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COX-1-derived PGE$_2$ and PGE$_2$ type 1 receptors are vital for angiotensin II-induced formation of reactive oxygen species and Ca$^{2+}$ influx in the subfornical organ

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HYPERTENSION POSES a significant challenge to public health and healthcare by increasing the risk for heart attack, stroke, and kidney failure. The "slow pressor" hypertension, a delayed increase in blood pressure in response to increased systemic angiotensin II (ANG II), is elicited through the subfornical organ (SFO), a small forebrain structure devoid of the blood-brain barrier (6, 22, 41, 49). SFO neurons express ANG-II type 1 receptors (AT$_1$Rs) and respond to circulating ANG II (11, 22) by sending signals through angiotensinergic and glutamatergic projections directly to the paraventricular nucleus (PVN) (3, 11, 13) and other autonomic nuclei that control sympathetic outputs and blood pressure (31). The ANG II-induced upregulation of sympathoexcitation, followed by an elevation of blood pressure, is mediated through reactive oxygen species (ROS) (10, 42, 53, 69) and Ca$^{2+}$ (15, 18, 33). It is well established that ANG II-mediated signals activate NADPH oxidase, leading to accumulation of ROS in the brain that are functionally coupled with hypertensive responses (67). This ANG II-mediated overproduction of ROS is also associated with the activation of voltage-gated L-type Ca$^{2+}$ channels in central neurons (52, 57, 58, 70) and other cell types (2, 55, 62). Activation of L-type Ca$^{2+}$ channels is critical to neurotransmission and long-term potentiation (LTP) in the central nervous system (60, 64).

Previous work has also revealed that the activation of AT$_1$R results in arachidonic acid (AA) release from membrane phospholipids by phospholipases A$_2$ (PLA$_2$) (54). AA is then metabolized to prostaglandin E$_2$ (PGE$_2$) by cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2). Accumulating evidence suggests that COX-derived PGE$_2$ plays important roles in the regulation of cardiovascular dysfunctions, including ANG II-induced hypertension (4, 6, 21, 39, 40, 45, 56). The PGE$_2$ receptor subtype 1 (EP$_1$R) is expressed in the SFO, and studies using genetically modified mice have shown that it plays an important role in signaling the onset of slow-pressor ANG-II hypertension (6, 21). As a member of the G protein-coupled receptor family (17, 25), EP$_1$R is involved not only in ANG II-elicted ROS production in the SFO (6) but also in the regulation of Ca$^{2+}$ influx through Ca$^{2+}$ channels in neurons and neuroendocrine cells (4, 24, 25, 34). To better understand the complex mechanisms underlying hypertension, it is essential to determine if and how PGE$_2$, EP$_1$R, ROS, and Ca$^{2+}$ are functionally linked to serve as key intermediate molecules in ANG II-mediated signaling in SFO neurons.

In this study, we sought to uncover the roles of key prostanooid-linked signals, including PLA$_2$, COX-1, COX-2, and EP$_1$R, in ANG II-elicted ROS production and voltage-gated Ca$^{2+}$ influx. Using electron microscopic immunolabeling, we found that COX-1 was coexpressed with AT$_1$R in SFO neurons. Consistent with this finding, ANG II-triggered PGE$_2$...
release from SFO cells was AT_{1}R and COX-1 dependent. In addition, ANG II induced ROS formation and L-type voltage-gated Ca^{2+} currents (L-VDCCs) via PL_{A2}, COX-1-derived PGE_{2}, and EP_{1}R. Finally, Nox2 was identified as the source of ANG II-induced, EP_{1}R-mediated ROS production in the SFO.

**MATERIALS AND METHODS**

*Materials.* All procedures were approved by the Animal Care and Use Committee at Weill Cornell Medical College. Adult (8–10 wk old) C57Bl/6 wild-type (WT) and transgenic mice, including AT_{1}R-enhanced green fluorescent protein (eGFP), EP_{1}R^{−/−}, COX-1^{−/−}, COX-2^{−/−}, and Nox2^{−/−}, were obtained from in-house colonies. ANG II, pronase, thermolysin, nifedipine, BAY K 8644, tetrodotoxin (TTX), α-conotoxin (Ctx)-GVIA, and N-(p-amylcyclamomoyl)anthranilic acid (ACA) were purchased from Sigma-Aldrich (St. Louis, MO). Mn[H]tetrazaki(4-benzoic acid)porphyrin chloride (MtxTBP) was procured from EMD Biosciences. PGE_{2}, SC51089, SC560, and NS398 were obtained from Cayman Chemical (Ann Arbor, MI). The AT_{1}R antagonist losartan was a gift from the DuPont Merck Pharmaceuticals Developmental (Deerpark, NJ). The NADPH oxidase polypeptide inhibitor gp91^{phox} docking sequence (gp91-ds) with a sequence of [H]GGGGCSTRIRRQL[NH2] was purchased from Bio-Synthesis (Lewisville, TX). Dihydroethidium (DHE) and calcein AM were purchased from Aves Laboratories (Tigard, OR).

