Renal Denervation Improves Exaggerated Sympathoexcitation in Rats With Heart Failure

Kaushik P. Patel, Bo Xu, Xuefei Liu, Neeru M. Sharma, Hong Zheng

Abstract—Renal denervation (RDN) has been postulated to reduce sympathetic drive during heart failure (HF), but the central mechanisms are not completely understood. The purpose of the present study was to assess the contribution of neuronal nitric oxide synthase (nNOS) within the paraventricular nucleus (PVN) in modulating sympathetic outflow in rats with HF that underwent RDN. HF was induced in rats by ligation of the left coronary artery. Four weeks after surgery, bilateral RDN was performed. Rats with HF had an increase in FosB-positive cells in the PVN with a concomitant increase in urinary excretion of norepinephrine, and both of these parameters were ameliorated after RDN. nNOS-positive cells immunostaining, diaphorase staining, and nNOS protein expression were significantly decreased in the PVN of HF rats, findings that were ameliorated by RDN. Microinjection of nNOS inhibitor G-monomethyl l-arginine into the PVN resulted in a blunted increase in lumbar sympathetic nerve activity (11±2% versus 24±2%) in HF than in sham group. This response was normalized after RDN. Stimulation of afferent renal nerves produced a greater activation of PVN neurons in rats with HF. Afferent renal nerve stimulation elicited a greater increase in lumbar sympathetic nerve activity in rats with HF than in sham rats (45±5% versus 22±2%). These results suggest that intact renal nerves contribute to the reduction of nNOS in the PVN, resulting in the activation of the neurons in the PVN of rats with HF. RDN restores nNOS and thus attenuates the sympathoexcitation commonly observed in HF. (Hypertension. 2016;68:175-184. DOI: 10.1161/HYPERTENSIONAHA.115.06794.)

Key Words: central nervous system ■ heart failure ■ neurons ■ nitric oxide synthase ■ sympathetic nervous system

Enhanced sympathetic nerve activity is a risk factor that influences the progression of heart failure (HF) and mortality in patients. Although most therapeutic pharmaceutical strategies target the peripheral symptoms of the disease, they may not influence the enhanced sympathetic nerve activity. Indeed, various studies have detailed the role of altered baroreceptor and chemoreceptor afferent mechanisms in the elevated sympathetic drive in HF,1,2 but these mechanisms do not fully account for the total sympathoexcitation observed in HF.

In the central nervous system, the paraventricular nucleus (PVN) of the hypothalamus mediates sympathetic nerve activity and influences the cardiovascular system.3,5 Our previous studies have shown that the PVN is activated in rats with HF in conjunction with enhanced glutamatergic tone and blunted nitric oxide (NO) mechanism within the PVN.6,7 Specifically, neurons within the PVN exhibit an increased hexokinase and FosB expression, which are markers of chronic neuronal activation.5,10-12 Furthermore, rostral ventrolateral medulla (RVLM) projecting PVN neurons are more active in rats with HF than in sham-operated controls, suggesting that presynaptic neurons within the PVN are activated and contributed importantly in initiating sympathoexcitation in HF.11 The discharge frequency of putative vasopressinergic magnocellular neurosecretory neurons in the PVN is increased during stimulation of afferent renal nerves (ARN) and during the activation of specific renal receptors.14 Recently, we have shown that ARN stimulation activates RVLM projecting PVN neurons.15 Stimulation of ARN also increases sympathetic activity and arterial pressure.15,16 In addition, neurons containing Fos-like immunoreactivity were observed to be increased after ARN stimulation in the PVN, indicated that the PVN neurons were activated by ARN stimulation.17 These observations suggest that afferent information from the kidney is important in the coordination of neural and hormonal activity concerned with body fluid balance and the regulation of arterial blood pressure in normal and disease conditions.17-21

Renal denervation (RDN) has been shown to reduce arterial pressure and sympathetic outflow in various animal models of hypertension and in patients with resistant hypertension.22-24 Catheter-based RDN has been shown to reduce blood pressure in treatment-resistant hypertensive patients for the primary end point at 6 months.25 In animal studies, RDN has been shown to decrease sympathetic activity and arterial pressure in a neurogenic form of hypertension produced by sectioning of aortic
depressor nerves. Concurrently, noradrenergic activity is altered in the hypothalamus after RDN, suggesting that the sympathoinhibitory effects of RDN are mediated centrally at the level of the hypothalamus.26 Studies in experimental models of HF have previously induced RDN either before or immediately after the induction of HF27,28 but not in the chronic stage of HF, which is clinically more relevant.

The present study was conducted to examine the hypothesis that the activation of the PVN in HF is mediated and conveyed by intact renal nerves. Furthermore, this renal nerve–mediated information contributes to the downregulation of neuronal NO synthase (nNOS) within the PVN of rats with HF, leading to the enhanced activation of neurons in the PVN that translates ultimately to the activation of sympathetic nervous system in HF.

Methods

Animals
All procedures used for this study were approved by University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health guiding principles for the research involving animals. Male Sprague–Dawley rats weighing 220 to 250 g were purchased from Sasco Breeding Laboratories (Omaha, NE). Animals were housed with a 12-hour light-dark cycle at ambient 22°C 30% to 40% relative humidity. Laboratory chow and tap water were available ad libitum. After acclimatization for 1 week, rats were assigned randomly to 1 of 4 groups: sham, HF, sham+RDN, HF+RDN (n=15–18 per group, total number of rats=66).

