Microinjection of ANG II into paraventricular nucleus enhances cardiac sympathetic afferent reflex in rats

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It is well known that there are potent interactions between the renin-angiotensin system and the autonomic nervous system (20, 31). Angiotensin (ANG) type 1 (AT1) receptors are densely distributed in the circumventricular organs, such as the subfornical organ (SFO), the organum vasculosum of the lamina terminalis, and the area postrema (15, 26, 32), and ANG II action on these regions can evoke cardiovascular responses (20, 31). Because the circumventricular organs have intimate neural connections with several integrative sites for sympathetic drive, it is believed that these organs are among the components that mediate ANG II modulation of sympathetic output. In addition, recent studies (8, 23, 31, 32) also have revealed that ANG II receptors and ANG II-immunoreactive nerve fibers, terminals, and cell bodies are localized in several discrete regions known as the integrative sites controlling sympathetic outflow. These sites include the paraventricular nucleus (PVN), the rostral ventrolateral medulla (RVLM), the caudal ventrolateral medulla, and the nucleus tractus solitarii. It has been demonstrated previously (3, 16, 21) that injection of ANG II into these regions alters levels of blood pressure, renal sympathetic nerve activity (RSNA), heart rate (HR), and vascular resistance, suggesting the activity of the sympathetic nervous system is modulated by ANG II. Although the origin of the sympathoexcitation is not completely understood, it is known that ANG II, when elevated, can lead to augmentation of sympathetic outflow.

One mechanism by which ANG II augments sympathetic output is through the modulation of cardiovascular reflexes. In addition to the arterial baroreflex (5, 17, 22), ANG II is also suggested to be involved in the cardiac sympathetic afferent reflex (CSAR), as reported in previous studies (11, 12, 28). It has been shown in our studies that the CSAR is enhanced in dogs with congestive heart failure. This enhancement was attenuated by the intracerebroventricular injection of the ANG II AT1-receptor antagonist losartan (11, 12, 28). These results suggest that chronic elevation of central ANG II can potenti ate the CSAR via central AT1 receptors. However, the central site in which ANG II is involved in augmented CSAR is not clear. One area in which ANG II appears to play an important role in modulation of sympathetic outflow and the CSAR is in the PVN, because ANG II receptors are densely distributed in the PVN (32), and AT1 receptor antagonists can easily block the effects of ANG II at this site (3). Other evidence has indicated a neural pathway between the PVN and the RVLM (23), thereby further supporting the notion that the PVN...
plays a modulatory role in CSAR and sympathetic outflow. The purpose of this study was to determine whether ANG II in the PVN modulates the CSAR induced by both chemical stimulation of afferent endings and electrical stimulation of cardiac sympathetic afferent nerves in normal rats. In addition, the ANG II-receptor subtype mediating this effect was also examined.

METHODS

Forty-seven male Sprague-Dawley rats, weighing between 350 and 450 g, were used in these experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska and were carried out under the guidelines of the AmericanPhysiological Society and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Surgical Instrumentation

Each rat was anesthetized with urethane (800 mg/kg ip) and α-chloralose (40 mg/kg ip). Supplemental doses of anesthesia were administered at 1/10 of the initial dose per hour. A midline incision in the neck was made, and the carotid sinus area was exposed bilaterally. Each carotid sinus nerve was identified and cut. All other nerve fibers that were visible in the area of the carotid sinus were also cut. The carotid bifurcation and the common carotid arteries were stripped of adventitial tissues from 4 mm below the bifurcation to 4 mm above. The vessels were painted with 10% phenol solution to destroy any remaining nerve fibers in this area. Each vagus was then identified in the neck, tied, and sectioned. A carotid artery was catheterized for measurement of mean arterial pressure (MAP) and HR. The effectiveness of baroreceptor denervation was determined by recording the change in HR to venous injection of phenylephrine (20 μg/kg). This dose evoked an increase in blood pressure between 25 and 40 mmHg. Baroreceptor denervation was assumed to be complete if HR did not change >5 beats/min in response to this intervention.

