TNF-α in Hypothalamic Paraventricular Nucleus Contributes to Sympathoexcitation in Heart Failure by Modulating AT1 Receptor and Neurotransmitters

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Proinflammatory cytokines, including tumor necrosis factor (TNF)-α, augment the progression of heart failure (HF) that is characterized by sympatheoexcitation. In this study, we explored the role of TNF-α in hypothalamic paraventricular nucleus (PVN) in the exaggerated sympathetic activity observed in HF. Heart failure rats were made by ligating the left anterior descending coronary artery. The expression levels of angiotensin type 1 receptor (AT1-R) and neurotransmitters were analyzed in the PVN of HF rats that received direct PVN infusion of a TNF-α blocker (pentoxifylline or etanercept) or vehicle. Sham-operated control (SHAM) or HF rats were treated for 4 weeks through PVN infusion with each TNF-α blocker or vehicle. Rats with HF had higher levels of glutamate, norepinephrine, AT1-R and tyrosine hydroxylase (TH), and lower levels of gamma-aminobutyric acid (GABA), neuronal nitric oxide synthase (nNOS) and the 67-kDa isoform of glutamate decarboxylase (GAD67) in the PVN when compared to SHAM rats. Plasma levels of cytokines, norepinephrine and angiotensin II and renal sympathetic nerve activity (RSNA) were increased in HF rats. PVN infusion of pentoxifylline or etanercept attenuated the decreases in PVN GABA, nNOS and GAD67, and the increases in RSNA and PVN glutamate, norepinephrine, TH and AT1-R observed in HF rats. We have developed a novel method for chronic and continuous infusion of drugs directly into the PVN and provided evidence that TNF-α in the PVN modulates neurotransmitters and the expression of AT1 receptor, which could account for exaggerated sympathetic activity in HF.

Keywords: tumor necrosis factor-alpha; hypothalamic paraventricular nucleus; neurotransmitters; angiotensin type 1 receptor; heart failure

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Increased sympathetic activity is a pathophysiological characteristic of chronic heart failure (HF) and a major contributor to morbidity and mortality in HF patients (Felder et al. 2003). Recent studies indicate that central nervous system (CNS) mechanisms contribute to the sympathetic abnormalities seen in HF (Felder et al. 2003, 2009). In the brain, the paraventricular nucleus (PVN) of the hypothalamus is reciprocally connected with other CNS areas involved in cardiovascular functions (Swanson and Sawchenko 1983). The PVN is, therefore, an important central site for integration of sympathetic nerve activity (Swanson and Sawchenko 1980) and regulation of cardiovascular functions (Boudaba et al. 1996).

Several neurotransmitters in the PVN influence its neuronal activity (Swanson and Sawchenko 1980). Norepinephrine (NE) and glutamate are well-known excitatory neurotransmitters in the central nervous system. Increased extracellular NE in the PVN is involved in the sympathetic hyperactivity in HF rats (Basu et al. 1996; Arabia et al. 2002). Functional glutamate receptors
expressed in the PVN (Hermes et al. 1996; Tasker et al. 1998) are involved in cardiovascular reflex control (Antonaccio et al. 1978; Brennan et al. 1983). Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter within the PVN (Cole and Sawchenko 2002; Chen et al. 2003). Patel et al. previously suggested that a GABA-mediated inhibitory mechanism within the PVN contributed to sympathoexcitation in HF rats (Zhang, Li et al. 2002). In addition, nitric oxide (NO) levels are inversely related to sympathetic activity and are downregulated in the PVN of HF rats. Overexpression of PVN neuronal nitric oxide synthase (nNOS) in HF rats attenuates sympathetic activity (Zhang and Patel 1998), suggesting that NO within the PVN acts as an inhibitory neuromodulator.

Recent studies suggest that proinflammatory cytokines are upregulated in the PVN, thereby leading to sympathoexcitation in HF (Kang et al. 2008a, 2008b). TNF-α, IL-1β, and IL-6 can all activate the hypothalamic-pituitary-adrenal (HPA) axis (Turnbull and Rivier 1999; Dunn 2000) and contribute to increased sympathetic nerve activity (Zhang et al. 2003). However, the mechanisms by which proinflammatory cytokines modulate sympathetic activity in HF are yet unclear. Therefore, we hypothesized that increased proinflammatory cytokines in the PVN of HF rats would cause an imbalance in the excitatory and inhibitory neurotransmitters in the PVN, thereby contributing to increased sympathoexcitation in HF. To test this hypothesis, we inhibited TNF-α in the PVN with infusion of TNF-α blockers directly into the PVN, and then measured neurotransmitters (glutamate, NE and GABA) and neuronal nitric oxide synthase in the PVN of rats with ischemia-induced HF and in sham-operated controls.

