Lack of central nitric oxide triggers erectile dysfunction in diabetes

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Am J Physiol Regul Integr Comp Physiol 292: R1158–R1164, 2007. First published November 9, 2006; doi:10.1152/ajpregu.00429.2006.—Erectile dysfunction is a serious and common complication of diabetes mellitus. The proposed mechanisms for erectile dysfunction in diabetes include central and autonomic neuropathy, endothelial dysfunction, and smooth muscle dysfunction. The paraventricular nucleus (PVN) of the hypothalamus is known to be involved in centrally mediated penile erection. This study was designed to examine the role of nitric oxide (NO) within the central nervous system component of the behavioral responses including erection in diabetic rats. N-methyl-D-aspartic acid (NMDA)-induced erection, yawning, and stretch through the PVN can be blocked by prior administration of NO synthase (NOS) blocker, L-NMMA, in freely moving, conscious male normal rats. Four weeks after streptozotocin (STZ) and vehicle injections, NMDA-induced erection, yawning, and stretch responses through the PVN are significantly blunted in diabetic rats compared with control rats. Examination of neuronal NOS (nNOS) protein by Western blot analysis indicated a reduced amount of nNOS protein in the PVN of rats with diabetes compared with control rats. Furthermore, restoring nNOS within the PVN by gene transfer using adenoviral transfection significantly restored the erectile and yawning responses to NMDA in diabetic rats. These data demonstrate that a blunted NO mechanism within the PVN may contribute to NMDA-induced erectile dysfunction observed in diabetes mellitus.

Erectile dysfunction is a serious and common complication of diabetes mellitus (12, 32). The proposed mechanisms for erectile dysfunction in diabetes include central and autonomic neuropathy, endothelial dysfunction, and smooth muscle dysfunction (13, 20). The coexistence of the neuropathic and angiopathic changes in the vast majority of diabetic patients (and in an array of experimental animal models) has complicated efforts to define their relative contributions to erectile dysfunction (20, 31). Thus, the precise pathophysiological mechanisms of erectile dysfunction in diabetic patients remain obscure.

In diabetic male patients Sildenafil (Viagra) appears to be therapeutic in 50% of the patients (4, 17). This suggests that there is another mechanism that may be important in erectile dysfunction in diabetic males. Apart from the peripheral actions of corporal smooth muscle relaxation, erectile dysfunction is related to a complex interaction between sensory and autonomic fibers (1). Penile erection is thought to involve parasympathetic, neurally mediated relaxation of blood vessels, as well as of the trabecular meshwork of smooth muscle that comprises the corpora cavernosa (1, 16). The activation of parasympathetic nerves involves central mechanisms responsible for penile erection, and the subsequent vasodilation that results involves underlying peripheral mechanisms. Erectile dysfunction is thought to involve the central nervous system component of this pathway.

The paraventricular nucleus (PVN) of the hypothalamus is an integration center between the central and peripheral autonomic nervous systems. It is involved in numerous functions from feeding, metabolic balance, blood pressure, and heart rate, to erectile function and sexual behavior. Some premotor neurons that project into the spinal proerectile neurons are present in the PVN of the hypothalamus (36).

Nitric oxide (NO) plays an important role in normal penile erection by virtue of its ability to potently relax corporal smooth muscle cells in the penis (1). In addition to this well-documented peripheral action, evidence is accumulating that indicates NO may also function as a neurotransmitter in the central nervous system to modulate sexual behavior and penile erection (15, 21). The PVN is a primary site within the forebrain that has been implicated in NO-mediated penile erection (9, 30). Delivering NO or NO donors to the PVN of conscious rats elicits episodes of penile erection and yawning (21), and electrical stimulation of the PVN in anesthetized rats elicits an increase in intracavernous pressure (8).

Penile erection, yawning, and stretch are behavioral responses that occur concomitantly in response to the administration of N-methyl-D-aspartic acid (NMDA) within the PVN (22). NMDA receptor activation causes an influx of calcium, which, in turn, is associated with the activation of NO synthase (NOS) in the forebrain, resulting in the synthesis/release of NO. Nitric oxide plays an important role in the expression of penile erection and yawning (21). Consistent with these observations, blockade of NOS with L-arginine (L-NMMA) prevented NMDA-induced erectile behavior (22).