The chest was opened through the fourth intercostal space in some rats. The pericardium was removed to expose the left ventricle. This preparation was used for epicardial application of a piece of filter paper (3 × 3 mm) containing bradykinin (BK) (0.04 or 0.4 μg in 2 μl). In other rats, the chest was opened through the left second intercostal space. The left ventral ansa, which contains cardiac sympathetic afferent nerves, was identified, tied, and ligated. A pair of stainless steel stimulating electrodes was placed on the central end of this nerve. The stimulus was delivered with a stimulator (Grass S88, Astro-Med; W. Warwick, RI) and a stimulus isolation unit. The rats were placed in a stereotaxic instrument (Stoelting; Chicago, IL) and the skull was exposed through an incision on the midline of the scalp. After the bregma was identified, a cannula was positioned in the PVN. The coordinates for the left PVN were determined from the Paxinos and Watson rat atlas (19), which is 1.8 mm posterior, 0.4 mm lateral to the bregma, and 7.9 mm ventral to the zero level. A cannula (outer diameter 0.5 mm and inner diameter 0.1 mm) connected to a microsyringe (0.5 μl; model 7000.5, Hamilton) was advanced into the left PVN with a manipulator (model 310, Stoelting).

At the end of the experiment, the rat was euthanized with an overdose of pentobarbital sodium (100 mg/kg iv). The brain was removed from the skull and placed in 10% forma-
mersed in a warm mineral oil bath and was placed on a pair of platinum-iridium recording electrodes. The signal was amplified with a Grass direct current preamplifier (model P18D, Astro-Med) with a low-frequency cutoff set at 30 or 100 Hz and a high-frequency cutoff at 1 or 3 kHz. The amplified discharge was monitored on a storage oscilloscope (model 121N, Tektronix; Beaverton, OR), and then imported to a computer system with other parameters. A voltage integrator (model 1801, Buxco Electronics) was used for quantifying the raw RSNA. Background noise was determined when nerve activity was completely inhibited by increasing arterial pressure (20 μg/kg iv; phenylephrine) before sinoaortic denervation (SAD) or after section of the central end of the renal nerve at the end of the experiment. This value was subtracted from all the integrated values of RSNA. The raw nerve activity, integrated nerve activity, MAP, and HR were recorded on a PowerLab data-aquisition system (model 16S, ADInstruments; Mountain View, CA) and stored on a disk until analyzed.

Experimental Protocols

Stimulation of cardiac sympathetic afferent nerves. Rats were divided into three groups: ANG II (n = 12), losartan (n = 11), and losartan + ANG II (n = 6). At 1 min after microinjection of ANG II (0.03, 0.3, and 3 nmol), losartan (50 nmol), or losartan (50 nmol) + ANG II (3 nmol) into the PVN, the responses of the RSNA to electrical stimulation of cardiac sympathetic afferent nerves were compared with controls. The frequencies of stimulation varied at 5, 10, 20, and 30 Hz with a constant voltage of 10 V. The pulse width was kept at 1 ms and each stimulus lasted 30 s. Stimuli were delivered in random sequences in each experimental protocol. The time period between each stimulus was at least 1 min. The volume of microinjection was 100 nl, and the controls for each group were injected with saline (100 nl).

Epicardial application of BK. The rats were divided into three groups: ANG II (n = 6), losartan (n = 6), and losartan + ANG II (n = 6). The volume of each microinjection was 100 nl, and the controls for each group were injected with saline (100 nl). The filter papers containing BK (0.04 and 0.4 μg in 2 μl) were applied to the epicardial surface of the anterior wall of the left ventricle. Each drug was applied for 40 s, the filter paper was then removed, and the epicardium was rinsed three times with 10 ml of warm normal saline (38°C). Successive applications of BK were separated by at least 15 min to avoid tachyphylaxis. After microinjection of ANG II (0.3 nmol), losartan (50 nmol), or losartan (50 nmol) + ANG II (0.3 nmol) into the PVN, the responses of the RSNA to epicardial application of BK were compared with controls.

