents and plays a critical role in cardiovascular regulation. Angiotensin II (Ang II) is a powerful signaling molecule in dmNTS neurons and exerts some of its biological effects by modulating Ca\(^{2+}\) currents via reactive oxygen species (ROS) derived from reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. We investigated whether a Nox2-containing NADPH oxidase is the source of the Ang II—induced ROS production and whether the signaling mechanisms of its activation require intracellular Ca\(^{2+}\) or protein kinase C (PKC). Second-order dmNTS neurons were anterogradely labeled with 4-(4-[didecylamino]styryl)-N-methylpyridinium iodide transported from the vagus and isolated from the brain stem. ROS production was assessed in 4-(4-[didecylamino]styryl)-N-methylpyridinium iodide–positive dmNTS neurons using the fluorescent dye 6-carboxy-2',7'-dichlorodihydro-fluorescein di(acetoxymethyl ester). Ang II (3 to 2000 nmol/L) increased ROS production in dmNTS neurons (EC\(_{50}\) = 38.3 nmol/L). The effect was abolished by the ROS scavenger Mn (III) porphyrin 5,10,20-tetrakis (benzoic acid) porphyrin manganese (III), the Ang II type 1 receptor antagonist losartan, or the NADPH oxidase inhibitors apocynin or gp91ds-tat. Ang II failed to increase ROS production or to potentiate L-type Ca\(^{2+}\) currents in dmNTS neurons of mice lacking Nox2. The PKC inhibitor GF109203X or depletion of intracellular Ca\(^{2+}\) attenuated Ang II–elicited ROS production. We conclude that the powerful effects of Ang II on Ca\(^{2+}\) currents in dmNTS neurons are mediated by PKC activation leading to ROS production via Nox2. Thus, a Nox2-containing NADPH oxidase is the critical link between Ang II and the enhancement of Ca\(^{2+}\) currents that underlie the actions of Ang II on central autonomic regulation. (Hypertension. 2006;48:482-489.)

Key Words: arterial hypertension • baroreflex • calcium channels • oxidative stress • blood pressure • autonomic nervous system

A select group of brain stem nuclei regulates the systemic circulation by modulating cardiac output, vascular resistance, and fluid balance.1,2 In particular, the dorsomedial region of the nucleus of the solitary tract (dmNTS), where vagal afferents from aortic baroreceptors and cardiorespiratory chemoreceptors terminate, plays a major role in cardiovascular regulation.3-5 There is increasing evidence that angiotensin II (Ang II) is a critical neuromodulator in central autonomic nuclei,6-9 including the dmNTS.10-13 Within the dmNTS, activation of Ang II type 1 (AT\(_1\)) receptors alters cardiorespiratory reflexes including baroreceptor excitability and ion channel permeability.10,14 These changes may contribute to Ang II–induced sympathoexcitation,15-18 hypertension,15,16 and heart failure.17,18 Superoxide generated by the enzyme NADPH oxidase has emerged as a key intermediary in the central and peripheral effects of Ang II.10,14-16,18-21 NADPH oxidase, initially described in neutrophils,22,23 is now known to be present in diverse cell types, including neurons.15,16,24-26 NADPH oxidase is composed of membrane-bound subunits, gp91\(^{phox}\) and p22\(^{phox}\), and cytoplasmic subunits, p47\(^{phox}\), p40\(^{phox}\), p67\(^{phox}\), and the small GTPase Rac1 and/or Rac2.22,23,27-30 The catalytic subunit gp91\(^{phox}\), also termed Nox2, has several homologues, Nox1 and Nox3 through Nox5, the location of which is cell-type specific.22,23 On stimulation of AT\(_1\) receptors by Ang II, p47\(^{phox}\) is phosphorylated resulting in the assembly of the enzyme and production of superoxide.23,31,32 In some cell types, protein kinase C (PKC) activation via intracellular Ca\(^{2+}\) is a critical step in p47\(^{phox}\) phosphorylation and subsequent enzyme assembly.26,33,34

