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Localization of Nitric Oxide Synthase in the Reproductive Organs of the Male Rat[1]

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ABSTRACT

Nitric oxide synthase (NOS), which catalyzes the production of nitric oxide (NO), was characterized within the reproductive tract of adult male Sprague-Dawley rats by means of biochemical and immunohistochemical techniques. Tissues examined included the testis, epididymis (caput, corpus, and cauda regions), vas deferens, ejaculatory duct, seminal vesicle, and coagulating gland. NOS activity was measured by use of an assay based on the stoichiometric conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline and NO, catalyzed by NOS. Low levels of NOS activity were detected in the testis and seminal vesicle (< 0.5 fmol [^{3}H]-L-citrulline formed/min/mg protein in each tissue). The highest levels of NOS activity were present in the cauda segment of the epididymis and in the vas deferens, each having a sevenfold greater amount of NOS activity than the testis ($p < 0.05$). Intermediate levels of NOS activity were detected in the coagulating gland (0.863 ± 0.248 fmol [^{3}H]-L-citrulline formed/min/mg protein), caput epididymidis (0.457 ± 0.180 fmol [^{3}H]-L-citrulline formed/min/mg protein), and corpus epididymidis (0.631 ± 0.215 fmol [^{3}H]-L-citrulline formed/min/mg protein).

NADPH diaphorase histochemistry and NOS immunohistochemistry localized NOS to neuronal fibers coursing throughout the smooth musculature and subepithelial regions of the epididymis, vas deferens, and ejaculatory duct. Endothelial cells and nerve plexuses within the adventitia of blood vessels supplying reproductive tissues were also positive for NOS. Additional localizations of NOS were within epithelial cells of the epididymis and coagulating gland. Taken together, these results support an extensive role for NO in contractile, hemodynamic, and secretory processes in the male reproductive tract and imply that this novel mediator participates in regulatory mechanisms required for male reproductive function.

INTRODUCTION

Nitric oxide (NO) is an important messenger molecule employed in a host of biological processes [1]. NO functions as an atypical neurotransmitter in the central nervous system and as an agent of non-adrenergic, non-cholinergic (NANC) autonomic neurotransmission in the periphery [2–4]. In the urogenital tract, NO subserves functions of the penis [5–11], urethra [12], and bladder [13], suggesting its probable role as a major physiological mediator of peripheral autonomic activity in the pelvis. Prime evidence in support of this prospect derives from investigations of penile erection. NO synthase (NOS), the enzyme that synthesizes NO, is localized to nerves within rat [11, 14], canine [11], bovine [15], and human [16] pelvic and genital structures. Additionally, electrically induced relaxation of isolated corporal smooth muscle tissue from rabbits [5, 8, 10] and men [6, 7, 9, 10] as well as penile nerve-stimulated erections in rabbits [17], rats [11], and dogs [18] are blocked by specific inhibitors of NO production whereas these effects are elicited with agents that facilitate NO pathways.

Because of the biochemical and functional evidence for NO in pelvic structures, one may propose that NO is widely involved in the autonomic control of the urogenital tract, including the structures required for male reproductive function. Although this hypothesis appears to conflict with the widely held belief that the primary origin of autonomic neuronal influence in mammalian reproductive organs is adrenergic, there is support for alternative neuronal sources of regulation in the pelvis and genitalia. For instance, while the vas deferens receives extensive adrenergic innervation [19], cholinergic effects have been shown [20], and there is further evidence for the existence of a NANC neuronal mechanism affecting the contractility of this organ [21–23]. In various structures, cholinergic innervation may be sparse, but in certain locations such as the bladder, its effects may predominate [19]. Furthermore, other neuronal effectors arising from peptidergic or purinergic pathways have been identified [19, 24, 25], contributing to the complexity of autonomic regulation in the urogenital tract.

As direct support for the significance of NO in the reproductive tract, we have shown previously that NOS-containing neurons exist in the mammalian pelvic plexus [11, 16]. Since the pelvic plexus serves as the center for autonomic regulation of pelvic viscera and to some extent even portions of the striated musculature within the pelvis [26, 27], it is reasonable to conjecture that the biological functions of any pelvic structure receiving neuronal fibers coursing from the pelvic plexus may involve NO mediation. Alternatively, there may exist in pelvic organs intrinsic localizations of NOS involving local neuronal pathways or other cellular sources that release NO in an autocrine or para-

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crine manner. Whichever the case may be, NO could function in the elaborate anatomic organization in the pelvis ranging from the vasa deferentia to accessory genital glands, thereby modulating diverse activities of the male sexual response.

