The Distribution of Nitric Oxide Synthase in Human Corpus Cavernosum on Various Impotent Patients

Young Deuk Choi, Sang Yol Mah, Zhong Cheng Xin, and Hyung Ki Choi

Recent evidence implicates NO (Nitric oxide) as the principal mediator in an erection. To investigate the role of NO in the human erectile function, we studied the distribution pattern of nitroaxergic fibers in the corpus cavernosum specimens obtained from 38 men using nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry. Diffusely scattered delicate nerve fibers showing blue color reaction after NADPH-d histochemical staining were observed in normal control specimens from potent men. The neurogenic impotence group showed a statistically-significant decrease in the number of positive fibers compared to the normal control group. The number of positive fibers in the non-neurogenic impotence group was decreased compared to the normal control group but was statistically insignificant. With nitric oxide synthase (NOS) immunohistochemical stain, immunoreactive nerve bundles were easily seen in normal control specimens from potent men. NOS immunoreactive nerve bundles were contained within the corpus cavernosa which stained with NADPH-d reaction. Our results suggest that nitric oxide, a potent smooth muscle relaxing neurotransmitter in the autonomic nervous system, plays a physiologic role in erectile function and NADPH-d enzyme histochemical staining on the biopsied corpus cavernosum may be used as an important diagnostic method in the evaluation of neurogenic impotence.

Key Words: Nitric oxide, nitric oxide synthase, impotence, erection, human
cyclic guanosine monophosphate (cGMP), and relaxation of rabbit corpus cavernosal muscles in vitro stimulation of NANC neurotransmission (Ignauro et al. 1990). Several reports have also produced evidence that supports NANC neurogenic relaxation of the human corpus cavernous muscle (Saenz de Tejada et al. 1988; Holmquist et al. 1991; Kim et al. 1993). However, only a few studies have evaluated the distribution of NO synthesizing nerve fiber in human corpus cavernosum. Thus, we investigated the innervation of the human cavernosum by NO-synthesizing nerves.

Production of NO from the terminal guanidine nitrogen of L-arginine has recently been shown to be a ubiquitous process through the action of enzyme nitric oxide synthase (NOS). Attempts at complete description of the location and amount of NO acting as a neurotransmitter have been hampered by its short half-life and gaseous nature. These characteristics have forced researchers to study more stable intermediates and the enzyme responsible for NO synthesis. Within the past year great efforts have been made by several groups to uncover the presence and distribution of NOS in various biologic systems.

Activities of NO could be demonstrated through immunohistochemical staining with antibodies against neuronal NOS, the enzyme responsible for NO generation from its precursor L-arginine (Burnett et al. 1993). Many investigators used the inexpensive and easily performed nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reaction as a marker for NOS in the autonomic nervous system (Brdt and Synder, 1990; Hope et al. 1991; Burnett et al. 1992). Vincent et al. (1992) and others have shown that the histochemical stain for enzyme NADPH-d specifically stained the same structures as those stained by a specific neuronal NOS antibody (Hope et al. 1991; Brock et al. 1993; Burnett et al. 1993). Dawson et al. have recently shown NADPH-d co-localization with antiserum directed against NOS, in all but a few exceptions, in the brain and peripheral nervous system of rats and monkeys (Dawson et al. 1991). Reports by several authors concerning the similarity in staining patterns between NADPH-d staining and NOS immunohistochemistry have provided us with the opportunity to evaluate the importance of NANC nerve fiber contribution to erectile function (Hope et al. 1991; Brock et al. 1993; Burnett et al. 1993). Therefore, to investigate the role of NO in human erectile function, we studied the distribution pattern of NO containing NANC fibers in the corpus cavernosum of men with and without erectile dysfunction using NADPH-d histochemistry.

**MATERIALS AND METHODS**

**Materials**

- Corpus cavernosal muscle biopsy samples were obtained from 38 men who underwent penile surgery and divided into 3 groups: potent controls, neurogenic impotence, and non-neurogenic impotence.

The control group (n=9, 55±13.3 (mean±SEM) years) was comprised of 2 men undergoing peneectomy for penile cancer, both of whom gave a history of normal erectile rigidity, and 7 men undergoing penile prosthesis implantation for psychogenic impotence (n=5) and Peyrion’s disease (n=2). All showed normal erectile events on nocturnal penile tumescence test.

Men who did not show normal erectile events in an erection response test to audiovisual stimulation and at least three nocturnal penile tumescence tests were classified as neurogenic impotence (n=14, 44±13.4 years) or non-neurogenic impotence (n=15, mean 50±9.0 years) according to their neurologic status of cavernous nerve.

