Blockade of Endogenous Angiotensin-(1–7) in the Hypothalamic Paraventricular Nucleus Reduces Renal Sympathetic Tone

Ana Quênia Gomes da Silva, Robson Augusto Sousa dos Santos, Marco Antônio Peliky Fontes

Abstract—In this study, we tested the hypothesis that angiotensin-(1–7) [Ang-(1–7)] acting in the neurons of paraventricular hypothalamic nucleus (PVN) contributes to the maintenance of sympathetic activity and blood pressure. For this purpose, the effects of microinjection of the A-779, the receptor Mas antagonist, into the PVN on mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) were evaluated. In rats anesthetized with urethane (1.2 to 1.4 g/kg IP), bilateral microinjections of A-779 (0.1 nmol) into the PVN resulted in a selective and significant decrease in RSNA (−26±6% versus −2±3% vehicle; saline 0.9%). The magnitude of the decrease in RSNA produced by A-779 was comparable to that observed after microinjection of muscimol (1 nmol; −26±4%), a powerful neuronal inhibitor. A higher dose of A-779 (1 nmol) caused a reduction in RSNA (−21±4%) that was comparable in magnitude to the reduction observed with the lower dose. When compared with vehicle solution, no significant changes in MAP or HR were observed with both doses of A-779 tested. A decrease in RSNA was also observed after microinjections into the PVN of the angiotensin II type 2 (AT2) receptor antagonist PD123319 (1 nmol; −18±4%). Microinjections of the AT1 antagonist losartan but not CV 11974 reduced MAP without changing RSNA. These results suggest that Ang-(1–7) Mas receptors and AT2 receptors in the PVN neurons play a role in mediating the tonic maintenance of RSNA. (Hypertension. 2005;46:341-348.)

Key Words: angiotensin antagonist ■ angiotensin ■ rats

The paraventricular hypothalamic nucleus (PVN) is 1 of the 5 major premotor neuron cell groups involved in the control of the sympathetic outflow.1 The PVN influences sympathetic activity via direct projections to sympathetic preganglionic neurons or via a synaptic relay with the rostral ventrolateral medulla (RVLM), another group of presympathetic neurons that play a pivotal role in cardiovascular regulation.1,2 Activation of PVN neurons by microinjection of the γ-aminobutyric acid (GABA) receptor antagonist bicuculline increases arterial pressure and sympathetic activity in conscious3 and anesthetized4 rats. On the other hand, inhibition of PVN neurons by microinjection of muscimol, a powerful neuronal inhibitor, into the PVN decreases sympathetic activity and blood pressure.5 This effect is more pronounced in the spontaneously hypertensive rat, suggesting that an imbalance in the sympathetic tonus generated from PVN neurons in this rat strain contributes to the elevated levels of blood pressure in this model of hypertension.5

Findings from several previous studies indicate that peptides of the renin-angiotensin system (RAS) may act as important neuromodulators in different central sites involved in sympathetic output control,6,7 and a dysfunction in the brain RAS may be implicated in the pathogenesis of hypertension.6

Angiotensin-(1–7) [Ang-(1–7)] is now considered a biologically active peptide of the RAS family.8 Many of the Ang-(1–7) actions are mediated primarily through the recently described receptor Mas9 and are selectively blocked by its specific antagonist A-779.10 Several studies suggest that apart from its peripheral actions, Ang-(1–7) exerts actions in central sites involved in cardiovascular control,8,11 including nucleus of solitary tract,12,13 RVLM,14–17 and PVN neurons.18 In fact, the first biological action described for Ang-(1–7) showed that this peptide was a potent secretagogue of arginine vasopressin in hypothalamic–hypophyseal explants.19 Immunoreactive staining for Ang-(1–7) is present in the PVN,20 specifically in the lateral parvocellular and posterior magnocellular subdivisions,21 and endogenous Ang-(1–7) in the rat hypothalamus is present in concentrations comparable to angiotensin I (Ang I) and Ang II.22 Previous studies showed that microiontophoretic application of Ang-(1–7) into the PVN augments the excitability of the neurons in this region,18,23,24 and this effect can be selectively blocked by A-779.24 Altogether, these findings provide anatomical

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and functional support for a possible neuromodulatory action exerted by Ang-(1–7) in the PVN.

