Angiotensin II type 2 receptor-coupled nitric oxide production modulates free radical availability and voltage-gated Ca\(^{2+}\) currents in NTS neurons

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The Nucleus of the Solitary Tract (NTS), the major site of termination of vagal afferents arising from aortic baroreceptors and cardiorespiratory chemoreceptors, plays a critical role in normal cardiovascular regulation and in the neurohumoral dysfunction associated with hypertension (7, 13, 21, 31). The octapeptide ANG II is heavily expressed in the medial NTS (mNTS) and contributes to cardiovascular regulation and dysregulation, in large part, by activation of ANG II type 1 receptors (AT1R) and production of reactive oxygen species (ROS). ANG II type 2 receptors (AT2R) induce production of nitric oxide (NO), which may interact with ROS and mediate AT1R signaling. We sought to determine whether AT2R-mediated NO production occurs in mNTS neurons and, if so, to elucidate the NO source and the functional interaction with AT1R-induced ROS or Ca\(^{2+}\) influx. Electron microscopic (EM) immunolabeling showed that AT2R and neuronal NO synthase (nNOS) are coexpressed in neuronal somata and dendrites receiving synapses in the mNTS. In the presence of the AT1R antagonist losartan, ANG II increased NO production in isolated mNTS neurons, an effect blocked by the AT2R antagonist PD123319, but not the angiotensin (1–7) antagonist D-Ala. Studies in mNTS neurons of nNOS-null or endothelial NO synthase (eNOS)-null mice established nNOS as the source of NO. ANG II-induced ROS production was enhanced by PD123319, the NOS inhibitor G-nitro-L-arginine (LNNA), or in nNOS-null mice. Moreover, in the presence of losartan, ANG II reduced voltage-gated L-type Ca\(^{2+}\) current, an effect blocked by PD123319 or LNNA. We conclude that AT2R are closely associated and functionally coupled with nNOS in mNTS neurons. The resulting NO production antagonizes AT1R-mediated ROS and dampens L-type Ca\(^{2+}\) currents. The ensuing signaling changes in the NTS may counteract the deleterious effects of AT1R on cardiovascular function.

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Electron microscopy. EM dual-immunolabeling has been described previously (50). Male wild-type mice were anesthetized with pentobarbital sodium, and their brains were fixed by perfusion with heparin-saline (1,000 U/ml) followed consecutively by 3.8% formaldehyde in 0.1 mol/l phosphate buffer. Coronal sections of 40 μm were cut through the mNTS starting at 7.32 caudal to bregma and ending at 7.76 caudal to bregma using a vibratome (Leica Microsystems), and all of the sections included were at the level of the area postrema (34). The tissue sections were incubated for 48 h at 4°C in 0.1 mol/l Tris-buffered saline/0.1% BSA solution containing a 1:200 dilution of a mouse monoclonal nNOS antibody for immunoperoxidase labeling and a 1:1,000 dilution of a rabbit polyclonal AT2R antiserum for immunogold-silver (9, 11). The nNOS antibody (cat. no. 610309) was against a sequence at the C-terminal (amino acids 1095–1289) of human nNOS. Western blot analysis showed that the purified antiserum recognizes a single band of ~155 kDa, corresponding to the molecular weight of nNOS (44). The AT2R antiserum was against a synthetic peptide sequence from the COOH-terminal domain (amino acids 349–363) of the rat AT2R (Abcam, cat. no. ab19134). This antiserum specifically recognizes AT2R in human cervical carcinoma (HeLa) cells transfected with AT2R, but not AT1R (11). After the primary antiserum incubation, the sections were placed for 30 min each in biotinylated secondary horse anti-mouse IgG (1:400; Invstar) and avidin-biotin peroxidase complex (ABC Elite Kit, Vector Laboratories). The bound peroxidase was visualized by reaction of the sections for 5 min in 3.3′-diaminobenzidine (Sigma-Aldrich) and hydrogen peroxide. For immunogold labeling, the sections were placed for 2 h in a 1:50 dilution of donkey anti-rabbit IgG conjugated with 1-nm colloidal gold, and a silver intense EM kit was used for 6 min to enhance bound gold particles (Amersham Biosciences). The sections were postfixed in 2% osmium tetroxide and embedded in EM bed-812. Single ultrathin sections (70 nm) containing the mNTS at the level of the area postrema were cut using an EM UC6 ultramicrotome (Leica Microsystems) and collected and dried on 400-mesh copper grids. Ultrathin sections were then counter-stained for ultrastructural analysis using a Philips CM10 transmission electron microscope.

