Protein and Gene Expression of Nitric Oxide Synthase Isoforms I and III in the Rat Penile Shaft

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ABSTRACT: Nitric oxide synthase (NOS) plays a key role in penile smooth muscle relaxation through the regulation of nitric oxide (NO). NO is a major neurotransmitter in the autonomic nervous system, and alteration of its activity has been implicated in erectile dysfunction. The objectives of this study were twofold: 1) to demonstrate and localize the NOS protein isoforms I and III in the normal rat penis, and 2) to identify and quantitate NOS I and III gene expression in the normal rat penis. The gene and protein product of NOS isoforms I and III are expressed in rat penile tissue. Protein expression of NOS I was confined primarily to neuronal tissue, while NOS III protein expression was identified primarily in both cavernosal smooth muscle and endothelium. The presence of both NOS I and III was confirmed in the penile shaft by Western blot. Quantitation of NOS I and III gene expression by reverse transcription–polymerase chain reaction revealed NOS III to be more highly expressed than that of NOS I in the rat penile shaft. NOS I and III protein and gene products are both expressed in normal rat penile tissue. Protein expression is localized primarily to neuronal tissue for NOS I, whereas NOS III is localized primarily to cavernosal smooth muscle and endothelium. NOS III gene expression is greater than that of NOS I in the normal rat penile shaft. These findings support the possibility that penile erection is regulated by different NOS isoforms released from neural, endothelial, and smooth muscle sources.

Key words: Nitric-oxide synthase, penile erection, penis, Western blotting, immunohistochemistry, gene expression.

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Penile erection is a both a hemodynamic and neuromuscular process. Normal erection of the penis requires a sustained increase in arterial flow as well as a coordinated decrease in venous outflow. Each of these processes is controlled by the autonomic nervous system (Lavoisier et al, 1986, 1988; Karacan et al, 1987; Garbán et al, 1995). Autonomic nerves that release both nonadrenergic/noncholinergic (NANC) and cholinergic neurotransmitters control the relaxation of cavernosal smooth muscle and thus facilitate penile erection. In addition, it has been shown that vascular endothelium, which lines the sinusoidal spaces of the corpora cavernosa, is involved in the release of endothelial-derived relaxing factors, namely nitric oxide (NO). This particular process appears to be an integral component in the normal physiology of penile erection (Furchgott and Zawadzki, 1980; Saenz de Tejada et al, 1988).

NO has been shown to be a major neurotransmitter in the autonomic nervous system's NANC neurotransmission (Burnett et al, 1992; Rajfer et al, 1992). Previous data demonstrate that both NANC neurotransmission and endothelial-derived NO are impaired in erectile dysfunction (ED; Saenz de Tajada et al, 1989; Rajfer et al, 1992; Seftel et al, 1997).

Because NO is a short-lived molecule whose level cannot easily be measured, NO activity is commonly estimated by measuring levels of nitric oxide synthase (NOS) and its enzyme activity. There are 3 known isoforms of NOS: type I, or neuronal NOS, which is located in neuronal and epithelial cells; type II, or inducible NOS, which is located in macrophages, cytokine-induced, and smooth muscle cells; and type III, or endothelial NOS, which is located in endothelial and smooth muscle cells. In humans, these isoforms are encoded by genes located on chromosomes 12, 17, and 7, respectively (Förstermann et al, 1994). We have used the designations of NOS I, NOS II, and NOS III rather than those of nNOS, iNOS, and eNOS, respectively. This is in accordance with previous reviews (Försterman et al, 1994; Schmidt and Walter, 1994).

Recent evidence has identified the expression and localization of these specific NOS isoforms to penile tissue. The NOS I isoform has been reported to be primarily localized to penile neurons innervating the corpora cavernosa, neuronal plexuses in the adventitial layer of penile arteries, and the dorsal penile nerves (Burnett et al, 1992; Jung et al, 1997). Likewise, the gene and protein expressions of the NOS II isoform has been reliably localized to penile smooth muscle cells (Garbán et al, 1995; Ra-

jasekaran et al, 1998). However, gene expression and protein distribution of NOS III in the penile shaft has not been well defined. Recently, Dail et al (1995) have shown that NOS I and III protein expression exists in rat penile erectile tissue; however, the gene expression of both isoforms and the specific protein distribution and localization of the NOS III isoform in the penile shaft were not defined. The authors demonstrated the presence of NOS I and III protein expression, yet they did not specifically localize the NOS III isoform in the rat penile shaft. Furthermore, they did not focus or expand on their protein findings with the demonstration and quantitation of NOS I and III gene expression in the rat penis.

