

Role of Nitric Oxide in Penile Erection

Hee Chang Jung¹, Ki Hak Mun¹, Tong Choon Park¹,
Yung Chang Lee², Jong Min Park³, Keun Huh³,
Do Hwan Seong⁴, and Jun Kyu Suh⁴

The present study was undertaken to investigate the role of nitric oxide (NO) in erectile physiology by correlating its action with the existence and activity of nitric oxide synthase (NOS), which produces NO. We applied Western blot analysis in both human and rat penile tissue. In the rat, reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining and spectrophotometric assay were also performed, in addition to in vivo electroerection study with pharmacological manipulation. Western blot analysis identified a protein of 155 KDa identical to the neural form of NOS in the human and rat penis. The NOS blot densities in the two species were similar, and both were lower than that in the rat cerebellum. Histochemical staining localized NOS to neurons innervating the corpora cavernosa, including the pelvic plexus, the cavernosal nerves and their terminal fibers within the corporeal erectile tissue, and dorsal penile nerves. NOS activity was also found in the cerebellum, urethra, penis, and urinary bladder, in decreasing order of intensity. Intracavernous injections of NOS inhibitor (L-NOARG or L-NAME in concentrations from $10^{-6}M$ to $10^{-3}M$ suppressed electrostimulation-induced erection in a concentration-dependent manner. Subsequent intracavernous injection of L-Arginine ($10^{-2}M$) partially restored the erection. The neural form of constitutive NOS in the corpora cavernosa synthesizes NO, which mediates penile erection. Determination of cavernosal NOS expression or activity may permit characterization of certain pathological conditions that cause impotence.

Key Words: Nitric oxide, nitric oxide synthase, penile erection

Nitric oxide (NO), a short-lived and freely diffusible molecule, is generated from L-arginine and exhibits several biological functions in mammalian cells and tissues. In blood vessels, it is responsible for non-adrenergic non-cholinergic (NANC) vasodilation (Furchgott, 1990; Moncada *et al.* 1991); in

macrophages, it mediates cytotoxicity. Also, NO functions as an atypical neurotransmitter in the central nervous system and as an agent of autonomic neurotransmission in the peripheral nervous system (Bredt and Snyder, 1992).

Penile erection involves parasympathetic, neuro-nally mediated relaxation of the blood vessels and trabecular meshwork of smooth muscle that constitutes the corpora cavernosa (de Tejada *et al.* 1988). The relaxation of the cavernous smooth muscle plays a critical role in an erection, which is largely nerve-mediated by a NANC mechanism (de Tejada *et al.* 1988). However, endothelium-dependent cholinergic neurotransmission may also mediate penile erection (Trigo-Rocha *et al.* 1993). Several *in vitro*

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Department of Urology¹ and Anatomy², School of Medicine, and Department of Pharmacology³, College of Pharmacy, Yeungnam University, Taegu, Department of Urology⁴, School of Medicine, Inha University, Incheon, Korea

Address reprint request to Dr. J.K. Suh, Department of Urology, Inha University Hospital, Shinheung-Dong 3-Ga, Choong-Gu, Incheon 400-103, Korea

studies have demonstrated that NO is responsible for the relaxation of human and rabbit cavernous smooth muscle (Ignarro *et al.* 1990; Azadzi *et al.* 1992), and recent *in vivo* studies in several species demonstrated that NO is an important mediator of penile erection (Trigo-Rocha *et al.* 1993; Wang *et al.* 1994). Our previous studies have also shown similar results in the rat electroerection model (Jang and Suh, 1995).

Nitric oxide synthase (NOS), which catalyzes NO formation, has been purified from the brain (Bredt and Snyder, 1990) and molecularly cloned. The enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, specifically stains at the same site as a specific neuronal NOS antibody (Vincent and Hope, 1992), and neuronal NADPH diaphorase is regarded as an NOS (Hope *et al.* 1991). Recently, NOS has been localized to nerves within the rat penis and spinal nuclei innervating spinal ganglia (Burnett *et al.* 1992) and has also identified in the human penis (Brock *et al.* 1993).

