Proinflammatory Cytokines Upregulate Sympathoexcitatory Mechanisms in the Subfornical Organ of the Rat

Shun-Guang Wei, Yang Yu, Zhi-Hua Zhang, Robert B. Felder

Abstract—Our previous work indicated that the subfornical organ (SFO) is an important brain sensor of blood-borne proinflammatory cytokines, mediating their central effects on autonomic and cardiovascular function. However, the mechanisms by which SFO mediates the central effects of circulating proinflammatory cytokines remain unclear. We hypothesized that proinflammatory cytokines act within the SFO to upregulate the expression of excitatory and inflammatory mediators that drive sympathetic nerve activity. In urethane-anesthetized Sprague–Dawley rats, direct microinjection of tumor necrosis factor (TNF-α) or interleukin (IL)-1β into SFO increased mean blood pressure, heart rate, and renal sympathetic nerve activity within 15 to 20 minutes, mimicking the response to systemically administered proinflammatory cytokines. Pretreatment of SFO with microinjections of the angiotensin II type-1 receptor blocker losartan (1 μg), angiotensin-converting enzyme inhibitor captopril (1 μg) or cyclooxygenase-2 inhibitor NS-398 (2 μg) attenuated those responses. Four hours after the SFO microinjection of TNF-α (25 ng) or IL-1β (25 ng), mRNA for angiotensin-converting enzyme, angiotensin II type-1 receptor, TNF-α and the p55 TNF-α receptor, IL-1β and the IL-1R receptor, and cyclooxygenase-2 increased in SFO, and mRNA for angiotensin-converting enzyme, angiotensin II type-1 receptor, and cyclooxygenase-2 increased downstream in the hypothalamic paraventricular nucleus. Confocal immunofluorescent images revealed that immunoreactivity for the p55 TNF-α receptor and the IL-1 receptor accessory protein, a subunit of the IL-1 receptor, colocalized with angiotensin-converting enzyme, angiotensin II type-1 receptor–like, cyclooxygenase-2, and prostaglandin E2 EP, receptor immunoreactivity in SFO neurons. These data suggest that proinflammatory cytokines act within the SFO to upregulate the expression of inflammatory and excitatory mediators that drive sympathetic excitation. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.05112.)

Key Words: cyclooxygenase-2 ■ cytokine receptors ■ paraventricular nucleus ■ sympathetic nervous system

Proinflammatory cytokines (PICs) are increased in cardiovascular disease states, and studies over the past decade have suggested that blood-borne and brain PICs contribute to the neurohumoral activation in heart failure (HF) and in some forms of hypertension. We recently demonstrated that the subfornical organ (SFO), a circumventricular organ that lacks a blood–brain barrier, is an important central nervous system sensor of peripheral inflammation, mediating the effects of circulating PICs on autonomic and cardiovascular function. However, the mechanisms by which PICs act within the SFO to influence neurohumoral excitation have not been examined.

The SFO is rich in angiotensin-converting enzyme (ACE) and in angiotensin II (ANG II) type-1 receptors (AT, R), key components of the brain renin–angiotensin system (RAS) that activates SFO neurons and drives sympathetic nerve activity in pathophysiological states like hypertension and HF. PICs contribute to upregulation of brain RAS activity in the hypothalamic paraventricular nucleus (PVN), another brain region that has been implicated in the sympathetic excitation and cardiovascular dysfunction in hypertension and HF. Cyclooxygenase (COX), the key enzyme regulating the production of prostaglandin E2 (PGE2), is also abundantly expressed in the highly vascularized SFO. PGE2 increases the firing rate of SFO neurons by disinhibiting inhibitory γ-aminobutyric acid inputs. ANG II infusion induced hypertension is reportedly dependent on the activity of constitutively expressed COX-1 in the SFO and PGE2 production in SFO might be expected to increase sympathetic nerve activity.

The present study was undertaken to determine whether the excitatory effects of PICs on cardiovascular function and sympathetic nerve activity are mediated by PIC-induced upregulation of RAS and COX-2 activity in the SFO. Because the SFO projects directly to the PVN, which has been implicated as an important source of augmented sympathetic and neuroendocrine activity in hypertension and HF, we also examined...
whether PIC activation of the SFO affects the neurochemical milieu downstream in the PVN.

