Enhanced angiotensin-mediated excitation of renal sympathetic nerve activity within the paraventricular nucleus of anesthetized rats with heart failure

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Abstract

Chronic heart failure (HF) is characterized by increased sympathetic drive. Enhanced angiotensin II (AngII) activity may contribute to the increased sympathoexcitation under HF condition. The present study was to examine sympathoexcitation by 1) the effects of AngII in the paraventricular nucleus (PVN) on renal sympathetic nerve activity (RSNA), and 2) the altered angiotensin type 1 (AT₁) receptor expression during HF. Left coronary artery ligation was used to induce HF. In the anesthetized-Sprague Dawley rats, microinjection of AngII (0.05 to 1 nmol) into the PVN increased RSNA, mean arterial pressure (MAP) and heart rate (HR) in both sham-operated and HF rats. The responses of RSNA and HR were significantly enhanced in rats with HF as compared to sham rats (RSNA: 64±8% versus 33±4%, P<0.05). Microinjection of AT₁ receptor antagonist losartan into the PVN produced a decrease of RSNA, MAP and HR in both sham and HF rats. The RSNA and HR responses to losartan in HF rats were significantly greater. (RSNA: -25±4% versus –13±1%, P<0.05). Using RT-PCR and Western blotting, we found that there were significant increases in the AT₁ receptor mRNA (∆186±39%) and protein levels (∆88±20%) in the PVN of rats with HF (P<0.05). The immunofluorescence of AT₁ receptors was significantly higher in the PVN of rats with HF. These data support the conclusion that an increased angiotensinergic activity on sympathetic regulation, due to the up-regulation of AngII AT₁ receptors within the PVN, may contribute to the elevated sympathoexcitation that is observed during HF.

Key words: angiotensin, sympathetic activity, blood pressure, heart failure
Introduction

It has been well established that increased sympathetic nerve activity is a characteristic feature of chronic heart failure (HF) (37, 41). This elevated sympathetic activity induces an increase in peripheral resistance that increases cardiac afterload and preload. Furthermore, continued elevation of cardiac sympathetic activation results in high levels of norepinephrine, which contributes to myocardial toxicity and apoptosis (49), resulting in a decrease in myocardial contractility. Some studies have shown that various cardiovascular reflex functions are impaired in HF (15, 23). Using an animal model of HF produced by coronary artery ligation, studies from this laboratory and work by others have revealed that renal sympathetic nerve activity (RSNA) is elevated in rats with HF (7, 43). However, peripheral blockade has been shown only partially reducing the effects of sympathoexcitation under HF condition (38). An increasing number of studies have suggested that altered central mechanism(s) may be responsible for the elevated neurohumoral drive in HF (6, 7, 42, 58).

The paraventricular nucleus (PVN) of the hypothalamus is an important central site for the integration of sympathetic nerve activity (53). Morphologic and electrophysiological studies have shown that the PVN is reciprocally connected to other areas of central nervous system, such as the nucleus tractus solitarii (NTS) and the rostral ventrolateral medulla (RVLM) in the brain stem. These brain sites are involved in cardiovascular regulation, which makes them the areas of interest for this study (13, 27, 51). Using retrograde tracing techniques, various studies have shown that the PVN is a major source of forebrain input to the sympathetic nervous system (50, 51). Stimulation of PVN has been shown to elicit increased discharge of several sympathetic nerves, including renal (18) and adrenal (20). Specifically, studies showed that the PVN plays an essential role in the mediation of RSNA under resting and reflex conditions (14). Our previous
studies have shown increased neuronal activity, as measured by hexokinase activity, in the PVN during HF (44), which points to the involvement of the PVN in the altered sympathetic activity in HF. However, it is still unknown the specific mechanisms within the PVN which are involved in regulating sympathetic nervous outflow.