The main aim of this study was to test the hypothesis that endogenous Ang-(1–7) acting in the PVN neurons may contribute to the maintenance of sympathetic activity and arterial pressure. For this purpose, we determined the effects of microinjection of the Ang-(1–7) antagonist A-779 into the PVN on mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA). The effects produced by A-779 were compared with the effects produced by microinjection into PVN of Ang II type 1 (AT₁) and AT₂ antagonists and with muscimol, a powerful GABA<sub>B</sub> agonist known to reduce sympathetic activity and MAP when injected into the PVN.<sup>5</sup>

### Methods

#### General Procedures

All experiments were performed on Wistar rats (250 to 350 g) bred at the animal facilities of the Biological Sciences Institute (CEBIO, UFMG, Belo Horizonte, MG, Brazil) and performed in accordance with the guidelines established by our local institutional animal welfare committee. Under urethane anesthesia (1.2 to 1.4 g/kg IP), catheters were placed in a femoral vein and artery, and the trachea was cannulated. The adequacy of anesthesia was verified by the absence of a withdrawal response to nociceptive stimulation of a hindpaw. Supplemental doses of urethane (0.1 g/kg IV) were administered if necessary. Temperature was monitored and maintained in the range of 36.5°C to 37.5°C with a heating lamp. The hindpaw was monitored and main-3; Figure 2). Strikingly, this effect was different from that observed during the same period after vehicle was not accompanied by significant changes in MAP or HR. The magnitude of the decrease in RSNA produced by 0.1 nmol of A-779 caused a progressive decrease in RSNA that began 5 minutes after the bilateral microinjection (Figures 1 and 2). The mean maximum decrease in RSNA during the 30-minute period after A-779 microinjection was 26±6%, which was significantly different from that observed during the same period after vehicle microinjection (2±3%; Figure 2). Strikingly, this effect was not accompanied by significant changes in MAP or HR. The magnitude of the decrease in RSNA produced by 0.1 nmol of A-779 was comparable to that observed after microinjection of muscimol (26±4%), which, in contrast, also caused simultaneous decreases in MAP (mean maximum changes 20±6 mm Hg versus 4±2 mm Hg control) and HR (17±12 bpm versus 3±3 bpm control; Figure 2).

In a second series of experiments, we compared the effects on cardiovascular variables produced by microinjection into the PVN of equimolar doses of A-779, losartan, and muscimol.

#### Experimental Procedures

For the duration of each experiment, HR, MAP, and RSNA were recorded continuously. After all surgical procedures were completed, there was a waiting period of 20 minutes to allow the measured cardiovascular variables to stabilize. The baseline MAP, HR, and RSNA were then recorded for a 20-minute period. After that, microinjections of different compounds were made into the PVN and cardiovascular variables were then recorded for an additional 30-minute period. Different angiotensin antagonists, Ang-(1–7), Ang II, muscimol, or vehicle, were injected in separated groups of rats, and each rat received only 1 bilateral microinjection into the PVN; therefore, there were no cumulative effects of the antagonists in the data interpretation. Before the end of some experiments (n = 10) with angiotensin antagonists or vehicle, bilateral microinjections of muscimol were also made into the PVN by using the same stereotaxic coordinates adopted for injections of the previous compound. Because muscimol reduces blood pressure and sympathetic activity when injected into PVN, this procedure was helpful to functionally confirm the injection sites. At the end of each experiment, a bilateral microinjection of 2% alcian blue dye (100nl) was made into the PVN and, in each rat, the location of injection sites within the PVN was confirmed histologically, as described previously.<sup>26</sup>