The present study was designed to examine NMDA/NO-induced penile erection, yawning, and stretch in streptozotocin (STZ)-induced diabetic rats. We hypothesized that the blunted behavioral responses to NMDA in diabetes reflects an impaired NO mechanism within the PVN. The involvement of NO mechanism in the NMDA-mediated behavioral response was also explored, as was the possibility that diabetes impairs the transduction of changes in NO-mediated behavioral responses.

METHODS

Induction of Diabetes

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

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National Institutes of Health and the American Physiological Society. Male Sprague-Dawley rats (180–200 g, Sasco) were maintained in a 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.

The rats were randomly injected with STZ (65 mg/kg ip in a 2% solution of 0.1 M citrate buffer) to induce diabetes. The rats not receiving STZ served as a control and were injected with citrate buffer only. Onset of diabetes was identified by polydipsia, polyuria, and blood glucose levels >250 mg/dl. Four weeks after the injection of STZ or vehicle, the experiments were performed on each of the rats.

**Surgical Procedures**

Four weeks after STZ and vehicle injection, each animal was implanted with a stainless steel cannula aimed at the PVN. The rats were anesthetized with pentobarbital (50 mg/kg ip) and then placed in a stereotaxic apparatus (Davis Kopf instruments, Tujunga, CA). A longitudinal incision was made on the skin on the scalp, and the bregma was exposed. The coordinates for the PVN were determined according to the atlas of Paxinos and Watson (26). A small burr hole was made in the skull. A stainless steel guide cannula (500-μm outer diameter; Microdialysis AB, Solna, Sweden) was implanted stereotaxically at the following coordinates: 1.5 mm posterior to the bregma, 0.4 mm lateral to midline, and 7.8 mm ventral to the dura. Two stainless-steel anchoring screws were fixed to the skull, and the cannula was secured in place by acrylic dental cement. The animals were then returned to their cages and allowed to recover for 3 days.

**Adenovirus Injections in the PVN**

On the day of the injections, the rats were placed individually into a Plexiglas cage and injected with adenoviral vectors (Ad.nNOS, final concentration, 1 x 10^10 pfu/ml) or adenoviral vectors encoding β-galactosidase (Ad.β-Gal) into the PVN in a volume of 200 nl using a microsyringe (0.5 μl; model 7000.5, Hamilton). After the injection, the animals were returned to their cages and allowed to recover for 3 days.

**Western Blot Analysis Assay**

Four weeks after STZ and vehicle injections, the rats were anesthetized with an overdose of pentobarbital and killed. 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, supraoptic nucleus (SON) and lateral hypothalamus (LH) area was punched bilaterally with a blunt needle (0.5 mm in diameter), according to the method of Palkovits and Brownstein (24). The protein in the PVN punches was extracted according to the Molecular Research Center published protocol. Protein content was determined using a protein assay kit (Pierce, Rockford, IL). Because of the higher sensitivity of the antibodies, 5 μg total protein is sufficient to generate a clear signal. Therefore, 6 μg protein was 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 being washed, the membrane was incubated with secondary antibody (goat anti-rabbit IgG, peroxidase conjugated, 1:5,000; Pierce) for 40 min at room temperature. The signals were visualized using an enhanced chemiluminescence substrate (Pierce) and detected by a digital image system (UVP, Upland, CA).

**Experimental Protocol**

**Experiment 1:** Effect of l-NMMA on NMDA-mediated behavioral responses in normal rats. Three days after surgery, NMDA-induced penile erection, yawning, and stretching within the PVN in freely moving, conscious male normal rats were measured. Rats were placed individually into a Plexiglas cage and injected with NMDA (25 ng) into the PVN in a volume of 100 nl. The vehicle solution was artificial cerebrospinal fluid (composition in mM: 132 NaCl, 3.0 KCl, 0.65 MgCl2, 1.5 CaCl2, 24.6 NaHCO3, and 3.3 glucose adjusted to pH 7.4). After NMDA injection, the rats were observed to count number of episodes of penile erection, yawning, and stretching over 20-min intervals for the next 80 min. This response to NMDA injection was repeated 2 days later, 5 min after l-NMMA administration (200 pmol in 200 nl, using 1 mM solution of l-NMMA over 2 min).

**Experiment 2:** Behavioral responses to NMDA or sodium nitroprusside administration in control and diabetic rats. The rats were injected with NMDA (25 ng) or sodium nitroprusside (SNP, 25 ng) into the PVN in a volume of 100 nl. After injection, the rats were observed to count episodes of penile erection, yawning, and stretching episodes at 20-min intervals for the next 80 min.

