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Evidence for the involvement of endothelial nitric oxide synthase from smooth muscle cells in the erectile function of the human corpus cavernosum

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Abstract Nitric oxide (NO) is an important mediator in the relaxation of cavernosal smooth muscle. The present study examines the existence and location of the constitutive isoform eNOS (endothelial NO synthase) accompanying the already substantiated neurogenic NOS (nNOS) in the human corpus cavernosum of men with and without erectile dysfunction. Activities of NOS enzymes were examined in specimens of 11 potent and nine long-term impotent patients by means of light and electron microscopy using NADPH-diaphorase staining and immunohistochemical eNOS-specific, smooth muscle actin-specific and nNOS-specific markers. Cavernosal smooth muscle shows a distinct expression of eNOS. In contrast to the weaker expression of eNOS and nitrinergic innervation found in larger veins, the small intracavernosal helicine arteries express large quantities of eNOS and possess a dense nitrinergic innervation. Long-term impotent patients display a broad heterogeneity in eNOS expression and nitrinergic innervation while no overall correlation between NOS expression and erectile function was observed. The expression of eNOS indicates eNOS as a main source of NO alongside nNOS. The differentiated localization of eNOS supports at least a role of this isoform in vascular regulation.

Key words Smooth muscle · Erectile dysfunction · eNOS · nNOS · Nitrinergic innervation

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Introduction

Non-adrenergic, non-cholinergic (NANC) neurotransmitters are essential for the relaxation of smooth muscle cells in the human corpus cavernosum, but the underlying mechanism of penile erection is not fully understood [11, 16]. There are few analytical methods explaining and evaluating neurogenic and non-neurogenic causes of impotence. Several years ago, nitric oxide (NO) produced by NO synthase (NOS)-containing nerve fibers was determined to be an important mediator of penile erection [11]. More recent studies implicate a reduction in NOS-containing nerve fibers as responsible for erectile dysfunction [2, 21]. Other sources of NO for penile erection subject to regulation by neurotransmitters have also been discussed, such as endothelium and smooth muscle [4, 12].

NO is generally considered to act as an important neuronal and non-neuronal mediator in the regulation of smooth muscle tone, blood flow and secretory function [1, 5]. NO is produced by NOS from L-arginine. Two constitutive isoforms of NOS exist, acting as regulators of physiological processes. The activities of these isoforms are regulated by a variety of humoral and neuronal mediators, such as vasoactive intestinal polypeptide (VIP) and acetylcholine [9]. Neuronal NOS (nNOS) must be differentiated from endothelial NOS (eNOS) [8]. NOS isoforms are located in endothelial, neuronal, epithelial and muscular structures [15, 18]. Preliminary studies demonstrated numerous nNOS-positive fibers in the corpus cavernosum [3, 19]. Neuronal NOS has been thought to be the essential origin of NO in the corpus cavernosum, while the relevance of eNOS remains unclear.

In order to study the sources of NO in the human corpus cavernosum, a non-selective histochemical method and selective antibodies for nNOS and eNOS were employed at light and electron microscopic levels. NADPH-diaphorase (NADPH-d) staining allows the determination of eNOS and nNOS distribution, due to

co-localization of NOS with NADPH-d in neuronal and endothelial tissues [10, 14]. NADPH-d is involved in the conversion of L-arginine to citrulline and NO.

The aim of this study was to substantiate the existence and differential distribution of the constitutive isoform eNOS alongside neurogenic nNOS in the human corpus cavernosum. Moreover, both NOS isoforms were examined in potent and impotent patients in order to elucidate possible differences between these groups.

Methods

Collection of tissue

Twenty human corpus cavernosum tissue specimens were obtained with consent from patients subjected to penile surgery. The specimens were immediately fixed in 4% paraformaldehyde for 4 h and then rinsed in 0.1 M phosphate-buffered saline (PBS) for 24 h. The tissue was stored for 12 consecutive hours in a PBS solution containing 18% sucrose for cryoprotection and then frozen to −80°C.