*Electron microscopy.* Tissue was processed and analyzed as previously described (19, 43). In brief, three males of AT_{1}R-eGFP and WT age-matched 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% acrolein (Polysciences) in 2% paraformaldehyde in 0.1 mol/l phosphate buffer and then 2% paraformaldehyde in 0.1 mol/l phosphate buffer. Coronal 40-μm vibratome sections were cut and collected onto thin bar copper grids from each AT1R-eGFP animal, all ImG particles in such fields (i.e., near to the tissue surface) in randomly selected grid interface (52x84). Tissue processed for dual immunolabeling was additionally incubated (52x154) nm) containing the SFO were cut and collected onto thin bar copper hyde (10 min) and silver intensification using an IntenSE-M Kit. Tissue processed for dual immunolabeling was additionally incubated (52x185) Tissue processed for dual immunolabeling was additionally incubated (52x215) Tissue processed for dual immunolabeling was additionally incubated (52x255) DHE fluorescence intensity relative to control samples (Ft/Fo).

*ROS detection.* Intracellular ROS levels in dissociated SFO cells were assessed using DHE. SFO cells were dissociated as described above, moved to a petri dish mounted on Nikon diaphot 300 inverted microscope (Nikon, Japan), and incubated with DHE (2 μmol/l) for 30 min. The fluorescence intensity was measured before (vehicle) and after application of ANG II or PGE2 using an ethidium bromide (DNA complex) filter (Chroma Technology, Bellows Falls, VT) with the spectrum of an excitation maximum 510 nm and an emission maximum 605 nm, which indicates the presence of superoxide (O_{2}^{-}) and possibly other ROS such as H_{2}O_{2}, hydroxyl radical, and peroxynitrite (47). To validate ROS measurement using this system, we tested the capability of exogenous H_{2}O_{2} to increase DHE signal in the SFO cells. The increase in DHE-derived fluorescence observed after treatment of SFO cells with H_{2}O_{2} is attributed to intracellular stimulation (24–26°C). The whole cell-patch recording. The dissociated SFO cells were placed in a 35-mm petri dish mounted on the Nikon inverted microscope and perfused with the oxygenated l-aCSF at room temperature (24–26°C). The whole cell-patch recording of isolated SFO neurons was performed using an Axopatch-200A patch-clamp amplifier (Molecular Devices) and a glass electrode pulled by a P-97 micropipette puller (Sutter Instruments) with tip resistance of 3–5 MΩ when filled with a pipette solution containing (in mmol/l): 145 CsCl, 1 MgCl_{2}, 10 HEPES, 2 ATP, and 0.2 cAMP (pH 7.3). Series resistance and membrane capacitance were compensated. Bipolar
SFO neurons were visualized following dissociation. TTX-sensitive voltage-gated Na\(^+\) currents were recorded to confirm that the patched cell was a neuron. Two millimole/liter Ca\(^{2+}\) in l-aCSF was used as a charge carrier for the voltage-gated Ca\(^{2+}\) currents elicited by a 500-ms depolarization pulse from a holding potential at −60 mV to stepping potentials between −50 and +20 mV. To selectively record the long-lasting (L)-type Ca\(^{2+}\) current, the selective voltage-gated Na\(^+\) channel blocker TTX (1 μmol/l) and N-type Ca\(^{2+}\) channel blocker Ctx-GVIA (1 μmol/l) were added to the perfusion buffer in some patched SFO neurons. The amplitude of L-VDCC was measured at the end of 500-ms pulse (57, 58). The amplitudes of Ca\(^{2+}\) currents were analyzed using pClamp 8 (Molecular Devices, Sunnyvale, CA).

