Contribution of Central Nervous System Endothelial Nitric Oxide Synthase to Neurohumoral Activation in Heart Failure Rats

Vinicia C. Biancardi, Sook J. Son, Patrick M. Sonner, Hong Zheng, Kaushik P. Patel, Javier E. Stern

Abstract—Neurohumoral activation, a hallmark in heart failure (HF), is linked to the progression and mortality of HF patients. Thus, elucidating its precise underlying mechanisms is of critical importance. Other than its classic peripheral vasodilatory actions, the gas NO is a pivotal neurotransmitter in the central nervous system control of the circulation. While accumulating evidence supports a contribution of blunted NO function to neurohumoral activation in HF, the precise cellular sources, and NO synthase (NOS) isoforms involved, remain unknown. Here, we used a multidisciplinary approach to study the expression, cellular distribution, and functional relevance of the endothelial NOS isoform within the hypothalamic paraventricular nucleus in sham and HF rats. Our results show high expression of endothelial NOS in the paraventricular nucleus (mostly confined to astroglial cells), which contributes to constitutive NO bioavailability, as well as tonic inhibition of presynaptic neuronal activity and sympathoexcitatory outflow from the paraventricular nucleus. A diminished endothelial NOS expression and endothelial NOS-derived NO availability were found in the paraventricular nucleus of HF rats, resulting, in turn, in blunted NO inhibitory actions on neuronal activity and sympathoexcitatory outflow. Taken together, our study supports blunted central nervous system endothelial NOS-derived NO as a pathophysiological mechanism underlying neurohumoral activation in HF. (Hypertension. 2011;58:454-463.)

Key Words: heart failure ■ NO ■ hypothalamus ■ astrocyte ■ sympathetic

In addition to its classic peripheral vasodilatory actions, the gas NO is a key neurotransmitter within the central nervous system (CNS), particularly in regions involved in the neurohumoral control of the circulation, including the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei. Constitutively produced NO tonically inhibits neurosecretory and preautonomic neuronal activity, restraining, in turn, sympathohumoral outflow to the circulation. Importantly, blunted CNS NO function is linked to neurohumoral activation in heart failure (HF). Despite this evidence, the precise cellular sources and NO synthase (NOS) isoforms involved remain unknown. Given the abundance of NO-producing neurons within the SON and PVN, it is generally implicit that constitutive NO arises from a neuronal (nNOS) source. This, however, has not been compellingly demonstrated, given that most studies were based on the use of nonselective NOS blockers. An alternative source of constitutive NO is the endothelial NOS (eNOS), shown recently to influence the CNS control of the circulation. Different from nNOS, eNOS is localized within endothelial cells in brain capillaries, although recent evidence supports eNOS expression in astrocytes as well. Because eNOS can synthesize NO in a sustained manner, it is a likely source of tonic ambient NO levels within the CNS. Still, whether eNOS contributes to tonic PVN NO levels and what its functional role is in the regulation of neuronal activity and neurohumoral outflow in physiological and pathological conditions, remain unknown. Here, we used a multidisciplinary approach to study eNOS cellular distribution and functional significance in the PVN of control and HF rats. We found eNOS to contribute to constitutive NO levels and to tonic inhibition of PVN neuronal activity and sympathoexcitatory outflow. Our results also support a role for eNOS in blunted NO availability and elevated sympathoexcitation during HF.

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

Animals
Male Wistar rats (180 to 220 g) were used. All of the procedures were approved by the institutional animal care and use committees at Georgia Health Sciences University and the University of Nebraska Medical Center.
Induction of HF
HF was induced by coronary artery ligation.21 Sham animals underwent the same procedure, but the coronary artery was not occluded. All of the animals were used 6 to 7 weeks after surgery. Transthoracic echocardiography (Vevo 770, Visual Sonics) was performed to evaluate cardiac parameters.

Retrograde Labeling of Rostral Ventrolateral Medulla-Projecting PVN Neurons
PVN neurons innervating the rostral ventrolateral medulla (RVLM; PVN-RVLM) were retrogradely labeled with rhodamine microspheres (Lumaflo) injected unilaterally (400 nL) within the RVLM, and the location and extension of the injections sites were confirmed histologically.22,23

Immunohistochemistry
Conventional immunofluorescence22 was used to characterize eNOS, phospho-eNOS (Ser1177 and Thr495), and its colocalization with nNOS, astroglial cells, microvessels, and oxytocin neurons. Confocal images were obtained and a densitometry analysis was used to compare eNOS immunoreactivity (ir) between sham and HF groups.22

Measurements of NO Availability

Hypothalamic Slices
NO was visualized in living hypothalamic slices using the NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2, Calbiochem).19 Slices were loaded with DAF-2 (2.5 µmol/L) in the presence or absence of relatively selective and nonselective eNOS and nNOS blockers. Confocal images were obtained, and DAF-2 was quantified in identified neurons and astrocytes.

Whole-Animal DAF-2 Infusion
Rats were intravenously injected with DAF-2 following modified methods described previously.23 Brains were then dissected, 20-µm hypothalamic sections were collected in a microscope slide, and images of DAF-2 were taken.

In Vitro Electrophysiological Recordings From PVN-RVLM Neurons
Whole-cell patch-clamp recordings of PVN-RVLM neurons were obtained from hypothalamic sections in HF and sham rats.5 The mean firing rate recorded during a 2 minutes period, before and after bath drug applications, was calculated and compared between groups.

Hemodynamic and Renal Sympathetic Nerve Activity Measurements
Renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP), and heart rate (HR) were monitored.9 The peak response of RSNA to the administration of drugs into the PVN was expressed as 1- or 2-way ANOVA, followed by Bonferroni post hoc tests, were used as indicated. Values of P<0.05 were considered statistically significant.