Induction of HF
Rats were randomly assigned to either a sham-operated control group or a HF group. HF was produced by left coronary artery ligation, as previously described.29 The rats were anesthetized with isoflurane gas starting in an anesthetia chamber from 3% to 4%. During the procedure, isoflurane (2%–2.5%, gas vaporizer) was used. The degree of left ventricular dysfunction and HF was determined by using both hemodynamic and anatomic criteria. Rats with both left ventricular end-diastolic pressure >15 mmHg and infarct size >30% of total left ventricular wall were included in the study. Six rats in the HF group had infarct sizes <30% and were excluded from data analysis.

Renal Denervation
Four weeks after ligation surgery, rats underwent RDN under anesthesia. Complete RDN was achieved by cutting bilaterally all the visible renal nerves from the renal artery and vein, and painting the vessels with 70% ethanol. This method has been shown to ablate the afferent and efferent renal nerves.30–32

One week after RDN (and 5 weeks after coronary ligation), rats were either euthanized to collect tissues for molecular or immunohistochemical studies or anesthetized for terminal electrophysiological studies to record PVN neuronal activity or lumbar sympathetic nerve responses to ARNs or lumbar sympathetic nerve responses to microinjections of N'-monomethyl L-arginine (L-NMMA) into the PVN. Blood was collected for analysis of angiotensin II and aldosterone levels. Urine was collected for analysis of norepinephrine excretion. Additional methodologies and detailed description of procedures including norepinephrine, angiotensin II and aldosterone measurements, immunohistochemistry, nicotinamide adenine dinucleotide phosphate-diaphorase activity, Western blot analysis, lumbar sympathetic nerve activity (LSNA) recording, microinjection into the PVN, extracellular single-unit recordings, electric stimulation of ARNs are available in Methods section in the online-only Data Supplement.

Statistical Analysis
Data were subjected to a 2-way ANOVA followed by a multiple range (for multiple comparisons) or Student–Newman–Keuls test. P<0.05 were considered to indicate statistical significance.

Results

General Characteristics
The Table presents morphological characteristics and left ventricular function parameters among the 4 experimental groups. The body weight, whole heart weight, and heart weight/body weight ratio were significantly increased in HF group. RDN had no significant effects on these parameters in both sham and HF groups. Only rats with >30% infarcts of the left ventricular wall were included in the study. Six rats in the HF group had infarct sizes <30% and were excluded from data analysis. Sham rats had no visible myocardial damage. Left ventricular end-diastolic pressure was significantly increased in the HF rats compared with both sham groups and HF+RDN group. The magnitude of +dP/dt and −dP/dt were significantly decreased in both HF+RDN rats compared with both sham

<table>
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<tr>
<th>Measures</th>
<th>Sham (n=10)</th>
<th>HF (n=10)</th>
<th>Sham+RDN (n=12)</th>
<th>HF+RDN (n=12)</th>
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<tr>
<td>Body weight, g</td>
<td>408±18</td>
<td>452±26*</td>
<td>386±10</td>
<td>426±21</td>
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<td>Heart weight, g</td>
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<td>0.47±0.04*</td>
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<tr>
<td>Infarct size (% of epicardial LV)</td>
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<td>36±6*</td>
<td>0</td>
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<td>LVEDP, mm Hg</td>
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<td>25±4*</td>
<td>1±1</td>
<td>12±3*†</td>
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<td>+dP/dt, mm Hg/s</td>
<td>6899±329</td>
<td>5016±378*</td>
<td>6856±274</td>
<td>5366±298*</td>
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<tr>
<td>−dP/dt, mm Hg/s</td>
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<td>−3895±277*</td>
<td>−6023±314</td>
<td>−4115±169*</td>
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<td>Plasma angiotensin II, pg/mL</td>
<td>68±12</td>
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<td>53±8</td>
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<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>1647±117</td>
<td>2254±74*</td>
<td>1753±91</td>
<td>2017±29*†</td>
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</tbody>
</table>

Data are means±SE. HF indicates heart failure; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; and RDN, renal denervation. *P<0.05 compared with sham. †P<0.05 compared with the group without denervation.
groups. Left ventricular end-diastolic pressure was partially reduced by RDN, whereas +dP/dt and −dP/dt were not significantly affected by RDN. The data confirmed that rats in the HF groups were experiencing cardiac dysfunction and that RDN may contribute to partial improvement in cardiac function.

**Urinary Norepinephrine Excretion Measurements**

Urinary norepinephrine excretion was significantly greater in HF rats than in sham-operated controls and was used as an index of overall sympathetic activation. RDN reduced the urinary excretion of norepinephrine in rats with HF (4.2±0.8 HF+RDN versus 8.1±0.3 μg/d HF; P<0.05). There was no significant change in the sham rats with RDN suggesting that the RDN per se did not change the excretion of norepinephrine in the urine in control conditions (Figure 1).

Kidney norepinephrine content was significantly greater in HF rats than in sham-operated controls (325±35 versus 168±36 ng/g; P<0.05). RDN reduced the kidney content of norepinephrine to almost undetectable level in both sham and HF rats, which confirms the completeness of RDN.

**Plasma Angiotensin II and Aldosterone Measurements**

Plasma angiotensin II and aldosterone were significantly greater in HF rats than in sham-operated controls. RDN significantly reduced the plasma angiotensin II and aldosterone in rats with HF (Table 1). RDN did not significantly change the levels of angiotensin II or aldosterone in the sham-operated controls.