Data analysis. Data are expressed as means ± SE. Multiple comparisons were evaluated by ANOVA, followed by post hoc analysis using Tukey’s test with paired comparison. Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

COX-1 is coexpressed with AT\(_1\)R in SFO neurons. The SFO contains a network of diversified neurons, neuronal processes, and glial cells. To establish a structural framework from which to best unravel key functional interactions, we performed ultrastructural labeling of COX-1 and AT\(_1\)R within the SFO. Examination of central portions of the SFO revealed that COX-1 ImG labeling was common and was concentrated in postsynaptic neuronal processes and glial processes (Fig. 1). Quantification of this distribution pattern (Fig. 1D) demonstrated that 60% of the ImG particles were associated with either neuronal somata or dendrites, and 30% were associated with glial processes in the neuropil (total number of ImG particles examined, 1,072). In addition, 68% of the COX-1 ImG-labeled postsynaptic neuronal processes (i.e., somal and dendritic) also displayed AT\(_1\)R-eGFP ImP labeling. The results confirm that AT\(_1\)R and COX-1 are spatially linked and provide anatomical support for the theory that COX-1 may be associated with ANG II-elicited generation of PGE\(_2\) in SFO neurons.

N-II-triggered endogenous PGE\(_2\) release from dissociated SFO cells is dependent on AT\(_1\)R, PLA\(_2\), and COX-1. Following dissociation of SFO cells, calcein AM (1 μmol/l) was loaded to isolated cells to check viability. As shown in Fig. 2A, one example of calcein-labeling of dissociated SFO cells lasted 1 h, indicating viability of the in vitro model. In addition, the time course of calcein-labeled SFO cells also indicates that most

Fig. 1. Cyclooxygenase-1 (COX-1) immunolabeling within the central subfornical organ (SFO) is highly concentrated within neuronal postsynaptic processes, most of which also display angiotensin-II (ANG II) type 1 receptor (AT\(_1\)R)-enhanced green fluorescent protein (eGFP) immunoperoxidase (ImP) labeling, and glial processes. A: AT\(_1\)R-eGFP-ImP-positive cell body (Soma) and dendrite (LDen), both near fenestrated capillaries (Fen Cap), contain COX-1-immunogold (ImG) particles (arrow). B: COX-1 ImG labeling (arrows) of a glial process (Glia) and an AT\(_1\)R-GFP-ImP-labeled dendrite (LDen). C: COX-1 ImP labeling (arrow) near a mitochondrion in an unlabeled dendritic process (Den). D: histogram of the percent distribution of all COX-1 ImG labeling by process type (Vasc glia, glial associated with fenestrated capillaries; Vasc epith, vascular epithelial cells), divided by whether the process did (dark grey) or did not (light grey) colabel for AT\(_1\)R-GFP. Scale bars = 0.5 μm.
isolated SFO cells, including neurons, remained viable following 50 min of calcein labeling (86.7 ± 4.7%, P > 0.05 vs. 0 min, cell number from each experiment = 48–54, number of experiments = 3) (Fig. 2B). Taken together, this proved the viability of the isolated SFO cells and neurons and allowed us to perform further functional studies in vitro.

Considering that COX-1 was present in SFO neurons, often in the same cells as AT₁R, we sought to determine whether ANG II would elicit PGE₂ production from dissociated SFO cells and, if so, what pathway might be involved in the PGE₂ release (6, 29, 54). As shown in Fig. 2C, the basal amount of endogenous PGE₂ released from WT SFO cells following incubation in vehicle for 30 min was 1.69 ± 0.2 ng/mg (N = 9). Incubation in ANG II (100 nmol/l) for 30 min elicited a significant increase in endogenous PGE₂ release from WT SFO cells (∆208 ± 38.5%, P < 0.01 vs. WT SFO vehicle, N = 5). This effect was blunted when the cells were preincubated with the AT₁R antagonist losartan (3 μmol/l, 58.8 ± 23.5%, P > 0.05 vs. WT SFO vehicle, N = 9) or the PLA₂ antagonist ACA (1 μmol/l, 88.2 ± 29.4%, P > 0.05 vs. WT SFO vehicle, N = 8). Furthermore, incubation of COX-1−/− SFO cells in ANG II did not increase PGE₂ release (∆64.5 ± 8.3%, P > 0.05 vs. WT SFO vehicle, N = 6). Incubation of COX-2−/− SFO cells in ANG II partially but significantly inhibited PGE₂ release (∆89.4 ± 12.4%, P < 0.05 vs. WT SFO vehicle, N = 5). As shown in Fig. 2D, the basal levels of endogenous PGE₂ released from WT PVN cells following incubation in vehicle and ANG II (100 nmol/l) were relatively low compared with WT SFO cells (vehicle, 0.75 ± 0.49 ng/mg N = 4; and ANG II, 0.57 ± 0.47 ng/mg; P > 0.05, N = 4). These results indicate that ANG II elicits endogenous PGE₂ release from SFO cells, and this PGE₂ production may be dependent on the conversion of AA by COX-1. Importantly, this provides mechanistic support for our previous finding that COX-1-dependent PGE₂ formation plays a key role in ANG II-induced hypertension in the SFO.

