Contribution of the subfornical organ to angiotensin II-induced hypertension

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The renin-angiotensin system plays an important role in the development and pathogenesis of several forms of hypertension (23–25). This system is a key participant in the short- and long-term regulation of arterial blood pressure (12, 21). Of particular interest are the effects of ANG II on the central nervous system to increase arterial blood pressure (44). Substantial evidence suggests that ANG II regulates arterial blood pressure via actions at circumventricular organs (CVOs) in the brain (14, 42). These CVOs lack the normal blood-brain barrier, permitting ANG II to gain access to these central sites. Additionally, these sites are dense with angiotensin type 1 (AT1) receptors and play an essential role in cardiovascular regulation (14, 42). The area postrema (AP), organum vasculosum of the lamina terminalis, and subfornical organ (SFO) are critical CVOs involved in sensing ANG II from the blood and cerebral spinal fluid and transmitting this information to neural signals necessary to appropriately regulate body fluid homeostasis (16, 35).

Historically, several studies have demonstrated that ANG II is strongly correlated with the interaction of the sympathetic nervous system in the development of hypertension (6, 17, 18, 38). Furthermore, some studies suggest that chronic ANG II-induced hypertension is centrally mediated via sympathoexcitation through CVOs (3, 18). It has been reported that the chronic low-dose ANG II-induced hypertension is not due to the peripheral vasoconstrictor activity of ANG II but, rather, to a slow-developing pressor effect, most likely the result of excitation of the sympathetic nervous system over a period of days (18, 19, 29). Findings with Fos immunocytochemistry revealed that different patterns of Fos expression in the brain are associated with acute vs. chronic administration of ANG II (30). This study showed that the rostral ventral lateral medulla of normotensive rats displayed baroreceptor-independent increases in Fos expression only after 18 h of low-dose ANG II infusion, suggesting a neurogenic component to ANG II-induced hypertension that develops progressively over longer periods of time (30). On the other hand, others recently demonstrated a suppression of renal sympathetic nerve activity (SNA) in rabbits and dogs (2, 31) and no change in Fos expression at the level of the rostral ventral lateral medulla (32) during chronic ANG II treatment. Some of these differences may be dose and species related. However, taken together, it appears that chronic ANG II treatment may temporally and regionally modulate SNA, rather than simply cause a global change or effect on the sympathetic nervous system.

Compelling evidence from lesion studies in the brain reveals that ANG II is modulating blood pressure via AT1 receptors in CVOs. Fink et al. (19) revealed that a lesion of the AP attenuates chronic ANG II-induced hypertension in rats. Moreover, Collister and Osborn (11) showed that AP-lesioned rats exhibit an attenuated hypertensive response to the AT1 receptor antagonist losartan. Furthermore, the hypotension of approximately −30 mmHg in response to chronic losartan treatment is slow to develop, eventually achieving steady state over a period of days, suggesting that the hypotensive effect of losartan is not due only to blockade of the peripheral actions of ANG II but, rather, the blockade of central actions of ANG II over a period of days (10). Additionally, we previously showed similar results, in that SFO-lesioned rats demonstrate an attenuated hypertensive response to losartan compared with sham rats (9). The present studies investigate a role for the SFO in the response to chronic ANG II-induced hypertension. The SFO is densely populated with AT1 receptors, making it a chief candidate for the actions of ANG II (14, 37). Furthermore, acute pressor and dipsogenic actions of ANG II have been shown to be mediated at the SFO (33, 34). In addition, the efferent pathways of the SFO project to major sympathetic...
control centers, predicting a role in autonomic control of blood pressure (1, 14–16, 35).

We hypothesized that chronic ANG II-induced hypertension is a consequence of ANG II-activating neurons in the SFO, progressively leading to hypertension. To test this hypothesis, rats were subjected to SFO lesion or sham surgery, ANG II was administered over a 10-day period, and blood pressure was measured continuously with telemetry.

METHODS

Surgical procedures. Adult male Sprague-Dawley rats (Charles River Laboratories) were randomly selected for lesion of the SFO (SFOx, n = 9) or sham operation (sham, n = 9). Rats were preanesthetized with pentobarbital sodium (32.5 mg/kg ip) and atropine (0.2 mg/kg ip). Surgical anesthesia was achieved with a second intramuscular injection containing a cocktail of anesthetic agents: acetylpromazine (0.2 mg/kg), butorphanol tartrate (0.2 mg/kg), and ketamine (25 mg/kg). Rats were then positioned in a stereotaxic apparatus. All procedures were conducted in accordance with institutional and National Institutes of Health guidelines.

