Imbalance of Angiotensin Type 1 Receptor and Angiotensin II Type 2 Receptor in the Rostral Ventrolateral Medulla
Potential Mechanism for Sympathetic Overactivity in Heart Failure

Lie Gao, Wei-Zhong Wang, Wei Wang, Irving H. Zucker

Abstract—Upregulation of angiotensin II type 1 receptors (AT₁R) in the rostral ventrolateral medulla (RVLM) contributes to the sympathoexcitation in the chronic heart failure (CHF). However, the role of angiotensin II type 2 receptor (AT₂R) is not clear. In this study, we measured AT₁R and AT₂R protein expression in the RVLM and determined their effects on renal sympathetic nerve activity, blood pressure, and heart rate in anesthetized sham and CHF rats. We found that (1) although AT₁R expression in the RVLM was upregulated, the AT₂R was significantly downregulated (CHF: 0.06±0.02 versus sham: 0.15±0.02, P<0.05); (2) simultaneously stimulating RVLM AT₁R and AT₂R by angiotensin II evoked sympathoexcitation, hypertension, and tachycardia in both sham and CHF rats with greater responses in CHF; (3) stimulating RVLM AT₁R with angiotensin II plus the specific AT₂R antagonist PD123319 induced a larger sympathoexcitatory response than simultaneously stimulating AT₁R and AT₂R in sham rats, but not in CHF; (4) activating RVLM AT₁R with CGP42112 induced a sympathoinhibition, hypotension, and bradycardia only in sham rats (renal sympathetic nerve activity: 36.4±5.1% of baseline versus 102±3.9% of baseline in artificial cerebrospinal fluid, P<0.05); (5) pretreatment with 5,8,11,14-eicosatetraynoic acid, a general inhibitor of arachidonic acid metabolism, into the RVLM attenuates the CGP42112-induced sympathoinhibition. These results suggest that AT₂R in the RVLM exhibits an inhibitory effect on sympathetic outflow, which is, at least partially, mediated by an arachidonic acid metabolic pathway. These data implicate a downregulation in the AT₂R as a contributory factor in the sympathoexcitation in CHF. (Hypertension. 2008;52:708-714.)

Key Words: angiotensin II type 1 receptor ■ angiotensin II type 2 receptor ■ rostral ventrolateral medulla ■ sympathetic outflow

It is well accepted that chronic heart failure (CHF) is characterized by heightened sympathetic tone. This excessive sympathetic outflow to the heart and peripheral vessels attempts to increase myocardial performance and increases peripheral resistance, thereby contributing to an increase in myocardial oxygen consumption leading to a further deterioration in cardiac function. It has been well established that activation of angiotensin II type 1 receptors (AT₁R) in the rostral ventrolateral medulla (RVLM) evokes sympathetic excitation and pressor effects in normal animals. Data from a previous study from our laboratory further suggested that the upregulated AT₁R expression in the RVLM and its enhanced intracellular signaling transduction plays a critical role in the sympathoexcitation in the CHF state. In addition, Ito et al demonstrated that activation of AT₁R in the RVLM appears to be important for the maintenance of hypertension in spontaneously hypertensive rats, another animal model of sympathoexcitation.

In contrast with the AT₁R, the functions of central angiotensin II type 2 receptors (AT₂R) regarding the regulation of autonomic system are not well understood. Although the AT₁R predominates in the tissues during fetal development, this receptor has been identified to exist in many adult mammalian tissues, including the brain. Further experiments have demonstrated that central regions related to sympathetic function such as the hypothalamus and brainstem exhibit positive AT₂R mRNA hybridization signals, implying the involvement of AT₂R in the regulation of sympathetic outflow. Kang et al found that, in the cultured neurons from newborn rat hypothalamus and brainstem, stimulation of AT₂R significantly increased neuronal voltage-gated potassium channel current (Iᵥ) and that the third intracellular loop of the AT₂R is a key component for this effect. This group further determined that the phospholipase A2/arachidonic acid/12-lipoxygenases pathway mediates the modulation of potassium currents by activation of the AT₂R. These data strongly suggest that the AT₂R exhibits an inhibitory effect on neuronal function by increasing potassium current and therefore decreasing excitability of neurons. Indeed, a recent study by Matsuura et al demonstrated an AT₂R-mediated hyper-
polarization and decrease in firing rate of RVLM presympatheic neurons using the whole-cell patch-clamp technique in AT1R knockout mice. However, there are no reports concerning the effects of activating RVLM AT2R on sympathetic outflow and cardiovascular function in either normal or pathological conditions. In the current experiment, we measured both AT1R and AT2R protein expression in the RVLM from sham and CHF rats. We also observed the effects of stimulating RVLM AT1R and/or AT2R on renal sympathetic nerve activity (RSNA), arterial blood pressure, and heart rate (HR) in the anesthetized sham and CHF rats to determine the physiological and pathological significance of AT2R on autonomic regulation.