**Experiment 3:** Effect of diabetes on nNOS protein in the PVN. Using micropunch followed by immunoblotting techniques, we determined the nNOS protein level in PVN, SON, and LH in STZ-induced diabetic rats compared with vehicle injected control rats.

**Experiment 4:** NMDA-mediated behavioral responses in diabetic rats after restoration of nNOS protein in the PVN with viral gene transfection. The rats were injected with NMDA (25 ng) into the PVN in a volume of 100 nl. After NMDA injection, the rats were observed to count episodes of penile erection, yawning, and stretching over 20-min intervals for the next 80 min. Three days after viral injections, these responses were repeated. These responses were repeated again 4 days later (7 days after viral injections).

**Brain Histology**

At the end of the experiment, Chicago blue dye (2%, 100 nl) was injected into the brain for histological verification of injection site, and the rats were killed. The brain was 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 then 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. 1.

**Statistical Analysis**

Data were subjected to one-way ANOVA followed by a multiple-range test for comparison of means among groups for behavioral responses. nNOS protein was compared between groups by using the unpaired t-test. All data are presented as means ± SE. P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**General Data**

The mean blood glucose level for the control group was significantly lower than the blood glucose level of diabetes (105 ± 19 mg/dl vs. 423 ± 27 mg/dl, P < 0.05). The control group had a higher body weight compared with the diabetic group (342 ± 15 g vs. 237 ± 16 g, P < 0.05).

Figure 1 illustrates the termination of injector tracts within the hypothalamus. Injector placements were found throughout the rostrocaudal extent of the PVN. Among the 36 injections...
targeting the PVN, 35 injections were in the PVN area, while 1 injection was outside, but adjacent to the PVN (not reported here). Among the 35 injections that were in the PVN, 7 injections were in normal rats, 14 injections were in control rats, and 14 injections were in rats with diabetes. The 100-nl injection volumes targeting the PVN would be expected to distribute the drug in or within /H11021 0.5 mm away from the rostrocaudal and mediolateral boundaries of the PVN (34).

**Effects of L-NMMA on NMDA-Mediated Behavioral Responses in Normal Rats**

There were increases in penile erection, yawning, and stretch in normal rats in response to microinjection of NMDA within the PVN. Prior administration of L-NMMA into the PVN in normal rats reduced the NMDA-induced erection, yawning, and stretch responses (Fig. 2) (P < 0.05). All of the microinjections were identified to be within the PVN regions in control and diabetic rats. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle. Scale bar represents 0.5 mm.

**Behavioral Responses to NMDA Administration in Control and Diabetic Rats**

Four weeks after STZ injection, microinjection of NMDA within the PVN of conscious, freely moving, diabetic rats demonstrated blunted erection, yawning, and stretch responses compared with control rats (Fig. 3) (P < 0.05). The proportion of responsive rats (those showing at least 1 erectile event) is dramatically different between control (85%; 6 out of 7 rats) and diabetic group (0%; 0 out of 7 rats). Vehicle injection within the PVN of control and diabetic rats did not induce penile erection, yawning, and stretch responses (data not shown).

**Behavioral Responses to SNP Administration in Control and Diabetic Rats**

We also tested whether administration of an NO donor, SNP, within the PVN could elicit erection, yawning, and...
stretch responses in control and diabetic rats (Fig. 4). Administration of SNP into the PVN produced erectile and yawning responses in diabetic rats unlike that obtained with NMDA administration. The proportion of diabetic rats showing at least one erectile event is increased to 50% (3 out of 6 rats) but still lower than control (100%; 5 out of 5 rats). It should be noted that the responses to SNP in diabetic rats were significantly blunted compared with control rats ($P < 0.05$).

**nNOS Protein Level in the PVN**

Using micropunch technique, we determined that the nNOS protein level is decreased in the PVN of STZ-induced diabetic rats compared with vehicle-injected control rats (Fig. 5). However, the levels of nNOS were not significantly different in other adjacent forebrain areas (SON and LH) that were examined.