The aim of the present investigation was to clarify the role of NO in penile erection and to correlate it with NOS. To this end, we applied immunoblotting and histochemical methods to localize NOS in cavernous tissue, in addition to *in vivo* electroerection to characterize the pharmacologic action of NO.

MATERIALS AND METHODS

Human penile tissues (n=3) were procured at prosthesis insertion for erectile impotence with venogenic cause and used for Western blot analysis. Adult male Sprague-Dawley rats (250~300 gm; n=60) were used for Western blot analysis, NADPH diaphorase histochemical stain, NOS assay, and *in vivo* electroerection. In the rat, anesthesia was induced by sodium pentothal (50 mg/Kg) intraperitoneally.

Western blot analysis

After anesthesia had been induced, the cerebellum and penis were selectively obtained from the rats. Rat and human corpus cavernosum were homogenized in 0.1 M phosphate buffer (pH 7.5), and the solution was centrifuged at 600×g to remove cell

debris. The cytosol fraction was separated from the mitochondrial fraction by centrifugation at 10,000×g. Prior to SDS-PAGE analysis, the cell lysates were adjusted to 15 mg of total protein from various lysates of human penis, rat penis and rat cerebellum. Electrophoresis in 7.5% SDS-denatured polyacrylamide gels was performed according to the procedure of Laemmli (Laemmli, 1970) using the Hofer Model SE 250 apparatus. The gels were stained with Comassie brilliant blue R250 (Aldrich, Milwaukee, Wisconsin, USA). Equivalent other gels were run and the proteins transferred onto nitrocellulose membranes using standard techniques. Western blot analysis was performed according to the procedure of Towbin and associates (Towbin *et al.* 1979) using rabbit polyclonal anti-brain NOS (Transduction Laboratories, Lexington, Kentucky, USA) as a primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) as a secondary antibody. The polyclonal anti-brain NOS was purified by affinity chromatography from rabbit cerebellum and has been shown to be a distinct gene product of 155 KDa. The blots were visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma, St. Louis, MO, USA). The molecular weight was confirmed by using 116 KDa (β -galactosidase) and 205 KDa (myosin) standards. The blot density of NOS was measured by a Macintosh computer program (NIH image V 1.57/68 K)

Histochemical staining for NADPH-diaphorase

After anesthesia, the rats were placed in a supine position, and the heart was exposed through a sternotomy and pericardiectomy. The heart was perfused with normal saline through the right atrial root for 5 min to remove the blood. The tissues were then fixed for 10 min in freshly prepared 2% paraformaldehyde/phosphate-buffered saline (PBS). With the aid of a Zeiss dissecting microscope, the pelvic tissue, including the major pelvic ganglion and cavernous nerve and the penis, were removed. Tissue specimens were cryoprotected overnight in 25% sucrose PBS at 4°C. Cryostat sections (6 μ m) were cut and allowed to adhere to charged slides, air-dried for 5 min, and hydrated for 10 min in PBS. Sections were incubated with NADPH, NBT, Triton-X, PBS

(pH 8.0) for 45 min at 37°C. The reaction was terminated by washing the sections in buffer; coverslips were applied with buffered glycerin as a mounting medium. The staining pattern was assessed by confirming the densely blue region present in four random 400× fields of the pelvic plexus, cavernous nerve, dorsal nerve and cavernous erectile tissue.

Spectrophotometric assay of NOS activity

The cerebellum, posterior urethra, penis, and bladder taken from six rats were homogenized in 0.25 M sucrose buffer, and cytosols were prepared as for Western blotting analysis.

The assay of NADPH diaphorase activity was performed as described by Bredt and Snyder (1990) with some modification. Briefly, enzyme assays contained 125 mL of each tissue cytosol and 125 mL of 100 mM arginine to 50 mL in buffer containing 50 mM HEPES (pH 7.4), 2 mM NBT, 1 mM NADPH, 1 mM EDTA, 1.25 mM CaCl₂ and 5,000 units of calmodulin per milliliter. After incubation for 5 min at 37°C, spectrophotometric assay was carried out at a wave length of 585 nm (Shimadzu model UV 1201, Kyoto, Japan).

The nitrite concentration in various cytosols was measured by spectrophotometric assay according to Griess reaction. A mixture of 180 mL of various cytosol, 10 mL of 2 mM NADPH, and 10 mL of 2 mM arginine was incubated for 1 hour at 37°C and then incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min. The absorbance was measured at 550 nm. Nitrite content was calculated using sodium nitrite as a standard. NOS activity was measured by colorimetric assay of NADPH diaphorase activity, which was determined as the degree of nitroblue tetrazolium formazan (NBTF) production. NADPH diaphorase calculations were based on a molar extinction coefficient for NBTF=12,600 M⁻¹.cm⁻¹.

Electroerection

After anesthesia, the rat was placed in a supine position, and the bladder and prostate were exposed through a midline abdominal incision. With the aid

of a Zeiss dissecting microscope, the major pelvic ganglion and cavernous nerve were identified posterolaterally to the prostate, and platinum wire electrodes were placed around the nerve for electrical stimulation. The penis was denuded of skin. Two 25 gauge needles were inserted into each corpus cavernosum, one for intracavernous pressure (ICP) recording and the other for intracavernous drug injection. Systemic arterial blood pressure was monitored via a 25 gauge cannula in the carotid artery. All fluid-filled lines were connected to a Statham pressure transducer and a Macpacq system (Biopac Systems, Santa Barbara, California, USA) and, finally, to a Macintosh computer for recording and data analysis.

In all experiments, cavernous nerve stimulation was performed. Parameters of stimulation delivered were 1 Hz, 3~6 Volt, square-wave pulses (1 msec pulse width) to obtain a full erectile response. The drugs were injected intracavernously at 0.1 mL/injection. Between injections, there were intervals of approximately 10~15 min. Chemicals and drugs used included N-nitro-L-arginine (L-NOARG); N-nitro-L-arginine methyl ester (L-NAME); and L-arginine hydrochloride (Sigma).

After determination of the full erectile response to nerve stimulation, L-NOARG was injected intracavernously in increasing concentrations of 10⁻⁶, 10⁻⁵, 10⁻⁴ and 10⁻³ M. Nerve stimulation was repeated 12 min after each dose (n=7). In the other group of rats (n=7), L-NAME was injected, and neurostimulation was repeated in the same manner.

After determining the effect of 10⁻³ M NOS inhibitor on the erection induced by nerve stimulation, 10⁻² M of L-arginine was injected intracavernously, and nerve stimulation was repeated 10 min later.

Calculations and statistics

Data on δ ICP (maximal ICP-basal ICP) are given as mean values \pm standard deviation. Statistical analyses were performed using the SPSS software program. Statistical significance was tested by repeated measures of ANOVA (Fig. 6) and by the student's t test for paired comparison (Fig. 7). P-values < 0.05 were considered significant.

RESULTS

Western blot analysis

Data of Western blot analysis are means of triplicate determinations from a representative experiment, which was repeated with similar results. Analysis of Coomassie blue-stained SDS-PAGE gels revealed various protein expression bands corresponding to a molecular mass between 116 KDa and 205 KDa (Fig. 1A). Western blot analysis demonstrated that the neuronal NOS blot strongly recognized a protein with an apparent molecular mass of 155 KDa both in human and rat corpus cavernosum, as well as in rat cerebellum (Fig. 1B). Relative to the rat cerebellum, the NOS blot density was 64% in the human penis and 63% in the rat penis. We also

performed immunoblotting analysis of both human and rat corpus cavernosum with a rabbit polyclonal or monoclonal anti-endothelial NOS (Transduction Laboratories, USA). However, it was difficult to discriminate endothelial NOS blot among many protein blots, reflecting that the eNOS antibody has a cross-reaction with other tissue proteins and is not appropriate for this study.

NADPH diaphorase

The presence of NADPH diaphorase-positive nerves is easily apparent as a highly localized, densely blue lesion. The center for autonomic innervation in the pelvis, the pelvic plexus, contains a network of nerves and ganglia that stained positively for NADPH (Fig. 2A). At the prostatomembranous junction, cavernous nerves arising dorsolateral to the urethra and extending to the penis stained positively (Fig. 2B).

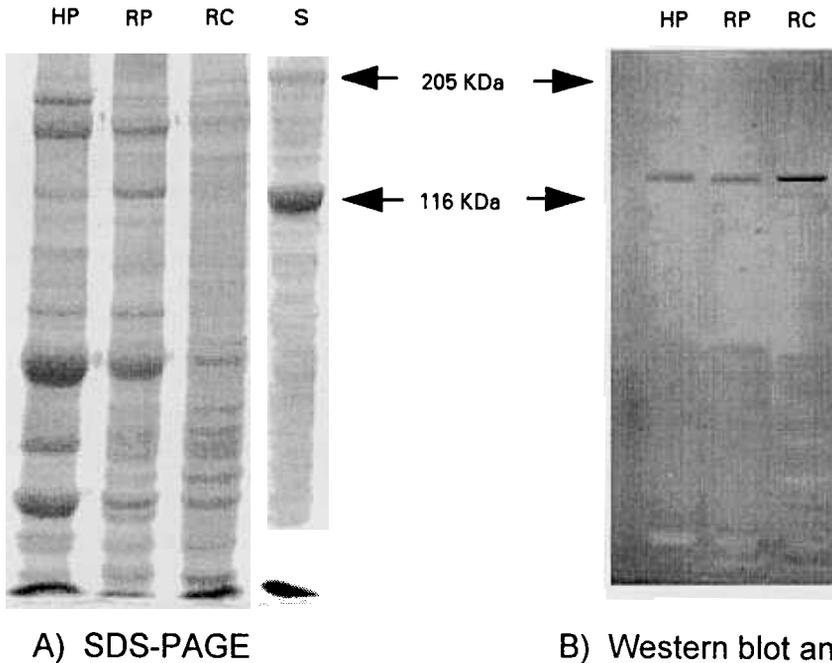


Fig. 1. Western blot analysis of various tissue cell lysate for the identification of nitric oxide synthase (NOS). A) 7.5% SDS-polyacrylamide gels run by the Laemmli method, and then stained with Coomassie blue. B) Western blot analysis was performed using rabbit polyclonal anti-brain NOS as a primary anti-body and goat anti-rabbit IgG conjugated with alkaline phosphatase as a secondary antibody, after running 7.5% SDS-polyacrylamide gel electrophoresis. The blots were visualized by BCIP and NBT. S: standard molecular marker (205 KDa: myosin, 116 KDa: β -galactosidase), HP: human penis, RP: rat penis, RC: rat cerebellum.

Within the corpora cavernosa, NADPH-positive staining was localized to terminal nerve fibers, especially around the cavernous vessel and its endothelium (Fig. 3A). The dorsal aspect of the penis exhibited a NADPH-positive staining pattern of the dorsal nerves (Fig. 3B). The distribution of the nerves in the rat pelvis and the cavernous erectile tissue staining positively for NOS corresponded to the autonomic innervation of the corpora cavernosa.

NOS activity

The mean values of NOS activity in the organs

are shown in Fig. 4. The activity of NADPH (nmol/mg/min.) was 88.2 ± 13.9 in the cerebellum (n=13) (Fig. 4), 69.2 ± 9.1 in the urethra (n=13), 54.8 ± 6.0 in the corpus cavernosum (n=13), 49.6 ± 5.6 in the bladder (n=13). The NADPH diaphorase activity relative to that in the cerebellum was $80.4 \pm 12.1\%$ in the urethra, $65.3 \pm 4.3\%$ in the penis, and $62.2 \pm 8.2\%$ in the bladder. The nitrite amount ($\mu\text{M}/\text{gm}$ of tissue) in the cerebellum, urethra, penis, and bladder were 0.80 ± 0.01 , 0.55 ± 0.05 , 0.36 ± 0.02 , and 0.14 ± 0.01 , respectively (n=6~8, Fig. 5). These values correlate well with the NADPH diaphorase activity.

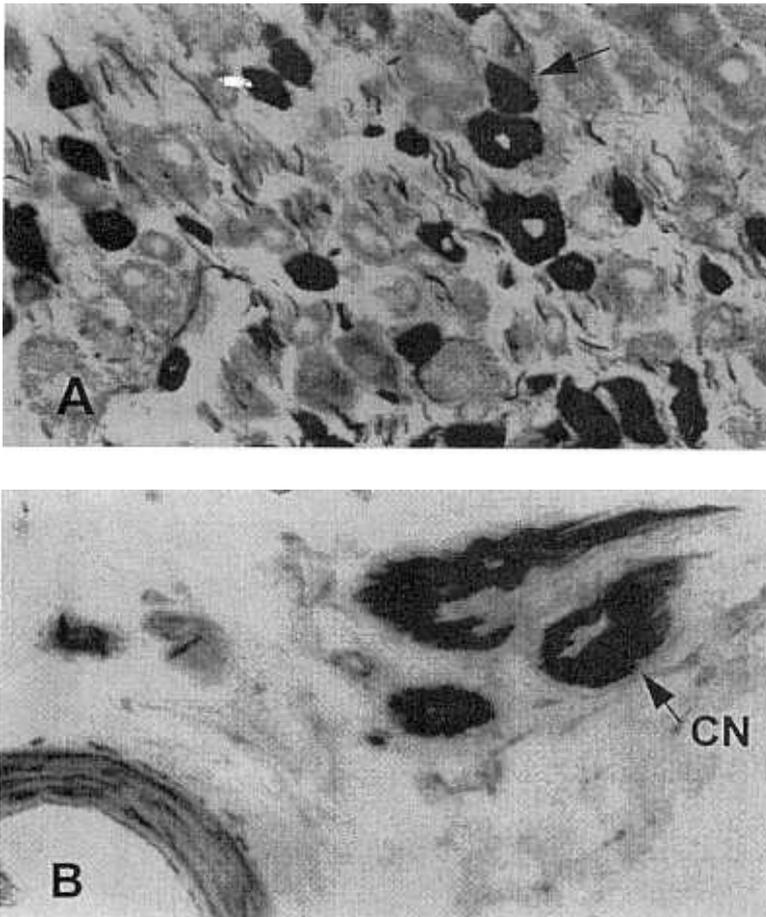


Fig. 2. Histochemical localization of nitric oxide synthase (NOS) with NADPH diaphorase staining in the rat penis. A) Histochemical localization of NOS in pelvic ganglia adjacent to the prostate. Individual ganglion cell bodies (arrow) and nerve fiber contain NOS. Reduced from $\times 400$. B) Cross-section through the penis proximally shows prominent nerve plexus staining of the cavernosal nerve (CN). Reduced from $\times 400$.

Electroerection

The increases in the ICP value after cavernous nerve stimulation were 95.6 ± 2.8 mmHg in the L-NOARG-treated group and 96.5 ± 2.8 mmHg in the L-NAME-treated group. Intracavernous injection of L-NOARG at concentrations from 10^{-6} to 10^{-3} M suppressed the electrostimulation-induced erection in a dose-dependent manner. Intracavernous injection of L-NAME at concentrations from 10^{-6} to 10^{-3} M also suppressed the nerve-induced erection in a

dose-dependent manner ($p < 0.05$; Fig. 6). Electro-stimulation-induced erection was mostly abolished by the injection of 10^{-3} M L-NOARG or L-NAME.

After nerve stimulation, the ICP was 4.3 ± 1.7 mmHg in the L-NOARG (10^{-3} M)-pretreated animals ($n=7$) and 9.2 ± 2.4 mmHg in the L-NAME (10^{-3} M)-pretreated group ($n=7$). Subsequent intracavernous injection of L-arginine hydrochloride (10^{-2} M) increased the ICP significantly both in the L-NOARG-pretreated group (23.6 ± 4.6 mmHg) and the L-NAME-pretreated group (32.9 ± 5.2 mmHg). Therefore, L-arginine partially restored penile erec-

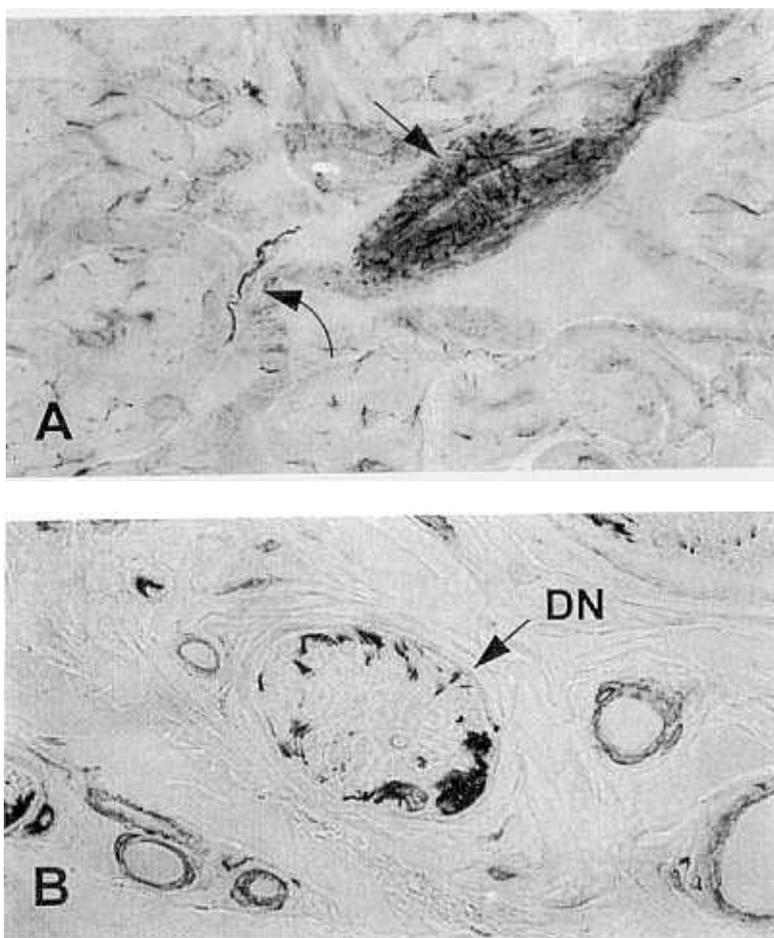


Fig. 3. Histochemical localization of nitric oxide synthase (NOS) with NADPH diaphorase staining in the rat penis. A) Cross-section through the penis proximally shows endothelial staining of the cavernosal vessel (arrow) and to nerves extending into erectile tissue (curved arrow). Reduced from $\times 400$. B) Cross-section through the penis distally shows nerve fiber staining of the dorsal nerve (DN). Reduced from $\times 400$.

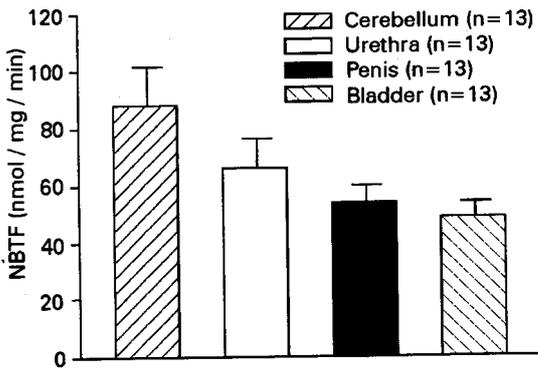


Fig. 4. Nitric oxide synthase (NOS) activity in various organs of the rat. NOS activity was measured by colorimetric assay of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity, which was determined as the degree of nitroblue tetrazolium formazane (NBTF) production. Values represent mean \pm SE.

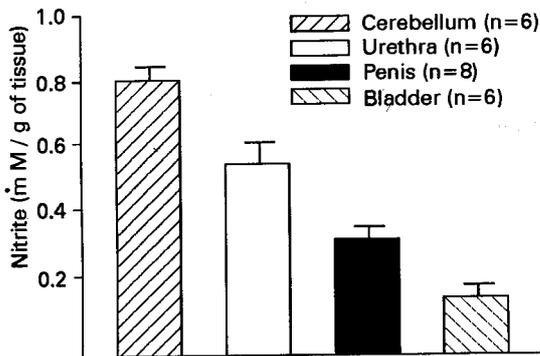


Fig. 5. Nitrite synthesis in various organs of the rat. Nitrite was measured by colorimetric assay based on Griess reaction. Values represent mean \pm SE.

tion suppressed by NOS inhibitor (Fig. 7).

DISCUSSION

In the past decade, intensive research has focused on the principal neurotransmitter, which is responsible for cavernous smooth muscle relaxation and penile erection. Many candidates have been identified, but recently, NO emerged as the most likely (Ignarro *et al.* 1990; Azadzoi *et al.* 1992; Wang *et al.*

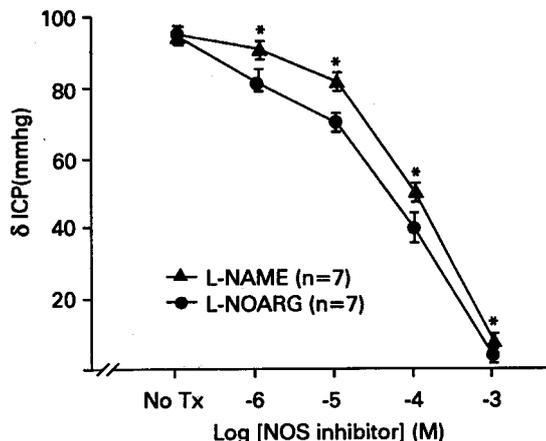


Fig. 6. Effect of L-NOARG and L-NAME on penile erection induced by cavernous nerve stimulation in the rat (n=7). Values represent mean \pm SE. L-NOARG: N ω -Nitro-L-arginine. L-NAME: N ω -Nitro-L-arginine methyl ester. δ ICP: maximal intracavernosal pressure-basal intracavernosal pressure. **p* < 0.05: significantly different from group with no treatment

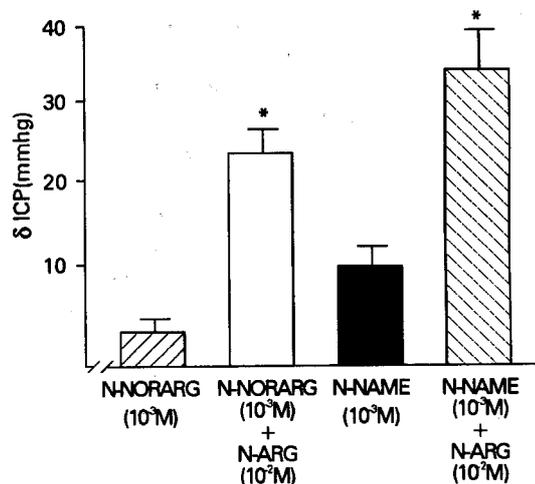


Fig. 7. Effect of L-arginine hydrochloride on penile erection suppressed by L-NOARG or L-NAME in the rat (n=7). Values represent mean \pm SE. L-NOARG: N ω -Nitro-L-arginine. L-NAME: N ω -Nitro-L-arginine methyl ester. L-ARG: L-arginine hydrochloride. δ ICP: maximal intracavernosal pressure-basal intracavernosal pressure. **p* < 0.05: significantly different from L-NOARG or L-NAME