The focus of this study was twofold: 1) to confirm the anatomic distribution and localization of NOS I and III protein expression in the rat penile shaft using specific monoclonal antibodies for immunohistochemistry (IHC) and Western blotting, and 2) to quantitate gene product expression for both NOS I and III in the penile shaft using the novel technique of competitive quantitative reverse transcription–polymerase chain reaction (RT-PCR). Elucidation of NOS I and III quantitative gene expression in the rat penis may provide previously unknown insight into the genetic regulatory process that governs normal erectile function.

Materials and Methods

NOS I and III Western Blot

One-hundred-day-old postpubertal adult male Sprague Dawley (250–300 g) rats were anesthetized with isoflurane followed by an intraperitoneal injection of ketamine (85 mg/kg) and xylazine (15 mg/kg). We performed intracardiac perfusion with 500 mL of phosphate-buffered saline (PBS) pH 7.5. The shaft of the penis was carefully dissected from the surrounding skin and fascia. Amputation of the penile shaft was performed at the level of the bulb of the penis. We then snap-froze the penile shaft in liquid nitrogen ($-80°C$) and cut the tissue into 20-μm sections on gelatin-coated slides. Animals were subsequently killed after removal of the penile shaft. Recent studies involving penile NOS localization have described either the use of penile shaft alone or the penile shaft along with the bulb of the penis (Penson et al, 1996; Shen et al, 1996; Garbán et al, 1997). We have utilized penile shaft alone in our studies for the following reasons: 1) in accordance with previously cited reports, en bloc penile shaft tissue represents a more uniform sample of NOS content in the normal penis as opposed to an isolated segment of penile tissue; 2) use of the entire penile shaft facilitates IHC and gene expression studies because of the larger mass of tissue to work with; and 3) use of the penile shaft avoids the excess urethral and periurethral tissue found in the distal urethra of the rat.

Rat penile shaft tissue was then homogenized in cold PBS containing protease inhibitors (1 mm phenylmethylsulfonylfluoride, EDTA, and leupeptin). Positive control tissue for NOS I included rat cerebellum and rat pituitary lysate. Positive controls

for NOS III included human endothelial lysate and rat cerebellum. The presence and activity of the NOS III isoform in both cerebellar and cerebral tissue has been well-described (Guo et al, 1997; Dumont et al, 1999).

Following a 3-minute centrifugation in a Fisherbrand picofuge, the supernatant was resuspended in equal volumes of 2× sodium dodecyl sulfate sample buffer (125 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 200 mM dithiothreitol, and 0.2% bromophenol blue) and boiled for 5 minutes. The protein content of each sample was then determined (Lowry et al, 1951). Electrophoresis was carried out on a 7.5% polyacrylamide gel under conditions of 200 mV and 100 mA for 4 hours, with 100 μg total protein loaded onto each lane (Laemmli et al, 1970). Full Range Rainbow Molecular Weight Markers (Amersham, Piscataway, NJ) were applied to one of the lanes. After electrophoresis the proteins were transferred to a 0.45-μm nitrocellulose membrane using a semidry blotting apparatus (Hoefer-Pharmacia Instruments, Piscataway, NJ) at 10 V and 250 mA for 2.5 hours (Towbin et al, 1979). Blocking solution (5% nonfat Carnation dry milk in PBS with 0.01% sodium azide) was applied to the membrane overnight. Following the blocking step, the membrane was incubated with either NOS I (1:250) or NOS III (1:1500), (mouse immunoglobulin G [IgG] monoclonal antibodies from Transduction Laboratories, Lexington, Ky) for 18 hours at 4°C. After 3 washes with PBS-Tween, the membranes were incubated with a secondary antibody, peroxidase-conjugated goat anti mouse IgG (Jackson Immunoresearch Labs, West Grove, Pa) for 4 hours. Following washes as described before, immunoreactive bands were visualized by incubating each membrane with an enhanced chemiluminescence detection reagent (ECL Western Blotting Reagents, Amersham). After incubation with ECL reagents, the membrane was exposed to X-AR2 film (Eastman Kodak, Rochester, NY) for exposure times ranging from 30 seconds to 5 minutes. Quantitation of protein bands was carried out by optical densitometry.

NOS I and III Immunohistochemistry

Sections of rat penile shaft tissue were immersed in 4% paraformaldehyde, washed in PBS, and fixed in paraffin-embedded sections. The penile shaft slides were then heated in a 1:200 dilution of antigen unmasking solution (Vector Laboratories, Burlingame, Calif) at 100°C for 20 minutes. The slides were then cooled and washed in PBS. Blocking serum (preimmune horse) was then applied for 30 minutes and removed. All tissues were then incubated overnight at 4°C with a 1:200 dilution of either mouse anti-NOS I or anti-NOS III monoclonal antibody (Transduction Laboratories). The slides were then washed in PBS and incubated with a 1:500 dilution of biotinylated antibody (horse anti-mouse IgG) for 30 minutes. After PBS wash, the slides were incubated in the Vectastain ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 30 minutes at room temperature. After a brief PBS wash, 3,3′-diaminobenzidine (DAB) and 3% H_2O_2 solution were placed on the tissue for 1 minute.