Results
Representative echocardiography images and mean cardiac functional data for sham and ligated rats (n=31 per group) are shown in Figure S1 and Table S1 (available in the online Data Supplement). Compared with sham rats, ligated rats showed an increased left ventricle internal diameter throughout the cardiac cycle and a decreased percentage in posterior wall thickening, ejection fraction, and fractional shortening (all P<0.05). Moreover, a macroanatomical examination of hearts in ligated rats revealed a dense scar and thinning of the anterior left ventricular wall (data not shown).

eNOS Expression in the PVN Localizes Primarily Within Astrocytes and Perivascular Elements
A dense eNOSir, with a noticeable contrasting pattern to that of nNOS, was observed in the PVN (Figure 1A through 1C; n=4 rats). Although nNOS was almost exclusively localized in principal neurons,5,11 eNOS was more diffusely distributed. No overlap between eNOSir and nNOSir (Figure 1D through 1F) or eNOS and oxytocin (Figure S2) was detected in the PVN and the SON. The specificity of the eNOS antibody is supported by the lack of staining in the absence of primary antibody (data not shown) and in brain tissue obtained from eNOS knockout mice (Figure S3).

Dual immunohistochemical studies for eNOS and a microvasculature (anti-endothelial cell antigen RECA-1) or astrocyte markers (glial fibrilary acidic protein [GFAP] and S100β for processes and cell bodies, respectively) were performed. Images were also obtained from the SON, in which astrocytes have a distinct anatomic distribution, with cell bodies congregated in the ventral glial laminae.24 Although eNOS was found closely associated with the PVN and SON microvasculature (Figure 1G through 1I), it did not overlap with RECA-1 but rather appeared to be present in perivascular profiles abutting on the vessel wall. Conversely, a high degree of colocalization was found between eNOS and perivascular astrocyte processes (GFAP, Figure 1J through 1L) and cell bodies (S100β; Figure 1M through 1O). Similar results were observed in larger microvessels (arterioles) of the cortex (Figure S4).

eNOS Contributes to Constitutive NO Availability and Influences Basal Sympathoexcitatory Outflow From the PVN
Hypothalamic brain sections were loaded with the NO-sensitive indicator DAF-2 in the presence or absence of the relatively eNOS-selective inhibitors (L-N5-(1-Iminoethyl) trifluoromethylphenyl) imidazole (TRIM; 100 µmol/L; Figure 2A through 2C).26 or cavatatin (10 µmol/L; Figure 2A through 2C).27 Preincubation of sections with l-NIO significantly decreased PVN DAF-2 staining (∼19% inhibition; P<0.05; Figure 2G). These effects were absent in eNOS knockout mice (Figure 2H). Similar results were obtained with cavatatin (∼18% inhibition; P<0.05). The nNOS selective inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM; 100 µmol/L; Figure 2D) reduced DAF-2 fluorescence to a similar extent (∼21% inhibition; P<0.05), whereas the nonselective NOS

Statistical Analysis
Data are presented as mean±SEM. Unpaired or paired t tests, as well as 1- or 2-way ANOVA, followed by Bonferroni post hoc tests, were
marker S100 (J) and the SON (L). Neuronal NO synthase (nNOS) staining (red) was confined to main neurons in both nuclei. O and F, Merged higher magnification images of the areas outlined by squares in B (PVN) and E (SON), showing lack of colocalization between eNOS (green) and nNOS (red) in both nuclei. G through I, eNOS (green) and the endothelial cell marker RECA-1 (red) immunoreactivities within the PVN (G) and SON (I). J through L, eNOS (green) and the glial marker GFAP (red) immunoreactivities within the PVN (J) and the SON (L). M through O, eNOS (green) and the glial marker S100β (red) immunoreactivities within the PVN (M) and SON (O). Details of PVN staining are shown in H, K, and N; higher magnification images of the PVN area outlined in G, J, and M, respectively. Note the dense eNOS staining in the SON ventral glial laminae (I, L, and O, arrows) and the colocalization (yellow color: green + red) with glial markers, but not with endothelial, RECA-1 positive cells. Scale bars: 50 μm. 3V indicates third ventricle; OT, optic tract.

Figure 1. Endothelial NO synthase (eNOS) is densely expressed in segregated cell populations in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). A and D, Immunostaining of eNOS (green) within the PVN and SON, respectively. B and E, Neuronal NO synthase (nNOS) staining (red) was confined to main neurons in both nuclei. C and F, Merged higher magnification images of the areas outlined by squares in B (PVN) and E (SON), showing lack of colocalization between eNOS (green) and nNOS (red) in both nuclei.

In vivo microinjections of L-NIO (50 to 200 pmol) into the PVN of sham rats (n=6) increased RSNA, MAP, and HR in a dose-dependent manner (Figure 2J; P<0.05 for all of the variables, 1-way ANOVA). No changes were observed when a similar volume of vehicle was administered (data not shown). In separate experiments, eNOS antisense was delivered directly within the PVN of sham rats (n=3), and the effects of intra-PVN injection of the nonselective NOS blocker Nω-monomethyl-L-arginine (L-NMMA; 200 pmol/200 nL) were assessed before and 2 hours after eNOS antisense delivery. We reasoned that if both eNOS and nNOS isoforms contributed to basal constitutive NO levels, a proportion of L-NMMA effect should be blocked by eNOS antisense pretreatment. As summarized in Figure 2J, the increases in RSNA and MAP evoked by L-NMMA under control conditions were significantly diminished 2 hours after eNOS antisense delivery (P<0.05 for RSNA and MAP, respectively; paired t test). Taken together, our studies support a contribution of eNOS to constitutive NO production and tonic regulation of sympathoexcitatory outflow from the PVN.

Diminished eNOS Expression in the PVN of HF Rats

We assessed then whether eNOSir, as well as its activation/inhibition phosphorylation sites, the phospho-eNOS-Ser1177 and eNOS-Thr495, respectively28 (n=3 per group), were altered in HF rats, in PVN subnuclei containing presympathetic (ventromedial and posterior parvocellular) and magnocellular neurosecretory neurons (lateral magnocellular).3 We found a lower eNOSir in the ventromedial parvocellular and lateral magnocellular PVN subnuclei (P<0.05). A strong tendency, while not reaching statistical significance, was observed in the posterior parvocellular subnucleus. The eNOS-Ser1177ir was lower in the ventromedial parvocellular and posterior parvocellular (P<0.05) but not in the lateral magnocellular subnuclei. Conversely, eNOS-Thr495ir was unaffected in all of the subnuclei (Figure 3).