The present study results from our basic premise that NO significantly influences various aspects of male reproductive activity. In investigating this possibility, we applied biochemical and histochemical methods to localize and characterize NOS in select reproductive organs of the male rat. Central to our studies is a precise immunohistochemical technique for NOS localization that was used originally to define the neuroanatomic basis for NO [2]. Therefore, this report provides initial evidence that a regulatory pathway involving NO exists in the male reproductive tract.

MATERIALS AND METHODS

Materials

[^{3}H]-L-Arginine (59 Ci/mmol) was obtained from Amersham Inc. (Arlington Heights, IL). Anti-NOS antibody was generated in the rabbit against the rat cerebellar NOS whole protein (a gift from the laboratory of Dr. Solomon Snyder). The specificity of the antibody was established for this investigation by preincubating the antibody with purified NOS protein, which abolished immunoblots and immunohistochemical staining of rat tissue described previously to contain NOS [2, 11]. Normal goat serum and the reagents required for the avidin-biotin-peroxidase immunohistochemical staining method were from Vector Laboratories Inc. (Burlingame, CA). Tissue-Tek O.C.T. compound was from Miles, Inc. (Elkhart, IN). Eluate columns were from Evergreen Scientific Inc. (Los Angeles, CA). Reagents and protein standards for the Pierce Coomassie Plus protein assay were purchased from Pierce (Rockford, IL). L-Nitroarginine methyl ester, paraformaldehyde, Dowex-50W cation exchange resin, diaminobenzidine, nitroblue tetrazolium, dimethylsulfoxide, NADPH, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Tissue

Adult male (325–350 g) Sprague-Dawley rats were killed by carbon dioxide overdose, and their reproductive organs were removed by anatomical dissection and weighed. These organs included testis, epididymis (divided into caput, corpus, and cauda segments), vas deferens, ejaculatory duct, and seminal vesicle and coagulating gland (both weighed separately after fluid expression). Rat cerebellum was removed specifically for NOS assay (see below). For NOS enzyme assays, each tissue (n = 2 animals/assay), excluding the ejaculatory duct, was homogenized in 10 vol (w/v) of 50 mM Tris (pH 7.4) containing 1 mM EDTA and 1 mM EGTA, and centrifuged at 10 000 × *g* for 4 min at 4°C. Supernatants were then collected and assayed for NOS activity as described below.

For diaphorase histochemistry, specimens were immersed immediately in liquid nitrogen and stored at −70°C until sectioned. For NOS immunohistochemistry, additional animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg BW) and then were whole-body perfuse-fixed with 4% freshly depolymerized paraformaldehyde in 0.1 M PBS, pH 7.4. After anatomical dissection, organs were immersed for 1 h in fixative and cryopreserved with 10% sucrose in 0.1 M PBS overnight. For histochemical and immunohistochemical studies, fresh-frozen and perfuse-fixed tissue specimens were embedded in Tissue-Tek O.C.T. compound and cut on a cryostat (−18°C) into 10-μm-thick sections that were affixed onto gelatin/chrome alum-coated slides.

NOS Activity Assay

NOS activity was measured by monitoring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described previously [28]. Briefly, enzyme assays contained 25 μl of tissue supernatant and 100 μl of 1 μCi/ml [^{3}H]-L-arginine, 1.2 mM NADPH, and 0.7 mM $CaCl_2$. After a 15-min incubation at room temperature, the assays were terminated by the addition of 3 ml of 20 mM Hepes (pH 5.5) with 2 mM EDTA and the entire mixture was applied to 0.5-ml columns of Dowex-50W cation exchange resin (Na^+ form) to remove unreacted [^{3}H]-L-arginine. [^{3}H]-L-Citrulline in the column eluate was quantified by liquid scintillation spectroscopy. The recovery rate of [^{3}H]-L-citrulline for the columns was measured for all urogenital tissues by preincubating each tissue supernatant with a known concentration of [^{3}H]-L-citrulline and then measuring the [^{3}H]L-citrulline in each column eluate. Column saturation studies were also done to ensure that all of the [^{3}H]-L-arginine was retained in the column. Additional assays were performed in the presence of excess L-nitroarginine methyl ester (L-NAME), a competitive inhibitor of NOS, to verify the specificity of the assay for production of [^{3}H]-L-citrulline by NOS catalysis. Interassay variations were controlled for by standardizing NOS activity measurements in urogenital tissues against the NOS activity measured in the NOS-rich rat cerebellum, which was analyzed in parallel for each assay. Protein concentration was measured by use of the Pierce Coomassie Plus Assay.