Eleven of the patients (17–64 years age; mean age 44 years) with normal erectile function suffered penis deviations. All patients were treated via Nesbit's surgical method. Normal erectile function was ascertained by anamnestic evaluation.

The other nine patients (34–50 years age; mean age 45 years) had shown complete erectile dysfunction for approximately 2 years. This group underwent implantation of flexible hydraulic protheses. One of the patients suffered from severe diabetes, another from Klinefelter's syndrome, one from impotence due to venous leakage and one from impotence following radical surgical therapy of a urinary bladder carcinoma. In five cases, impotence was of unknown origin.

NADPH-diaphorase staining

Tissue samples were sliced using a cryostat (Reichert-Jung) into 20-μm sections and rinsed with 0.1 M PBS for a minimum of 30 min. Incubation followed in a TRIS-buffered solution (pH 8.0) containing 83 mg nicotinamide adenine dinucleotide phosphate (β-NADPH), 40 mg nitroblue tetrazolium chloride (NBT) and 0.1% Triton-X 100 in 100 ml. The control sections were subjected to the same procedure without β-NADPH. Sections from rat brains were stained as positive controls. The specimens were incubated for 1 h at 37°C. To stop the reaction, the sections were rinsed in PBS (pH 7.3) three times, then thaw-mounted onto glass slides and covered with Kaiser's glycerol gelatin. The specimens were then studied microscopically using an Axiophot microscope (Zeiss, Oberkochen, Germany) and subsequently photographed.

Immunohistochemistry

Prior to immunohistochemical examination tissue sections (20 μm) were placed in a bathing solution of 3% H_2O_2 and 60% methanol PBS for 30 min, then permeabilized with 0.2% Triton-X 100 in 0.1 M PBS. The sections were then treated with 5% normal goat serum (NGS) and 5% bovine serum albumin (BSA) solution in PBS. Before each step, the sections were rinsed three times in PBS buffer. Incubation with the primary antibody occurred in a PBS-based solution of 0.8% BSA and 20 mM NaN_3 for 12 h at 4°C. The anti-eNOS antibody was applied in a dilution of 1:2000, the anti-smooth muscle actin antibody at 1:800 and the anti-nNOS antibody at 1:2000. After rinsing with PBS the sections were incubated with the corresponding secondary biotinylated antibody for 1 h at room temperature. A streptavidin–horseradish peroxidase complex was then applied as a detection system (1:100 dilution) for 1 h. Finally the staining was developed for 3–5 min with 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M TRIS-HCl buffer and 0.1% H_2O_2. Counterstaining was performed using methyl green. Sections of rat brains were stained to provide a positive control for the effectiveness of the nNOS antibody. Negative control sections were incubated without the primary antibody.

Electron microscopy

For ultrastructural examination, slices stained by NADPH-d reaction were postfixed for 20 min with 2% osmium tetroxide in 0.1 M PBS at 4°C. This procedure led to the formation of poorly soluble osmium-coordinated complexes with the formazan, generated from NBT via NADPH-d activity, appearing as black spots at the ultrastructural level. Following thorough rinsing in 0.1 M PBS buffer for 30 min, the slices were conventionally dehydrated in a graded ethanol series and finally infiltrated with and embedded in Araldite. Ultra-thin sections (30–60 nm) were obtained using a diamond-bladed Reichert ultramicrotome, placed on copper grids, and examined with a Zeiss EM 902A electron microscope (Zeiss, Wetzlar, Germany).

Point-counting

A point-counting method was employed to obtain comparable parameters for the density of nerve fibers in the NADPH-d-stained sections. The specimens were examined under ×400 magnification with a simple 100 test-point square lattice projected into the field of vision. Only those areas not directly connected to the tunica albuginea were morphometrically analyzed. An area of 2.5 mm^2 was analyzed in each specimen. Each intersection of a single nerve fiber and a test-point was counted in 10 fields of view for each section. Large bundles of nerve fibers were excluded.

Data analysis

The density of nitrinergic nerve fibers was measured via point-counting as described above. Values are expressed as intersections with the square lattice per field. Data were expressed as the mean ± SD of n experiments. Statistical significance was tested using Student's t-test for unpaired values.

