Gene Transfer of Neuronal Nitric Oxide Synthase to the Paraventricular Nucleus Reduces the Enhanced Glutamatergic Tone in Rats With Chronic Heart Failure

Hong Zheng, Xuefei Liu, Yifan Li, Neeru M. Sharma, Kaushik P. Patel

Abstract—Our previous studies have shown that the decreased NO and increased glutamatergic mechanisms on sympathetic regulation within the paraventricular nucleus (PVN) may contribute to the elevated sympathoexcitation during chronic heart failure (CHF). In the present study, we investigated the effects of neuronal NO synthase (nNOS) gene transfer on N-methyl-d-aspartic acid receptor subunit NR1 in the rats with a coronary ligation model of CHF. Adenovirus vectors encoding nNOS (AdnNOS) or adenovirus vectors encoding β-galactosidase were transfected into the PVN in vivo. Five days after application of AdnNOS, the increased expression of nNOS within the PVN was confirmed by NADPH-diaphorase staining, real-time PCR, and Western blot. In anesthetized rats, AdnNOS treatment significantly enhanced the blunted renal sympathetic nerve activity, blood pressure, and heart rate responses to NO synthase inhibitor N²-monomethyl-L-arginine in the rats with CHF compared with CHF-adenovirus vectors encoding β-galactosidase group. AdnNOS significantly decreased the enhanced renal sympathetic nerve activity, blood pressure, and heart rate responses to N-methyl-d-aspartic acid in the rats with CHF (renal sympathetic nerve activity: 44%±2% versus 79%±6%; P<0.05) compared with CHF-adenovirus vectors encoding the β-galactosidase group. AdnNOS transfection significantly reduced the increased NR1 receptor mRNA expression (Δ35%±5%) and protein levels (Δ24%±4%) within the PVN in CHF rats. Furthermore, in neuronal NG-108 cells, NR1 receptor protein expression decreased in a dose-dependent manner after AdnNOS transfection. According to our results, nNOS downregulation enhances glutamate transmission in the PVN by increasing NR1 subunit expression. This mechanism may enhance renal sympathetic nerve activity in CHF rats. (Hypertension. 2011;58:966-973.)

Key Words: glutamatergic ■ sympathetic activity ■ blood pressure ■ heart failure

Increased sympathetic nerve activity is a characteristic symptom of chronic heart failure (CHF). The elevated sympathetic activity induces vasoconstriction and an increase in peripheral resistance that increases cardiac afterload. In addition, peripheral vasoconstriction and sodium and water retention lead to increased cardiac preload. All of these aggravate the morbidity and raise the mortality in the CHF. Peripheral adrenergic blockade cannot completely eliminate the state of sympathoexcitation. Recent studies have suggested that altered central mechanism(s) may be responsible for these impaired reflex regulations and may contribute to the elevated neurohumoral drive in CHF.

The paraventricular nucleus (PVN) of the hypothalamus is an important central site for the integration of sympathetic nerve activity. Using retrograde tracing techniques, the studies have shown that the PVN is a major source of forebrain input to the sympathetic nervous system. In the PVN, a number of neurotransmitters, excitatory and inhibitory, converge to influence its neuronal activity. Among them are NO and glutamate. Previously we have shown that the increased activity of PVN neurons associated with CHF is attributed to an increase in glutamatergic mechanism and a decrease in NO mechanism within the PVN.

The interaction of NO and the glutamate receptor is important for neuronal development and function. The NO system acts as a negative feedback system for the excitatory glutamatergic system. Glutamate through N-methyl-d-aspartic acid (NMDA) receptors activates NO synthase (NOS) in neurons and induces an increase in NO production. On the other hand, NO elicits a regulatory effect on glutamate receptor activity. In the PVN, NO and glutamate interact to regulate neuronal function. NO inhibits NMDA-mediated increases in the renal sympathetic nerve activity (RSNA) within the PVN. This indicates a short loop inhibition by NO of excitation by NMDA receptor activation to increase RSNA within the PVN. This may be an important interaction in dictating sympathetic outflow in CHF known to have altered NOS
and glutamate activity in the PVN with a concomitant increase in basal sympathetic tone.