As a negative control, sections were incubated with the secondary antibody only and stained with DAB (data not shown). The staining was visualized with brightfield microscopy.

*Primers used in competitive quantitative RT-PCR**

Gene	Oligonucleotide Sequence	3′ Location	Product
NOS I (sense)	5′-GGATACCAGCCTGATCCATGGAA-3′	2483	602 bp
NOS I (antisense)	5′-TCCTCCAGGAGGGTGTCCACCGCATG-3′	3062	
NOS III (sense)	5′-AGGCTGCTGCCCGAGATATCTTCA-3′	275	261 bp
NOS III (antisense)	5′-TTGGGTGGGCACACACCTATGTGG-3′	495	

Gene Expression Using Quantitative Competitive RT-PCR

RNA Isolation—RNA was extracted and purified from the penile shaft tissue of 10 different 100-day-old male Sprague Dawley rats (250–300 g) using the Tri Reagent protocol (Trizol, Molecular Research Center, Cincinnati, Ohio). RNA was precipitated with cold isopropanol and placed at −70°C overnight. The RNA was then spun at $14\,000 \times g$ for 15 minutes, and the pellet was washed twice with cold ethanol. The pellet was resuspended in 50 μL of autoclaved water.

DNAse Treatment—Each sample was treated with RQ1 DNAse (Promega, Madison, Wis) and incubated in a 37°C water bath for 30 minutes. RNA was recovered after equal volume phenol:chloroform treatment and subsequent equal volume chloroform recovery. The RNA solution was then buffered with 3M sodium acetate added to 100% ethanol and precipitated at −80°C for 30 minutes. The RNA was spun at $14\,000 \times g$ for 15 minutes, and the pellet was washed with 70% ethanol. The pellet was resuspended in 25 μL of autoclaved water. All prepared RNA was checked for DNA contamination through RT-PCR (Perkin-Elmer, Norwalk, Conn).

Competitive Quantitative RT-PCR—The NOS competitive primers were constructed using the PCR MIMIC Construction Kit (Clontech, Palo Alto, Calif). This technique uses a nonhomologous DNA fragment that has both NOS gene-specific end sequences. The MIMIC is constructed through a series of PCRs. This specific construct allows the MIMIC complementary DNA to compete with native NOS during the PCR. Both NOS I and III primers were synthesized at the Northwestern University Biotechnology Facility. RT-PCR was performed using the Gene Amp RNA PCR kit (Perkin-Elmer). The primers used are listed in the Table.

RT was performed by adding 1 μg of total RNA from the desired rat penile tissue with 0.5 μL of Moloney murine leukemia virus reverse transcriptase. The mixture was then incubated at 42°C for 30 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. From this solution a 25-μL PCR reaction mix was prepared (5 μL from the previous RT reaction and 1 μL constituting the serial dilutions of the MIMIC construct) to amplify the NOS I (602 base pair [bp]) fragment and the NOS III (261 bp) fragment. The NOS I (555 bp) and the NOS III (602 bp) MIMIC constructs were incubated along with the native penile RNA as indicated earlier. PCR was performed as follows: incubation for 2 minutes at 95°C, 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C for 35 cycles in the presence of *Taq* Gold Polymerase (Perkin Elmer). The thermocycler used was a MJ Research model (PTC-100). Native NOS cDNA and MIMIC cDNA construct samples were run together on a 1.5% agarose (Sigma Chemical Company, St Louis, Mo; wide range/standard 3:1) gel containing 0.5 μg/mL ethidium bromide. The bands were then subjected to densitometry using Molecular Analyst software (Bio-Rad, Hercules, Calif). Native penile shaft NOS concentration was then calculated using the quantitative competitive RT-PCR method with the known serial dilutions of the MIMIC construct. The values were then plotted as the concentration of the MIMIC construct (X axis) against the ratio of the band densities of the MIMIC construct to the native NOS band densities (Y axis). Native NOS was then calculated by interpolation of the resultant linear regression to the equivalence point of the MIMIC construct (ie, where the ratio of the MIMIC construct and NOS gene product was 1.0; Piatak et al, 1993; Siebert et al, 1993).

Results

NOS I and III Western Blot

Western blotting was used to confirm the presence of both NOS I and III protein immunoreactivity in the rat penile shaft. The Western blots confirmed the selective immunoreactivity of both NOS I and III proteins using the same monoclonal antibodies as that for IHC. Optical densitometry was used to determine that probing with the monoclonal anti-NOS I antibody demonstrated an immunoreactive band at approximately 160 kd. Likewise, optical densitometry demonstrated NOS III immunoreactivity with the monoclonal anti-NOS III antibody, which was present at approximately 140 kd (Figure 1A and B).