Dissociation of the mNTS neurons. As described in detail elsewhere (49, 50), rats were killed by CO2, the brain stem was quickly removed and transferred to a chamber containing ice-cold sucrose-artificial cerebrospinal fluid (aCSF) oxygenated with a mixed gas containing 95% O2 and 5% CO2 (39, 40). Although hypercapnia and hypoxia at death could influence NTS chemoreceptive neurons, these changes are likely to be reversed by equilibration of the tissue in 95% O2 and 5% CO2. Coronal slices (300 μm in thickness) were obtained and incubated with 0.02% pronase and 0.02% thermolysin at 36°C in the oxygenated lactic acid (l)-aCSF. With the area postrema as a landmark, the mNTS region was punched, and its cells were mechanically dissociated. The mNTS neurons were identified based on their typical cellular morphology, e.g., small round or oval bipolar cells with long thin processes (49, 50).

ROS or NO detection. Methods for ROS and NO detection have been described in detail elsewhere (20, 24–26, 39, 49) and are briefly summarized. NO and ROS were detected using DAF-FM (20, 24–26) labeling and a 1:1,000 dilution of a rabbit polyclonal AT2R antiserum (red arrows) is seen in the neuropil. An immunogold-labeled axon terminal (AT2-t) is seen in the neuropil. A single AT2R gold particle (red arrows) located in the synaptic portion (yellow curved arrow) of the plasma membrane contacted by an unlabeled axon. An immunogold-labeled axon terminal (AT2-t) is seen in the neuropil. Scale bars = 0.5 μm.
and C-DCDHF-DA (39, 49), respectively. The mNTS neurons were incubated with C-DCDHF-DA (5 μmol/l) or DAF-FM (5 μmol/l) in oxygenated l-aCSF for 30 min, and then rinsed in control buffer. Time-resolved fluorescence (FTTC filter) was measured at 30-s intervals with a Nikon diaphot 300 inverted microscope equipped with CCD digital camera (Princeton Instruments) and using IPLab software (Scanalytics) (49). Recordings were started after a stable baseline measurement was achieved, usually 10 min. For all experiments, concurrent vehicle recordings were performed. In some experiments, the cell type(s) producing NO was identified by immunofluorescence labeling with antibodies for the neuronal marker NeuN (1:500), the astrocytic marker GFAP (1:500), the microglial marker Iba1 (1:500), or the endothelial marker CD31 (1:500).

**Whole-cell patch recording.** Voltage-gated Ca\(^{2+}\) currents were elicited from whole-cell patched NTS neurons. An Axopatch-200A patch-clamp amplifier was used with Cs\(^{+}\) electrode solution (49, 50). Using 2 mM Ca\(^{2+}\) as a charge carrier in oxygenated l-aCSF, we observed that inward Ca\(^{2+}\) currents were elicited by a 500 ms depolarizing pulse from a holding potential of −60 mV to −10 mV using pClamp 8 (Axon Instruments). The amplitude of inward currents measured at the end of depolarizing pulses is considered as that of L-type Ca\(^{2+}\) currents (49, 50).

