Role of the Subfornical Organ in the Chronic Hypotensive Response to Losartan in Normal Rats

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Abstract—Angiotensin II is known to act at a unique set of brain regions known as the circumventricular organs. These structures lack the normal blood–brain barrier and are therefore thought to participate in the central nervous system processing of neuroendocrine signals. We have reported that chronic treatment with the angiotensin type 1 (AT1) receptor antagonist, losartan, decreases arterial pressure in normotensive rats. Furthermore, this hypotension is attenuated in area postrema–lesioned rats, suggesting a role of endogenous angiotensin II at this circumventricular organ. Another circumventricular organ, the subfornical organ (SFO), has also been shown to mediate actions of angiotensin II. The present study tested the hypothesis that the SFO is a central site of action of endogenous angiotensin II at AT1 receptors. Adult male Sprague-Dawley rats were anesthetized and placed in a stereotaxic apparatus, and the SFO was sham or electrolytically lesioned. One week later, rats were instrumented with venous catheters and radiotelemetry pressure transducers for continuous infusion and monitoring of mean arterial pressure, respectively. After 3 days of control, losartan was administered intravenously (10 mg·kg⁻¹·d⁻¹) for 10 days in both SFO-lesioned and sham rats. By day 4 of losartan administration, mean arterial pressure had decreased to 75±2 mm Hg in sham rats (n=9) but had only fallen to 83±2 mm Hg in lesioned rats (n=10). This attenuated hypotensive response in SFO-lesioned rats continued through day 10 of losartan treatment. These results support the hypothesis that the SFO mediates part of the hypotensive effects of chronic AT1 receptor blockade in the normotensive rat. (Hypertension. 2003;41:576-582.)

Key Words: antagonists, angiotensin □ autonomic nervous system □ blood pressure □ brain □ receptors, angiotensin II □ renin-angiotensin system □ circumventricular organ

The role of the renin-angiotensin system (RAS) in chronic blood pressure regulation is still not fully understood and continues to be studied. Much of our current understanding of this system is based on the response of arterial pressure to drugs that block the RAS, including ACE inhibitors and angiotensin type 1 (AT1) receptor antagonists. We have demonstrated a profound chronic hypotensive response to the AT1 receptor antagonist losartan in normotensive, salt-replete rats. After 10 days of losartan treatment, rats consuming a normal-salt diet demonstrated a progressive hypotensive response of ∼35 mm Hg. This clearly shows an important role of this hormone in the maintenance of arterial pressure in normal animals, and yet, we do not fully understand the mechanism of this response.