NOS I and III Immunohistochemistry

Using the same anti-NOS I monoclonal antibody that was used for the corresponding Western blot, intense NOS I immunoreactivity was observed in the dorsal penile nerves along with their corresponding branches. Minimal staining was localized to the endothelium of the dorsal vascular structures. Likewise, minimal staining of the corpora cavernosal smooth muscle tissue and the periurethral area was seen. No staining was seen in the sections incubated with secondary antibody only (Figure 2A through C).

Using the same anti-NOS III monoclonal antibody that was used for the corresponding Western blot, intense NOS III staining localized to the corpora cavernosal smooth muscle tissue. Moderate staining was localized to the endothelium of the dorsal vascular structures. Staining of the periurethral area was also seen. No staining was
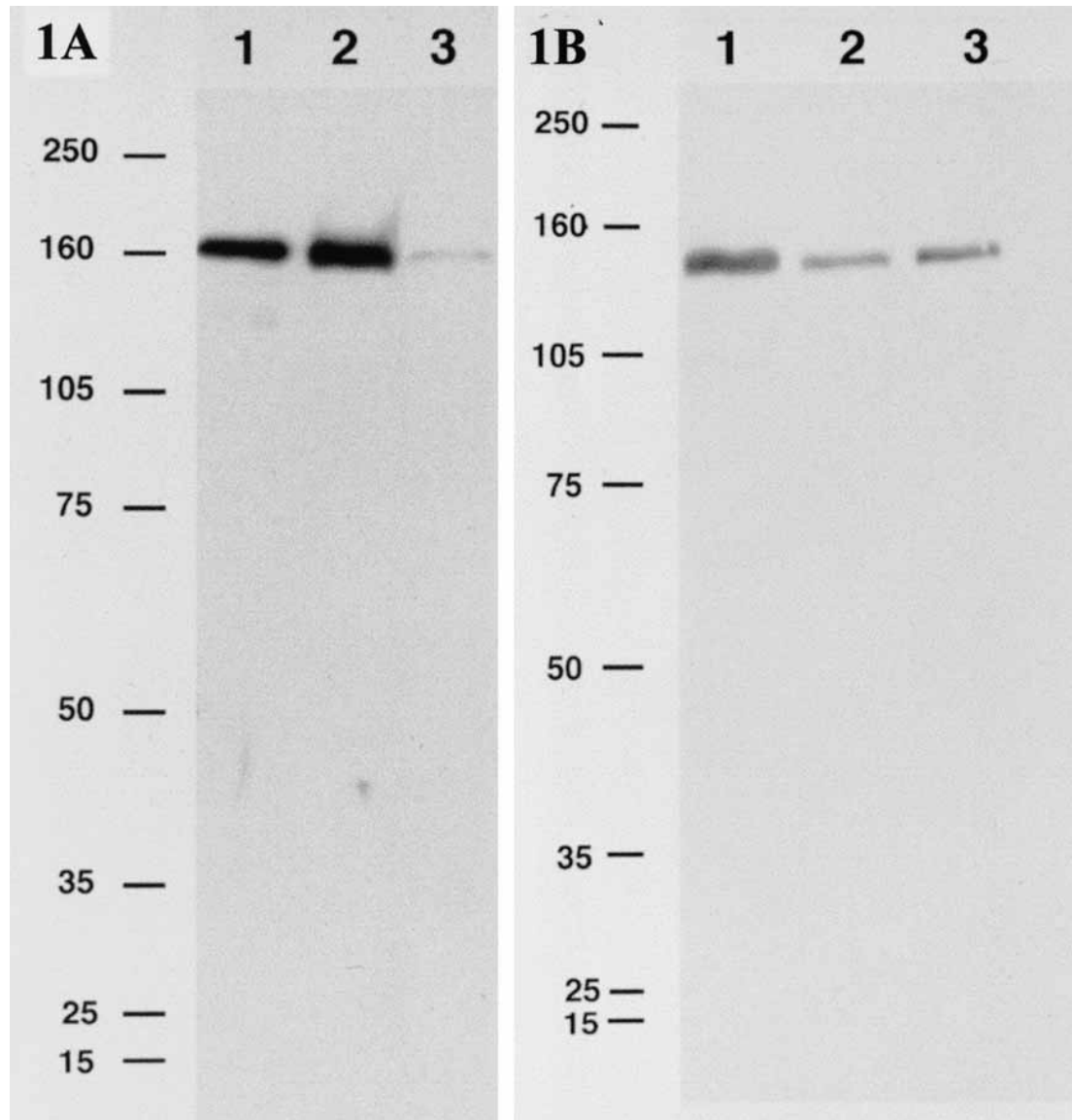
Figure 1. NOS I and III Western blot of rat penile shaft. **(A)** Western blot detecting the presence of NOS I with an anti-NOS I monoclonal antibody. Immunoreactive bands, 160 kd in size, were identified in lanes 1–3. Lanes 1 and 2 represent the control tissue; rat pituitary lysate and rat cerebellum, respectively. Lane 3 is rat penile shaft tissue. **(B)** Western blot detecting the presence of NOS III with an anti-NOS III monoclonal antibody. Immunoreactive bands, 140 kd in size, were identified in lanes 1–3. Lanes 1 and 2 represent the control tissue; human endothelial lysate, and rate cerebellum, respectively. Lane 3 is rat penile shaft tissue.