**FosB Immunohistochemistry in the PVN**

The number of cells in the PVN (rostral-caudal level of the PVN) that stained positive for the neuronal activation marker, FosB, was increased in HF compared with the sham group (Figure 2). This enhanced activation was attenuated in the HF+RDN rats compared with the HF rats. These data indicate that the PVN is in an activated state during HF, perhaps contributing to the enhanced sympathetic activity. After RDN, the activation in the PVN is attenuated, paralleling the decrease in overall sympathetic activation as measured by urinary norepinephrine excretion (Figure 1).

**Expression of the nNOS (Immunohistochemistry, Diaphorase, and Protein) During HF and After RDN**

The number of cells in the PVN that stained positive for the nicotinamide adenine dinucleotide phosphate-diaphorase activity was decreased in HF compared with the sham group. This reduction in NOS activity was attenuated in the HF rats after RDN (Figure 3). Immunostaining showed that the nNOS signaling in the PVN was decreased in HF compared with the sham group. This reduction in nNOS immunoreactivity was decreased 48% in HF that was abrogated by RDN (Figure 4A and 4B). Furthermore, Western blot data showed similar pattern for reduction in the PVN; nNOS expression was decreased 48% in HF that was abrogated by RDN (Figure 4C). There was no difference in nNOS expression in the PVN after RDN between the sham and HF groups.

**L-NMMA Microinjection Into the PVN**

The basal LSNA was significantly increased in rats with HF compared with the sham rats (3.1±0.3 versus 2.0±0.7 μV·s; P<0.05). RDN significantly reduced the basal LSNA in HF group rats (2.0±0.1 HF+RDN versus 3.1±0.3 μV·s HF; P<0.05; Figure 5A). To examine endogenous NO tone from the PVN, we blocked NOS within the PVN with microinjection of L-NMMA.
L-NMMA. L-NMMA microinjection into the PVN increased LSNA in both sham and HF groups as expected and shown previously while recording renal sympathetic nerve activity. The percentage change in LSNA from basal values was smaller in HF than in sham (11±2% versus 24±2%; P<0.05; Figure 5B and 5C). Mean arterial pressure and HR responses to L-NMMA were blunted in the HF condition. However, after RDN, these parameters were all normalized in HF rats.

Responses in RVLM Projecting PVN Neurons to Stimulation of ARN
In 40 spontaneously active neurons recorded in the PVN, 10 units (5 from sham and 5 from HF rats) were antidromically activated from the RVLM in 6 rats. Stimulation of the ARN increased firing of RVLM projecting PVN neurons in both sham and HF groups. We have previously shown that RVLM projecting PVN neurons have a higher basal firing rate in the HF group. Furthermore, here we show that the percentage increase in firing of PVN/RVLM neurons from basal values was enhanced in HF compared with sham (22.7±3.1% versus 9.0±1.3%; P<0.05; Figure 6A). These data show that RVLM projecting PVN neurons in HF may have an exaggerated response to ARN stimulation.

Responses in LSNA to Stimulation of ARN
Stimulation of the ARN increased LSNA in both sham and HF groups. The percentage change in LSNA from basal values was enhanced in HF compared with sham (45±5% versus 22±2%; P<0.05; Figure 6B and 6C). Similarly, mean arterial pressure

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**Figure 3.** The effect of renal denervation (RDN) on nicotinamide adenine dinucleotide phosphate-diaphorase in the paraventricular nucleus (PVN) of rats with heart failure (HF). Representative pictures of PVN with nitric oxide synthase (NOS)–positive staining (A) in 4 groups of rats: sham, HF, sham+RDN and HF+RDN. Scale bar, 100 μm. B, Mean values of NOS-positive cells in the PVN. *P<0.05 vs sham; #P<0.05 vs without RDN.

**Figure 4.** A, Representative pictures of paraventricular nucleus (PVN) with neuronal nitric oxide synthase (nNOS) staining (A) in 4 groups of rats: sham, heart failure (HF), sham+renal denervation (RDN), and HF+RDN. Scale bar, 100 μm. B, Mean values of relative nNOS intensity in the PVN. C, nNOS protein expression in the sham and HF rats with/without RDN. *P<0.05 vs sham; #P<0.05 vs without RDN.
and HR responses to ARN were also elevated in the HF condition (11±4 versus 3±1 mmHg and 7±2 versus 4±1 bpm; P<0.05, respectively; Figure 6B and 6C). These data show that in HF there is an exaggerated response to ARN stimulation.

**Discussion**

The present study shows that removal of renal nerves by RDN abrogates the elevated neuronal activity in the PVN and the concomitant increase in global sympathetic outflow in HF, as indicated by the elevated excretion of norepinephrine in the urine. At the same time, RDN restored the endogenous nNOS in the PVN that had been decreased in rats with HF. Consistent with these observations, RDN normalized the blunted LSNA response to inhibition of endogenous NOS within the PVN observed in HF rats. Electric stimulation of ARN resulted in an enhanced neuronal firing of RVLM projecting PVN neurons in rats with HF. As a corollary, ARN stimulation elicits a potentiated activation of LSNA in rats with HF. We propose that potentially tonic activation of ARN (caused by the HF condition) contributes to the activation of preautonomic neurons within the PVN during HF. These results demonstrate a critical role for the PVN in relaying neural signals from the kidney that may be tonically active in the HF condition and are abrogated by RDN. A possible mechanism for the therapeutic effects of RDN during HF may be through an NO-dependent mechanism within the PVN.

