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Nitroxergic innervation of the human ureterovesical junction

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Abstract Nitric oxide synthase (NOS) immunohistochemistry and nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry were used to investigate the distribution of nitroxergic, i.e., nitric oxide-synthesizing, neuronal perikarya and processes in the human ureterovesical junction (UVJ). Tissue specimens obtained from two cadaver kidney donors and two patients undergoing radical cystectomy for bladder cancer were examined. Clusters of NOS-immunoreactive neurons were localized in extramural ureterovesical ganglia. NOS-containing nerve fibers traveled within large extramural nerve trunks and marched among smooth muscle bundles. Extramural and intramural blood vessels were encircled by varicose NOS-positive axonal processes. The distribution of NOS immunoreactivity paralleled the staining pattern for NADPH-d activity. Urothelium stained strongly for NADPH-d activity but showed no NOS immunolabeling. Specimens from all four patients investigated showed similar staining patterns. Our results suggest that nitric oxide, a potent smooth-muscle-relaxing neurotransmitter in the autonomic nervous system, plays a physiologic role in opening the human UVJ.

Key words Ureterovesical junction · Nitric oxide
Innervation · Immunohistochemistry · Human

The ureterovesical junction (UVJ) acts as a one-way valve in protecting the kidney from the deleterious consequences of chronic reflux, namely infection and scarring. Besides the two "classical" neurotransmitters norepinephrine and acetylcholine [18], several neuroactive peptides, such as vasoactive intestinal peptide and calcitonin-gene-related peptide, have recently been localized immunohistochemically in the human UVJ [5]. However, the physiologic role of these substances in regulating the motility of the terminal and intravesical ureter is still obscure.

Nitric oxide (NO), a neurotransmitter that is widespread in the autonomic nervous system [7, 10, 14, 15, 17, 19], is a physiologic relaxor of penile [11], urethral [16] and bladder neck [16] smooth muscle. With regard to the strong inhibitory effects of NO on ureteral contraction [3], we investigated innervation of the human UVJ by NO-synthesizing (nitroxergic) nerves.

Nitroxergic nerves were demonstrated through immunohistochemical staining with antibodies against neuronal nitric oxide synthase (NOS) [12], the enzyme responsible for NO generation from its precursor L-arginine [12, 15]. Instead of this technique, many investigators use the inexpensive and easily performed NADPH-diaphorase (NADPH-d) reaction as a marker for NOS in the autonomic nervous system [4, 6, 9, 22]. Therefore, we studied colocalization of NOS-immunoreactive and NADPH-d-positive neurons in the human UVJ.

Materials and methods

We obtained specimens of the terminal ureter (3 cm) and bladder cuff (diameter about 1.5 cm) from two cadaver kidney donors (a 19-year-old man and a 54-year-old women) and two patients undergoing radical cystectomy for invasive bladder cancer (a 63-year-old man and a 74-year-old women). Specimens were dissected only from unaffected areas. No patient had a history of voiding disorder. The samples were immersed in 4% formaldehyde, buffered with 0.1 M sodium phosphate at a pH of 7.4 for 18–24 h at 4 °C, washed in 0.1 M phosphate-buffered saline (PBS) containing 15% sucrose for 24–48 h at 4 °C, and snap frozen in liquid N_2. Sections (10–20 µm) were cut in a cryostat (Frigocut 2700 Reichert-Jung, Heidelberg, FRG), mounted onto chromalum/gelatin-coated glass slides, and