Material

The nicotinamide dinucleotide phosphate (NADPH) and nitroblue tetrazolium (NBT) used for the NADPH-d staining were produced by Biomol (Hamburg, Germany). The primary rabbit anti-eNOS and mouse anti-smooth muscle actin antibodies were also from Biomol. The anti-nNOS antibody was obtained from Transduction Laboratories (Lexington, Ky.). The secondary biotinylated goat anti-rabbit and goat anti-mouse antibodies were from Vector Laboratories (Burlingame, Calif.). BSA was purchased from Sigma (Deisenhofen, Germany), which also provided the normal goat serum as well as the necessary chemicals required for staining with the avidin-biotin-peroxidase complex.

Results

As described by Tamura et al. [19], NADPH-d staining reveals a dense nitrinergic innervation by large nerve bundles (50–100 μm in diameter) in the central area of the corpora cavernosa as well as a network of single fibers with varicosities in the fibromuscular stroma innervating the bulk of smooth muscle bundles (Fig. 1a, b). Further, distinct histochemical staining of

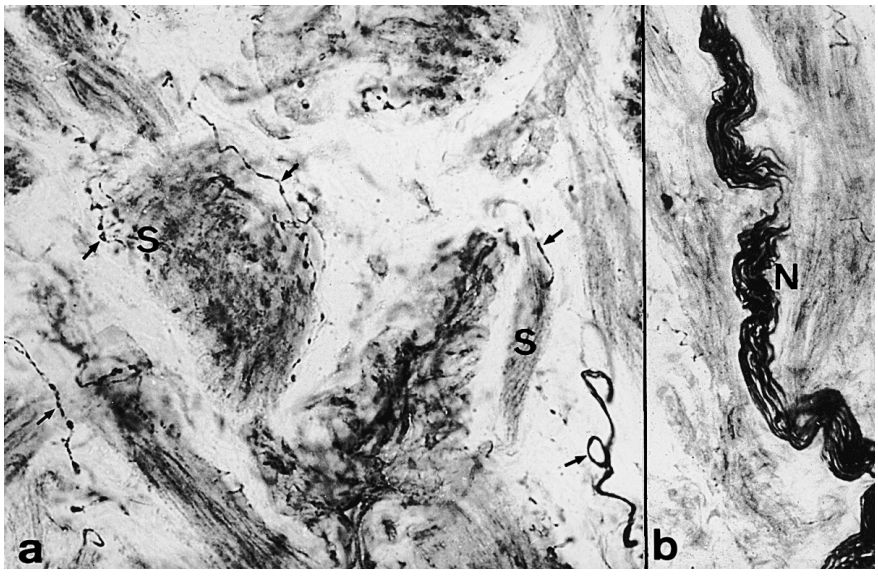

Fig. 1a,b Light micrographs of a 20-μm-thick cryostat section of the human corpus cavernosum; NADPH-diaphorase (NADPH-d) reaction. **a** The dark reaction product of NADPH-d staining visible in nerve fibers (*arrows*) distributed throughout the fibromuscular stroma (*S*) also shows a dark coloring after NADPH-d staining ×400, **b** Thick nitrinergic nerve bundles (*N*) are located in the cavernosal stroma (*S*). × 630