### Statistical Analysis

The baseline values of MAP, HR, and RSNA were measured as the average values of these variables for the 1-minute period immediately preceding microinjections into the PVN. Experimental values were obtained from a mean across 1 minute and collected at each 5-minute interval after the bilateral injection into the PVN. Comparisons between responses evoked by microinjections of different compounds into the PVN were determined by 2-way ANOVA followed by Bonferroni post hoc test. Student's t test was also used when appropriate. A value of P < 0.05 was taken to indicate a statistically significant difference. All values are presented as mean±SEM.

#### Results

The Table shows the baseline values of MAP and HR observed in the different groups evaluated before microinjections into the PVN. When compared with microinjections of vehicle, microinjections of A-779 into the PVN evoked significant changes in sympathetic activity. In particular, microinjections of 0.1 nmol of A-779 caused a progressive decrease in RSNA that began within 5 minutes after the bilateral microinjection (Figures 1 and 2). The mean maximum decrease in RSNA during the 30-minute period after A-779 microinjection was 26±6%, which was significantly different from that observed during the same period after vehicle microinjection (2±3%; Figure 2). Strikingly, this effect was not accompanied by significant changes in MAP or HR. The magnitude of the decrease in RSNA produced by 0.1 nmol of A-779 was comparable to that observed after microinjection of muscimol (26±4%), which, in contrast, also caused simultaneous decreases in MAP (mean maximum changes 20±6 mm Hg versus 4±2 mm Hg control) and HR (17±12 bpm versus 3±3 bpm control; Figure 2).

### Baseline Values for MAP and HR Before Bilateral Microinjections Into PVN

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (100 nl)</td>
<td>5</td>
<td>91±6</td>
<td>347±12</td>
</tr>
<tr>
<td>Ang-(1–7) (25 pmol)</td>
<td>6</td>
<td>80±4</td>
<td>350±10</td>
</tr>
<tr>
<td>Ang II (25 pmol)</td>
<td>4</td>
<td>97±5</td>
<td>369±11</td>
</tr>
<tr>
<td>A-779 (0.1 nmol)</td>
<td>5</td>
<td>96±4</td>
<td>357±9</td>
</tr>
<tr>
<td>A-779 (1 nmol)</td>
<td>6</td>
<td>87±4</td>
<td>323±14</td>
</tr>
<tr>
<td>A-779 + Ang-(1–7)</td>
<td>5</td>
<td>92±6</td>
<td>359±5</td>
</tr>
<tr>
<td>Losartan (1 nmol)</td>
<td>6</td>
<td>86±7</td>
<td>335±11</td>
</tr>
<tr>
<td>CV 11974 (1 nmol)</td>
<td>6</td>
<td>80±5</td>
<td>363±4</td>
</tr>
<tr>
<td>PD123319 (1 nmol)</td>
<td>6</td>
<td>89±2</td>
<td>349±7</td>
</tr>
<tr>
<td>Muscimol (1 nmol)</td>
<td>5</td>
<td>90±7</td>
<td>356±17</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SEM.

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and PD123319 (1 nmol each group; Figure 3). As observed with the lower dose, bilateral microinjections of 1 nmol of A-779 into the PVN resulted in a gradual decrease in baseline RSNA (mean maximum decrease \( -21\pm4\% \)). With this dose, the decrease in RSNA was accompanied by a decrease in MAP (\( -13\pm5 \) mm Hg), but the decrease was not significantly different from that evoked by control microinjections. No consistent changes in HR were observed after A-779 microinjections into the PVN (Figure 3). Microinjections of PD123319 (1 nmol) also evoked a significant cardiovascular response when compared with the control microinjections (Figure 3). The magnitude of the sympathoinhibitory effect evoked by PD123319 (\( -18\pm3\% \)) was comparable to that observed with an equimolar dose of A-779. However, contrary to that observed with A-779, the fall in RSNA evoked by PD123319 reached statistical significance only 25 minutes after the injection. Bilateral microinjections of 1 nmol of losartan into the PVN caused a significant fall in MAP (\( -13\pm2 \) mm Hg) without significant changes in RSNA (Figure 3). On the other hand, microinjection of CV 11974 caused a significant increase in RSNA that was observed only at 5 minutes after microinjection of this compound into the PVN (Figure 3).