A growing body of evidence indicates that components of the RAS are increased both centrally and peripherally in cardiovascular disease and contribute to its pathophysiology. Recent studies also suggest that proinflammatory cytokines and the renin-angiotensin system (RAS) can contribute to these changes (Kang et al. 2008a, 2008b, 2009a, 2009b). Our laboratory and others have reported that cytokine blockade in the brain decreases components of the RAS (Kang et al. 2008b, Guggilam et al. 2008) and that RAS blockade attenuates production of proinflammatory cytokines (Kang et al. 2008a, 2009b). We inhibited cytokine synthesis in the brain with centrally infused pentoxifylline (PTX) and found that lowering hypothalamic levels of TNF-α and IL-1β without changing plasma cytokine levels is associated with reductions in brain renin, ACE, and AT1-R, strongly suggests that proinflammatory cytokines are intrinsically involved in regulating brain RAS, and thus sympathetic nerve activity and extracellular fluid volume, in rats with HF. Animals were intracerebroventricularly (ICV) treated with the angiotensin type 1 receptor (AT1-R) antagonist losartan and we found that plasma levels of NE, angiotensin II and proinflammatory cytokines were reduced (Guggilam et al. 2008; Kang et al. 2008a, 2008b, 2009a).

However, it is unknown whether proinflammatory cytokines in the PVN alter angiotensin type 1 receptor (AT1-R) within the PVN to modulate neurotransmitters and contribute to sympathoexcitation in HF. Therefore, we hypothesized that increased PVN proinflammatory cytokines in HF rats would cause an imbalance between excitatory and inhibitory neurotransmitters within the PVN, thereby contributing to the increased sympathoexcitation in HF. To test this hypothesis, we inhibited TNF-α in the PVN with infusion of TNF-α blockers directly into the PVN, and then measured AT1-R and nNOS expression, along with renal sympathetic nerve activity (RSNA) and neurotransmitter concentrations (glutamate, NE, and GABA), in the PVN of HF or SHAM rats, in order to further delineate the interaction between proinflammatory cytokines and the increased sympathetic activity observed during heart failure.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (275-300 g) obtained from Harlan (Indianapolis, IN) were used for all experiments. Rats were housed in temperature- (23 ± 2°C) and light-controlled (12 h light/dark cycle), animal quarters and were provided rat chow and tap water ad libitum. The Institutional Animal Care and Use Committees of Xi’an Jiaotong University, Louisiana State University and Shanxi Medical University approved all protocols. This investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

General experimental protocol

Rats underwent implantation of bilateral PVN cannulae and were allowed a week for recovery. Coronary artery ligation was then performed, and ischemia confirmed using echocardiography. Subsequently, osmotic minipumps were implanted subcutaneously and connected to the cannulae for the continuous infusion of TNF-α blockers or vehicle (artificial cerebrospinal fluid, aCSF) directly into the PVN. Four sets of animals were used in this study: the first set was used for tissue neurotransmitter measurements (n = 5-6 rats/group), the second set was used for Western blot (n = 6 rats/group), the third set was used for mRNA measurement (n = 5-6 rats/group) and the fourth set for immunohistochemistry (n = 5-6 rats/group). At the conclusion of the study, conscious renal sympathetic activity measurements were obtained in animals used for Western blot.

Implantation of bilateral PVN cannulae for chronic infusion studies

The method for PVN cannulation has been previously described (Francis et al. 2000). Each rat was anesthetized (ketamine + xylazine, ip), and the head placed into a stereotaxic apparatus. A skin incision was made, the skull opened and the dura carefully dissected parallel to the sinus vein. A stainless steel double cannula (Plastics One Inc, Roanoke, Virginia) with a center-to-center distance of 0.5 mm, was implanted into the PVN using an introducer, according to stereotoxic coordinates (1.8 mm posterior to the bregma and 8.5 mm ventral from the skull surface) (Paxinos and Watson 1987). The cannula was fixed to the cranium using dental acrylic and two stainless steel screws. Animals received buprenorphine (0.01 mg/kg, sc) immediately following surgery and 12 h post-operation. The success rate of bilat-
eral PVN cannulation is 65%. At the completion of the study, brains were sectioned to verify location of cannulae, and only animals with verifiable bilateral PVN injection sites were used in the final analysis.

**Coronary ligation**

Rats underwent sterile surgery under anesthesia (ketamine + xylazine, ip) for induction of HF by ligation of the left anterior descending coronary artery, or the same surgery without vessel ligation (SHAM), as previously described (Kang et al. 2006, 2008a, 2008b, 2009a; Guggilam et al. 2008).