The purpose of the present study was to test the effect of the interaction between NO and the NMDA-glutamate receptor in the PVN on the subsequent regulation of sympathetic nerve activity and cardiovascular responses in CHF. We hypothesize that restoration of neuronal NOS (nNOS) with adenoviral gene transfer ameliorates the NMDA receptor and subsequently the excitatory mechanisms within the PVN. This approach has the potential to elucidate the mechanism(s) responsible for the increased neurohumoral drive in the CHF state.

**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 (SASCO, NE) weighing 200 to 220 g were assigned to 4 groups (sham-adenovirus vectors encoding β-galactosidase [AdβGal], CHF-AdβGal, sham-adenovirus vectors encoding nNOS [AdnNOS], and CHF-AdnNOS).

CHF was produced by left coronary artery ligation, as described previously. Echocardiograms were performed after 6 to 7 weeks of ligature surgery. Rats with elevated left ventricular end-diastolic pressure (>15 mm Hg), infarct size >30% of total left ventricle wall, significant reductions in dp/dtmax, and ejection fraction (>40%) were considered to be in CHF.

**Adenovirus Transfection in the PVN**

Seven to 8 weeks after cardiac surgery, each rat was anesthetized. A 200-nL solution (1 × 10⁹ pfu/mL) of AdnNOS or AdβGal was injected into the PVN. With a concentration of 1 × 10⁹ pfu/mL, no damage to the neurons within the PVN was observed from light microscopic evaluation. These adenovirus vectors were generously provided by Channon and colleagues at Oxford.19,20

**General Surgery for Hemodynamic, RSNA Measurement, and Microinjection**

Five days after viral injection, the rat was anesthetized with urethane (750 mg/kg, IP) and α-chloralose (70 mg/kg, IP) and instrumented for recording arterial pressure (AP) and heart rate (HR), as described previously. The changes in integration of the nerve discharge during the experiment were expressed as a percentage from basal value. An NOS inhibitor N⁶-monomethyl-L-arginine or NMDA was injected into the PVN in 3 doses (50, 100, and 200 pmol in 100 nL) in random order. The responses in RSNA, mean AP (MAP), and HR over 30 minutes were recorded.

**NADPH-Diaphorase Activity as a Marker of NOS Activity**

The rat was perfused with heparinized saline followed by 4% paraformaldehyde. The brain sections in nitroblue tetrazolium solution were then placed in an oven at 37°C for 1 hour. 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.

**Micropunch of the PVN**

In the other 4 groups of rats (sham-AdβGal, CHF-AdβGal, sham-AdnNOS, and CHF-AdnNOS), after the rats were euthanized, the brains were removed and frozen at −80°C. The PVN were punched bilaterally with a blunt needle (ID: 0.5 mm). The punched tissue was put in TRI reagent (Molecular Research Center Inc) or protein extraction buffer.

**Real-Time RT-PCR for the Measurement of nNOS and NR1 Receptor mRNA**

Total RNA extracted from the punched tissue was subjected to reverse transcription. The cDNA was amplified by real-time quantitative RT-PCR with the BioRad iCycler IQ system (Biorad Laboratories). Relative mRNA expression of nNOS or NR1 receptor was calculated using the Pfaffl equation which relates expression of the target gene to expression of a reference gene (ribosomal protein L19, rp19).

**Western Blot Assay of nNOS and NR1 Receptor Protein**

The punched tissues were incubated with protein lysis buffer. Then the fractionized proteins on the gel were electrophoretically transferred onto the polyvinylidene fluoride membrane. The membrane was probed with primary nNOS and NR1 receptor antibodies. The signals were visualized using an enhanced chemiluminescence substrate (Pierce) and detected by digital image system (UVP Biolaging). The expression of nNOS or NR1 receptor protein was calculated as the ratio of intensity of the nNOS or NR1 receptor band relative to the intensity of GAPDH band.