**ANG II and PGE₂ induce increased ROS production in SFO cells.** It has been established that the SFO mediates systemic ANG II-dependent hypertension via increased production of ROS (6, 66, 68). Moreover, we have recently reported that a single dose (100 nmol/l) of ANG II causes a significant increase in ROS formation in WT SFO cells in vitro, a response that was absent in either EP₁R−/− or COX-1−/− SFO cells, but was intact in COX-2−/− SFO cells (6). Using the EP₁R antagonist SC51089, we also confirmed that ANG II-induced ROS formation in WT SFO cells was via EP₁R. In addition, PGE₂ (100 nmol/l) elicited increases in ROS formation to a similar extent as ANG II (6). These data indicate that PGE₂, via EP₁R, serves as a downstream signal in ANG II-induced ROS formation. Using DHE as a fluorescence ROS indicator, we thus focused on the roles of other critical elements in ANG-II signaling pathway, including AT₁R, PLA₂, and NADPH oxidase, in ANG II- and PGE₂-induced ROS formation in SFO cells.

As shown in Fig. 3, both exogenous ANG II (N = 8–24 per individual dose) and PGE₂ (N = 6–18 per individual dose) increased the DHE signal in a dose-dependent manner in dissociated WT SFO cells. Coaplication of the AT₁R antagonist losartan (3 μmol/l) significantly inhibited the increase in DHE induced by ANG II, but not by PGE₂ (Fig. 3, B and C).

As shown in Fig. 4, ANG II (100 nmol/l) induced an increase in DHE signal (+25 ± 3%, P < 0.01 vs. vehicle, N = 9), a response that was blocked by the general PLA₂ inhibitor ACA (1 μmol/l), the COX-1 inhibitor SC560 (10 μmol/l), the NADPH oxidase peptide inhibitor gp91-ds (1 μmol/l) and in Nox2−/− cells (Fig. 4A), but not by s-gp91-ds. PGE₂ (100 nmol/l) also induced a similar increase in ROS formation (+32 ± 8.2%, P < 0.01 vs. vehicle, N = 7) that was blocked by gp91-ds (1 μmol/l) and in Nox2−/− cells. This effect, however, was not blocked by ACA (1 μmol/l), SC560 (10 μmol/l), or s-gp91-ds (Fig. 4B). Taken together, these results indicate that PLA₂-mediated PGE₂ production is downstream of AT₁R but upstream of COX-1 and that
and the AT1R antagonist Los (3 nM) and other central neurons (Wang et al. 2004). Such results indicate that ANG II-elicted potentiation of the dihydropyridine-sensitive L-VDCC in SFO neurons is mediated by AT1R.

PLA2, COX-1, and EP1R are involved in ANG II-induced L-VDCC in SFO neurons. Since the EP1R is also involved in presynaptic Ca2+ influx (24) and glutamate release in the central nervous system (4), we used pharmacological and electrophysiological approaches to determine whether the PLA2/COX-1/EP1R signaling molecules that link to ANG II-induced ROS generation also play a role in the ANG II-mediated potentiation of L-VDCC in SFO neurons. As shown in Fig. 6, A and B, ANG II (100 nmol/l)-induced enhancement of L-VDCC (P < 0.01 vs. vehicle, N = 11) was abolished by pretreatment with the general PLA2 inhibitor ACA (1 μmol/l, P > 0.05 vs. vehicle, N = 5), coapplication of the COX-1 inhibitor SC560 (10 μmol/l, P > 0.05 vs. vehicle, N = 6), and the EP1R antagonist SC51089 (10 μmol/l, P > 0.05 vs. vehicle, N = 11) but were unaffected by coapplication of the COX-2 inhibitor NS398 (10 μmol/l, P < 0.01 vs. vehicle, N = 5). Moreover, ANG II-induced potentiation in Nox2 is the source of ANG II and PGE2-induced ROS formation in SFO cells.

ANG II potentiates the L-VDCC in SFO neurons via AT1R.