Methods
Forty-seven male Sprague-Dawley rats (Sasco, Wilmington, Mass) weighing between 290 and 380 g were used in these experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of Rat CHF Model
CHF was induced by coronary artery ligation as previously described. See the online data supplement available at http://hyper.ahajournals.org for the details.

Acute Experiments
The acute experiment, including the general animal preparation, recording of RSNA, and RVLM microinjection procedures, were carried out as previously described. See the online data supplement for details.

Preparation of RVLM and Western Blot Analysis
In a separate group of rats than those used for the previously mentioned microinjections, brains were removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned at 100-μm thickness in a cryostat. The RVLM was punched using the technique of Palkovits and Brownstein and homogenized in RIPA buffer. Protein extraction from homogenates was used to analyze AT1R and AT2R expression by Western blot. The concentration of protein extracted was measured using a protein assay kit (Pierce, Rockford, Ill) and adjusted to the same with equal volumes of 2× SDS sample buffer. The samples were boiled for 5 minutes followed by loading on a 7.5% SDS-PAGE gel (10 μg protein/30 μL per well) for electrophoresis using a Bio-Rad mini gel apparatus at 40 mA/gel for 45 minutes. The fractionated protein on the gel was transferred onto a polyvinylidene fluoride membrane (Millipore) and electrophoresed at 300 mA/gel for 90 minutes. The membrane was probed with primary antibodies (AT1R rabbit polyclonal antibody, Santa Cruz, 1:1000; AT2R rabbit polyclonal antibody, Santa Cruz, 1:1000) and secondary antibody (goat antirabbit IgG-HRP, Santa Cruz, 1:2500) and then treated with enhanced chemiluminescence substrate (Pierce) for 5 minutes at room temperature. The bands in the membrane were visualized and analyzed using UVP BioImaging Systems.

Statistical Analyses
All data are described as the mean±SEM. The integrated RSNA before agent intervention was set as a 100% of baseline. The change in RSNA induced by a given agent was described as a percent of baseline. A 1-way or 2-way analysis of variance was used followed by either the Newman-Keuls or Bonferroni post hoc analysis where appropriate. Statistical analysis was done with the aid of SAS software. P<0.05 was considered statistically significant.

Table. Hemodynamic and Morphological Characteristics of Sham and CHF Rats

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Sham (n=16)</th>
<th>CHF (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>392±16</td>
<td>403±20</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.6±0.2</td>
<td>2.2±0.2*</td>
</tr>
<tr>
<td>Wet lung weight, g</td>
<td>2.1±0.1</td>
<td>3.1±0.2*</td>
</tr>
<tr>
<td>Infarct size, % of left ventricle</td>
<td>0</td>
<td>41.5±3.6*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>92.4±3.9</td>
<td>89.7±5.7</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>354±21</td>
<td>372±19</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>83±5.9</td>
<td>46±7.1*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>1.4±1.1</td>
<td>15.3±2.1*</td>
</tr>
</tbody>
</table>

Values are means±SE. LVEDP indicates left ventricular end diastolic pressure. *P<0.05 compared with sham.

Results
Baseline Physiological Parameters of Sham and CHF Rats
The Table summarizes the baseline characteristics of sham and CHF rats used in the present experiment. In the rats with CHF, gross examination revealed a dense scar in the anterior ventricular wall and the mean infarct area was 41.5±3.6% of the left ventricular area. No infarcts were found in sham-operated rats. Corresponding to this morphological alteration was a marked decrease in cardiac function of CHF rats. Left ventricular end diastolic pressure was significantly elevated and ejection fraction was significantly lowered in the CHF rats compared with sham rats. Moreover, many CHF rats exhibited pulmonary edema, hydrothorax, and ascites. However, there were no significant differences in the arterial blood pressure and heart rate between sham and CHF rats.