seen in neural tissue or in the sections incubated with the secondary antibody only (Figure 3A through C).

Gene Expression Using Competitive Quantitative RT-PCR

Gene expression of both NOS I and III was identified in normal rat penile shaft tissue. Using the competitive quantitative RT-PCR (PCR MIMIC) technique and optical densitometry, NOS III gene expression was greater than that of NOS I. Optical densitometry was used to quantitate all gene products (Figure 4A and B).

Discussion

In this study we identified both protein and gene expression of NOS I and III in the rat penile shaft. Competitive, quantitative RT-PCR was used to quantify gene expression for both NOS I and III, whereas protein expression of each isoform was localized in the penile shaft with IHC. The authenticity of our NOS I and III IHC staining was supported by our Western blotting using the same monoclonal anti-NOS I and III antibodies. The use of monoclonal antibodies for NOS I and III in both IHC and
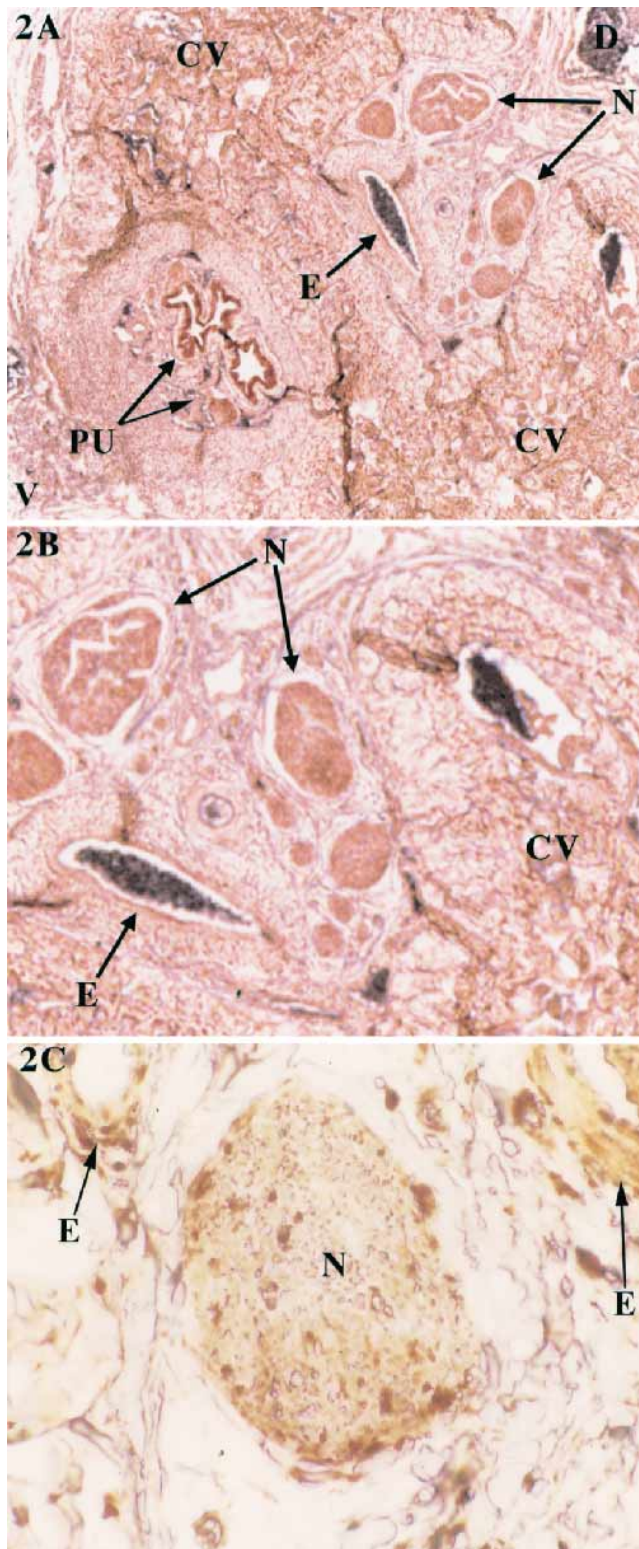
Figure 2. NOS I immunohistochemistry in the rat penile shaft. Anti-NOS I monoclonal antibody concentration was 1:200 for all staining. **(A)** Low-power view (4×) of penile shaft cross-section reveals NOS I staining of the penile dorsal neural structures (N). Minimal staining was also evident in the smooth muscle of the cavernosal tissue (CV) and in the endothelium (E) of the vascular structures. Staining of the periurethral tissue (PU)