In the PVN, a number of inputs that use different neurotransmitters converge to influence its neural activity (53). Angiotensin II (AngII) has been found to act as a neurotransmitter in the central nervous system and is involved in the regulation of sympathetic activity to the cardiovascular system (22). In autonomic areas of the brain, such as PVN, RVLM and NTS, Ang II has shown as a contributor to sympathoexcitation and sympathoexcitatory reflexes, such as the cardiac sympathetic afferent reflex (CSAR), baroreflex and arterial chemoreflex in HF (1, 10, 54). Components of the angiotensin system including angiotensinogen, angiotensin-converting enzyme and AT1 receptors have been found to exist in the PVN (24, 46). Electrophysiological studies have demonstrated that AngII influences neurons in the PVN (3, 32) and is also involved in cardiovascular reflexes (59), which suggests that AngII within the PVN plays a role in regulating sympathetic nerve activity and cardiovascular function. Our recent study has shown that injection of AngII into the PVN significantly increased RSNA (30). AT1 receptors in the PVN have also been found to be involved in central mechanisms regulating cardiovascular function during hypertension and chronic HF (26, 58). Therefore, we hypothesize that altered AngII activity and AngII receptor expression in the PVN is involved in sympathetic dysfunction in HF. To test this hypothesis, we examined if 1) the endogenous AngII is partially responsible for the elevated RSNA in rats with HF, and 2) AT1 receptor mRNA and protein levels are increased in the PVN of rats with HF.
Methods

Animals

This study was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee, and conformed to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society. Male Sprague-Dawley rats, weighing 200-220g, were obtained from SASCO Breeding Laboratories (Omaha, NE) and were assigned to two groups (sham-operated and HF group).

HF was produced by left coronary artery ligation, as previously described (57). The degree of left ventricular dysfunction and HF was determined by using both hemodynamic and anatomic criteria. Left ventricular end-diastolic pressure (LVEDP) was measured by using a Mikro-Tip catheter (Millar Instruments, Houston, TX) at the time of the terminal experiment. To measure infarct size, the heart was dissected and the atria and right ventricle were removed. A digital image of the left ventricle was captured using a digital camera (Kodak, Rochester, NY). The percentage of infarct area to total left ventricle area was quantified using SigmaScan Pro (Aspire Software International, Ashburn, VA). Rats with both LVEDP > 15mmHg and infarct size > 30% of total left ventricular wall were considered to be in HF.

General Surgery for Hemodynamic, RSNA Measurement and Microinjection

On the day of the experiment (6–8 weeks after cardiac surgery), the rat was anesthetized with urethane (0.75g/kg, i.p.) and α-chloralose (70mg/kg, i.p.) and instrumented for recording
arterial pressure (AP) and heart rate (HR) as previously described (57). The rat was then placed in a stereotaxic apparatus, and a cannula connected to a microsyringe (0.5µl) was introduced into the PVN (1.5mm posterior, 0.4mm lateral to the bregma and 7.8mm ventral to the dura).

The left kidney was exposed through a left retroperitoneal flank incision, and a branch of the renal nerve was isolated from the adipose and connective tissues. The distal end of the nerve was ligated, and the nerve was placed on a bipolar platinum electrode. The nerve-electrode junction was insulated electrically from the surrounding tissues with Wacker gel (St Louis, MO). The electrical signal from the electrode was linked via a high impedance probe (H1P5) to a Grass P511 band-pass amplifier (gain, 10000) with high- and low-frequency cutoffs of 1,000 Hz and 100 Hz. The output from the Grass amplifier was directed to a Grass integrator, which rectifies the signal and integrates the raw nerve discharge. The output of the Grass integrator was displayed as an integrated voltage that is proportional to the renal nerve discharge. The average rectified signal [resistor-capacitor circuit (RC) filtered with a time constant of 0.5 s] was then recorded and stored for later analysis in a computer-based data-acquisition system (Mac-Lab).

Efferent RSNA at the beginning of the experiment was defined as basal nerve discharge. The RSNA recorded at the end of the experiment (after the rat was injected with hexamethonium, 30mg/kg, iv.) was defined as background noise. The value of RSNA was calculated by subtracting the background noise from the actual recorded value and changes found in integration of the nerve discharge during the experiment were expressed as a percentage from basal value. Responses of mean AP (MAP) and HR were expressed as the difference between the basal value and the value after each dose of a drug.
Experimental Protocols

In six sham-operated rats and six HF rats, three doses of AngII (0.05, 0.5 and 1nmol in 100nl) in a random order were injected into the PVN at intervals of 30min. The responses in RSNA, AP and HR over 30min were recorded after each dose of AngII. The vehicle control was performed using artificial cerebral spinal fluid (aCSF, 100nl, composition in mM: 132 NaCl, 3.0 KCl, 0.65 MgCl₂, 1.5 CaCl₂, 24.6 NaHCO₃, and 3.3 glucose, pH 7.4) microinjected into the PVN. To substantiate the concept that any responses of RSNA, AP and HR to AngII were not a peripheral action, intravenous injection of 0.5nmol (in 100nl) of AngII were examined (sham, n=5; HF, n=5).