**Echocardiographic assessment of left ventricular function**

Echocardiography was performed under ketamine sedation to assess left ventricular (LV) function as previously described (Kang et al. 2006, 2008a, 2008b). Ischemic zone (IZ) was estimated by planimetry of the region of the LV endocardial silhouette which demonstrated akinesis or dyskinesis, and expressed as a percentage of the whole (%IZ). From these measurements, %IZ, LV ejection fraction (LVEF), and LV end-diastolic volume (LVEDV), indices of the severity of congestive heart failure, were reported.

**Drug infusion**

Within 24 h of coronary ligation or sham operation, anesthetized (ketamine + xylazine, ip) rats underwent subcutaneous implantation of osmotic minipumps (Alzet, DURECT Corporation, Cupertino, California). Minipumps were connected to the bilateral PVN cannulae for continuous infusion of PTX, which inhibits the production of TNF-α, at a total dose of 10 or 30 μg/h; ETN, which binds with circulating TNF-α and prevents it from binding to its receptor, at a total dose of 2.5 or 7.5 μg/h; or aCSF over a 4-week period. The doses used in this study were determined from preliminary studies based on a previous report from our laboratory (Kang et al. 2009a). The locations of the microinjection sites in the PVN areas are shown in Fig. 6. Microinjection sites were localised within the visual boundaries of PVN. There was no correlation between the responses and the placement of the cannula in the sub-regions of the PVN.

**Tissue microdissection**

Palkovits’s microdissection procedure was used to isolate the PVN, as previously described (MohanKumar et al. 1998; Kang et al. 2009a). The tissues were collected from both sides of the PVN of individual rat used.

**Measurement of PVN tissue levels of glutamate, GABA and NE, and of plasma NE**

Tissue concentrations of glutamate and GABA were measured using HPLC with electrochemical detection (EC/300, Eicom Corporation, Cupertino, California). Tissue NE concentration was measured using HPLC with electrochemical detection (HT/500, Eicom Corporation, Japan) as previously described (Barber et al. 2003; Yang et al. 2008; Kang et al. 2009a). Plasma NE was measured using HPLC as previously described (Guggilam et al. 2007, 2008; Siramula et al. 2008; Kang et al. 2009b).

**Western blot**

Measurement of PVN protein was performed as previously described (Guggilam et al. 2008). Briefly, protein extracted from the PVN was used for measurements of tyrosine hydroxylase (TH; Abcam Inc, Cambridge, Massachussetts), the 67-kDa isoform of glutamate decarboxylase (GAD67; Abcam Inc), AT1-R (Abcam Inc) and nNOS (Santa Cruz Biotechnology Inc, Santa Cruz, California) expression by western blot. Protein loading was controlled by probing all blots with β-actin antibody (Santa Cruz Biotechnology Inc) and normalizing their protein intensities to that of β-actin. The bands were analyzed using NIH Image J software.

**Immunohistochemistry**

Coronal sections from brains were obtained from the region approximately 1.80 mm from the bregma. Immunohistochemical labeling was performed in floating sections as previously described (Kang et al. 2006, 2009a) to identify Fra-like (Fra-LI, a marker of chronic neuronal activation; Santa Cruz Biotechnology Inc) and AT1-R (Abcam Inc) expression. For each animal, the positive neurons within the bilateral borders of the PVN were manually counted in three consecutive sections and an average value was reported.

**ELISA studies**

Plasma and tissue cytokine levels were measured using ELISA (Invitrogen Corporation, Carlsbad, California) techniques, as previously described (Kang et al. 2008a, 2008b, 2009b). Plasma ANGII was measured using an EIA kit (Cayman Chemical Company, Ann Arbor, Michigan) as previously described (Kang et al. 2008a).

**Electrophysiological recordings and anatomical measurements**

Arterial pressure (AP), heart rate (HR) and renal sympathetic nerve activity (RSNA) were recorded. The general methods for recording and data analysis have been described previously (Kang et al. 2008a, 2009a). Maximum RSNA was detected using an intravenous bolus administration of sodium nitroprusside (SNP, 10 μg) (Nagura et al. 2004; Kang et al. 2009a). At the end of the experiment, the background noise, defined as the signal-recorded postmortem, was subtracted from actual RSNA and subsequently expressed as percent of maximum (in response to SNP) (Liu et al. 2000; Guggilam et al. 2007). The left ventricular end-diastolic pressure (LVEDP), the right ventricle (RV)/body weight (BW) ratio and lung/BW ratio were measured as previously described (Kang et al. 2008a, 2009a).

**Statistical analysis**

All data are expressed as mean ± SEM. Data were analyzed by two-way ANOVA. Multiple testing was corrected for by using Tukey’s test post hoc. Echocardiography data were analyzed with repeated measures ANOVA. A p-value of 0.05 was considered significant.

**Results**

**Echocardiography**

Echocardiography performed within 24 hours of coronary artery ligation revealed that HF rats had a lower LVEF, a higher LVEDV, and a higher LVEDV/M ratio than SHAM rats (Table 1). The infarct sizes in this study ranged from 40%-50% of the LV. The %IZ, LVEF, LVEDV, and...
LVEDV/M ratio were matched among rats assigned to vehicle versus drug treatment. At 4 weeks, LVEDV and LVEDV/mass ratio were significantly higher than the 24 h-baseline values in the PTX-, ETN- and aCSF-treated HF rats, and LVEF was significantly lower in the aCSF-treated HF rats (Table 1). At 4 weeks, LVEF was higher in the high dose of PTX- or ETN-treated HF rats when compared with aCSF-treated HF rats. However, there were no significant differences in LVEDV, LVEDV/mass ratio or %IZ among PTX, ETN and aCSF-treated HF rats at 4-weeks (Table 1). These results suggest that bilateral PVN infusion of the high dose of PTX or ETN improved LVEF, but did not affect LVEDV, LVEDV/mass ratio and %IZ of HF rats.

**PVN Neurotransmitters**

Rats with HF had higher levels of NE and glutamate, and lower levels of GABA in the PVN. Four-week bilateral infusions of low doses of PTX or ETN into the PVN attenuated, and high doses of PTX or ETN prevented, the decrease in PVN GABA and the increases in PVN glutamate and NE in HF rats (Fig. 1). In animals where only one side of the PVN was cannulated, infusions of high doses of PTX or ETN improved LVEF, but did not affect LVEDV, LVEDV/mass ratio and %IZ of HF rats.

**TH, AT1-R and GAD67 protein expression in the PVN**

Western blot analysis showed that HF rats had higher TH and AT1-R levels, and lower GAD67 levels, in the PVN, when compared with SHAM rats (Fig. 2). Bilateral PVN infusion of high doses of PTX or ETN for 4 weeks prevented the decrease in PVN GAD67, and the increases in TH and AT1-R in the PVN of HF rats (Fig. 2). These results suggest that TNF-α in the PVN alters AT1-R and neurotransmitter related rate-limiting enzymes within the PVN, thereby contributing to the exaggerated sympathetic activity in HF.

**nNOS in the PVN**

The protein (Fig. 2) and mRNA (Fig. 3A) expression of nNOS in the PVN was reduced in HF rats as compared to SHAM rats. Bilateral PVN infusions of high doses of PTX

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**Table 1. Echocardiographic measurements (n = 20 rats/group).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVEDV (ml)</th>
<th>LVEDV/Mass</th>
<th>LVEF</th>
<th>IZ (%)</th>
<th>LVEDV (ml)</th>
<th>LVEDV/Mass</th>
<th>LVEF</th>
<th>IZ (%)</th>
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<td><strong>HF</strong></td>
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<tr>
<td>bilateral PVN infusion of 10 μg/h PTX</td>
<td>0.76 ± 0.09*</td>
<td>1.03 ± 0.08*</td>
<td>0.36 ± 0.05*</td>
<td>46.3 ± 4*</td>
<td>1.12 ± 0.09*</td>
<td>1.43 ± 0.12±</td>
<td>0.40 ± 0.05*</td>
<td>45.6 ± 5*</td>
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<td>bilateral PVN infusion of 2.5 μg/h ETN</td>
<td>0.74 ± 0.08*</td>
<td>1.08 ± 0.09*</td>
<td>0.38 ± 0.05*</td>
<td>46.5 ± 4*</td>
<td>1.14 ± 0.10±</td>
<td>1.45 ± 0.13±</td>
<td>0.38 ± 0.04*</td>
<td>46.0 ± 5*</td>
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<tr>
<td>bilateral PVN infusion of 30 μg/h PTX</td>
<td>0.78 ± 0.09*</td>
<td>1.06 ± 0.08*</td>
<td>0.37 ± 0.04*</td>
<td>46.9 ± 5*</td>
<td>1.04 ± 0.08±</td>
<td>1.29 ± 0.12±</td>
<td>0.51 ± 0.06±</td>
<td>43.7 ± 5*</td>
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<td>bilateral PVN infusion of 7.5 μg/h ETN</td>
<td>0.77 ± 0.09*</td>
<td>1.05 ± 0.07*</td>
<td>0.39 ± 0.05*</td>
<td>47.2 ± 5*</td>
<td>1.07 ± 0.09±</td>
<td>1.33 ± 0.13±</td>
<td>0.49 ± 0.05±</td>
<td>44.9 ± 5*</td>
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<td><strong>SHAM</strong></td>
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<td>bilateral PVN infusion of 10 μg/h PTX</td>
<td>0.36 ± 0.05</td>
<td>0.58 ± 0.06</td>
<td>0.82 ± 0.07</td>
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<td>0.33 ± 0.05</td>
<td>0.54 ± 0.07</td>
<td>0.83 ± 0.06</td>
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<tr>
<td>bilateral PVN infusion of 2.5 μg/h ETN</td>
<td>0.35 ± 0.04</td>
<td>0.57 ± 0.06</td>
<td>0.83 ± 0.08</td>
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<td>0.34 ± 0.04</td>
<td>0.53 ± 0.07</td>
<td>0.84 ± 0.07</td>
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<tr>
<td>bilateral PVN infusion of 30 μg/h PTX</td>
<td>0.36 ± 0.04</td>
<td>0.54 ± 0.07</td>
<td>0.83 ± 0.06</td>
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<td>0.32 ± 0.04</td>
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<td><strong>SHAM</strong></td>
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<tr>
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<td>0.57 ± 0.07</td>
<td>0.81 ± 0.07</td>
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<td>0.35 ± 0.05</td>
<td>0.58 ± 0.08</td>
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<tr>
<td>bilateral PVN infusion of 30 μg/h PTX</td>
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<td>46.8 ± 4*</td>
<td>1.10 ± 0.09±</td>
<td>1.41 ± 0.12±</td>
<td>0.43 ± 0.05*</td>
<td>45.8 ± 5*</td>
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<tr>
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<td>1.07 ± 0.09*</td>
<td>0.37 ± 0.06*</td>
<td>47.0 ± 5*</td>
<td>1.12 ± 0.10±</td>
<td>1.44 ± 0.13±</td>
<td>0.41 ± 0.04*</td>
<td>46.2 ± 5*</td>
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<tr>
<td>SHAM</td>
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<tr>
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<td>0.57 ± 0.08</td>
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SHAM, sham-operated control; HF, heart failure; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; IZ %, percent ischemic zone. Values are mean ± SEM. *P < 0.05 versus SHAM + Treated or SHAM + PVN aCSF. †P < 0.05 HF + Treated versus HF + PVN aCSF. ‡P < 0.05, 4-weeks versus 24-hours value.
Fig. 1. Norepinephrine, glutamate and GABA in the PVN. PVN levels of norepinephrine (NE), glutamate and GABA in heart failure (HF) and sham operated (SHAM) rats treated for 4 weeks with PVN PTX, PVN ETN or PVN aCSF. PVN levels of (A) NE, (B) glutamate, and (C) GABA were lower in HF rats treated with PVN PTX or PVN ETN. *P < 0.05 versus SHAM + Treated or SHAM + PVN aCSF. † P < 0.05 HF + Treated versus HF + PVN aCSF.

Fig. 2. Western blots of TH, AT1-R, GAD67 and nNOS in the PVN. Rats with HF had higher levels of TH and AT1-R, and lower levels of GAD67 and nNOS when compared with SHAM rats. Bilateral PVN infusions of high doses of PTX or ETN decreased expression of TH and AT1-R, and increased GAD67 and nNOS expression in the PVN of HF rats. *P < 0.05 versus SHAM + Treated or SHAM + PVN aCSF. † P < 0.05 HF + Treated versus HF + PVN aCSF.
or ETN for 4 weeks prevented the decreases in protein and mRNA expression of nNOS within the PVN of HF rats; however, unilateral PVN infusions of high doses of PTX or ETN for 4 weeks only attenuated the decrease in mRNA and protein expression of nNOS within the PVN of HF rats. These results suggest that TNF-α in the PVN of HF rats downregulates nNOS in the PVN, contributing to sympathoexcitation.

**Fra-LI activity and AT1-R immunoreactivity in the PVN**

Compared with SHAM rats, HF rats had higher levels of Fra-LI activity (black dots, Fig. 4), AT1-R mRNA expression (Fig. 3B) and AT1-R immunoreactivity (pinkish red, Fig. 4) in the PVN. Bilateral PVN infusions of high doses of PTX or ETN for 4 weeks prevented the increases in Fra-LI and AT1-R immunoreactivity and AT1-R mRNA expression in the PVN of HF rats.

**PVN levels of proinflammatory cytokines**

PVN levels of TNF-α, IL-1β and IL-6 were higher in HF rats than in SHAM rats. PVN levels of TNF-α, IL-1β and IL-6 were lower in HF rats that received bilateral PVN infusions of PTX or ETN, when compared to aCSF-treated HF rats (Table 3). Bilateral PVN infusions of low doses of PTX or ETN attenuated, and high doses of PTX or ETN prevented, the increases in PVN proinflammatory cytokines seen in HF rats, while unilateral PVN infusions of high doses of PTX or ETN merely attenuated the increases in PVN proinflammatory cytokines (Table 3).

**Humoral indicators of heart failure**

Plasma levels of NE, ANGII, TNF-α, IL-1β and IL-6 were all higher in HF rats than in SHAM rats. Bilateral PVN infusions of low doses of PTX or ETN attenuated, and high doses of PTX or ETN prevented, the increases in plasma levels of TNF-α, IL-1β, IL-6, NE and ANGII in HF rats (Table 3). Unilateral PVN infusions of high doses of PTX or ETN only attenuated these increases in HF rats.

**Renal sympathetic nerve activity (RSNA)**

At the conclusion of the study, conscious RSNA was measured 5 h after rats recovered from anesthesia, HF rats exhibited higher RSNA (% of max) when compared to SHAM rats; bilateral PVN infusions of PTX or ETN inhibited RSNA in HF rats in a dose-dependent manner (Fig. 5). These results suggest that increased TNF-α in the PVN contributes to the exaggerated sympathetic activity in HF rats.

**Functional/anatomical indicators of heart failure**

Compared with aCSF-treated SHAM rats, aCSF-treated HF rats had higher LVEDP, RV/BW and lung/BW ratio. PTX- or ETN-treated HF rats had lower LVEDP and lung/BW ratios than aCSF-treated HF rats (Table 2).
The major observation of this study is that, in rats with HF, increased TNF-α in the PVN alters AT1-R and induces an imbalance between excitatory and inhibitory neurotransmitters and their rate-limiting enzymes within the PVN, thereby contributing to the exaggerated sympathetic activity seen in HF. Infusions of TNF-α blockers into the PVN decreased AT1-R expression within the PVN and attenuated neurotransmitter imbalance, ultimately decreasing sympathetic hyperactivity in HF rats. These results suggest that TNF-α in the PVN regulates AT1-R and neurotransmitters within the PVN and contributes to the exaggerated sympathetic activity in HF rats.

Discussion

The major observation of this study is that, in rats with HF, increased TNF-α in the PVN alters AT1-R and induces an imbalance between excitatory and inhibitory neurotransmitters and their rate-limiting enzymes within the PVN, thereby contributing to the exaggerated sympathetic activity seen in HF. Infusions of TNF-α blockers into the PVN decreased AT1-R expression within the PVN and attenuated neurotransmitter imbalance, ultimately decreasing sympathetic hyperactivity in HF rats. These results suggest that TNF-α in the PVN regulates AT1-R and neurotransmitters within the PVN and contributes to the exaggerated sympathetic activity in HF rats.

Sympathoexcitation is the hallmark of congestive heart failure (Zucker et al. 1995). Both humans and experimental animals display elevated sympathetic activity in HF. However, the mechanism contributing to this increased sympathetic activity is not fully understood. It is well established that central nervous system mechanisms contribute to this increased sympathetic drive. In the brain, the PVN is a key regulator of fluid homeostasis and vasopressin release, and an integrator of sympathetic outflow to major organs, including the heart and kidneys. Descending projections from the PVN directly target the brain stem, notably the rostral ventrolateral medulla, and the intermediolateral column of the spinal cord. Through these pathways, the PVN regulates changes in systemic sympathetic activity (Shafton et al. 1998; Cham and Badoer 2008).

The neuronal activity of the PVN plays an important role in the regulation of sympathetic tone. Neurohormones, peptides and neurotransmitters (both excitatory and inhibitory) have been demonstrated to contribute to this sympathetic tone. These include norepinephrine, GABA and glutamate. The PVN receives dense catecholaminergic innervations from the caudal medulla, which serve mainly
to relay sensory information. GABA is the principal inhibitory neurotransmitter in the PVN. GABA inputs originate mainly from local hypothalamic sources and are thought to impart limbic and cortical influences on PVN mechanisms. Further, GABA has been shown to evoke sympatho-inhibitory response within the PVN. Blockade of the GABAergic system in the PVN with the GABA antagonist bicuculline increases sympathetic activity, indicating that there is a strong tonic GABA-mediated inhibition (Li et al. 2006). Conversely, glutamate is the dominant excitatory neurotransmitter involved in neuroendocrine regulation, where injection of glutamate into the PVN cause increases in sympathetic activity (Li et al. 2006). A number of afferent mediators of PVN responses, including cytokines and neuropeptides, have been identified in HF. However, less attention has been directed towards identifying neurotransmitter systems involved in sympathetic regulation in HF. Interestingly, in HF, the sympathetic tone is in disarray and is accompanied by increased neuronal activation as evidenced by increases in hexokinase activity and neuronal firing in animals (Kang et al. 2006, 2009a). Recent studies have demonstrated that increases in both cytokines and ANGII have been shown to up-regulate neuronal activity within the PVN (Zhang, Francis et al. 2002; Zhang et al. 2003; Kang et al. 2008b).

Increased PVN activity in HF results from interplay of several neurotransmitters and neuromodulators in these sympathetic neurons. Interestingly, a recent study showed that intracarotid artery injection of cytokines activated PVN and RVLM neurons and that this was accompanied by increased sympathetic activity, indicating that cytokines within the cardiovascular regulatory centers contribute to the exaggerated sympathetic response (Zhang et al. 2003). In addition, we also recently demonstrated that cytokine blockade within the CNS attenuated sympathetic activity in HF rats (Dunn 2000; Kang et al. 2008b). These findings indicate that brain cytokines contribute to sympathetic hyperactivity in HF. In the present study, we found that RSNA is increased, along with PVN Fra-LI expression (an indicator of chronic neuronal activation), in rats with HF. This increase in Fra-LI activity was significantly reduced in HF rats receiving PVN infusions of TNF-α blockers, suggesting a role for TNF-α in the chronic neuronal excitation and increased sympathetic activity seen in HF.

Functional studies suggest that NO modulates sympathetic outflow by enhancing synaptic GABAergic function. The PVN, an important site for autonomic and endocrine homeostasis, constitutes an important center mediating NO actions on sympathetic outflow (Watkins et al. 2009). Moreover, the inhibitory effect of NO donors in the PVN on

![Graph](image-url)
the sympathetic nervous system was eliminated by GABA blockade, while the sympathoexcitatory effect of NO inhibitors was eliminated by activation of the GABA system, suggesting that the inhibitory effects of NO in the PVN on RSNA were mediated via the GABA neurotransmitter system (Zhang and Patel 1998). NO can act as a retrograde messenger molecule released by PVN-spinal neurons to excite GABA neurons that subsequently depress synaptic transmission in the PVN-spinal pathway via an inhibitory feedback mechanism present in the spinal sympathetic network. Thus, both NO and GABA together contribute to sympathetic activity. Here, we also demonstrate that animals with HF had decreased mRNA and protein expression for nNOS within the PVN. We also show a decrease in the expression of GAD67, a marker that recognizes GABAergic neurons and that signifies the relative level of GABA, within the PVN. Treatment with a TNF-α inhibitor within the PVN restored nNOS and GABA, indicating that cytokines within the PVN modulate these neurotransmitters and contribute to exaggerated sympathetic activity in HF.

In this study, we also found that levels of the excitatory neurotransmitters glutamate, NE, and its rate limiting enzyme, TH, were increased in the PVN of HF rats. The elevated basal level of NE concentration in the PVN of HF rats may reflect a marked stimulation of ascending noradrenergic drive to PVN or alternatively may be a result of impaired re-uptake of NE. Bilateral microinjection of glutamate into the PVN increases plasma NE and epinephrine levels (Zhang and Patel 1998), thus, it is reasonable to suggest that the increase in glutamate might play an important role in activating TH in the PVN neurons, thereby increasing catecholamine synthesis and release. It is worth noting that TNF-α inhibition in the PVN led to reduced TH, NE and glutamate in the PVN. Thus, our findings indicate that increased TNF-α within the PVN alters the delicate balance between excitatory and inhibitory neurotransmitters within the PVN, thereby contributing to the exaggerated sympathetic activity in HF rats.

The myocardial infarction model in the rat mimics the most common cause of HF in humans. Using this model, we and others have shown that cytokine and RAS is upregulated in the PVN of heart failure rats (Bains and Ferguson 1995; Kang et al. 2008b). Blockade of RAS components modulates PVN neurotransmitters and decreases sympa-
thetic activity, indicating a role for the central nervous system RAS in sympathoexcitation in HF (Zhang, Francis et al. 2002; Dampney et al. 2005). Findings from our laboratory and others indicate that cytokines interact with RAS both in the central and peripheral nervous systems (Guggilam et al. 2008; Kang et al. 2008a, 2008b, 2009a). Cytokine blockade decreases circulating ANGII levels and, conversely, RAS blockade attenuates circulating cytokine levels (Patel 2000; Gao et al. 2008; Kleiber et al. 2008). Interestingly, both cytokines and AT1-R are up-regulated in the PVN during HF. Therefore, it is plausible to suggest that an interaction between TNF-α and AT1-R within the PVN might modulate neurotransmitters and contribute to sympathoexcitation in HF. This is supported by our findings that TNF-α blockade within the PVN decreases AT1-R expression and modulates neurotransmitters thereby attenuating sympathoexcitation in HF rats.

Clinically, most drugs for the treatment of HF, including ACE inhibitors or AT1-R blockers, are aimed at reducing afterload and intravascular volume. In this study, we demonstrate that blockade of TNF-α in the PVN also reduced pulmonary congestion, as demonstrated by reduced wet lung/BW ratio and reduced LVEDP in HF rats.

Although the results of clinical trials targeting TNF-α in HF were inconclusive, it is clear that increased sympathetic activity in HF is a complex phenomenon involving the inter-

<table>
<thead>
<tr>
<th>Groups</th>
<th>PVN (pg/mg protein)</th>
<th>Plasma (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
<td>IL-1β</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bilateral PVN infusion of 10 μg/h PTX</td>
<td>5.3 ± 0.4*</td>
<td>34.5 ± 2.9*</td>
</tr>
<tr>
<td>bilateral PVN infusion of 2.5 μg/h ETN</td>
<td>5.4 ± 0.5*</td>
<td>37.3 ± 3.5*</td>
</tr>
<tr>
<td>bilateral PVN infusion of 30 μg/h PTX</td>
<td>3.7 ± 0.4*</td>
<td>20.9 ± 2.6*</td>
</tr>
<tr>
<td>bilateral PVN infusion of 7.5 μg/h ETN</td>
<td>4.0 ± 0.5*</td>
<td>21.8 ± 2.7*</td>
</tr>
<tr>
<td>unilateral PVN infusion of aCSF</td>
<td>7.3 ± 0.5*</td>
<td>55.6 ± 4.8*</td>
</tr>
<tr>
<td>bilateral PVN infusion of 10 μg/h PTX</td>
<td>3.5 ± 0.3</td>
<td>18.2 ± 1.9</td>
</tr>
<tr>
<td>bilateral PVN infusion of 2.5 μg/h ETN</td>
<td>3.7 ± 0.4</td>
<td>19.4 ± 1.8</td>
</tr>
<tr>
<td>bilateral PVN infusion of 30 μg/h PTX</td>
<td>3.4 ± 0.5</td>
<td>19.1 ± 2.4</td>
</tr>
<tr>
<td>bilateral PVN infusion of 7.5 μg/h ETN</td>
<td>3.5 ± 0.6</td>
<td>20.2 ± 2.5</td>
</tr>
<tr>
<td>SHAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unilateral PVN infusion of 30 μg/h PTX</td>
<td>5.1 ± 0.4*</td>
<td>35.1 ± 2.9*</td>
</tr>
<tr>
<td>unilateral PVN infusion of 7.5 μg/h ETN</td>
<td>5.3 ± 0.5*</td>
<td>38.9 ± 3.7*</td>
</tr>
<tr>
<td>unilateral PVN infusion of aCSF</td>
<td>7.5 ± 0.8*</td>
<td>54.6 ± 4.9*</td>
</tr>
<tr>
<td>SHAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unilateral PVN infusion of aCSF</td>
<td>3.8 ± 0.4</td>
<td>20.1 ± 2.7</td>
</tr>
</tbody>
</table>

*P < 0.05 versus SHAM + Treated or SHAM + PVN aCSF. †P < 0.05 HF + Treated versus HF + PVN aCSF.
action of several neuromodulators and neurotransmitters in the PVN. Data from this study, along with data from other studies targeting cytokines in HF, support the assertion that anti-cytokine agents can be used in conjunction with traditional HF therapies.

In summary, the present study demonstrates that administration of PTX or ETN bilaterally into the PVN of HF rats attenuates proinflammatory cytokines, AT1-R, glutamate and NE in the PVN. Further, bilateral infusion of PTX or of ETN into the PVN normalizes the HF-induced decreases in GABA and nNOS in the PVN. Our results suggest that TNF-α inhibition may decrease sympathoexcitation in HF by decreasing AT1-R and excitatory neurotransmitters, while concurrently increasing inhibitory neurotransmitter levels, thereby restoring the delicate balance between the two within the PVN (Fig. 7). Further studies are needed to determine the mechanisms by which these interactions occur.

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