Western blotting of penile tissue is a novel technique. Previous to this the successful use of anti-nNOS (NOS I) and anti-eNOS (NOS III) monoclonal antibodies in different human and animal tissues has been reported (Leger et al, 1998; Tsuyama et al, 1999). Furthermore, the specificity of monoclonal antibodies may be superior to that of polyclonal antibodies for NOS protein identification and localization, although comparative studies are few (Lewis et al, 1996). Regardless, in accordance with previous reports, our Western blotting revealed immunoreactive bands at 160 and 140 kd for NOS I and III, respectively (Förstermann et al, 1994).

Consistent IHC localization of NOS I was found in the dorsal penile neural complexes. The localization of NOS I staining to the dorsal neural complexes most likely represents the presence of neuronal axons, which have been found to produce or store large quantities of this specific NOS isoenzyme (Burnett et al, 1992; Jung et al, 1997). Conversely, localization of NOS III to the dorsal penile nerves was not seen with our IHC staining. This NOS III staining characteristic correlates with previous reports that have not localized this isoenzyme to neural tissue (Dail et al, 1995; Jung et al, 1997; Rajasekaran et al, 1998). Most interesting was the strong presence of NOS III staining noted in the corporal cavernosal smooth muscle. Given the important role that lacunar smooth muscle has in the regulation of erections, confirmation of the presence of NOS III in this tissue may suggest a physiologic role for this particular isoform. In addition to NOS III localization to corporal smooth muscle, minimal amounts of NOS I staining was also localized to the corporal smooth muscle. This staining most likely represents the neural complexes innervating the corpus cavernosa, which is consistent with previous reports (Burnett et al, 1992; Jung et al, 1997); however, it may also represent direct corporal cavernosal staining. These findings do differ with those reported in previous work and may be a function of the more precise localization characteristics of the anti-NOS I monoclonal antibody used in this particular study. Likewise, staining of both NOS I and III was noted in the endothelium of the dorsal arteries, arterioles, and venules. The endothelial findings in our IHC are supported by the fact that the same endothelium that exists in vessels also exists in lacunar smooth muscle. Thus, the corporal smooth muscle staining of both NOS I and III may also represent the endothelial staining of these two NOS iso-

