Angiotensin II–Dependent Hypertension Requires Cyclooxygenase 1–Derived Prostaglandin E$_2$ and EP$_1$ Receptor Signaling in the Subfornical Organ of the Brain

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Hypertension is a global health problem, affecting nearly one third of the population and predisposing to serious diseases affecting the brain, heart, and kidneys. Cyclooxygenase (COX)-derived prostanoids, endogenous fatty acid metabolites known to play critical roles in a wide variety of biological processes, have long been implicated in blood pressure (BP) regulation. Clinical use of prostanoid synthesis-inhibiting nonsteroidal anti-inflammatory drugs are associated with hypertension, suggesting that endogenous prostanoids generally reduce blood BP. However, recently a more complex picture has emerged in which specific components of the prostanoid system have divergent effects and can be prohypertensive. For example, the major prostanoid prostaglandin E$_2$ (PGE$_2$) and its receptor subtype 1 (EP1R), one of 4 G protein–coupled receptors (EP$_1$–4R) mediating the effects of PGE$_2$, are now considered key players in hypertension and related end-organ damage. In particular, recent studies using mice with global targeted disruption of EP1R revealed a critical role for this receptor subtype in systemic angiotensin (Ang) II–dependent hypertension. However, the underlying mechanisms involved in this, including the enzymatic source of PGE$_2$, that is, COX-1 or COX-2, and its site(s) of action are not known. The subfornical organ (SFO) is a key forebrain region that mediates systemic Ang II–dependent HTN via reactive oxygen species (ROS). We tested the hypothesis that cross-talk between PGE$_2$/EP$_1$R and ROS signaling in the SFO is required for Ang II HTN. Radiotelemetric assessment of blood pressure revealed that HTN induced by infusion of systemic “slow-pressor” doses of Ang II was abolished in mice with null mutations in EP$_1$R or COX-1 but not COX-2. Slow-pressor Ang II–evoked HTN and ROS formation in the SFO were prevented when the EP$_1$ antagonist SC-51089 was infused directly into brains of wild-type mice, and Ang-II-induced ROS production was blunted in cells dissociated from SFO of EP$_1$R$^{-/-}$ and COX-1$^{-/-}$ but not COX-2$^{-/-}$ mice. In addition, slow-pressor Ang II infusion caused a $\sim$3-fold increase in PGE$_2$ levels in the SFO but not in other brain regions. Finally, genetic reconstitution of EP$_1$R selectively in the SFO of EP$_1$R-null mice was sufficient to rescue slow-pressor Ang II–elicited HTN and ROS formation in the SFO of this model. Thus, COX 1–derived PGE$_2$ signaling through EP$_1$R in the SFO is required for the ROS-mediated HTN induced by systemic infusion of Ang II and suggests that EP$_1$R in the SFO may provide a novel target for antihypertensive therapy.
ROS production evoked by Ang II in this brain region and contributing to neural dysregulation and hypertension remain poorly understood.

Here we sought to determine whether COX-derived PGE$_2$ and EP$_1$R signaling in the SFO provide an essential link among Ang II, ROS, and the central neural changes that give rise to slow-pressor Ang II hypertension. Using genetic and pharmacological tools to selectively target distinct components of the prostaglandin system in mice, we provide evidence that COX–1–derived PGE$_2$, signaling through EP$_1$R in the SFO is required for the ROS-mediated hypertension induced by systemic infusion of Ang II.

Methods
An expanded Materials and Methods section is available in the online-only Data Supplement.

Animals
Adult EP$_1$R-null, COX-1–null, and COX-2–null mice (8- to 10-weeks old) were obtained from in-house colonies. Mice were congenic with the C57Bl/6 strain, and age-matched C57Bl/6 mice were used as wild-type (WT) controls. All of the procedures were approved by the Cornell University Animal Care and Use Committee. Care of the mice met or exceeded standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, US Department of Agriculture regulations, and the American Veterinary Medical Association Panel on Euthanasia.

Pharmacological Agents
Inhibitors of EP$_1$R (SC-51089), COX-1 (SC-560), COX-2 (NS398), and Ang II type 1 receptors (losartan) were used.

BP Studies
Mice were anesthetized and instrumented with radiotelemetry devices as described. After 7 days recovery, baseline BP measurements were taken over 3 to 4 days, after which mice were implanted SC with osmotic minipumps loaded with the slow-pressor dose of Ang II (600 mg/kg per minute, 14 days) as described. BP was recorded daily for 3 weeks to monitor the effects of Ang II during the entire infusion period, as well as several days postinfusion. In studies using ICV infusion of SC-51089, mice were instrumented with ICV cannulas during the same surgical session as radiotelemetry implantation. For these studies, at the time of Ang II pump installation, a second 14-day osmotic minipump containing SC-51089 (144 μg/d) was implanted and connected to the ICV cannula, and BP monitoring was carried out as described above.

Measurement of Dipsogenic Responses
WT mice were implanted with osmotic minipumps loaded with the 2-week slow-pressor dose of Ang II (see above) or saline. Mice were euthanized at 3, 7, or 14 days after the start of infusions and brains flash frozen. Micropunches of SFO, paraventricular nuclei, somatosensory cortex, and cerebellum were collected from 2 mice per biological sample and weighed. Samples were processed and PGE$_2$ concentration was determined using an enzyme immunoassay kit as described.