In an additional series of experiments, we observed that microinjections of Ang-(1–7) (25 pmol) into the PVN evoked a significant cardiovascular response compared with control microinjections (Figure 4). In particular, there was an increase in RSNA (\( 24\pm8\% \)), accompanied by a small but not significant increase in HR (\( 14\pm4 \) bpm). The magnitude of the increase in RSNA evoked by Ang-(1–7) was similar to that observed after an equimolar injection of Ang II (\( 22\pm2\% \)); however, contrary to that observed with Ang-(1–7), Ang II produced a sustained effect. Neither Ang-(1–7) nor Ang II caused significant changes in blood pressure (Figure 4). The increase in RSNA produced by this dose of Ang-(1–7) was completely blocked by the lower dose of A-779 used in the present study (0.1 nmol; Figure 5).

Postmorten histology confirmed that the majority of the injection sites were located within or in the borders of the PVN, extending from the level 1.4 to 2.1 mm caudal to bregma (Figure 6). For clarity, the histological analysis shown in Figure 6 refers only to the animals belonging to the groups of experiments shown in Figure 2. According to a previous study,5 microinjections of muscimol in the same regions were effective in reducing RSNA, which indicates...
that the sympathoexcitatory PVN area was reached. In the present study, from 10 experiments in which muscimol was injected into the PVN after antagonists or vehicle, only 1 animal was excluded from the analysis on the basis of an absence of response to muscimol injection into the PVN limits.

**Discussion**

The main finding of this study was that microinjection of Ang-(1–7) antagonist A-779 into the PVN reduces RSNA. The decrease in RSNA was consistent, observed with both doses tested, and similar in magnitude to the effect observed with muscimol, a powerful neuronal inhibitor.28 We also observed that microinjection of Ang-(1–7) into the PVN increases RSNA, and this effect was blocked by its selective antagonist, A-779. The observations are consistent with previous findings showing an excitatory action for Ang-(1–7) in the PVN neurons.18,23,24 In addition, the current study shows that blockade of AT2 receptors in the PVN reduces sympathetic activity, suggesting a role for AT2 receptors in the PVN to the tonic maintenance of sympathetic activity. Finally, based on the data obtained with microinjection of the GABA\(_A\) agonist, muscimol, our results also confirm recent findings that the PVN exerts a tonic effect on the control of sympathetic vasomotor tone under basal conditions in anesthetized rats.5

A possible excitatory action for Ang-(1–7) in the PVN neurons has been suggested previously; however, the involvement of Ang-(1–7) in the PVN to the maintenance of sympathetic output was never evaluated. Electrophysiological studies demonstrated that most neurons in the PVN are excited by Ang-(1–7),18,23 and the Ang-(1–7)–induced firing rate increase of PVN neurons is effectively blocked by A-779.24 Conversely, the Ang II–induced firing rate increase of PVN neurons is not altered in the presence of A-779,24 indicating that the Ang-(1–7) excitatory effect in the PVN neurons is mediated by a selective receptor, which corroborates our findings. Therefore, these previous studies, together with the present results showing that bilateral microinjection of A-779 into the PVN results in a substantial decrease in...
RSNA, suggest an excitatory role for Ang-(1–7) in the PVN neurons in the tonic regulation of sympathetic output.

