

Nitric Oxide Synthase—Its Distribution and Alteration in the Intramural Ganglia of the Urinary Bladder in Normal and Urethra-obstructed Guinea Pigs

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Abstract

Nitric oxide (NO) has been proposed to function as an inhibitory neurotransmitter in the lower urinary tract. This study investigates the distribution of NO-containing neurons and its changes following urethral obstruction in the guinea-pig. By using nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry and NO synthase (NOS) immunohistochemistry, the highest frequency of NO-containing neurons was observed in the bladder base. Double labelling studies showed that 70.9% of NADPH-d reactive neurons co-expressed NOS immunoreactivity. Acetylcholinesterase reactivity was present in the majority of the intramural neurons with 54% of them expressed NOS immunoreactivity. NADPH-d reactivity was colocalized with vasoactive intestinal polypeptide, calcitonin gene-related peptide and substance P immunoreactivities in both neurons and fibres. Colocalization study also revealed that NADPH-d reactive neurons formed a distinct cell population from tyrosine hydroxylase-positive neurons. At 12 hours after urethral obstruction, NADPH-d reactivity in the intramural ganglion cells was noticeably enhanced and this was sustained till 24 hours whence some intensely stained neurons appeared to undergo degenerative changes. Neuronal degeneration was more drastic at 48 hours so that the number of NADPH-d-positive neurons was significantly reduced. The present study suggests that NO is an important neurotransmitter in the urinary bladder and that it may be involved in the relaxation activity in the bladder base during micturition. It is speculated that the increased NADPH-d reactivity in intramural ganglion cells elicited by urethral obstruction may be responsible for the cell death. It is suggested that the resulting cell loss or bladder denervation may account for the urinary dysfunction such as frequency and urgency of micturition in patients with urethral obstruction.

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Introduction

Nitric oxide (NO) has been recognised as a nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter at various sites in the nervous system.^{1,2} Its function as a NANC inhibitory transmitter has been documented in muscular and sphincter relaxation in the cardiovascular,³ digestive⁴ and urogenital system.^{5,6} The possibility that NO is involved in inhibitory transmission in the lower urinary tract has recently been investigated in various species including human.⁷⁻⁹ Morphologically, NO-containing neurons have been detected immunohistochemically using an antibody against nitric oxide synthase (NOS),¹⁰ and histochemically by localization of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d).² NOS or NADPH-d positive neurons and fibres have been dem-

onstrated in the neural pathways of the lower urinary tract.^{11,12}

Recently, studies on neuronal NOS have focused on its plasticity in response to some pathological events.^{13,14} For example, NADPH-d reactivity or NOS immunoreactivity has been induced in the motoneurons of the ventral horn following spinal root avulsion¹⁵ and urethral obstruction.¹⁶ Upregulated expression of neuronal NOS and NADPH-d has been also detected in dorsal root ganglia (DRG) after pelvic nerve injury,¹⁷ chronic chemical irritation of the bladder¹⁸ and urethral obstruction.¹⁹

Bladder outlet obstruction is a major urologic problem and has been the subject of many clinical and experimental studies.^{20,21} It has been reported that the obstruction may result in structural and functional alterations of the

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bladder including detrusor hypertrophy, elevated voiding pressures and detrusor instability.²¹ The latter dysfunction was attributed to partial denervation in the bladder wall,²⁰⁻²² changes in the peptide contents in the bladder nerves,²³ and alterations of response of detrusor muscle to autonomic transmitters.²¹ Previous experimental study has shown that within the first 24 hours after acute complete urethral obstruction the initial bladder overdistension could lead to marked damage in bladder nerves in rabbits.²⁴

Although extensive studies on localization of numerous neurochemicals and their functions in the intramural ganglia of the urinary bladder have been reported, relatively little is known about that of NO in these ganglia. There is apparent lack of a systematic study on the topographic distribution of NO-containing intramural ganglion cells in the urinary bladder. In fact, virtually nothing is known about the effect of urethral obstruction on expression of NADPH-d and NOS in these cells. This review summarises the results obtained in our recent series of studies dealing with the distribution of NO-containing neurons,²⁵ and colocalization of NADPH-d and NOS with some common neurochemicals, especially the peptides²⁶ in the guinea-pig urinary bladder. The effect of acute urethral obstruction on the expression NADPH-d reactivity in the intramural neurons is also considered.²⁷

Materials and Methods

Animals

Seventy-five adult male guinea pigs (Laboratory Animal Centre, NUS, Singapore) weighing between 300 and 350 g were used in the present study.

In the handling and care of all animals, the International Guiding Principles for Animal Research as stipulated by the World Health Organization²⁸ and as adopted by the Laboratory Animal Centre, National University of Singapore were followed.

Anaesthesia

Prior to surgery or perfusion, all animals were deeply anaesthetized by an intraperitoneal injection of 7% chloral hydrate (5 ml/kg body weight) administered in the lower abdomen.

Surgical Procedures (Urethral Ligation)

Following anaesthesia, the peritoneal cavity was exposed through a vertical midline incision at the lower abdomen. The delicate connective tissue over the anterior aspect of the bladder neck was cleared with a pair of fine scissors. The bladder neck and proximal urethra were then exposed and secured. A silk thread was passed around the proximal urethra which was then ligated tightly. The abdominal incision was closed in two layers with silk suture. The same surgical procedure

was carried out in sham-operated (control) animals except for ligation of the urethra. Signs of successful ligation of urethra in the experimental animals included the absence of urination and postoperative distension of the lower abdomen postoperatively. None of these signs were observed in the control animals. All animals were sacrificed within 48 hours postoperatively, since the animals with urethral ligation beyond 48 hours after operation invariably died from bladder rupture.