In five sham-operated rats and five HF rats, the AT₁ receptor antagonist losartan was injected (50nmol in 100nl) into the PVN. The responses in RSNA, AP and HR over the 30min were recorded after each dose administration. Intravenous injection of 50nmol (in 100nl) of losartan were examined (sham, n=5; HF, n=5).

Brain Histology

For all of the microinjection placements in the PVN, at the end of the experiments, monastral blue dye (50nl) was injected into the brain for histological verification of the location. The brains were removed, frozen, sectioned, and processed for histology as described previously (57). The location of the center of dye spot was transferred to a histological map based on the rat atlas (45).
**Micropunch of the PVN**

In the other group of sham-operated and HF rats, after the rat was euthanized by pentobarbital (65mg/kg, i.p.), the brain was removed and frozen at -80°C. Six consecutive 100µm-thick coronal sections were cut with a cryostat (-18°C). The PVN and the supraoptic nucleus (SON) were punched bilaterally with a blunt needle (ID: 0.5mm) according to the method of Palkovits and Brownstein (39). The punched tissue was put in 0.5ml of TRI Reagent (MRC Inc, OH) or protein extraction buffer (1% sodium dodecyl sulfate, 0.1% Triton, 10% phenylmethylsulphonyl fluoride, 1mM EDTA, 10mM Tris). The total RNA and protein in the homogenate were extracted respectively.

**Semi-quantitative RT-PCR for the Measurement of AT₁ Receptor mRNA**

Total RNA extracted from the punched tissue was subjected to reverse transcription for 40min at 37°C in presence of 400U of MMLV reverse transcriptase (USB, OH). Each 1µl of cDNA was used as a template for PCR amplification. The following primers were used: for AT₁ receptors (GenBank accession: NM 030985), the sense primer was AGAGGATTCGTGGCTTGAG, the antisense primer was AGGGATCATGACAAATATG; for β-actin (GenBank accession: NM 031144), the sense primer CACGGCATTTGAACTAATG, the antisense primer was TCTCAGCTGTGGGTGTAAG. 25µl PCR reaction mixture contained 0.7µM forward and reverse primers and 1U of Taq polymerase (Invitrogen, CA). The amplification was performed with 30 thermal cycles of 94°C for 1min, 56°C for 1min and 72°C for 1min and a final extension at 72°C for 10min. Then PCR products were fractionated in a 1%
agarose gel and visualized by ethidium bromide staining and UV-transillumination. The visualized DNA bands were captured by a digital camera. Density analysis of DNA bands was performed with Kodak 1D image analysis software. Data were expressed as the ratio of AT₁ receptors and β-actin densities.

**Western Blotting**

The protein extraction from homogenates mentioned above was used for Western blotting analysis. The protein concentration was measured using a protein assay kit (Pierce, IL). Samples were adjusted to the same concentration of protein and treated at 95°C for 5 min before loading on the 7.5% SDS-PAGE gel (5μg protein/30μl per well) to be subjected to electrophoresis at 40mA/each gel for 60min. Then the fractionized proteins on the gel were electrophoretically transferred onto a PVDF membrane (Millipore, MA) at 300mA for 90min. The membrane was probed with primary antibody [rabbit polyclonal AT₁ receptor and AngII type II (AT₂) receptor antibody, 1:1000, Santa Cruz, CA; rabbit polyclonal β-tubulin antibody, 1:2000, Santa Cruz, CA] and secondary antibody (Peroxidase-conjugated goat anti rabbit IgG, 1:5000, Pierce, IL). AT₁ receptor primary antibody is rabbit polyclonal affinity purified antibody raised against a peptide mapping within an N-terminal extracellular domain of AT₁ of human origin. The signals were visualized using an enhanced chemiluminescence substrate (Pierce, IL) and detected by a digital image system (UVP Inc, CA). AT₁ receptor and AT₂ antibody protein level were normalized using β-tubulin. As a test for the specificity and sensitivity of the AT₁ receptor antibodies we used two different antibodies from two different companies, the two different antibodies were targeted at different ends of the protein, to show that they result in similar bands, the
antibody from SantaCruz for protein detection, and the antibody from Abcam for protein detection and immunostaining. This two antibodies target different ends of AT₁ receptor protein. SantaCruz AT₁ receptor primary antibody is raised against a peptide mapping within an N-terminal extracellular domain of AT₁ of human origin, and Abcam AT₁ receptor primary antibody is corresponding to C terminal amino acids of Human AT₁ receptor.