**Cell Culture**

The NG108-15 (neuroblastoma × glialoma) hybrid cells were grown in DMEM. The cells were then treated with AdnNOS, in a dose-dependent manner (10⁴ to 10⁸ pfu/mL) for 24 hours. Lysates (30–40 µg) were processed to measure nNOS and NR1 receptor protein by Western blot.

**Immunofluorescent Staining of NG108 Cells**

Adherent NG108 cells were fixed and incubated with primary nNOS and NR1 receptor. Secondary antibody consisted of Cy2-conjugated donkey antinoise IgG, and Cy3-conjugated antigoat was used. Labeled cells were visualized by Olympus fluorescence microscope equipped with digital camera.

**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 post hoc analysis of significance (Statview II, Abacus Inc, Berkeley, CA). P<0.05 was considered statistically significant.

An expanded Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

**Results**

**General Data**

Table S1 (available in the online Data Supplement) summarizes the salient morphological and hemodynamic characteristics of rats used in the present study. Any rats subjected to coronary artery ligation that displayed myocardial infarcts <30% of the left ventricular wall were excluded from the study (5 of 40 rats with coronary artery ligation surgery). The infarction area in CHF group was ∼40% of the endocardial surface. Sham rats had no observable damage to the myocardium. Heart weight was significantly greater in CHF rats than in sham rats. Left ventricular end-diastolic pressure was significantly elevated in CHF rats compared with sham rats. AdnNOS injections did not change the left ventricular end-diastolic pressure and infarcted size in both sham and CHF groups.

Basal MAP, HR and RSNA are also presented in Table S1. Although the level of raw RSNA in rats with CHF trends to be higher than in sham-operated rats, it did not reach statistical significance. Because an increase in RSNA and norepinephrine is present in conscious rats with CHF, it
would seem possible that the anesthetics may affect the expression of the increase in RSNA. Also, it is not strict to reliably compare multifiber sympathetic nerve activity recordings between groups of rats because of differences in numbers of the fibers on the electrode and damage to the fibers being recorded. There were no statistically significant differences in basal MAP or HR between the sham and CHF groups. AdnNOS injections did not change the basal MAP, HR, and RSNA in both sham and CHF groups.

**Adenoviral Gene Transfer of nNOS Within the PVN**

We evaluated the efficacy of AdnNOS gene transfer in the PVN by comparing the NADPH-diaphorase staining of the PVN. An example of the differences in staining of the infected versus uninfected PVN is shown in Figure 1A. There was a significant increase in the number of diaphorase-positive cells in the AdnNOS-infected PVN compared with the contralateral uninfected PVN in sham (increased 53%) and CHF (increased 136%) groups (*P<0.05* vs sham group. *#P<0.05* vs noninfected contralateral PVN).

We also evaluated the efficacy of AdnNOS gene transfer in the PVN by comparing nNOS mRNA and protein levels in the PVN. There was a significant increase in the mRNA (Figure 2A) and intensity of the protein bands of nNOS (Figure 2B) in the AdnNOS-infected PVN compared with the AdβGal-infected sham and CHF groups (*P<0.05*). AdβGal-injected groups demonstrated no significantly increased diaphorase-labeled cells in the injected side of the PVN in both the sham and CHF groups (Figure 1B).

We also evaluated the efficacy of AdnNOS gene transfer in the PVN by comparing nNOS mRNA and protein levels of the PVN. There was a significant increase in the mRNA (Figure 2A) and intensity of the protein bands of nNOS (Figure 2B) in the AdnNOS-infected PVN compared with the AdβGal-infected sham and CHF groups (*P<0.05*). AdβGal-injected groups demonstrated no significantly increased diaphorase-labeled cells in the injected side of the PVN in both the sham and CHF groups (Figure 1B).

**Effects of Microinjection of NMDA Into the PVN on RSNA, AP, and HR**

RSNA, MAP, and HR responses to microinjection of NMDA (200 pmol) into the PVN were significantly potentiated (RSNA: 79±6% versus 37±4%; MAP: 19.2±1.6 versus 13±2.8 mm Hg; HR: 46.8±6.7 versus 19.0±4.8 bpm; *P<0.05*) in the rats with CHF compared with sham rats. AdnNOS significantly decreased the enhanced RSNA, MAP, and HR responses to NOS inhibitor Nω-monomethyl-L-arginine in the rats with CHF compared with the CHF-AdβGal group (Figure 3).

**Figure 1.** A, NADPH-diaphorase–labeled neurons in the paraventricular nucleus (PVN) of 4 groups of rats: sham-adenovirus vectors encoding β-galactosidase (AdβGal), chronic heart failure (CHF)-AdβGal, sham-adenovirus vectors encoding neuronal NO synthase (AdnNOS), and CHF-AdnNOS. Right side of the PVN is the viral infected side. Left side is the noninfected side. B, Number of NOS-positive cells in the PVN in 4 groups of rats: sham-AdβGal, CHF-AdβGal, sham-AdnNOS, and CHF-AdnNOS. Values represent mean±SE. *P<0.05* vs sham group. *#P<0.05* vs noninfected contralateral PVN.

**Figure 2.** A, Mean data of relative mRNA expression of neuronal NO synthase (nNOS) to rpl19 mRNA in the punched paraventricular nucleus (PVN) tissues measured by real-time RT-PCR. B, Example of visualized bands of nNOS and GAPDH protein. Mean data of band densities of nNOS normalized by GAPDH. *P<0.05* vs sham group. *#P<0.05* vs adenovirus vectors encoding β-galactosidase (AdβGal)–injected group.
MAP, and HR responses to NMDA in the rats with CHF (RSNA: 44±2% versus 79±6%; MAP: 13.2±2.0 versus 19.2±1.6 mm Hg; HR: 21.6±3.8 versus 46.8±6.7 bpm; \( P<0.05 \)) compared with the CHF-AdβGal group (Figure 4B). In the sham-AdnNOS group, AdnNOS injection also decreased RSNA significantly. However, there were no significant changes of MAP and HR in the sham group after viral transfer. AdβGal demonstrated no significant effects on the response of RSNA, MAP, and HR to the NMDA in both the sham and CHF groups (Figure 4).

**Measurements of NR1 Receptor Expression in the PVN**

Result of real-time RT-PCR experiments indicated that NR1 receptor mRNA expression in the punched PVN tissues from the CHF rats was significantly increased compared with sham rats (Figure 5A). However, in the CHF-AdnNOS group, relative NR1 receptor expression was significantly lower than CHF-AdβGal and not different from the sham-AdβGal or sham-AdnNOS groups. Consistent with these results, Western blot showed that NR1 receptor protein levels were also significantly higher in CHF rats compared with sham rats (Figure 5B). In the CHF-AdnNOS group, relative NR1 receptor expression was significantly lower than CHF-AdβGal and not different from the sham-AdβGal or sham-AdnNOS groups. Sample gels showing NR1 receptor and GADPH protein in the 4 experimental groups are presented in Figure 5B.

**nNOS and NR1 Receptor Expression in NG108 Cell Line**

To determine the effects of overexpression of nNOS on the regulation of NR1 receptor expression in vitro, neuronal NG108 cells were treated with AdnNOS or AdβGal. The changes in nNOS and NR1 receptor protein expression were analyzed by Western blot after 24 hours of viral transfection (Figure 6A). NR1 receptor protein expression was downregulated significantly even at the lower concentrations of AdnNOS compared with the control (without virus). The highest dose showed an \( \sim 50\% \) decrease in NR1 receptor protein expression compared with the control dose.

Using immunofluorescent staining, nNOS and NR1 receptor interaction and their subcellular localization were studied
in the NG108 cells (Figure 6B). The intensity of nNOS staining was increased, whereas NR1 receptor staining was decreased with AdnNOS treatment. The AdnNOS-treated cell shows more nNOS and NR1 receptor colocalization in the cytoplasm as compared with the control (AdβGal-treated cells).