Expression of AT1R and AT2R in the RVLM of Sham and CHF Rats
Figure 1 shows the AT1R and AT2R protein expressions in the RVLM of sham and CHF rats. CHF rats exhibited higher AT1R expression than that in sham rats similar to results we found previously in CHF rabbits. Interestingly, from the same RVLM tissue sample, we found a lower AT2R expression from CHF RVLM rats compared with sham rats. These data suggest that, in the CHF state, although AT1R expression in the RVLM was upregulated, AT2R expression was significantly downregulated. In normal conditions, the level of AT2R expression in the RVLM is only half of AT1R expression. In the CHF state, this disparity of AT1R and AT2R expression was increased due to the upregulated AT1R and the downregulated AT2R. Angiotensin II (Ang II) activates both AT1R and AT2R. The ratio of these 2 angiotensin receptors therefore determines the final effects of Ang II in a specific tissue or organ. From Figure 1D, it can be seen that in CHF rats, the ratio of AT1R to AT2R was markedly increased compared with that from sham rats (13.8±0.7 versus 2.1±0.4, respectively, P<0.01; Figure 1D).

Effects of Stimulating RVLM AT1R and/or AT2R on RSNA and BP in Sham and CHF Rats
Functional Location of RVLM by l-Glutamate
Figure 2 shows the functional identification of the RVLM by microinjection of l-glutamate. A transient pressor,
tachycardia, and sympathoexcitatory response was induced by microinjection of L-glutamate (5 nmol in 50 nL) unilaterally into the RVLM. This functional test was routinely carried out using one barrel of a 3-barreled micropipette before each reagent was given.

Simultaneously Activating AT1R and AT2R by Ang II

Figure 3 shows the cardiovascular and sympathetic responses to microinjection of Ang II (50 pmol in 50 nL) unilaterally into the RVLM to stimulate AT1R and AT2R. Ang II induced sympathoexcitation in both sham (146.7 ± 9.4% of baseline) and CHF (235.3 ± 11.6% of baseline) rats with a significantly larger response in the CHF rats (P < 0.01). The enhanced sympathetic response to Ang II in the CHF state may be due to upregulation of AT1R expression, downregulation of AT2R expression, or both. The increase in mean arterial pressure (MAP) and HR were however similar between the 2 groups (MAP: 24.7 ± 3.6 mm Hg, ΔHR = 21.3 ± 6.4 beats/min for sham and ΔMAP = 28.1 ± 2.3 mm Hg, ΔHR = 26.9 ± 8.2 beats/min for CHF rats).

Activating AT1R by Ang II Plus PD123319

Figure 4A shows the cardiovascular and sympathetic responses to microinjection of Ang II (50 pmol in 50 nL) plus PD123319 (1 nmol in 50 nL) unilaterally into the RVLM in sham and CHF rats. From Figures 4B and 4D it can seen that, in both sham and CHF rats, this treatment also produced an increase in the arterial blood pressure (ΔMAP: 38.2 ± 6.5 mm Hg for sham, 31.6 ± 4.7 mm Hg for CHF; P < 0.05), HR (ΔHR: 31.4 ± 8.1 beats/min for sham, 27.6 ± 5.8 beats/min for CHF; P < 0.05), and RSNA (ΔRSNA: 193.2 ± 6.8% of baseline for sham, 241.6 ± 10.4% of baseline for CHF; P < 0.05), suggesting that selective stimulation of AT1R in the RVLM also evoked a cardiovascular and sympathetic excitation. Compared with Ang II alone, microinjection of Ang II plus PD123319 into the RVLM elicited a larger hypertension and sympathoexcitation only in sham but not in CHF rats. The mean data of RSNA responses to the activation of AT1R and AT2R, or only AT1R, are shown in Figure 4B.
Figure 3. Effects of microinjecting Ang II into the RVLM on sympathetic outflow, HR, and arterial pressure in sham and CHF rats. A, A representative trace and (B) the group data showing the maximal change in RSNA. **P<0.01 compared with the sham. n=7 per group.