**Data analysis.** The electron microscopy images were obtained exclusively at the surface (1–2 μm) of the flat-embedded tissue, where penetration of the immunoreagents is optimal. Profiles containing two or more gold particles were considered to be immunogold-labeled. Peroxidase-immunoreactive profiles had a granular, electron-dense precipitate greater than that seen in any other morphologically similar profiles in the surrounding neuropil. Illustrations were prepared by importing digital images directly into Adobe Photoshop. ROS or NO fluorescence data are expressed as the ratio Ft/Fo, where Ft is fluorescence following 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. There was no relationship between baseline fluorescence (Fo) and the fluorescence increase induced by ANG II (Ft) under the experimental conditions studied (20, 49). Data (mean ± SE) were statistically evaluated by the paired Student’s t-test, or ANOVA, as appropriate. P values < 0.05 were considered significant.
**RESULTS**

*AT₂R are coexpressed with nNOS in somata and dendrites of the mNTS*. Immunogold labeling for AT₂R was seen mainly within somatodendritic profiles, some of which also contained diffuse immunoperoxidase reaction product for nNOS in the mNTS of mouse (Fig. 1, A–C) or rat (data not shown). In the soma, AT₂R immunogold was detected away from the plasma membrane and near cytoplasmic organelles, including endomembrane of smooth endoplasmic reticulum and Golgi lamellae (Fig. 1A). In dendrites, AT₂R were localized to intracellular, as well as surface membranes. The surface labeling was evident on nonsynaptic, as well as synaptic portions of the plasmalemmal surface (Fig. 1, B and C). Occasionally, AT₂R gold particles were observed on the plasma membrane beneath a postsynaptic membrane specialization (Fig. 1C). Dendrites containing AT₂R and nNOS were among those that received synaptic contacts from axon terminals. Some of these terminals contained sparse AT₂R immunolabeling (Fig. 1B) and were typical of glutamatergic visceral vagal afferents, forming multiple dendritic contacts (3). These results provide ultrastructural evidence that AT₂R and nNOS are present in the same neurons.

*Activation of AT₂R increases NO production from nNOS*. We used DAF-FM imaging to determine whether the AT₂R activation results in NO production in the mNTS neurons. In the presence of control buffer (1-aCSF), ANG II (10–4,000 nmol/l) had little effect on the NO signal (Fig. 2B). However, in the presence of the AT₁R antagonist losartan (3 μmol/l), ANG II increased the NO signal markedly (Fig. 2B). To determine the cellular source of NO, we examined NO-dependent fluorescence in neurons, microglia, astrocytes, and endothelial cells identified using cell-specific markers. We found that ANG II-induced NO fluorescence (P < 0.01 vs. control) was colocalized only with neurons (n = 26) (Fig. 2C). The enhancement of NO-dependent fluorescence by coapplication of ANG II with losartan was blocked by the AT₂R antagonist PD123319 (30 μmol/l, P > 0.05 vs. ANG II alone, n = 9).

NO-dependent fluorescence was also blocked by the cell-permeable NO scavenger PTIO (51) (50 μmol/l, P > 0.05 vs. ANG II, n = 14) or the NOS inhibitor LNNA (1 mmol/l, P > 0.05 vs. ANG II, N = 6), attesting to the specificity of the NO signal. The ANG-(1–7) receptor antagonist D-Ala (2 μmol/l) did not attenuate the increase in NO-dependent fluorescence induced by ANG II (P > 0.05 vs. losartan, n = 8) (Fig. 3A). Moreover, ANG-(1–7) (100 nM) did not increase NO-dependent fluorescence (P > 0.05 vs. losartan, n = 9) (Fig. 3B). We then used eNOS or nNOS-null mNTS neurons to examine the enzymatic source of NO. In the presence of losartan, ANG II (100 mmol/l) increased NO production in the mNTS neurons of wild-type mice (P < 0.05 vs. control, n = 6) or eNOS-null mice (P < 0.05 vs. control, n = 9), but not in nNOS-null mice (P > 0.05 vs. control, n = 9) (Fig. 4). Therefore, nNOS is required for the NO production evoked by activation of AT₁R.