**Renal Denervation Normalizes the Global Sympathoexcitation in HF**

RDN in most experimental forms of hypertension and drug-resistant hypertensive patient has been shown to reduce arterial pressure and sympathetic activity. Because exaggerated sympathoexcitation is characteristic of both hypertension and HF, the efficacy of RDN to reduce sympathoexcitation has been explored in both ischemia-induced and pacing heart models of HF and patients with HF. In previous studies that examined the effects of RDN in HF, RDN was performed either before or immediately after the induction of HF. Therefore, we investigated whether RDN reduces global sympathetic tone after the development of HF to further increase the clinical relevance. The results from the present study show that bilateral RDN was sufficient to reduce global sympathetic outflow, as evident by a
reduction in urinary excretion of norepinephrine and in basal level of LSNA.

Renal Denervation Abrogates Activation of the PVN During HF

This study found that concomitant with the increase in global sympathetic outflow, there was an activation of the PVN in HF. In HF rats, there was a 2-fold increase in FosB-positive cells in the PVN compared with sham. In addition, HF rats exhibited greater protein expression of ΔFosB, which is a stable splice variant of FosB that is specifically upregulated during chronic neuronal activation. Other studies have reported PVN activation using Fra-like immunoreactivity after induction of HF. Extracellular recordings in the PVN have also shown that RVLM projecting PVN neurons are more active in rats with HF at 4 weeks. Afferent neural information from renal sensory receptors has been shown to influence the activity of neurons at different levels of the neuraxis. Furthermore, ARN stimulation has also been shown to activate the PVN. In addition, a time course study examining inducible transcription factors in the PVN found that the expression of neuronal activity marker cFOS was rapidly increased after ARN stimulation. Therefore, because renal afferents are known to influence neuronal activity within the PVN, it could be postulated that the enhanced FosB expression observed in the PVN of HF rats may be related to the potential tonic activation of ARNs during the HF condition. The level of this tonic activation and the exact modality of the stimulus from the kidney that activates the renal afferents in this disease condition remains to be elucidated.

Figure 6. A, Discharge of rostral ventrolateral medulla projecting paraventricular nucleus neurons to afferent renal nerves (ARN) stimulation. Peristimulus histogram of spike occurrence triggered by electric stimulation of the afferent renal nerve with 100 sweeps, bin = 0.02 s. Segments of original recordings of changes in discharge after ARN stimulation in sham and heart failure (HF) rats. B, Original recordings showing changes in lumbar sympathetic nerve activity (LSNA), mean arterial pressure (MAP), and heart rate (HR) before and after ARN stimulation (40 Hz) in individual sham and HF rats. C, Mean percent change of LSNA, MAP, and HR to ARN stimulation in sham and HF groups. *P<0.05 vs sham. ARNs indicates afferent renal nerve stimulation.
It should be noted that there are multiple triggers in the HF condition that have the potential for increased activation of the renal afferent nerve activity, including reduced perfusion pressure, increased venous pressure, increased inflammation, increased oxidative stress to name a few.18

In addition, HF rats exhibit increased sympathetic and hemodynamic responses to activation of the PVN.46 In the current study, FoxB3 results suggest that the PVN is activated during HF. Perhaps this activation of the PVN may be because of an exaggerated response to ARN activation during HF. This increased activity within the PVN translates to increased overall sympathetic outflow. However, RDN caused a decrease in the index of overall sympathetic activity, urinary excretion of norepinephrine in HF rats, whereas this response was minimal or absent in the sham group. One plausible interpretation of these data is that there may be a tonic level of ARN activity during the HF condition, which may contribute to the state of activation of the PVN resulting in an increased overall sympathoexcitation. RDN did improve overall sympathetic drive, as indicated by a decrease in excretion of urinary norepinephrine levels and reduction in baseline LSNA in HF rats. Reducing norepinephrine is important, as HF patients with lower levels of plasma norepinephrine are given a better prognosis.46 These results suggest that RDN may alter the activity of neurons in central cardiovascular regulatory sites, such as the PVN, thereby contributing to the improvement in sympathetic tone. Indeed, our results indicate that ARN-mediated sympathetic and hemodynamic responses are improved after RDN.

Renal Denervation Improves nNOS Expression and Functional NOS Activity in the PVN During HF

NO, which is well known to inhibit neurons, is decreased in terms of mRNA and protein expression and diaphorase staining in the PVN during HF;47,48 Sympathoexcitatory and hemodynamic responses to endogenous blockade of NO within the PVN were impaired in HF,7 suggesting that the PVN contributes to the increased sympathoexcitation exhibited in HF via an altered NO mechanism. This study shows that RDN restores the levels of nNOS within the PVN of rats with HF, suggesting that RDN restores the endogenous inhibitory nNOS mechanisms in rats with HF. The levels of diaphorase positive neurons are also restored in rats with HF, suggesting that the NOS activity index is also restored after RDN. Consistent with these observations, the functional activity of NOS within the PVN of rats with HF is also restored. The improved LSNA responses to microinjection of L-NMMA into the PVN are concomitant with improved nNOS levels in the PVN of rats with HF after RDN.