Activating AT₂R by CGP42112

Figure 5 shows the cardiovascular and sympathetic responses to microinjection of CGP42112 unilaterally into the RVLM in normal rats. CGP42112 (50 pmol in 50 nL) evoked a decrease in blood pressure (ΔMAP = –31.3±4.6 mm Hg; P<0.05), HR (ΔHR = –26.7±5.2 beats/min; P<0.05), and RSNA (ΔRSNA = 36.4±11.1% of baseline; P<0.05), suggesting that activation of AT₂R in the RVLM depressed sympathetic outflow. These effects of CGP42112 were abolished by pretreatment with PD123319, a specific AT₂R antagonist. Moreover, pretreatment with 5,8,11,14-eicosatetraynoic acid (a general inhibitor of arachidonic acid metabolism, 10 pmol in 50 nL) partially attenuated the CGP42112-induced hypotension, tachycardia, and sympathoinhibitory responses. In the CHF rats, we did not find significant changes in blood pressure (ΔMAP = –5.1±3.2 mm Hg, P>0.05), HR (ΔHR = –3.8±4.4 beats/min, P>0.05), and RSNA (ΔRSNA = 89.9±13.6% of baseline, P>0.05) after CGP42112 was microinjected into the RVLM, which is shown in Figure 6.

Discussion

It has long been known that activation of AT₁R in the RVLM evokes an increase in sympathetic outflow.4,17 AT₁R activation in the RVLM plays a critical role in the sympathoexcitation in the CHF state.6 However, the role of AT₂R in modulation of sympathetic outflow in CHF is completely unknown. The major novel findings from the present study are that (1) AT₂R protein expression in the RVLM of CHF rats was significantly lower than that in sham rats; (2) microinjection of Ang II plus the AT₁R antagonist, PD123319, into the RVLM produced a larger hypertension and sympathoexcitation than Ang II alone in sham but not in CHF rats; and (3) microinjection of the AT₂R agonist, CGP42112, into the RVLM evoked hypotension, bradycardia, and sympathoinhibition in sham rats, but not in CHF rats. These results document that AT₂R expression in the RVLM is downregulated in CHF rats and that AT₂R stimulation in the RVLM exhibited an inhibitory effect on sympathetic outflow in the normal condition. These data suggest that suppressed AT₂R signaling may be involved in the sympathetic overactivity in the CHF state.
Sympathetic nerve activity is regulated at several central loci, including the subfornical organ, the area postrema, the paraventricular nucleus in the hypothalamus, and the nucleus of solitary tract and RVLM in the medulla. Of these structures, the RVLM is an important region in maintaining tonic activity of sympathetic nerve outflow. By directly projecting to sympathetic preganglionic neurons of the spinal cord and receiving inputs from other sympathetic-related central nuclei, the RVLM acts as a final common pathway in transferring signals from more rostral structures to peripheral sympathetic nerves. A series of studies from our laboratory have demonstrated that the upregulated AT1R expression and the enhanced AT1R-related intracellular signaling pathway in the RVLM play a critical role in the sympatho-excitation in the CHF condition, similar to that observed in hypertension. Interestingly, in the current study, we found that although AT1R protein expression in the RVLM of CHF rats was upregulated, the AT2R was significantly downregulated and therefore greatly increased the ratio of AT1R to AT2R protein expression from 2.1/0.4 in sham to 13.8/0.7 in CHF (Figure 1). Although the physiological roles of central AT2R in whole animals are unclear, the patch clamp data from the cultured individual neurons of the hypothalamus and brainstem clearly demonstrated an increase in the potassium current induced by this receptor, an effect contrary to that of the AT1R on neuronal channel function. The functions and intracellular signaling pathways of the AT2R in most peripheral tissues and organs also are opposite to that of the AT1R. For example, stimulating AT2R induces vasodilation, stimulates nitric oxide production, and inhibits reactive oxygen species generation. Taken together, these data led us to postulate that the balance between AT1R and AT2R in the RVLM may be critical to maintain sympathetic tone in normal conditions and that the downregulated AT2R combined with the upregulated AT1R in the RVLM may contribute to the sympatho-excitation in the CHF state. The mechanism for the ameliorating effects of AT2R stimulation on the responses to AT1R stimulation in the present experiments is not completely clear; however, a potential decrease in neuronal potassium current induced by the downregulation of AT1R expression (ie, dominance of AT1R expression) may

Figure 5. Effects of microinjection of CGP42112 into the RVLM on sympathetic outflow, HR, and arterial pressure in sham rats. A, A representative trace and (B) the time course of group data showing the change in RSNA. *P<0.05 compared with the artificial cerebrospinal fluid group; #P<0.05 compared with the CGP42112 group. n=7 to 8 per group.

Figure 6. Effects of microinjecting CGP42112 into the RVLM of CHF rats on sympathetic outflow, HR, and arterial pressure. A, A representative trace and (B) the group data showing the maximal change in RSNA. n=7 per group.
imply facilitated neuronal excitability and exaggerated sympathetic outflow.

