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Immunocytochemical distribution of nitric oxide synthase in the human corpus cavernosum: an electron microscopical study using the tyramide signal amplification technique

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Abstract Nitric oxide has proven to be an important mediator in the relaxation of human cavernosal smooth muscle. Nevertheless, there are many inconsistencies in the literature regarding the cellular and subcellular distribution of endothelial nitric oxide synthase in the human penis. The purpose of this study was to reexamine the localization of eNOS and nNOS in the cellular anatomy of the human cavernous body by means of electron microscopical immunocytochemistry in combination with the tyramide signal amplification technique (TSA). Using specific antibodies against eNOS and nNOS, the NAPDH-diaphorase reaction and advanced protocols for fixation and staining procederes, the occurrence of NOS isoenzymes eNOS and nNOS were examined in cavernosal specimens of ten male patients who were subjected to surgery for penile deviation. eNOS immunoreactivity and NADPH-d staining was seen to be significantly present in the endothelial cells covering the cavernous spaces and in the endothelium of helicine arteries. In endothelial cells, the NADPH-d reaction product BSPT-formazan was abundantly detectable attached to membranes of the endoplasmatic reticulum and the mitochondria whereas posititve eNOS immunostaining was seen in the endothelial cells throughout their cytoplasm without any particular relation to organelles. No considerable eNOS immunoreactivity was detectable in the trabecular smooth muscle cells. nNOS staining was found in nerve fibers innervating the cavernous body and cavernosal arteries. Our results counteract the hypothesis of the cavernous smooth muscle as a local source of NO and underline the importance of an intact endothelial function for penile erection and the contribution of eNOS to this process.

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Introduction

Investigative work laid by several groups during recent years clearly implicated that nitric oxide (NO) is the main non-adrenergic, non-cholinergic (NANC) mediator to induce the relaxation of penile arteries and the trabecular meshwork of cavernous smooth muscle in the mechanism of penile erection [4, 12]. Since the NO-cGMP pathway plays such a prominent role in normal erectile function, therapeutic efforts for the treatment of impotence have been guided towards preserving or enhancing this pathway [20, 21]. Moreover, NO is generally considered to act as an important regulator of smooth muscle tone, blood flow and secretory function in the lower urinary tract of mammals [1, 9]. NO is released from NANC nerve terminals and the endothelium of penile blood vessels and the cavernous spaces. NO is synthetized as a product of the catalytic conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). Three major NOS isoenzymes have yet been described that differ in their molecular structure and biological properties: constitutive and Ca^{2+}-dependent neuronal and endothelial isoenzymes b/nNOS and eNOS, and the inducible, Ca^{2+}-independent iNOS, which is typically associated with macrophages and other cells of immune function and is dependent upon tetrahydrobiopterin as a main cofactor. More recently, a novel nNOS isoform has been found to be expressed in the rat penis, urethra and prostate [13]. The activities of these isoenzymes are regulated by androgens [22] and neuronal mediators, such as acetylcholine and vasoactive intestinal polypeptide [19]. Since the detection of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) activity is regarded as a suitable histochemical marker for the localization of NOS in cellular structures, this method as well as the use of various commercially available NOS antibodies which were subjected to differing histochemical protocols have

been applied to describe the distribution of NOS isoenzymes in the human corpus cavernosum. Thus, there are many conflicting reports on the cellular distribution of NADPH-d and eNOS in the human penis, and various potential sources of NO within the human erectile tissue, e.g. the smooth musculature itself, have been stated in the literature [2, 5]. Although transgenic mice lacking the nNOS gene preserve erectile function [6], nNOS has been thought to be the most essential origin of NO in the cavernous tissue while the relevance of eNOS still remains controversial. This prompted us to reexamine the occurrence of eNOS and nNOS in human erectile tissue by light and electron microscopical immunocytochemistry applying optimized fixation and staining protocols and the tyramide signal amplification (TSA) technique to enhance the sensitivity of the cytochemical staining procedure, especially for eNOS. The TSA method was first described by Bobrow et al. [3] and has been proven to be much more sensitive than conventional light microscopical immunoperoxidase staining [8, 15]. In the present communication, we have modified this method and adapted it to the electron microscopical level in order to substantiate the distribution of constitutive eNOS alongside nNOS in human corpus cavernosum. The results are compared to those of the NADPH-d reaction.