Blunted eNOS-Derived NO in Presympathetic Regions of the PVN in HF Rats

Basal DAF-2 staining and changes evoked by the eNOS blocker L-NIO were measured in 3 different PVN subnuclei of sham and HF rats (n=6 per group; Figure 4A through 4C). In the presympathetic ventromedial and posterior parvocellular subnuclei of sham rats, we found a higher basal DAF-2 staining (compared with HF rats), which was diminished by L-NIO (P<0.05 in both cases, Figure 4D, 4E, 4G and 4H). Conversely, L-NIO failed to affect DAF-2 in HF rats (Figure 4F and 4I). In the lateral magnocellular subnucleus, we found no differences in basal DAF-2 between sham (Figure 4J and 4K) and HF rats, which was similarly diminished by L-NIO in both groups (P<0.05; Figure 4L). Similar results were obtained with cavatrin in a subset of sham and HF rats (n=2 per group, 697 cells sampled; data not shown).
Figure 2. Endothelial NO synthase (eNOS) contributes to constitutive NO availability and actions in the paraventricular nucleus (PVN). Photomicrographs of PVN 4,5 diaminofluorescein diacetate (DAF-2) staining in control rats in (A) artificial cerebrospinal fluid (ACSF), (B) eNOS inhibitor L-N5-(1-iminoethyl)-ornithine2HCl (L-NIO; 10 μmol/L), (C) eNOS inhibitor cavtratin (10 μmol/L), (D) neuronal NO synthase (nNOS) inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM; 100 μmol/L), (E) nonselective NOS inhibitor N G-nitro-L-arginine methyl ester (L-NAME; 200 μmol/L), and (F) combined L-NAME+NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO; 200 μmol/L). G, Summary data showing mean DAF-2 intensity in each group. *P<0.05 vs: ACSF; †L-NIO, cavtratin and TRIM, ‡L-NAME; n=809 neurons from 18 rats. H, Summary data showing the effects of L-NIO (10 μmol/L) in wild-type and eNOS-knockout (KO) mice.*P<0.05 vs ACSF; n=195 neurons from 6 rats. I, Summary data showing dose-dependent increases in renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP), and heart rate (HR) after microinjection of L-NIO (50, 100, and 200 pmol) into the PVN of control rats (n=6; P<0.05). J, RSNA, MAP, and HR responses to N-monomethyl arginine (L-NMMA; 200 pmol/200 nL) before and 2 hours after microinjection of eNOS antisense oligonucleotide (AS-ODN; 100 pmol/100 nL) into the PVN in a sham rat. The L-NMMA–mediated increases in RSNA and MAP were significantly reduced when L-NMMA was reapplied 2 hours after the AS-ODN microinjection. *P<0.05 vs before AS-ODN; n=3. Scale bars: 50 μm. AU indicates arbitrary units.
Diminished In Vivo Parenchymal Perivascular PVN NO Production in HF Rats

Given the lack of evident DAF-2 staining in parenchymal vessels in brain sections, we used an alternative in vivo approach, which efficiently detects perivascular DAF-2 staining in the CNS.23 Systemic intravenous infusions of DAF-2 (100 μL of 5 mmol/L solution, 15 minutes) readily stained parenchymal perivascular NO-induced fluorescence without staining other neuropile elements (Figure 5), as described previously in the cortex.23 We found PVN perivascular DAF-2 to be diminished in HF rats (≈34%; P<0.05 versus sham rats; n=3 per group).

Blunted eNOS Contribution to Tonic NO Inhibition of PVN Neuronal Activity and RSNA in HF Rats

Last, we assessed whether the blunted eNOS-derived NO resulted in blunted NO inhibition of presympathetic PVN neuronal activity and, consequently, enhanced sympathoexcitatory drive in HF rats.8,9 In vitro patch-clamp recordings from retrogradely labeled PVN-RVLM neurons showed that blockade of eNOS (t-NIO, 10 μmol/L) increased neuronal activity in sham (≈55.0%; n=10 cells; P<0.05) but not in HF rats (≈5.5%; n=12 cells; Figure 6A and 6B). Similarly, in vivo studies showed that the increase in RSNA (expressed as percentage of change from baseline), MAP, and HR evoked by microinjections of t-NIO into the PVN in sham rats was diminished in HF rats (n=5 per group; P<0.05, sham versus HF, 2-way ANOVA; Figure 6C).

Discussion

A large body of evidence supports an integral role for CNS NO in the control of the circulation,1–3 as well as a contribution of blunted NO function to neurohumoral activation in HF.8,9 Most of these studies, however, focused on the targets and outcomes of NO actions, whereas only a few specifically addressed cellular sources and isoforms mediating NO actions.10,14,24 Given that the strength and specificity of NO actions are influenced by the spatial distribution and efficacy of NO isoforms and their sensitivity and proximity to their targets,2 elucidating the cellular sources and isoforms contributing to NO availability is of critical importance. Results from the present studies show the following: (1) in addition to nNOS, eNOS is abundantly expressed in the SON and PVN; (2) nNOS and eNOS display a segregated although spatially interrelated cellular distribution (neuronal and astroglial, respectively); (3) eNOS contributes to constitutive NO production and tonic NO-dependent inhibition of neuronal activity and sympathetic outflow from the PVN; and (4) eNOS is involved in blunted NO availability and actions in HF rats.
Taken together, our studies support eNOS-derived NO as a critical neuromodulator of presympathetic PVN neuronal activity and sympathoexcitatory outflow from the PVN and indicate that blunted CNS eNOS function contributes to sympathoexcitation in HF.