**NMDA-Mediated Behavioral Responses in Diabetic Rats After Restoration of nNOS Protein in the PVN With Viral Gene Transfection**

Three days after adenoviral transfection, penile erection (Fig. 6, left) and yawning episodes (Fig. 6, middle) in response to NMDA injection within the PVN were dramatically increased in diabetic rats. These responses were again blunted in diabetic rats at 7 days after Ad.nNOS transfection. Three days after adenoviral transfection stretch episodes (Fig. 6, right) in response to NMDA within the PVN were not changed in diabetic rats.

Ad.nNOS injection within the PVN of control rats did not alter the NMDA-mediated penile erection (Fig. 6, left), yawning (Fig. 6, middle), and stretch episodes (Fig. 6, right) at either 3 or 7 days after Ad.nNOS transfection.
Ad-β-Gal injection within the PVN of control and diabetic rats did not alter the NMDA-mediated penile erection, yawning, and stretch episodes at either 3 or 7 days after adenoviral transfection. For the penile erection, the maximum response to NMDA injection is 1.8 ± 0.4 (control rats) vs. 0.3 ± 0.2 (diabetic rats). Three days after Ad-β-Gal injection, the maximum erection response to NMDA injection is 1.9 ± 0.3 (control rats) vs. 0.3 ± 0.1 (diabetic rats).

**DISCUSSION**

The results of the present study indicate that blockade of endogenous NO production within the PVN with L-NMMA blunts penile erection, yawning, and stretch responses to NMDA within the PVN. NMDA-induced erection, yawning, and stretch responses are significantly blunted in diabetic rats compared with control rats. Consistent with the results, nNOS protein levels in the PVN were decreased in rats with diabetes. Adenoviral injection of nNOS within the PVN normalized the penile erectile and yawning responses to NMDA in diabetic rats.

The PVN is involved in numerous behavioral functions, including feeding, erectile function, and sexual behavior. In particular, a group of neurons originating in this nucleus and projecting to extra-hypothalamic brain areas control penile erection in male rats. Activation of these neurons by dopamine, NMDA, oxytocin, or by electrical stimulation leads to penile erection, while their inhibition by GABA or opioid peptides inhibits this sexual response (2). Erection can occur alone or associated with yawning and stretching (3). The physiological significance of yawning and stretching is considered an ancestral vestige surviving through evolution, indicating arousal, although its role is far from clear (5, 23). These behavioral responses are under the control of several neurotransmitters and neuropeptides at the central level. Some of the compounds interact in the PVN of the hypothalamus to control erection, yawning, and stretching. In the current study, we were able to inhibit NMDA-mediated penile erection, yawning, and stretching by prior administration of L-NMMA, an NOS inhibitor, into the PVN. The data suggest that NMDA induces penile erection, yawning, and stretch by activation of NOS in the PVN. The results are consistent with experiments showing that NMDA-mediated NO within the PVN is critically involved in penile erection in rats (15, 21). Furthermore, this finding is consistent with the observation that rats treated with Nω-nitro-L-arginine methyl ester (L-NAME), another inhibitor of NOS, show a severely impaired libido, as measured by precoital sexual behavior, mounting, intromission, and ejaculation (14, 28).

Administration of NMDA within the PVN demonstrated a decreased response in penile erection, yawning, and stretch in diabetic rats. This is further supported by the observation that the levels of nNOS protein are decreased in rats with diabetes compared with control rats. In the present study, we further measured penile erection, yawning, and stretch before and after the administration of adenoviral transfection of nNOS gene into the PVN of control and diabetic rats. Li et al. (19) have shown that injection of Ad.nNOS within the PVN produces an overexpression of nNOS within the PVN, as determined by the increased number of NADPH diaphorase positive cells (70%), increased levels of nNOS protein (80%) within the PVN, and functional tests for restoration of nNOS 3 days after transfection. However, the intensity and number of NADPH-positive cells in the PVN of Ad.nNOS-injected rats was decreased in rats examined 6 days after the transfection. The present study shows that restoration of nNOS within the PVN of rats with diabetes may correct the behavioral responses (erection and yawning) mediated by microinjection of NMDA after 3 days viral transfection. This suggests an abnormality in a central NO mechanism involved in the erectile response in diabetic rats.

The presence of the penile erectile and yawning responses to SNP administration in the PVN in diabetic rats, albeit smaller, imply that the peripheral mechanisms involved in the overall response are relatively intact and operational. However, because the response to SNP was blunted in diabetic rats, these results could not differentiate between an additional peripheral abnormality and a central abnormality in the diabetic rats. The results of the studies with viral transfection showed an improvement in the NMDA-mediated responses. However, it should be noted that the responses were slightly smaller (although statistically not significant) than those in control rats consistent with an altered peripheral mechanism as well.