In addition to generation of ROS, the AT1R is also involved in voltage-dependent Ca2+ influx in the central nervous system (58, 70). Since the L-VDCC is a key signal for the regulation of short- and long-term potentiation and neurotransmitter release (12, 16, 46, 60, 64), we next sought to determine whether the AT1R-mediated signals were linked with the L-VDCC in SFO neurons. The VDCC in the mouse SFO neurons are composed of transient fast-inactivating and slowly inactivating components (59). Representative traces show that following application of TTX (1 μmol/l) block voltage-gated Na+ currents and Ctx-GVIA (1 μmol/l) to inhibit the transient, fast inactivating N-type Ca2+ currents in SFO neurons, the L-VDCCs were well distinguished (Fig. 5A). Biophysically, the current-voltage curves of this L-VDCC show that it was maximally elicited by a depolarization pulse from the holding potential at −60 mV to the stepping potential at −10 mV (Fig. 5B). Pharmacologically, it was activated by ANG II (100 nmol/l), which is in agreement with previous observation in the SFO (61) and other central neurons (Wang et al. 2004). Such effect was attenuated by coapplied losartan (3 μmol/l) (Fig. 5, A and C). However, further coapplication of the L-type Ca2+ channel activator BAY K 8644 (2 μmol/l, P < 0.01 vs. vehicle, N = 16) restored increased currents, but the L-VDCC was completely inhibited by the L-type Ca2+ channel blocker nifedipine (2 μmol/l, P < 0.01 vs. vehicle, N = 6) (Fig. 5, A and C) and nonselective Ca2+ channel blocker Cd2+ (100 μmol/l, P < 0.01 vs. vehicle, N = 6) (12, 58) (Fig. 5C). Taken together, ANG II (100 nmol/l) triggered a potentiation of the L-VDCC in SFO neurons (+53.3 ± 5.8%, P < 0.01 vs. vehicle, N = 9), a response that was blocked by the AT1R antagonist losartan (3 μmol/l, −15.7 ± 7.3%, P < 0.05 vs. vehicle, N = 9) (Fig. 5C). These results indicate that ANG II-elicted potentiation of the dihydropyridine-sensitive L-VDCC in SFO neurons is mediated by AT1R.

Fig. 3. A: representative dihydroethidium (DHE) images of the dissociated WT SFO cells are shown in the presence of vehicle and 100 nmol/l ANG II. B and C: dose-response curves show effects by ANG II (N of cells = 8–24/each dose; and N of experiments = 5) and PGE2 (N of cells = 6–18/each dose; and N of experiments = 4) on the relative intensity of DHE in the presence of Veh and the AT1R antagonist Los (3 μmol/l) in WT SFO cells. *P < 0.05 vs. Veh; **P < 0.01 vs. Veh.

Fig. 4. A: histogram shows the relative intensity of DHE in the presence of 100 nmol/l ANG II (N = 9) and coapplied ACA (1 μmol/l, N = 17), SC560 (10 μmol/l, N = 26), gp91-ds-tat (1 μmol/l, gp91, N = 8), and scrambled gp91-ds (1 μmol/l, s-gp91, N = 22) in WT SFO cells and in Nox2−/− SFO cells (N = 18) in the presence of ANG II (100 nmol/l). B: histogram summarizes the relative intensity of DHE in the presence of 100 nmol/l PGE2 (N = 7) and coapplied ACA (1 μmol/l, N = 17), SC560 (10 μmol/l, N = 7), gp91-ds-tat (1 μmol/l, gp91, N = 9), and scrambled gp91-ds-tat (1 μmol/l, s-gp91, N = 20) in WT SFO cells and in Nox2−/− SFO cells in the presence of PGE2 (100 nmol/l) (N = 18). **P < 0.01 vs. Veh.
L-type Ca\(^{2+}\) currents was abolished in EP\(_{2}R^{-/-}\) SFO neurons (\(P > 0.05\) vs. vehicle, \(N = 7\)) (Fig. 6B). Figure 6C shows the time course of ANG II-induced enhancement in the amplitude of L-VDCC currents in one single isolated SFO neuron. The ANG II-enhanced L-VDCC was reversed by the coapplied EP\(_{1}R\) antagonist SC51089 (10 \(\mu\)mol/l) (Fig. 6C). These results establish that L-VDCC is downstream of the ANG-II signaling pathway, and they are downstream of the ANG-II signaling pathway. Since ROS are known modulators of L-VDCC and they are downstream of the ANG-II signaling pathway, we were curious as to whether Nox2-derived ROS are involved in ANG II-induced L-type Ca\(^{2+}\) influx. We examined the effects of the ROS scavenger MnTBAP and Nox2 \(^{-/-}\) SFO neurons on ANG II-elicited L-VDCC. As shown in Fig. 7A, ANG II-induced potentiation in L-VDCC in WT SFO neurons (\(P < 0.01\) vs. vehicle, \(N = 6\)) was abolished by pretreatment with MnTBAP (100 \(\mu\)mol/l, \(P > 0.05\) vs. vehicle, \(N = 6\)). This response was also abolished in Nox2 \(^{-/-}\) SFO neurons (\(P > 0.05\) vs. vehicle, \(N = 4\)), implying that Nox2-derived ROS are downstream of the ANG-II signaling pathway.
required for the ANG-II potentiation of L-VDCC in SFO neurons.