The mechanism by which blockade of the endogenous RAS lowers arterial pressure chronically is difficult to determine because of the numerous actions of angiotensin (Ang) II, which include vasoconstriction,2 renal retention of sodium and water,3–5 sympathoexcitation,6 and vascular hypertrophy.7 It has been reported that chronic low-dose Ang II hypertension is not solely caused by peripheral Ang II vasoconstrictor activity.8 The observation that the acute pressor effects of Ang II are blocked within the first hour of losartan treatment, during which time there is no effect of blood pressure, suggests that this too was not caused by blockade of the peripheral vasoconstrictor actions of Ang II alone. In addition, the progressive response to losartan we observed occurred over a 10-day period and was slow to develop. Furthermore, we have never observed any natriuresis or diuresis in these animals and therefore do not suspect a profound role of the kidney in this response.3 In addition, we have recently shown that Ang II acting at angiotensin type 2 (AT2) receptors does not play a role in this chronic hypotensive response to losartan.9 Animals treated with losartan combined with the AT2 antagonist PD 123,319 did not display an attenuated hypotensive response compared with that of animals treated with losartan alone. In contrast, we have reported that the circumventricular organ, the area postrema, does play a role in this response. In rats with lesions of the area postrema, the chronic hypotensive steady-state response to losartan was attenuated by ∼40%.10 The area postrema has previously been suggested as a primary site of the sympathoexcitatory effects of circulating Ang II,11–18 and this observation is consistent with the idea that basal levels of sympathetic activity and arterial pressure are dependent on endogenous Ang II acting at the area postrema.
In addition to the area postrema, the subfornical organ (SFO), another circumventricular organ, has been implicated in mediating effects of Ang II. In terms of cardiovascular regulation, there is ample evidence that Ang II acts at the SFO. Direct application of Ang II at the SFO elicits a pressor response that is blocked by prior treatment with the Ang II antagonist saralasin.19 Intravenous Ang II causes a pressor response that can be blocked by lesion of the SFO.20,21 Interestingly though, chronic Ang II hypertension is exacerbated in rats with lesions of the SFO,22 suggesting that the effects of Ang II at the SFO are quite different, depending on the chronicity. In 1970, the idea that the pressor response to Ang II at the SFO was sympathetically mediated was proposed.23 The known efferent projections of the SFO support this idea. The SFO projects to the median preoptic nucleus, the organum vasculosum of the lamina terminalis, the nucleus tractus solitarius, and both the paraventricular (PVN) and supraoptic nuclei of the hypothalamus.24 Of particular interest, are recent studies involving effects of SFO excitation on PVN neurons projecting to sympathetic control centers. SFO neurons identified as projecting to the PVN are excited by Ang II administration,25 and this is blocked by prior treatment with saralasin.26 Likewise, lesions of the PVN decrease pressor responses to SFO stimulation.27 Furthermore, PVN cells projecting to the intermediolateral cell column are excited by SFO stimulation.28 Lastly, PVN or rostral ventral lateral medulla pretreatment with an Ang II antagonist blocks pressor responses seen with Ang II injection at the SFO.29

Taken together, it seems probable that basal levels of Ang II could be acting at the SFO to drive sympathetic nerve activity and basal arterial pressure. Likewise, blockade of AT1 receptors at the SFO could therefore be involved with the long-term hypotensive response to losartan described above. In the present study, experiments were designed to test the hypothesis that the long-term hypotensive actions of losartan are the result of blockade of the actions of endogenous Ang II at the SFO. Arterial pressure and heart rate responses to chronic losartan treatment were measured in normal rats and in rats with lesions of the SFO.

Methods

Adult male Sprague-Dawley rats (275 to 300 g) were used in all experiments. All procedures were conducted in accordance with institutional and National Institutes of Health guidelines.

Surgical Procedures

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were randomly selected for either lesion of the SFO (SFOx) or sham operation. Rats were preanesthetized with pentobarbital (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 (acepromazine, 0.2 mg/kg; butorphanol tartrate, 0.2 mg/kg; and ketamine, 25 mg/kg). Rats were then placed in a stereotaxic apparatus.

Lesion of the SFO was accomplished through the method of Fitts et al.30 A dorsal midline incision was made through the skin of the skull. Bregma and lambda landmarks were exposed, and a 3-mm hole was made in the skull 0.8 mm posterior to bregma. A Teflon-insulated monopolar tungsten electrode was lowered to 4 predetermined coordinates, and a current of 1 mA was passed for 7 seconds. Sham operations were identical to lesions with the exception that ventral coordinates were 1.5 mm less so as to not damage the SFO, and no current was passed. The hole in the skull was repaired with bone wax, and the skin was closed with 3-0 silk suture. After surgery, all rats were given an intramuscular antibiotic injection of 2.5 mg gentamycin and a subcutaneous injection of 0.075 mg butorphanol tartrate for analgesic purposes.