In dmNTS neurons, NADPH oxidase subunits are present in close association with AT\(_1\) receptors.25 Furthermore, reactive oxygen species (ROS) scavengers and NADPH oxidase inhibitors attenuate Ang II–elicited enhancement of Ca\(^{2+}\)
currents in these neurons.\(^{25}\) These findings have raised the possibility that NADPH oxidase-derived ROS are involved in the effects of Ang II on Ca\(^{2+}\) currents in dmNTS. However, direct evidence linking AT\(_1\) receptors to NADPH oxidase–dependent ROS production through intracellular Ca\(^{2+}\) and PKC activation in dmNTS is missing. Furthermore, it is not known whether Nox2 is the catalytic subunit of NADPH oxidase that mediated the ROS production. In the present study, we used ROS imaging, whole-cell patch clamping, and Nox2-null mice to determine whether Ang II induces ROS production in dmNTS neurons and, if so, whether Nox2 plays a role in Ang II–induced ROS production and in the attendant changes in Ca\(^{2+}\) currents.

**Methods**

All of the experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee at Weill Medical College of Cornell University.

**Materials**

Fluorescent dyes 4-(4-[didecylamino]styryl)- N-methylpyridinium iodide (DiA) and 6-carboxy-2',7'-dichlorofluorescein di-acetoxyethyl ester (C-DCDHF-DA) were purchased from Molecular Probes. Rosanin was a gift from Merck and DuPont. Nox2-null mice were obtained from an in-house colony.\(^{26,27}\) C57Bl/6J mice were used as wild-type (WT) controls. The polypeptide gp91ds-tat and its scrambled version\(^{35}\) were synthesized by Bio-Synthesis.

**Anterograde Labeling of NTS Neurons Receiving Vagal Afferents**

Second-order vagal afferent neurons in the NTS were labeled with DiA in male Sprague–Dawley rats (2 to 4 weeks old) or in the male adult mice, as described previously.\(^{25,36,37}\) Briefly, animals were anesthetized by a mixture of 8 mg/kg of ketamine and 8 mg/kg of xylazine. The right vagus nerve was isolated from the surrounding tissues, and a few crystals of DiA were placed on the nerve at the level of the carotid bifurcation and caudal to the nodose ganglion. The region was sealed with silicone elastomer (World Precision Instruments) to prevent dye leakage.

**Dissociation of Second-Order NTS Neurons**

Seven to 10 days after labeling, animals were euthanized by CO\(_2\), and the brain stem was quickly removed and transferred to a chamber containing ice-cold sucrose-artificial cerebrospinal fluid.\(^{25}\) Coronal slices were obtained and incubated with 0.02% pronase and thymolysin at 36°C in oxygenated lactate-containing artificial cerebrospinal fluid.\(^{25}\) Using the area postrema as a landmark, the dmNTS was punched, and neurons were isolated. DiA-labeled presynaptic boutons from first-order neurons, remaining attached to second-order NTS neurons, were identified using a Nikon Diaphot 300 inverted fluorescence microscope. Unlabeled dmNTS neurons were also obtained from the contralateral NTS region of the same animal. The criteria for identification of the unlabeled dmNTS neurons were based on their anatomic location in the dorsomedial portion of the NTS and their typical cell morphology, for example, small round or oval bipolar cells with long thin processes.\(^{25,36–40}\)

**Immunofluorescent Labeling**

Dissociated dmNTS neurons were fixed in 4% paraformaldehyde. Neurons were permeabilized, treated with 3% BSA and incubated in a primary antisera mixture including rabbit anti-AT\(_1\) receptor (1:100)\(^{25,33,35}\) and goat anti-gp91phox (1:50).\(^{25}\) The neurons were then incubated in a mixture containing anti-rabbit Texas Red and anti-goat fluorescein isothiocyanate antisera (Jackson ImmunoResearch) and were visualized using the Nikon Diaphot 300 microscope.