Materials and methods

Tissue source and fixation protocol

Cavernosal specimens were obtained from ten men who underwent Nesbit surgery for correction of penile deviation. The tissue was stored in isopentane at −80 °C or was immediately frozen in isopentane chilled by liquid nitrogen, sectioned with a cryostat, mounted on glass slides, and fixed with ice-cold acetone for 10 min followed by a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.1 phosphate-buffered saline (PBS) for 1–12 h.

Electron microscopical NADPH-d histochemistry

Free-floating sections, sliced with a cryostat to 20–70 μM, were incubated for 90 min at 37 °C in PBS containing 0.2 mg/ml of the tetrazolium salt 2-(2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl)-tetrazolium chloride (BSPT), 1 mg/ml β-NADPH and 0.3% Triton X-100 (pH 7.4). The histochemical reaction was terminated by washing with PBS at room temperature. Sections were then postfixed with 1% osmium tetroxide, en bloc contrasted with 1% uranyl acetate, and flat-embedded in Durcupan. Sections were examined with a Zeiss 900 electron microscope (Zeiss, Oberkochen, Germany).

Immunocytochemical assays

Assays were performed as has been described comprehensively [17, 18]. Following thorough specificity tests (Western blotting and immunocytochemistry using bovine aorta as a positive control) anti-eNOS and anti-nNOS monocolonal antibodies from Transduction Laboratories (Lexington, USA) were chosen. Primary antibodies were diluted 1:100,000 to incubate the sections (10–20 μM) overnight at 4 °C. To visualize NOS-protein the TSA Indirect Kit (DuPont NEN, Boston, USA) in combination with the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, USA) with 3,3′-diaminobenzidine (DAB) according to the instructions of the manufacturers was used. The method uses horseradish peroxidase bound to a secondary antibody to catalyze the covalent binding of biotinylated tyramide at the antigen-antibody binding locus extremely close at the enzyme site. Labeled streptavidin is then used to bind to the deposited biotin and allows detection of the antigen. The following protocol was applied:

- Rinsing the sections thoroughly in PBS to remove fixative Incubation in 50% ethanol with 0.3% H_2O_2 for 10 min.
- Rinsing in TPBS [10 mM TRIS and 0.05% Thimerosal in 10 mM phosphate buffer (pH 7.4) with 1% normal horse serum (NHS)].
- Incubation of the sections in TPBS with 3% NHS to block nonspecific binding.
- Incubation with the primary antibody in appropriate dilution in TPBS with 3% NHS at room temperature for 24–36 h.
- Rinsing in TPBS with 1% NHS for 3 × 5 min.
- Incubation with goat anti-mouse antiserum (Elite Kit) diluted 1:200 in TPBS with 2% normal rat serum for 60 min.
- Rinsing 3 × 5 min with TPBS.
- Transferring the sections to avidin-biotin solution for 60 min.
- Rinsing in TPBS 3 × 5 min.
- Incubation with biotinylated tyramide (BT) diluted in TPBS (10 ml TPBS, 10 μl BT, 10 μl 5% H_2O_2).
- Rinsing 3 × 5 min with TPBS.
- Incubation in avidin-biotin solution at room temperature for 60 min.
- Rinsing thoroughly 5 × 5 min with TPBS.
- Staining with DAB solution (7.5 mg diaminobenzidine in 15 ml PBS and 0.375 ml of freshly prepared H_2O_2 solution consisting of 40 μl 30% H_2O_2 and 1.5 ml aqua dest.) at room temperature for 60 min.
- Rinsing 3 × 10 min with TPBS.
- Preparation for EM embedding as described for NADPH-d.

Results

EM NADPH-d histochemistry and eNOS immunocytochemistry in sections of human corpus cavernosum

In cavernous trabecular endothelial cells, NADPH-d reaction product BSPT-formazan was detectable as distinct electron dense precipitates at membranes of the endoplasmic reticulum and mitochondria. Labeled membranes were seen in most of the endothelial cells and also, to a certain degree, in smooth muscle cells (Fig. 1). The results of the specificity controls are displayed in Fig. 2a–e. Under the electron microscope, the eNOS- and nNOS-immunoreaction products showed a variable intracellular distribution and were rather seen to be dispersed in the cytoplasm than attached to small vesicles, ribosomes, the nuclear envelope, and the plasmalemma. eNOS staining intensity was seen to be significantly present in the endothelial cells covering the cavernous spaces and the muscular tunica of arteries branching into the corporeal body. Ultrathin sections of human cavernous tissue immunostained with the eNOS antibody combined with TSA revealed DAB precipitates in the endothelial cells throughout their cytoplasm without any particular relation to the nuclear envelope and the membranes of mitochondria, endoplasmic reticulum, and the Golgi apparatus (Figs. 3, 4). Examin-