Subjects with neurogenic impotence had cavernous nerve abnormalities in neurologic examination: 14 men with sacral lesions such as spinal cord injury (n=10; paraplegia) and myelopathy (n=4). All of them had underlying neurologic disease and demonstrated abnormal physical neurologic examination. We measured bulbocavernous reflex latency and dorsal nerve somatosensory evoked potential using an electrophysioograph (Excell, Cadwell AB., Inc., Kennewick, Washington, U.S.A). We adopted 33.8~42.6 msec. for the normal values of short latency somatosensory evoked potential stimulating dorsal nerve and 25.2~39.6 msec. as normal bulbocavernous reflex latency. The latency of bulbocavernous reflex and somatosensory evoked potential stimulating dorsal nerve was prolonged in every man with
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neurogenic impotence.

Subjects with non-neurogenic impotence totalled of 15 men. The causes for impotence were DM in 5, hypogonadism 2, hypertension 3 and unknown 5. All men had a consistently poor response to intra-cavernosal prostaglandin E1 (10 µg) injection but a normal bulbocavernous reflex latency and somatosensory evoked potential stimulating dorsal nerve. Further investigation using a combination of duplex ultrasonography or cavernoinsomay/cavernoosography had demonstrated failure of venous occlusion in 6 patients, penile arterial insufficiency in 2 and mixed venous/arterial abnormalities in 3.

Methods

Tissue preparation

All specimens were obtained at operation and were taken from the deep penoscrotal portion of the cavernosum and were at least 1 to 3 mm thick. Every tissue was immediately frozen in liquid nitrogen. At the time of staining, thin sections (6 µm thick) were cut on a cryostat (−18°C) and mounted onto gelatin/chrome alum-coated slides.

NADPH diaphorase staining

Slide-mounted sections were initially incubated in 4% paraformaldehyde in 0.05 M phosphate buffered saline (pH 8.0) for 15 minutes. The sections were incubated in a mixture of 1mM β-NADPH (Sigma Chemical Co. St. Louis, MO, U.S.A.), 0.2 mM nitroblue tetrazolium (Sigma) and 0.2% Triton X-100 in 0.1 M phosphate buffer (pH 7.4) for 30 minutes at 37°C, then washed with buffer before mounting with buffered glycerol.

Staining was assessed by counting the number of NADPH-d positive nerve fibers present in 10 random fields (power ×200).

NOS immunohistochemistry

Slide-mounted tissue sections were incubated in 4% paraformaldehyde in 0.1M phosphate buffered saline for 15 minutes and then permeabilized in 0.2% Triton X-100 for 15 minutes. The sections were treated with 0.3% hydrogen peroxide solution for 10 minutes to inactivate endogenous peroxidase. All sections were subsequently treated with 1.5% normal goat serum for 30 minutes at 4°C and then incubated overnight at 4°C with 1:50 dilution of an anti-NOS rabbit affinity-purified polyclonal antibody (Transduction Labs., Lexington, Kentucky, U.S.A.). Sections were then stained with an avidin-biotin complex system (Vector Lab., Burlingame, CA, U.S.A.) with diaminobenzidine as the chromogen and then counter-stained with hematoxylin.

S-100 protein immunohistochemistry

To confirm the identification of nerves, immunohistochemical staining using antibody to S-100 protein was performed in specimens from potent controls and neurogenic impotence. All specimens were fixed in 10% formaline for 24 hours, processed routinely and embedded in paraffin. Slide sections 6µm-thick were treated with 0.3% hydrogen peroxide solution for 10 minutes to inactivate endogenous peroxidase. All sections were subsequently treated with 1.5% normal goat serum for 30 minutes at 4°C and then incubated with anti-S-100 protein antibody (Dako Corp., Carpinteria, CA, U.S.A.; used at a 1:300 dilution) for 60 minutes at room temperature. Sections were incubated with HRP-labeled secondary antibodies (Dako Corp., Carpinteria, CA, U.S.A.) for 60 minutes at room temperature. After further washing in PBS, they were reacted with AEC, and then counter-stained with hematoxylin.

Staining was assessed by counting the number of S-100 protein antibody positive nerve fibers present in 10 random fields (power ×200).

Statistics

Results were expressed as mean±standard error of mean with n showing number of patients. The distribution of results was skewed and statistical significance was tested using Student’s t-test for comparison between groups. A result was deemed statistically significant if p≤0.05.

RESULTS

NADPH-d histochemical staining revealed pos-
Fig. 1. Photograph of a biopsy specimen obtained from a potent man (A, B) and a patient with neurogenic impotence (C). A: positively-stained nerve bundles for NADPH diaphorase showing blue color reaction (arrows). B: diffusely scattered positively-stained fine nerve fibers for NADPH diaphorase (arrows). C: reduced number of positively-stained fine nerve fibers for NADPH diaphorase compared to that of a potent man (arrows).

Table 1. Number of NADPH-d positive staining nerve fibers in the corpus cavernosal tissue

<table>
<thead>
<tr>
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<th>Potent control (n=9)</th>
<th>Non-neurologic impotence (n=15)</th>
<th>Neurogenic impotence (n=14)</th>
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<tr>
<td>NADPH-d</td>
<td>269.44 ± 73.22</td>
<td>208.46 ± 56.16*</td>
<td>126.85 ± 43.80**</td>
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@: Mean ± SEM of NADPH-d positive staining nerve fibers in 10 random fields
*: p>0.05 vs. potent control group
**: p<0.001 vs. potent control group

Among the 15 samples from men with non-neurogenic impotence, the average number of positively-staining fibers (mean 208.46 ± 56.16 fibers/10 random fields) was slightly reduced in comparison with potent men.

The number of NADPH-d positively-staining fi-
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**Fig. 3.** Nitric oxide synthase histochemistry (A) and immunohistochemistry (B) in corpus cavernosum. Photomicrographs depict tissue sections containing a representative nerve bundle. A: NADPH diaphorase histochemistry. B: immunohistochemistry with purified anti-nitric oxide synthase antibody. NADPH diaphorase positive staining nerve bundle is also immunohistochemically stained by NOS antibody.

**Fig. 4.** Staining of S-100 protein in a biopsy specimen from a potent man (A) and a patient with neurogenic impotence (B). It demonstrates diffusely scattered positively-stained fine nerve fibers for S-100 protein within the corpus cavernosum. Note no significant difference in distribution of S-100 protein staining between the specimen from a potent man and a patient with neurogenic impotence.

bers from potent men displayed a range from 182 to 403, whereas those of men from the neurogenic impotence group ranged from 61 to 212 (Fig. 2). There existed a small degree of overlap of the NADPH-d positively-staining fiber numbers between the groups. The number of NADPH-d positively-staining fibers in biopsy samples taken from men with non-neurogenic impotence was between the number in the potent control group and neurogenic impotence group (Fig. 2).

With NOS immunohistochemical stain, immunoreactive nerve bundles were easily seen in specimens from potent men and their distribution was similar to those stained by NADPH-d reaction (Fig.
Table 2. Number of S-100 protein antibody positive staining nerve fibers in the corpus cavernosal tissue

<table>
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<th>Potent control (n=5)</th>
<th>Neurogenic impotence (n=5)</th>
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<td>1496.8 ± 69.25</td>
<td>1190.3 ± 249.81*</td>
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*: Mean ± SEM of S-100 protein antibody positive staining nerve fibers in 10 random fields
*: p>0.05 vs. potent control group

3). Immunostain with antibody to S-100 protein, an excellent general nerve stain, demonstrated diffusely scattered delicated nerve fibers within the corpus cavernosum in biopsy specimens of men from the potent control group. The number of positively-stained fibers was slightly decreased in specimens from men with neurogenic impotence, but no significant differences were found in distribution of S-100 protein staining between the control and neurogenic impotence groups (Table 2)(Fig. 4).

DISCUSSION

NOS catalyzes the synthesis of NO from L-arginine via a Ca²⁺/calmodulin-dependent mechanism. NO is believed to act as a neurotransmitter, eliciting smooth muscle relaxation by stimulating the formation of cGMP through activation of guanylyl cyclase. This cGMP synthesis in turn results in a decrease in intracellular Ca²⁺ and subsequent smooth muscle relaxation and erection of the penis (Moncada, 1992). NADPH-d enzyme histochemical staining occurs as a blue precipitate with nitroblue tetrazolium in the presence of NADPH. NOS requires NADPH as a cofactor for conversion of L-arginine to citrulline and NO.

The recent availability of an antibody specific for NOS and the several recent reports concerning the similarity in staining pattern between the aforementioned enzyme histochemical method and immunohistochemical method have provided us with the opportunity to evaluate the contribution of the NANC nerve fiber to erectile function by applying both methods (Hope et al. 1991; Brock et al. 1993; Burnett et al. 1993). With immunohistochemical stain, immunoreactive nerve bundles were easily seen in specimens from the potent control group. NOS immunoreactive nerve bundles were scattered within the corpus cavernosa and their distribution was similar to those stained by enzyme histochemical method.

The ability to define the level of sexual function on the basis of NANC nerve fiber density and location within the sinusoidal space has previously been described by a few researchers (Brock et al. 1993). Brock et al. reported that the intensity of staining NADPH-d in cavernosal tissue has been shown to be decreased in patients with cavernosal nerve injury (Brock et al. 1993).

Our results revealed a sharply distinct difference in staining pattern for NADPH-d reaction between the potent control group and neurogenic impotence group. In men from potent control, an abundant number of positively-stained fibers were recorded, but in men from the neurogenic impotence group, a fairly-reduced number of NADPH-positive nerve fibers were seen. So enzyme histochemical staining to NADPH-d might provide an important insight into the functional integrity of the human cavernous nitroxynergic nerve and thus might be used as a diagnostic method in the evaluation of neurogenic impotence.

S-100 protein is a dimeric, acidic protein with a molecular mass of 21kD, and has been considered to be specific to the nervous system (Bishop et al, 1985). The nerves stained positively S-100 protein immunostaining are nonselective and all the adrenergic, cholinergic, and NANC neuroeffector systems are known to be stained. Our results on S-100 immunohistochemical staining demonstrated that there was no significant difference in the distribution and number of positively-stained fibers between the studied groups. This result supports the selective decrease of NADPH-d stained nerve fibers in the neurogenic impotence group.

We defined the neurogenic impotence group as those who had presumed cavernous nerve injury, but there were other types of erectile dysfunction regardless of nerve status. We studied men with non-neurogenic impotence who did not have neurologic abnormalities. Results of 15 samples from the non-
neurogenic impotence group showed that the number of NADPH-d positively-staining fibers displayed a range between the numbers shown in the potent control group and neurogenic impotence group. This implies that erectile dysfunction may stem from the reduced function of NADPH-d fibers even if there are no distinct clinical abnormalities in neurologic examination. Therefore, a defect in the NO-mediated corporeal relaxation may be the critical mechanism of many clinical forms of erectile dysfunction. Lugg et al. showed that aging in rats resulted in a decreased erectile response to electric field stimulation, which was accompanied by a reduction in penile NOS activity in senescent rats (Lugg et al. 1995). In addition, several animal models (e.g., with diabetes, peripheral neuropathy induced by cavernous nerve neurectomy, hypercholesterolemia, testosterone deprivation, aging) have shown the alteration of NOS activity (Burnett et al. 1995; Carrier et al. 1995; Garban et al. 1995; Vernet et al. 1995; Zavara et al. 1995). In our study, the NADPH-d stained fiber number displayed a variable range according to their etiologic factors. In some cases the presence of positively-stained nerve bundles was easily visible, but in DM cases without neurologic abnormalities, NADPH-d positive nerve fibers were markedly reduced. Positively-stained fibers were also slightly reduced in the 3 hypertension and 2 hypogonadism patients. However, since we presently lack a sufficiently-large sample to define these changes according to the etiologic factors, larger studies are still needed.

The various staining patterns evident from our study provide additional support to the large body of scientific evidence already accumulating that NO is an important mediator of penile erection.

To date, no method exists to test the integrity of the penile autonomic innervation directly. Diagnosis of a neurogenic cause for impotence requires measurement of the somatosensory penile innervation, usually achieved by recording a bulbocavernous reflex, although the validity of this test has been doubted by some (Lavoisier et al. 1989). Neurogenic impotence remains a diagnosis of exclusion based on a normal vascular assessment in a man with no evidence of psychologic risk factors and in whom a neurologic impairment is likely. Recently, several researchers have suggested that recording electromyographic activity from the tissue of the corpus cavernosum may detect penile neuropathy (Stief et al. 1991; Junemann et al. 1993). But the results are still unclear and many studies are required to validate the value of the electromyographic activity from the corpus cavernosum. Based on the results of our study, NADPH-d enzyme histochemical staining on biopsied cavernosal tissue may be used as a minimally invasive diagnostic tool that can directly assess the cavernous nerve function.

In conclusion, our results support the presumption that nitroxyergic transmission of the corpus cavernosum exists in humans and indicates a role for NO as a physiologic neurotransmitter that induces penile erection. NADPH-d activity within nerve fibers in the corpus cavernosum indicates that the easily performed and inexpensive NADPH-d technique can be used as a valid diagnostic tool for neurogenic conditions of the corpus cavernosum.

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