NOS Immunohistochemistry

Immunohistochemical localization of NOS in the reproductive tract was performed as previously described [2]. Slide-mounted tissue sections were permeabilized in 0.1% Triton in 0.1 M PBS for 15 min, blocked with 1% (v/v) normal goat serum and 1 mg/ml BSA for 1 h at 4°C, and then incubated in 1% (v/v) normal goat serum and 1 mg/ml BSA containing affinity-purified NOS antiserum (1:50 dilution) overnight at 4°C. Sections were then stained with an avidin-biotin-peroxidase system with diaminobenzidine as the

chromogen. For controls, specimens were processed in the presence of preimmune serum or in the absence of primary antibody. As additional controls, select specimens were incubated with anti-NOS antibody inactivated with serial dilutions of excess NOS protein.

NADPH Diaphorase Staining

NADPH diaphorase histochemical staining was performed to confirm NOS localizations in the reproductive tract. Diaphorase histochemical staining occurs when the reduced form of NOS is reoxidized in the presence of the electron acceptor, nitroblue tetrazolium, and NADPH [29]. Slide-prepared tissue sections were initially incubated in 4% freshly depolymerized paraformaldehyde in PBS for 15 min, and then incubated for 30 min at room temperature with 1 mM NADPH and 0.2 mM nitroblue tetrazolium in 50 mM Tris (pH 7.4) and 12% (v/v) dimethylsulfoxide.

Statistical Analysis

Multiple-comparison tests were used to evaluate the distribution of NOS activity measurements in tissues studied by NOS assay. A p value less than 0.05 was considered statistically significant.

RESULTS

NOS Activity Assay

NOS activity was variably measured in discrete structures of the male reproductive tract (Fig. 1). NOS activity was minimally detected in the testis, with less than 0.5 fmol [^{3}H]-L-citrulline formed/min/mg protein. Substantial levels of NOS activity were measured in all three regions of the epididymis. The cauda epididymidis was particularly NOS-rich, containing more than seven times the amount of NOS activity in the testis ($p < 0.05$). This statistical difference did not exist for the other two epididymal regions. Similar to the cauda, the vas deferens showed a high level of NOS activity, exceeding the level measured in the testis ($p < 0.05$). NOS activity in the seminal vesicle was comparable to that in the testis, whereas NOS activity in the adjacent coagulating gland was approximately threefold greater than that measured in the testis ($p > 0.05$).

The recoverability of [^{3}H]-L-citrulline in all urogenital tissues was 85–90%. [^{3}H]-L-Citrulline production in these tissues was specifically inhibited in the presence of L-NAME. Concentrations of this competitive NOS inhibitor ranging between 0.1 and 5 mM inhibited [^{3}H]-L-citrulline production by greater than 90% in all tissues except the testis and seminal vesicle. In the latter tissues, only about 50% maximal inhibition could be achieved, which was associated with their minimally assayable NOS activity levels. Specific inhibition of NOS activity in these tissues validated NOS activity determinations.