**Discussion**

In the present study, we observed that, in rats with CHF, AdnNOS normalizes the potentiated increase in RSNA, AP, and HR in response to microinjection of NMDA into the PVN, accompanied by downregulated NR1 receptor message and protein in the PVN. The results indicate that the effects of endogenous nNOS on the glutamatergic mechanism within the PVN may play an important role in the altered balance and tone of sympathetic outflow in the CHF condition.

The AdnNOS and AdβGal used in the present study were constructed in Dr Channon’s laboratory at Oxford. Their findings demonstrate AdnNOS to be a versatile and efficient tool for nNOS gene transfer in vascular cells and gene therapy. They also demonstrated that upregulation of nNOS via gene transfer provided a novel method for increasing cardiac vagal function. We have demonstrated the efficacy of adenoviral gene transfer of nNOS into cells of the PVN of rats by using the same AdnNOS. It should be noted that this viral upregulation of nNOS is not specifically targeted to just preautonomic neurons in the PVN. As a consequence, this upregulation occurs in other neuronal types, as well as in the glia and vascular cells in the PVN. Because we were examining the effects on RSNA, we are attributing the changes to effects on the preautonomic neurons in the PVN. AdnNOS infects cells in the PVN and leads to a functional effect on RSNA mediated by the PVN. The results provide a novel approach to restore neuronal levels of NOS, thus providing a potentially important candidate gene for cardiovascular gene therapy in disease states, such as CHF and hypertension, lacking central nNOS.

The PVN is one of the major central nervous sites that directly controls sympathetic outflow. CHF is characterized by elevated systemic sympathetic activity and salt and water retention, and both involve the function of the PVN. We have found significantly increased hexokinase activity, an index of neuronal activity, in the parvocellular PVN and magnocellular PVN of rats with CHF compared with the control rat.

A study has confirmed this finding in the same model, using Fos related-antigen–like activity at 2 and 4 weeks after coronary occlusion. This has also been confirmed more recently by Zhang et al by direct recording of
increased firing of neurons within the PVN in rats with myocardial infarction.

Inhibitory mechanism of sympathetic regulation within the PVN via NO\(^10\) was reduced, whereas the excitatory mechanism-regulated NMDA NR\(_1\) receptor was enhanced\(^8\) in CHF rats. These alterations may induce an imbalance of the inhibitory and excitatory mechanisms in this area and influence sympathetic outflow. We have shown that there is an interaction between the glutamatergic system and the NO system within the PVN.\(^{13}\) It appears that the NO system acts as a negative feedback system for the excitatory glutamatergic system. Because both the NO and NMDA systems are altered in CHF, it remains to be examined whether the link between the glutamatergic system and the NO system is altered in CHF leading to sympathoexcitation.

In the present study, AdnNOS significantly decreased the enhanced RSNA, BP, and HR responses to NMDA in the rats with CHF. These observations support the contention that an overexpression of nNOS within the PVN may be responsible for the increased suppression of sympathetic outflow. The endogenous NO-mediated effect in the PVN of AdnNOS-treated rats is more effective in suppressing RSNA compared with Ad\(\beta\)Gal-treated rats. Meanwhile, we also found that overexpression of nNOS into the PVN suppressed the sympathetic activity response to NMDA in sham rats but less than in CHF rats. The explanation for this is that AdnNOS transfer is more effective on sympathoinhibition in rats with CHF lacking central nNOS. Overexpression of NOS in the central nervous system attenuated sympathoexcitation in the CHF state. Using the CHF mice model, Sakai et al\(^{31}\) transferred adenoviral vectors encoding either endothelial NOS or Ad\(\beta\)Gal into the nucleus tractus solitarius to examine the effect of increased NO production in the nucleus tractus solitarius on the enhanced sympathetic drive in CHF. After the gene transfer, they found that urinary norepinephrine excretion was reduced in adenoviral vectors encoding endothelial NOS-transfected CHF mice, and overexpression of eNOS in the nucleus tractus solitarius attenuates the enhanced sympathetic drive in this model.

NMDA NR\(_1\) receptor mRNA expression and protein level in the PVN are significantly increased in CHF,\(^9\) which may contribute to the elevated sympathoexcitation during CHF. In the CHF state, many central and peripheral humoral factors are significantly altered. The upregulation of NMDA receptor and the subsequent increase in glutamate activity within the PVN may be part of the compensatory responses. NO has been recognized as a factor eliciting a wide-range regulation of gene expression.\(^{32}\) We have shown that the numbers of NOS-positive cells in the PVN were decreased in rats with CHF.\(^{30}\) In the present study, we found that NO may elicit an inhibitory action on the NMDA NR\(_1\) receptor to produce an inhibition of sympathetic nerve activity in CHF states. In an isolated cell culture system, incubation of AdnNOS with NG108 cells, which have been known to have both NOS and NR\(_1\) receptors,\(^{33,34}\) causes a dose-dependent decrease in the expression of the NR\(_1\) receptor protein. These data are consistent with the idea that increased nNOS in rats with CHF may have caused the decrease in NR\(_1\) receptor protein expression within the PVN of rats with CHF.

Bains et al\(^{11,35}\) using whole cell recording from the hypothalamic slice preparation in rats, observed that application of NO increased NMDA-induced inhibitory postsynaptic potentials. Conversely, blocking NO with \(\text{L}-\text{nitro-L-arginine methyl ester}\) elicited more pronounced NMDA-induced depolarization but no accompanying increase in inhibitory postsynaptic potentials. These results provide evidence suggesting that there is a negative feedback mechanism between the NO and glutamate systems within the PVN. Our results suggest that there is an ongoing endogenous inhibitory NO mechanism that opposes the excitatory NMDA mechanism in the PVN. With regard to the effect of NO on NMDA receptors, some recent studies\(^{35}\) have shown that, as a radical molecule, NO and its derivative can redox glutamate-NMDA receptors and reduce the activity of the receptors, which are believed to be one of mechanisms of modulation of NO on the nervous system. The chemical reactions of NO are largely dictated by its redox state. Recent data suggest that NO can react with critical sulphydryl group(s) of the NMDA receptor to downregulate its activity.\(^{36}\)
Perspectives

We have demonstrated that induction of nNOS within the PVN of rats with CHF not only ameliorates NO-mediated sympathoexcitation but also improves glutamate-mediated sympathoexcitation that is observed in rats with CHF. The ability of AdnNOS to cause an increase in nNOS within the PVN appears to be primarily responsible for the downregulation of NR1 receptors and consequent attenuation of enhanced responses to glutamatergic tone. The findings underscore the importance of targeting nNOS within the central sites, such as the PVN, to attenuate the sympathoexcitation and subsequent cardiovascular risk in conditions with enhanced sympathoexcitation, such as CHF and hypertension.

Sources of Funding

This work was supported by the National Institutes of Health program grant HL62222.

Disclosures

None.

References


Gene Transfer of Neuronal Nitric Oxide Synthase to the Paraventricular Nucleus Reduces the Enhanced Glutamatergic Tone in Rats With Chronic Heart Failure
Hong Zheng, Xuefei Liu, Yifan Li, Neeru M. Sharma and Kaushik P. Patel

Hypertension. 2011;58:966-973; originally published online October 3, 2011;
doi: 10.1161/HYPERTENSIONAHA.111.176222
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/58/5/966

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/10/04/HYPERTENSIONAHA.111.176222.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Gene Transfer of nNOS to the Paraventricular Nucleus Reduces the Enhanced Glutamatergic Tone in Rats with Chronic Heart Failure

Hong Zheng¹, Xuefei Liu¹, Yifan Li², Neeru M. Sharma¹, and Kaushik P. Patel¹
¹Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE 68198-5850; ²Division of Basic Biomedical Science, College of Medicine, University of South Dakota, Vermillion, SD 57069

Address for correspondence: Dr. Kaushik P. Patel Ph.D.
Department of Cellular and Integrative Physiology
University of Nebraska Medical Center
985850 Nebraska Medical Center
Omaha, NE 68198-5850
Phone: (402) 559-8369
Fax: (402) 559-4438Email: kpatel@unmc.edu
Methods

General Surgery for Hemodynamic, RSNA Measurement and Microinjection

On the day of the experiment (5 days after viral injection), the rat was anesthetized with urethane (0.75g/kg, i.p.) and α-chloralose (70mg/kg, i.p.). The left femoral vein was cannulated with polyethylene tubing for injection of supplemental anesthesia. The left femoral artery was cannulated and connected via a pressure transducer (Gould P23 1D) to a computer-based data recording and analyzing program (PowerLab) to record mean arterial blood pressure (MAP) and heart rate (HR).

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 MAP and HR were expressed as the difference between the basal value and the value after each dose of a drug.

For placement of microinjection cannulas into the PVN, the anesthetized rat was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A longitudinal incision was made on the head and bregma was exposed. A small burr hole was made in the skull to allow access to the PVN. The coordinates for the PVN, determined using the Paxinos and Watson atlas, were 1.5mm posterior to bregma, 0.4mm lateral to midline, and 7.8mm ventral to the dura. A thin needle (0.2mm OD) connected to a 0.5µl microsyringe (Hamilton) was lowered into the PVN. An inhibitor of NOS, L-NMMA, or NMDA was injected into the PVN in three doses (50, 100, and 200pmol) in random order. Subsequent injections were made at least 20min after prior injections to allow MAP, HR, and RSNA to return to basal levels. In a separate group rats (n=5), the vehicle control, 100nl of artificial cerebrospinal fluid (aCSF) was microinjected into the PVN. The effects on RSNA, MAP and HR were observed.

In a separate group of rats, the vehicle control, 100nl of artificial cerebrospinal fluid (aCSF) was microinjected into the PVN and RSNA, MAP and HR were monitored. The vehicle control, 100nl of aCSF microinjected into the PVN, had no effects on RSNA, MAP and HR. At the end of the experiment the brains were subjected to histological evaluation to determine the injection sites. Injections within the boundaries of the PVN were used in the data presented. Typically injections outside the boundaries
of the PVN did not produce any changes in RSNA, MAP and HR. The spread of the dye was typically within the boundaries of the PVN.

**NADPH-diaphorase Activity as a Marker of NOS Activity**

The rat was perfused through the left ventricle of the heart with heparinized saline followed by 4% paraformaldehyde. The brain sections of 30µm were cut with a cryostat. Every third section was kept from the anterior commissure (0.4mm posterior to bregma) posterior to where the optic tracts were observed to be in their most lateral position on the ventral surface of the brain (2.6mm posterior to bregma). The sections were collected in 0.1M phosphate, containing 0.3% Triton X-100, 0.1mg/ml nitroblue tetrazolium and 1.0mg/ml β-NADPH. The sections were placed in an oven at 37°C for 1hr. Following the reaction, the sections were rinsed in phosphate buffer and 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.

**Real-time RT-PCR for the Measurement of nNOS and NR1 Receptor mRNA**

Total RNA extracted from the punched tissue was subjected to reverse transcription. The cDNA was amplified by real-time quantitative RT-PCR with the BioRad iCycler IQ system (Biorad Laboratories). All primer pairs were designed using BeaconDesign 4.0 (Biorad Laboratories). For nNOS (GenBank: NM 052799), the sense primer was 5’-GCGGAGCAGAGCGGCCTTAT-3’, the antisense primer was 5’-TTTGGTGGGAGGACCAGGGG-3’; for NR1 receptor (GenBank: NM 017010), the sense primer 5’-ATAGTGACAATCCACCAAGAGCC-3’, the antisense primer was 5’-GTAGCTCGCCCCATCATTTCCGT-3’; for rpl19 (GenBank: NM 031103), the sense primer 5’-CCCCAATGAAACCAACGAAA-3’, the antisense primer was 5’-ATGGACAGTCAGGCTTC-3’. Relative mRNA expression of nNOS or NR1 receptor was calculated using the Pfaffl equation relates expression of the target gene to expression of a reference gene (rpl19).