Sexual dysfunction is a well-recognized consequence of diabetes mellitus in men (18, 33). Erectile dysfunction, retrograde ejaculation, and loss of seminal emission have been described in male diabetic patients. STZ-induced diabetic rats provide an interesting and relevant model to study the effects of diabetes on male sexual dysfunction, since they exhibit several deficits in copulatory behavior similar to those in diabetic men (35, 37). Four weeks-STZ-induced diabetic rats have shown an endocrine and metabolic disorder often associated with erectile dysfunction and peripheral neuropathy (11). Diabetic rats show significant deficits in mount, intromission,
and ejaculatory behaviors, suggesting that both the sexual arousal (libido) and potency components of male sexual behavior are adversely affected by diabetes (13, 35). Examination of these responses early in the diabetic condition, as done in this experiment, also further emphasizes the importance of these observations since minimal/none of the chronic functional effects of metabolic dysfunction or complications are evident at this early stage.

Various reports have suggested that the diminished sexual behavior in STZ-induced diabetic rats is related to changes in hypothalamic-pituitary gonadal axis (27, 29). However, gonadectomy did not change the urethrogenital reflexes, suggesting that androgen changes are unlikely as the cause of the sexual dysfunction in this model (10). The results of our study demonstrate a severe male sexual dysfunction of central neuropathic origin in diabetic rats. We have demonstrated a marked derangement of the central NO mechanism within the PVN of diabetic rats that leads to a lack of NMDA-mediated penile erection. These findings suggest a direct impact of diabetic neuropathy within specific central sites, such as the PVN, on male sexual dysfunction and make this a useful model to study mechanisms by which neurological dysfunction may cause sexual dysfunction in males. Our findings suggest that central neuropathic processes (within specific sites, such as the PVN) may cause diabetic penile erectile dysfunction and that manipulations of these processes (upregulating nNOS within the PVN by gene therapy) could alleviate the sexual dysfunction commonly observed in diabetes.

The involvement of central NO in penile erection has clinical implications in diabetes (6). The use of drugs involved in manipulations of the NO pathway, such as Sildenafil, an oral phosphodiesterase inhibitor, improves penile erectile function in patients with erectile dysfunction (6). It is of importance to note that Sildenafil requires the occurrence of sexual stimulation to elicit this effect. This implies that one possible action for the drug may be via improving the neurotransmission of central NO within the hypothalamic nuclei. That 50% of the diabetic males that have erectile dysfunction cannot be helped by Sildenafil (4, 17) also suggests that some other factor (other than a purely peripheral vascular abnormality) is involved in diabetic erectile dysfunction, perhaps a central component involving nNOS in the PVN. Therefore, there is certainly a need to develop basic and applied research to identify the central targets of such neuromodulation. Understanding these mechanisms would greatly enhance our ability to treat erectile dysfunction in various disease conditions, such as diabetes.

The results of these studies may have a more general implication of a central NO mechanism within the PVN involved in erectile dysfunction. This is consistent with the observation that mice that lack neuronal NO display grossly normal appearance, locomotor activity, long-term potentiation, and long-term depression, yet have abnormal sexual behavior (7). This implies that central NO, specifically within the PVN, may be a major mediator of sexual behavior that may be affected in various disease states that are associated with erectile dysfunction in various disease conditions. At present,
the potential mechanism of how diabetes decreases NO is still unknown. Modulation of NO synthesis and the interaction of NO with ANG II, known to be elevated in diabetes, may be an important mechanism. Our preliminary studies in NG108 cultured neuronal cell line shows that ANG II downregulates nNOS in these cells. Consistent with these observations, Park at el (25) have shown that blockade of angiotensin receptors (AT1) restores erectile capacity in normotensive aged rats (25). Thus it is possible that an elevated angiotensin level during diabetes is responsible for the downregulation of nNOS and subsequent centrally mediated erectile dysfunction observed in diabetes. This remains to be elucidated.

In summary, we have demonstrated that erectile dysfunction in diabetes is due to a selective defect in the NO mechanisms within the PVN. This defect is a result of a loss in the synthetic enzyme for the production of NO within neurons of the PVN. Restoring this synthetic enzyme may have a significant therapeutic value in diabetes mellitus in humans.

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