ROS Detection
ROS production was assessed in dissociated SFO cells and in SFO-containing tissue sections using dihydroethidium (DHE) as an indicator. For in vitro ROS detection, WT, COX–1–null, COX–2–null, or EP$_1$R-null mice were euthanized, brains removed, and coronal sections containing the SFO obtained. SFO cells were dissociated and incubated with DHE, and time-resolved fluorescence was measured every 30 s (before vehicle) and after the addition of Ang II (100 nmol/L), as described. Additional in vitro studies were performed the same way, except pretreatment with SC-51089 (10 μmol/L) or losartan (3 μmol/L) was used, and PGE$_2$ (100 nmol/L) was also applied. For in situ ROS detection, brains were removed on day 16 of Ang II or vehicle infusions, frozen sections were incubated with DHE, and fluorescence was visualized and quantified as described. Data are expressed as DHE fluorescence intensity relative to control samples.

Adenoviral-Mediated Reconstitution of EP$_1$R in EP$_1$R-Null Mice
A recombinant adenoviral vector encoding murine EP$_1$R tagged with hemagglutinin on the N terminus (AdEP$_1$R; Figure S4A, available in the online-only Data Supplement) was engineered and then generated by the Iowa Gene Transfer Vector Core. AdEP$_1$R potency and stability were validated both in vitro and in vivo (Figure S4). An adenoviral vector encoding green fluorescent protein (AdGFP) obtained from Iowa Gene Transfer Vector Core was used as the control vector. EP$_1$R-null mice underwent SFO-targeted injection of AdEP$_1$R (5×10$^{10}$ plaque-forming units/mL; 500 nL) or titer-matched AdGFP, as described. During the same surgical session, radiotelemeters were implanted as described above. Nine days later, osmotic minipumps loaded with the 2-week slow-pressor Ang II dose were implanted. BP recording and ROS measurements were performed as described above.

Data Analysis
Data are expressed as mean±SEM. Comparisons between 2 groups were evaluated using the Student t test. Multiple comparisons were evaluated by ANOVA followed by the Dunnett or Tukey test. Differences were considered statistically significant at P<0.05.

Results
Genetic Disruption of EP$_1$R Prevents Hypertension During Slow-Pressor Ang II Infusion
EP$_1$Rs are implicated in the BP elevation induced by high doses of Ang II. Here, using EP$_1$R-null and WT mice, we determined whether EP$_1$Rs are involved in hypertension caused by chronic slow-pressor doses of Ang II, a model thought to mimic human hypertension and in which there is a strong central nervous system (CNS) component. Baseline mean arterial pressure (MAP) was not different between the groups (EP$_1$R$^{-/-}$: 98±2 mmHg; WT: 97±3 mmHg; P>0.05). In accordance with previous studies, Ang II induced a gradual rise in MAP in WT mice that peaked at ~30 mmHg above baseline after 2 weeks of Ang II infusion (Figure 1A). In contrast, this Ang II–induced rise in BP was absent in EP$_1$R-null mice (Figure 1A).
Ang II Slow-Pressor Hypertension Is Ameliorated in COX-1–Null But Not COX-2–Null Mice

Data in Figure 1A suggest that PGE₂ signaling is needed for slow-pressor Ang II hypertension. Because PGE₂ is a major reaction product of both COX isozymes,20 we next sought to determine the enzymatic source of PGE₂ involved in the slow-pressor effects of Ang II using mice with null mutations in either COX-1 or COX-2. Baseline MAP did not differ between the groups (COX-1⁺/−: 93±2 mmHg; COX-2⁻/⁻: 96±7 mmHg; WT: 98±3 mmHg; P>0.05). Similar to WT mice in Figure 1A, Ang II induced the classic slow rise in MAP, which peaked during the second week of infusion (Figure 1B). This response was abolished in COX-1–null mice, whereas it remained intact in COX-2–null mice (Figure 1B).

EP₁R and COX-1 in the CNS Are Implicated in Ang II–Induced Cardiovascular and Dipsogenic Effects

Data in Figure 1 suggest that COX-1–derived PGE₂ and EP₁R are involved in the rise in BP during slow-pressor Ang II infusion, but the use of global knockouts prevents us from pinpointing the site(s) of these effects. To test the hypothesis that slow-pressor Ang II hypertension is caused by a PGE₂/EP₁R mechanism operating in the CNS, we used 3 separate approaches. First, given abundant evidence that slow-pressor Ang II hypertension is mediated via CNS-driven increases in sympathetic activity,21 power spectral analysis was used to assess slow-pressor Ang II–induced sympathetic responses in EP₁R-null versus WT mice. Increased low-frequency/high-frequency oscillations of arterial pressure reflect increased sympathetic activity.21 Consistent with previous findings, Ang II infusions caused a doubling of the low-frequency/high-frequency ratio in WT mice by the end of the 2-week infusion period (pumps empty completely on day 16; Figure 2A) when the hypertensive response is maximal (see Figures 1 and 2B). In contrast, the low-frequency/high-frequency ratio was unchanged in EP₁R⁻/⁻ mice over the course of Ang II infusions (Figure 2A). Second, using chronic infusion of the EP₁R antagonist SC51089 into brains (ICV) of WT mice, data shown in Figure 2B demonstrate that the slow-pressor Ang II–induced rise in MAP observed in ICV vehicle-treated controls was prevented by blockade of EP₁R in the CNS. It should be noted that ICV infusions at this volume do not escape into the peripheral circulation.11 Third, given the well-established role of the CNS in mediating Ang II effects on dipsogenesis,16,22 we used the classic assay of bolus injection of Ang II in the brain (ICV) coupled with measurement of drinking responses in WT mice pretreated (IP) with either SC51089 or the selective inhibitors of COX-1 (SC-560) or COX-2 (NS-398). As seen in Figure 2C, Ang II elicited the well-established dipsogenic response in vehicle-treated mice. This response was intact in mice treated with the COX-2 inhibitor, whereas it was markedly attenuated in mice treated with either the COX-1 inhibitor or the EP₁R antagonist.