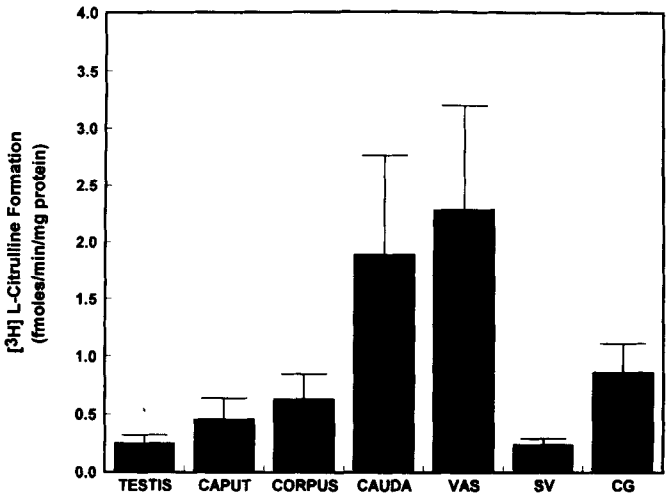

FIG. 1. Distribution of NOS activity in rat male reproductive tissues. All values are expressed as fmol [^{3}H]-L-citrulline formed per minute per milligram protein. Supernatants of tissue homogenates were prepared, and NOS activity was measured as the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as described in *Materials and Methods*. Each bar represents the mean of four assays (2 samples/assay) ± standard error. VAS, vas deferens; SV, seminal vesicle; CG, coagulating gland.

NOS Immunohistochemistry and NADPH Diaphorase Histochemistry

Controls. No tissues incubated in the absence of primary antibody showed staining. In the presence of preimmune serum, NOS staining was not observed in any tissue except for the epididymis which exhibited light staining in the basal regions of the epididymal tubules (Fig. 2A). Select specimens incubated with anti-NOS antibody inactivated with serial dilutions of excess NOS protein also showed no staining (for example, see Fig. 4A).

Testis. In the testis, NADPH diaphorase activity and NOS immunoreactivity were confined to the vascular endothelium. Interstitial Leydig cells, and the Sertoli and germ cells within the seminiferous epithelium did not react with either stain. Additionally, spermatozoa throughout the epididymis and vas deferens were devoid of diaphorase staining and NOS immunoreactivity.

Epididymis. Throughout the epididymis, vascular endothelial cells were positive for both NADPH diaphorase activity and NOS immunoreactivity. Additional localizations for NOS were to nerve fibers coursing in the periphery of blood vessels.

NOS staining was also positive in the epithelium, varying in intensity and distribution within the different regions of the epididymis. The presence of epithelial NOS was not shown with either staining technique in the initial segment and the proximal caput epididymidis. In contrast, epithelium in the mid-to-distal caput epididymidis was stained distinctly for NADPH diaphorase and NOS; this staining resembled that in the corpus and cauda epididymides (see depictions of cauda epididymidis in Fig. 2). NOS staining within the epithelium of the mid-to-distal caput epididym-