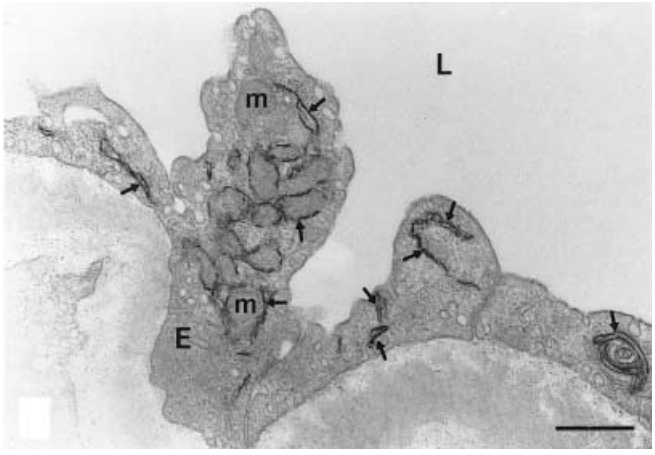

Fig. 1 Electron micrograph illustrating NADPH-d localization in the cavernous endothelium. BSPT-formazan deposits are located at portions of mitochondrial and endoplasmic membranes (*arrows*; *E* endothelial cells, *m* mitochondria, *L* cavernous space). *Scale bar* 0.5 μM

ation of numerous sections at different levels of the specimens showed that about 80% of endothelial cells were immunopositive for eNOS. NADPH-d staining and immunohistochemistry revealed eNOS expression in the endothelium of helicine arteries of the corpus cavernosum. No considerable eNOS immunoreactivity or formazan precipitation was detectable in the trabecular smooth muscle cells. nNOS staining was abundantly found in nerve fibers innervating the cavernous body and the cavernosal arteries. Some axon profiles within the bundles presented intense nNOS immunostaining whereas others appeared unlabeled (Fig. 5).

Discussion

A fundamental principle in the mechanism of penile erection is the relaxation of the corporeal smooth musculature. Several biochemical, histochemical and physiological studies indicated NO as the most essential mediator in erectile function. Since there are many inconsistencies in the literature about the cellular and subcellular distribution of eNOS in the anatomy of the human penis, we reinvestigated the localization of this isoenzyme using advanced protocols for tissue fixation and staining. The present study is the first to provide evidence of eNOS and nNOS expression in the cavernous body using the TSA technique and EM. We have optimized the TSA method, screened the specificity of the immunolabeling for EM by omission of the primary eNOS antibody as well as by using normal horse and rabbit serum instead of the eNOS antibody. Furthermore, double-labeling procedure was applied to prevent cross-reactivity. The application of the TSA method allowed to lower the antibody concentration to about fivefold, thus the signal-to-noise ratio was greatly improved and hardly any background staining occurred.