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was also demonstrated. Anatomic labeling for dorsal (D) and ventral (V) orientation of the tissue is included. **(B)** Higher-power view (10×) of intense NOS I immunoreactivity of the dorsal nerves (N) of the rat penis. Minimal staining is also noted in the endothelium (E) of surrounding vessels and in the cavernosal smooth muscle (CV). **(C)** High-power view (40×) of NOS I immunoreactivity in the dorsal nerve neural bundles (N). Moderate staining of vessel endothelium (E) is also noted.
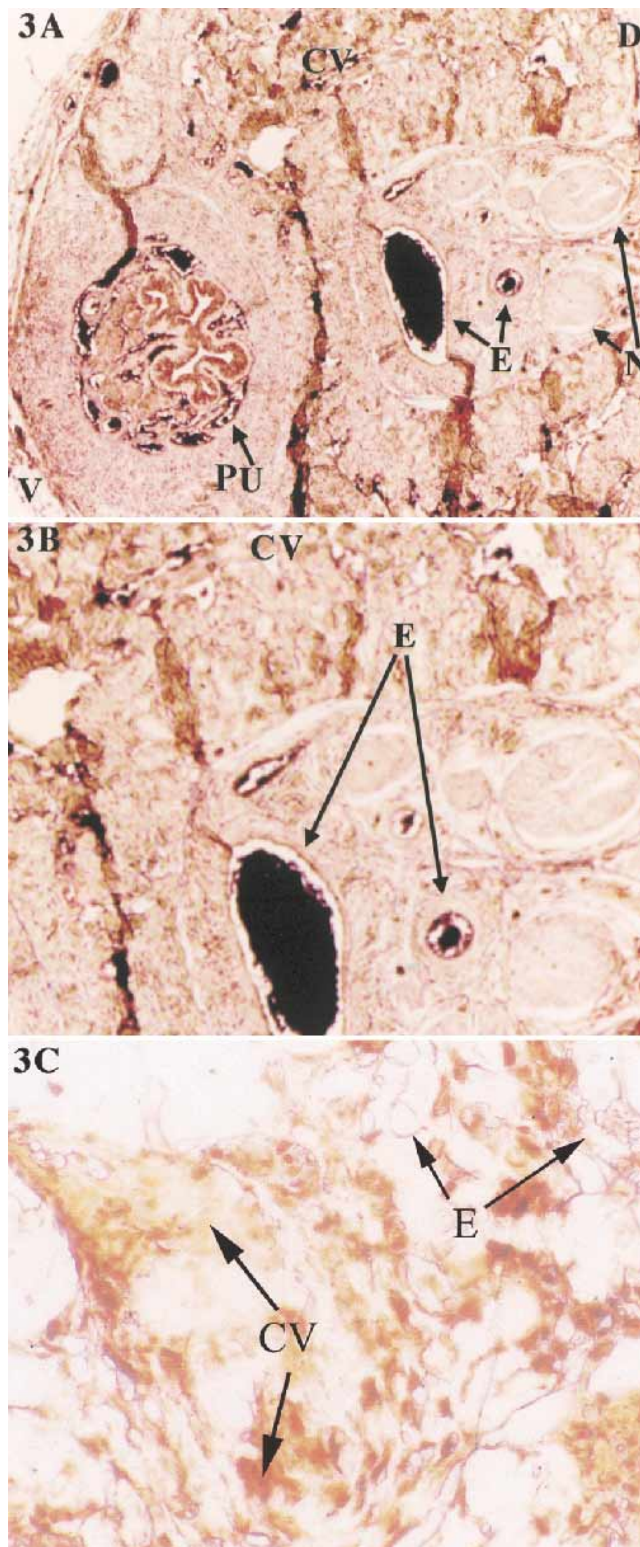
forms. These findings correspond with previous reports that have also identified penile co-localization of both NOS I and III in the penile shaft (Dail et al, 1995). However, unlike the study by Dail et al, we have specifically localized the NOS III isoform to both corporal smooth muscle and endothelium, and have identified the presence of NOS I staining in the endothelium and cavernosal smooth muscle. In addition, we have incorporated the novel technique of competitive, quantitative RT-PCR studies, demonstrating NOS I and III gene expression in the rat penile shaft, which will be discussed later. The consistent presence of NOS I and III in the penile dorsal vascular structures, corporal cavernosal smooth muscle, and endothelium support the possibility that an integrative network exists among these particular isoforms of NOS.

In accordance with our smooth muscle and endothelial findings, co-localization of NOS I and III staining was also observed in the periurethral area. Penile periurethral NO staining has been reported from several other investigators who have demonstrated the presence of NOS isoforms in this location (Vizzard et al, 1994; Radziszewski et al, 1996; Smet et al, 1996). In accordance with these previous reports the periurethral NOS I staining we found most likely represents both autonomic and motor innervation to the urethra, whereas periurethral NOS III staining most likely represents the smooth muscle and endothelial staining of the rich vascular complex that surrounds the urethra. The significance of these finding remains to be determined and is beyond the scope of the present manuscript.

Competitive quantitative RT-PCR was used to determine NOS I and III gene expression in the rat penis. The identification and quantification of NOS I and III gene expression in the penile shaft compliments and supports our protein expression findings. The novel technique of competitive quantitative RT-PCR was used to directly compare the relative quantity of NOS I and NOS III gene expression in the rat penile shaft. Consistently, NOS III gene expression was greater than that of NOS I. The identification and quantification of NOS gene expression in the rat penile shaft represents data that have not been previously reported. Further study is needed to determine how this greater amount of NOS III gene expression effects the penile shaft protein expression of each respective NOS isoform in the penile shaft.