Statistical Analysis

The RSNA was expressed as the percent change from control (before stimulation). The percent changes in the RSNA induced by cardiac sympathetic afferent nerve stimulation were plotted in each group and were used as an index of the central sensitivity of the CSAR. Ten seconds of the integrated RSNA, MAP, and HR immediately before cardiac sympathetic afferent stimulation or before administration of BK. The last 10 s of the responses were similarly averaged. A two-way repeated-measures analysis of variance, followed by the Newman-Keuls test for post hoc analysis, was used when multiple comparisons were made. All statistical analyses were done using computer software (SigmaStat, SPSS; Chicago, IL). All data are expressed as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Baseline MAP, HR, and RSNA

Baseline MAP, HR, and RSNA were measured in controls and after microinjection of ANG II into the PVN. Both middle and high doses of ANG II induced significant increases in MAP and RSNA. Microinjection of losartan into the PVN induced a slight decrease in MAP, but did not affect the baseline RSNA (Table 1).

Effect of microinjection of ANG II and losartan into PVN on CSAR evoked by electrical stimulation of cardiac sympathetic afferent nerves. Three doses of ANG II (0.03, 0.3, and 3 nmol) or saline were microinjected into the PVN in SAD and vagotomized rats. The RSNA responses to electrical stimulation of the central end of the left cardiac sympathetic nerve and the central gain of the CSAR were measured after microinjection of saline (control) or ANG II. A representative recording is shown in Fig. 2. As can be seen, the RSNA response to the stimulation was enhanced after microinjection of ANG II (3 nmol). Figure 3 shows average RSNA responses to electrical stimulation of the left cardiac sympathetic nerve in control (saline) and for each dose of ANG II. Both low and middle doses of ANG II had a tendency to increase the RSNA responses to stimulation of the cardiac sympathetic nerves, but only the high dose of ANG II evoked a statistically significant increase in RSNA with both the 20- and 30-Hz stimuli (Fig. 3). The effect of losartan on the enhanced CSAR by microinjection of ANG II into the PVN was examined in 11 rats. Figure 4 shows that RSNA responses to stimulation were significantly augmented after ANG II (3 nmol) into the PVN. Losartan (50 nmol) microinjection normalized the CSAR.

Effects of Microinjection of ANG II and Losartan Into PVN on CSAR Evoked by Epicardial Application of BK

Figure 5 shows a representative recording of the RSNA response to epicardial application of BK. The RSNA response to epicardial application of BK (0.4 μg) was significantly enhanced after microinjection of ANG II (0.3 nmol). The effects of ANG II (0.03, 0.3 nmol) or saline were microinjected into the PVN on baseline RSNA (Table 1). The baseline RSNA was expressed as the percent change from control (before stimulation). The percent changes in the RSNA induced by cardiac sympathetic afferent nerve stimulation were plotted in each group and were used as an index of the central sensitivity of the CSAR. Ten seconds of the integrated RSNA, MAP, and HR immediately before cardiac sympathetic afferent stimulation or before administration of BK. The last 10 s of the responses were similarly averaged. A two-way repeated-measures analysis of variance, followed by the Newman-Keuls test for post hoc analysis, was used when multiple comparisons were made. All statistical analyses were done using computer software (SigmaStat, SPSS; Chicago, IL). All data are expressed as means ± SE. P < 0.05 was considered statistically significant.

Table 1. Effects of microinjection of ANG II and losartan into paraventricular nucleus on baseline change of MAP, HR, and RSNA

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>RSNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>12</td>
<td>−1.0 ± 0.3</td>
<td>−0.5 ± 0.1</td>
<td>0.2 ± 2.1</td>
</tr>
<tr>
<td>ANG II (0.03 nmol)</td>
<td>8</td>
<td>6.0 ± 2.7</td>
<td>−0.1 ± 0.1</td>
<td>4.7 ± 2.4</td>
</tr>
<tr>
<td>ANG II (0.3 nmol)</td>
<td>12</td>
<td>10.3 ± 2.3*</td>
<td>0.1 ± 0.2</td>
<td>9.2 ± 2.0*</td>
</tr>
<tr>
<td>ANG II (3 nmol)</td>
<td>12</td>
<td>18.6 ± 2.8*</td>
<td>−0.6 ± 0.7</td>
<td>16.2 ± 2.4*</td>
</tr>
<tr>
<td>After ANG microinjection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>11</td>
<td>−0.3 ± 0.3</td>
<td>−0.5 ± 0.1</td>
<td>−0.1 ± 2.4</td>
</tr>
<tr>
<td>Losartan (50 nmol)</td>
<td>11</td>
<td>−4.2 ± 1.2*</td>
<td>2.1 ± 2.1</td>
<td>−4.5 ± 2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. MAP, mean arterial pressure; HR, heart rate; RSNA, renal sympathetic nerve activity; ANG II, angiotensin II.
Fig. 2. Representative recording shows an enhanced renal sympathetic nerve activity (RSNA) response to electric stimulation of the left cardiac sympathetic afferent nerve (10 V, 0.5 ms, and 30 Hz) in control (saline; left) and angiotensin II (ANG II)-injected (right) rats. Bars show cardiac sympathetic afferent nerve stimulation. ABP, arterial blood pressure; MAP, mean arterial pressure.