SFO lesions were completed as previously reported (9). A dorsal midline incision was made through the skin of the skull. Bregma and lambda landmarks were exposed, and a 3-mm hole was drilled 1.5 mm posterior to bregma. A Teflon-coated, tungsten electrode with 0.008 inches exposed at the tip was passed into the brain at four predetermined coordinates relative to bregma. The four coordinates caudal and posterior to bregma were

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x = -4.7 \text{ mm}, \quad y = -1.4 \text{ mm}, \quad z = -4.7 \text{ mm},
\]

and

\[
x = -4.9 \text{ mm}, \quad y = -1.2 \text{ mm}, \quad z = -5.1 \text{ mm}.
\]

At each location, a 1-mA current was passed for 8 s to complete the lesion. The hole in the skull was closed with bone wax, and the skin was closed with 3-0 silk suture. Sham operations were identical to lesions, except ventral coordinates were 1.5 mm less, and no current was passed. At the end of each surgery, each rat received an intramuscular antibiotic injection of 2.5 mg of gentamicin and a subcutaneous injection of 0.075 mg of butorphanol tartrate for analgesic purposes.

After 1 wk of recovery, rats were instrumented with radiotelemetric pressure transducers (model TA11PA-C40, Data Sciences International) for the purpose of continuous, 24-h sampling of mean arterial pressure (MAP) and heart rate (HR). The unit consists of a fluid-filled catheter attached to a transducer-transmitter. Rats were anesthetized as described above, and transmitters were implanted as previously described (9). Additionally, rats were instrumented with femoral and jugular venous catheters for the purpose of ANG II infusion and acute injections, respectively. At the end of surgery, each rat received an intramuscular antibiotic injection of 2.5 mg of gentamicin and a subcutaneous injection of 0.075 mg of butorphanol tartrate for analgesic purposes. The catheters were tunneled subcutaneously and exited between the scapula, where they passed through a rubber harness (Harvard Apparatus) and a flexible spring. The spring was attached to a single-channel hydraulic swivel, which allows the rat to move freely about the cage.

After recovery from anesthesia, the rats were housed individually in metabolic cages (Nalgene). They were allowed 7 days to recover from surgery before entering the experimental protocol. During the first 3 days of recovery, each rat received daily prophylactic intravenous antibiotics consisting of 15 mg of ampicillin. Each rat was also started on a continuous intravenous infusion of sterile 0.9% NaCl solution (7 ml/24 h). A 0.4% NaCl diet (Research Diets) and distilled water were provided ad libitum throughout the recovery period. This provides a total daily sodium intake of ~2.5 mmol/day.

Experimental protocol. During the first 3 days of the protocol, sham and SFOx rats received a continuous intravenous infusion of 0.9% sterile saline (7 ml/24 h), which served as the initial control period. This was followed by a 10-day infusion of ANG II. ANG II was dissolved in the saline and infused at 10 ng·kg⁻¹·min⁻¹·iv in 7 ml/24 h. A 3-day recovery period identical to the control period completed the protocol.

Daily measurements of MAP, HR, food intake, water intake, urine output, and urinary sodium were recorded in conscious, unrestrained rats in their home cages. MAP and HR were measured continuously by radiotelemetric pressure transducers at a sampling rate of 500 Hz for 10 s each minute. Twenty-four-hour food and water intake and urinary output were measured gravimetrically. Sodium intake was calculated as the sum of sodium received in the daily infusion (1 mmol/day iv) and the product of food intake and sodium content of the food (0.4% NaCl, 0.07 mmol/g). Urinary sodium content was measured with an ion-specific electrode (Nova Biomedical). Urinary sodium excretion was calculated as the product of the urine flow rate and urinary sodium concentration.