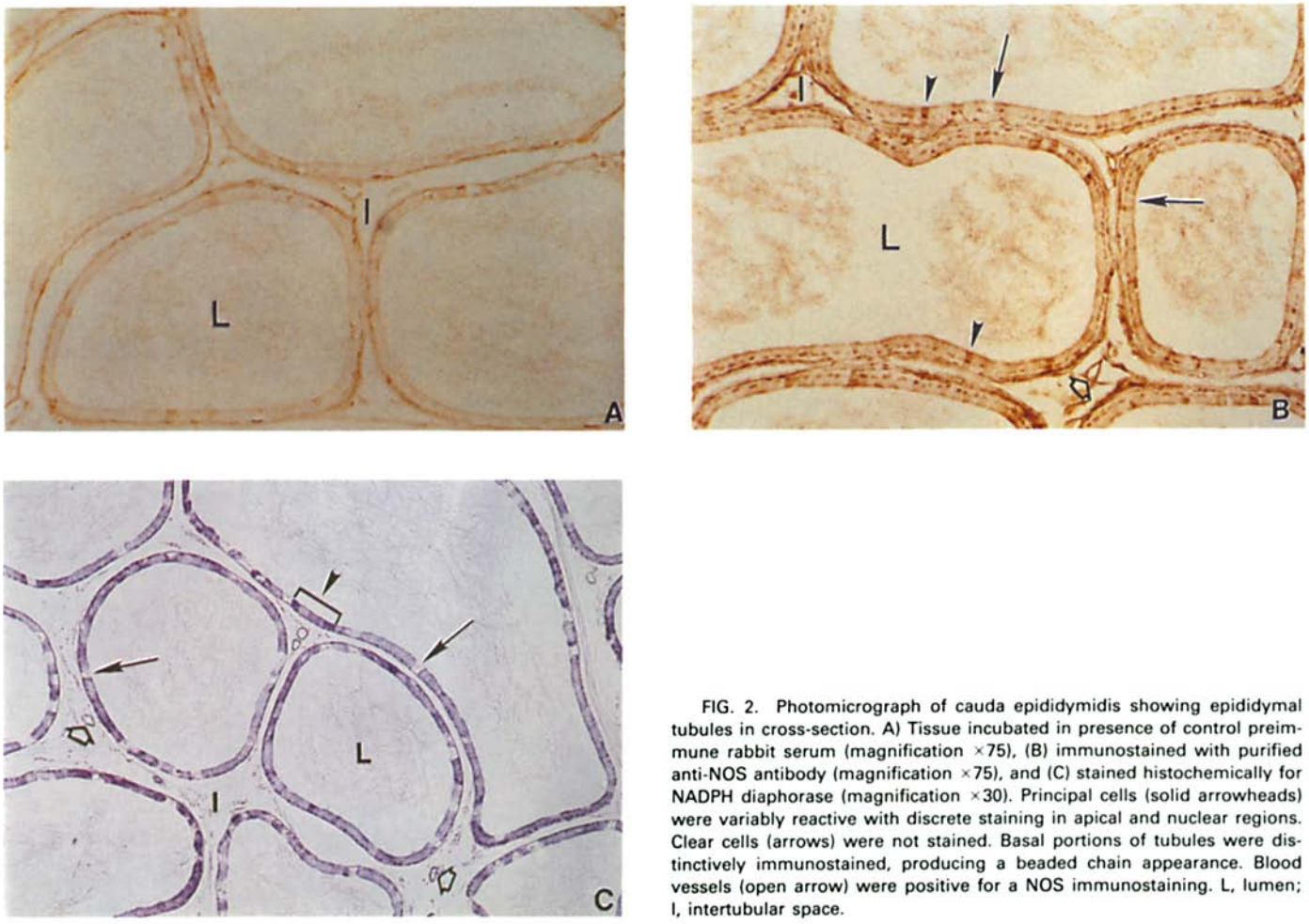

FIG. 2. Photomicrograph of cauda epididymidis showing epididymal tubules in cross-section. A) Tissue incubated in presence of control preimmune rabbit serum (magnification ×75), (B) immunostained with purified anti-NOS antibody (magnification ×75), and (C) stained histochemically for NADPH diaphorase (magnification ×30). Principal cells (solid arrowheads) were variably reactive with discrete staining in apical and nuclear regions. Clear cells (arrows) were not stained. Basal portions of tubules were distinctively immunostained, producing a beaded chain appearance. Blood vessels (open arrow) were positive for a NOS immunostaining. L, lumen; I, intertubular space.