Interestingly, microinjection of CGP42112, a specific AT2R agonist, into the RVLM evoked a significant hypotension, bradycardia, and decreased RSNA, a completely opposite effect to the well-known role induced by central AT1R stimulation. These data provide direct evidence showing physiological significance of brain AT2R in the regulation of autonomic function. Moreover, the effects of AT2R in the RVLM were completely blocked by pretreatment with the AT2R antagonist, PD123319, demonstrating the specificity of this effect. Support for our hypothesis comes from mice lacking AT1Rs. Siragy et al24 reported that AT1R-null mice had slightly elevated systolic blood pressure compared with that of wild-type control mice. Infusion of a subpressor dose of Ang II failed to induce a change of blood pressure in wild-type mice but significantly increased blood pressure in AT2R knockout mice. Moreover, Li et al25 found that injection of Ang II into the cerebral ventricle evoked a larger increase in blood pressure in AT2R knockout mice than that in wild-type mice. In wild-type mice, central injection of Ang II plus PD123319 initiated a greater pressor response than that induced by Ang II alone. The majority of neuronal AT2R intracellular signaling pathways are mediated by different mediators from that of AT1R.

It has been demonstrated in neurons cultured from neonatal rat hypothalamus and brainstem that inhibitory G proteins, phospholipase A2, and protein phosphatase 2A are involved in the AT2R-dependent increase in potassium current.21-27 AT2R activation stimulates protein phosphatase 2A activity28 and protein phosphatase 2A may directly participate in a dephosphorylation-mediated activation of the potassium channel. Zu et al13 explored the involvement of a series of arachidonic acid metabolites in the AT2R-evoked increase in the potassium current in cultured neurons and demonstrated that the pathway of arachidonic acid metabolism is responsible for the modulation of potassium currents by AT2R. Indeed, in the current study, we found that pretreatment with 5,8,11,14-eicosatetraynoic acid, a general inhibitor of arachidonic acid metabolism, partially attenuated the activation of AT2R-induced suppression of sympathetic outflow (Figure 5).

In the current study, we found that microinjection of Ang II into the RVLM evoked a significant pressor, tachycardia, and sympathoexcitiation in both sham and CHF rats with a greater sympathoexcitatory response in CHF rats (Figure 3). This differential effect of Ang II in the RVLM between sham and CHF animals appears to be due to the increased AT1R and decreased AT2R expressions in the CHF state compared with sham. Indeed, when Ang II was microinjected into the RVLM after pretreatment with PD123319, we found enhanced sympathoexcitatory responses only in sham but not in CHF rats (Figure 4). These data suggest that, in the normal condition, simultaneously activating AT1R and AT2R in the RVLM produced smaller responses than that induced by stimulating AT1R alone. This implies that the opposing effects of AT1R and AT2R in the RVLM play a role in the maintenance of sympathetic outflow. On the other hand, in the CHF state, loss of this opposing influence due to the downregulation of AT2R may contribute to the sympathoexcitatory.

Stimulation of AT2R in the RVLM by CGP42112 induced inhibition of cardiovascular activity and sympathetic outflow exhibits regional specificity. In normal rats, microinjection of CGP42112 into the vicinity of RVLM, which had no response to L-glutamate, evoked no change in blood pressure, HR, and RSNA. Interestingly, microinjection of CGP42112 into the caudal ventrolateral medulla where L-glutamate often generates inhibition of sympathetic nerve activity evoked pressor and sympathoexcitatory effects (data not shown).

**Perspectives**

In a recently published paper29 we reported a decrease in nocturnal arterial pressure coincident with a decrease in urine concentration of noradrenaline and 24-hour noradrenaline excretion in normal, conscious rats after RVLM overexpression of AT2R by adenoviral transfection. In the current experiment, we documented the negative influence of stimulating endogenous AT2R in the RVLM on sympathetic outflow in normal rats and the weakened AT2R pathway in the RVLM of CHF rats, providing further insights into the physiological and pathological significance of AT2R in the neural control of autonomic and circulatory function. In future experiments, we will observe the potential beneficial effects of AT2R overexpression in the RVLM of CHF rats on heart failure state by AT2R-adenoviral transfection. Moreover, the first selective nonpeptide AT2R agonist, Compound 21, has been synthesized recently by Wan et al.30 It is intriguing to speculate substances such as this may hold potential and promise for the treatment of such diseases characterized by sympathoexcitation such as CHF and hypertension.

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**Disclosures**

None.

**References**


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