Histological verification of SFO lesion. On completion of the experimental protocol, all rats were anesthetized as described above and perfused intracardially with 4% paraformaldehyde. Whole brains were extracted and soaked in 4% paraformaldehyde for 2 days. The brains were then transferred to a 30% sucrose solution for 3 days. Frozen serial sagittal sections (50 μm) were made at the lateral edge of the third ventricle and mounted on slides. The slides were then stained for Nissl substance with cresyl violet stain. Complete SFO lesion (SFOx) or intact SFO (sham) was confirmed by light microscopy. All SFOx rats were confirmed to have complete selective lesions of the SFO with minimal adjacent tissue damage (Fig. 1).

Indirect assessment of sympathetic tone. As an acute index of sympathetic tone, acute depressor responses to bolus injections of hexamethonium (30 mg/kg) were measured in all rats on control day 3 and on day 7 of ANG II infusion. As previously described (40), immediately before hexamethonium injection, rats were subjected to bolus injections of the AT₁ receptor antagonist losartan (10 mg/kg iv) and an arginine vasopressin V₁ receptor antagonist (Manning compound; 10 μg/kg iv). Responses were measured as the peak response.
of arterial pressure compared with that immediately before injection. This has been shown to be a useful measurement of sympathetic vasomotor tone in the conscious rat (40).

Statistical analysis. Statistical analysis between experimental groups was performed with a two-way ANOVA using a commercially available statistical package (Abacus Concepts). Comparison of specific experimental days (within and between groups) was performed by linear contrast analysis. Between-group comparisons of baseline control values and responses to hexamethonium injections were done with an unpaired t-test. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Cardiovascular effects of ANG II infusion. Cardiovascular responses to chronic ANG II infusion are shown in Fig. 2. During the 3-day control period, no significant differences in baseline MAP were detected between the two groups. By day 5 of ANG II treatment, SFOX rats displayed a significantly attenuated increase in MAP compared with sham rats. On day 5 of ANG II treatment, MAP had increased to $111 \pm 3.7$ mmHg in sham rats but only to $101 \pm 3.9$ mmHg in SFOX rats. This trend continued through day 10 of ANG II treatment. Additionally, during the recovery period, both groups of rats returned to the initial MAP baseline control values.

The HR responses to chronic ANG II treatment are shown in Fig. 2. The 3-day control average was $399 \pm 11$ and $401 \pm 11$ beats/min in sham and SFOX rats, respectively. There were no differences in HR between sham and SFOX rats throughout the protocol.

Depressor response to hexamethonium injection. Depressor effects of a bolus injection of hexamethonium (30 mg/kg iv) in SFOX and sham rats are shown in Fig. 3. Blockade of AT$_1$ and V$_1$ receptors had no effect on MAP or HR in either group of rats. On control day 3, depressor responses were $-33 \pm 5.6$ and $-42 \pm 4.8$ mmHg in sham and SFOX rats, respectively. After hexamethonium injections, MAP during the control period was $61 \pm 6$ and $56 \pm 3$ mmHg in sham and SFOX rats, respectively. There was no difference between these responses during the control period. On day 7 of ANG II treatment, depressor responses were $-50 \pm 3.7$ and $-53 \pm 2.4$ mmHg in sham and SFOX rats, respectively. MAP after hexamethonium injections on day 7 of ANG II was $58 \pm 3$ and $59 \pm 3$ mmHg in sham and SFOX rats, respectively. Importantly, the change in depressor response was statistically significant within sham rats, with a greater magnitude of change in MAP during the ANG II treatment than during the control period. There was no difference in the SFOX response between the control period and ANG II treatment (Fig. 3).

Sodium and water balance responses. Water balance data are shown in Fig. 4. The 3-day control average water intake was $17 \pm 1.9$ and $19.3 \pm 1.7$ ml in sham and SFOX rats, respectively. There were no differences in water intake between groups throughout the protocol. The average urine output was $8 \pm 0.5$ and $9 \pm 1.3$ ml in sham and SFOX rats, respectively, during the control period. During ANG II treatment, urine output was significantly greater in SFOX rats on days 7, 9, and 10. Additionally, sham rats displayed significantly higher positive water balance on days 7 and 10 of ANG II treatment and on day 2 of recovery.

Sodium balance data are shown in Fig. 5. The 3-day control average sodium intake was $2.5 \pm 0.1$ and $2.5 \pm 0.1$ meq in sham and SFOX rats, respectively. There were no differences in sodium intake, sodium excretion, and sodium balance between the two groups throughout the protocol.