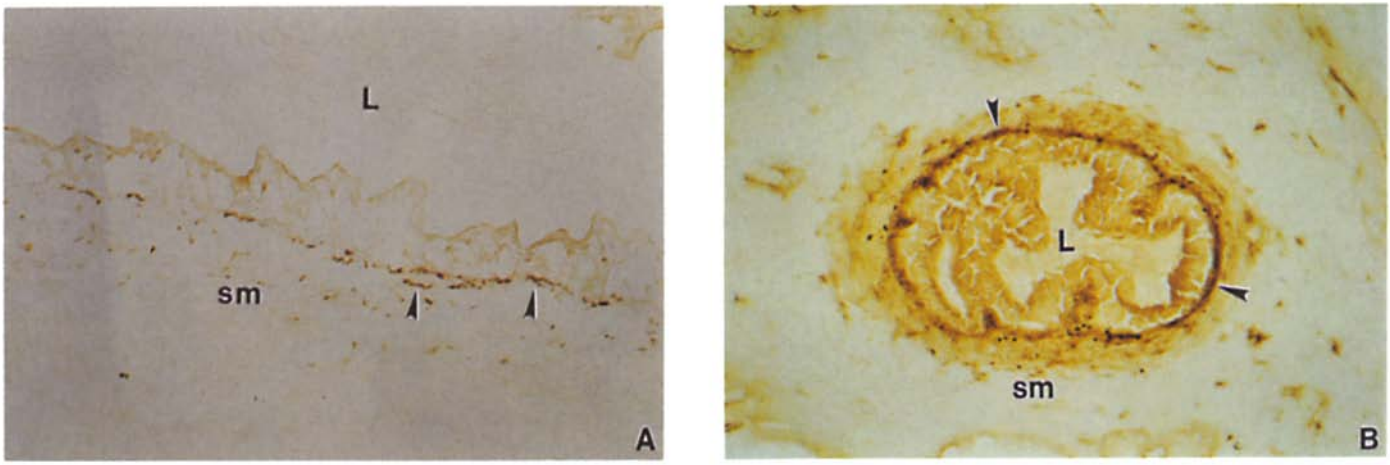

FIG. 3. Photomicrograph of vas deferens (A) in longitudinal section and (B) in cross-section immunostained with purified anti-NOS antibody. Immunoreactivity was localized to nerve plexuses (arrowheads) situated in lamina propria. sm, smooth muscle; L, lumen. Magnification ×187.

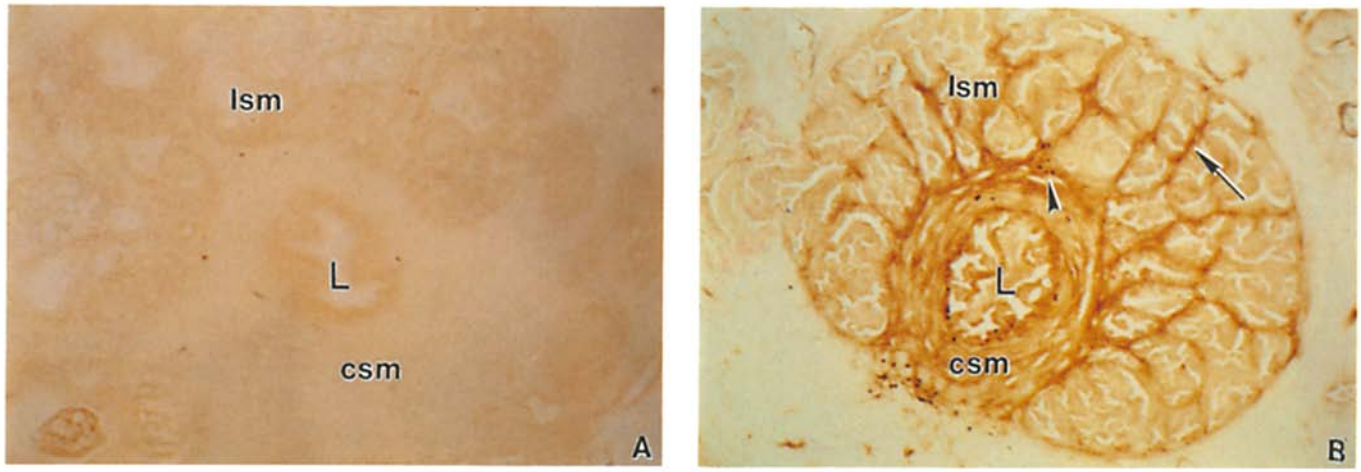

FIG. 4. Photomicrograph of ejaculatory duct in cross-section. A) Tissue incubated with anti-NOS antibody preabsorbed with NOS protein, and (B) immunostained with purified anti-NOS antibody. Distinctive pattern of staining shows nerve fibers (arrow and arrowhead) interspersed with smooth muscle bundles of circular layer (csm) and between smooth muscle bundles of longitudinal layer (lsm). L, lumen. Magnification ×75.