Activation of the PVN by ARN Stimulation

The kidneys have a dense afferent sensory and efferent sympathetic innervation and are thereby strategically positioned to be the origin and target of sympathetic nervous system activation.9,33,49 Many studies utilizing anterograde tract tracing of fluorescent dyes, horseradish peroxidase transport, or pseudorabies virus injected into the kidneys and electrophysiological evidence indicate that the renal afferent information is transmitted to sites within the spinal cord that relay information to the central nervous system associated with cardiovascular regulation, including nucleus tractus solitaries, RVLM, preoptic area, subfornical organ, lateral hypothalamus, and the PVN in the hypothalamus.19,21,50 Renal afferent nerve signals can elicit both inhibitory renal reflexes and long looped supramedullary renoeexcitatory responses.21,51 Although the inhibitory renal reflex is blocked by RDN or spinal cord transection at C2, it was unaffected by transection at pontinemedullary junction,51 suggesting that the inhibitory reflex operates at the spinomedullary level. Electrophysiological and morphological evidence suggest that majority (70%) of the fibers in the ARN are small unmyelinated C-fiber or finely myelinated A-delta fibers.18,52,53 It is postulated that most of the chemoreceptor input is carried by these fibers. In addition, it is thought that the electrical stimulation (used in this study) mainly elicits the response caused by the activation of this group of small fibers causing an excitatory response.14,18,21 Stimulation of the myelinated large fibers causes the inhibitory response.19

Renal afferent nerve signals are centrally integrated and their activation results in an increase in sympathetic tone, which is directed toward not only the kidneys but also other organs that have a dense sympathetic innervation resulting in an increase in sympathetic outflow to cause a rise in blood pressure.20,21,54–56 Renal afferent signals are also involved in spinal feedback loops, termed renorenal reflexes, whereby afferent activity from 1 kidney can modulate ipsilateral and contralateral effferent renal nerve activity to regulate diuresis and natriuresis to balance overall renal function between the 2 kidneys.19 In this regard, it is of interest to note that such inhibitory renorenal reflexes are reported to be blunted in rats with HF.57 Furthermore, it was proposed that this blunting was mediated by angiotensin II. This could be interpreted to imply a role for renal efferents in potentially reducing inhibitory input from the kidney. These data are also consistent with the concept that this reduced inhibitory input may be overwhelmed by excitatory input generated within the kidneys of rats during the HF condition because RDN was able to abrogate the increase in global sympathoexcitation. Therefore, the kidney is not only a target of sympathetic outflow but also a source of signals that have the potential to directly modulate overall sympathetic outflow in disease conditions such as HF and hypertension.19

Evidence for excitatory reflexes originating in diseased kidneys is derived from studies in rats with chronic renal failure and patients with renal failure.19 There is also considerably strong evidence that the diseased kidneys exert an excitatory effect on sympathetic nerve activity in various pathological conditions involving renal injury, including hypertension, HF, chronic renal failure, diabetes mellitus, and obesity.19,49,58–61 It has been proposed that renal inflammation is prevalent in many of these pathological conditions and may contribute to the increased sympathetic outflow via activation of ARNs.19

The PVN is an important site that integrates and responds to a variety of neural and humoral signals regulating sympathetic drive and extracellular fluid volume.31,65 Previous study demonstrated that the discharge frequency of neurons in the PVN was increased during stimulation of ARN.14 In this respect, it is of interest to understand whether preautonomic neurons in the PVN respond to ARN stimulation and how the PVN integrates signals from the ARN. In the present study, electrical stimulation of the ARN activated RVLM projecting PVN neurons to a greater extent in rats with HF.
than sham rats. These data suggest that afferent signal from the kidneys of HF rats are more potent in eliciting activation of preautonomic neurons within the PVN. It is of interest to note that basal levels of RVLM projecting PVN neurons are also increased in HF rats, suggesting that there is a potential for tonic ongoing activation by the ARN during HF condition, which may contribute to the state of activation of the PVN that would then translate into an increased overall sympathoexcitation under basal conditions. However, to conclusively demonstrate that renal afferents are activated tonically in the HF condition, either direct renal afferent recordings or a selective interruption of renal afferents is required and remains to be explored.

**Activation of the ARN Produces Exaggerated LSNA Responses in Rats With HF**

Renal afferent information has been shown to influence baroceptors and cardiac afferent reflexes to influence sympathetic tone. It should be noted that the cardiac afferent reflex is shown to have enhanced sympathoexcitatory responses in HF and hypertension. Consistent with our findings of an overactive sympathoexcitatory state originating from the PVN during HF, the potentially tonic ARN stimulus may be a critical contributor during the HF condition. Previous studies have demonstrated the importance of the PVN in cardiovascular regulation during HF. More recently, we have shown that there is a specific activation of RVLM projecting PVN by stimulation of ARN. It is of interest to note that all the neurons that were responsive to ARN stimulation in that study were also sensitive to cardiac sympathetic afferents, a source of sympathoexcitatory input. It may well be that this convergence of input from the kidney and heart are synergistically driving these preautonomic PVN neurons in the HF condition. ARN stimulation induces sympathoexcitation via the PVN by a glutamatergic mechanism. Therefore, the ARN-induced activation of the PVN may influence the neuronal activity in the RVLM among other sites and thereby contribute to the overall regulation of sympathetic activity. This does not also preclude the possibility that ARN may influence spinal cord-projecting PVN neurons to play an equal or greater role in the ARN-induced activation of the PVN and subsequent sympathoexcitation. It may well be that the presence of input from any of these afferents (renal, cardiac, or chemoreceptors) may be sufficient to activate the PVN to drive the sympathetic overactivation because specific cardiac denervation or chemodenervation have also been shown to alleviate the overactive sympathoexcitation observed in HF.

Direct electrical stimulation of the renal afferent nerves in animals has been shown to produce sympathoexcitation in various vascular beds and an increase in arterial pressure. It is possible that a pathological signal, which remains to be identified, from the kidney may initiate an increase in ARN activity, whereby causing an increase in overall sympathetic tone which in turn exuberates the pathological signal (positive feedback loop). In the present study, stimulation of the ARN produced an increase in LSNA and these responses were exaggerated in rats with HF, suggesting that the renal-mediated activation of the sympathoexcitation is more potent in rats with HF, possibly contributing to the enhanced sympathoexcitation commonly observed in the HF condition.