One week later the rats were instrumented with radio telemetry blood pressure transducers and both femoral and jugular venous catheters. The telemetry unit consists of a fluid-filled catheter attached to a transducer/transmitter. A midline abdominal incision was made to expose the descending aorta. After clamping the aorta proximally, the catheter was implanted directly into the aorta via a 21-gauge needle. The catheter was advanced cranially so the tip was located just distal to the renal arteries. The catheter was glued in place with medical adhesive, and the aortic clamp was removed. The body of the transmitter was secured to the abdominal wall during closure of the body cavity with 3-0 silk suture. The skin was closed with surgical staples. Rats were then instrumented with femoral and jugular venous catheters. The catheters were tunneled subcutaneously and exited behind the scapulae. The catheters were passed through a flexible spring connected to a single-channel hydraulic swivel to which the femoral venous catheter was attached. The springs were attached to the rats via a rubber harness (Harvard Apparatus), which the rats wore for the remainder of the protocol. After surgery, all rats were given an intramuscular antibiotic injection of 2.5 mg gentamycin and a subcutaneous injection of 0.075 mg butorphanol tartrate for analgesic purposes. During the first 3 days of postsurgical recovery, the rats received daily prophylactic intravenous antibiotics of 15 mg ampicillin. The rats were also started on a continuous intravenous infusion of sterile 0.9% saline (7 mL/24 h). The rats were housed individually in metabolic cages. A 0.4%-NaCl diet (Research Diets) and distilled water were provided ad libitum. Rats were allowed 1 week to recover before entering the experimental protocol.

Experimental Protocol

The first 3 days of the protocol served as a control period in which the rats received a continuous intravenous infusion of 0.9% sterile saline (7 mL/24 h). This was followed by a 10-day infusion of the AT1 receptor antagonist losartan (10 mg·kg⁻¹·d⁻¹). The losartan was dissolved in 0.9% sterile saline and infused at a rate of 7 mL/24 h. A final recovery period of 3 to 4 days identical to the control period completed the protocol. The infusions were administered through a 0.2-μm syringe filter.

Daily measurements of mean arterial pressure (MAP), heart rate, food intake, water intake, urine output, and urinary sodium were recorded in conscious unrestrained rats in their home cages. MAP and heart rate were measured continuously by radio telemetry pressure transducers (model No. TA11PA-C40, Data Sciences International) at a sampling rate of 500 Hz for 10 seconds 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/d), plus 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 urine flow rate and urinary sodium concentration. The protocol was conducted in both experimental groups: SFOx (n=10) and sham rats (n=9).

Measurement of Baseline Plasma Renin Activity and Tests of AT1 Receptor Blockade

Plasma renin activity (PRA) was measured in rats on the second control day. Blood (500 μL) was collected via the jugular catheter and placed into a chilled 1-mL syringe containing 1 mg EDTA in 20 μL. The whole blood was centrifuged, and plasma was collected and stored at −70°C for later radioimmunoassay, as previously described31.

To test the efficacy of the AT1 receptor blockade, acute pressor responses to bolus injections of Ang II (30 ng IV) were measured on
day 3 of the control period and day 7 of the losartan infusion. Responses were measured as the peak increase of arterial pressure.

**Functional Testing of Lesion of the SFO**

To assess the functional presence of the SFO, we performed a drinking response test to an infusion of Ang II. It has been previously reported that normal rats will respond by drinking approximately 2 to 4 mL of water in response to a slow infusion of Ang II over 90 minutes, and this response is significantly attenuated in animals with lesions of the SFO. On control day 3, all rats were infused with Ang II at a rate of 1.8 μg/h for 90 minutes. Water intake was measured during this time in all rats.

**Histological Verification of SFOx**

On completion of the protocol, all rats were anesthetized as described above and perfused intracardially with 4% paraformaldehyde. Whole brains were dissected and soaked in 4% paraformaldehyde for 2 days. The brains were then transferred to a 30% sucrose solution for a minimum of 2 days. Frozen serial sagittal sections (50 μm) were made at the lateral edge of the third ventricle and mounted on slides. The slides were stained for Nissl substance with cresyl violet stain. Confirmation of complete SFO lesion or intact SFO (sham-operated rats) was made under light microscopy. All SFOx rats included in the final analysis of the data were confirmed to have complete lesions of the SFO.