Perfusion and Tissue Preparation for Light and Electron Microscopy

Under anaesthesia the animals were perfused with Ringer's solution (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (for light microscopy) or 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 via the intracardiac route. After perfusion, the whole bladder was removed and postfixed with the similar fixative for an additional 2 hours and kept in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose overnight at 4°C. The fixed and fully distended bladder was divided into 3 tissue pieces: caudal pole, middle portion, and cranial pole as described by Gabella.²⁹ The mucosa of bladder was stripped off from the muscular layers.

NADPH-d Histochemistry

For light microscopy, the NADPH-d reaction medium contained 0.3% Triton X-100 (Sigma, St. Louis, USA), 0.3 mM nitroblue tetrazolium (Sigma) and 1.2 mM β -NADPH (Sigma) in 0.1 M Tris buffer, pH 7.6. The reaction was performed at 37°C for 30 minutes and stopped by washing in 0.1 M Tris buffer (pH 7.6). The tissue samples were air dried overnight and coverslipped with Permount.

For electron microscopy, the whole-mount tissue preparations were incubated at 37°C for 2 hours in a medium containing 1.2 mM 2-(2'-benzothiazolyl)-5-styryl-3-(4'-pathalhydrazidyl) tetrazolium chloride (BSPT, Sigma) which was dissolved in 3 to 4 drops of dimethylformamide and 1.2 mM β -NADPH (Sigma) in 0.1 M phosphate buffer, pH 7.6. The reaction was stopped by washing in phosphate buffer (pH 7.8). The areas of the whole-mount preparations containing NADPH-d reactive neurons were identified and trimmed into small pieces of tissue samples under the light microscope. The tissue samples were postfixed in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 1 hour, then dehydrated in a graded series of ethanol and finally embedded in Araldite. Ultrathin sections were double stained with lead citrate and uranyl acetate and viewed under a Philips 400T or JEOL 1200EX electron microscope.

Acetylcholinesterase (AChE) Histochemistry

The tissue samples were first rinsed in ice-

cold isotonic sodium sulphate for 10 minutes before transferring to a medium containing the substrate acetylthiocholine iodide (Sigma) and tetraisopropylpyrophosphoramidate (ISO-OMPA, an inhibitor of non-specific cholinesterase). Following 4 hours of incubation, the tissue samples were treated in a sodium sulphide solution in 0.2 N acetic acid (pH 5.5) for 3 minutes and the reaction was stopped by washing the tissues in 0.1 M phosphate buffer (pH 7.4). The whole-mount preparations were air-dried overnight at room temperature (25°C) and then mounted in Permount.

Immunohistochemical Studies

The tissue samples were incubated in the following primary antibodies: polyclonal antisera from rabbit against NOS (Transduction Lab, USA), neuron specific enolase (NSE) (Polysciences, USA), vasoactive intestinal polypeptide (VIP), substance P (SP) (Incstar, USA) and calcitonin gene-related peptide (CGRP) (Amersham, UK), and monoclonal antiserum from mouse against tyrosine hydroxylase (TH) (Boehringer Mannheim, Germany).

Subsequent antibody detection was carried out using the Vectastain ABC-kit (PK-4001, Vector Laboratories, Burlingame, CA) against rabbit IgG with 3,3'-diaminobenzidine (DAB, Sigma) as a peroxidase substrate.

For immunofluorescence, subsequent antibody detection was carried out by using antirabbit or antimouse antisera labelled with fluorescein isothiocyanate (FITC, Sigma).

Double Labelling Studies

For colocalization of NOS and AChE, the tissue samples were initially processed for NOS immunofluorescence and subsequently stained for AChE histochemistry according to the method described above (see *AChE Histochemistry*).

For colocalization of NADPH-d and NOS, VIP, TH, CGRP, SP and NSE, the tissue samples were first processed for NOS, VIP, TH, CGRP, SP and NSE immunofluorescence and then further histochemically reacted for NADPH-d according to the method described above (see *NADPH-d Histochemistry*).

NOS, VIP, TH, CGRP, SP and NSE immunofluorescent neurons double stained for NADPH-d or AChE in the same region were examined and then photographed under a microscope (Aristoplan) using an Olympus ploempak N2 filter for FITC (emission maximum 437 nm; excitation maximum 436 nm) and a normal filter, respectively.

Quantitative Study

Cell counts of positively-stained neurons in each group were performed on whole-mount preparations of the bladder under a microscope (Aristoplan) by using a

normal or an Olympus ploempak N2 filter for FITC (emission maximum 437 nm; excitation maximum 436 nm). All profiles of positively stained cells were identified and counted in each group of animals. The numbers of positive neurons in different regions of the bladder of the respective animal groups were expressed as mean $\pm$ SD. Since the NADPH-d positive neurons showed marked gradation in staining for the enzyme, they were divided into 3 categories: intense, moderate and weak. The numbers of double-labelled cells expressing NOS/NADPH-d, VIP/NADPH-d, and NOS/AChE were also counted. The proportions of double-labelled cells versus the total NADPH-d or AChE positive cells were determined.

Results

1. Distribution and Total Number of NADPH-d and NOS Positive Neurons

1.1 Topographic Distribution of NADPH-d and NOS Positive Neurons

Numerous NADPH-d positive neurons were distributed in the muscular lamina, whereas such NADPH-d neurons were negligible in the overlying mucosa lamina that was stripped off. The majority of the NADPH-d cells were localized in ganglia of different sizes consisting of 2 to 40 neurons each. The ganglia which contained 20 to 40 cells were located preferentially at the base of the bladder (Fig. 1A), others with 10 to 20 cells, or 2 to 10 cells (Fig. 1B), were distributed mainly in the body and dome, respectively. NADPH-d positive neurons showed a marked gradation of reactivity for the enzyme, about one-third of them was heavily stained while the remainder being moderately or weakly stained (Fig. 1). Amongst the NADPH-d positive cells were some unstained neurons (Fig. 1). NADPH-d positive fibres, often showing varicosities, tended to occur in bundles linking the ganglia (Fig. 1) as well as extending into the smooth muscles of the bladder wall.

NOS-IR was also detected in the intramural ganglion cells in the muscular lamina. The ganglia containing about 20 to 30 immunoreactive cells were located preferentially in the bladder base (Fig. 2A), while those containing 10 to 20 cells and 2 to 10 cells (Fig. 2B), were distributed in the body and dome, respectively. Compared with the NADPH-d staining, the intensity of NOS immunoreactive neurons was relatively more uniform. The NOS positive neurons often displayed long processes which tended to form nerve bundles (Fig. 2).

1.2 Number of NADPH-d and NOS Neurons Against Total Neuronal Population

All the intramural neurons were immunostained for NSE. The largest ganglia were detected in the bladder

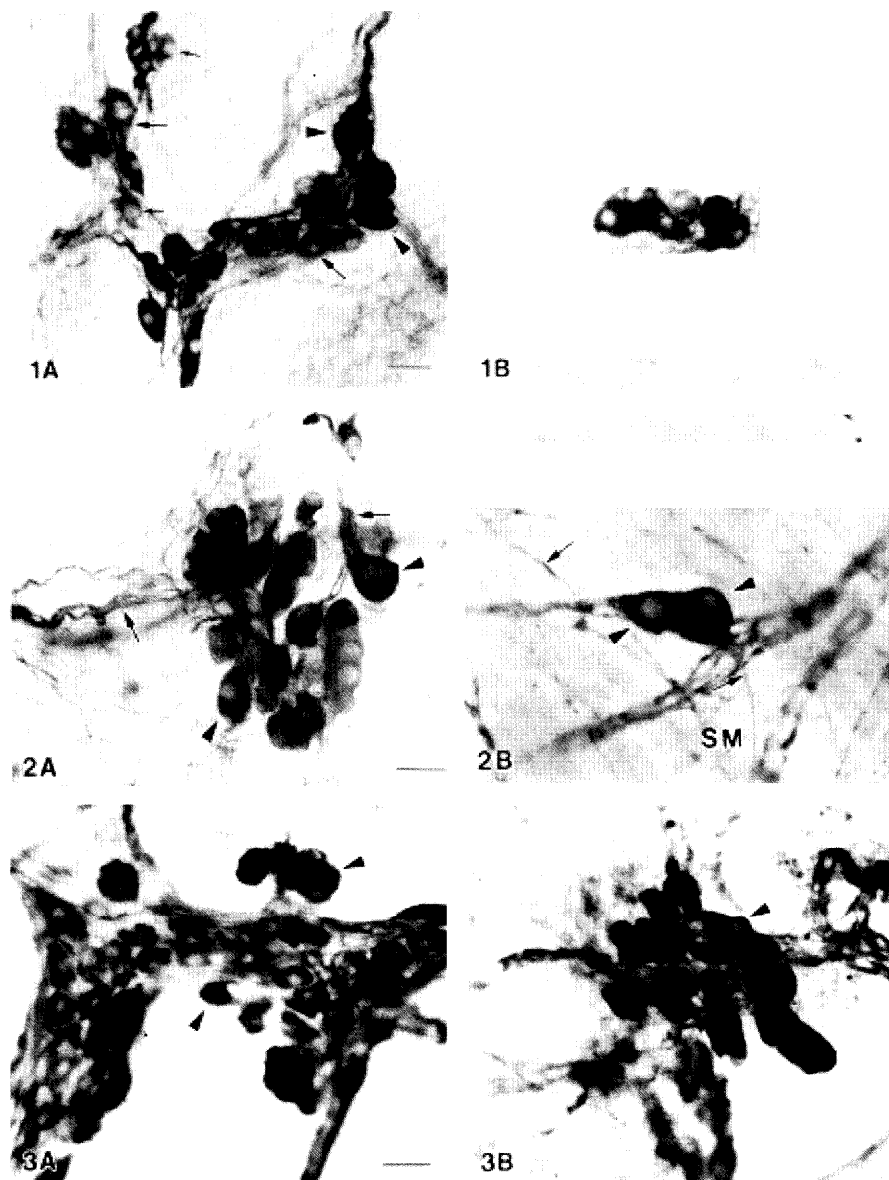


Fig. 1(A,B) Ganglion containing NADPH-d positive neurons in the bladder base (A). Note that some neurons are intensely stained (arrowheads) while others are moderately labelled (arrows) or weakly stained (small arrows) Ganglion containing 6 neurons in the bladder dome (B). The cells also exhibit different intensities of NADPH-d. Bar, 50 μ m.
 Fig. 2(A,B). Ganglion containing about 20 cells (A, arrowheads) expressing NOS immunoreactivity in the bladder base. Several NOS positive processes occur in bundles (arrows). Ganglion with 2 NOS positive neurons (B, arrowhead) in the bladder dome. Their processes (arrows) appear to project into the smooth muscle (SM) of the bladder wall. Bar, 50 μ m.
 Fig. 3(A,B) Large ganglion in the bladder base (A, arrowheads) stained for NSE immunohistochemistry. Ganglion in the bladder body stained for NSE (B, arrowhead). Note that the number of cells labelled for NSE is greater than those labelled by other the 2 methods. Bar, 50 μ m.

base (Fig. 3A), while the medium-sized ganglia were distributed in the body (Fig. 3B), and the small-sized ganglia were in the dome. The number of neurons expressing NSE-IR was more than that of neurons labelled for NADPH-d or NOS (Table I). The results of cell counts showed that the total number of NSE positive neurons was 3260 ± 421 , being 1668 ± 307 in the base, 1122 ± 210 in the body and 482 ± 130 in the dome, respectively (Table I). The present cell counts showed that the number of NADPH-d positive neurons in the entire bladder was 2750 ± 150 , whereas the number of NOS was about 1470 ± 221 (Table I). This result revealed that NADPH-d

positive neurons accounted for 84% while NOS immunoreactive neurons made up 45% of the total neuronal population (NSE as reference 100%) in the whole bladder.

2. Double Labelling Studies

2.1 Colocalization of NOS and NADPH-d

Although most of the intramural neurons were double labelled with NOS and NADPH-d, some NADPH-d positive cells were NOS negative (Fig. 4 A,B). Cell counts showed that 70.9% of the NADPH-d positive neurons in the whole bladder expressed NOS-IR (Table II).

TABLE I: NUMBER (MEAN $\pm$ SD) OF NADPH-d, NOS AND NSE POSITIVE INTRAMURAL NEURONS IN DIFFERENT REGIONS OF THE URINARY BLADDER

Staining	n	Base	Body	Dome	Whole bladder
NADPH-d	4	1650 $\pm$ 216	894 $\pm$ 206	206 $\pm$ 40	2750 $\pm$ 150
NOS	4	810 $\pm$ 114	555 $\pm$ 79	105 $\pm$ 71	1470 $\pm$ 221
NSE	4	1668 $\pm$ 307	1122 $\pm$ 210	482 $\pm$ 130	3260 $\pm$ 421

2.2 Colocalization of NSE and NADPH-d

In tissue samples processed sequentially for NSE immunofluorescence and NADPH-d histochemistry, the majority of the NSE immunoreactive neurons (Fig. 5A)

TABLE II: NUMBER (MEAN $\pm$ SD) AND PROPORTION OF DOUBLE STAINED (NOS/NADPH-d, VIP/NADPH-d AND NOS/AChE)INTRAMURAL NEURONS AGAINST THE NUMBER OF NADPH-D* OR AChE** POSITIVE CELLS IN THE URINARY BLADDER

Double staining	n	Number of double-stained cells	Number of cells stained with NADPH-d* or AChE**	Percentage of double-stained cells
NOS/NADPH-d	3	1425 $\pm$ 276	2008 $\pm$ 367*	70.9%
VIP/NADPH-d	3	465 $\pm$ 74	2179 $\pm$ 298*	21.3%
NOS/AChE	3	1333 $\pm$ 221	2594 $\pm$ 394**	51.4%

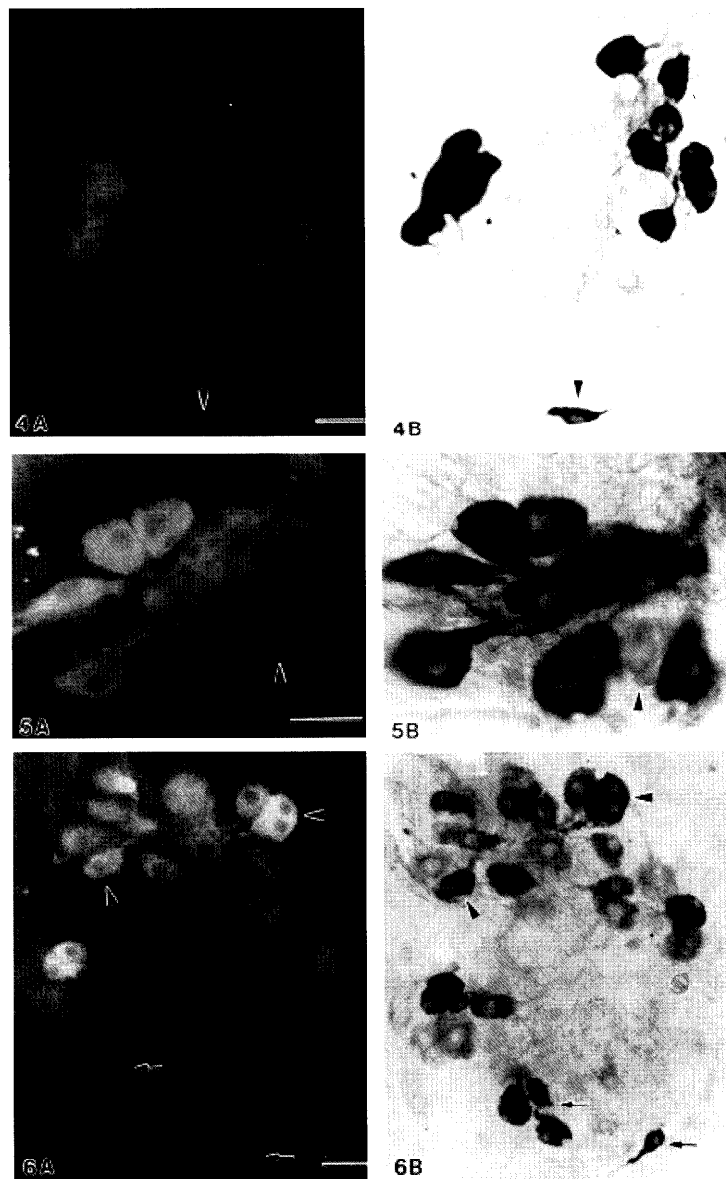


Fig. 4(A, B). Fluorescence (A) and bright-field (B) photomicrographs Of same ganglia in the bladder base labelled for NOS and NADPH-d, respectively. Note majority of the neurons co-express NOS immunoreactivity and NADPH-d reactivity. Some NADPH-d positive neurons, however, are NOS negative (arrowheads). Bar, 50 μ m.
 Fig. 5(A,B). Ganglia stained for NSE (A) and NADPH-d (B), respectively. Note that one neuron (A, arrowhead) in the ganglion is NADPH-d negative (B, arrowhead). Bar, 50 μ m.
 Fig. 6(A, B). Fluorescence (A) and bright-field(B) photomicrographs of the same ganglion in the bladder base labelled for NOS (A) and AChE (B), respectively. Most neurons are double labelled with NOS and AChE (A, arrowheads) with some cells showing only AChE reactivity (B, arrows) Bar, 50 μ m.

were also stained for NADPH-d (Fig. 5B). Some NSE positive neurons were unstained for NADPH-d (Fig. 5A,B).

2.3 Colocalization of NOS and AChE

The majority of the intramural ganglion cells were stained for AChE, and of these, 51.4% of them co-expressed NOS immunoreactivity (Fig. 6A,B, Table II).

2.4 Localization of TH and NADPH-d

Although TH immunoreactivity and NADPH-d reactivity occurred in cells of the same ganglion, the two enzymes were always localized in separate neuronal populations (Fig. 7A,B). Often, TH immunofluorescent fibres appeared to surround the NADPH-d positive neuronal somata.

2.5 Colocalization of VIP and NADPH-d

VIP was also colocalized with NADPH-d in some intramural neurons and fibres (Fig. 8A,B). Cell counts revealed that about 21.3% of the NADPH-d reactive cells co-expressed VIP immunoreactivity (Table II).

2.6 Colocalization CGRP and NADPH-d

The fibres stained for CGRP appeared to project into NADPH-d positive ganglia and surrounded the NADPH-d positive cell bodies (Fig. 9A,B). Colocalization of NADPH-d and CGRP was seen in some segments of the nerve fibres (Fig. 9A,B).

2.7 Colocalization of SP and NADPH-d

SP positive fibres were also closely associated with NADPH-d reactive cell bodies (Fig. 10A,B). The SP positive fibres, often beaded, tended to occur in bundles linking the ganglion cells. Some fibres which exhibited colocalization of NADPH-d were present between ganglion cells.

3. Alteration Of NADPH-d Reactivity in the Intramural Ganglia Following Urethral Obstruction

3.1 Light Microscopy

In sham-operated control animals, NADPH-d reactivity in the intramural ganglion cells of the urinary bladder was comparable to that of normal animals (Fig. 11A).

At 12 hours after urethral obstruction, the overall NADPH-d reactivity in the intramural ganglia was enhanced (Fig. 11B). The frequency of intensely stained NADPH-d neurons in this group was significantly higher than that in the control animals ($P < 0.01$, Table III). The neurites of some cells were grossly dilated (Fig. 11B).

At 24 hours after urethral ligation, some NADPH-d positive neurons notably the intensely stained cells appeared to undergo degenerative changes (Fig. 11C). The outlines of these cells became extremely irregular (Fig. 11C) due to eruptions of cytoplasm from the cell surface.

The frequency of intensely stained NADPH-d positive neurons in this group was sustained at a higher level than in the controls (Table III).

The most dramatic feature in animals sacrificed at 48 hours postoperatively was the wide occurrence of neurons in advanced stages of degeneration in different parts of the bladder (Fig. 11D). Cellular debris or fragments were strewn among seemingly normal ganglion cells and along the nerve processes (Fig. 11D). Cell counts showed that the total number of NADPH-d positive neurons in the entire bladder was significantly reduced when compared with that in the control animals (Table III). The proportion of intensely stained NADPH-d positive neurons was relatively lower than that in the controls (Table III).

3.2 Electron Microscopy

With NADPH-d staining, the reaction product was deposited on the membranes of various subcellular organelles including certain segments of the nuclear envelop, the rough endoplasmic reticulum, Golgi saccules and outer membranes of some mitochondria (Fig. 12).

At 12 hours after urethral ligation, more NADPH-d reaction product was present in the intramural neurons and it tended to be associated with mitochondria which appeared swollen.

At 24 hours after urethral obstruction, mitochondrial swelling was more pronounced affecting virtually every one of them. The Golgi saccules appeared disorganized.

At 48 hours, most mitochondria appeared vacuolated and lysis as a result of disruption of their cristae. The Golgi saccules were extremely distorted. On the other hand, the cisternae of rough endoplasmic reticulum

TABLE III: NUMBER (MEAN $\pm$ SD) AND PERCENTAGE (IN PARENTHESIS) OF INTENSELY STAINED NADPH-d POSITIVE INTRAMURAL NEURONS AGAINST TOTAL POPULATION OF NADPH-d POSITIVE INTRAMURAL NEURONS IN THE URINARY BLADDER IN CONTROL AND URETHRA-OBSTRUCTED ANIMALS

Groups of animals	n	Number and percentage of intensely stained NADPH-d neurons	Total number of NADPH-d neurons
Control	8	884 $\pm$ 142 (32.2%)	2743 $\pm$ 221
12 hours after operation	4	1462 $\pm$ 232 (46.9%)**	2934 $\pm$ 335
24 hours after operation	4	1136 $\pm$ 146 (40.1%)**	2834 $\pm$ 256
48 hours after operation	4	426 $\pm$ 156 (22.9%)**	1858 $\pm$ 347*

* $P < 0.05$ (ANOVA test), ** $P < 0.01$ (U-test)

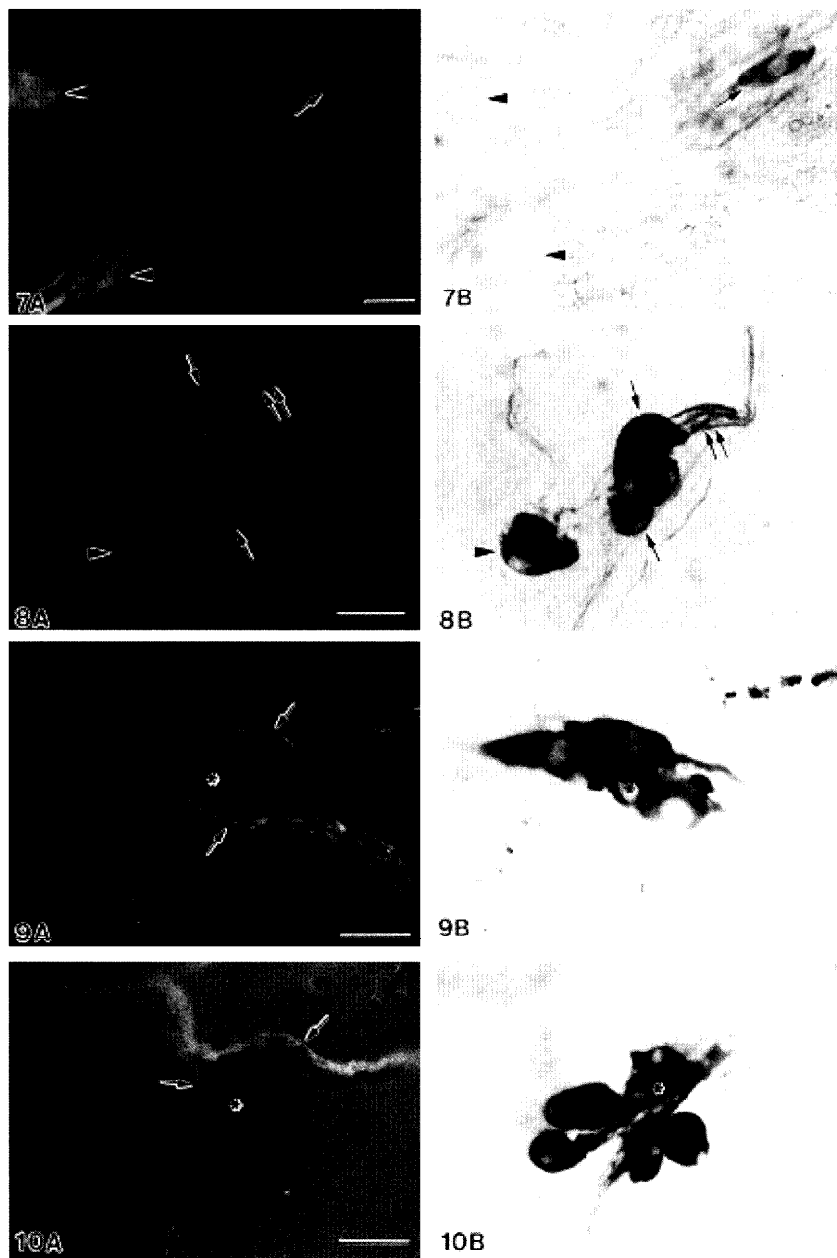


Fig. 7(A, B). Fluorescence (A) and bright-field (B) photomicrographs of the same ganglion labelled for TH (immunofluorescence, arrowheads) and NADPH-d (darkly labelled, arrows), respectively in the bladder base. A is taken by simultaneous illumination of incident fluorescent light and lower-level transmitted white light. Note that TH immunofluorescent neurons (A, arrowheads) represent a separate cell population from the NADPH-d positive cells (B, arrow) in the same ganglion. Bar, 50 μ m.

Fig. 8(A, B). Double staining of VIP (A) and NADPH-d (B) in the same ganglion in the bladder base. Three of the neurons (A, B, arrows) are double stained for VIP (immunofluorescence) and NADPH-d (dark staining). One NADPH-d positive neuron is VIP negative (A, B, arrowheads). Note some nerve fibres also co-express VIP and NADPH-d (double arrows). Bar, 50 μ m.

Fig. 9(A, B). Double labelling of CGRP (A) and NADPH-d (B) in the same intramural ganglion in the bladder base. CGRP immunofluorescence is localized only in the nerve fibres (A, arrows) which appear to encircle the NADPH-d positive ganglion (A, B, asterisks). Bar, 50 μ m.

Fig. 10(A, B). Double labelling of SP (A) and NADPH-d (B) in the same intramural ganglion in the bladder base. Note that some SP immunofluorescent fibres (A, arrows) appear to surround the NADPH-d reactive neuronal somata (A, B, asterisks). Bar, 50 μ m.

remained relatively intact (Fig. 13). Degenerative changes such as clumping of synaptic vesicles and mitochondrial swelling were also seen in axon terminals near the neuronal somata (Fig. 14).

Discussion

1. Topographic Distribution and Population of NADPH-d

and NOS Positive Neurons

The intramural ganglia embedded in the bladder wall have been considered to constitute part of the pelvic ganglia.³⁰ In the latter, it has been described that both the sympathetic and parasympathetic information is integrated for regulating the function of the bladder.³⁰ The intramural ganglia show considerable species variation,

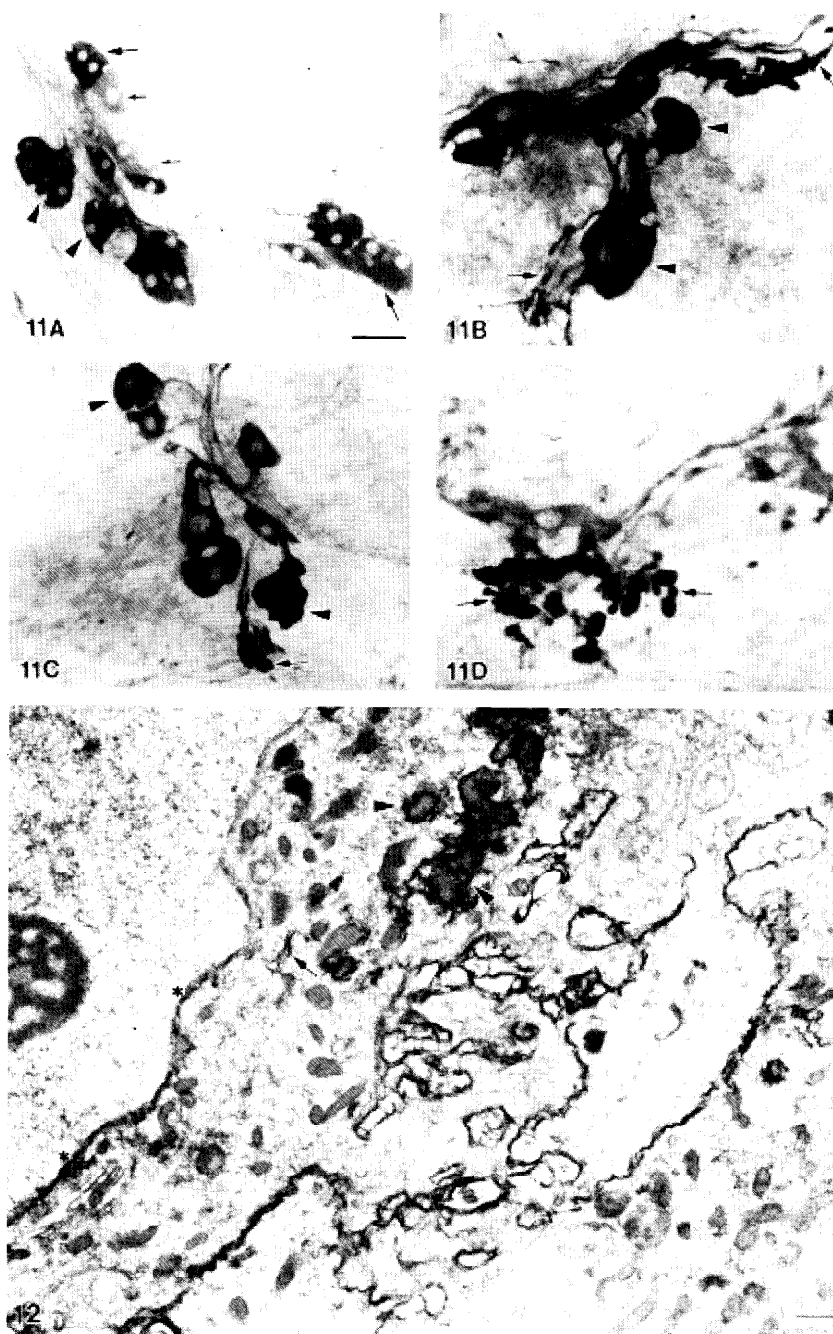


Fig. 11 (A, B, C, D). Intramural ganglia in the bladder base of sham-operated control animals (A). Note that some neurons are intensely stained (arrowheads), others are moderately labelled (arrows) or weakly stained (small arrows).