**Detection of Intracellular ROS**

ROS production was assessed using C-DCDHF-DA.\(^{41–43}\) C-DCDHF-DA loses its diacetyl groups by cleavage via intracellular esterases and is oxidized by ROS to dichlorofluorescein (DCF). The isolated neurons were incubated with 5 μmol/L C-DCDHF-DA for 30 minutes. Time-resolved fluorescence was measured every 30 s using IPLab, an image analysis software from Synalitics Inc. Recordings were started after a stable baseline was achieved. In all of the experiments, concurrent vehicle recordings were performed. No differences in the increase of DCF fluorescence induced by Ang II were observed between the DiA-labeled and DiA-unlabeled neurons selected from the contralateral dmNTS according to morphological criteria. Therefore, in all of the subsequent experiments, results from labeled and unlabeled neurons were pooled.

**Electrophysiology**

Voltage-gated Ca\(^{2+}\) currents were elicited using the whole-cell configuration of patch clamping.\(^{25,39,40,44}\) An Axopatch-200A patch clamp amplifier was used with the Cs\(^+\) electrode solution.\(^{25}\) Using 2 mmol/L Ca\(^{2+}\) as a charge carrier, Ca\(^{2+}\) currents were elicited by 500-ms pulses from a holding potential of −60 mV to −20 mV using pClamp 8 (Axon Instruments).

**Data Analysis**

Data are expressed as mean±SEM. Paired or unpaired Student t test was performed. ROS data are expressed as the ratio Ft/Fo, where Ft is fluorescence after the application of Ang II in a given cell, and Fo is the baseline fluorescence of the same cell immediately before application of Ang II. Fo ranged from 105.2 to 169.7 relative fluorescence units. There was no relationship between baseline fluorescence (Fo) and the fluorescence increase induced by Ang II (Ft) under the experimental conditions studied.

**Results**

**DIA-Labeled dmNTS Neurons Express AT\(_1\) Receptors and Nox2**

To determine whether AT\(_1\) receptors and Nox2 were present in the same dmNTS neurons in which ROS were assessed, we examined AT\(_1\) receptor and Nox2 immunoreactivity in DiA-labeled rat dmNTS neurons. DiA-labeled presynaptic boutons from first-order vagal afferent neurons were observed on isolated second-order dmNTS neurons (Figure 1A). The majority of DiA-labeled neurons examined were immunoreactive for AT\(_1\) and/or Nox2 (Table). Thus, AT\(_1\) receptors and Nox2 coexist in dmNTS neurons receiving vagal afferents.

**Ang II Increases ROS Production and Ca\(^{2+}\) Currents in dmNTS Neurons**

We investigated effects of Ang II on ROS production and Ca\(^{2+}\) currents in rat dmNTS neurons. Ang II was applied at concentrations from 3 mmol/L, comparable to endogenous Ang II concentrations in brain tissues.\(^{45}\) In vehicle-treated neurons, DCF fluorescence remained stable during the monitoring period (Figure 1B and 1C). Ang II dose-dependently increased DCF intensity, reflecting an increase in ROS. The effect was initially observed at 3 mmol/L (P<0.05 versus control; n=8) and reached a plateau at 100 mmol/L with an EC\(_{50}\)=38.3 mmol/L (Figure 1D). In subsequent studies we tested the effect of Ang II at concentrations of 30 to 100 mmol/L. Ang II (100 mmol/L) was also able to enhance the nifedipine-sensitive L-type Ca\(^{2+}\) current (P<0.05 versus control; n=4; Figure 2). Hydrogen peroxide (2 mmol/L), a product of the dismutation of superoxide by superoxide dismutase, also increased the
L-type Ca\(^{2+}\) current in dmNTS neurons \((P<0.05 \text{ versus control; } n=4; \text{ Figure 2})\). Finally, the ROS scavenger Mn (III) porphyrin 5,10,15,20-tetakis (benzoic acid) porphyrin manganese (III) (MnTBAP)\(^{46}\) (30 \(\mu\)mol/L) blocked the Ang II–induced ROS production (Figure 3A). These observations indicate that nanomolar concentrations of Ang II increase ROS production and Ca\(^{2+}\) currents in dmNTS neurons.