Although the effect produced by A-779 on RSNA seems modest (~20% to 25% reduction), it is important to point out that this effect was comparable in magnitude to the effect produced by muscimol. Muscimol is a powerful neuronal inhibitor acting as an agonist at GABA_A receptors, which virtually all neurons possess, so that muscimol is thought to be effectively a universal inhibitor of neurons. In addition, equimolar doses of AT_1 antagonists (used in the present study) or even higher doses used in previous studies did not cause an effect of similar magnitude on RSNA in baseline conditions. The kidney is very sensitive to RSNA changes; therefore, changes of this magnitude are enough to cause changes in kidney function, for example.

It could be questioned whether the doses of antagonists used in the present study block the actions of its respective agonists. In this regard, in additional series of experiments, we observed that microinjection of Ang-(1–7) into the PVN results in an increase in RSNA, and this effect was completely blocked by its selective antagonist A-779 with the lower dose used in the present study (ie, 100 pmol). In addition, it has been shown that the pressor effect evoked by microinjection of Ang-(1–7) into the RVLM was also prevented by administration of A-779 with the same dose used in the present study but not by AT_1 or AT_2 receptor antago-
nists in a higher concentration (200 pmol). The same can be observed for Ang II, of which the pressor effects in the RVLM were blocked by 1 nmol of losartan but not by 1 nmol of PD123319. Therefore, the finding of the present study, together with the results from previous studies, suggest that the doses of angiotensin antagonists used in the present study are likely enough to block the actions of its respective agonists.

During the last decade, studies using the selective Ang-(1–7) antagonist A-779 provided pharmacological evidence for an Ang-(1–7) receptor distinct from the classical Ang II receptors AT1 and AT2. These indirect evidences were confirmed recently by the identification of an Ang-(1–7) receptor: the G-protein–coupled receptor (GPCR) Mas. This receptor is expressed predominantly in the mouse and rat brain and particularly in the forebrain. However, further detailed studies are necessary to confirm the expression and distribution of Mas receptor in the hypothalamus and specifically in the PVN. Despite that, the findings of the present study showing that bilateral microinjection of A-779 into the PVN markedly reduces sympathetic activity and that the pressor effect produced by Ang-(1–7) is blocked by A-779, together with the results from previous studies, suggest that the actions of endogenous Ang-(1–7) in the PVN are mediated by a selective receptor.

On the basis of the present and previous findings, the question could be raised as to what is the origin of Ang-(1–7) in the PVN neurons. Angiotensin-converting enzyme 2 (ACE 2) directly converts Ang II to Ang-(1–7); however, as far as we know, there are no reports in the literature demonstrating the presence of ACE 2 specifically in the PVN. Despite that, an important characteristic of Ang-(1–7) is that this peptide can be formed directly from Ang I by enzymatic pathways not involving ACE. In this regard, enzymes such as prolyl endopeptidase, which is present in the hypothalamus, could be involved in the generation of Ang-(1–7). Combined high-performance liquid chromatography–radioimmunoassay techniques demonstrated that endogenous Ang-(1–7) in hypothalamus and brain stem are present in concentrations comparable to Ang I and Ang II. In addition, Ang-(1–7) immunoreactivity is present in the rat hypothalamus, including in cells and fibers associated with central vasopressinergic pathways. These findings give further support to the hypothesis that Ang-(1–7) plays a role as a neuromodulator in the hypothalamus, which seems to be particularly evident with regard to the PVN. Altogether, these studies indicate the presence of Ang-(1–7) in the rat hypothalamus; however, the process by which Ang-(1–7) is generated or incorporated in neurons is currently unknown. In fact, even the Ang II formation routes in the brain are unknown.