PGE₂ Synthetic Enzymes and Receptors Are Expressed in the SFO Under Basal Conditions, and PGE₂ Production Is Augmented in This Brain Region During Slow-Pressor Ang II Infusion

Data in Figure 1 and 2 suggest that COX-1–derived PGE₂ acting at EP₁R in the CNS is important in slow-pressor Ang II hypertension. Because the SFO is a key region of the CNS mediating this form of hypertension,11,12 we next examined the capacity of the SFO for PGE₂ formation and signaling. Real-time quantitative PCR was performed to determine basal mRNA levels of COX isozymes, PGE synthases, and EP receptor subtypes in SFO tissue harvested from adult WT mice. First, COX-1 was expressed at ≈7-fold higher levels in SFO than COX-2 (Figure 3A). Second, of the PGE synthases that convert PGH₂ to PGE₂, that is, PGES-1, PGES-2, or cytosolic PGES, the latter was expressed at much higher levels in the SFO than either of the other 2 isoforms (Figure 3B). Third, of the 4 receptor subtypes mediating PGE₂ effects, mRNA levels of EP₁R were >10-fold higher than EP₂R, EP₃R, or EP₄R in SFO tissue (Figure 3C). Importantly, all 4 of the EPR subtypes were expressed at very low levels in organum vasculosum of the lamina terminalis (Figure S1). Finally, we sought to directly evaluate the effects of slow-pressor Ang II infusions on PGE₂ formation in the SFO. As seen in Figure 3D, Ang II caused a significant increase in

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Image: Figure 1. Slow-pressor angiotensin (Ang) II hypertension is prevented in mice with null mutations in EP₁ receptor (EP₁R) or cyclooxygenase (COX) 1 but not COX-2. A. Summary of mean arterial pressure (MAP) before, during, and after 2 weeks of slow-pressor Ang II infusions in wild-type (WT; n=5) and EP₁R⁻/⁻ (n=7) mice. B. MAP throughout slow-pressor Ang II in WT (n=7), COX-2⁻/⁻ (n=4), and COX-1⁻/⁻ (n=5) mice. *P<0.05 vs baseline (WT in A and B, COX-2 in B); †P<0.05 vs WT or COX-2⁻/⁻.. Arrow indicates start of Ang II infusions.
levels were observed in any of these regions compared with controls (Figure S2).

**PGE₂/EP₁R Signaling Is Coupled to Ang II–Induced ROS Accumulation in the SFO**

Slow-pressor Ang II infusion causes increased ROS formation in the SFO. Thus, several experiments were performed to test the hypothesis that PGE₂/EP₁Rs are required for ROS formation in the SFO in response to Ang II. First, ROS production was assessed in vitro in single cells dissociated from the SFO of adult WT and null mice. Consistent with earlier reports, Ang II caused a significant increase in DHE signal in WT SFO cells compared with control (Figure 4A). This response was absent in SFO cells dissociated from either EP₁R−/− or COX-1−/− mice, whereas it was intact in COX-2−/− SFO cells (Figure 4A). Pharmacological studies using the EP₁R antagonist SC-51089 in SFO cells dissociated from WT mice confirmed the results observed in the EP₁R−/− mice (Figure S3) and further verified earlier data that Ang II–induced increases in ROS formation are sensitive to losartan (Figure S3). Interestingly, PGE₂ elicited increases in DHE intensity to a similar extent as Ang II, and although this response was inhibited by SC-51089, it was unaffected by losartan (Figure S3). Next, in situ DHE microfluorography was used to assess Ang II–induced ROS production in the SFO of WT mice receiving systemic slow-pressor Ang II infusions concomitant with ICV infusions of either SC-51089 or vehicle. Consistent with earlier reports, DHE fluorescence intensity was ~2.5-fold higher in the SFO of mice receiving Ang II (day 16) compared with untreated mice (Figure 4B). This response was abolished in mice receiving ICV infusions of SC-51089 (Figure 4B).

**Virally Mediated Reconstitution of EP₁R Selectively in the SFO Rescues Slow-Pressor Ang II Hypertension and ROS Formation in EP₁R-Null Mice**

Our data thus far show that global knockout of EP₁R prevents slow-pressor Ang II–induced hypertension, and the CNS, particularly the SFO, may be involved. To directly test the hypothesis that SFO-selective expression of EP₁R is sufficient to induce gradual hypertension elicited by slow-pressor doses of Ang II, we used a genetic rescue approach to reconstitute EP₁R selectively in the SFO with AdEP₁R in EP₁R-null mice. First, the potency and stability of AdEP₁R were evaluated. AdEP₁R increased exogenous EP₁R mRNA levels as measured by quantitative PCR in Neuro2A cells in a concentration-dependent manner (Figure S4B). Further studies using the EP₁R antagonist SC-51089 in SFO cells dissociated from either SC-51089- or vehicle-treated mice (Figure 4B). This response was abolished in mice receiving ICV infusions of SC-51089 (Figure 4B).