Fig. 3. Effects of microinjection of three doses of ANG II (0.03, 0.3, and 3 nmol) into the PVN on the RSNA responses to cardiac sympathetic afferent nerve stimulation. The RSNA responses were significantly enhanced after microinjection of the middle dose and high doses of ANG II into the PVN. *P < 0.05 compared with control (saline).

Fig. 4. Losartan (Los; 50 nmol in the PVN) normalizes the enhanced cardiac sympathetic afferent reflex. RSNA responses to cardiac sympathetic afferent nerve stimulation in the control (saline), ANG II (3 nmol), and ANG II + losartan (50 nmol groups). *P < 0.05 compared with saline; †P < 0.05 compared with losartan + ANG II.
II into the PVN. Figure 6 shows that average RSNA responses to epicardial application of two doses of BK (0.04 and 0.4 μg), which were used to evaluate the CSAR. The RSNA response evoked by epicardial application of high dose of BK (0.4 μg) was larger than a low dose of BK (0.04 μg). In addition, microinjection of ANG II (0.3 nmol) into the PVN (n = 6) significantly enhanced the RSNA responses to the high dose of BK (0.4 μg) compared with controls (Fig. 6A). Pretreatment with the AT1 receptor antagonist losartan (50 nmol in the PVN) abolished the enhanced CSAR induced by microinjection ANG II into the PVN (n = 6). Microinjection of losartan (50 nmol) alone into the PVN had no significant effect on the RSNA responses to BK (n = 6) (Fig. 6B). The data suggest that endogenous ANG II mechanisms are not tonically involved in the BK-mediated response.

DISCUSSION

CSAR is known as a sympathoexcitatory reflex. The sympathetic afferent endings innervating the heart are chemosensitive and mechanosensitive. The CSAR has been demonstrated in several species. Either epicardial application of hydrogen peroxide or intrapericardial BK stimulates the nerve endings in the heart and evokes an excitatory CSAR in rats and cats (9, 27). Our previous study (11) showed that stimulation of cardiac sympathetic afferent nerves induced the CSAR in dogs and that intracerebroventricular infusion of ANG II augmented this reflex. The present study demonstrates the following: 1) microinjection of ANG II into the PVN augmented the CSAR-induced increases in RSNA in rats, 2) these enhanced responses were blocked by microinjection of losartan into the PVN, and 3) blockade of endogenous ANG II in the PVN has no effect on CSAR-induced RSNA response.

There are numerous ANG II binding sites in the brain that have been shown to influence cardiovascular function and sympathetic activity (15, 26, 32). Microinjections of ANG II into the SFO and organum vasculosum of the lamina terminalis elicit an elevation in blood pressure (4). The PVN is an important integrative site within the brain to control cardiovascular activity (6). It is well known that the PVN contains neurons that project to the intermediolateral cell column of the thoracolumbar spinal cord and the RVLM and control sympathetic nerve activity and blood pressure (1, 2, 7). The PVN has been shown to be involved in mediating the cardiovascular response elicited by electrical stimulation of the SFO. Microinjection of ANG II into the PVN resulted in an increase in MAP in rats, and the response was significantly attenuated after systemic administration of losartan (3). ANG II infused into the RVLM shifts the RSNA-MAP response.


In conclusion, ANG II via the AT1-receptor in the PVN is involved in the central pathways of the CSAR leading to raised neuronal excitability and/or facilitated synaptic transmission. It suggests that central ANG II may play an important role in the augmented CSAR previously observed in the heart failure state.

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REFERENCES