**Statistical Analysis**

Statistical comparison within and between experimental groups was performed with a 2-way ANOVA with a commercially available statistical package (Abacus Concepts). Comparisons of specific experimental days (within and between groups) were performed by linear contrast analysis. For clarity in data presentation, only between-group differences are shown in figures. Between-group comparisons of baseline control values were performed by comparing the average of the 3 control days with an unpaired t test. In addition, between-group comparisons of PRA, pressor responses to Ang II, and water intake during the Ang II infusion test were made with an unpaired t test. A value of P<0.05 was considered statistically significant for all tests. All values are reported as mean±SE.

**Results**

**Functional Testing of Lesion of the SFO and Histological Verification of SFOx**

On control day 3, water intake responses to a slow infusion of Ang II (30 ng/min for 90 minutes) were measured in SFOx and sham rats. Sham rats drank 2.6±0.7 mL, whereas SFOx rats only drank 1.0±0.2 mL of water. These results were statistically different.

Histological verification of SFO lesion was confirmed in all SFOx rats. Typical examples of a sham lesion (left side) and SFO lesion (right side) are shown in Figure 1. In all SFOx rats, there was minimal destruction of the adjacent tissue at the light microscopic level.

**PRA Levels and Tests of AT₁ Receptor Blockade**

PRA was measured in all rats on the second control day. PRA was not different between the groups (SFOx, 7.0±1.2 ng Ang I·mL⁻¹·h⁻¹; SHAM, 5.9±0.7 ng Ang I·mL⁻¹·h⁻¹).

The efficacy of AT₁ receptor blockade was assessed by measuring the pressor responses to 30 ng of Ang II on the third control day and day 7 of losartan. Control responses were similar in sham (57±4 mm Hg) and SFOx (60±2 mm Hg) rats. On day 7 of losartan, this response was abolished (0±0 mm Hg) in both groups.

**Cardiovascular Responses to Losartan Infusion**

Shown in Figure 2 (top) are the MAP responses to losartan in SFOx and sham rats throughout the protocol. There were no significant differences observed between baseline MAP in SFOx and sham rats during the initial 3-day control period (SFOx, 101±2 mm Hg; SHAM, 99±2 mm Hg). By day 2 of losartan treatment, both SFOx and sham groups demonstrated significant decreases in MAP compared with baseline data (SFOx, −15±3 mm Hg; SHAM, −20±3 mm Hg). Both groups of rats continued to show progressive hypotensive
responses to losartan, but this response was markedly attenuated in SFOx rats by day 4 of losartan. By day 8 of losartan, MAP had fallen 33±3 mm Hg in sham rats but only 23±2 mm Hg in SFOx rats. This trend continued through day 10 of losartan treatment. Throughout the recovery period, both groups demonstrated increasing levels of MAP, such that both groups had returned to near control levels by the end of the recovery period.

The heart rate responses to losartan are shown in Figure 2 (bottom). The 3-day control average heart rate was 425±4 bpm in sham rats and 408±10 bpm in SFOx rats. There were no statistical differences seen between heart rates in sham and SFOx groups throughout the protocol. However, sham rats tended to display slightly greater heart rates compared with heart rates for SFOx rats, which were observed on all days of the protocol.

**Sodium and Water Balance Responses**

The water balance data are shown in Figure 3. The 3-day average control water intake was 20±2 mL/24 h in sham rats and 19±1 mL/24 h in SFOx rats. Urine output was higher in sham rats compared with SFOx rats on control day 3 (SFOx, 11.4±1.8 mL/24 h; SHAM, 16.9±3.4 mL/24 h). Otherwise, there were no differences observed in water intake, urine output, and water balance between the groups throughout the protocol.

The sodium balance data are shown in Figure 4. The 3-day average control sodium intake was 2.4±0.1 mEq/24 h in sham rats and 2.5±0.1 mEq/24 h in SFOx rats. There were no differences in sodium intake, sodium excretion, and sodium balance seen between the 2 groups throughout the protocol.

**Discussion**

There were 2 major findings in this study. First, baseline arterial pressure was not affected by lesion of the SFO 1 week after the lesion. Secondly, the long-term steady-state hypotensive response to losartan was attenuated by approximately one third in SFOx rats, suggesting this circumventricular organ as a site that mediates effects of endogenous Ang II, as well as an important target site for this AT1 receptor antagonist.