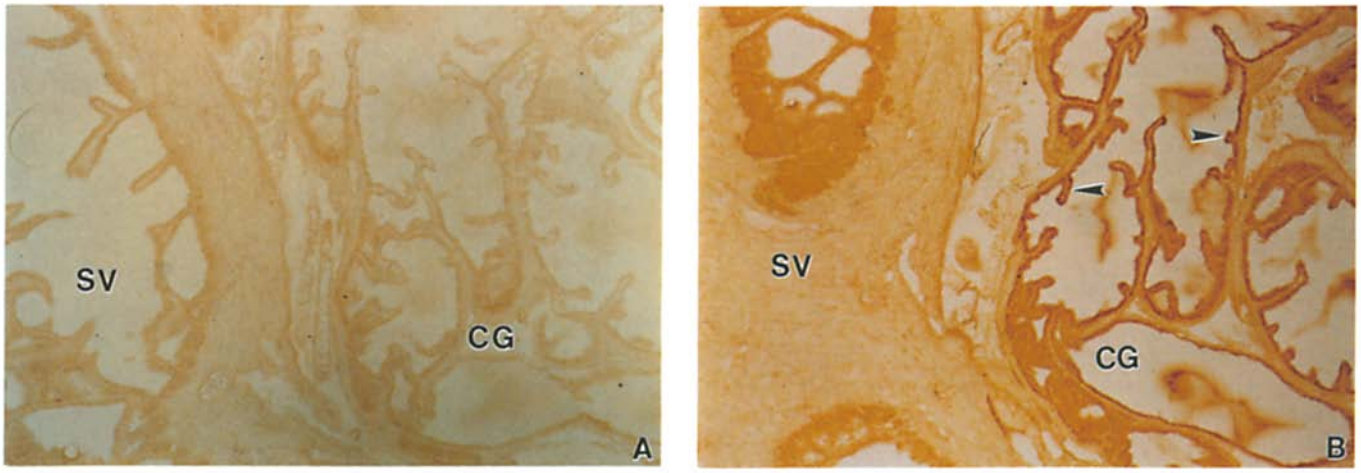

FIG. 5. Photomicrograph of seminal vesicle and coagulating gland in apposition. A) Tissue incubated in presence of control preimmune rabbit serum, (B) immunostained with purified anti-NOS antibody. Coagulating gland (CG) exhibited strong epithelial staining throughout (arrowhead), whereas only moderate staining was evident in the seminal vesicle (SV). Magnification ×75.

idis had a characteristic checkerboard pattern resulting from principal cells that were variably stained and clear cells that were not stained. The basal portion of the epididymal tubule stained intensely; a characteristic bilaminar appearance was observed consistent with subepithelial and epithelial staining. In addition, distinct NOS immunoreactivity was observed in the apical and nuclear regions of epithelial cells.

Similar to the staining in the mid-to-distal caput epididymidis, NOS immunostaining and NADPH diaphorase staining in the corpus and cauda regions were detected in the luminal epithelium and in the basal portion of the epididymal tubule. NADPH diaphorase staining was less intense in the corpus and cauda epididymides than in the mid-to-distal caput; this regional difference was not observed with NOS immunohistochemistry.

In the distal region of the cauda epididymidis and at the junction of the epididymis and vas deferens, intense staining for diaphorase and NOS was present in nerves contained within the smooth muscle surrounding the tubule. However, smooth muscle cells were devoid of staining.

Vas deferens. NADPH diaphorase activity and NOS immunoreactivity occurred in fine nerve fibers distributed throughout the smooth musculature of the vas deferens and in discrete nerve plexuses coursing throughout the lamina propria (Fig. 3). NOS staining was also apparent in the vascular endothelium. Smooth muscle cells were devoid of staining.

Ejaculatory duct. Nerves coursing through the circular muscle layer of the ejaculatory duct and spreading axially in the longitudinal layer of the duct were intensely stained for NOS (Fig. 4B) and NADPH diaphorase (not shown).

Seminal vesicle and coagulating gland. The seminal vesicle exhibited only light NOS staining (Fig. 5B) and was devoid of NADPH diaphorase staining. In contrast, the glandular epithelium of the coagulating gland exhibited intense staining for both NOS (Fig. 5B) and NADPH diaphorase (not shown). Where present, endothelial cells were NOS-positive in these tissues.

DISCUSSION

In the present study, we have demonstrated the distribution of NOS, the enzyme that synthesizes NO, throughout the reproductive tract of the adult male Sprague-Dawley rat. Parallel findings were obtained with a catalytic assay showing relative levels of NOS activity within reproductive organs and NOS immunohistochemistry showing precise localizations of NOS within these same structures. NADPH diaphorase histochemical results closely matched NOS localizations verified immunohistochemically.

On the basis of the widespread distribution of NOS in the male reproductive tract, we suggest the potential role for NO in the mediation of various reproductive functions. NOS localizations included nerves and vascular endothelium. These results are consistent with our previous findings in the penis [11, 16] and imply that NO modulates smooth muscle activity in other reproductive organs. The presence of NOS in neuronal plexuses within the adventitia and endothelium of arteries supplying reproductive organs indicates that NO regulates the vasoactivity of, and hence blood delivery, to these organs. NOS in neuronal fibers within the musculature and in neuronal plexuses within the subepithelial regions and/or lamina propria suggests that NO may mediate contractility of reproductive organs. Alternatively, subepithelial localizations of NOS-containing neuronal fibers might indicate that NO is involved in neuronally mediated secretory processes; this role resembles a putative function for regulatory neuropeptides in the rat urogenital tract [30, 31].