An integration network consisting of these 2 particular

Figure 3. NOS III immunohistochemistry in the rat penile shaft. Anti-NOS III monoclonal antibody concentration was 1:200 for all staining. **(A)** Low-power view (4×) of penile shaft cross-section reveals intense NOS III staining localized to the cavernosal tissue (CV). Moderate staining was also demonstrated in the endothelium of the dorsal vascular structures (E). Staining of the periurethral tissue (PU) was also demonstrated. Anatomic labeling for dorsal (D) and ventral (V) orientation of the tissue is included. **(B)** Higher-power view (10×) reveals intense NOS III immunoreactivity of cavernosal tissue (CV) with moderate staining of the endothelium (E). **(C)** High-power view (40×) reveals intense NOS III immunoreactivity of cavernosal smooth muscle tissue (CV) and endothelium (E).
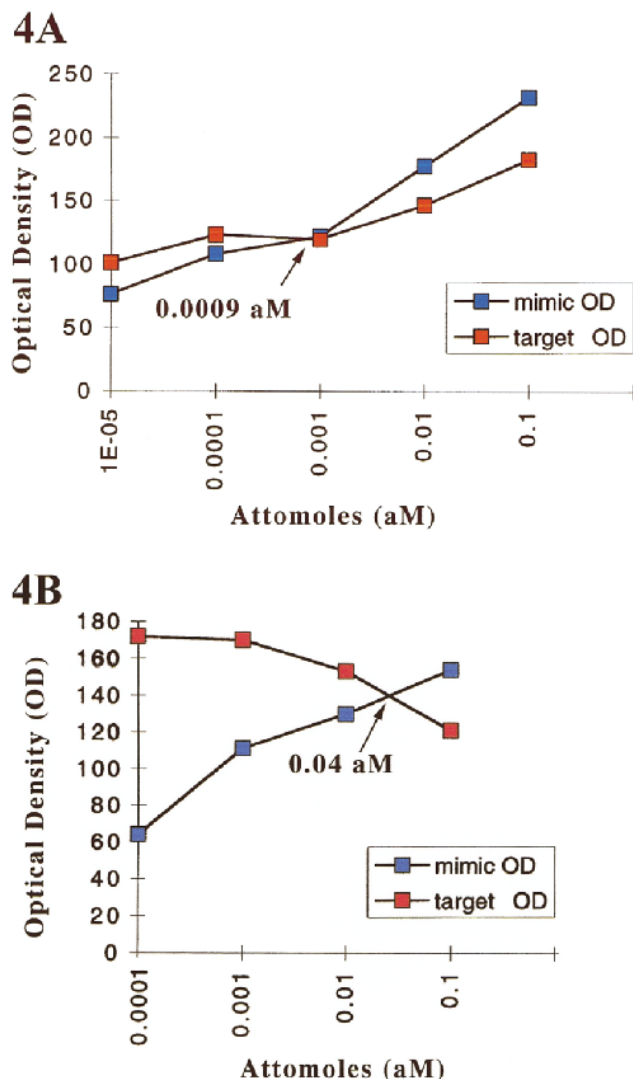
Figure 4. Gene Expression of NOS I and III using competitive quantitative RT-PCR. **(A)** Competitive RT-PCR of penile NOS I and the corresponding MIMIC mRNA. Convergence of penile and MIMIC mRNA occurs at the equivalence point of 0.0009 attomoles (aM). Values were plotted as the gene product concentration of the MIMIC construct (X axis) versus the gene product concentration of the wild-type NOS I gene expression (Y axis). The convergence of the band densities (the equivalence point) for native NOS I and its corresponding MIMIC is at 0.0009 aM. All values are expressed in aM (10^{-15} moles). **(B)** competitive RT-PCR of penile NOS III and the corresponding MIMIC mRNA. Convergence of penile and MIMIC mRNA occurs at the equivalence point of 0.04 aM. Values were plotted as the gene product concentration of the MIMIC construct (X axis) versus the gene product of the wild-type NOS III gene expression (Y axis). The convergence of the band densities (the equivalence point) for native NOS III and its corresponding MIMIC is at 0.04 aM. All values are expressed in aM (10^{-15} moles).

isoenzymes may play a controlling role in the overall erectile function of the rat. Sexual function control involving different NOS isoenzymes suggests that ED may occur secondary to some consequence of NOS isoenzyme dysfunction. This ED may occur at several different levels or through a lack of integration between the individual

levels. This proposed network of NOS isoforms controlling or influencing sexual function also offers the opportunity for the development of target-specific drug therapy. It is possible that one NOS isoform may compensate or augment the dysfunction of another NOS isoform in the penis. Compensation of one NOS isoenzyme for another NOS isoenzyme that has been genetically deleted or inhibited via a specific disease process has been reported (Burnett et al, 1996). In this report a compensatory increase of NOS III protein expression in NOS I knockout mice occurred; however, this study had some limiting design flaws, mainly that the protein content of NOS III as compared with that of NOS I was not corrected for the β actin content of the penile tissue. This may have affected the reported observations that NOS III protein expression exhibits a 20% compensatory increase in NOS I knockout mice. Further investigation with knockout models or gene therapy may assist in the determination of whether changes in a single isoform are accompanied by changes in alternative sources of nitric oxide. What is not known is whether there is a coordination of function or control between the various NOS isoforms.

In conclusion, we have demonstrated the presence of NOS I and NOS III in the rat penile shaft using correlative IHC, Western blotting, and quantitative gene expression studies. These results offer the possibility that penile erection is regulated by different NOS isoforms released from neural, endothelial, and smooth muscle sources.

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