We also performed Western blots on different tissues including brain, kidney, heart and muscle, and found varying amounts of same molecular band in various tissues. We examined specific areas in the brain suspected of varying amounts of AT₁ receptors, and the results showed that the level of AT₁ receptor protein varies from tissue to tissue (incidentally we saw a 10 fold difference in protein levels in the SON compared to PVN) and close to undetectable levels in the optic tract tissue (negative control).

**Immunofluorescent Staining**

The rats were anesthetized with pentobarbital (65mg/kg) and perfused transcardially with 150ml of heparinized saline followed by 250ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer. The brain was removed and postfixed at 4°C for 4hrs in 4% paraformaldehyde solution and then placed in 20% sucrose. The brain was blocked in the coronal plane and sections 30µm in thickness were cut with a cryostat. The sections were incubated with 10% normal donkey serum in PBS for 1hr at room temperature and then incubated with primary antibody against AT₁ receptor (mouse monoclonal antibody, 1:200, Abcam, MA) overnight at 4°C. This antibody recognizes isoforms AT₁A and AT₁B of the AT₁ receptor in rat. After washing with PBS, the sections were incubated with Cy3-conjugated donkey anti-mouse secondary antibody (1:400, Jackson ImmunoResearch, PA) for 2hr at room temperature. The nuclei were
stained by Hoechst 33258 (Molecular Probes, CA). After washing with PBS and drying, the sections were cover-slipped with fluoromounting-G (SouthernBiotech, AL). Distribution of AT₁ receptor immunofluorescence within the PVN and SON was viewed using an Olympus fluorescence microscope (Japan) equipped with a digital camera (Qimaging, Canada). Openlab software 4.0.3. (Improvision Inc, MD) was used to identify the total intensity of positive staining with Cy3. Three alternate sections (1.80±0.1mm posterior to bregma) representing the PVN and the SON were analyzed in this way, and then the mean data was calculated.

**Statistical Analysis**

Data are presented as mean±SE. Differences between groups were determined by a 2-way ANOVA followed by the Newman–Keuls test for posthoc analysis of significance (Statview™ II, Abacus Inc, Berkeley, CA). P<0.05 was considered statistically significant.

**Results**

Table 1 and Table 2 summarize the salient morphological and hemodynamic characteristics of sham-operated and HF rats utilized in the present study. For all the experiments, any rats subjected to coronary artery ligation that displayed myocardial infarcts <30% of the left ventricular wall, were excluded from the study (7 out of 36 rats with coronary artery ligation surgery). Accordingly, the infarction area in the chronic HF group was approximately 43% of the left ventricle. Conversely, sham rats had no observable damage to the myocardium. Heart weight was significantly greater in HF rats than in sham rats (P<0.05), suggesting compensatory hypertrophy of non-infarct regions of the myocardium. LVEDP was
significantly elevated in HF rats compared to sham rats. Rats with LVEDP >15mmHg were chosen for the HF group, while sham rats did not exhibit an increased LVEDP.

The dynamic characteristics of basal MAP, HR and RSNA are also presented in Table 1. Although the level of raw RSNA in rats with HF trends to be higher than sham operated rats, it did not reach statistical significance. Similarly, there were no statistically significant differences in basal MAP or HR between the sham and HF groups.

Figure 1 illustrates the brain histological data. Among the 22 injection sites that were in the PVN, 12 injection sites belonged to microinjection of AngII, and 10 injection sites belonged to microinjection of losartan. 6 out of 28 injections missed PVN, 4 injections belonged to microinjection of AngII and 2 injections belonged to microinjection of losartan.

Effects of Microinjection of AngII and Losartan into the PVN on RSNA, MAP and HR in HF and Sham Rats

An example of the responses in RSNA, MAP, and HR to administration of AngII (1nmol) into the PVN in a sham-operated rat and a rat with HF is illustrated in Figure 2A. Microinjections of 0.05, 0.5, and 1nmol of AngII elicited increases in RSNA, MAP, and HR, reaching 33±4%, 7±2mmHg and 18±6 beats/min, respectively, at the highest dose in sham-operated rats. The RSNA and HR responses were significantly elevated in the HF rats compared to the sham-operated rats, reaching 64±8% and 37±8 beats/min, respectively, at the highest dose (P<0.05) (Figure 2B).