NOS immunoreactivity in the vas deferens was particularly distinctive, matching the high amounts of NOS activity measured catalytically. In the proximal vas, staining for NOS was discretely localized to subepithelial neuronal plexuses and to neuronal fibers sparsely distributed within the smooth musculature. This pattern of staining increased towards the distal end of this organ and extended prominently in the region of the ejaculatory duct. The increased NOS-positivity that occurred in the distal-most portions of the vas is consistent with regional differences in the contractility of the vas, which may be explained by neuronal mechanisms other than the classical adrenergic influences [21–23, 32].

In the epididymis, NOS staining was localized to nerves and to the luminal epithelium. NO derived from nerves in the periphery of blood vessels and surrounding the epididymal tubule in regions near its junction with the vas deferens may be involved in the regulation of vasoactivity within the epididymis and the tubular contractility involved in the movement of spermatozoa from the cauda epididymidis into the vas deferens. This putative function is analogous to that proposed for NANC neurotransmitters in the intestine [33] and esophagus [34]. NOS staining localized to principal cells and to the basal region of the epididymal tubule suggests that NO may be involved in secretory and absorptive processes characteristic of this epithelium [35]. The distribution of NOS in the epididymis resembles the distribution of pseudocholinesterases in the epididymis [32] and exemplifies the distinct properties of the different regions of the epididymis. Further functional studies are warranted to clarify the role of NO in the epididymis.

With respect to the seminal vesicle and the adjacent coagulating gland, NOS activity and immunoreactivity were present predominantly in the latter. Similar to the epididymis, in the coagulating gland NOS was localized to the epithelium. These data support the notion that NO may play a unique role in glandular activity, as suggested in previous proposals for NO derived from epithelial sources [36,37]. Whether the epithelial staining shown here represents a unique NOS isoform (different from macrophage, endothelial, or neuronal NOS) or reflects a cross-reactivity with neuronal NOS is not clear. Other effector systems including peptidergic, aminergic, and cholinergic systems are proposed to influence function in the seminal vesicle [38].

NOS immunoreactivity in the testes was localized to the vascular endothelium. This localization suggests that NO may influence testicular perfusion and consequently the delivery of gonadotropin to Leydig cells as well as the transport of androgen from the testis. Previous studies have described the modulation of Leydig cell function by testicular vasculature [39].

The findings reported in this study are compatible with previous descriptions of autonomic control of reproductive functions [19]. Tonic activities such as penile flaccidity, bladder neck tone, and antegrade propulsion of seminal fluid through the ductal system are believed to be adrenergically mediated [26]. The seminal emission and ejaculation phases of the "ejaculatory process" are also thought to be primarily mediated by catecholamines or other adrenergic substances [26]. However, the adrenergic effects governing these processes may be modulated by NO, as in its proposed role in penile erection [16]. Since the present study shows copious NOS-positive neuronal fibers in the terminal portion of the vas deferens and ejaculatory duct, NO possibly acting via inhibitory neurotransmission may mediate the passage of seminal fluid. NO may synergize with the adrenergic system as well as with other neuromodulators such as vasoactive intestinal peptide [33, 40, 41] in order to facilitate the appropriate sequence of reproductive events. An earlier proposal that the coordination of secretion and contraction associated with sexual arousal in the male genital tract involves the antagonistic interplay of

cholinergic and adrenergic responses supports this possibility [42].

In conclusion, we have characterized NOS in discrete locations in the male rat reproductive tract both catalytically and immunohistochemically, leading us to infer additional functional roles for NO. Our description indicates that NO in the pelvis derives from neurons, endothelium, and epithelium. NO may regulate various local hemodynamic, contractile, and secretory processes of reproductive organs, although additional functional studies are needed to confirm these conclusions. The present results and previous localizations of NOS to neuronal elements of structures that participate in penile erection [11, 16–18] support an emerging view that NO is an important factor in the control mechanisms involved in male sexual function.

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