**Methods**

**Animals**

Adult male Sprague–Dawley rats (300–350 g) were purchased from Harlan (Indianapolis, IN). Animals were housed in Animal Care Facility at the University of Iowa and fed rat chow ad libitum. All experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. The experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Experimental Protocols**

1. Urethane-anesthetized rats underwent electrophysiologic and hemodynamic recording studies to determine the sympathetic responses to SFO microinjections of tumor necrosis factor (TNF-α) (25 ng) or interleukin (IL)-1β (25 ng), preceded by 10 minutes by SFO microinjection of vehicle (VEH), the AT1 receptor blocker losartan (1 µg), the ACE inhibitor captopril (1 µg), or the COX-2 activity inhibitor NS-398 (2 µg).

2. Urethane-anesthetized rats received an SFO microinjection of TNF-α (25 ng), IL-1β (25 ng) or VEH and were euthanized 4 hours later to collect SFO and PVN tissue to determine the mRNA expression of AT1R, ACE, COX-2 and COX-1, TNF-α, IL-1β, the p55 TNF-α receptor (TNFRI), and the IL-1β receptor (IL-1R).

3. Urethane-anesthetized rats were transcardially perfused with heparinized saline followed by 4% paraformaldehyde in 0.01 mol/L PBS to collect brain tissue for immunofluorescent staining to determine whether TNFRI and IL-1 receptor accessory protein (IL-1RACp) immunoreactivity was colocalized with AT1R-like, ACE, COX-2, and PGE, receptor EP3 immunoreactivity in the SFO.

**Drug Administration**

SFO microinjection of TNF-α, IL-1β, losartan, captopril, and NS-398 was performed via a 35-gauge cannula inserted in a 30-gauge guide cannula that was placed 0.9 mm posterior to bregma, along the midline of the skull (see online-only Data Supplement for details). TNF-α and IL-1β were purchased from Fitzgerald (Acton, MA) and Millipore (Billerica, MA), respectively. Losartan and captopril were purchased from Sigma (St. Louis, MO). All these drugs were dissolved in artificial cerebrospinal fluid for SFO microinjection. NS-398 was purchased from Tocris (Ellisville, MO), and was first dissolved in dimethyl sulfoxide and then diluted in artificial cerebrospinal fluid to make a 5% final dimethyl sulfoxide concentration.

**Statistical Analysis**

All values are expressed as the means±SEM. The significance of differences among groups was analyzed by 2-way repeated-measure ANOVA followed by post hoc Fisher’s test. Student’s t test was used to determine statistical significance between paired data for a single comparison. *P*<0.05 was considered to indicate statistical significance.

**Results**

**Hemodynamic and Sympathetic Effects of SFO Microinjections**

**TNF-α**

SFO microinjection of TNF-α (n=6; Figure 1A, 1E, 1F, and 1G) elicited significant increases in mean blood pressure (MBP), heart rate (HR), and renal sympathetic nerve activity (RSNA) in the rats pretreated with SFO microinjections of VEH. These excitative responses began within 15 to 20 minutes after the TNF-α microinjection. The maximum responses of MBP (19.8±2.7 mm Hg), HR (75.2±8.5 bpm), and RSNA (81.9±9.2% change) occurred 2 to 3 hours after the TNF-α microinjection and remained at higher than baseline level for at least 5 hours. Pretreatment with SFO microinjection of losartan (Figure 1B, 1E, 1F, and 1G), captopril (Figure 1C, 1E, 1F, and 1G), or NS-398 (Figure 1D, 1E, 1F, and 1G) significantly reduced the MBP (10.9±2.6, 12.7±2.9, and 9.5±2.6 mm Hg, respectively), HR (44.8±8.5, 48.7±8.6, and 38.8±8.2 bpm, respectively), and RSNA (49.1±9.4%, 53.7±6.2%, and 45.1±9.3% changes, respectively) responses to the TNF-α microinjection. SFO microinjections of an equal volume of vehicle (artificial cerebrospinal fluid) had no noticeable effect on baseline MBP (98.3±2.9 mm Hg), HR (323±13 bpm), or integrated RSNA (11.2±3.5 mV).