**Removal of Efferent Renal Innervation**

The potential effects of interrupting the renal efferent nerves should be considered as an alternative explanation for the changes in the nitroxidergic mechanisms within the PVN of rats with HF. It has been well documented that angiotensin II levels are elevated in HF and also in this model. One well-known function of the renal nerves is to stimulate renin release from the kidney and renal nerve activity has been reported to be elevated in HF. It is conceivable that this activation of efferent renal nerves may be causally related to increased levels of angiotensin II in HF. It has also been shown previously that angiotensin II causes an increased activation of the sympathoexcitation via the PVN in HF. Furthermore, we have observed that angiotensin II may also cause a decrease in levels of nNOS in vitro and in the PVN of rats with HF. So it is conceivable that the changes in the PVN are influenced by the removal of this angiotensin II component by removal of renal efferent nerves. The decline in circulating levels of angiotensin II and aldosterone after RDN in rats with HF is consistent with this overall hypothesis. However, it should be noted that the levels of angiotensin II are still significantly elevated after RDN in rats with HF, suggesting that it might contribute to this response but may not be totally responsible for the changes observed after RDN. The causal role for angiotensin II in the effects of RDN on central regulation of sympathoexcitation in HF remains to be explored.

**Activation of the PVN**

Another intriguing alternative possibility for these observations is that because there was a slight improvement in cardiac function with RDN, this improvement (in cardiac function) may elicit a signal to improve central nNOS mechanism within the PVN, which in turn would alleviate the enhanced sympathoexcitation in rats with HF. Nevertheless, the improvement in centrally mediated sympathoinhibition by RDN seems to be mediated via the nitroxidergic mechanism within the PVN. As to the source of this signal or its modality remains to be examined but seems to be associated with intact renal nerves.

**Perspectives**

The current study offers insight into the mechanism of the positive effects of RDN during HF. To date, the mechanisms for the normalization of sympathetic outflow by RDN during HF have not been fully elucidated. Here, we found that RDN normalizes the activation of the PVN during HF, likely through an NO-dependent mechanism. RDN reversed FosB activation of the PVN during HF and ARN-mediated sympathoexcitation. In addition, there was increased response to L-NMMA microinjection after RDN in HF rats, suggesting restoration of nNOS inhibition in the PVN by RDN.

In conclusion, the increased activation of the PVN via possible activation of ARN during HF condition may contribute to the enhanced sympathetic drive exhibited in HF, and the PVN mediates the RDN effect of restoring sympathetic activity during HF. In addition, we conclude that restoration of nNOS levels with RDN reverses enhanced PVN activation, as well as exaggerated systemic sympathetic activity commonly observed in the HF condition.
None.

References


**Novelty and Significance**

**What Is New?**

- The present study shows that removal of renal nerves by renal denerva-tion (RDN) abrogates the elevated neuronal activity in the paraventricular nucleus (PVN) and the concomitant increase in global sympathetic out-flow in heart failure (HF) rats.
- RDN restored the decreased endogenous neuronal nitric oxide synthase in the PVN of rats with HF.
- Stimulation of afferent renal nerves produced an exaggerated increase in preautonomic PVN neurons and lumbar sympathetic nerve activity in rats with HF.

**What Is Relevant?**

- A conceptually novel approach to reducing the enhanced sympathoexcita-tion in HF was to investigate the influence of RDN on PVN-mediated sympathoexcitation in HF. Specifically, we hypothesized that increased renal afferent input in HF activates the preautonomic sympathetic neu-rons in the PVN leading to enhanced sympathoexcitation.
- Studies utilizing RDN in hypertensive patients have provided exciting new insight into reduction of sympathetic tone. However, the mechanism of action for these results remains unknown. This present study was specif-ically designed to elucidate the nitric oxide mechanism within a specific site, the PVN, to give further insight into potential driving mechanism and treatments for hypersympathetic states such as HF and hypertension.

**Summary**

Our studies reveal a possible mechanism for the therapeutic effects of RDN during HF and provide insight into how neuronal nitric oxide synthase within the PVN, specifically may contribute to improving the elevated sympathetic tone during HF, likely through an nitric oxide–dependent mechanism.
Renal Denervation Improves Exaggerated Sympathoexcitation in Rats With Heart Failure: A Role for Neuronal Nitric Oxide Synthase in the Paraventricular Nucleus
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Renal Denervation Improves Exaggerated Sympatho-excitation in Rats with Heart Failure:
A Role for nNOS in the Paraventricular Nucleus (PVN)

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Methods
Materials and Methods

Animals
All procedures used for this study were approved by University of Nebraska Medical Center Institutional Animal Care and Use Committee and conducted according to the NIH guiding principles for the research involving animals. Male Sprague-Dawley rats weighing 220 to 250 g (age 7-8 weeks) were purchased from Sasco Breeding laboratories (Omaha, NE). Animals were housed with a 12-hour light-dark cycle at ambient 22°C 30-40% relative humidity. Laboratory chow and tap water were available ad libitum. After acclimatization for 1 week, rats were assigned randomly to one of four groups: sham, HF, sham+RDN, HF+RDN.