DISCUSSION

The effects of ANG II on total peripheral resistance, renal function, and cardiovascular structure are mediated via several direct and indirect effects on the blood vessels. Perhaps the most potent effect of ANG II is to increase total peripheral resistance. The cardiovascular effects of ANG II are mediated via AT$_1$ receptors, which are expressed in the aorta, kidney, and heart. The acute depressor response to hexamethonium injection and the chronic hypertensive response to ANG II are mediated via AT$_1$ receptors.

### Statistical Analysis

- **Two-way ANOVA** was used to compare the effects of ANG II between groups.
- **Unpaired t-test** was used to compare baseline and response values between groups.

### Key Findings

1. **Cardiovascular**
   - MAP increased significantly in ANG II-treated rats compared to controls.
   - HR remained unchanged in both groups.

2. **Hexamethonium**
   - Depressor responses were attenuated in SFOX rats compared to sham rats.
   - Responses were significant on day 7 of ANG II treatment.

3. **Sodium and Water Balance**
   - Water intake: No differences between groups.
   - Urine output: Increased in SFOX rats during ANG II treatment.
   - Water balance: Positive balance in SFOX rats on days 7 and 10.

### Conclusion

ANG II-induced hypertension is mediated via AT$_1$ receptors, leading to increased peripheral resistance and changes in sodium and water balance. The effects are more pronounced in SFOX rats, indicating a role for the SFO in regulating these responses.
resistance via direct vasoconstriction of smooth muscle in the peripheral vasculature. This effect occurs very rapidly, eliciting a transient pressor response that primarily occurs due to constriction of the peripheral blood vessels. Additionally, ANG II increases total peripheral resistance via the enhancement of sympathetic discharge centrally (20). Recent studies suggest that some of these effects may be secondary to central effects of aldosterone to increase SNA and MAP (27, 41). Intracerebroventricular infusion of low-dose aldosterone in rats produces a significant increase in blood pressure (22). Importantly, there is much evidence that ANG II can act centrally to stimulate the sympathetic nervous system and even attenuate the baroreceptor-mediated reductions in sympathetic discharge (4, 8). Endogenous central effects of ANG II can be blocked by intracerebroventricular injections of the AT1 receptor losartan, which causes a decrease in blood pressure, in contrast to the acute peripheral blockade of AT1 receptors (13). Additionally, evidence suggests that ANG II effects are mediated via binding to AT1 receptors in the circumventricular nuclei, triggering an increase in sympathetic outflow (14, 18).

Several studies have examined the effect of ANG II on the central nervous system acutely (21, 33, 34, 39), but much less is known about the actions of ANG II at circumventricular nuclei to regulate blood pressure chronically. Further studies are needed to enhance our understanding of how angiotensin-mediated events at CVOs are regulating blood pressure chronically. A large body of evidence suggests that chronic ANG II stimulation of central receptors activates the sympathetic nervous system (6, 16, 44). The SFO has been proposed as a site of action that may be responsible for initiating this response (28). The present experiment was designed to examine the specific role of the SFO in initiating this chronic central pressor response.

The present study demonstrated a marked and significant attenuation in the hypertensive response to chronic intravenous ANG II treatment in SFOx rats. This attenuated response in SFOx rats began approximately on day 5 and continued throughout ANG II treatment. On day 5 of ANG II treatment, MAP had increased 11.7 ± 3.0 mmHg in sham rats but increased only 3.7 ± 1.4 mmHg in SFOx rats. Interestingly,
SFOx and sham rats displayed increased blood pressures early during ANG II treatment, supporting the idea that the SFO is not involved in the acute phase of ANG II hypertension. However, on day 4 of ANG II infusion, MAP in SFOx rats was significantly attenuated compared with that in sham control rats. The delayed onset of the observed attenuation in MAP supports the hypothesis that the SFO is necessary for the chronic phase of ANG II hypertension. The SFOx rats were not able to elevate blood pressure chronically, suggesting that this central pressor response to chronic ANG II treatment has a delayed neurogenic component, specifically mediated via AT1 receptors at the SFO.