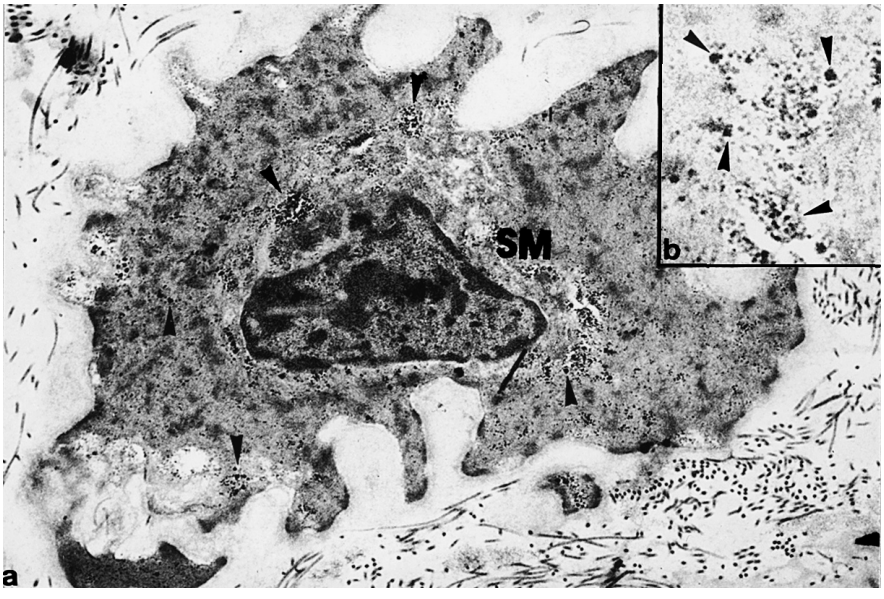

Fig. 2 **a** Ultrastructural localization of formazan precipitates in human corpus cavernosum following NADPH-d staining. Formazan complexes appear as dark spots (*arrowheads*) in smooth muscle (*S*) cells. ×15 000, **b** Osmium-complexed formazan appears as dark spots (*arrowheads*). ×45 000

fibromuscular stroma can be observed. Specimens were prepared for ultrastructural investigation in order to elicit the structures stained by NADPH-d. NADPH-d reaction products can easily be identified ultrastructurally as dark precipitates distributed in smooth muscle (Fig. 2) and endothelial cells. Expression of NADPH-d staining in the smooth muscle cells of the cavernosal stroma is confirmed by positive immunohistochemistry with eNOS antibodies, but not so with the nNOS antibody (Fig. 3a, b). Smooth muscle cells were identified immunohistochemically with an anti-smooth muscle actin antibody in consecutive sections (Fig. 3c). The tunica albuginea exhibits few nitrinergic nerve bundles, while nitrinergic vessel innervation and eNOS in the large vessels are extremely rare.

Differential studies of nitrinergic innervation and eNOS expression provide evidence of local differences, which can be confirmed by NADPH-d staining and immunohistochemistry via eNOS and nNOS antibodies (Figs. 4, 5). NADPH-d staining and immunohistochemistry reveal the distinct eNOS expression in the endothelium of helicine arteries of the corpus cavernosum (Figs. 4, 5a). The endothelium of the cavernosal sinus and subalbugineal veins showed lower activities and stained only faintly positive (Fig. 5b). Nitrinergic innervation varies in the same manner as the distribution of eNOS expression, dependent upon the type of vessel. Arteries show a dense nitrinergic innervation (Figs. 4, 5c), while nitrinergic innervation of veins remains sparse (Fig. 5d).

Specimens from impotent patients with different primary diseases do not show a uniform expression of eNOS and nNOS. A high degree of heterogeneity, ranging from normal levels to a decrease in either nitrinergic innervation, eNOS expression or both can be observed (Fig. 6). The patient who had suffered severe