PGE₂ levels in the SFO as early as day 3 of the infusion compared with saline controls. This was sustained through 7 days of the infusion, but by day 14, PGE₂ levels were not different from controls. PGE₂ levels were also measured in paraventricular nuclei, somatosensory cortex, and cerebellum of WT mice infused with slow-pressor doses of Ang II or vehicle but no significant Ang II–induced changes in PGE₂...
virus, EP,R −/− mice underwent SFO-targeted injections of AdEP,R or control vector AdGFP. Before initiating slow-pressor Ang II infusions 9 days after viral transduction, baseline MAP was not different in the 2 groups (AdGFP: 97 ± 5 mmHg, AdEP,R: 105 ± 2 mmHg; P > 0.05). In control AdGFP-treated EP,R −/− mice, there was no change in MAP at any time throughout the Ang II infusion (Figure 5A), verifying data in Figure 1 showing that slow-pressor Ang II hypertension is abolished in EP,R −/− mice. In contrast, the classic gradual rise in MAP was restored in AdEP,R-treated

Figure 3. Prostaglandin (PG) E2 synthetic enzyme and receptors are expressed at high levels in the subfornical organ (SFO) under basal conditions, and PGE2 levels are increased in the SFO early after slow-pressor angiotensin (Ang) II infusion. A through C, Basal mRNA levels of PGE2 receptors EP1–4R, cyclooxygenase (COX) isozymes, and PGE synthases in adult wild-type (WT) SFO tissue (n = 3), as analyzed by quantitative real-time PCR. †P < 0.05 vs EP2–4R (A), vs COX-2 (B), vs PGE synthase (PGES) 1 and PGES2. D, PGE2 levels measured by ELISA in micropunches of SFO from WT mice at days 3, 7, and 14 of slow-pressor Ang II (n = 3) or saline infusion (n = 3). *P < 0.05 vs saline. In all of the assays, 2 brains were pooled for per biological sample.

Figure 4. Prostaglandin (PG) E2/EP1 receptor (EP1R) signaling is required for angiotensin (Ang) II–induced reactive oxygen species (ROS) formation in the subfornical organ (SFO). A, Summary of the effects of Ang II vs vehicle (Veh) on ROS production as measured by dihydroethidium (DHE) fluorescence intensity in cells dissociated from SFO of adult wild-type (WT; n = 24), EP1R −/− (n = 9), cyclooxygenase (COX) 1 −/− (n = 9) and COX-2 −/− (n = 9) mice. *P < 0.05 vs Veh; NS indicates not significant. B, left, Representative confocal images showing DHE fluorescence in SFO tissue at day 16 of slow-pressor Ang II infusion in mice treated concomitantly with either ICV vehicle or SC-51089. Mice left untreated served as controls. Right, Summary of DHE fluorescence intensity in SFO tissue of mice with no treatment (n = 7) or at day 16 of slow-pressor Ang II infusions treated concomitantly with either ICV Veh (n = 6) or SC-51089 (n = 7). †P < 0.05 vs untreated; #P < 0.05 vs Ang II + ICV Veh. Scale bar: 50 μm.
EP1R−/− mice (Figure 5A). This was accompanied by low-frequency/high-frequency ratios that were similar to those observed in WT mice at the end of the 2-week infusion period (1.72 ± 0.6; P < 0.05). To determine whether this was accompanied by restoration of Ang II–induced ROS accumulation in the SFO, a separate cohort of EP1R−/− mice with SFO-targeted AdEP1R or AdGFP were subjected to DHE studies, as described above. In AdGFP-treated EP1R−/− mice, DHE fluorescence intensity in the SFO did not change from baseline, confirming in this null strain findings in Figure 4B obtained using the EP1R antagonist. In contrast, ROS levels in the SFO of AdEP1R-treated EP1R−/− mice were re-established to that of AngII–treated WT mice (see Figure 4B), with an ∼3-fold increase in DHE intensity compared with controls (Figure 5B).

Discussion

Brain Ang II, prostanoids, and ROS have each been proposed as important mediators of BP regulation and hypertension.2,9,23 Here we provide evidence that these factors are mechanistically linked in the pathogenesis of slow-pressor Ang II–elicited hypertension. We show that elevations in BP during slow-pressor Ang II infusions are abolished in mice with global null mutations of EP1R or COX-1 but not COX-2. Pharmacological inhibition of EP1R selectively in the CNS prevents slow-pressor Ang II hypertension, and central Ang II–driven sympathetic and dipsogenic responses are also mediated by brain EP1R. Markedly elevated levels of COX-1, cytosolic PGES, and EP1R are observed in the SFO relative to other PGE2 synthetic enzymes and receptors, making this forebrain structure an ideal platform for COX-1–derived PGE2 signaling through the EP1R. Indeed, slow-pressor Ang II infusions induce early robust PGE2 production in the SFO. Both in vitro and in vivo inhibition of EP1R prevents Ang II–induced ROS accumulation in the SFO, a response that is known to have a causative role in slow-pressor Ang II hypertension.11,15 Finally, virally mediated reconstitution of EP1R selectively in the SFO of EP1R−/− mice restores hypertension and SFO ROS formation in response to slow-pressor Ang II infusions. This provides the first evidence that COX-1–derived PGE2/EP1R signaling in the SFO is required for the ROS-mediated hypertension elicited in the slow-pressor Ang II model.