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either processed for anti-NOS immunofluorescence detection [8] or for NADPH-d activity detection [9]. For immunohistochemical analysis, the sections were incubated in a rabbit polyclonal antiserum against NOS purified from porcine cerebellum [12] at a dilution of 1:1000 in 0.1 M PBS containing 0.3% (v/v) Triton X-100 for 18 h at room temperature. The bound anti-NOS antibody was detected by indirect immunofluorescence with Cy3-conjugated goat anti-rabbit IgG (Jackson, West Grove, USA) at a dilution of 1:80 for 1 h at room temperature. PBS, instead of the primary antiserum, served as negative control. After photography, the cover slips were removed, and the representative sections were stained for NADPH-d activity. The histochemical technique consisted of incubating the sections in 0.1 M TRIS-HCl buffer containing 2.2 mM β-NADPH (Biomol, Hamburg, FRG), 0.3 mM nitroblue tetrazolium (Serva, Heidelberg, FRG), and 0.3% Triton X-100 (v/v) for 30–60 min at 37 °C. The reaction was stopped by rinsing the sections in ice-cold PBS. The sections were embedded in a mixture of PBS and glycerol at a 1:1 ratio.

Results

NADPH-d-reactive (Fig. 1) and NOS-immunoreactive (NOS-IR) nerve fibers were contained in nerve trunks which approached the adventitial layer of the distal ureter. Fine varicose fibers marched in the connective tissue septa in the muscle layer of the terminal and intravesical ureter. Bundles of NADPH-d-reactive and NOS-IR (Fig. 2) fibres were set running parallel to smooth muscle fascicles. A well-developed plexus of mostly smooth NADPH-d-reactive and NOS-IR (Fig. 3) axons was found in the lamina propria of the mucosa. NADPH-d-reactive (Fig. 4) and NOS-IR nerve terminals were observed around extramural and intramural blood vessels (mostly arteries). NADPH-d-reactive (Fig. 5) and NOS-IR nerve cells encompassed a subpopulation of neurons in extramural ureterovesical ganglia. Double-staining experiments showed that the neuronal elements labeled by NOS antibody could equally well be stained by the NADPH-d reaction. All the NADPH-d-labeled neuronal structures that were encountered contained NOS immunoreactivity; this suggests a one-to-one colocalization of NOS protein with NADPH-d activity in neurons (Fig. 6). Strong reactivity for NADPH-d was found in the urothelium, but no NOS immunoreactivity. Specimens from all four patients investigated showed similar staining patterns.

Discussion

NADPH-d-positive perikarya and nerve fibers, which are particularly numerous at the UVJ, were recently described in the mouse lower urinary tract [6]. Although distribution of neurons staining positive for NADPH-d usually resembles the staining pattern of NOS immunoreactivity in the autonomic nervous system [4, 9, 23], Belai et al. [1] were not able to detect all NOS-positive neurons in the rat gut with NADPH-d staining. Therefore, NADPH-d staining of neuronal structures cannot automatically be considered as a valid substitute for NOS immunohistochemical analysis. A parallel staining pattern with the use of both techniques has not yet been demonstrated in animal or human UVJ. Strict colcalization of NOS immunoreactivity and NADPH-d activity within nerve cell bodies and fibers in the UVJ indicates that the easily performed and inexpensive NADPH-d technique can be used as a valid diagonostic tool for pathologic conditions of the UVJ. In the case of megaureter, for example, this technique might be able to demonstrate a possible selective loss of relaxing nitroxergic nerves distal to ureteral dilation, as has been shown in similar diseases of the gastrointestinal tract, such as achalasia [13], infantile pyloric stenosis [10, 22] and megacolon (Hirschsprung's disease) [23].

Demonstration of nitroxergic axons surrounding blood vessels around the UVJ supports the presumption that neuronal NO release not only regulates smooth muscle tone of the UVJ itself but also controls local blood flow by inducing vasodilation. Nitroxergic innervation of dog mesenteric and cerebral arteries was shown by Toda and Okamura [20]. The vascular tissue of human corpus cavernosum [2, 11] and, apparently, cavernous artery [2] also has thin innervation pattern.