**eNOS of a Likely Glial Location Contributes to Basal NO Bioavailability**

We found eNOS immunoreactivity in the PVN to be largely localized in astrocyte cell bodies and processes. Given that eNOS did not colocalize with nNOS, which is exclusively expressed in neurons,⁵,¹¹ our studies support a cellular segregation between these 2 isoforms. Still, the precise cellular distribution of eNOS in the CNS remains controversial. Although eNOS was reported both in astrocytic cultures²⁹ and brain tissue,³⁰,³¹ including recently in the nucleus tractus solitarius,¹⁷ others failed to detect eNOS in astrocytes.¹⁶ In addition, although we found eNOS staining in close association with the local microvasculature, it did not overlap with endothelial cells but rather with processes in contact with the abluminal side of the microvessels. These processes were immunoreactive for the glial-specific marker GFAP, likely

![Figure 4. Blunted endothelial NO synthase (eNOS) contribution to constitutive NO in the paraventricular nucleus (PVN) of heart failure (HF) rats. A, Diagram depicting PVN subnuclei and projection targets (modified from Reference 4). B and C, Toto-3 counterstaining (nuclear marker, white) depicting the lateral magnocellular (1), ventromedial parvocellular (2), and posterior parvocellular (3) subnuclei in the PVN. Images were taken at −1.8 to 2.1 (B) and 2.1 to 2.3 mm (C) caudal to bregma. Representative confocal images of 4,5-diamidino-2-phenylindole (DAF-2) fluorescence in the ventromedial parvocellular (D and E), posterior parvocellular (G and H), and lateral magnocellular (J and K) PVN subnuclei in the absence or presence of 10 μmol/L of L-NAME (L-NIO), respectively, in a sham rat. F, I, and L, Summary data of DAF-2 intensity in neurons for each subnucleus in sham and HF rats. *P < 0.05 vs respective ACSF. †P < 0.05 sham ACSF vs HF ACSF; n = 6 per group. Scale bar: 50 μm.](image-url)
endothelium dye loading under our experimental conditions. In fact, using an in vivo approach shown previously to efficiently load vascular structures, we showed peripheral DAF-2 staining in the PVN. Thus, quantification of DAF-2 in vitro was restricted to neurons, in which we found a diminished DAF-2 fluorescence after eNOS blockade. Our results indicate that, despite eNOS segregated cellular distribution, the close proximity among neurons, astrocytes, and the local microvasculature in these nuclei, along with the ability of NO to freely diffuse from its site of production, ensues that eNOS-derived NO from either astrocytes or microvessels contributes to NO availability and actions in nearby neurons. This is important given previous controversial reports, showing that, whereas NO efficiently diminished the activity of most presympathetic PVN neurons, only a limited proportion of them expressed detectable levels of nNOS. Taken together, these studies suggest that NO produced by and diffusing from an alternative isoform (i.e., eNOS) contributes to NO actions on PVN neuronal activity and sympathetic outflow.

**Figure 5.** Diminished paraventricular nucleus (PVN) perivascular NO availability in heart failure (HF) rats after in vivo infusions of 4,5-diaminofluorescein diacetate (DAF-2). A and B, Epifluorescence images of DAF-2 fluorescence in the posterior paravascular subnucleus in a sham (A) and HF (B) rat. C and D, Higher magnification image of the PVN area outlined in A and B, respectively. Arrows point to typical examples of staining in rounded, coronally cut microvessels. E, Summary data showing a diminished perivascular DAF-2 staining in the PVN of HF rats. *P<0.05, n=3 per group. Scale bar: 50 μm. 3V indicates third ventricle.

representing astrocytic endfeet. This result is somewhat inconsistent with previous studies showing eNOS in brain endothelium. Thus, methodological dissimilarities, including antibodies used, fixation procedures, and overall sensitivity, could explain such reported differences.

To determine whether eNOS contributed to constitutive NO availability, we monitored NO using DAF-2, a well-established NO-sensitive fluorescent indicator. Our results showing a diminished basal DAF-2 in slices pretreated with L-NIO or cavatrin, 2 different eNOS selective blockers, support a tonic contribution of eNOS to PVN NO levels. The lack of L-NIO effects in eNOS knockout mice support its lack of L-NIO effects in eNOS knockout mice support its significance of these 2 isoforms, it is important to consider other properties as well. Based on their distinct cellular sources and bioactive properties, it is likely that NO originating from these alternative sources mediates distinct functions. For example, antagonistic effects were reported in the medulla, where eNOS and nNOS mediated inhibition and excitation of baroreflex function, respectively. Conversely, results from our laboratories indicate that both isoforms inhibit the firing activity of hypothalamic neurosecretory and presympathetic neurons, as well as sympathoex-
citatory outflow to the circulation.\textsuperscript{6,7,10} This raises questions about the functional significance of the presence of, in principle, 2 similar NO sources. One possibility is that each NO source is activated by different signaling mechanisms and/or conditions. This is supported by the presence of at least two NO-mediated signaling modalities, phasic (rapid and transient) and tonic (sustained), mediated likely by nNOS- and eNOS-derived NO, respectively. Thus, activation of nNOS via associated N-methyl-D-aspartate (NMDA) receptors in dendritic spines results in a brief, low-amplitude NO transient, which is spatially and temporally restricted to the site of production.\textsuperscript{19,42} Therefore, this NMDA-nNOS phasic modality is better suited to act in a synapse-specific manner.\textsuperscript{20} Conversely, eNOS is able to synthesize NO in a sustained manner, even at resting cytosolic calcium concentrations.\textsuperscript{18,19} supporting eNOS as the likely primary source of tonic ambient NO levels, mediating more widespread effects of NO within CNS networks.\textsuperscript{20,43} Taken together, our results further support the notion that eNOS contributes to sustained NO bioavailability and actions within the PVN. However, whether PVN eNOS and nNOS are activated under different conditions or by different signals remains to be determined.