The significance of these findings lies in the complex picture that has emerged recently concerning specific components of the prostanoid system and their divergent effects.
on BP. COX-inhibiting nonsteroidal anti-inflammatory drugs are among the most widely prescribed classes of therapeutic agents, many of the effects of which are mediated by their actions in the CNS. However, the ubiquitous tissue distribution and biological complexity of the prostanoid system, coupled with recent evidence that certain components are prohypertensive, underscores the importance of understanding how prostanoids influence BP regulation, particularly as newer agents with a higher selectivity of action within the prostanoid system are being developed. Our data bolster the emerging concept that endogenous PGE2-mediated EP1R activation contributes to Ang II–dependent hypertension and related end-organ damage. Although the study by Guan et al established that the pressor effects of high-dose systemic Ang II are blunted in EP1R−/− mice, the enzymatic source of PGE2 and the tissue site(s) of action remained poorly defined. Here, using tissue-specific reconstitution of EP1R in EP1R-null mice, our data now point to a key role for PGE2/EP1R signaling in the CNS, particularly the SFO, in mediating systemic Ang II–dependent hypertension. Although these studies do not rule out the possibility that other EPR subtypes and/or other tissues sites are involved in this model of Ang II hypertension, the complete restoration of the slow-pressor response, coupled with the markedly higher levels of EP1R expression in SFO compared with the other subtypes, strongly supports this concept. This is important information in considering EP1R as a novel target for the treatment of hypertension.

Another major finding of the present study is that we identified COX-1 in the SFO as the sole source of the PGE2 required for Ang II slow-pressor hypertension. Although it is well established that Ang II stimulates PGE2 synthesis, the enzymatic source of PGE2 in Ang II–evoked hypertension has remained poorly defined. Our data showing an absence of slow-pressor Ang II–evoked hypertension in COX-1−/− mice but an intact response in COX-2−/− mice implicates COX-1 as the source of PGE2 and the mediator of hypertension in this model. The relative roles of COX-1- versus COX-2–derived products in Ang II–dependent responses have been controversial, but our findings are consistent with previous studies showing that pharmacological blockade or genetic deletion of COX-1 but not COX-2 reduced the acute pressor effects of Ang II in mice. In addition, Capone et al showed recently that selective pharmacological inhibition of COX-1 but not COX-2 prevented the cerebrovascular effects of Ang II and that COX-1 is the major source of PGE2 in the somatosensory cortex. Our evidence that COX-1 is expressed at much higher levels than COX-2 in the SFO and that the COX-1–coupled PGE synthase cytosolic PGES is the predominant isoform in the SFO suggests COX-1 as the source of increased PGE2 levels in this brain region during slow-pressor Ang II infusion. The importance of COX-1 is further suggested by our in vitro data demonstrating that Ang II–evoked ROS formation in dissociated SFO cells is prevented by inhibition of COX-1 but not COX-2. Thus, our data suggest that, at least in the SFO, COX-1 predominates under basal conditions. Further studies will be required to define the cell-type localization of these enzymes in the SFO before and after Ang II infusion to better understand the cellular mechanisms involved.

It is notable that systemic Ang II induced increases in PGE2 production in the SFO but not in other brain regions, including the paraventricular nuclei, cortex, and cerebellum, despite these regions being enriched in PGE2 synthesis enzymes and receptors. This suggests that the links between Ang II and PGE2 are specific rather than attributed to generalized CNS activation in this model and are also consistent with the fact that the SFO lacks a blood-brain barrier and can be accessed by circulating Ang II via Ang II type 1 binding. Indeed, Ang II has been shown to elicit PGE2 synthesis in cultured CNS cells via stimulation of Ang II type 1 receptors and, interestingly, autoradiographic studies have demonstrated intense PGE2 binding in the anteroventral region of the third ventricle, a region that encompasses the SFO. It is also important to note that the Ang II–induced increase in PGE2 production in the SFO occurred early in the infusion period (3 and 7 days), before a significant rise in sympathetic outflow and BP. Furthermore, PGE2 levels had returned to baseline by 14 days, a time when hypertension is at its peak. This suggests that PGE2 in the SFO, per se, is not directly causing neural changes leading to hypertension in this model but rather serves as a critical signaling intermediate that then triggers downstream pathways involved in central Ang II–mediated hypertension. Indeed, essential hypertension has a slow and insidious onset, and its underlying pathophysiological mechanisms precede the elevation in BP. Increasingly, adaptive changes in CNS neurons are considered highly relevant to hypertension. Determining whether early induction of PGE2 in the SFO by slow-pressor Ang II is involved in such changes in CNS circuities involved in the delayed sympathetic activation and hypertension in this model will require further investigation.

We and others have demonstrated that Ang II induces ROS formation in the SFO via the activation of NADPH oxidase, and this is a key signaling event in the hypertensive and dipsogenic actions of Ang II in the brain. A key finding of the present study demonstrates that COX-1–derived PGE2 and EP1R are required for this Ang II–evoked ROS formation in the SFO in vitro and in vivo. This is consistent with recent studies demonstrating a similar mechanism in Ang II–mediated ROS formation in cerebral blood vessels, which likely involves NADPH oxidase. Given that Ang II–induced NADPH oxidase activation in neurons is Ca2+ dependent, along with evidence that activation of EP1R results in inositol triphosphate (IP3)-mediated release of intracellular Ca2+ and reduced Ca2+ efflux through the Na/Ca2+ exchanger, it is reasonable to speculate that NADPH oxidase mediates PGE2/EP1R-mediated ROS formation in the SFO through Ca2+ signaling. Further studies will be required to elucidate the detailed cellular mechanisms linking Ang II, prostanoids, ROS, and neuronal signaling in the SFO. For example, it will be important to define the relationship between early prehypertensive induction of PGE2 in the SFO by slow-pressor Ang II and ROS accumulation in this region during the later hypertensive phase in the model.
Perspectives

COX-derived prostanoid signaling has long been implicated in the pathogenesis of Ang II–dependent hypertension, and this study provides evidence for the first time that a mechanism involving increased COX-1–dependent PGE2 formation and EP1-R signaling in the SFO region of the forebrain is a key underlying mechanism. Determining how these various players are spatially and functionally linked in the SFO to provide the substrate for adaptive neural changes that lead to gradually developing Ang II hypertension is a critical next step. However, in the meantime, this is important information as new therapeutic agents targeting the prostanoid system are being developed.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Angiotensin-II-dependent Hypertension Requires Cyclooxygenase 1-derived Prostaglandin E₂ and EP₁ Receptor Signaling in the Subfornical Organ of the Brain

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Detailed Methods

Animals. All procedures were approved by the Animal Care and Use Committee at Cornell University. Studies were conducted in adult (8-10 wks old) male COX-1 null, COX-2 null, and EP1R null mice which were obtained from in-house colonies1-3. Mice were congenic with the C57Bl/6 strain and age-matched C57Bl/6 mice (Harlan Laboratories) were used as wild-type (WT) controls. Mice were fed standard chow and water ad libitum. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the AVMA Panel on Euthanasia.