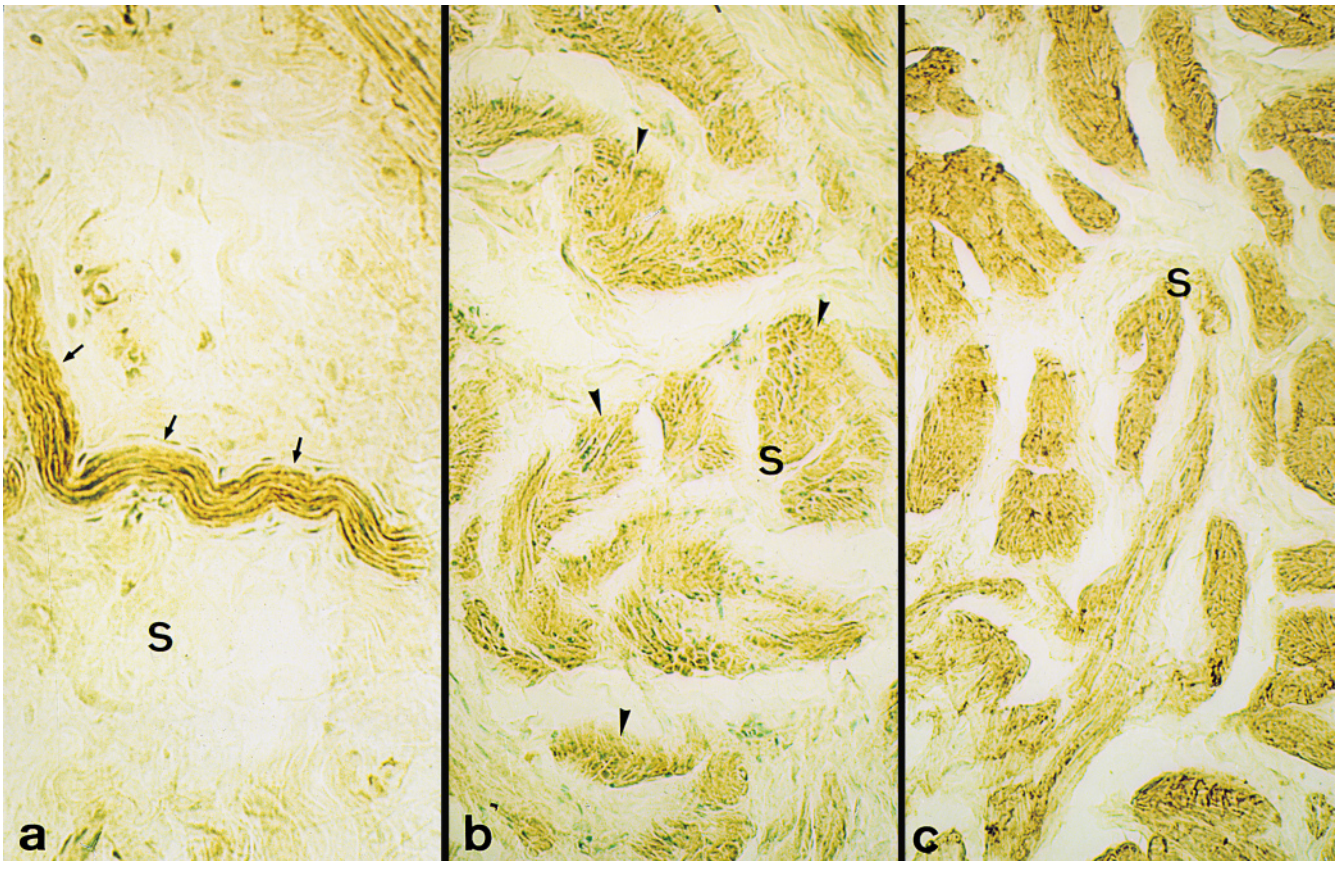

Fig. 3a–c Sections of human corpus cavernosum tissue from a potent man immunostained by antibodies for neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS). Sections are counterstained with methyl green. **a** Only nitrinergic nerve fibers (*arrowheads*), innervating the fibromuscular stroma, are stained by nNOS. The fibromuscular stroma is devoid of immunostaining. ×630. **b** A distinct part of the fibromuscular stroma is positive for eNOS immunoreaction (*arrowheads*). **c** Immunostaining with an anti-smooth muscle actin antibody shows a comparable distribution of immunoreactivity as observed with the eNOS antibody. ×200

diabetes mellitus for 15 years makes a microscopically "potent" impression, i.e., there are abundant nerve fibers and distinct expression of NOS in the endothelium and smooth muscle cells. Specimens from a patient who had undergone radical surgery were almost devoid of NOS-positive nerve fibers, while the endothelium and smooth muscle cells were less intensely stained than those of potent patients. In contrast, tissue sections of a patient with Klinefelter's syndrome were stained very faintly, especially the smooth muscle cells; weak nitrinergic innervation can, however, be found.