al. 1994; Jang and Suh, 1995). The source of NO - nerve endings, endothelium, or smooth muscle - has been a matter of controversy, and localization has been hampered by NO's short half-life and gaseous nature. These characteristics have forced researchers to study more stable intermediates and the enzyme responsible for NO synthesis (Nathan, 1992). This enzyme has been found in the brain and selected neuronal sites in the periphery (Bredt and Snyder, 1992). Several isoforms have been identified (Nathan, 1992), the most important of which are the constitutive NO synthases present in the endothelium and neural tissue and the inducible enzyme formed in activated immune cells and vascular smooth muscle. Recent localization of NOS in a variety of organs, including the penis, has indicated that NO has a neural source and role.

In the present study, we performed immunoblotting analysis of human cavernous tissue with a rabbit antibrain antibody that is highly selective for NOS in human and rat tissue. The enzyme, a distinct gene product of about 155KDa, was expressed in human and rat cavernosum with similar blot density. These findings confirm the existence of neuronal NOS in the corpus cavernosum.

To further specify the localization of NOS, we performed NADPH diaphorase staining. This method is very useful in evaluating NOS activity, based on the theory that NOS is identical to NADPH diaphorase and requires NADPH as a cofactor for conversion of L-arginine to citrulline and NO. NADPH diaphorase positive nerves were widely distributed, from major pelvic ganglion to axonal fiber innervating rat corpus cavernosum, compatible with previous findings of NOS localization (Burnett *et al.* 1992; Brock *et al.* 1993). This distribution of NOS nerves suggests a role for mediation of penile erection. The dorsal nerve in the rat seems to have an autonomic component as it showed a positive staining pattern. However, it requires further investigation to elucidate its role on penile erection in pathologic conditions. In contrast to the distinctive staining pattern of nerve fibers, that of vascular endothelium and cavernous smooth muscle was too variable to confer additional information, although these tissues usually stained faintly. Relative levels of NOS activity showed a regional predominance within the rat pelvis, which was compatible with the

findings by measurement of nitrite, as an indirect assay of NOS activity, in the same structure. The localization of NOS to neuronal fibers and the blood vessels of the penis suggests a role for NO as a neuronal mediator of erection.

We attempted to correlate the neural role of NO with the *in vivo* erectile response to the administration of NOS inhibitor of the NO substrate. For this work, the rat model has been well established (Quinlan *et al.* 1989). In our earlier experience, we also found that cavernous manipulation with drugs can be accomplished without difficulty (Suh *et al.* 1995). The NOS inhibitors of L-NOARG or L-NAME suppressed penile erection induced by cavernous nerve stimulation. In addition, L-arginine, the substrate for NO synthesis, partially restored the erection suppressed by the inhibitors. These findings fit with recent *in vivo* and *in vitro* experiments (Ignarro *et al.* 1990; Azadzi *et al.* 1992; Wang *et al.* 1994; Jang and Suh, 1995). Therefore, the selective localization of NOS in penile neurons, as well as the ability of NOS inhibitors to block physiologic erection, strongly indicate that NO is the major neurotransmitter in penile erection.

The involvement of NO, produced by the catalytic action of neuronal NOS, in an erection has some clinical implications. First, the etiology or pathogenesis of impotence can be elucidated. For example, neurogenic impotence may be diagnosed by the quantitative evaluation of functioning NOS (Brock *et al.* 1993). Diabetes mellitus, the disease that most commonly causes impotence, is a subject to be intensively studied. From our unpublished data, quantitative reduction of NOS containing nerve fibers is present in the cavernous tissue of rats with streptozotocin-induced diabetes. Second, as a treatment of impotence, an NO donor (Stief *et al.* 1992) or perhaps NO delivered in a stable preparation directly to the corpus cavernosum, could induce erection.

In conclusion, the present study shows that the neural form of constitutive NOS in the corpora cavernosa synthesizes NO, which mediates penile erection. Determination of cavernous NOS expression or activity may allow us to characterize certain pathologic conditions which cause erectile impotence.

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