Despite the well-known peripheral actions of Ang II, there is now an abundance of evidence that some of the long-term hypertensive effects of Ang II are centrally mediated to cause...
sympathoexcitation.\textsuperscript{16,33} Much of the central actions of Ang II are thought to be mediated through the central nervous system sites known as the circumventricular organs.\textsuperscript{34,35} In particular, the SFO and area postrema are known sites of action of some of the effects of Ang II.\textsuperscript{13} Both of these circumventricular organs have also been implicated in the sympathoexcitatory effects of Ang II.\textsuperscript{11,19,21,23,36} Because of these effects of Ang II, it is quite possible that many of the well-known blood pressure–lowering effects of ACE inhibitors and AT\textsubscript{1} receptor antagonists are due to blockade of central effects of Ang II as well. As reported previously,\textsuperscript{1,10} we have again demonstrated in the present study a pronounced chronic hypotensive effect of 30 to 35 mm Hg in normal rats treated with the AT\textsubscript{1} receptor antagonist losartan (10 mg \textbullet kg\textsuperscript{-1} \textbullet d\textsuperscript{-1}). This clearly demonstrates a major role of the RAS in the control of arterial pressure in the normal animal, and a clear understanding of the mechanism of this response will contribute to our overall understanding of long-term control of arterial pressure.

In the present study, we tested the hypothesis that the hypotensive response to losartan was the result of blocking the tonic effect of circulating Ang II binding to AT\textsubscript{1} receptors at the SFO. We believe the dose of losartan used effectively blocks AT\textsubscript{1} receptors in the SFO, because the dypsogenic effects of Ang II (which are thought to be mediated thorough the SFO), as administered in the Methods section, are completely blocked by this dose of losartan. In other words, we predicted that the effect of SFOx would block some or all of the hypotensive effects of chronic losartan treatment in the normal rat. Theoretically, ablation of the SFO would abolish the hypotensive effects of losartan if this mechanism were solely responsible for the decrease in arterial pressure. Indeed, SFOx rats demonstrated an attenuated decrease in arterial pressure by day 4 of losartan treatment. This trend continued through day 10, whereby the steady-state decrease in arterial pressure was attenuated by \textasciitilde 30\%. This observation suggests that an intact SFO is necessary for the complete expression of the hypotensive effects of losartan. We suspect this is caused by blockade of central sympathetic actions of Ang II originating at the SFO. The neuroanatomical projections of the SFO (reviewed in the Introduction), i.e., the paraventricular nucleus that projects to primary sympathetic control centers such as the rostral ventral lateral medulla and intermediolateral cell column of the spinal cord, support this idea. Furthermore, we have demonstrated an attenuated hypotensive response to losartan in rats with a “clamped” sympathetic nervous system caused by chronic treatment with hexamethonium and phenylephrine.\textsuperscript{37} These results further support the notion that part of the blood pressure–lowering effect observed with chronic losartan treatment is due to central sympathoinhibition. However, the full chronic hypotensive effects of losartan cannot be explained by this mechanism alone.

There are several reasons why ablation of the SFO did not completely abolish the hypotensive response to losartan. First, SFOx rats could have a different level of basal activity of the RAS. We have previously demonstrated that this hypotensive response is abolished in rats consuming an 8\%-NaCl diet in which the RAS was suppressed.\textsuperscript{1} In that study, basal PRA levels were nearly zero in this group of animals. For this reason, we measured basal PRA levels in SFOx rats, and it was no different than PRA levels in sham rats. Therefore, we do not believe that SFOx rats had an altered level of peripheral RAS activity, which could explain the attenuated response observed.

Second, it is possible that non-AT\textsubscript{1} receptor–mediated events could play a role in the hypotensive response to losartan. This too would explain why the lesion itself did not cause a lowering of blood pressure. One possibility is that there is much evidence that Ang II can exhibit a vasodilatory role at AT\textsubscript{2} receptors.\textsuperscript{38,39} During chronic losartan treatment, circulating Ang II is increased because of a lack of negative feedback at AT\textsubscript{1} receptors. This increased level of Ang II could therefore be acting at AT\textsubscript{2} receptors to mediate part of the hypotensive response to chronic losartan. We have recently tested this hypothesis and found that rats treated with the AT\textsubscript{2} receptor antagonist PD 123,319, in combination with losartan, demonstrated no lesser degree of hypotension compared with that of rats treated with losartan alone.\textsuperscript{9} Additionally, the Ang II metabolite, Ang-(1-7), has been implicated as having a vasodilatory role.\textsuperscript{40} Several studies have suggested that some of the antihypertensive effects of ACE inhibitors are due to the actions of rising concentrations of Ang-(1-7) during ACE inhibitor treatment.\textsuperscript{41,42} Again, during losartan treatment, Ang I and Ang II levels are increased and therefore give rise to an increase in Ang-(1-7) in this model, which could in turn play a role in the mechanism of the chronic hypotensive response to losartan. We are currently investigating this possibility.

Third, we must consider other nonneural mechanisms that could play a role in the hypotensive response to losartan. Renal effects of AT\textsubscript{1} receptor blockade must be considered. For example, intrarenal infusion of the AT\textsubscript{1} antagonist valsartan has been shown to decrease arterial pressure in the spontaneously hypertensive rat at a dose that had no effect systemically.\textsuperscript{93} This does suggest a renal site of action for this AT\textsubscript{1} antagonist. However, in the present study, we did not observe any diuresis or natriuresis that could explain the profound chronic hypotensive response. Furthermore, the fact that rats were in a steady-state sodium and water balance despite arterial pressures of \textasciitilde 75 mm Hg means that the renal function curve was reset to a lower pressure level.\textsuperscript{44,45} It is also possible that this dose of losartan caused structural changes in resistant vessels. This is thought to be a more long-term (weeks) process and is probably not important because the steady-state hypotensive response was observed in just 5 to 6 days.

Fourth, other neural mechanisms need to be considered in this response. As previously described, we have demonstrated a role of the area postrema in the hypotensive response to losartan.\textsuperscript{1} Although we have now demonstrated the involvement of both the SFO and area postrema in the response to losartan, the pattern of the attenuated response was not identical. SFOx rats reached a steady-state attenuated hypotensive response by day 4 of losartan treatment, whereas area postrema–lesioned animals did not show a difference from sham rats until day 8 of losartan treatment.\textsuperscript{10} Nevertheless, the steady-state pressure of both SFOx and area postrema–
lesioned rats after losartan treatment was nearly the same at
≈80 mm Hg. Other circumventricular organs could also play a role in this response. The organum vasculosum of the lamina terminalis is another circumventricular organ that has been shown to mediate actions of Ang II and has connections to major autonomic regulatory centers. It too is a likely candidate to be playing a role in this response. Moreover, losartan can cross the blood–brain barrier and therefore block brain RAS, or gain access to other brain control centers containing AT₁ receptors, such as the rostral ventral lateral medulla. The roles of these mechanisms in the hypotensive response to losartan remains to be investigated.

Perspectives

This report reemphasizes the central nervous system actions of endogenous Ang II in the long-term control of arterial pressure. Ang II has been implicated in the pathogenesis of many forms of experimental and clinical hypertension. We have demonstrated a pronounced chronic hypotensive response in the normal rat in response to chronic blockade of AT₁ receptors. In the present study, we have demonstrated a role of the SFO in this response. As such, we currently report that the SFO is a critical site for the long-term actions of endogenous Ang II in the normal rat. We have now clearly established independent roles of 2 circumventricular organs (area postrema and SFO) in the blood pressure regulatory actions of endogenous Ang II. Our future directions will allow us to look more closely at the combined roles of these circumventricular organs to better gain an understanding of their contributions to long-term control of arterial pressure through the actions of Ang II.

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