Point-counting

The quantification of nerve fibers in the cavernosal tissues as a whole shows no significant decrease in nitrinergic fibers in specimens from impotent patients. The mean value for "potency" is 1.55 ± 1.32 intersections per field whereas the value for "impotence" is 1.01 ± 1.09 countable nerve fibers per field of vision. The fewest nerve fibers were counted in an impotent patient who had undergone radical surgery.

Discussion

The present study is the first to provide evidence of eNOS expression within smooth muscle cells of the fibromuscular stroma and vessels of the human corpus cavernosum. Further, we were able to demonstrate local variances in the density of so-called nitrinergic nerve fibers containing nNOS and the expression of eNOS in endothelium. Differences between the distinct expression of eNOS in extensively innervated arteries and the weaker eNOS immunoreactivity and nitrinergic innervation of veins are of functional interest. These findings must be discussed in association with the general consensus regarding the hemodynamic events leading to erection, namely increased arterial flow, increased intracorporal pressure, and a decrease in venous outflow [7]. The investigation of patients with non-uniform forms of impotence provides evidence of the great heterogeneity in eNOS expression and nitrinergic innervation. It is therefore impossible to correlate alterations in NOS expression with erectile dysfunction independent of their origin.

Penile erection is evoked by neurally induced relaxation of cavernosal corpus smooth muscle. Several

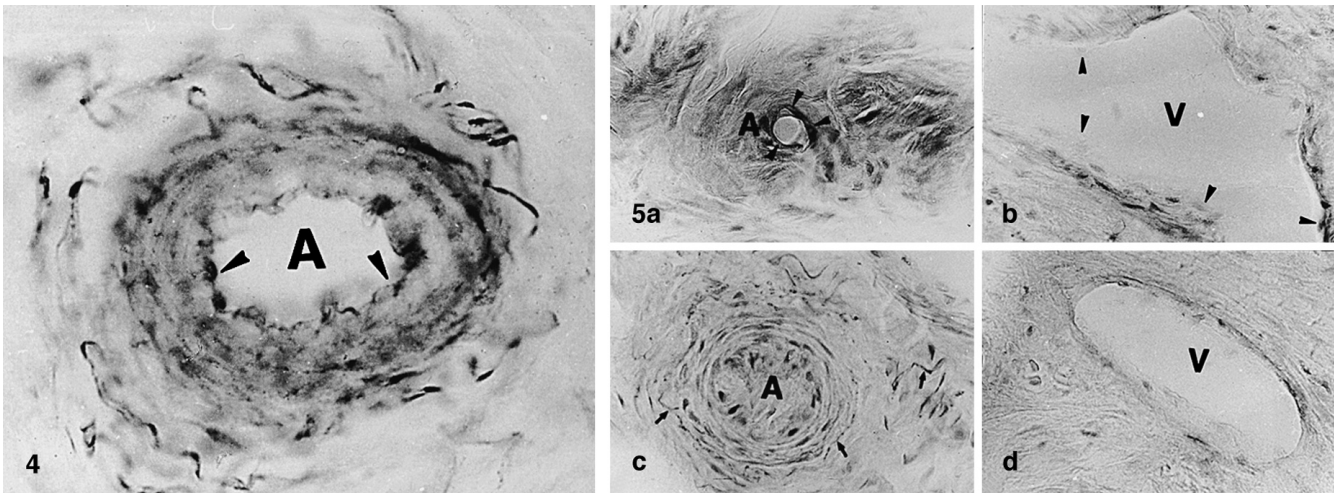

Fig. 4 NADPH-d staining of a 20-μm-thick cryostat section of corpus cavernosum tissue from a potent patient photographed under a light microscope. There is distinct coloring of the endothelium (*arrowheads*) of a corporal artery (*A*) densely innervated by nitrinergic nerve fibers (*arrows*). ×630