Induction of heart failure
Rats were randomly assigned to either a sham-operated control group or a HF group. HF was produced by left coronary artery ligation, as previously described. The rats were anesthetized with isoflurane gas starting in an anesthesia chamber from 3-4%. The rats had an endotracheal tube inserted orally to facilitate artificial ventilation. During the procedure, isoflurane (2 to 2.5%, gas vaporizer) was used. After the surgery, analgesics buprenorphine (0.01-0.05 mg/kg, sq, at the time of surgery and every 8-12 hours) was administered for two days. 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 under anesthetized condition. 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. The percentage of infarct area to total left ventricle area was quantified using SigmaScan Pro (Aspire Software International, Ashburn, VA). Rats with both LVEDP > 15 mmHg and infarct size > 30% of total left ventricular wall were considered to be in HF. 20% animals with surgery were excluded due to LVEDP < 15 mmHg and infarct size < 30% of total left ventricular.

Renal denervation
Four weeks after ligation surgery, rats underwent RDN under anesthesia (ketamine, 48 mg/kg; xylazine 12 mg/kg, ip). Briefly, the kidneys were exposed through a retroperitoneal flank incision. Complete RDN was achieved by cutting both sides of the visible renal nerves from the renal artery and vein, and painting the vessels with 70% ethanol. This method has been shown to ablate the afferent and efferent renal nerves. The experiments were performed at 1 weeks after RDN (5 weeks after the coronary ligation). Renal tissue norepinephrine (NE) content were measured to confirm the completeness of RDN.

Urinary norepinephrine excretion measurements
Urinary NE excretion was measured as an index of overall sympathetic activation. Five weeks after surgery, rats from all groups were placed in metabolic cages and urine was collected for 24 hours. Urinary norepinephrine concentration of thawed samples was measured using a commercially available ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany), following the manufacturer’s instructions. The limit of detection of the assay is 1.5 ng/ml norepinephrine in urine.

Plasma angiotensin II and aldosterone measurements
Five weeks after surgery, blood samples from all groups were collected and plasma angiotensin II and aldosterone was measured using commercially available ELISA kits (angiotensin II: RayBiotech, Norcross, GA; aldosterone: Alpco Diagnostics, Salem, NH). Sensitivity of the angiotensin II assay was 2.62 pg/ml. Sensitivity of the aldosterone assay was 15 pg/ml.

**Immunohistochemistry for FosB and nNOS**

Rats were anesthetized with pentobarbital (150 mg/kg, ip) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde (n = 6/group). The brain was removed and post-fixed at 4°C for 4 hours in 4% paraformaldehyde, and then placed in 30% sucrose for 72 hours. The brain was sectioned (30 μm) in the coronal plane with a cryostat. The sections were blocked with 10% goat serum and then incubated with goat anti-FosB primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for two days. The sections were incubated with biotinylated anti-goat secondary antibody, incubated with avidin-biotin complex (1:200; Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA), stained with DAB solution (Vector Laboratories), and mounted for visualization with light microscopy (100x, Leica, Buffalo Grove, IL). Image J software (NIH) was used to quantify FosB-positive cells in the PVN. The number of nuclei stained with FosB within a 200-μm radius from the center of the PVN was counted, blindly, using the Find Maxima function in Image J, which was validated by manual counting of the nuclei. Four to five sections of the PVN were averaged for each rat.

Immunofluorescence was used to assess localization of nNOS expression in the PVN. PVN sections were obtained as described above, and were incubated with 10% goat serum in 0.02% Triton X. Then, tissues were incubated with anti-rabbit nNOS primary antibodies (1:500, Santa Cruz Biotechnology) overnight at 4°C. After washing, the sections were incubated with Cy3-conjugated goat anti-rabbit (1:500, Jackson ImmunoResearch, West Grove, PA) for 2 hours. After washing, the sections were mounted on slides, and coverslips were placed with fluoromounting-G (SouthernBiotech, Birmingham, AL). Distribution of immunofluorescence within the PVN was viewed using an Olympus fluorescence microscope (Japan) equipped with a digital camera (Qimaging, Canada). Quantification of the intensity of the fluorescence was done using ImageJ.

**NADPH-diaphorase activity as a marker of NOS activity**

PVN sections were obtained from the same animals used in the immunohistochemistry study and collected in 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 1.0 mg/ml β-NADPH. The sections in nitroblue tetrazolium solution were then placed at 37°C for 1 hour. After the reaction, the sections were mounted onto slides. The presence of NADPH-diaphorase in the PVN was examined under a microscope. The density of the staining was evaluated by counting the number of cells that were positively stained for NADPH-diaphorase. The number of NADPH-diaphorase-stained cells was counted at the similar coronal level as FosB staining. Three adjacent sections were considered to represent one coronal level because the numbers of cells counted were within 5% of each other. The number of cells in the middle section was taken to represent the number of cells within a given nucleus.

**Western blot analysis for nNOS protein**

The protein extraction was used for Western blot analysis of nNOS in samples obtained from fresh punched PVN tissue from separate groups of rats (n = 4/group) 5. The sample was loaded onto the 7.5% SDS-PAGE gel for electrophoresis. The fractionated proteins on the gel were
electrophoretically transferred onto the polyvinylidene difluoride membrane. The membrane was incubated with primary antibody (anti-rabbit nNOS antibodies, Santa Cruz Biotechnology, 1:1,000) overnight. Then the membrane was incubated with secondary antibody (goat anti-rabbit IgG, peroxidase conjugated, Pierce, 1:5,000), treated with enhanced chemiluminescence reagent). An enhanced chemiluminescence substrate (Pierce) was applied to the membrane, followed by an exposure within an Epi Chemi II Darkroom (UVP BioImaging, Upland, CA) for visualization with the Worklab digital imaging system. Kodak 1D software was used to quantify the signal. The expression of nNOS was calculated as the ratio of intensity of the nNOS band, respectively, relative to the intensity of the β-actin band.