Pharmacological agents. The COX-1 inhibitor SC-560 (C17H12CIF3N2O), the COX-2 inhibitor NS-398 (C13H18N2O5S) and the EP1R inhibitor SC-51089 (C22H19CIN4O3•HCL) (Enzo Life Sciences, Plymouth Meeting, PA) were dissolved in dimethylsulfoxide (DMSO) and diluted with saline to the desired concentration. The final DMSO concentration was <0.2%, which does not affect physiological parameters tested. The Ang-II type 1 receptor (AT1R) antagonist losartan potassium (C22H22CIKN6O) (Sigma-Aldrich, St. Louis, MO) and PGE2 (C20H32O5) (Cayman Chemical, Ann Arbor, MI) were diluted in sterile saline. Specificity of SC-51089, SC-560 and NS-398 at similar doses used herein has been confirmed previously1.

Blood pressure studies. Mice were anesthetized (ketamine, 150 mg/kg + xylazine, 15 mg/kg, ip) and instrumented with radiotelemetry probes (TA11PA-C10, Data Sciences International, Arden Hills, MN) as previously described4, 5. Briefly, the catheter of the telemeter was implanted in the thoracic aorta via the left common carotid artery, and the body of the probe was placed in a subcutaneous pocket created in the right flank. The wound was closed and sutured, and body temperature was maintained at 37°C using a heating pad until sternal recumbency was recovered. Mice remained undisturbed in their home cages for 7 days to achieve full recovery of normal circadian rhythm and cardiovascular parameters4 before baseline BP recording over 3-4 days. Mice were then implanted subcutaneously with 14-day osmotic minipumps (ALZET®; Durect Corporation, Cupertino, CA) loaded with the slow-pressor dose of Ang-II (600ng/kg/min) as described5. BP was recorded daily for 2 hours (10am-12pm) for 3 weeks to monitor the effects of Ang-II during the entire infusion period as well as several days after infusion. It should be noted that although 14-day pumps were used, the actual calculated infusion times for these pumps ranges from 16 to 19 days (0.22-0.25μl/hr, fill volume of 100 ± 6μl).

In studies using intracerebroventricular (i.c.v.) infusion of SC-51089, mice were instrumented with i.c.v. cannulae (brain coordinates relative to bregma: 0.3mm caudal, 1.00 from midline, 3.3mm ventral)5 during the same surgical session as radiotelemetry implantation. After 7 days of recovery and 3 days of baseline recording, two osmotic minipumps were implanted subcutaneously. One contained the 14-day slow-pressor dose of Ang-II (600ng/kg/min) as described above. The other one was loaded with SC-51089 (144μg/day, 14 days) and was connected to i.c.v. cannulae using
MicroRenathane® tubing (Braintree Scientific, Braintree, MA). BP monitoring was carried out as described above.

**Power spectral analysis.** Power spectral analysis of arterial pressure variability was performed as described⁶-⁸. Briefly, spectral power of mean arterial pressure (MAP) in the frequency domain was determined using custom-written functions in HemoLab Analyzer and Batch Processor software (version 9.3, provided by Dr. Harald Stauss, University of Iowa, Iowa City, IA). An average spectrum using 4096 point Fast Fourier Transforms (FFT) with 50% overlap was computed for a compact spectrum display⁶. Spectra were divided into the following frequency ranges: low frequency (LF: 0.4-1.0 Hz) and high frequency (HF: 1.0-3 Hz)⁸. Data were expressed as LF/HF in relation to baseline.

**Measurement of dipsogenic responses.** WT mice were instrumented with i.c.v. cannulae as described above and allowed 7 days recovery. Mice were administered either vehicle, SC-51089 (10μg/kg), SC-560 (10mg/kg) or NS-398 (10mg/kg) by intraperitoneal injection (200 nl) 30 minutes prior to i.c.v. bolus administration of Ang-II (200ng, 200nl). Water drinking responses were measured over 1 hour as described⁹, ¹⁰.

**Quantitative real-time PCR detection of prostanoid-related transcripts.** WT mice were decapitated and brains were removed and immediately placed on dry ice. The SFO was isolated by micropunch (0.75mm, Stoelting Co., Wood Dale, IL) as described¹⁰. Two SFO samples were used per biological sample. Total RNA was isolated by Trizol® (Invitrogen, Carlsbad, CA) extraction and reverse transcribed using random hexamer primers. Template samples (25 ng) were subjected in triplicate to real-time qPCR (ABI 7500FAST system) using Power SYBR Green (Applied Biosystems, Foster City, CA) as described¹⁰. All primers were derived from *Mus Musculus* gene (National Center for Biotechnology Information GenBank) and are shown in Table S1. Serial dilution was performed for each set of primers to determine qPCR amplification efficiency before the experimental run. A dissociation protocol (60-95°C melt) was performed at the end of each run to verify that only one amplicon was formed during the process of amplification. No RT and no template controls were performed during each run to ensure no contamination was present. β-actin was used as a normalizer gene in all experiments. Relative fold-change was calculated using the comparative ΔΔCt method as described¹⁰.

**PGE₂ assay.** WT mice were implanted with osmotic minipumps loaded with the 14-day slow-pressor dose of Ang-II (600ng/kg/min, see above) or saline. Mice were decapitated at 3, 7 or 14 days after start of infusions and brains were removed and immediately flash frozen in liquid nitrogen for 10 sec. Micropunches of SFO, paraventricular nuclei (PVN), somatosensory cortex (CTX) and cerebellum (CBM) were collected from 2 mice per biological sample and weighed. Samples were then homogenized and prostanoids extracted as previously described¹, ¹¹. PGE₂ concentration was determined using an enzyme immunoassay kit (Cayman Chemical)¹, ¹¹.

**ROS detection.** ROS production was assessed in dissociated SFO cells and in SFO-containing tissue using dyhidroethidium (DHE) as an indicator. For *in vitro* ROS detection in SFO cells¹², WT, COX-1-null, COX-2-null or EP₃R-null mice were sacrificed.
using CO₂, and the brains were removed and quickly transferred to a chamber containing ice-cold sucrose artificial cerebrospinal fluid (s-aCSF) composed of (in mM): 26 NaHCO₃, 1 NaH₂PO₄, 3 KCl, 5 MgSO₄, 0.5 CaCl₂, 10 glucose, and 248 sucrose, oxygenated with 95% O₂ and 5% CO₂, pH 7.35. Coronal slices (300μm) were then obtained using a Vibratome (Leica) and stored in a chamber filled with oxygenated lactic acid (l)-aCSF composed of (in mM): 124 NaCl, 26 NaHCO₃, 5 KCl, 1 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 10 glucose, 4.5 lactic acid, pH 7.35. The SFO region was dissected and transferred to an oxygenated (l)-aCSF buffer containing 0.02% pronase and 0.02% thermolysin, and then incubated at 35°C for 1.5 hrs. Isolated SFO cells were then transferred to a glass-bottom Petri dish and perfused with the oxygenated (l)-aCSF and incubated with 2μM DHE (Molecular Probes) for 30 minutes in the dark and were continuously perfused with 2μM DHE containing (l)-aCSF. Following 100-150 ms exposure to mercury light, time-resolved fluorescence was measured every 30s after addition of vehicle, Ang-II (100nM) or PGE₂ (100nM) using IPLab (Scanalytics Inc.). Recordings were initiated after a stable baseline was achieved. In a subset of in vitro studies, SC-51089 (10μM) or losartan (3μM) (in oxygenated (l)-aCSF) were applied 30 min prior to Ang-II or PGE₂.

ROS production in SFO tissue was assessed by DHE microfluorography as described⁵. Brains were removed on day 16 of Ang-II or vehicle infusions (peak of hypertension), flash frozen and coronal sections (20μM) were taken onto chilled microscope slides. Sections were then thawed at room temperature, rehydrated with phosphate-buffered saline (PBS), and incubated for 5 min in the dark with DHE (1μM) followed by 2 min wash with PBS. DHE fluorescence was visualized by confocal microscopy (Zeiss LSM 510 or Leica SP5). Detector and laser settings were kept constant across all samples within individual experiments, and control and experimental samples were always processed in parallel. Fluorescence intensity was quantified using ImageJ software and normalized to fluorescence levels observed in control samples as described⁵.

Adenoviral-mediated reconstitution of EP₁R in EP₁R-null mice. A recombinant adenoviral vector encoding murine EP₁R tagged with HA on the N-terminus was engineered and then generated and characterized by the Iowa Gene Transfer Vector Core (IGTVC)¹³. An Ad vector encoding green fluorescent protein (AdGFP) was obtained from IGTVC and used as a control. Briefly, adenoviruses were based on the human Ad serotype 5, from which the E1a and E1b replication genes had been deleted¹³. The titer of both viruses was ~ 5x10¹⁰ pfu/mL. For AdEP₁R, HA (YPYDVPDYA) was N-terminally tagged to full length cDNA of mus musculus EP₁R gene (GenBank ID: NM013641). HA-EP₁R was under the control of the CMV promoter. In the same construct, a reporter gene GFP was driven off the RSV promoter (Fig S3A). To validate AdEP₁R potency and stability, several experiments were performed. First, Neuro2A cells were infected with serial dilutions of AdEP₁R (0-500 multiplicity of infection). 48 hrs after infection, cells were collected and qPCR analysis was performed as described above using a primer set spanning HA and EP₁R: Forward 5'-CCCATACGACGTACCAGATTACGCTAG-3'; Reverse 5'-GCAGGCACGCCACGCACTTTG-3' (Fig S3B). Second, for in vivo validation, WT mice underwent SFO-targeted injection of AdEP₁R (500nl) as described⁵,¹⁰,¹⁴ and HA-EP₁R expression was verified in regional micropunches and in situ. In situ expression of
AdEP$_1$R-induced HA-EP$_1$R in EP$_1$R null mice was examined using immunohistochemistry (Fig S3C). 9 days after SFO-targeted injection of titer-matched AdEP$_1$R or AdGFP, mice were perfused with 37°C saline followed by ice-cold 4% paraformaldehyde. Brains were removed and stored in 30% sucrose overnight before 20μm coronal sections were taken onto glass slides. Immunostaining was performed using a rabbit polyclonal HA antibody (2.5μg/ml, Abcam) followed by DyLight™549-conjugated AffiniPure goat anti-rabbit IgG (3μg/ml, Jackson ImmunoResearch Laboratories, Inc.). AdEP$_1$R and AdGFP-treated samples were assayed on the same slides. Images were taken using a confocal microscope (Zeiss LSM 510). Transgene expression was limited to the SFO in all samples except in one animal in which a few cells in organum vasculosum of the lamina terminalis were transduced. In separate studies, brains were collected 9 or 28 days after SFO-targeted AdEP$_1$R injection (a time-frame that covered the entire Ang-II infusion period in the following experiments). Micropunches from SFO, PVN, RVLM and CTX (0.75mm, Stoelting Co., Wood Dale, IL) were harvested and subjected to qPCR analysis using the HA-EP$_1$R primer set listed above (Fig S3D). β-actin was used as the normalizing gene. CTX samples from day 9 post-injection were used as the calibrator. Real-time qPCR was performed as described above. Finally, for BP and ROS studies, EP$_1$R-null mice underwent SFO-targeted injection of titer-matched AdGFP or AdEP$_1$R (500nl). During the same surgical session, radiotelemeters were implanted as described above. Nine days later, osmotic minipumps loaded with the 14-day slow-pressor Ang-II dose were installed as described above. BP recording, spectral analysis and ROS measurements were performed as described above.