In the present study, we observed that microinjections into the PVN of the AT1 antagonist losartan caused a small but significant decrease in MAP without changing baseline RSNA. This finding is in agreement with recent published studies showing that even larger doses of losartan microinjected into PVN, ranging from 50 to 80 nmol, caused a small reduction in MAP without altering resting HR or RSNA. In fact, electrophysiological experiments have shown that isolated application of losartan does not change baseline activity of PVN neurons. Conversely, we observed that microinjection of CV 11974 into the PVN caused an acute increase in RSNA without altering baseline MAP or HR. The reason for these differences between losartan and CV 11974 is unclear at present, but such discrepant physiological effects between both antagonists in the central nervous system have been reported previously. Despite that, it is important to point out that our study does not discard the possibility of a contribution of AT1 receptors in the PVN neurons to the maintenance of sympathetic tone and blood pressure. Given the fact that a high density of AT1 receptor binding is found on neurons in the PVN and exogenous Ang II excites PVN neurons, the question arises as to their functions. Indeed, it has been demonstrated recently that AT1 receptors in the PVN might contribute to the sympathetic output under some conditions such as during hyperosmolality or in heart failure.

We found that microinjection of the AT2 blocker PD123319 into the PVN reduced the baseline sympathetic activity. This result suggests that AT2 receptors are possibly mediating an endogenous sympathoexcitatory action of Ang II in the PVN neurons. In this regard, it is interesting to note that in cultured neurons from rat hypothalamus, AT1 and AT2 receptors are expressed, and activation of these receptors by Ang II has opposite effects on potassium channels. Addition, it was demonstrated previously that the excitatory action of Ang II on PVN neurons is antagonized by an AT2 antagonist. In a recent study, Chen and Tone found that microinjections of PD123319 into the PVN failed to acutely alter resting values of MAP, HR, and RSNA. The simplest explanation for the different result obtained in our study and in the Chen and Toney study is that in the present study, we used a dose 10-fold lower than the one that Chen and coworkers used previously. Therefore, a nonspecific action of PD123319 caused by the high dose used previously possibly masked a contribution of AT2 receptors in the PVN to the tonic maintenance of sympathetic activity. However, based on our results, we cannot discard the possibility of an interference of PD123319 with Mas, as has been observed in the kidney tissue. This interference could be explained by oligomerization or through a cross-talk mechanism between Mas and AT2 receptors, particular characteristics of GPCRs. However, it needs to be highlighted that the time course of the sympathoinhibitory effect in response to PD123319 microinjection into the PVN only reached significance after 20 to 25 minutes. This pattern of response was different from that observed after A-779 microinjection.

In a recent study, Li et al found that exogenous Ang II decreases the spontaneous miniature inhibitory postsynaptic currents (mIPSCs) to spinally projecting PVN neurons. This effect was blocked by losartan but not by PD123319, providing evidence that Ang II excites spinally projecting PVN neurons by attenuation of GABAergic synaptic inputs through activation of presynaptic AT1 receptors. These findings should not be considered to be in conflict with our observations. First, although the author mentions that losartan alone had no effect on the spontaneous mIPSCs, and this indicates the absence of effect of losartan on baseline activity of PVN neurons, the effect produced by PD123319 alone on
the mPSCs was not described. Second, the effect of other Ang peptides, such as Ang-(1–7) or Ang III, peptides known to excite PVN neurons, was not tested in the presence of PD123319, which could unmask a possible non-speciﬁc angiotensinergic action mediated by AT2 receptors. Therefore, based on the report by Li et al., we cannot discard the possibility that AT2 receptors are involved in the synaptic modulation of PVN neurons. Clearly, a possible contribution of AT2 receptors in the PVN to the maintenance of sympathetic activity needs to be investigated further.

Perspectives

In this study, we obtained evidence that the Ang-(1–7) receptor Mas and AT1 receptors in the PVN play a signiﬁcant role in modulating peripheral sympathetic activity. Evidence was also provided for a primary role of Ang-(1–7) receptors in the modulation of RSNA. For future studies, it remains to be determined the contribution of Ang-(1–7) in the PVN during altered physiological conditions, which could alter plasma levels of Ang-(1–7). The contribution of Ang-(1–7) in the PVN to the maintenance of sympathetic output in experimental models of hypertension, such as in spontaneously hypertensive rats, also deserves investigation.

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References


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