**AT\(_1\) Receptors Mediate ROS Production by NADPH Oxidase in dmNTS Neurons**

To determine the Ang II receptor subtype\(^{47}\) responsible for the ROS production in rat dmNTS neurons, we examined the effects of the AT\(_1\) receptor inhibitor losartan or the AT\(_2\) receptor inhibitor PD123319.\(^{48}\) Losartan (3 \(\mu\)mol/L) blocked the Ang II–induced increase in DCF \((P>0.05 \text{ versus control; } n=8)\), whereas PD123319 (40 \(\mu\)mol/L) did not (Figure 3B). Pretreatment (30 minutes) with the NADPH oxidase assembly inhibitor apocynin (1 mmol/L)\(^{49}\) or the peptide inhibitor gp91ds-tat (1 \(\mu\)mol/L)\(^{35}\) blocked the Ang II–induced increase in DCF \((P>0.05 \text{ versus control})\). In contrast, the 1 \(\mu\)mol/L scrambled version of gp91ds-tat had no effect (Figure 3C). These data indicate that Ang II induces ROS production in dmNTS neurons via AT\(_1\) receptors and NADPH oxidase.

**Figure 1.** Ang II potentiates ROS production in rat dmNTS neurons. A, AT\(_1\) receptors and Nox2 immunoreactivities are coexpressed in the same DiA-labeled dmNTS neuron. B, Ang II (100 nmol/L) increases DCF fluorescence in a DiA-labeled dmNTS neuron. C, Time course of DCF intensity in a single dmNTS neuron treated with vehicle (open symbol) or Ang II (closed symbol). D, Dose-response curve of the Ang II–induced potentiation of DCF (control: \(n=17\); Ang II: 3 nmol/L, \(n=8\); 10 nmol/L, \(n=8\); 30 nmol/L, \(n=17\); 100 nmol/L, \(n=17\); 2000 nmol/L, \(n=16\)). Scale bars, 10 \(\mu\)m. *\(P<0.05\) vs vehicle; **\(P<0.01\) vs vehicle.
Nox2 Is Critical for Ang II–Induced Ca\(^{2+}\) Currents and ROS Production in dmNTS Neurons

To obtain direct evidence that Nox2 is involved in Ang II–induced L-type Ca\(^{2+}\) current or superoxide production, we compared Ang II–induced L-type Ca\(^{2+}\) currents and ROS production in dmNTS neurons isolated from WT and Nox2-null mice. Ang II significantly potentiated L-type Ca\(^{2+}\) currents of dmNTS neurons in WT mice (\(P<0.05\) versus control; \(n=4\)) but not in Nox2-null mice (Figure 4B and 4C). Similarly, Ang II increased DCF intensity in WT mice (\(P<0.01\) versus control; \(n=6\)) but not in Nox2-null mice (Figure 4D). These results suggest that the enhancement of both L-type Ca\(^{2+}\) currents and ROS production by Ang II depends on ROS derived from Nox2.

To examine the role of intracellular Ca\(^{2+}\) in Ang II–induced ROS production, rat dmNTS neurons were treated continuously with thapsigargin (10 \(\mu\)mol/L) to deplete intracellular Ca\(^{2+}\) stores. \(^{50}\) Pretreatment with thapsigargin (for 30 minutes) partially attenuated the increase in DCF elicited by Ang II (\(P<0.05\) versus control; \(n=6\)). Pretreatment with thapsigargin in conjunction with removal of extracellular Ca\(^{2+}\) (for 30 minutes), however, completely blocked the Ang II–induced ROS production (\(P<0.05\) versus control; \(n=7\); Figure 5). To determine whether Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels plays a role in the ROS production, we used the L-type Ca\(^{2+}\) channel blocker nifedipine (5 \(\mu\)mol/L) or a combination of N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxinGVIA (600 nmol/L) and P/Q-type Ca\(^{2+}\) channel blocker AgaIVA

AT\(_{1}\) Receptor and/or Nox2 Immunoreactivities in DiA-Labeled or -Unlabeled Rat dmNTS Neurons

<table>
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<th>DiA-Unlabeled</th>
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<tr>
<td>(\text{Nox2}) only</td>
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**Figure 2.** Ang II and H\(_{2}\)O\(_{2}\) potentiate L-type Ca\(^{2+}\) currents in rat DiA-labeled dmNTS neurons. A, DiA-labeled dmNTS neurons used for patch clamping. B, Left: Ca\(^{2+}\) currents in the presence of vehicle (C), Ang II (A), or nifedipine (N). Right: Ca\(^{2+}\) currents in the presence of vehicle (C), H\(_{2}\)O\(_{2}\) (H), or nifedipine (N). C, Histograms illustrating effects of Ang II (left) or H\(_{2}\)O\(_{2}\) (right) on the L-type Ca\(^{2+}\) current (\(n=4\) group). \(^*P<0.05\) vs control; \(^{\dagger\dagger}P<0.01\) vs Ang II or H\(_{2}\)O\(_{2}\).**

**Figure 3.** AT\(_{1}\) receptors and NADPH oxidase mediate the ROS production induced by Ang II in rat dmNTS neurons. A, The Ang II–induced increase in DCF (\(n=8\)) was abolished by MnTBAP (30 \(\mu\)mol/L; \(n=9\)). B, Losartan (3 \(\mu\)mol/L; \(n=8\)) but not PD123319 (40 \(\mu\)mol/L; \(n=6\)) blocked DCF signals induced by Ang II. C, The Ang II–induced increase in DCF was blocked by the NADPH oxidase assembly inhibitor apocynin (1 mmol/L; \(n=9\)) or the NADPH oxidase peptide inhibitor gp91ds-tat (1 \(\mu\)mol/L; \(n=10\)) but not by its scrambled version (1 \(\mu\)mol/L; \(n=10\)). \(^*P<0.05\) vs control; \(^{**}P<0.01\) vs control.

**Ang II–Elicited ROS Production Depends on Intracellular Ca\(^{2+}\) and PKC**

To examine the role of intracellular Ca\(^{2+}\) in Ang II–induced ROS production, rat dmNTS neurons were treated continuously with thapsigargin (10 \(\mu\)mol/L) to deplete intracellular Ca\(^{2+}\) stores. \(^{50}\) Pretreatment with thapsigargin (for 30 minutes) partially attenuated the increase in DCF elicited by Ang II (\(P<0.05\) versus control; \(n=6\)). Pretreatment with thapsigargin in conjunction with removal of extracellular Ca\(^{2+}\) (for 30 minutes), however, completely blocked the Ang II–induced ROS production (\(P>0.05\) versus control; \(n=7\); Figure 5). To determine whether Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels plays a role in the ROS production, we used the L-type Ca\(^{2+}\) channel blocker nifedipine (5 \(\mu\)mol/L) or a combination of N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxinGVIA (600 nmol/L) and P/Q-type Ca\(^{2+}\) channel blocker AgaIVA.
These inhibitors failed to alter the Ang II–mediated increase in DCF (Figure 5). We then examined the role of PKC in the Ang II–induced ROS production. Pretreatment with the PKC inhibitor GF109203X \( (15 \mu\text{mol/L}) \) abolished the Ang II–induced DCF increase \( (P>0.05 \text{ versus control}; \ n=11; \ Figure \ 5) \). Taken together, these results suggest that intracellular Ca\(^{2+}\) and PKC are involved in the Ang II–induced, Nox2-dependent ROS production in dmNTS neurons.

### Discussion

There are several new findings in this study. First, using an ROS-sensitive dye, we demonstrated that Ang II, at nanomolar concentrations, elicits AT\(_1\) receptor-dependent ROS production coupled with potentiation of Ca\(^{2+}\) currents in dmNTS neurons. The EC\(_{50}\) of the ROS production (38.3 nmol/L) was virtually identical to that reported previously for the effects of Ang II on the L-type Ca\(^{2+}\) current (37.4 nmol/L).\(^{25}\) Second, using Nox2-null mice, we provided the first demonstration that the Ang II–mediated increase in ROS production and related potentiation of L-type Ca\(^{2+}\) currents involve an oxidase containing Nox2 as the catalytic subunit. Third, we demonstrated that intracellular Ca\(^{2+}\) and PKC activation are critical for the ROS production evoked by Ang II in dmNTS neurons. These findings collectively provide new evidence indicating that AT\(_1\) receptor–induced Nox2 activation and production of ROS leads to potentiation of L-type Ca\(^{2+}\) currents in dmNTS neurons, which may play a role in the vascular dysregulation mediated by central autonomic networks.

To measure ROS, we used the ROS-sensitive dye C-DCDHF-DA, which has been thoroughly tested and extensively used in studies of NADPH oxidase–dependent ROS production.\(^{41-43}\) C-DCDHF-DA is oxidized by a variety of ROS, including \( \text{H}_2\text{O}_2 \), peroxynitrite, and superoxide.\(^{41-43}\) The validity of the ROS detection method used in the present study is demonstrated by the observations that the DCF signal is blocked by: (1) the ROS scavengers MnTBAP, (2) a NADPH oxidase peptide inhibitor, and (3) constitutive genetic inactivation of Nox2. These observations also rule out
the possibility that ROS-independent formation of DCF is attributable, for example, to auto-oxidation of C-DCDHF-DA or conversion of C-DCDHF-DA to DCF by cytochrome c.52

Production of superoxide by NADPH oxidase is dependent on assembly of the cytosolic regulatory subunits with the membrane-bound subunits of the enzyme. A key step in this process is regulated by protein phosphorylation, particularly the PKC-dependent phosphorylation of p47phox.31–33 Our finding that a PKC inhibitor blocks Ang II–mediated ROS production in dmNTS neurons suggests that PKC, presumably by phosphorylating p47phox, is essential for the NADPH activation induced by Ang II. We also found that the ROS production evoked by Ang II requires Ca2+, suggesting that the PKC involved is Ca2+-dependent. Concerning the sources of Ca2+, our findings with thapsigargin suggest that Ca2+ release from intracellular stores is needed for the full expression of the ROS increase. An involvement of Ca2+ from intracellular stores is also suggested by the study of Gebke et al.,53 who showed in mixed cultures of subfornical organ and organum vasculosum of the lamina terminalis neurons that Ang II increases intracellular Ca2+ in the absence of extracellular Ca2+. Furthermore, Summers et al.34 provided evidence that in hypothalamic neurons Ang II induces Ca2+ release from intracellular stores. Although we cannot completely rule out the role of extracellular Ca2+ in the ROS production, Ca2+ influx via voltage-gated Ca2+ channels does not seem to contribute to the ROS production triggered by Ang II. Ang II does activate the L-type Ca2+ channels, but our data suggest that this effect is mediated by Nox2-dependent ROS production.

A previous study in the Neuro-2A cell line showed that the increase in ROS produced by Ang II is not affected by removal of Ca2+.34 This is in contrast to our findings in dmNTS neurons in which Ca2+ was an absolute requirement for the ROS production. The reasons for this discrepancy are not entirely clear. In addition to intrinsic differences between freshly isolated dmNTS neurons and Neuro-2A cells, the Ang II concentration is likely to be an important factor, because higher concentrations of Ang II (2 µmol/L) elicit increases in ROS in dmNTS neurons that are not blocked by Ca2+ removal (Supplemental Figure I, available online at http://hyper.ahajournals.org). Considering that, in the study of Zimmerman et al.,34 Ang II was applied at 5 µmol/L, it is conceivable that the discrepancy in the results is because of the difference in Ang II concentration used.

An important consequence of ROS production by Ang II in central autonomic neurons is the resultant increase in intracellular Ca2+ levels.25,34,40,55,56 The present data are consistent with previous reports that the elevation of intracellular Ca2+ induced by Ang II is secondary to the action of ROS on L-type voltage-gated Ca2+ channels.25,34 However, the mechanisms underlying the effects of Nox2-derived ROS on voltage-gated Ca2+ influx remain unclear. One possibility is that oxidative stress modulates voltage-gated Ca2+ channels. Indeed, it is well established that ROS increase voltage-gated Ca2+ currents in neurons.55 Our finding that H2O2 mimics the effect of Ang II on Ca2+ currents in dmNTS neurons supports this possibility, albeit indirectly.

The Ang II–induced potentiation of voltage-gated Ca2+ currents elicited by NADPH oxidase-dependent superoxide production is an important modulator of the excitability of dmNTS neurons.25,39,40 The NTS is a crucial coordinator of cardiorespiratory processes and an important component of the central renin–angiotensin system.1–5 Within the dmNTS, neurons or glia have been shown to express renin, angiotensinogen, or angiotensin-converting enzyme, as well as Angs I, II, and III and angiotensin AT1 receptors.10 Using dual-labeling immunofluorescence electron microscopy, we demonstrated colocalization of AT1 receptors and Nox2 in single DiA-labeled NTS neurons, indicating that a population of second-order dmNTS sensory neurons contains the AT1 receptor and Nox2.25 Thus, AT1 receptors are located in functional surface membrane sites in Nox2-containing neurons that are contacted by vagal-like and nonvagal afferents in the dmNTS.

Long-term modulation of baroreflex function and disturbances in the renin–angiotensin system are each associated with experimental and clinical hypertension.11,57 The mechanisms mediating adaptations in the baroreflexes presumably involve complex peripheral and central processes in which Ang II plays a role. For example, Ang II–dependent systemic hypertension is associated with increased sympathetic nerve activity,6 whereas Ang II–induced sympathoexcitation has also been linked to NADPH oxidase-derived ROS.15–19 Thus, our findings suggest that Ang II–induced ROS production and L-type Ca2+ currents might contribute to the central autonomic effects of Ang II.58,59 Moreover, considering the well-established link among L-type Ca2+ channels, ROS, and cellular plasticity,60,61 heightened activation of AT1 receptors in dmNTS neurons may also play a role in the reorganization of autonomic function accompanying hypertension.
Perspectives

The dmNTS is a critical central relay for cardiopulmonary afferents regulating cardiovascular homeostasis. We have demonstrated that Nox2 and AT$_1$ receptors are present in second-order dmNTS neurons in which exposure to Ang II induces ROS production and activation of Ca$^{2+}$ currents. The Ang II–induced ROS production and Ca$^{2+}$ currents were not observed in mice lacking Nox2. Furthermore, we have shown that Ang II–dependent ROS production requires intracellular Ca$^{2+}$ and PKC. The data indicate that Ang II induces ROS production in second-order dmNTS neurons through a Nox2-containing NADPH oxidase. The NADPH oxidase–derived ROS, in turn, activate L-type Ca$^{2+}$ channels. These findings provide the mechanistic basis for the powerful actions of Ang II in dmNTS and support the concept that ROS derived from Nox2 are critical signaling molecules in central autonomic neurons. They also support the growing evidence that ROS derived from Nox2 play a vital role in both central and peripheral mechanisms of hypertension.

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Disclosures

None.

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