**Diminished eNOS Expression and Function Contribute to Blunted NO Actions in HF Rats**

Previous studies showed elevated PVN neuronal activity\textsuperscript{44} and blunted PVN NO actions as major contributing factors to increased sympathoexcitatory outflow in HF.\textsuperscript{9,12} However, whether eNOS contributes to blunted CNS NO function in HF remained unexplored. This is supported by several lines of evidence in this work. First, we found a diminished PVN eNOS immunoreactivity in HF rats. Given that most eNOSir was localized in perivascular structures and that a diminished in vivo perivascular DAF-2 was observed in HF rats, it is likely that a diminished perivascular eNOS expression occurred in HF rats. Second, eNOS activity can be efficiently regulated by phosphorylation of various sites, particularly the Ser1177 and the Thr495, resulting in increased and decreased activity, respectively.\textsuperscript{28} Our results showing diminished staining for Ser1177 in HF rats suggest that, in addition to changes in eNOS expression, dysfunctional phosphorylation at Ser1177 also contributes to blunted eNOS-derived NO in HF rats. Interestingly, a diminished Ser1177 is commonly observed in the vasculature of hypertensive rats.\textsuperscript{45} Lastly, we found a blunted effect of eNOS blockade on NO bioavailability, PVN-RVLM firing activity, RSNA, MAP, and HR in HF rats. Thus, in addition to previously reported diminished eNOS function in the peripheral vasculature during HF,\textsuperscript{46} our study supports a contribution of blunted CNS eNOS function to elevated neuronal activity and sympathoexcitation in this condition.

**Perspectives**

Increased neurohumoral drive, characterized by sympathoexcitation, and elevated circulating neurohormones constitute a common finding in humans and experimental animal
models of HF,\textsuperscript{47,48} Given that sympathoexcitation increases the progression and mortality in HF,\textsuperscript{47} there is a great deal of interest in elucidating mechanisms underlying sympathoexcitation in HF. Our results showing the involvement of eNOS in blunted NO availability and actions during HF support eNOS as an important underlying pathophysiological mechanism in neurohumoral activation in HF, as well as a promising therapeutic target for the treatment of this disease.

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None.

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CONTRIBUTION OF CENTRAL NERVOUS SYSTEM eNOS TO NEUROHUMORAL ACTIVATION IN HEART FAILURE RATS

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Short Title:
eNOS in the PVN in Heart Failure rats

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**Expanded material and methods:**

**Animals.** Male Wistar rats (180-220g) from Harlan Laboratories (IN, USA) were maintained under a 12h:12h light-dark cycle and given free access to food and water. All procedures were carried out in agreement with the Medical College of Georgia and the University of Nebraska Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the respective committees.

**Induction of Heart Failure.** HF was induced by coronary artery ligation, a widely-used experimental animal model of heart failure\(^1,^2\). Briefly, animals were anesthetized with Isoflurane 4% and the trachea was cannulated with a 16G cannulae for mechanical ventilation and maintenance of anesthesia (isoflurane 2%). A left thoracotomy through the 5\(^{th}\) intercostal space was performed and the heart exteriorized. The ligation was placed on the main diagonal branch of the left anterior descending coronary artery using a 6.0 monofilament propylene suture. The heart was returned to the preceding position, and the chest cavity incision was closed. Buprenorphine (0.05 mg/kg, sc. Butler Schein/NLS, Dublin, OH, USA) was given immediately after surgery to minimize post surgical pain. Sham animals underwent the same procedure but the coronary artery was not occluded. All animals were used 6 to 7 weeks after surgery, a stage at which neurohumoral activation is already established\(^2\).

**Echocardiographic assessment of left ventricular function.** Trans-thoracic echocardiography (Vevo 770 system, Visual Sonics, Toronto, CAN) was performed four weeks after surgery under light anesthesia (isoflurane 2%). Long axis images at the level of the mitral and aortic valves were taken from the longest length of the left ventricle. Perpendicular to its image, short axis view of the left ventricle at the level of chordae tendineae muscle was recorded to evaluate cardiac parameters. Both anatomic imaging views were recorded in brightness and motion mode (m-mode), to determine the location, and to assess anatomical measurements, respectively.

Measurements from left ventricle internal diameter; left ventricle posterior wall and left ventricle anterior wall were obtained throughout the cardiac cycle from the short axis m-mode imaging. Mean values of 3 measurements for each parameter were acquired for systole and diastole. Automatic calculation using the parameters described was obtained for ejection fraction and fractional shortening.

Representative echocardiography images and mean cardiac functional data for Sham and ligated rats (n=31 each group) are shown in Fig. S1 and Table S1.

**Retrograde labeling of rostral ventrolateral medulla (RVLM) projecting PVN neurons.** Five weeks after surgeries, PVN neurons innervating the RVLM (PVN-RVLM) were labeled as previously described\(^3,^4\). Briefly, a 2 mm burr hole was made in the skull of the rat and the fluorescent tracer rhodamine labeled microspheres (Lumaflor, Naples, FL, USA) was pressure injected unilaterally (400nL) using the coordinates Bregma -11.96, Lateral: 2.0, Dorsal: 8.0 mm. Five-seven days after surgery, animals were used for in vitro electrophysiology experiments. The location and extension of the injections sites were confirmed histologically, and usually extended from the caudal pole of the facial nucleus to ~1 mm more caudal, and were ventrally located with respect to the nucleus ambiguous\(^3\).
**Immunohistochemistry.** Rats were deeply anesthetized with sodium pentobarbital (100mg/kg i.p.) and the brain was dissected and post fixed overnight in 4% phosphate-buffered paraformaldehyde (4% PFD) followed by cryoprotection in 0.01mol/L phosphate-buffered saline (PBS) containing 30% sucrose for 3 days at 4°C. Immunohistochemical labeling to visualize eNOS was performed using a biotin-streptavidin amplification method. Hypothalamic coronal sections were cut at 30μm thickness using a cryostate. To block unspecific sites, sections were pre-incubated in 10% horse serum for 1h followed by a 15min streptavidin/biotin blocking kit (Vector, Burlingame, CA, USA). Sections were then incubated in a purified rabbit anti-eNOS polyclonal primary antibody (1:10000. Cell Signalling, Beverly, MA, USA) for 48h at room temperature. Tissue was rinsed in 0.01mol/L PBS and incubated with a series of secondary antibodies raised against the species of the primary antibody, as follows: 1) anti-rabbit biotin-SP conjugate antibody (1:250) for 2h; 2) fluorescein-conjugated streptavidin (1:400) for 4h; 3) biotinylated anti-streptavidin antibody (1:250) for 2h (Vector) 4) Fluorescein (DTAF)-conjugated streptavidin (1:400) secondary antibody for 4h. Every step was preceded by rinses in 0.01mol/L PBS for 3 times. Immunohistochemistry for eNOS phosphorylation sites Ser1177 and Thr495 were performed in a similar manner using purified rabbit polyclonal primary antibodies against Phospho-eNOS (Ser1177) and Phospho-eNOS (Thr495) (both 1:1000. Cell Signalling, Beverly, MA, USA). Sections were mounted in vectashield and coverslipped.

To test for colocalization between eNOS and nNOS, astroglial cells, neurons and endothelial cells, the eNOS antibody was combined with one of the following primary antibodies: nNOS: monoclonal anti-nitric oxide synthase-brain (bNOS) Clone NOS-B1 (nNOS – 1:400. Sigma–Aldrich, St. Louis, MO, USA); astroglial cells: mouse anti-gliarial fibrillary acidic protein polyclonal antibody (GFAP - 1:5000. Millipore, Billerica, MA, USA) or monoclonal anti-S-100 (β-subunit) (S100β – 1:1000. Sigma–Aldrich, St. Louis, MO, USA); endothelial cells: mouse anti rat RECA-1 (RECA – 1:1000. AbD Serotec, Oxford, UK). To identify the different PVN subnuclei, sections were stained with a guinea pig anti-oxytocin serum (OT - 1:100000. Bachem, San Carlos, CA, USA), or counterstained with a fluorescent DNA marker, Toto-3 iodide (1:10000. Molecular probes, Invitrogen, Carlsbad, CA USA). The respectively secondary antibody (Cy3 or Cy5-conjugated donkey anti mouse or guinea pig, 1:250) were added only in the last step of the secondary reaction, following the same procedures. All antibodies and blocking agents were diluted in PBS 0.01mol/L containing 0.01% Triton X-100 and 0.04% NaN3. Secondary antibodies, except when stated otherwise, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The specificity of the used antibodies was determined by (a) the characteristic differential immunoreactive topographical distribution, and (b) by the absence of staining when the primary antibody was omitted (not shown). The specificity of the eNOS antibody was also confirmed by the absence of staining in brains obtained from eNOS knockout mice (eNOS KO - B6.129P2-Nos3<tm1Unc>/J) or respective wild-type mice (C57BL/6J), purchased from The Jackson laboratory (Bar Harbor, MA, USA).

**Confocal Imaging acquisition and quantification of eNOS immunoreactivity.** Acquisition and quantification of images containing eNOS/Phospho-eNOS immunoreactivities (ir) were done as previously described3. Briefly, images from 4 consecutive optical focal planes (4μm thick, 2μm interval for immunoreactivity) at various rostro-caudal PVN levels were taken using a Zeiss LSM 510 Confocal
scanning microscope (Carl Zeiss, Oberkochen, GER). Each optical section was averaged three times, and a projection image of the sections was generated. A laser argon-krypton was used to excite eNOS/Phospho-eNOS ir at 488nm, and OT-ir was acquired in the CY5 wavelength. Images from Sham and HF groups were digitized with identical acquisition settings for further comparison. A densitometry analysis, based on a threshold paradigm (2.5 times above background fluorescence), was used to compare eNOS ir between Sham and HF groups. Regions of interest were then traced within the different rostral caudal subnuclei of the PVN (lateral magnocellular, ventromedial parvocellular and posterior parvocellular subnucleus), identified using the third ventricle, fornix and the easily recognized ball-shaped lateral magnocellular subnucleus (OT or TOTO staining) as landmarks (see also Fig.5). The density of eNOS/Phospho-eNOS signals within each region was calculated, and results expressed as % area within the subnucleus occupied by threshold ir. Since all eNOS antibodies used were raised in the same species, we were unable to assess the relative expression of the activation/inhibition eNOS phosphorilation sites within the same rats. Moreover, in our hands, the efficiency of each antibody was different, forcing us to use different acquisition parameters for each type, in order to optimize the acquired image. This further prevented us from comparing the relative expression of eNOS and Phospho-eNOS sites within each experimental condition. Thus, quantitative comparisons were limited to comparing each respective eNOS ir between Sham and HF rats.

Fluorescent measurements of nitric oxide availability using the NO fluorescent indicator DAF-2. A-hypothalamic slices: NO was visualized in living hypothalamic slices using membrane-permeant form of the NO-sensitive dye 4,5 diaminofluorescein diacetate (DAF-2. Calbiochem, Gibbstown, NJ, USA), whose fluorescent intensity is linearly proportional to NO availability in the tissue studied. The calibration and the efficiency of this approach was previously documented in our lab. Sham and HF were anesthetized with pentobarbital (100mg/kg IP), rapidly decapitated and 180µm sections through the hypothalamus containing SON and PVN, between Bregma - 1.80 and - 2.12 mm were cut with a vibroslicer (Leica VT 1200 S, Leica, Buffalo Grove, IL, USA). Slices were collected in a holding chamber in standard artificial cerebrospinal fluid solution (ACSF) solution containing in mmol/L: NaCl 126; KCl 2.5; MgSO4 1; NaHCO3 26; NaH2PO4 1.25; D-glucose 20; CaCl2 1; and saturated with 95% O2–5% CO2 (pH 7.3 – 7.4) until used. Slices were then cut in half (parallel to the third ventricle) and divided in 2 groups, which were then pre-incubated in the presence or absence of the relatively specific eNOS inhibitor L-N5-(1-Imoethyl)-ornitine.2HCl (L-NIO – 10µmol/L. Alexis, San Diego, CA, USA) or the caveolin-1 scaffolding domain peptide derivative cavtratin (CAV - 10µmol/L. Calbiochem, La Jolla, CA, USA) for 30min and kept oxygenated (95% O2 and 5% CO2) at room temperature. Subsequently, the slices were loaded with DAF-2 (2.5µmol/L, diluted in fresh ACSF or ACSF plus the respective eNOS blocker previously oxygenated and placed inside a CO2 water jacketed incubator (Nuaire, Plymouth, MN, USA) for another 30min, at 37°C. Slices were then washed in fresh media solution for 3x during 45min, and then fixed in 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC - 40mg/mL) for 3h followed by 4% PFD overnight. Slices were then counterstained using Toto-3 iodide (1:10000) for anatomical orientation. The nNOS inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM – 100µmol/L. Calbiochem, La Jolla, CA, USA), the non specific NOS inhibitor N (G)-nitro-l-arginine methyl ester (L-NAME – 200µmol/L. Sigma–Aldrich, St. Louis,
MO, USA) and the NO scavenger Carboxy-PTIO (C-PTIO – 100µmol/L. Alexis, San Diego, CA, USA) were also used, as indicated. Confocal images were acquired as described above. Given technical limitations related to our ability to immunoidentify astrocytes in conjunction with DAF-2, and the lack of DAF-2 staining in microvessels in slices in vitro (see Discussion), quantification of DAF-2 was limited to PVN neurons, identified using an area size criteria (Toto-3 profiles ≥ 120µm²). This was established in separate sets of studies combining Toto-3 with neuronal and astroglial markers (not shown, neurons: 225±1.6µm² and astrocytes: 67±0.7µm², n=1394 and 382, respectively). Individual PVN neuronal somata within the different subnuclei were manually traced, and the cell size and mean gray value intensity of the fluorescence signal contained within the cells were measured (Image Pro software, Media Cybernetics, Bethesda, MD, USA) and expressed as fluorescence arbitrary units ranging from 0 (absolute black) to 255 (absolute green). Background fluorescence was subtracted from all images. A mean value per animal was calculated, and then the overall mean for each experimental condition was used for statistical comparisons.

B- Whole-animal DAF infusion: To determine in situ NO production in vivo, Sham and HF rats were intravenously injected with DAF-2 following modified methods previously described10, 11. Animals were anesthetized with Isoflurane 4% and a femoral vein was cannulated to deliver DAF-2 (100µL of 5mmol/L solution in dimethylsulfoxide) in a single bolus injection. Fifteen min after injection animals were perfused transcardiatically with 0.01mol/L PBS (50mL) followed by EDAC (40mg/mL; 50mL) and 4% PFD (150mL). The brain was dissected, post fixed in 4% PFD (1h) following by cryoprotection in 0.01mol/L PBS containing 30% sucrose for 1 day at 4°C. 20µm sections throughout the hypothalamus were collected in a microscope slide. The fluorescence obtained through this method was very labile, preventing us from performing a counterstaining approach, or from obtaining confocal images. Thus, sections were mounted and visualized using an Olympus BX51 microscope, and only images from the posterior region of the PVN were obtained, to ensure quantification in a region enriched with presympathetic neurons12.

In vitro electrophysiological recordings from PVN-RVLM neurons. Conventional whole-cell patch clamp recordings of retrogradely-labeled PVN-RVLM neurons were obtained from acute hypothalamic slices obtained from HF and Sham rats, as previously reported4. Briefly, hypothalamic slices were cut (300µm) and incubated in standard ACSF as described above. All recordings were performed at 30-32°C. Patch pipettes (4-8MΩ) were pulled from thin-wall (1.5mm outer diameter, 1.17mm inner diameter) borosilicate glass (GC150T-7.5, Clark, Reading, UK) on a horizontal electrode puller (P-97, Sutter instruments, Novato, CA). The pipette internal solution contained (in mmol/L) 140 K-gluconate, 10 KCl, 10 HEPES, 0.9 MgATP, 20 Phosphocreatine (Na+), 0.3 NaGTP, and 0.2 EGTA, pH 7.3). Electrical recordings were obtained using a multiclamp 700A amplifier (Axon Instruments, Foster city, CA, USA). The series resistance was monitored throughout the experiment, and data was discarded if series resistance increased >25MΩ. The voltage output was digitized at 16-bit resolution (10KHz) in conjunction with pClamp 9 software (Digidata 1320, Axon Instruments, Foster City, CA, USA). The firing activity of PVN-RVLM neurons was recorded in the current clamp mode, and the mean firing rate was calculated during a 2min baseline period before drug additions, and during a period comprising one minute before and one minute after the peak of the drug effect.
**PVN microinjections, hemodynamic and renal sympathetic nerve activity (RSNA) measurements.** On the day of the experiment, rats were anesthetized with urethane (0.75g/kg i.p.) and α-chloralose (70mg/kg i.p.) and the left femoral artery was cannulated and connected to a computer-driven data recording and analyzing system (PowerLab, ADI instruments, Colorado springs, CO, USA) via a pressure transducer (Gould P23 1D) for recording arterial blood pressure and heart rate. The anesthetized rat was placed in a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA). A longitudinal incision was made on the head and the bregma was exposed. The coordinates for the PVN were determined from the Paxinos and Watson Atlas. They were 1.5mm posterior to the bregma, 0.4mm lateral to the midline, and 7.8mm ventral to the dura. A small burr hole was made in the skull. For the microinjections, a thin needle (0.5mm OD and 0.1mm ID) connected to a micro syringe (0.5ml; model 7000.5 Hamilton micro syringe) was lowered into the PVN. Injections were made using the following coordinates: 1.8mm posterior, 0.4mm lateral to the bregma, and 7.8 mm ventral to the dura. After each experiment, Chicago blue dye was injected into the brain to verify that the injection site was located within the PVN. The brains were removed, fixed in 10% formalin for at least 24 h, sectioned (30 μm) on a cryostat, and processed for histology. Sections were mounted on gel-coated microscope slides and stained using 1% neutral red. The location of the injection was visualized on a microscope, and injections with terminations within the boundaries of the PVN were considered to be appropriately targeted to the PVN. RSNA was recorded as described previously. Briefly, the left kidney was exposed through a retroperitoneal flank incision. A branch of the renal nerve was isolated from the fat and connective tissue and was placed on a pair of thin bipolar platinum electrodes. The nerve-electrode junction was insulated electrically from the surrounding tissue with a silicone gel (Wacker Sil-Gel, 604 A B). The electrical signal was amplified (10000 times) with a Grass amplifier (P55) with a high- and low-frequency cutoff of 1000 and 100Hz, respectively. The output signal from the Grass amplifier was directed to a computer-run data acquisition system (PowerLab, ADI instruments, Colorado springs, CO, USA) to record and integrate the raw nerve discharge. The signal recorded at the end of the experiment (after the rat was dead) was deemed as background noise. The basal value of the nerve activity was defined by subtracting the background noise from the actual nerve activity value before the administration of drugs into the PVN. The peak response of RSNA to the administration of drugs into the PVN during the experiment (averaged over a period of 20-30s) was subsequently expressed as a percent change from baseline.

**eNOS antisense delivery into the PVN.** To silence eNOS in the PVN, we use the antisense (AS) oligodeoxynucleotide (ODN) technique. The AS-ODN and mismatch ODN was synthesized according to the rat eNOS mRNA sequence (GenBank accession number NM021838). The antisense sequence targeted to the eNOS mRNA is 5'-ATGGGCAACTTGAAGAG-3'. To avoid non-specific binding with mRNA of other genes, the specificity of the chosen antisense sequence has been checked using a BLAST search of GenBank. The result indicated that the chosen sequence had no significant overlap with other rat mRNAs. ODNs were modified with phosphorothioate oligodeoxynucleotides to improve their stability. Animals were anesthetized with urethane (0.75g/kg, i.p.) and α-chloralose (70mg/kg, i.p.). The ODNs were dissolved in the ACSF (1mmol/L) and were administered into the PVN by unilateral microinjections of 100nL of solution (100pmol/100nL). This dose and
protocol were based on our previous successful use of AS-ODN against nNOS within the PVN\textsuperscript{15}.

\textbf{Statistical Analysis.} Data are presented as mean ± SEM. Unpaired or paired \textit{t}-tests, as well as one or two way analysis of variance (ANOVA), followed by Bonferroni posthoc tests, were used as indicated. All analyses were performed using Graphpad prism software (Graphpad Software). Values of \textit{P}<0.05 were considered statistical significant.
References


**Table S1.** Cardiac parameters of heart failure (HF) and Sham-operated rats

<table>
<thead>
<tr>
<th>Variable measured</th>
<th>Sham</th>
<th>HF</th>
</tr>
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<tbody>
<tr>
<td>LVD,d (mm)</td>
<td>7.87±0.16</td>
<td>10.46±0.17*</td>
</tr>
<tr>
<td>LVD,s (mm)</td>
<td>3.49±0.25</td>
<td>8.84±0.19*</td>
</tr>
<tr>
<td>PW,d (mm)</td>
<td>2.28±0.14</td>
<td>2.02±0.10*</td>
</tr>
<tr>
<td>PW,s (mm)</td>
<td>3.85±0.15</td>
<td>2.51±0.12*</td>
</tr>
<tr>
<td>PW thickening, %</td>
<td>68±4</td>
<td>28±4*</td>
</tr>
<tr>
<td>LVEDV (mL)</td>
<td>0.30±0.01</td>
<td>0.61±0.02*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>82±3</td>
<td>30±2*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>56±2</td>
<td>16±1*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>329±6</td>
<td>331±9</td>
</tr>
</tbody>
</table>

Values are means ± SE. LVD,d/s: left ventricle diameter in diastole and systole, respectively; PW, d/s: posterior wall in diastole and systole, respectively; LVEDV: left ventricle end diastolic volume; EF: ejection fraction; FS: fractional shortening; HR: heart rate. *P < 0.01 vs. Sham; n = 31 each group.
Figure S1 - Echocardiographic assessment of left ventricular function.
Representative echocardiograph images in brightness-mode, long axis view in a sham (A, B) and a heart failure (HF) (D, E) rat; Movement-mode short axis view in Sham (C) and HF (F) rats. IVS: intra ventricular septum; AW: anterior wall; LV: left ventricle; PW: posterior wall; LVD,d/s: left ventricle dimension in diastole and systole. Note the dilated LVD,d/s in HF rats as well as the decreased contractility during systole in the AW.
Figure S2 - eNOS expression in the SON and PVN does not overlap with oxytocin neurons. A and D: Representative immunostaining of eNOS (green) within the PVN and SON, respectively. B and E: Oxytocin staining (white) in both nuclei. C and F: Merged image showing that eNOS (green) does not co-localize with oxytocin (white). Scale bars: 50µm. 3V: third ventricle; OT: optic tract
**Figure S3 - Lack of eNOS immunofluorescence in eNOS knock-out mice.**
Representative photomicrographs of eNOS immunoreactive in the SON of control wild type (A) and eNOS knockout (B) mice (n=2/group). Scale bars 50µm. 3V: third ventricle; OT: optic tract.
Figure S4 – eNOS immunoreactivity in cortical vessels. **A-C,** Representative example of a cortical vessel showing the endothelial marker RECA-1 (red, **A**) and eNOS (green, **B**) immunoreactivities. In **C:** both images were superimposed. **D-F:** Representative example of a cortical vessel showing the glial marker GFAP (red, **D**) and eNOS (green, **E**) immunoreactivities. In **F:** both images were superimposed. Scale bars 20µm.