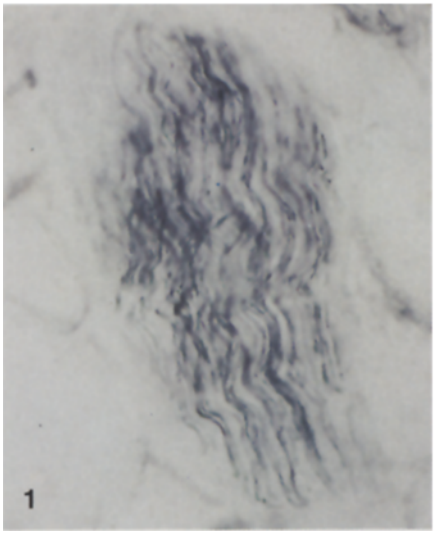
The observation that urothelium displayed a strong staining for NADPH-d but showed no NOS immunoreactivity might be attributed to mucosal NADPH-d that represents an isoform of NOS that is not readily detectable with the antiserum against neuronal NOS (NOS-I) used in our study [12]. Alternatively, selective staining of urothelium for NADPH-d only might be caused by other reducing urothelial enzymes that have no NOS activity at all.

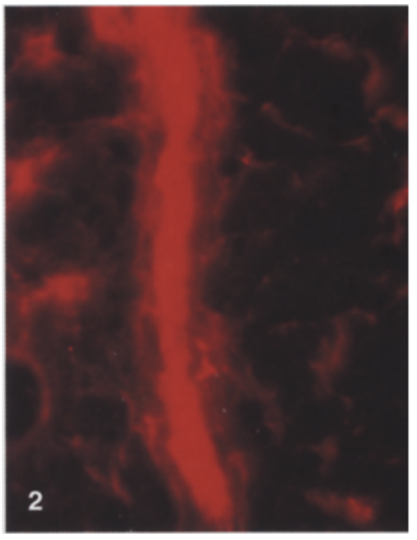
Our immunohistochemical and histochemical results support the presumption that nitroxergic innervation of the UVJ exists in humans. Besides histologic evidence, demonstration of neuronal NO release is required to confirm a neurotransmitter role of NO in the UVJ. However, neuronal NO release has not yet been shown with electric field stimulation [11, 21] of isolated tissue from the human UVJ. The bladder trigone, a muscular continuation of the distal ureter and its sheet of Waldeyer [24], relaxes after neurogenically induced NO release [16]. The ureter itself can be strongly relaxed by NO [3]. In conclusion our histologic results indicate a role for NO as a *physiologic* neurotransmitter that opens the human UVJ. Our

Fig. 1 NADPH-d-reactive fiber bundles in adventitial nerve trunk

Fig. 2 Longitudinal section of terminal ureter showing NOS-IR axonal processes in the muscle layer

Fig. 3 Longitudinal section of distal ureter showing a plexus of NOS-IR nerve fibers in lamina propria of the mucosa. *E*, epithelium, *LP*, lamina propria