**Data analysis.** Data are expressed as mean±SEM. Comparisons between two groups were evaluated using the Student’s t test. Multiple comparisons were evaluated by ANOVA followed by Dunnett’s or Tukey’s test. Differences were considered statistically significant at $p<0.05$. 

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References

Table S1

qPCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>GenBank ID</th>
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| EP₁R  | F: TTTATTAGCCTTGGGCTCGTGGA  
       | R: ATTCACACTAATGCCTGCAGGA | NM_013641 |
| EP₂R  | F: GATGAAGCAACCACAGCAGAC  
       | R: CAGAGAGGACTCCACATGA | NM_008964 |
| EP₃R  | F: GGTCATCCTCGTGTAACCTGTC  
       | R: GTGATGGTTAGCCCAAGGA | NM_011196 |
| EP₄R  | F: GCCCTCCTCCTGCAATATAAC  
       | R: TTTCAACACTCCAGCCTGAAC | NM_008965 |
| COX-1 | F: CACTGGTGGATGCCTTCTCT  
       | R: TCTCGGAAGCTCCTTGAAC | NM_008969 |
| COX-2 | F: GGGTGCTGGTGTCATGAGTG  
       | R: GGGTCATCCGGTGTCCTGGTG | NM_011198 |
| cPGES | F: TGCTGTACTTCACAGGAAACCT  
       | R: AAGTCACAACACTTTGCGTGC | NM_019766 |
| PGES1 | F: TTTCTGCTCTCGACAGCAGACT  
       | R: CACATCTGGGTCACTCCGATGA | NM_022415 |
| PGES2 | F: GAGGACAAGGCCCACACTAC  
       | R: ATCCCTGTCAACAAAGGACTT | NM_133783 |
| β-actin | F: CATCCTCCTCCTCCCTGGAGAAGA  
        | R: ACAGGATTCCATACCCCAAGGAAGG | NM_007393 |
Supplemental Figures

Figure S1. A) Basal mRNA levels of EPR$_{1,4}$ in adult WT organum vasculosum of the lamina terminalis (OVLT) tissue (n=3) as analyzed by quantitative real-time PCR. B) Comparison of basal mRNA levels of EPR$_{1,4}$ in SFO vs OVLT. *p<0.05 vs. EP$_1$R in OVLT and EP$_2$-4R in SFO and OVLT.
Figure S2. PGE$_2$ levels in WT mouse brain regions during slow-pressor Ang-II infusion. PGE$_2$ levels measured by ELISA in micropunches of PVN (A), CTX (B) and CBM (C) at 3, 7 and 14 days of slow-pressor Ang-II ($n=3$) or vehicle infusions ($n=3$). $p>0.05$ vs vehicle at all time-points in all regions. Two brains were pooled per biological sample for all regions.
Figure S3. Ang-II- and PGE$_2$-induced increases in ROS formation in cells dissociated from the SFO of WT mice. Histogram showing the effects of Ang-II or PGE$_2$ on ROS formation as measured by DHE fluorescence intensity in cells dissociated from SFO of WT mice (n=9-24) before and after vehicle (Veh), losartan (LS) or SC-51089. All data are expressed as a ratio of DHE fluorescence relative to baseline (control). *p<0.05 vs. control; †p<0.05 vs. vehicle; n.s., not significant.
Figure S4. Ad-mediated EP1R transgene expression in the SFO in vitro and in vivo. A) Schematic of AdEP1R vector containing HA-tagged full-length murine EP1R gene driven off CMV and enhanced green fluorescent protein (eGFP) driven off RSV. B) qPCR data using a primer set spanning the HA tag and EP1R gene revealed concentration-dependent (0-500 multiplicity of infection) effects of AdEP1R on HA-EP1R transcript levels in Neuro2A cells. C) Representative immunohistochemistry of SFO in EP1R−/− mice with SFO-targeted injections of AdEP1R (n=3) or AdGFP (n=3) using an antibody targeting the HA tag. D) Real-time RT-PCR data showing HA-EP1R mRNA levels in the SFO, PVN, RVLM and CTX of WT mice at day 9 (n=3) and 28 (n=3) after SFO-targeted AdEP1R injection. *p<0.05 vs. day 9 and 28 PVN, RVLM, CTX. Two brains were pooled per biological sample for each region.