**IL-1β**

Microinjection of IL-1β into the SFO (n=6; Figure 2A, 2E, 2F, and 2G) also induced substantial and long-lasting increases in MBP, HR, and RSNA in the rats pretreated with SFO microinjections of VEH. The excitative responses to IL-1β began within 15 to 20 minutes of the IL-1β microinjection. The peak increase in MBP (20.0±2.5 mm Hg) occurred at 1 to 2 hour and was sustained at a higher than baseline level for the remainder of the 3 to 5 hour recording period. The peak increase in HR (81.2±10.5 bpm) and RSNA (83.5±10.6% change) occurred 2 to 3 hours after the IL-1β microinjection and lasted for at least 5 hours. Pretreatment with SFO microinjection of losartan (Figure 2B, 2E, 2F, and 2G), captopril (Figure 2C, 2E, 2F, and 2G), or NS-398 (Figure 2D, 2E, 2F, and 2G) significantly attenuated the MBP (11.2±2.4, 11.8±2.2, and 10.7±2.5 mm Hg, respectively), HR (44.0±9.0, 49.7±5.9, and 48.7±8.2 bpm, respectively), and RSNA (42.2±7.3%, 52.0±9.4%, and 47.5±5.5% changes, respectively) responses to SFO microinjection of IL-1β.

**Histological Confirmation of SFO Microinjection**

At the conclusion of each experiment, SFO microinjection sites were examined by light microscopy. Microinjection sites were confirmed by the presence of Pontamine sky blue throughout the SFO, with no dye present in the 3rd ventricle (Figure S1).

**Effects of SFO Microinjections on Excitatory Milieu of SFO and PVN**

**TNF-α**

Real-time PCR analysis of brain tissue obtained 4 hours after the SFO microinjection of TNF-α revealed that mRNA levels for the RAS components, AT1R and ACE, were significantly increased in the SFO (AT1R, 3.32±0.34 versus 1.03±0.12
fold; ACE, 2.87±0.30 versus 1.05±0.13 fold) and the PVN (AT1R, 2.18±0.23 versus 1.04±0.10 fold; ACE, 2.21±0.33 versus 1.03±0.11 fold), compared with SFO microinjection of VEH (Figure 3A and 3B). There was also a significant increase in COX-2 mRNA in both SFO (4.07±0.42 versus 1.02±0.09 fold) and PVN (2.98±0.24 versus 1.01±0.08 fold), but not in COX-1 mRNA (SFO, 1.44±0.27 versus 1.02±0.12 fold; PVN, 1.39±0.16 versus 1.02±0.11 fold), compared with SFO microinjection of VEH (Figure 3C and 3D).

Similarly, 4 hours after SFO microinjection of TNF-α, TNF-α and IL-1β mRNA were significantly increased in the SFO (2.58±0.22 versus 1.00±0.09 and 2.30±0.38 versus 1.03±0.09 fold, respectively) and the PVN (2.34±0.16 versus 1.03±0.12 and 2.00±0.13 versus 1.01±0.07 fold, respectively), compared with microinjection of VEH (Figure 3E and 3F). TNFR1 and IL-1R mRNA levels were also increased in both SFO (2.81±0.25 versus 1.01±0.11 and 2.11±0.34 versus 1.00±0.09 fold, respectively) and PVN (2.55±0.18 versus 1.03±0.12 and 1.83±0.12 versus 1.09±0.11 fold, respectively), compared with SFO microinjection of VEH (Figure 3G and 3H).

**IL-1β**

The IL-1β microinjection, like the TNF-α microinjection, significantly increased AT1R and ACE mRNA in the SFO (AT1R, 3.06±0.32 versus 1.00±0.10; ACE, 2.65±0.27 versus 1.01±0.12 fold) and PVN (AT1R, 2.01±0.23 versus 1.08±0.09; ACE,
2.05±0.30 versus 1.01±0.11 fold), compared with the SFO microinjection of VEH (Figure 4A and 4B). COX-2 mRNA was also significantly increased in both SFO (4.23±0.34 versus 1.03±0.09 fold) and PVN (3.25±0.26 versus 1.05±0.10 fold), but COX-1 mRNA was unaffected (SFO, 1.44±0.27 versus 1.12±0.18 fold; PVN, 1.50±0.27 versus 1.08±0.17 fold, compared with SFO microinjection of VEH (Figure 4C and 4D).

TNF-α and IL-1β mRNA levels were significantly elevated in the SFO (2.39±0.27 versus 1.00±0.10 and 3.02±0.43 versus 1.10±0.15 fold, respectively) and the PVN (2.17±0.25 versus 1.03±0.11 and 2.33±0.16 versus 1.04±0.16 fold, respectively) compared with VEH (Figure 4E and 4F). TNFR1 and IL-1R mRNA levels were also augmented in SFO (2.60±0.23 versus 1.06±0.09 and 2.66±0.45 versus 1.10±0.12 fold, respectively) and PVN (2.37±0.17 versus 1.09±0.12 and 2.30±0.16 versus 1.00±0.11 fold, respectively), compared with VEH (Figure 4G and 4H).

SFO microinjection of TNF-α and IL-1β had no significant effects on the mRNA levels of any of these RAS and inflammatory elements in the cortex (Figures 3 and 4).
Immunofluorescent studies revealed intense, evenly distributed expression of TNFR1 and IL-1RAcP immunoreactivity in the SFO of normal rats (Figure 5; Figures S2 and S3 in the online-only Data Supplement). AT1R-like, ACE, COX-2, and EP3 receptor immunoreactivity was also denser expressed in the SFO (Figure 5; Figures S2 and S3). Confocal immunofluorescent images indicated that TNFR1 and IL-1RAcP immunoreactivity colocalized with AT1R-like, ACE, COX-2, and EP3 receptor immunoreactivity on SFO neurons (Figure 5; Figures S2 and S3). Some colocalization was also apparent in undefined cellular elements surrounding SFO neurons. AT1R immunostaining is reported...
as AT1R-like to acknowledge the lack of specificity of the AT1R antibody.

Discussion

Our previous work has demonstrated that blood-borne PICs induce sympathetic activation and a pressor response and that the SFO plays an important role in mediating those effects. The present study examined potential mechanisms in the SFO, and downstream in the PVN, that might mediate cardiovascular and autonomic responses to circulating PICs. Novel findings of this study are (1) localized microinjections of TNF-α and IL-1β into the SFO increase BP, HR, and RSNA, closely mimicking the effects of systemically administered TNF-α and IL-1β; (2) pretreating the SFO with microinjections of agents that counter RAS and COX-2 activity attenuates the cardiovascular and sympathetic responses to SFO microinjections of PICs; (3) TNF-α and IL-1β receptor immunoreactivity is colocalized with AT1R-like, ACE, COX-2, and EP3 receptor immunoreactivity on SFO neurons; and (4) SFO microinjections of TNF-α and IL-1β upregulate mRNA for key components of the RAS (ACE and AT1R) and mediators of central inflammation (TNF-α and IL-1β), their receptors and COX-2 in both SFO and PVN. These findings suggest that the SFO-mediated acute sympathoexcitatory response to PICs depends on the ambient level of RAS and COX-2 activity and that PICs act within the SFO to increase RAS and COX-2 activity. This study provides new insights into the central mechanisms driving neurohumoral excitation in cardiovascular disorders like HF and hypertension.

Central nervous system markers of inflammation and RAS activity are dramatically upregulated in cardiovascular autonomic regions of the brain in experimental models of HF and hypertension and contribute to the increased sympathetic activity characteristic of those conditions. However, the signals that direct the brain to upregulate these excitatory systems are still poorly understood. Afferent neural and humoral signals relaying compromised cardiac, vascular, and renal status likely both contribute. As a forebrain circumventricular organ that is exposed to blood-borne humors and projects directly to cardiovascular autonomic nuclei, the SFO is uniquely positioned to regulate central neural responses to peripheral stresses. Previous work from our laboratory has demonstrated that blood-borne ANG II upregulates AT1R mRNA and protein in the SFO. The present study demonstrates that TNF-α and IL-1β can also upregulate AT1R mRNA in the SFO, as well as the mRNA for ACE, TNF-α, and IL-1β and their receptors and COX-2, the rate limiting enzyme in the production of PGE2. Thus, in conditions like hypertension and HF in which circulating ANG II and PICs are both increased, both may contribute to neuro-excitation in the SFO.

In addition, the present study demonstrates that PICs, acting on their receptors in the SFO, also increase RAS and COX-2 activity downstream in the PVN, a source of presynaptic pathways innervating preganglionic sympathetic neurons in the intermediolateral column of the spinal cord both directly and indirectly via presynaptic neurons in the rostral ventrolateral medulla. Thus, the effects of PICs in the SFO may increase the excitability of PVN neurons to a variety of ascending and descending neural inputs that ultimately determine the level of sympathetic nerve activity.

The present study demonstrates that PIC receptors are present on SFO neurons expressing RAS components and inflammatory mediators and that mRNA expression of these elements in the SFO and in the PVN is increased when measured 4 hours after local microinjection of PICs. Although these changes may well contribute to the increases in sympathetic drive observed in HF and hypertension, in which circulating levels of PICs are chronically elevated, they cannot account for the SFO-mediated acute (within 10–20 minutes) increases in sympathetic drive and arterial pressure induced by intravenous, intracarotid, and direct SFO microinjection of PICs in normal rats. There are several potential explanations for these early excitatory responses. PIC binding to G-protein coupled receptors in the SFO may activate G-protein-dependent signaling cascades, such as protein kinase A and protein kinase C, to modulate both ionotropic and metabotropic receptors. PICs can activate mitogen-activated protein kinase signaling,
which has been reported to have acute and chronic influences on sympathetic nerve activity in normal and pathophysiological conditions. For example, extracellular signal-regulated protein kinases 1 and 2 can acutely phosphorylate the voltage-gated potassium channel (KV) subunit Kv4.2 in neurons, reducing the transient outward potassium current that modulates neuronal excitability. PICs may also interact with fast neurotransmitter systems to modulate neuronal excitability and synaptic transmission. TNF-α can increase local glutamate concentration in the synaptic cleft by stimulating glutamate release from microglia or inhibiting the glutamate retake by astrocytes and can potentiate glutamate-mediated neuronal excitation by modulating NMDA and GABA receptor function. Finally, like ANG II, PICs may induce ER stress, which has been associated with ANG II–induced hypertension. One or more of these mechanisms may account for acute sympathetic and cardiovascular responses to PICs, and all may contribute to chronic sympatho-excitation in pathophysiological states.

A novel finding of this study is the ability of pretreatment with agents that block RAS and COX-2 activity to substantially reduce the acute sympathetic and cardiovascular responses to the SFO PIC microinjections. This effect occurs far too early to be explained by a reduction in the activity of PIC-stimulated production of ACE, AT, R, and COX-2. A more likely explanation might be that ambient levels of ANG II and PGE2 sustain a basal level of neuronal excitability that is reduced by pretreatment with the inhibitors of RAS and COX-2 activity, rendering presympathetic neurons less responsive to acutely administered PICs.

Perspectives

The SFO is an active interface between peripheral and central inflammation. We previously demonstrated a critical role for the SFO in the cardiovascular and sympathetic response to blood-borne PICs. The present study offers some initial insights into the mechanisms by which PICs, which do not directly activate ion channels to excite neurons, might act within the SFO to induce sympathetic excitation. We found that the acute excitatory response to PICs is substantially reduced by agents that inhibit RAS and COX-2 activity, suggesting that the PIC response is dependent on a basal state of neuronal excitability sustained by these 2 mechanisms. We also observed colocalization of AT, R-like, ACE, COX-2 and EP, receptor immunoreactivity with PIC receptor immunoreactivity on SFO neurons, and a PIC-induced upregulation of mRNA for ACE, AT, R, TNF-α, TNFR1, IL-1β, IL-1R, and COX-2 not only in the SFO but also downstream in the PVN, suggesting that PICs may act within the SFO to induce a chronic, sustained increase in the production of RAS and inflammatory mediators in these 2 critical cardiovascular regulatory regions of the brain. By doing so, PICs may facilitate the central nervous system actions of other excitatory mediators, like ANG II and aldosterone, that circulate in chronic cardiovascular disease states like hypertension and HF. Because it lacks a blood–brain barrier, the SFO is potentially an ideal target for systemic actions of other excitatory mediators, like ANG II and PICs, which do not act within the SFO to induce sympathetic excitation. We found one or more of these mechanisms may account for hypertension, HF, and other cardiovascular-related diseases with an inflammatory component.

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Disclosures

None.

References


Novelty and Significance

What Is New?

• Subfornical organ (SFO) microinjection of tumor necrosis factor (TNF-α) or interleukin-1β induces pronounced increases in blood pressure, heart rate, and renal sympathetic nerve activity that are largely reduced by pretreatment with the angiotensin II type-1 receptor blocker losartan, the angiotensin-converting enzyme inhibitor captopril, or the cyclooxygenase-2 activity inhibitor NS-398.

• SFO microinjection of TNF-α or interleukin-1β increases the mRNA expression of angiotensin-converting enzyme, angiotensin II type-1 receptor, cyclooxygenase-2, TNF-α, interleukin-1β, the p55 TNF-α receptor TNFR1, and the interleukin-1 receptor in the SFO and paraventricular nucleus.

What Is Relevant?

• Interaction of proinflammatory cytokines with renin–angiotensin system and cyclooxygenase-2/prostaglandin E2 in the SFO may be an important mechanism contributing to the neurohumoral activation in hypertension and heart failure.

• Proinflammatory cytokines, acting on SFO, upregulate excitatory and inflammatory mediators downstream in the paraventricular nucleus, a source of presynaptic neurons.

Summary

The data demonstrate that proinflammatory cytokines act within the SFO to elicit a sympathoexcitatory response mediated by the renin–angiotensin system and by cyclooxygenase-2/prostaglandin E2 and to upregulate the expression of components of the brain renin–angiotensin system and mediators of inflammation in the SFO and downstream in the paraventricular nucleus. These mechanisms may contribute to the sympathoexcitatory influence of circulating proinflammatory cytokines in cardiovascular disease states.
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SUPPLEMENT

to

Pro-inflammatory Cytokines Upregulate Sympathoexcitatory Mechanisms in the
Subfornical Organ of the Rat

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Running Title: Cytokines and sympathetic activation
MATERIALS AND METHODS

Acute electrophysiological and hemodynamic recording

The surgical preparation for electrophysiological recording had been detailed in our previous publications.1,2 Briefly, rats were anesthetized with urethane (1.5 g/kg, IP), supplemented as needed (0.1 g/kg, IV). Blood pressure (BP) was measured via a catheter implanted in the left femoral artery. Body temperature was maintained at 37 ± 1°C with a heating pad and heat lamp. A renal nerve was exposed and was dissected free from surrounding tissue via a left flank incision. The renal nerve was placed on bipolar silver wire recording electrodes to record renal sympathetic nerve activity (RSNA). The incisions were sutured after nerve and electrodes were stabilized with Kwik-Cast silicon sealant (WPI, INC, Sarasota, FL). The recording session began when the baseline RSNA and BP were stable, at least 60 minutes following completion of the surgical preparations.

The method for SFO microinjection has been described in detail previously.3 Briefly, the skull was exposed by a midline incision and leveled between the bregma and lambda. One 30-gauge guide cannula was placed 0.9 mm posterior to bregma, along the midline for SFO microinjection. The cannula tip was advanced to a final position 2.7 mm ventral to the cranial surface. A 35-gauge injection cannula connected with a 1-μl Hamilton microsyringe was inserted into the guide cannula and extended 2 mm beyond the tip of the guide cannula for SFO microinjection (0.2 μl over 10 s). The microinjection sites were verified by 2% Pontamine sky blue (100 nl) that was injected in the same locations at the end of experiments.

Data were acquired with a Cambridge Electronics Design laboratory interface (CED, model 1401; Cambridge, UK) connected to a personal computer. RSNA was initially processed with a Paynter filter (20-ms time constant, BAK Electronics; Germantown, MD) to rectify and integrate the raw multifiber signal. The BP signal was passed to the CED 1401 via a Gould TA240S chart recorder (Gould Instruments, Valley View, OH). Heart rate (HR) was derived from the frequency of the BP pulses. Digitized data were stored for subsequent off-line analysis with Spike2 software (CED).

Histology

At the conclusion of the experiments, the animals that underwent the SFO microinjection were deeply anesthetized with overdose of urethane and decapitated. The brain was rapidly removed and fixed with 4% paraformaldehyde for 48 hours. Frozen serial coronal sections (30 μm) through the SFO region were obtained with a cryostat. The SFO microinjection sites were confirmed by light microscopy.

Real-time PCR

Rats anesthetized with urethane (1.5 g/kg, IP) were decapitated to obtain brain tissue for real-time PCR measurement. The brains were immediately removed, frozen in liquid nitrogen and stored at −80 °C for subsequent use. The frozen brains were cut into
300-μm coronal sections. Target tissues including SFO, PVN and cortex were obtained using a punch device (inner diameter 1.5 mm, Stoelting, Wood Dale, IL). The mRNA levels of ACE, AT1R, COX-2, COX-1, TNF-α, IL-1β, TNFR1 and IL-1R in SFO, PVN and cortex were measured with real-time PCR following reverse transcription of total RNA. The sequences for primers and probe used are shown in Table S1. Real-time PCR was performed using the ABI prism 7700 Sequence Detection System (Applied Biosystems). GAPDH mRNA was quantified as an internal control for each sample. The value for each sample was normalized to GAPDH and expressed as a fold difference relative to the control.

Immunofluorescence

Rats anesthetized with urethane (1.5 g/kg, IP) were transcardially perfused with 4% paraformaldehyde. Brains were removed and embedded with OCT and rapidly frozen in acetone chilled by dry ice. Coronal forebrain sections (16 μm) of target tissues were made using a cryostat and stored at −80 °C for subsequent staining.

Immunofluorescent staining was used to examine the co-localized expression of cytokine receptors TNFR1 and IL-1RAcP (a component of IL-1R) with renin-angiotensin system components AT1R and ACE as well as COX-2 and PGE2 EP3 receptors in the subfornical organ. The sections were incubated with the primary antibodies, the rabbit polyclonal antibodies to TNFR1 (Cat# ab19139, 1: 200, Abcam), IL-1RAcP (Cat# I8153, 1: 200, Sigma-Aldrich), and the mouse monoclonal antibodies to AT1R (Cat# ab9391, 1:100, Abcam), ACE (Cat# ab11738, 1:100, Abcam), COX-2 (Cat# sc-166475, 1:100, Santa Cruz) and EP3 (Cat# sc-57105, 1:100, Santa Cruz) followed by secondary antibodies Alex Fluor 488 goat anti-rabbit IgG (Cat# A-11070, 1:200, Invitrogen) and Alex Fluor 568 goat anti-mouse IgG (Cat# A-11003, 1:200, Invitrogen). Immunofluorescent staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 710, Carl Zeiss, Inc).

Because of the known non-specificity of commercially available AT1R antibodies, including the one used here, immunostaining for AT1R is designated AT1R-like.

REFERENCES


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<td>IL-1R (TaqMan)</td>
<td>Forward primer: 5'-GCCACCGGAATGAGACG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GCCCGTGACGTGGGAGA-3’</td>
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<tr>
<td></td>
<td>Probe: 5'-AGCTGACCCAGGATACACAGACTG-3’</td>
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<tr>
<td>ACE (TaqMan)</td>
<td>Forward primer: 5'-GGAGACGACTTACAGTGAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-CACACCCAAAGGAATTCAGC-3’</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-AATGGGCCACGTCCCGGAAT-3’</td>
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Figure S1. Histological confirmation of SFO microinjection sites. SFO microinjections of TNF-α and IL-1β were verified by injection of 2% Pontamine sky blue (0.2 µl) at the conclusion of the experiments. A: blue dye evenly distributed in the SFO. B: blue dye is not in the SFO.
Figure S2. Laser confocal images showing the co-localized expression of TNF-α receptor 1 (TNFR1: A, C) and IL-1 receptor accessory protein (IL-1RAcP: B, D) immunoreactivity with the angiotensin II type-1 receptor-like (AT$_1$R: A, B) and angiotensin converting-enzyme (ACE: C, D) immunoreactivity in SFO of normal rats. Red, TNFR1 or IL-1RAcP; Green: AT$_1$R-like, ACE; Yellow: Merged.
Figure S3. Laser confocal images showing the co-localized expression of TNF-α receptor 1 (TNFR1: A, C) and IL-1 receptor accessory protein (IL-1RAcP: B, D) immunoreactivity with cyclooxygenase (COX)-2 (A, B) and prostaglandin E2 receptor EP₃ (C, D) immunoreactivity in SFO of normal rats. Red, TNFR1 or IL-1RAcP; Green: COX-2 or EP₃; Yellow: Merged.