**Western Blot Assay of nNOS and NR1 Receptor Protein**

The punched tissues were incubated with lysis buffer (10mmol/L Tris, 1mmol/L EDTA, 1% sodium dodecyl sulfate, 0.1% Triton X-100 and 1mmol/L phenylmethanesulfonylfluoride). Samples were loaded on the 7.5% SDS-PAGE gel to be subjected to electrophoresis. Then the fractionized proteins on the gel were electrophoretically transferred onto the PVDF membrane. The membrane was probed with primary antibodies (rabbit anti-nNOS, NR1 receptor and GAPDH antibody, Santa Cruz, CA) overnight at 4°C. The membrane was incubated with a peroxidase-conjugated secondary antibody (Pierce). The signals were visualized using an enhanced chemiluminescence substrate (Pierce) and detected by digital image system (UVP BiolImaging). The signals were quantified by Kodak 1D software (Eastman Kodak Company). The expression of nNOS or NR1 receptor protein was calculated as the ratio of intensity of the nNOS or NR1 receptor band relative to the intensity of GAPDH band.

**Cell Culture**
The NG108-15 (neuroblastoma X glioma) hybrid cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G and streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 Cells were seeded in 6 well plates and grown until 60-70% confluent before treatment with indicated concentrations of with AdnNOS, in a dose-dependent manner (10^4~10^8 pfu/ml, 24hrs) for 24hrs. Cells were homogenized in lysis buffer (10mmol/L Tris, 1mmol/L EDTA, 1% sodium dodecyl sulfate, 0.1% Triton X-100 and 1mmol/L phenylmethanesulfonfylfluoride). Lysates (30-40μg) were processed to measure nNOS and NR1 receptor protein by Western blot.

Immunofluorescent Staining of NG108 cells

Adherent NG108 cells were grown on laminin coated 6mm Transwel-Clear™ inserts (Corning, Costar) overnight. Cells were fixed and then permeabilized with 0.2% Triton X-100 for 20min. 10% normal donkey serum was used for blocking for 1hr followed by incubation with primary antibody at 4°C overnight. All antibodies used were diluted in 1% blocking solution. For nNOS and NR1 receptor double immunostaining, dilutions of 1:200 for mouse anti-nNOS and 1:200 for goat anti-NR1 receptor were used. Secondary antibody consisted of a 1:200 dilution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated anti-goat was used for 2hrs. Coverslips were then mounted onto slides using Fluoromount G (Southern Biotechnology). Labeled cells were visualized by Olympus fluorescence microscope equipped with digital camera.
## Results

**Table S1: Baseline values of morphology and hemodynamics in rats with heart failure and sham-operated rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham-AdβGal (n=10)</th>
<th>CHF-AdβGal (n=10)</th>
<th>Sham-AdnNOS (n=10)</th>
<th>CHF-AdnNOS (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>372±16</td>
<td>396±21</td>
<td>367±19</td>
<td>391±12</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.1±0.1</td>
<td>1.8±0.3*</td>
<td>1.2±0.1</td>
<td>1.9±0.3*</td>
</tr>
<tr>
<td>Infarct size (% of epicardial LV)</td>
<td>0</td>
<td>41±6*</td>
<td>0</td>
<td>42±7*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2±2</td>
<td>26±2*</td>
<td>3±2</td>
<td>23±4*</td>
</tr>
<tr>
<td>Basal mean blood pressure (mmHg)</td>
<td>92±5</td>
<td>87±3</td>
<td>89±3</td>
<td>86±4</td>
</tr>
<tr>
<td>Basal heart rate (bpm)</td>
<td>329±18</td>
<td>359±39</td>
<td>336±19</td>
<td>351±21</td>
</tr>
<tr>
<td>Basal integrate RSNA (µV.s)</td>
<td>4.3±0.7</td>
<td>5.4±0.2</td>
<td>4.1±0.1</td>
<td>4.8±0.4</td>
</tr>
</tbody>
</table>

Values are presented as mean±SE; * indicates $P<0.05$ versus sham rats. LV: left ventricle; wt: weight.