**General surgical procedures**

5 weeks after the coronary ligation, each rat was anesthetized by an injection of urethane (0.75 g/kg ip) and α-chloralose (70 mg/kg ip) (n = 5/group). The right femoral artery and femoral vein were cannulated for the recording of arterial blood pressure and administration of chemicals, respectively. Mean arterial pressure (MAP) and heart rate (HR) were simultaneously recorded on a PowerLab data-acquisition system (8SP, AD Instruments).

**Lumbar sympathetic nerve activity recording**

5 weeks after the coronary ligation, rats were anesthetized with α-chloralose (70 mg/kg, ip) and urethane (0.75 g/kg, ip). The lumbar sympathetic nerve was isolated through a ventral midline incision. The lumbar nerve was isolated and placed on a bipolar platinum electrode. The electrical signal from the electrode was amplified with a Grass amplifier with a high and low frequency cutoff of 1000 Hz and 100 Hz. The signal was recorded with the MacLab. Efferent lumbar sympathetic nerve activity (LSNA) at the beginning of the experiment was defined as basal nerve discharge. The LSNA recorded at the end of the experiment (after the rat was injected with hexamethonium, 30 mg/kg, iv) was defined as background noise. The value of LSNA was calculated by subtracting the background noise from the actual recorded value. The changes in integration and frequency of the nerve discharge during the experiment were subsequently expressed as a percentage from basal value.

**L-NMMA microinjection into the PVN**

Anesthetized rats were placed in a stereotaxic apparatus for microinjections into the PVN. A longitudinal incision was made on the head to expose the bregma and a small burr hole was made in the skull to access the PVN. A thin needle (0.2 mm, OD) connected to a 0.5 μl microsyringe (Hamilton, Reno, NV) was lowered into the PVN. LSNA, MAP, and HR were recorded before and after microinjection of 100-200 pmol (50-100 nl) of L-NMMA dissolved in artificial spinal fluid (aCSF) into the PVN. The injections were randomized and given at 30-45 min intervals. Vehicle injections of aCSF were given to control for volume responses. At the completion of the experiment, monastral blue dye (2% Chicago blue, 30nl) was injected into the brain for histological verification (Figure S1). Sympatho-excitation after drug injection was calculated as percent change in LSNA from baseline and as absolute changes in MAP and HR from baseline. Baseline and peak responses were averaged over a 30-second time interval.

** Extracellular single-unit recordings in the PVN**

5 weeks after the coronary ligation, rats were placed in a stereotaxic apparatus. The stereotaxic coordinates for the PVN were determined according to Paxinos and Watson’s atlas 6. Typically,
three tracks were explored for extracellular recording in each rat, from -1.4 to -2.1 mm caudal to the bregma, 0.4 mm lateral (right side) to the midline, and with a depth of 7.4-8.6 mm ventral to the dorsal surface. Extracellular single-unit recording was carried out using a single micropipette (resistance: 5-15M) filled with 0.5 M sodium acetate containing 2% pontamine sky blue. The glass micropipettes were advanced using a microdrive controller (Type 860, Hugo Sachs Elektronik) into the PVN. The spontaneous activity of neurons was amplified (gain: 1,000) with an alternating current/direct current differential amplifier (model IX1, Dagan) with a low-frequency cutoff at 30 Hz and a high frequency cutoff at 3 kHz. Neuronal discharge was recorded on a PowerLab data-acquisition system (8/30, AD Instruments). The frequency of the neuronal discharge was analyzed with special software (SpikeHistogram, AD Instruments). PVN-RVLM neurons were identified by antidromic stimulation and collision test as previously described.

Histology of recording sites within the PVN
At the end of the experiment, pontamine sky blue was iontophoresed (-15 μA, 10 min) to mark the site of the last recorded neuron, and other recording sites were extrapolated from the marked point according to Paxinos & Watson’s atlas. Then, the rat was euthanized with an overdose of anesthesia. The rat brains were removed fresh, fixed in the 4% formalin and sectioned. The dye spots for recording sites in the PVN and the sites of electrolytic lesion in the RVLM were identified with a light microscope. Rats whose recording sites were within the boundaries of the PVN were used for data analysis. The location of the center of the dye spot was transferred to a histological map according to Paxinos & Watson’s atlas (Figure S2).

Stimulation of afferent renal nerve
To stimulate ARN, the left kidney was exposed through a left retroperitoneal flank incision. A branch of the renal nerve was isolated through a retroperitoneal incision under an operating microscope. 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 gel. The stimulus to ARN consisted of brief trains of stimuli (3-5 pulses at 200 Hz, 2-3 ms pulse durations, constant current 0.2 mA) applied once every 2 s as per previous studies. Onset latency of response to the stimulation of the ARN was determined as the time interval from the stimulus artifact to a 30% change with respect to control in discharge frequency on peristimulus time histograms. This criterion was based on previous studies from other laboratories.

Reference:


S1: 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. Filled circle represents the site of termination of an injection that is considered to be within the PVN region in sham group; “+” represents that in HF group. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus. Bar = 5mm.
S2: a-c Approximate locations of neurons that were antidromically activated from the rostral ventrolateral medulla (RVLM). The distance (in mm) posterior to bregma is shown for each section. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus.