Blunted nitric oxide-mediated inhibition of sympathetic nerve activity within the paraventricular nucleus in diabetic rats


Blunted nitric oxide-mediated inhibition of sympathetic nerve activity within the paraventricular nucleus in diabetic rats. Am J Physiol Regul Integr Comp Physiol 290: R992–R1002, 2006. First published December 1, 2005; doi:10.1152/ajpregu.00363.2005.—Recent evidence suggests that a central mechanism may be contributing to the sympathetic abnormality in diabetes. Nitric oxide (NO) has been known as a neurotransmitter in the central nervous system. The goal of this study was to examine the role of the endogenous NO system of the paraventricular nucleus (PVN) in regulation of renal sympathetic nerve activity (RSNA) in streptozotocin (STZ)-induced diabetic rats. The change in number of NADPH-diaphorase-positive neurons [a marker for neuronal NO synthase (nNOS) activity] in the PVN was measured. Diabetic rats were found to have significantly fewer nNOS positive cells in the PVN than in the control group (120 ± 11 vs. 149 ± 13, P < 0.05). Using RT PCR, Western blotting and immunofluorescent staining, it was also found that nNOS mRNA expression and protein level in the PVN were significantly decreased in the diabetic rats. Furthermore, using an in vivo microdialysis technique, we found that there was a lower NO release from the PVN perfusates in rats with diabetes compared with the control rats (142 ± 33 nM vs. 228 ± 29 nM, P < 0.05). In α-chloralose- and urethane-anesthetized rats, an inhibitor of NO synthase, L-NMMA, microinjected into the PVN produced a dose-dependent increase in RSNA, mean arterial pressure (MAP), and heart rate (HR) in both control and diabetic rats. These responses were significantly attenuated in rats with diabetes compared with control rats (RSNA: 11 ± 3% vs. 35 ± 3%, P < 0.05). On the other hand, an NO donor, sodium nitroprusside (SNP), microinjected into the PVN produced a dose-dependent decrease in RSNA, MAP, and HR in the control and diabetic rats. RSNA (17 ± 3%, vs. 41 ± 6%, P < 0.05) and MAP in response to SNP were significantly blunted in the diabetic group compared with the control group. In conclusion, these data indicate an altered NO mechanism in the PVN of diabetic rats. This altered mechanism may contribute to the increased renal sympathetic neural activity observed in diabetes.

neuronal nitric oxide synthase; neuromodulator; neurohumoral drive; renal sympathetic nerve activity

VARIous TYPeS OF AUTONOMIC reflex abnormalities have been observed in diabetic patients and rats (5, 37). Both sodium retention and a decreased diuretic response are characteristic of diabetes (21, 25). The blunted natriuretic and diuretic responses can be normalized by denervating the kidney or by treating with insulin to control blood glucose levels (29, 48). Renal sympathetic nerve recordings have revealed a blunted renal sympathto-inhibition in streptozotocin (STZ)-induced diabetic rats when challenged with acute volume expansion (30). Although there has been some progress in elucidating the peripheral mechanism involved in these abnormalities, the role of central mechanisms has not been explained.

Various sites in the brain are involved in autonomic regulation (13, 35, 36). Areas of the hypothalamus are known to be involved in fluid balance (4, 38). The paraventricular nucleus (PVN) and the supraoptic nucleus (SON) produce AVP, which is a humoral factor involved in fluid balance (4, 38). Studies have shown increased levels of circulating AVP in diabetic patients (41, 43). The PVN has also been shown to be involved in the regulation of sympathetic outflow and specifically affect the heart and kidney, as there are direct projections from the PVN to intermediodorsal cell column of the spinal cord at levels at which cardiac and renal postganglionic sympathetic nerves originate (1, 6, 38).

The PVN is a nucleus upon which a number of neurotransmitters converge to influence its neuronal activity. The coordinated actions of excitatory and inhibitory neurotransmitters in the PVN determine the neuronal activity of the PVN. There are complex interactions between specific PVN neurotransmitters for altering sympathetic nerve activity (11, 12). Either the increased excitatory stimuli or decreased inhibitory stimuli into the PVN would lead to the increased neuronal activity of the PVN, and consequent increased sympathetic outflow (38, 39).

Evidence has indicated the PVN is involved in the blunted renal sympatho-inhibition in response to acute volume expansion in the STZ-induced diabetic rat. Krukoff and Patel (16) assessed the neural activity in discrete regions of the brain of STZ-induced diabetic rats by histochemical localization and photodensitometric quantification of the metabolic enzyme hexokinase as a marker of neuronal activity. The data revealed significant increases in hexokinase activity in the PVN in diabetes. Our recent study has also shown that there is increased basal neuronal activity in the PVN and SON in diabetic rats. This suggests that the neurons in the PVN and SON are activated during diabetes and may contribute to the autonomic dysfunction during diabetes (49).

Nitric oxide synthase (NOS) has been localized in many discrete areas of the brain (3, 20, 22). Increasing amounts of evidence indicate that nitric oxide (NO) acts as a nonconventional neurotransmitter in the central nervous system. A growing body of evidence supports the hypothesis that NO acts to decrease sympathetic output to the periphery (15). Zhang et al. (45) demonstrated that microinjection of the NO donor sodium nitroprusside (SNP) into the PVN produced a significant decrease in renal sympathetic nerve activity (RSNA). Con-
versely, the NOS inhibitors, l-NMMA or l-NAME, produced increases in RSNA. These data suggest that NO in the PVN has an inhibitory effect on RSNA.

The diabetic condition is known to produce attenuated vasodilation in response to agonists known to operate via a NO mechanism (19, 26). Diabetic state impairs NOS-dependent reactivity of peripheral and cerebral blood vessels. However, very little is known about the NO system in the central nervous system in the diabetic state. Reagan and McEwen. (32) found that the expression of nNOS mRNA and protein in the hippocampus of STZ-induced diabetic rats were significantly decreased when the rats were subjected to restraint stress. Yu et al. (42) conducted experiments in the cerebrocortex and found that the activity of NOS determined by conversion of [3H]l-arginine to [3H]l-citrulline was markedly decreased in STZ-induced diabetic rats.

We hypothesized that there is an altered NO mechanism within the PVN in the regulation of sympathetic outflow during diabetes and that this altered mechanism may contribute to the increased sympathetic nerve activity during diabetes. To test this hypothesis, we examined whether 1) there was a decreased number of NOS-positive cells in the PVN of diabetic rats, 2) the nNOS mRNA expression and protein level in the PVN were significantly decreased in rats with diabetes, 3) the amount of NO released from the PVN perfusates was lower in rats with diabetes, and 4) the effects of both endogenous and exogenous NO within the PVN contribute to the regulation of sympathetic outflow in rats with diabetes.

METHODS

Induction of Diabetes

This study protocol 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 (180–200 g, Sasco) were maintained in vivarium with a 12:12-h light-dark cycle. The temperature was maintained at 20–22°C, and the humidity was maintained at 30–40%. Standard laboratory chow and tap water were available ad libitum.

Randomly selected rats were injected with STZ (65 mg/kg in a 2% solution of 0.1 M citrate buffer, ip) to induce diabetes. Onset of diabetes was identified by polydipsia, polyuria, and blood glucose levels >250 mg/dl. Experiments were performed 3 wk after the injection of STZ or vehicle.

NADPH-Diaphorase Activity as a Marker of nNOS Activity

Three weeks after injection of STZ or vehicle, the rats were anesthetized with pentobarbital sodium (65 mg/kg). The rats were perfused transcardially with 150 ml of heparinized saline followed by 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brain was removed and postfixied at 4°C for 4 h in 4% paraformaldehyde solution and then placed in 20% sucrose. The brain was blocked in the coronal plane, and 30-μm-thick sections were cut with a cryostat. Every third section was kept from the anterior commissure (0.40 mm posterior to bregma) posterior to where the optic tracts were observed to be their most lateral position on the ventral surface of the brain (2.60 mm posterior to bregma). The sections were collected in 0.1 M phosphate, containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml β-NADPH. The sections were placed in an oven at 37°C for 1 h. After the reaction, the sections were rinsed in phosphate buffer and mounted onto slides.

The presence of NADPH-diaphorase in discrete regions of the brain was examined under a light 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 for each individual nucleus at the same coronal level. Three adjacent sections were considered to represent one coronal level as the numbers of cells counted were within 5% of each other. The average number of cells in the sections (1.80 ± 0.1 mm posterior to bregma) was taken to represent the number of cells within a given nucleus. The PVN, SON, and the lateral hypothalamus (LH) were counted unilaterally. Cells within the identified boundaries of PVN and SON were counted. Cells lateral to the third ventricle and dorsal to the SON represented cells in LH.

Semiquantitative RT-PCR

The rats were euthanized with an overdose of pentobarbital sodium. The brain was removed, frozen on dry ice, and then stored at −70°C. Six consecutive 100-μm-thick coronal sections were cut with a cryostat (−18°C), and the PVN area was punched bilaterally with a blunt needle (0.5 mm in diameter), according to the method of Palkovits and Brownstein (27). The punched tissue was put in 0.5 ml of Tri-Reagent and homogenized by a Sonicator (Sonic & Materials Inc, Newtown, CT). RNA was isolated followed by reverse transcription for 40 min at 37°C in the presence of random hexamers and MMLV-Rtase. The product was used for nNOS cDNA amplification. The sequence of the oligonucleotide primers and their location in the public sequence are as follows: nNOS, 5'-GGGAGCGAGGCGGCCTTA (sense) (GenBank NM-052799, bp 255–274) and 5'-TTGTTGGGAGGACCGAGGG (antisense) (GenBank NM-052799, bp 494–475); β-actin, 5'-GGG AAAATCTGCGTGACATT (sense) (GenBank BC063166, bp 698–717) and 5'-CCGATGTGCAAGTC ACTT (antisense) (GenBank BC063166, bp 950–931). β-actin was coamplified with each receptor cDNA as an internal control. The PCR mixture contained 0.7-μM primers, dNTP, BSA, and 1 U of Taq DNA polymerase (Promega). After 10 min of denaturing at 94°C, the amplification was performed at 94°C for 1 min, at 56°C for 1 min and at 72°C for 1 min for 30 cycles with the final extension at 72°C for 10 min. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining and UV-transillumination. The visualized DNA bands were captured by Kodak digital camera.

The relative intensities of the bands were quantified by densitometric analysis (Kodak 1D software, New York, NY). The nNOS band was normalized to the β-actin band, and the values were expressed as percent change from the control group.

Western Blotting Assay

The punches containing PVN were subjected to a protein extraction procedure. The protein sample was mixed with 4% SDS sample buffer. The sample was boiled for 5 min and then loaded onto the 7.5% SDS-PAGE gel for electrophoresis. The proteins were then electrophoretically separated for 1 h at 80 mA and then transferred onto the polyvinylidene difluoride membrane. The membrane was incubated with 5% milk-Tris-buffered saline-Tween solution for 30 min at room temperature. Then, the membrane was incubated with primary antibody (rabbit anti-rat nNOS polyclonal antibody, 1:1,000; rabbit anti-rat tubulin polyclonal antibody, 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After washing, the membrane was incubated with secondary antibody (goat anti-rabbit IgG, Peroxidase conjugated, 1:5,000, Pierce Biotechnology, Rockville, IL) for 40 min at room temperature. The signals were visualized using an enhanced chemiluminescence substrate (Pierce) and detected by a digital imaging system (UVP, Upland, CA).
Immunofluorescent Staining

The perfused brain samples were prepared using the NADPH-diaphorase staining protocol. The brain was then frozen, and serial transverse sections (30 μm) were cut. After a rinse with PBS, the sections were incubated with 10% normal donkey serum in PBS for 1 h at room temperature. The sections were incubated with primary antibodies against nNOS (rabbit anti-rat nNOS polyclonal antibody, 1:200, Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS, the sections were incubated with Cy5-conjugated donkey anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. After second antibody incubation, the nucleus was stained by Hoechst 33258 (Molecular Probes). After washing with PBS and drying, the sections were cover-slipped with fluoromounting-G (SouthernBiotech, Birmingham, AL). Distribution of nNOS immunofluorescence within the PVN was viewed using a Olympus fluorescence microscope (Japan) equipped with a digital camera (Qimaging, Burnaby, Canada) and initially assessed descriptively.

Digital images of the PVN were captured, and the labeling was quantified using computer-assisted image analysis. Openlab software 4.0.3. (Improvement, Lexington, MA) was used to identify the total intensity of positive staining. Three alternate sections (1.80 ± 0.1 mm posterior to bregma) representing the PVN were analyzed in this way, and then the mean data were calculated.

In Vivo Microdialysis and NO\textsubscript{a} Measurement

In vivo microdialysis studies were performed according to the method described by Horn et al. (9). The rats were anesthetized with α-chloralose (70 mg/kg ip) and urethane (0.75 g/kg ip). A burr hole was drilled in the skull such that a microdialysis probe (0.24-mm diameter, Microdialysis AB, Solna, Sweden) could be positioned into the PVN. The probes were attached to an infusion pump and the PVN was perfused with Ringer solution. Three consecutive 20-min perfusion collections were then performed. All perfusate samples were collected in 1 μl of 0.5 M perchloric acid, frozen at −70°C and retained for assay of NO\textsubscript{a} metabolites.

We used an NO analyzer (Model 280, Sievers) that used chemiluminescence for quantification of NO and its reaction products (an average of the three samples). The sensitivity of this instrument allowed picomolar detection of total NO\textsubscript{a} in biological fluids.

Recording of Renal Sympathetic Nerve Activity, Arterial Pressure, and Heart Rate

On the day of the experiment, the rat was anesthetized with α-chloralose (70 mg/kg ip) and urethane (0.75 g/kg ip). The femoral vein was cannulated for administration of supplemental anesthesia and saline. Additional anesthesia was administered as needed (urethane, 0.25 g/kg and α-chloralose 20 mg/kg ip). This supplemental anesthesia was not different between the groups. The femoral artery was cannulated and connected to the MacLab via a pressure transducer for recording arterial pressure (AP) and heart rate (HR). The left kidney was exposed through a left retroperitoneal flank incision. The renal 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 1,000 Hz and 100 Hz, respectively. The signal was recorded with the MacLab. Effferent 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, 30 mg/kg iv) was defined as background noise. The value of RSNA 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. Responses of mean arterial blood pressure (MAP) and HR were expressed as the difference between the basal value and the value after each dose of a drug.

Stereotaxic Placement of Microsyringe and Injection into the PVN

The rat was placed in a stereotaxic device. An incision was made on the midline of the scalp, and bregma was exposed. With the use of a brain atlas by Paxinos and Watson (31), the coordinates of the right PVN with reference to bregma were calculated as being 1.3 mm posterior, 0.4 mm lateral, and 7.8 mm ventral to the dura. A small burr hole was placed in the skull, and a needle (0.5 mm OD; 0.1 mm ID) that was connected to a microsyringe (0.5 μl) was lowered into the PVN. At the completion of the experiment, Chicago blue dye (2%, 100 nl) was injected into the brain for histological verification of injection site.

Brain Histology

At the end of the experiment, the brain was carefully removed and fixed in 4% formaldehyde. The brain was then frozen, and serial transverse sections (30 μm) were cut using a cryostat. The sections were thaw mounted on slides. The sections were stained using 1% aqueous neutral red. Presence of blue dye within the PVN was determined using a light microscope. Dye that was located in the PVN was considered to be “hits” of the PVN. The results of these injections are shown in Fig. 5.

Microinjection Experiments Protocol

Experiment 1. RSNA, AP, and HR were measured in control (n = 8) and diabetic (n = 8) rats. An inhibitor of nitric oxide synthase, N\textsuperscript{\textalpha}-monomethyl-L-arginine (L-NMMA), was injected into the PVN in three doses (50, 100, and 200 pmol in 50–200 nl). The doses were given consecutively at intervals of 20 min in a random order. Maximum changes in RSNA, MAP, and HR were determined.

To substantiate the notion that any responses of AP, HR, and RSNA to L-NMMA are mediated by the blockade of NO\textsubscript{a} within the PVN and not by a peripheral action, the responses of changes in MAP, HR, and RSNA were recorded after intravenous injection of 500 pmol of L-NMMA in five normal rats.

Experiment 2. RSNA, AP, and HR were measured in control (n = 8) and diabetic (n = 8) rats. An NO donor, SNP, was injected into the PVN in three doses (5, 10, and 20 nmol in 50–200 nl). The doses were given consecutively at intervals of 20 min. Maximum changes in RSNA, MAP, and HR were determined.

Experiment 3. The responses of AP, HR, and RSNA to L-NMMA and SNP were collected in a group of rats (n = 5) in which the injection site was not in the area of PVN. This group is termed the anatomical control group.

Data Analysis

Data were subjected to a two-way ANOVA followed by a Student-Newman Keuls test. P < 0.05 were considered to be significant.

RESULTS

General Data

Table 1 summarizes the salient baseline characteristics of control and diabetic rats used in the present study. The mean blood glucose level for the control group was significantly lower than the blood glucose level of the diabetic group (P < 0.05). Before the microinjection of L-NMMA into the PVN, MAP of the control group was not significantly different from the MAP found in the diabetic group (P > 0.05). The control group had a higher baseline HR compared with the diabetic group (P < 0.05).
NADPH-diaphorase Activity as a Marker of nNOS Activity

The PVN (Fig. 1A), SON, and LH stained positive for NADPH-diaphorase activity. The number of nNOS positive cells in the PVN of diabetic rats was significantly less than that found in the control group (120 ± 11 vs. 149 ± 13, P < 0.05). No significant difference in the number of nNOS-positive cells was found in the SON or the LH of diabetic compared with control rats (Fig. 1B).

nNOS mRNA and Protein Level in the PVN

The result of RT-PCR experiments indicated that there was a significantly lower level of nNOS mRNA in diabetic rats (ratio of intensity: 0.63 ± 0.06 vs. 0.80 ± 0.08, P < 0.05) (Fig. 2A). Western blotting showed 155-kDa bands in the PVN of control and diabetic rats. Diabetic rats had significantly lower protein level of nNOS compared with the control group (ratio of intensity: 0.76 ± 0.04 vs. 0.92 ± 0.06, P < 0.05) (Fig. 2B).

As an in situ confirmation of the alteration in nNOS within the PVN, the immunofluorescence for nNOS was lower in the PVN from rats with diabetes compared with control rats (Fig. 3).

Endogenous NO\textsubscript{x} Levels in the PVN

The average levels of NO\textsubscript{x} release within the PVN in control and diabetic rats are presented in Fig. 4. The basal NO\textsubscript{x} release from the PVN was significantly lower in diabetic rats compared with control rats (142 ± 33 nM vs. 228 ± 29 nM, P < 0.05).

Microinjection of \textit{l}-NMMA and SNP into the PVN

Figure 5 illustrates the brain histological data. Among the 36 injections that were in the PVN area, 12 injection sites belong to microinjection of \textit{l}-NMMA, 14 injection sites belong to microinjection of SNP, and 8 injection sites belong to microdialysis of the PVN in control and diabetic rats.

Experiment 1. Administration of \textit{l}-NMMA (50, 100, and 200 pmol in 50–200 nl) in the PVN elicited increases in RSNA, MAP, and HR in both groups (Fig. 7B). The diabetic group showed an increase in RSNA that was significantly blunted compared with control rats. At the highest dose, RSNA increased 11 ± 3% above baseline in diabetic rats compared with an increase of 35 ± 3% in control rats (P < 0.05). An example of the peak responses in RSNA, MAP, and HR to \textit{l}-NMMA in the two groups is illustrated in Fig. 6A and Fig. 7A. These data indicate that blockade of endogenous NO synthesis in rats with diabetes is less effective in raising the RSNA, MAP, and HR in rats with diabetes.

| Table 1. Characteristics of control and diabetic rats in the microinjection experiments |
|----------------------------------------|----------------|
|                                     | Control | Diabetic |
| Body weight, g                       | 338±18  | 227±17*  |
| Blood glucose, mg/dl                 | 101±13  | 410±20*  |
| Basal MAP, mmHg                       | 94±7    | 90±6     |
| Basal HR, bpm                        | 392±11  | 337±19*  |

Values are expressed as means ± SE. n represents number of animals; n = 15 for control and diabetic rats. *Significant difference between groups, P < 0.05.
Fig. 2. *A*: gene expression of neuronal nitric oxide synthase (nNOS) in the samples of punches containing PVN. *Top*: example of visualized electrophoresis bands of amplification of nNOS and β-actin; *Bottom*: mean data of band densities of nNOS normalized by β-actin in control and diabetic rats. *B*: Western blot analysis for nNOS in the samples of punches containing PVN. *Top*: example of visualized electrophoresis bands of nNOS and tubulin; *Bottom*: mean data of band densities of nNOS normalized by tubulin in control and diabetic rats. *P* < 0.05 compared with control group.

Fig. 3. *A*: immunofluorescence photomicrographs from the sections of the PVN region stained for nNOS. Number of nNOS-positive neurons (green) is decreased in diabetic rats compared with control rats. Blue spot shows the nucleus stained by Hoechst 33358. Scale bar = 200 μm. *B*: total intensity of nNOS-positive staining in the PVN. Values represent the means ± SE in each group. *P* < 0.05 compared with control group.
In normal rats, we also examined the possibility that the observed effects of NO synthase inhibitors were mediated by a peripheral effect. Intravenous administration of 500 pmol of L-NMMA elicited no significant change in RSNA (0.6 ± 4.4%), MAP (5.0 ± 0.3 mmHg), or HR (1 ± 2 bpm).

Experiment 2. Administration of SNP (5, 10, and 20 nmol in 50–200 nl) in the PVN elicited decreases in RSNA, MAP, and HR in both groups (Fig. 8B). The diabetic group showed a decrease in RSNA that was significantly less than the decrease found in the control group. This blunted response was significantly different at all three doses of SNP. At the highest dose, RSNA decreased 17 ± 3% in diabetic rats compared with a decrease of 41 ± 6% in the control rats (P < 0.05). A blunted decrease in MAP was also observed in the diabetic group. The response of HR was not significantly different between the groups. An example of the peak responses in RSNA, AP, and HR to SNP in the two groups is illustrated in Fig. 6B and Fig. 8A.

Experiment 3. In rats that were administered L-NMMA outside the PVN (missed the PVN), we did not see any response. Similarly, microinjection of SNP outside the PVN did not change RSNA, MAP, or HR (data not shown).

DISCUSSION

The results of this study indicate that in the diabetic rats, there was 1) a decreased number of NOS-positive cells in the PVN, 2) a decreased nNOS mRNA expression and protein level in the PVN, 3) a lower NO release from PVN. In addition, 4) microinjection of the NOS inhibitor, L-NMMA, into the PVN elicited a blunted increase in RSNA, MAP, and HR, and 5) microinjection of the NO donor, SNP, elicited a blunted decrease in RSNA and MAP. These data suggest that the reduced renal sympathoinhibition mediated by a lack of endogenous NO within the PVN may contribute to the elevated sympathetic nerve activity during diabetes.

In these experiments, we used similar-aged rats in control and diabetic groups. Because of the induction of diabetes with STZ, there was a significant difference in the weight of the two the groups at the time of the experiments. One could contend that the change in body weight itself (or something secondary to it), rather than diabetes per se may contribute to the observations. This is not likely because normal control rats with weights similar to those observed in diabetic rats show responses similar to those observed in the control rats (with higher body weights) used in the current experiment (data not shown).

Because Garthwaite et al. (8) first demonstrated that NO was acting as a neuronal messenger in cerebellar granule cells, NO has been reported (7) to be involved in various physiological activities as a nonconventional neurotransmitter. The functional evidence is supported by direct measurement of RSNA (14, 45), showing that endogenous NO within the PVN is involved in the regulation of sympathetic outflow. NO is a gaseous molecule that easily diffuses through cell membranes. Its presence in neurons not only means that it can influence neuronal function via release from the terminal endings and act
postsynaptically, it can also affect the activity of neighboring neurons (10).

We hypothesize that NO is inhibitory to renal sympathetic outflow via its actions in the PVN, and this NO input into the PVN decreases during diabetes and, hence, contributes to increased neuronal activity of the PVN and the elevated sympathetic nerve activity. This can probably be attributed to either the decreased activity of nNOS (47) and/or production of nNOS (28) and, hence, the reduced synthesis of NO within PVN during diabetes. It might also be possible that the reduced renal sympatho-inhibition by NO in the PVN is accounted for by the reduced response of neurons in the PVN to NO during diabetes.

The NADPH-diaphorase histochemical technique identifies an enzyme that can catalyze the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble and visible formazan. This technique was used because it has been reported that NADPH-diaphorase accounts for the function of NOS in neuronal tissue (3, 33, 34). While there are at least eight other enzymes known to be responsible for NADPH-diaphorase activity (2), all but NOS are sensitive to formaldehyde. In this study the number of NADPH-diaphorase-positive cells in the PVN of STZ-induced diabetic rats was significantly lower than that observed in the control group. The data suggest that nNOS, responsible for the synthesis and production of NO, is deficient in diabetic rats. Consistent with this result, the NO synthesis in the perfusates, nNOS mRNA expression, and protein level within the PVN was reduced in the diabetic rats. These abnormalities may lead to a reduced tonic release of endogenous NO in diabetic rats.

Consistent with this idea, the present study demonstrates a blunted increase in RSNA after microinjection of the NO antagonist L-NMMA into the PVN of STZ-induced diabetic rats. This data indicate that RSNA is under less tonic inhibition from NO in diabetic rats than that seen in the control rats. The effect of L-NMMA in the present study is consistent with findings by Zhang et al. (45) who observed an increase in RSNA after microinjection of similar doses of L-NMMA into the PVN. Although MAP and HR increased in both groups, the increase in MAP and HR was significantly blunted in the diabetic rats.
The present study also shows a decrease in RSNA, MAP and HR with administration of SNP, an NO donor, into the PVN. However, in STZ-induced diabetic rats there was a blunted decrease in RSNA and MAP after the administration of SNP into the PVN. The response of HR was not significantly different between the groups. HR is heavily influenced by the parasympathetic nervous system while AP is to a lesser extent, and a response in AP may not always coincide with the response observed in HR. The blunted decline in RSNA in diabetics suggests that the effect of NO within the PVN is reduced. This is probably due to reduced efficacy of NO on neuronal activity downstream from synthesis and release of NO. The precise downstream mechanism for this blunted effect remains to be determined.

At present, the precise cellular targets and mechanisms through which NO acts within the PVN to inhibit sympathetic outflow are unknown. The action of NO could be direct or indirect. Horn et al. (9) observed that perfusion of the PVN with NO-CSF led to an increase in the concentrations of some amino acids in the perfusate, including GABA (40). Zhang and Patel (46) found that within the PVN, the inhibitory effect of endogenous NO on RSNA was mediated by GABA. The central GABA system exerts a tonic inhibitory influence over the sympathetic nervous system. Effects of sympatho-excitation by L-NMMA within the PVN could also be blocked through a GABAergic mechanism. Thus it was proposed that the effect of NO within the PVN might be mediated by the release of GABA. Using perforated patch-clamp recording, Li et al. (17) found GABAergic synaptic activity in the PVN neurons was potentiated by NO donors, and the NO-mediated inhibition of firing activity was blocked by the GABA receptor antagonist. This suggests that the production of GABA, or the actions of GABA within the PVN, may be blunted because of decreased NO synthesis within the PVN and/or blunted response of the PVN neuron to NO in diabetes.

Another possible mechanism through which NO acts within the PVN to inhibit sympathetic outflow is mediated by glutamate-activated NMDA receptor. Our recent study has shown that NO in the PVN, which is released by activation of the NMDA receptor, also inhibits NMDA-mediated increases in sympathetic nerve activity (18). This negative feedback of NO on the glutamatergic system within the PVN may play an important role in maintaining the overall balance and tone of the sympathetic nervous system.
sympathetic outflow in normal and pathophysiological conditions known to have increased sympathetic tone.

The importance of neuronal NO in many sites that participate in central autonomic regulation leads to the prediction that disease states associated with altered autonomic outflow may also exhibit altered central NO mechanisms. The work from our laboratory has indicated that the NO system within the PVN involved in controlling autonomic outflow is also altered during heart failure and may contribute to the elevated levels of renal sympathoexcitation commonly observed in heart failure (44, 47). Both heart failure and diabetes are characterized by an increased sympathetic nerve activity. It may well be that the altered central NO mechanism may be responsible for the elevated sympathetic outflow observed under both conditions. In broad terms, it may well be that central NO mechanisms dictate overall sympatho-excitation.

The present study suggests a downregulation of the NO system within the PVN during diabetes that leads to increased RSNA. We speculate that this abnormality is also responsible for altered fluid balance, mainly sodium retention, in diabetic patients. Sodium retention is believed to occur early in diabetic patients and is thought to be relatively common (23–25). It has been suggested that diabetics who develop nephropathy are those who are unable to compensate for this excess sodium. Furthermore, hypertension and its complications usually occur with the progression of kidney disease. The present study provides evidence of a specific central malfunction leading to altered renal sympathetic function in diabetes. It is hoped this information will enhance the possibility of a future pharmacologic intervention that will prevent the complications discussed.

In conclusion, we postulate that the decreased levels of nNOS within the PVN of STZ-induced diabetic rats leads to a decreased production of NO within the PVN, which produces an altered renal sympatho-inhibition in response to acute volume expansion in diabetic rats. This altered mechanism may contribute to the increased renal sympathetic neural activity observed in diabetes.

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