**PGE₂ potentiates L-VDCC via EP₁R and Nox2 in SFO neurons.** PGE₂ is known to induce an increase in Ca²⁺ influx in the central nervous system (4, 32). Therefore, we further analyzed whether PGE₂, as a downstream signal of ANG-II pathway, is capable of inducing L-VDCC in SFO neurons and, if so, whether it involves the activation of EP₁R/Nox2 pathway. We examined the effect of applying antagonists for AT₁R, PLA₂, and EP₁R or deleting EP₁R and Nox2 on PGE₂-induced L-VDCC in SFO neurons. As shown in Fig. 7B, PGE₂ (100 nmol/l) induced potentiation of L-VDCC in SFO neurons (55.6 ± 9.9%, P < 0.01 vs. vehicle, N = 16), a response that was unaffected by losartan (3 μmol/l, P < 0.01 vs. vehicle, N = 4) or ACA (1 μmol/l, P < 0.01 vs. vehicle, N = 5). However, coapplied SC51089 (10 μmol/l, SC, N = 7) or genetic deletion of EP₁R (N = 5) inhibited L-VDCC enhancement induced by PGE₂ (P > 0.05 vs. vehicle) (Fig. 7B). Moreover, coapplied MnTBAP (100 μmol/l) or genetic deletion of Nox2 (N = 5) also abolished the L-VDCC potentiated by PGE₂ (P > 0.05 vs. vehicle) (Fig. 7B). These results indicate that the EP₁R and Nox2 serve as critical signals linking PGE₂ to L-type Ca²⁺ channels in the SFO.

Paracrine regulation of L-VDCC in SFO neurons by endogenous PGE₂. Our ultrastructural data show that COX-1 labeling is concentrated in postsynaptic neuronal processes in the SFO. Assuming that endogenous PGE₂ derived by postsynaptic COX-1 activates presynaptic EP₁R (24), which may be responsible for ANG II-induced potentiation of L-VDCC in isolated SFO neurons, we next sought to investigate whether locally generated PGE₂ derived from COX-1 in neighboring cells potentiates L-VDCC in the patched neuron. Adding SC560 (200 nmol/l) to the pipette buffer to inhibit intracellular COX-1 in the SFO neuron patched via the whole cell configuration (Fig. 8A), we found that exogenously applied ANG II (100 nmol/l) still increased L-VDCC (P < 0.01 vs. vehicle, n = 9). However, this response was blocked by extracellular coapplication of SC51089 (10 μmol/l, P > 0.05 vs. vehicle, n = 6) (Fig. 8B). Further coapplication of BAY K 8644 (2 μmol/l) reversed the inhibition of L-VDCC potentiation (P < 0.01 vs. vehicle, n = 4) in the same patched neuron, indicating that the L-type Ca²⁺ channels retained their functionality (Fig. 8B). These results indicate that endogenous PGE₂ derived from ANG II-activated COX-1 in neighboring SFO cells could exert paracrine action by potentiating L-VDCC through EP₁R. This assumption is further supported by our ultrastructural labeling studies that indicate extensive expression of COX-1 in SFO neuronal somata, dendrites, and glial cells.

DISCUSSION

COX-derived prostanoids and other endogenous fatty acid metabolites are involved in a wide variety of biological processes (1, 8, 27, 45, 50) and have been implicated in blood pressure regulation (20, 45). The SFO (11, 14), PGE₂/EP₁R signaling (6, 7, 21), ROS (6, 66, 68), and voltage-gated Ca²⁺ channels (37, 52, 70) have each been independently proposed as important mediators in the regulation of hypertension. Here we provide the first in vitro evidence that all of these factors are transducingly linked in ANG II-mediated signaling in the
SFO. We also demonstrate that COX-1 is highly concentrated in neuronal postsynaptic processes in the central SFO, particularly in the processes of cells that coexpress the AT1R and that COX-1 is the major source for the ANG II-activated PGE2 release in the SFO. Furthermore, ANG II-evoked PGE2 and ROS formation in dissociated SFO cells is prevented by deletion of COX-1 and partially attenuated by deletion of COX-2. ANG II or PGE2-potentiated ROS and Ca2+ currents are dependent on PLA2, EP1R, and ROS. These results support the notion that COX-1-derived PGE2 and EP1R signaling in the SFO are critical for slow-pressor ANG-II hypertension (6) and provide a detailed signaling pathway linking ANG II with ROS and L-VGCC, as summarized schematically in Fig. 9. As previously reported (53, 66), we speculate that this ANG II-induced intracellular increase in O2 may also lead to an increase in extracellular H2O2. Additionally, the present in vitro data also support the in vivo results recently published by our group (6).

Ultrastructural labeling of SFO neurons supports paracrine action of PGE2. The neuronal location of AT1R in the SFO was illustrated by GFP labeling in postsynaptic somata and dendrites, many of which also contained COX-1. The colocalization of AT1R and COX-1 in the SFO provides valuable ultrastructural support for the functional interaction seen between ANG II and COX-1 in dissociated SFO cells. In dual-labeled somatodendritic profiles, COX-1 ImG labeling was present in the cytoplasmic compartment of SFO neurons. Plasmalemmal labeling of COX-1 was also seen in small- and medium-sized dendrites receiving synaptic input from one or more axon terminals. Postsynaptic coexpression of COX-1 and AT1R in SFO neurons indicates that COX-1 is likely to be associated with AT1R-elicited generation of PGE2 and that release of PGE2 from postsynaptic sites may modulate EP1 receptors at presynaptic sites (24). In addition to COX-1, our recent EM data indicate that COX-2 is also present in SFO neurons that express AT1R (unpublished data).

It is notable that PGE2 is also produced and released from glial cells (29). Our electron microscopic data demonstrate that processes of glial cells in the SFO express about 30% of the COX-1 ImG labeling, which might also express AT1R. Furthermore, the fact that inhibition of COX-1 with intracellular SC-560 did not affect ANG II-induced, EP1R-mediated L-type Ca2+ currents in whole-cell patched SFO neurons indicates that COX-1 activation leading to PGE2 release is of exogenous origin. Thus these data raise the possibility that ANG II triggers PGE2 release from the neighboring cells, including neurons or glial cells such as ependymal cells, microglia, and astrocytes, and PGE2 exerts a paracrine action in the SFO.

ANG II, PLA2, COX-1, and EP1R-mediated ROS in the SFO. COX-1, PGE2, and NADPH oxidase-derived oxidative stress are involved in inflammatory responses (63) and EP1Rs were recently linked to ANG-II hypertension (6, 7). However, if or how these key signaling molecules interacted with one another had not been established. In this study, we found that inhibition of PLA2, COX-1, and EP1R prevented ANG II-elicited ROS production in the SFO, raising the possibility that there is molecular cross talk between COX-1/EP1R and Nox/Ros signaling in SFO neurons. Since the SFO is composed of neurons and nonneuronal glial cells such as ependymal cells, microglia, astrocytes, and vascular cells, ROS production detected in this in vitro study could come from dissociated SFO neurons or glial and vascular cells. However, further investigation is needed to identify different cell types and the exact mechanisms underlying EP1R-mediated ROS formation via Nox2-NADPH oxidase in the SFO.

EP1R and Ca2+ influx. The role of Nox2-dependent ROS in the regulation of ANG II-potentiated L-type Ca2+ currents has been previously addressed in central neurons and other cell types in peripheral tissue (2, 23, 57, 58, 70). However, a link between PGE2/EP1R signaling and ANG II-potentiated L-VGCC in central neurons had not been established. Our data provide strong evidence that PGE2 can potentiate L-VGCC through EP1R. EP receptors are a family of G protein-coupled receptors that regulate intracellular free Ca2+, and PGE2 is known to regulate Ca2+ influx (4, 5, 25–28, 32, 35, 36) through EP receptors in neurons and other cell types. EP1R and EP3R

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Fig. 9. A summary schematic overview highlights the signaling pathway proposed in the present study. Note: Nox2-containing NADPH oxidase is composed of 5 subunits, the membrane anchored catalytic subunits gp91phox (Nox2) and p22phox and the cytosolic subunits p47phox, p40phox, and p67phox. It is speculated that the intracellular increase in superoxide (O2) leads to an increase in extracellular H2O2 (53, 66). P, phosphorylated; AA, arachidonic acid; PKC, protein kinase C; [Ca2+]i, intracellular Ca2+ concentration.
couple to Go/11 protein and activate phospholipase C (38). However, contrary to potential Ca^{2+}-mobilizing actions by EP, R, the EP, R-coupled regulatory effects on Ca^{2+} homeostasis in adrenal medulla chromaffin cells and melanotrophs are inhibitory (25, 36). Nevertheless, because of lack of validated antibodies against AT1, R and selective antagonists for other subtypes of EP receptors, the present study has not ruled in coexpression of AT1, R and EP, R in the SFO or ruled out the possible involvement of other EP receptor subtypes in the regulation of ANG-II hypertension in this model.