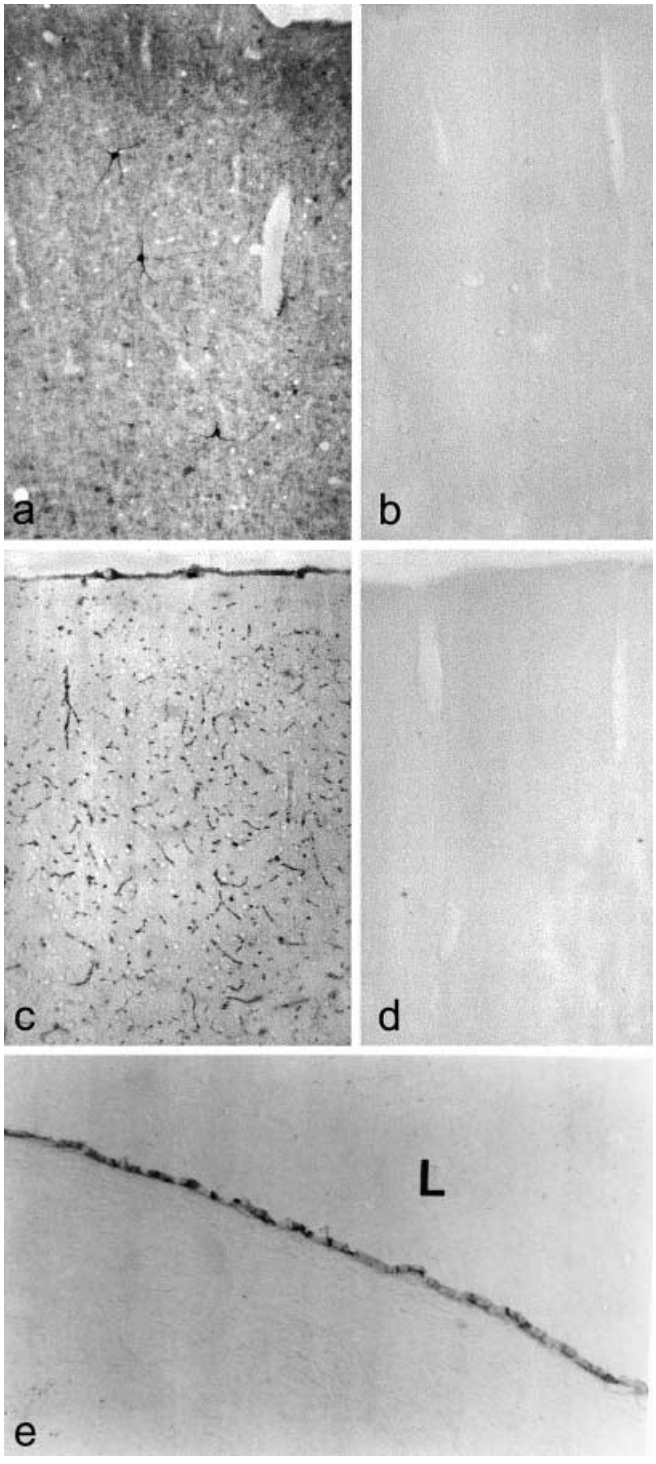

Fig. 2 Specificity controls for nNOS (**a,b**) and eNOS (**c–e**) immunostaining, **a–d** displaying sections through the rat brain cortex. **a, c** Positive control, note heavily stained solitary neurons in **a** and selective immunoreaction of vascular endothelium in **c**. **b, d** Negative control, omitting the primary antibodies. **b** The result of immunostaining using rabbit normal serum instead of primary antibodies. **e** Immunostaining of bovine aorta endothelium. Cryosections were fixed for 10 min with cold acetone followed by a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde. Section was labeled with monoclonal eNOS antibody (*L* arterial lumen)

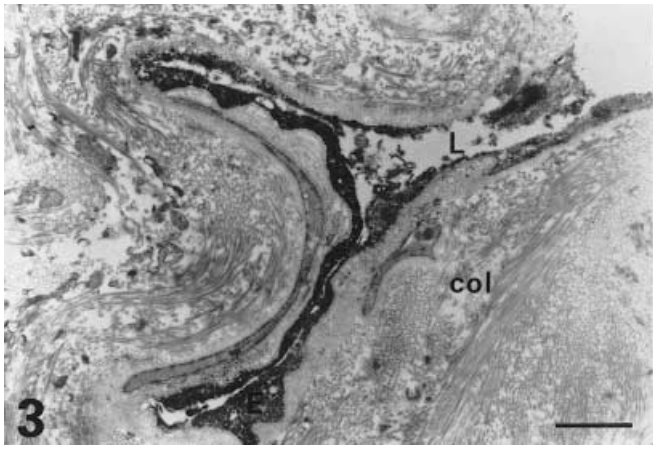

Fig. 3 Electron micrograph of cavernosal tissue section after eNOS immunostaining in combination with TSA. Endothelial cells show labeling with the DAB reaction product throughout their cytoplasms. Note the comprehensive labeling of endothelial structures after TSA application, whereas the background staining remains at a low level (*Col* collagen fibers, *E* endothelial cells, *L* cavernous space). *Scale bar* 1.5 μM

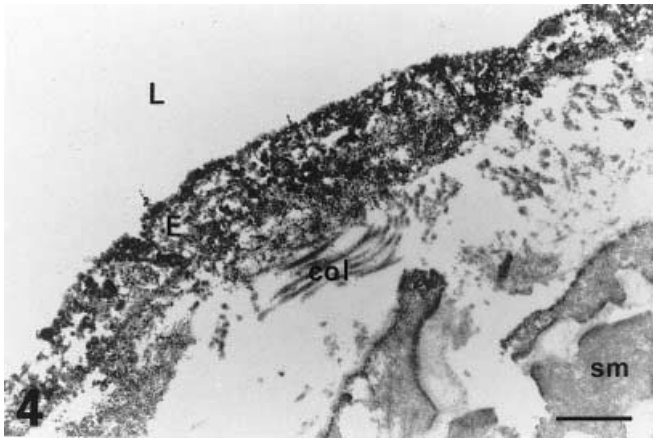

Fig. 4 Higher magnification of an endothelial cell showing eNOS staining irregularly distributed throughout the cell (*sm* smooth muscle cell, *Col* collagen fibers, *E* endothelial cells, *L* cavernous space). *Scale bar* 1 μM

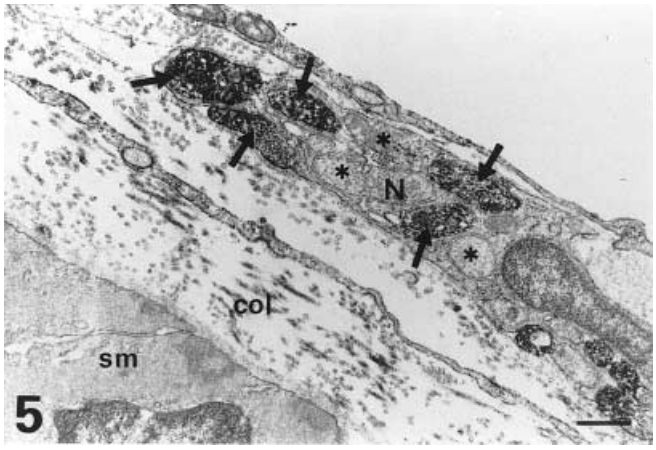

Fig. 5 Electron micrograph of nNOS immunolabeled perivascular nerve fibers innervating a cavernosal artery. Some axon profiles within the bundle present nNOS immunostaining (*arrows*) whereas others appear unlabeled (*asterisks*; *N* nerve bundle, *sm* smooth muscle cell, *Col* collagen fibers). *Scale bar* 0.5 μM

We were able to demonstrate that the majority of NADPH-d reaction in human cavernous tissue is represented by eNOS. At the EM level, we discovered the NADPH-d reaction product, BSPT-formazan, in endothelial cells covering the cavernous spaces exclusively as membrane-bound deposits on organelles. These findings are confirmed by the results of other authors who observed that eNOS is partly attached to the Golgi apparatus, endoplasmic membranes and the plasma membrane [10, 14]. While BSPT-formazan bound to endocellular membranes, distinct eNOS immunoreactivity, indicated by the DAB reaction product, was present throughout the cytoplasm of the endothelial cells. Since there is no tendency of BSPT-formazan to dislocate and to attach artifactually to lipophilic structures, as has been described by other investigators [16], a possible explanation is that only the active form of the enzyme is labeled by the tetrazolium salt. This conclusion is supported by biochemical studies where NOS in endothelial cells was found to be predominantly associated with the particulate fraction, but only 10–30% with the cytosol [11]. In contrast to other investigators, we were unable to detect considerable amounts of either eNOS or nNOS immunoreactivity within smooth muscle cells of the fibromuscular stroma [2]. The fact that, in the case of NADPH-d staining, BSPT-formazan was also not seen in substantial amounts makes it likely that cavernous smooth muscle cells do not contain NOS of any isoform. We suppose dense eNOS signals in cavernous smooth muscle cells might be due to the methods used for tissue fixation and incubation that influence the specificity and intensity of the immunostaining procedere in several ways. Thus, the hypothesis of the cavernous smooth muscle itself as another source of local NO release appears doubtful. On the other hand, we were able to confirm the expression of nNOS in nitrinergic fibers innervating the cavernous body. Moreover, we demonstrated local differences in nNOS labeling of axon profiles in distinct nerve bundles. The quantities of NOS in the human cavernosal tissue raise the question whether a decline in the density of the NOS isoenzymes contributed to the development of erectile dysfunction. While some authors reported a decrease in the number of NADPH-d-positive nerve fibers in cavernosal biopsies from a group of patients with neurogenic impotence, others concluded from their findings that erectile dysfunction cannot be solely reduced to pathological findings in penile NOS density [2, 7]. Despite the importance of the presence of NADPH-d and nNOS activity in discrete neuronal locations, our results underline the importance of an intact endothelial function for penile erection and the contribution of eNOS to this process.

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