The vehicle control, 100nl of aCSF microinjected into the PVN, had no effects on RSNA, MAP and HR both in HF or sham rats (Figure 2B). Intravenous injection of 0.5nmol of AngII
had also no effect on these parameters (Figure 2B). Similarly, microinjection of AngII outside
the PVN did not change RSNA, MAP, or HR (data is not shown).

An example of the responses in RSNA, MAP, and HR to administration of losartan
(50nmol) into the PVN in a sham-operated rat and a rat with HF is illustrated in Figure 3A. Microinjection of losartan into the PVN decreased RSNA, MAP and HR in both sham and HF
rats. The responses of RSNA and HR to losartan in HF rats were significantly greater than the
responses of sham rats (RSNA: -25±4% versus -13±1%; HR: -21±3 beats/min versus -10±1
beats/min, P<0.05). Figure 3B shows the mean data of RSNA, MAP and HR responses to
losartan microinjection in the PVN. Intravenous injection of 50nmol of losartan had also no
effect on these parameters (Figure 3B). Microinjection of losartan outside the PVN did not
change RSNA, MAP, or HR (data is not shown).

Measurements of AT1 Receptor Expression in the PVN

Results of RT-PCR experiments indicated that AT1 receptor mRNA expression in the
punched PVN tissues from the HF rats was significantly increased compared with sham rats
(P<0.05) (Figure 4). Western blotting showed that AT1 receptor protein levels were also
significantly higher in HF rats compared with sham rats, which was consistent with the previous
results. (P<0.05) (Figure 5A).

In contrast to the PVN, however, PCR and western blotting results did not show
significant changes in AT1 receptor mRNA (Figure 4) and protein level (Figure 5B) in the SON,
another important hypothalamic area in control of cardiovascular and body fluid homeostasis
(52), in HF rats compared with sham rats. This suggests that the change in AT1 receptors within
the PVN in HF is region-specific. Moreover, western blotting results did not show a significant change in AT_2 receptor protein level within the PVN in HF rats compared with sham rats (Figure 6). The AT_1 receptor/ AT_2 receptor ratio within the PVN is significantly higher in HF rats compared to the sham rats (0.87±0.18 versus 0.55±0.09, P<0.05).

As an in situ confirmation of the alteration in AT_1 receptors within the PVN, the immunofluorescence for AT_1 receptors was significantly increased in the PVN from rats with HF compared with sham rats (Figure 7A, 7B). The immunofluorescence for AT_1 receptors within the SON (Figure 7C) was not altered from rats with HF compared with sham rats. Figure 7D shows the quantification data of immunofluorescence signal in the PVN and the SON.

**Discussion**

In the present study, we observed that, in rats with HF, the responses of RSNA and HR to microinjection of AngII into the PVN were augmented, accompanied by up-regulated AngII AT_1 receptor message and protein in the PVN area. There is a significant difference in the response to AT_1 receptor antagonist losartan in the PVN in HF, namely the fact that RSNA and HR responses to losartan in HF rats were significantly greater than the responses observed in sham rats. These results suggest that enhanced AT_1 receptor-mediated angiotensin action in the PVN on sympathetic outflow may contribute to sympathetic dysfunction in HF. Although there was a tendency towards a change in responses of arterial pressure to AngII, particularly at the high doses they were not statistically significant. The changes in arterial pressure are dependent on a number of factors including RSNA mediated reno-vasoconstriction. Activation of PVN produces a differential change in different sympathetic outflows (21) and that may account in part for the
lack of changes in blood pressure responses in this study. In order to test if RSNA burst frequency increased by increases in HR and reduced by falls in HR, we have conducted further experiments (unpublished data) to prevent the change in HR and then monitored the changes in RSNA. After using β-receptor blocker metoprolol to prevent the changes of HR, we still saw the effects of Ang II in the PVN on the RSNA. Therefore, we think the effects of Ang II in the PVN are due to the direct effect on RSNA, not due to the effects from HR.