Fig. 5a–d Light microscopic immunohistochemical detection of nNOS and eNOS in 20-μm-thick cryostat sections of human corpus cavernosum tissue from men with normal erectile function. **a** The endothelium (*arrowheads*) of a small arteriole (*A*) is distinctly immunoreactive for eNOS. **b** There is only a partlial eNOS immunoreactivity in the endothelium (*arrowheads*) of corpus cavernosum veins (*V*). **c** nNOS-immunoreactive nerve fibers (*arrows*) were observed around a helicine artery (*A*). **d** Around a vein (*V*) of the corpus cavernosum no immediately adjacent nNOS-containing nerve fibers were stained. ×500

studies have indicated NO as an essential mediator in erectile function [3, 11, 20]. Initially nitrinergic nerve fibers were assumed to be the source of NO. Increasing evidence of non-neuronal NO release in the corpus cavernosum as a mediator of erectile function has recently been found [3]. As demonstrated on strips of isolated cavernosal smooth muscle in rabbits, the endothelium provides additional NO mediation of cavernosal smooth muscle relaxation [12]. The endothelial release of NO is evoked by several neurotransmitters involved in neurotransmission of penile erection, such as acetylcholine and VIP [5, 13]. Aside from its substantiated neuronal and endothelial production, NO release from cavernosal smooth muscle cells must also be considered [2, 4]. Brock et al. [2] also demonstrated positive NADPH-d staining of smooth muscle cells. The question of NOS expression in these cells must therefore be raised [2]. NO release from smooth musculature has been demonstrated in isolated gastric smooth muscle cells evoked by NANC neurotransmission [9]. The observation of eNOS expression confirmed by NADPH-d staining and eNOS-specific immunostaining also support the suspicion of cavernosal smooth muscle cell involvement in NO mediation of erectile function. The fibromuscular stroma and the walls of the small helicine arteries contain large quantities of muscle cells capable of NO production. The combination of a distinct endothelial eNOS expression and dense nitrinergic innervation is important for the initiation of a rigid erection, which requires increased arterial blood flow through relaxed vessels. In contrast, the venous outflow vessels are significantly more sparsely innervated by nitrinergic fibers and almost devoid of eNOS. These data confirm findings by Dail et al. [6] in penile tissues of rats. Varying expression in the vascular tree must correspond with functional requests. These include an increase in arterial inflow and a simultaneous decrease in venous outflow.

The intense nitrinergic innervation and the quantities of eNOS in human cavernosal tissue both raise the question of whether a decline or other pathological change in the various NOS isoforms might be a contributing factor in the development of erectile dysfunction. Despite the small number of specimens from impotent patients with erectile dysfunction of inhomogeneous origin we must stress the fact that no common morphological or morphometric alterations in these tissues can be found in comparison with potent patients.

The tissue from a patient with a 15-year history of severe insulin-dependent diabetes and specimens from two further impotent patients were more densely innervated than average, while the eNOS expression in vascular endothelium and smooth muscle cells appeared to be normal. These findings support the conclusion that in these three patients reductions in cavernosal NOS are of no relevance to the symptoms involved in these cases, which we were only able to examine in terms of NOS quantities, not the substance's activity and quality. Vernet et al. [21] examined rats with diabetes mellitus type I and II – most of which developed erectile dysfunction – and noted a marked decrease in penile nNOS activity with less of a reduction in nNOS content. Few of the diabetic rats appeared to be healthy with regard to NOS content in comparison with the control group.

In two patients remarkable alterations, in comparison with potent controls, were found which could have contributed to their history of erectile dysfunction. First nitrinergic innervation was reduced to a minimum (no countable nerve fibers in the specimen of the patient with bladder cancer and radical surgery according to the

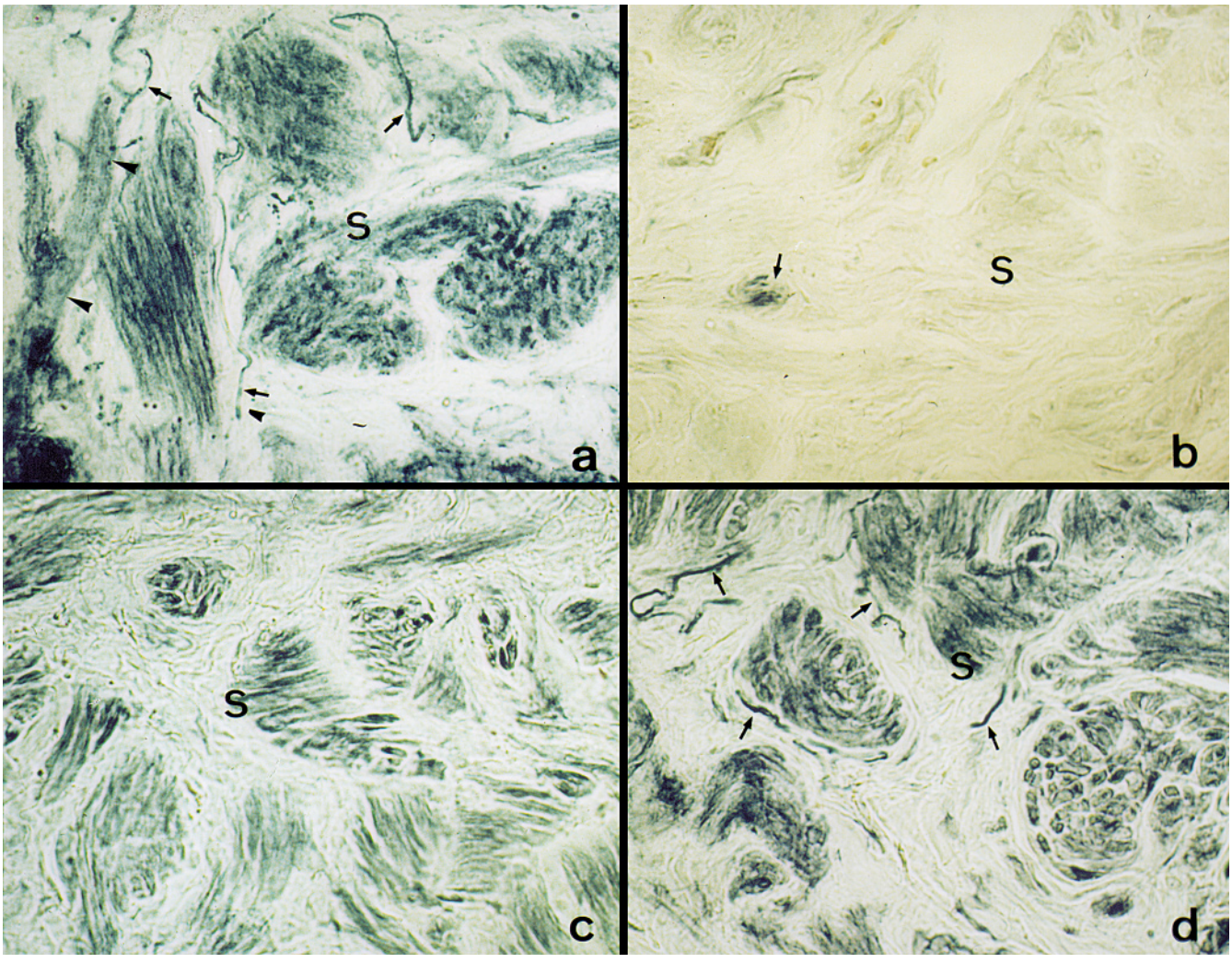

Fig. 6a–d Photomicrographs of the human corpus cavernosum from a patient with normal erectile function and from patients with erectile dysfunction. **a** Corpus cavernosum from a potent patient with distinct staining of smooth muscle (*S*), endothelium (*arrowheads*) and nerve fibers (*arrows*). **b** An impotent patient with Klinefelter's syndrome shows a nearly total absence of NADPH-d staining. **c** A patient who was impotent following radical surgery shows a marked decrease of nitrinergic innervation. **d** Corpus cavernosum of an impotent diabetic patient providing no evidence of alterations in eNOS or nNOS expression. ×400

point-counting method) and secondly the bulk of the smooth muscle cells within the erectile tissue stained only faintly positive. This suggests that the distinct reduction in both isoforms of NOS played a major role in the development of impotence in these cases. It is our conclusion that erectile dysfunction cannot be solely reduced to pathological findings in penile NOS.

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