**Mechanisms underlying ROS-enhanced gating of L-type Ca^{2+} channels.** Our results demonstrate that EP, R/ROS signaling is required for ANG II and PGE2 potentiation of L-VDCC in SFO neurons, but EP, R/ROS signaling is not necessary for direct gating induced by L-type channel activator BAY K 8644. This indicates that ROS has different binding sites from those of BAY K 8644. Recent reports suggest a novel mechanism for the regulation of Ca^{2+} influx and excitability. Using a TIRF imaging-based approach, a pathway has been characterized that functionally links subplasmalemmal generation of endogenous ROS that precede and colocalize with enhanced L-type Ca^{2+} channel activity via activation of PKCα in cerebral arterial smooth muscle (2). Further studies are needed to determine how ROS are functionally linked to L-VDCC potentiation in the SFO.

**Potential contribution of L-type Ca^{2+} currents to SFO function.** Though the mechanisms underlying ANG II-induced sympathoexcitation via SFO neuronal projections to other autonomic nuclei remain elusive, it is likely that voltage-gated L-type Ca^{2+} influx is involved in presynaptic glutamate release and therefore synaptic plasticity, such as LTP (16, 46, 64). For LTP mediated by increased release of glutamate in the corticalolateral nucleus of the amygdala, L-VDCCs are necessary for the persistent expression, and possibly the induction, of presynaptic cortico-LA LTP. Whereas L-VDCCs only weakly contribute to baseline release, they contribute significantly to increased synaptic transmission (16). On the other hand, the electrical stimulation-induced LTP of synapses onto the excitatory narrow-field vertical neurons of the sSc requires postsynaptic Ca^{2+} elevation by both NMDARs and L-type Ca^{2+} channels during induction but not maintenance (64). Therefore, L-type Ca^{2+} channels might be required for both induction and persistent expression of presynaptic LTP.

**The role of SFO L-type voltage-dependent Ca^{2+} channels in ANG II-induced hypertension.** The functional link between ANG II and voltage-gated Ca^{2+} influx in central neurons, including sympathetic neurons, has been previously reported (15, 33, 61, 70). In vivo, it is evident that the SFO sends the ANG-II peptidergic efferent to the PVN neurons (13, 30, 31) and that the ANG II-induced hypertension mediated via the SFO is attenuated by intracerebroventricular administration of the L-VDCC blocker nifedipine (48). In vitro, L-VDCC plays an important role in the regulation of peptidergic neurotransmitter release from presynaptic axonal terminals of central neurons (59). Therefore, it can be speculated that AT1, R in SFO sensory neurons are activated by circulating ANG II, which increases Ca^{2+} influx mediated by L-VDCC through EP, R and ROS pathways. Since increments in intracellular calcium concentration lead to ANG-II release from axonal terminals in the PVN-projecting SFO neurons (18), L-VDCC potentiation may cause excitation of presynaptic neurons in the PVN, leading to hypertension.

**Perspective and significance.** The in vitro results described here, along with our recently published in vivo results (6), provide strong evidence that EP, R-mediated ROS formation via Nox2-NADPH oxidase in the SFO may provide useful targets for the treatment of hypertension. SFO-targeted treatment with cytotoxic superoxide dismutase has proven to be as effective in ameliorating ANG-II hypertension as chronic intracerebroventricular infusion of the AT1, R antagonist losartan (68). Peripheral administration of the AT1, R antagonist losartan may also target the AT1, R expressed in the SFO because of its lack of a normal blood-brain barrier (9). In addition, blocking the ANG-II/PGE2/L-VDCC pathway in the SFO might be beneficial for the treatment of hypertension (51).

In conclusion, the present study used diverse cutting-edge technologies, including electron microscopy, EIA, ROS imaging, patch recording, and null mutant mice to comprehensively determine that ANG II-mediated PLA2 and PGE2 signals are coupled with ROS formation and intracellular Ca^{2+} homeostasis in SFO neurons. Additionally, as PGE2 is also released from glial cells, future studies should focus on the identification of possible distinct contributions by different cell types to ANG II-triggered PGE2 release. Taken together, our findings are a critical step toward a more complete understanding of ANG-II signaling in the SFO and may provide novel targets for future antihypertensive therapy.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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