Heart failure is characterized by elevated systemic sympathetic activity and salt and water retention (34). Previously, work from this laboratory has shown that inhibitory mechanisms of sympathetic regulation within the PVN via nitric oxide (NO) (56) and GABA (57) were reduced, while the excitatory mechanism regulated by NMDA NR1 receptors was enhanced (28) in HF rats. These alterations may induce an imbalance of the inhibitory and excitatory mechanisms in this area and influence sympathetic outflow. In the present study we concentrated on the excitatory mechanism of renal sympathetic nerve regulation within the PVN. As a major excitatory neurotransmitter, AngII has been found to regulate sympathetic nerve activity in several brain areas, including the hypothalamus. Using whole-cell recording from hypothalamic slice preparations obtained from the rat, it was observed that AngII induced depolarization of type II neurons in the PVN (pPVN) (3). AT1 receptor is the major AngII receptor mediating the excitatory signal of AngII in the PVN (25). We observed that stimulation of AngII receptors induced a sympathetic excitation, further confirming that AT1 receptors in the PVN mediate sympathetic outflow. Dibona et al also found in another brain area, angiotensin system influenced basal RSNA and arterial baroreflex regulation. Microinjection of AT1 receptor antagonists into the RVLM of anesthetized rats significantly improved the arterial baroreflex regulation of RSNA (6).
Although the angiotensin system does not appear to contribute significantly to the generation of resting tonic activity in neurons, it is shown to contribute significantly to the tonic activity of the neurons under certain conditions. These conditions include salt deprivation or spontaneous hypertension in which the endogenous levels of AngII or AT1 receptors are up-regulated (4). In the present study, after microinjection of AT1 receptor antagonist losartan into the PVN, the responses of RSNA and HR in HF rats were significantly changed as compared to sham rats. This result supports the hypothesis that enhanced endogenous AngII effects in the PVN contribute to the elevated sympathoexcitation in HF. The greater reduction of RSNA by losartan in the HF group suggests that there was increased angiotensinergic tone in the PVN stimulating RSNA. However, our basal measurements of RSNA did not show the significant increase in HF. It should be noted that comparison of basal RSNA levels between animals is wrought with a number of concerns such as variability in number of fibers recorded from the nerve, the precision of nerve dissection from the surrounding tissue, the amount of fat surrounding the nerve, the proximity of the nerve segment to the electrode hooks, and nerve damage during handling and dissection(5). It is also possible that there are counter-regulatory mechanisms including glutamatergic and GABA mechanisms that are offsetting the effect of the sensitized angiotensinergic mechanisms in the PVN.

In the present study, we did not measure the circulating AngII levels. In the chronic HF state, plasma Ang II levels are found to be increased (33, 60). Although AngII does not cross the blood brain barrier, circulating levels of AngII may effect the central nervous system including the PVN through some of the circumventricular organs (CVOs), such as the subfornical organ and the organum vasculosum of the lamina terminalis (OVLT). The neurons in the CVOs have projections to the PVN. We think through this pathway, the circulating AngII could effect the
local Ang II levels in the central nervous system (36). Many studies also show AT$_1$ receptor up-regulation under the condition of higher level of circulating Ang II (10, 40, 55). Our results of AT$_1$ receptor up-regulation are consistent with these previous observations in the PVN. However, one observation indicated that AT$_1$ receptor protein level, not mRNA, increased in the PVN in rats with HF (17). Our data shows both AT$_1$ receptor mRNA and protein are up-regulated in the PVN, consistent with the observation that losartan can block the enhanced Ang II activity through the effects on the AT$_1$ receptors.

As an in situ confirmation of the alteration in AT$_1$ receptors within the PVN, the results of immunofluorescence for AT$_1$ receptors showed a significant increase in the PVN from rats with HF compared with sham rats. According to Pan et al.’s study, the immunoreactivity of AT$_1$ receptors was colocalized with a presynaptic marker, synaptophysin, in the PVN (25). However, according to a study by Oldfield and colleagues, AT$_1$ receptor immunostaining appears to occur in hypophysiotrophic neurons in the PVN that subserve anterior pituitary function but not in preautonomic cells (35). Further confirmation is needed regarding the immunoreactivity of AT$_1$ receptors in the PVN.