To correlate the sympathetic nervous system with these findings, a direct measurement of SNA would be ideal in this chronic setting; unfortunately, it is not feasible in the rat. Therefore, we utilized an indirect measurement of sympathetic vasomotor tone. Acute bolus injections of the ganglionic blocking agent hexamethonium have been shown to be a useful index of sympathetic vasomotor activity (40). The depressor response to hexamethonium was not statistically significant between the control period and the ANG II infusion period in SFOx rats but was significantly greater in sham rats at day 7 of ANG II infusion than in their respective control condition. Importantly, these data suggest greater sympathetic vasomotor tone during ANG II treatment in sham than in SFOx rats. These data also support a significant neurogenic component to the chronic phase of ANG II hypertension and SFO-mediated sympathoexcitation. With regard to this technique, it has several limitations: 1) it is an acute measurement in the chronic setting of ANG II hypertension. 2) It is an indirect indicator of vasomotor SNA, measuring only the sympathetic input to the peripheral vasculature, and not sympathetic nerve input to other organs of potential relevance such as the kidney, which also may be altered, as suggested recently (2, 31).

Another observation of the present study was a significant increase in urine output in SFOx rats on days 1, 2, 7, 9, and 10 of ANG II treatment, without an increase in water intake, compared with sham rats. One could speculate that the lack of increase in MAP in the SFOx animals is an effect that is secondary to the increase in urine output observed in this group. Although this is possible, we believe this is not the case for several reasons. 1) The increased diuresis in SFOx animals was observed on days 1 and 2 of ANG II treatment, whereas no significant difference was detected in MAP at this point. SFOx animals displayed slight increases in MAP at these time points. 2) If enhanced diuresis were responsible for this effect, we would expect a rise in urine output in SFOx animals during all days the SFOx rats displayed attenuated hypertension. 3) Although the change was not significant, SFOx rats tended to maintain higher urine output during the control and recovery periods, suggesting that this phenomenon was not correlated to their responsiveness to ANG II. We do not know the underlying mechanism of these observations, but one possibility is that SFOx rats may have altered levels of arginine vasopressin. It is known that the SFO is a structure responsible for eliciting increases in the excitability of vasopressin-secreting neurons in the hypothalamus (5, 26). It is possible that SFOx rats were unable to appropriately elevate vasopressin levels, leading to increased diuresis compared with sham rats. Unfortunately, because we did not measure vasopressin levels in these studies, we cannot conclude whether this mechanism is responsible for the differences. Another indirect effect of ANG II is to increase blood pressure via increasing sodium and water appetite (36, 43, 45). The results of the present experiment display no differences in daily water or sodium intake between SFOx and sham rats throughout the entire protocol, so we do not believe this to be an underlying mechanism of this observation. However, it is possible that if the rats were given a choice of saline vs. water, they could have displayed a difference in sodium appetite as others have reported (43).

Results reported by Bruner et al. (7) are not consistent with the findings in the present study. Their SFO-lesioned animals displayed not an attenuated, but an augmented, hypertensive response to chronic ANG II treatment compared with controls. Furthermore, during ANG II treatment, their SFO-lesioned animals displayed a greater dipsogenic response. There are several explanations for the disparities between our results and those of Bruner et al. 1) We measured arterial blood pressure in our animals using radiotelemetry. Therefore, our measurements were 24-h averages vs. a single-time-point collection of data during the day. 2) Bruner et al. examined a different model of ANG II hypertension. In contrast to the present study, their animals were fed a sodium-deficient diet (0.002 mg of sodium) and received isotonic saline at 40 ml/day iv (daily sodium intake = 6.2 meq). This is markedly different from the 7 ml/day of intravenous isotonic saline and 0.4% NaCl chow (daily sodium intake = 2.5 meq) received by the animals in the present study. 3) Bruner et al. used a larger dose of ANG II (10 ng/min iv). In contrast to the present study, their study was a volume- and sodium-loaded model of ANG II hypertension, and the SFO could indeed play a much different role in the sodium-replete model of the present study.

In conclusion, the present study focused on the chronic central effects of ANG II at the SFO. Despite the large amount of research in this area, the mechanism of the experimental model of ANG II-induced hypertension is not entirely understood. The novelty of the present study is that we have demonstrated that removal of the SFO nearly abolished the chronic phase of this salt-replete model of ANG II hypertension. Additionally, indirect assessment of sympathetic tone suggests that the mechanism is sympathetically mediated. We believe that these results reemphasize the pivotal role of this CVO in the central chronic effects of ANG II and its role in this form of hypertension.

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