*AT₂R-dependent NO production modulates AT₁R-dependent ROS production*. NO rapidly interacts with the ROS superoxide to produce peroxynitrite and other nitrated species (19, 32). Therefore, we sought to determine whether the NO produced by activation of AT₂R counteracts the ROS produced by activation of AT₁R. In agreement with previous results (49, 50), ANG II, at a concentration producing maximal effects (1 μmol/l), increased the ROS signal in rat mNTS neurons (P < 0.01 vs. control, n = 16). The increase was blocked by the ROS scavenger Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (30 μmol/l, P > 0.01 vs. control, n = 11), but it was facilitated by PD123319 (30 μmol/l, P < 0.05 vs. ANG II alone, n = 18), or LNNA (1 mmol/l, P < 0.05 vs. ANG II, n = 7) (Fig. 5A). ANG II (1 μmol/l) also increased ROS production in wild-type mouse mNTS neurons (P < 0.01 vs. control, n = 7). The ROS increase was blocked by losartan (3 μmol/l, P > 0.05 vs. control, n = 8) and was not observed in Nox2-null mice (P < 0.05 vs. control, n = 9), confirming

![Figure 3. AT₂R- and NOS-dependent NO signals in rat mNTS neurons.](image-url)
Nox2 as the source of ROS. In contrast, the ROS production was enhanced in nNOS-null mNTS neurons (P < 0.05 vs. WT, n = 9) (Fig. 5B). These results indicate that the NO produced by AT2R activation suppresses the Nox2-derived ROS evoked by AT1R receptor activation.

**AT2R-mediated NO production down-regulates L-type Ca2+ currents.** ANG II increases voltage-gated L-type Ca2+ currents in the mNTS neurons via AT2R activation and Nox2-derived ROS (49, 50). Ca2+ current enhancement in the mNTS plays a role in the mechanisms by which ANG II induces the neurohumoral dysfunction underlying hypertension (45). To gain insight into the functional implications of the interplay between NO and ROS in the mNTS neurons, we examined the effect of the AT2R-mediated NO production on the Ca2+ currents evoked by ANG II. Without losartan, ANG II (100 nmol/l) increased the amplitude of L-type Ca2+ current (P < 0.01 vs. control, n = 5) (Fig. 6, A and F). However, in the presence of losartan, ANG II decreased the amplitude of L-type Ca2+ current (P < 0.01 vs. control; n = 7) (Fig. 6, B and F), an effect blocked by PD123319 (30 μmol/l) (Fig. 6, C and F) or LNNA (1 mmol/l) (Fig. 6, D and F). We then used the NO donor SNAP to determine whether NO itself was able to downregulate ANG II-induced Ca2+ currents. Consistent with this hypothesis, SNAP (2 μmol/l) decreased the Ca2+ current (P < 0.01 vs. control; n = 6) (Fig. 6, E and F). These findings indicate that NO produced by AT2R activation downregulates the ANG II-induced Ca2+ current.

**DISCUSSION**

We have shown that AT2R are coexpressed with nNOS in somata and dendrites of mNTS neurons, and NO production by these neurons is markedly enhanced by AT1R inhibition. Such NO increase is prevented by an AT2R antagonist, suggesting the involvement of AT2R in the signaling pathway. Results in rats were generally comparable to those obtained in mice. Using NOS-null mice, we demonstrated that nNOS, not eNOS, is the source of NO. Interestingly, experiments with ANG-(1–7) and D-Ala suggested that, at variance with findings in other systems (54), ANG-(1–7) or its receptor does not affect NO production in mNTS. Because of the close interaction between NO and ROS, we examined the effect of AT2R activation on ROS production. We found that AT2R inhibition or NOS inhibition enhances the ROS production evoked by ANG II. Similarly, ROS production was enhanced in nNOS-null mNTS neurons, an effect attenuated by AT1R inhibition. To determine the functional implications of the AT2R-dependent NO production, we tested its effect on voltage-gated L-type Ca2+ currents, an important signaling mechanism in the mNTS neurons (45, 49, 50). AT2R inhibition suppressed the Ca2+ currents evoked by ANG II, an effect dependent on AT2R and NO. Taken together, these findings provide evidence of a reciprocal interaction between AT2R-dependent NO production and AT1R-dependent ROS production. The resulting balance between NO and ROS has an impact on voltage-gated Ca2+ channels, which are critically involved in the functional output of the mNTS neurons (22).

![Graph](image1.png)

**Fig. 4.** AT2R-induced NO production is dependent on nNOS in mouse mNTS neurons. In the presence of losartan (3 μmol/l), ANG II (100 nmol/l), potentiated NO production in wild-type (WT) (n = 6) and eNOS-null mNTS neurons (n = 9). However, the ANG II (100 nmol/l)-induced increase in NO was not observed in nNOS-null neurons (n = 9). *P < 0.05; paired Student’s t-test or ANOVA.

![Graph](image2.png)

**Fig. 5.** AT2R-induced NO production antagonizes AT1R-induced reactive oxygen species (ROS) in the mNTS neurons. A: in rat mNTS neurons, ANG II (1 μmol/l) increases the ROS signal (n = 16), an effect blocked by the ROS scavenger Mn(III) tetakis(4-benzoic acid)porphyrin chloride (30 μmol/l; n = 10) or the AT1R antagonist losartan (Los, 3 μmol/l; n = 11), but potentiated by the AT2R antagonist PD123319 (PD, 30 μmol/l; n = 18) or the NOS inhibitor LNNA (1 mmol/l; n = 7). B: in mouse wild-type mNTS neurons, ANG II (1 μmol/l) potentiated the ROS signal (n = 7), an effect blocked by losartan (Los, 3 μmol/l; n = 8). However, ANG II (1 μmol/l) reduced the ROS signal in Nox2-/− mNTS neurons (n = 10). ANG II (1 μmol/l) enhanced the ROS signal in NOS-/- mNTS neurons (n = 9), an effect completely blocked by pretreatment with losartan (Los; 3 μmol/l; n = 6). *P < 0.05; **P < 0.01; paired Student’s t-test or ANOVA.
The present study sought to compare the findings from rats and mice, including genetically modified mice, to integrate all information derived from these two different models to generate a synergistic platform for understanding the roles of AT2R in nNOS- vs. eNOS-derived NO. Although it is not unlikely that the mechanisms underlying the AT2R-mediated NO release in the rat NTS is different from that in the mouse NTS, the similarities between rat and mice, from physiology to genomics, allows us to explain present findings obtained from the rat NTS neurons.

Somatodendritic profiles in the mNTS contain both AT2R and nNOS. The primary location of AT2R in the mNTS was in postsynaptic somata and dendrites, many of which also contained nNOS. The colocalization of AT2R and nNOS provides ultrastructural support for the functional interaction between ANG II and NO. In dual-labeled somatodendritic profiles, AT2R were present in the cytoplasmic compartment, associated with membranous structures involved in synthesis and intracellular trafficking of proteins to and from the surface (35, 43). Plasmalemmal labeling of AT2R was occasionally seen in small- and medium-sized dendrites receiving synaptic input from one or more axon terminals. The presence of AT2R at the synapses in the nNOS-containing neuron suggests that activation of postsynaptic AT2R may result in generation of NO that presynaptically modulates the release of inhibitory and/or excitatory transmitters.

Age-dependent expression of AT2R. In contrast with early observations of mRNA of AT2R expression at very high levels in the fetus and its rapid regression to low levels in adults (37), recent publications using Western blot analysis shows that the brain stem from rat and mouse fetuses and neonates exhibit a significantly lower AT2R protein expression compared with adult rats (15, 17, 53). These papers also show that 2–3-wk-old brain stem expresses considerable amounts of AT2R protein. Therefore, AT2R are also present in the adult brain stem, highlighting their physiological relevance (15, 17).

AT2R induce nNOS-dependent NO production. NO is an important signaling molecule in the NTS, but its enzymatic source(s) and relationships to AT2R activation have not been clearly defined (17). Some studies have provided evidence that NTS neurons are endowed with AT2R (27, 29, 36). In addition, other reports show that NTS neurons express nNOS but not eNOS (14, 28), whereas others have suggested that eNOS in the NTS plays a role in the regulation of blood pressure in spontaneously hypertensive rats (33, 48, 52). The identification of the enzymatic sources of NO is complicated by the difficulties in selectively inhibiting specific NOS isoforms, especially eNOS (4). To overcome this problem, in the present study, we used nNOS and eNOS-null mice and identified nNOS as the sole source of NO in the mNTS. However, on the basis of our observations in dissociated NTS cells in which AT1R were blocked, we cannot support or contradict the
findings in vivo demonstrating the involvement of eNOS-derived NO in the dampening of baroreceptor sensitivity induced by exogenous ANG II (33, 48, 52). Our immunoelectron microscopy studies demonstrated a colocalization of nNOS and AT2R, providing ultrastructural evidence supporting a functional interaction between these proteins. However, the signaling pathways by which AT2R activate nNOS remain to be established. Activation of bradykinin receptors has been implicated in AT2R-induced NO production (42, 46), a finding not confirmed by other studies (1). Therefore, the mechanisms linking AT2R activation to NO production remain to be elucidated.

**Interaction between AT1R-derived ROS and AT2R-derived NO.** NO reacts avidly with ROS to generate a nitrated species, some of which contribute to its physiological and pathological effects (8, 22, 23). Our findings suggest that when neurons are exposed to ANG II, AT2R-induced NO is scavenged by AT1R-derived ROS. Thus, robust increases in NO are observed only if ROS production is suppressed by AT1R inhibition. Conversely, the ROS production induced by AT2R activation is dampened by the NO produced by AT1R activation. In the normal state, ROS production predominates over NO production, and ROS can be detected, even in the absence of AT2R inhibition. However, as demonstrated here, AT2R inhibition reduces NO and potentiates AT1R-dependent ROS generation, suggesting that loss of NO enhances ROS availability. This effect is unlikely to be related to NO-induced Nox2 inhibition because NO does not suppress, but promotes, Nox2-dependent ROS formation (20). Therefore, ROS and NO can reciprocally influence their bioavailability and biological activities in the mNTS neurons. Previous studies have demonstrated that AT2R facilitate vasodilation through NO/NO-mediated signal after chronic AT1R antagonism and that AT1R antagonists increase both AT2R expression and cGMP (6, 40). These effects may contribute, in part, to the beneficial actions of AT1R blockers in the treatment of hypertension. Thus, our finding that blockade of AT1R in NTS neurons may significantly enhance the AT2R-mediated NO release in vivo may have clinically relevant implications.

**Effects on voltage-gated Ca2+ channels.** Voltage-gated L-type Ca2+ channels play an important role in neurotransmission and long-term potentiation, and, as such, are critical to neuronal function in the NTS. AT2R have been proposed to modulate neuronal Ca2+ channel activity by NO through two different mechanisms, cGMP and S-nitrosylation (2). The present results suggest that AT2R activation suppresses Ca2+ currents through NO production, whereas AT1R receptor activation enhances Ca2+ currents via Nox2-derived ROS (49, 50). The reduction in Ca2+ currents by NO shown here is in agreement with similar findings in other neuronal types (5). The mechanisms of the effects of NO on L-type Ca2+ channels have not been elucidated in full, but they may include activation of soluble guanylyl cyclase or S-nitrosylation of Ca2+ handling proteins (2).

**Perspectives and Significance**

We investigated the role of AT2R in the regulation of NO and ROS homeostasis in mNTS neurons. We found that AT2R and nNOS are colocalized at postsynaptic sites and that AT2R activation enhances NO production via activation of nNOS, while reducing the availability of ROS generated by AT1R activation via Nox2. These effects on NO and ROS have an impact on L-type Ca2+ currents since AT2R-mediated NO attenuates the Ca2+ currents triggered by AT1R activation. These results suggest that AT2R-mediated NO production may offset the deleterious effects of AT1R-mediated ROS in the mNTS, possibly, by suppressing L-type Ca2+ currents. Our findings also unveil a previously unrecognized interaction between AT2R and AT1R in the NTS. Such interaction is relevant to clinical conditions, such as hypertension and cardiac failure, in which the balance between central AT1R and AT2R is altered (16). Furthermore, AT2R-mediated NO may help explain the beneficial effects of AT1R inhibitors for the treatment of hypertension (12, 41). Finally, the functional significance of the interaction between mNTS AT1R and AT2R in the regulation of sympathetic outflow and blood pressure remains to be defined (12). Further studies using in vitro and in vivo approaches will be required to address these outstanding questions.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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