Changes in excitatory synaptic functions can occur by presynaptic mechanisms such as altered neurotransmitter release and/or postsynaptic mechanisms. We observed that AT$_1$ receptors were up-regulated in the PVN in HF rats, which is associated with the enhanced AngII receptor function. This may be one of the central mechanisms underlying the elevated sympatoexcitation in HF, as the up-regulation of AT$_1$ receptors has been observed in other studies in response to different challenges. For example, AT$_1$ receptors have been shown to be involved in the integration of neuroendocrine function in the forebrain region. AT$_1$ receptor antagonists inhibited osmotic-dependent vasopressin release from hypothalamic tissues. In the
intact animal, intracerebroventricular application of losartan induced a significant reduction in drinking and plasma vasopressin level in dehydrated rats (1, 16). These observations suggest that alteration of the AT$_1$ receptors in the hypothalamus may play a role in adaptation of the body to different homeostatic challenges. The significantly higher gene expression of AT$_1$ receptors in the PVN also has been observed in some other disease states, such as spontaneous hypertension (47). The spontaneously hypertensive rat are found to have significantly higher numbers of AT$_1$ receptor subtype. Central angiotensinergic pathways may use AT$_1$ receptors and play a role in the function of sympathetic pathways maintaining arterial pressure.

The AT$_2$ receptor is another important mediator for the action of AngII. Both AT$_1$ and AT$_2$ receptor subtypes in the PVN are involved in AngII-related sympathetic activity. AT$_1$ receptors exert excitatory responses to administered AngII into the PVN, while AT$_2$ receptors exert inhibitory effects (2). The net effect of AngII therefore may depend on the cellular AT$_1$/AT$_2$ receptor ratio (48). The functions and intracellular signaling pathways of the AT$_2$ receptor in most peripheral tissues and organs are opposite to that of the AT$_1$ receptor. For example, stimulating AT$_2$ receptor induces vasodilation, stimulates NO production, and inhibits reactive oxygen species generation (19). Recently, Gao L et al found that AT$_1$ receptor protein expression in the RVLM of CHF rats was up-regulated, while AT$_2$ receptor was significantly down-regulated and therefore greatly increased the protein expression of AT$_1$/AT$_2$ receptor ratio in HF (11). These data led them to postulate that the balance between AT$_1$ receptor and AT$_2$ receptor may be critical to maintain sympathetic tone in normal conditions and that the imbalance of AT$_1$ receptor and AT$_2$ receptor may contribute to the sympathoexcitation in the HF state. In our study, we did not observe a significant change in the expression of AT$_2$ receptors within the PVN in HF rats compared with sham rats. There was increased AT$_1$/AT$_2$ receptor ratio
in our study as well that could account for the enhanced excitatory effects of AngII receptor in the HF state.

The cause(s) of up-regulation of AT₁ receptors within the PVN in HF remain to be examined. In the HF state, the cardiac sympathetic afferents and chemosensitive afferents are sensitized and result in an increased sympathetic drive (9, 31). The increased afferent input to the autonomic centers including the PVN may be part of the reason for the up-regulation of AT₁ receptors within the PVN in HF (54, 59). Also many central and peripheral humoral factors such as aldosterone and TNF-α are significantly altered in HF (8, 12). All of these alterations may induce the compensatory responses in the cardiovascular and autonomic centers. Further studies are also required to determine the cellular mechanisms of action of AngII in the PVN and its interactions with other neurotransmitters in that region. For example, we have observed that AngII reduces the level of neuronal NO synthase (nNOS) in cultured NG108 neuronal cells (29). This would indicate that the enhanced AngII action which produces excitatory effects can also exaggerate this effect by reducing the inhibitory effects of NO.

In conclusion, the results of the present study indicate that altered AngII system within the PVN due to the up-regulation of the AT₁ receptors in the PVN may contribute to the elevated sympathetic nerve activity in the HF state. Because sympathoexcitation contributes to the deterioration of the HF state, a comprehensive understanding of the sympathoexcitation will help to uncover new therapies for the sympathetic dysfunction in HF.

**Perspective and Significant**

In the PVN, there are multiple other excitatory neurotransmitters/substances that are involved in the sympathetic outflow and cardiovascular function, including AngII,
norepinephrine, serotonin, dopamine, cytokines and reactive oxygen species (8, 12). Increased AngII action in diseases such as hypertension and HF may contribute to increased sympathetic outflow. This study shows that altered AngII system within the PVN due to the up-regulation of the AT\(_1\) receptors may contribute to the elevated sympathetic nerve activity in the HF state. Similar alterations in the central AngII system may contribute to sympathoexcitation in various other disease states such as hypertension.

References


Figure Legends

Fig. 1. A-C: Schematic representations of serial sections from the rostral (-1.4) to the caudal (-2.1) extent of the region of the PVN. The distance (in mm) posterior to bregma is shown for each section. Each filled circle represents the site of termination of an injection that is considered to be within the PVN region in the AngII injection experiments; "+" represents that in losartan injection experiments. D: Histological photo showing the injection site (arrow) in the PVN of one rat. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus.