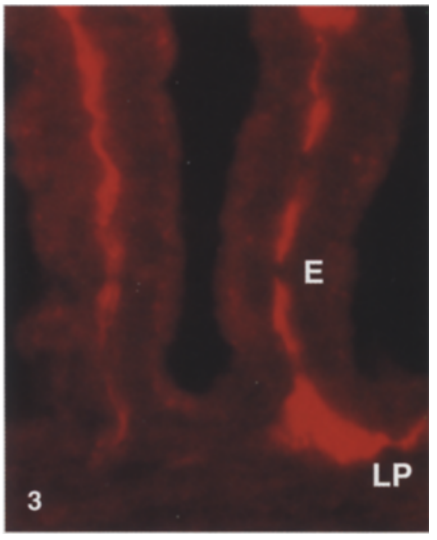


Fig. 4 NADPH-d-positive axons surround extramural small artery

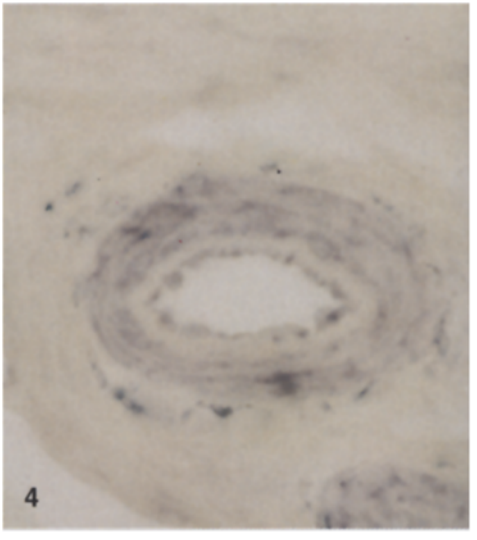


Fig. 5 NADPH-d-reactive neuronal perikarya within ureterovesical ganglion. Population of neurons in this ganglion is not stained; most contain lipofuscin pigment

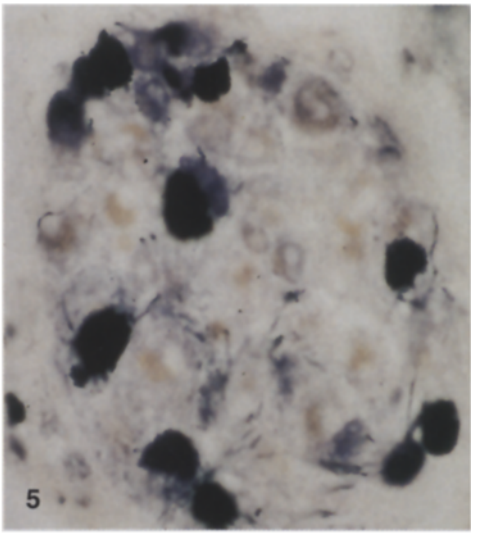
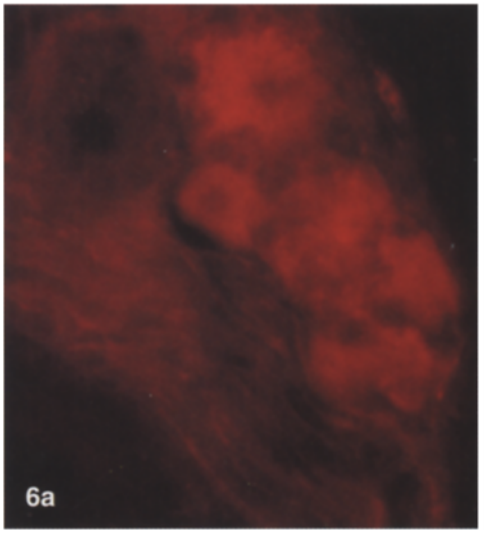
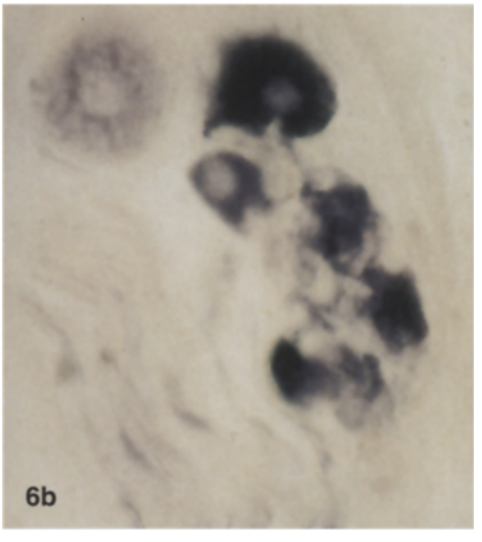


Fig. 6a, b Paired micrographs of ureteric ganglion. NOS-IR neurons (a) are also histochemically stained by NADPH-d activity (b)





results might have clinical implications: treatment of prevesical ureteral stones and colic with NO-releasing drugs applied through a percutaneous-nephrostomy catheter or through retrograde instillation into the affected ureter might have therapeutic value.

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