Fig. 2. A: Segments of original recordings from an individual sham-operated rat and a rat with HF, demonstrating the representative response to HR, MAP, integrated RSNA (int. RSNA) and RSNA to the microinjections of AngII into the PVN. B: The mean data of changes in HR, MAP and RSNA after microinjections of AngII and aCSF into the PVN and venous in sham and HF rats. *: P<0.05 versus group of sham.

Fig. 3. A. Segments of original recordings from an individual sham-operated rat and a rat with HF, demonstrating the representative response to HR, MAP, int. RSNA and RSNA to the microinjections of losartan into the PVN. B. The mean data of changes in RSNA, MAP and HR after microinjections of losartan into the PVN and venous in sham and HF rats. *: P<0.05 versus group of sham.
Fig. 4. A: Gene expression of AT$_1$ receptors in the PVN and SON tissues measured. Top: Example of visualized bands of AT$_1$ receptors and $\beta$-actin of sham and HF rats. Bottom: Mean data of band densities of AT$_1$ receptors normalized by $\beta$-actin in sham and HF.

Fig. 5. Protein level of AT$_1$ receptors in the PVN (A) and SON (B) tissues. Top, example of visualized bands of AT$_1$ receptors and $\beta$-tubulin. Bottom: Mean data of band densities of AT$_1$ receptors normalized by $\beta$-tubulin in sham and HF. *: $P<0.05$ versus sham group

Fig 6. Protein level of AT$_2$ receptors in the PVN tissues. Top, example of visualized bands of AT$_2$ receptors and $\beta$-tubulin. Bottom: Mean data of band densities of AT$_2$ receptors normalized by $\beta$-tubulin in sham and HF.

Fig. 7. Immunofluorescent photomicrographs from the sections of the PVN (A, B) and SON (C) region stained for AT$_1$ receptors with/without AT$_1$ receptors blocking peptide. The intensity of AT$_1$ receptor staining (red) is increased within the PVN in HF rats compared with sham rats. Blue spot shows the nucleus stained by Hoechst 33258. 3V: third ventricle; OX: optic tract. D: the quantification data of immunofluorescence signal in the PVN and the SON. *: $P<0.05$ versus sham group.
### Table 1: Baseline values of morphology and hemodynamics in rats with heart failure and sham-operated rats in the microinjection experiments

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=11)</td>
<td>(n=11)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>365±17</td>
<td>360±20</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>1138±64</td>
<td>1521±88*</td>
</tr>
<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>3.1±0.3</td>
<td>4.2±0.4*</td>
</tr>
<tr>
<td>Infarct size (% of LV)</td>
<td>0</td>
<td>44.5±5.5*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2±2</td>
<td>21±4*</td>
</tr>
<tr>
<td>Basal mean arterial pressure (mmHg)</td>
<td>91±9</td>
<td>87±8</td>
</tr>
<tr>
<td>Basal heart rate (beats/min)</td>
<td>334±31</td>
<td>368±45</td>
</tr>
<tr>
<td>Basal integrated RSNA ((µV.s)</td>
<td>44.2±8.8</td>
<td>51.3±11.2</td>
</tr>
</tbody>
</table>

Values are presented as mean±SE; * indicates P<0.05 vs. sham rats. LV: left ventricle; LVEDP: left ventricular end-diastolic pressure; RSNA: renal sympathetic nerve activity.
**Table 2: Characteristics of sham and heart failing rats in the molecular biological experiments**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>378±26</td>
<td>393±24</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>1159±78</td>
<td>1614±87*</td>
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<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>3.1±0.4</td>
<td>4.1±0.3*</td>
</tr>
<tr>
<td>Infarct size (% of LV)</td>
<td>0</td>
<td>40.2±6.8*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>3±1</td>
<td>26±7*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SE; * indicates P<0.05 vs. sham rats. LV: left ventricle; LVEDP: left ventricular end-diastolic pressure.
Figure 1
Figure 2
Figure 2
Figure 3
Figure 3
Figure 4
Figure 5

A.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
<th>Sham</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R 43KD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin 55KD</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

![Bar graph showing ratio of intensity for AT1R in Sham and HF groups.](image)

Sham (n=5) vs. HF (n=5)
Figure 5
Figure 6
Figure 7
Figure 7