Intramural ganglia in the bladder base of urethral obstructed animals 12 hours after operation (B). Majority of the containing cells exhibit intense staining for NADPH-d (B, arrowheads). The neurites of some neurons are markedly diluted (B, arrows).

Intramural ganglia in the bladder base of urethral obstructed animals 24 hours after operation (C). The intramural ganglion cells appear to undergo degenerative changes as evidenced by the fragmentation of the cytoplasm. A portion of the cytoplasm appears to have detached from the soma of a neuron (C, arrow). Two other cells display irregular outlines (C, arrowheads).

Intramural ganglia in the bladder base of urethral obstructed animals 48 hours after operation (D). Showing total disintegration and lysis of intensely stained NADPH-d neurons (D, arrows). Bar, 50 μ m.

Fig. 12. Electron micrograph showing portions of NADPH-d reactive intramural neurons in the bladder base of sham-operated animal. The NADPH-d reaction product is deposited on the membrane of rough endoplasmic reticulum (arrow), mitochondria (arrowheads) and some segments of the nuclear envelope (asterisks). Bar, 1 μ m.

being numerous in the guinea pig,²⁹ fewer in cats, rabbits, ferrets and absent in rats and mice.³⁰ Their existence in the human bladder has also been reported.³¹ The intramural nervous control of the urinary bladder is

more complex than has been hitherto assumed.³² In addition to the classical transmitters noradrenaline and acetylcholine, numerous neuromodulators including VIP, ENK, SOM, CGRP, SP and, more recently, NO have

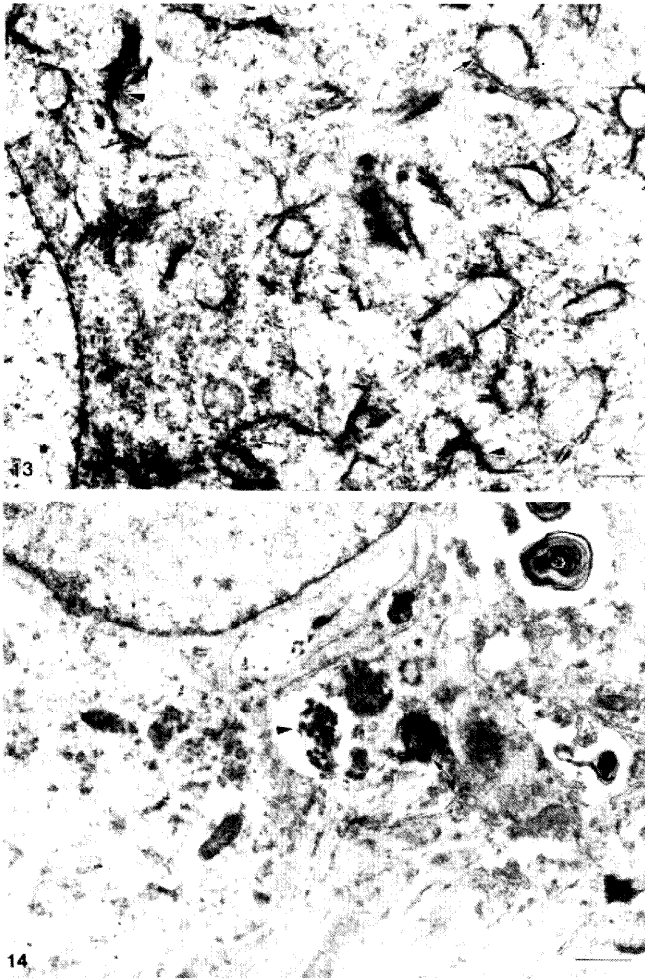


Fig. 13. Electron micrograph showing portion of an NADPH-d reactive intramural neuron in the bladder base 48 hours after urethral obstruction. Virtually all mitochondria (arrows) appear vacuolated. The Golgi saccules (arrowheads) appear disorganized. Bar, 1 μ m.

Fig. 14. Electron micrograph showing degenerative changes in an axon and its varicosities. Note the clumping of synaptic vesicles (arrowhead) and contents of dense inclusions (arrow) and myelinated figure (asterisks). Bar, 1 μ m.

been identified in nerves supplying the bladder and urethra.^{12,32}

Just as in the neonatal guinea pig urinary bladder,¹² present results have revealed that NADPH-d reactive neurons were the most prevalent neuronal subpopulation in the urinary bladder of adult guinea pig. Besides NADPH-d, NOS immunoreactivity was also localized in the intramural ganglion cells in the wholemount preparations. Since the majority of the NADPH-d or NOS positive neurons were concentrated in the bladder base, it is suggested that nitric oxide might be involved in the relaxation activity in the base during micturition. This would be consistent with recent pharmacological studies which demonstrated that NO was a mediator for the neurogenic dilation of the bladder neck during the micturition reflex in different species.⁷⁻⁹

The total number of intramural ganglion cells of the guinea pig urinary bladder has been studied using NADH diaphorase which showed the presence of 2000 to 2500 neurons in the female guinea pig bladder.²⁹ In the present study, the total number of neurons was estimated by NSE immunohistochemistry. NSE is an isoenzyme of the glycolytic enzyme enolase which has been found to be localized specifically within neuronal perikarya, dendrites and axons of both the central and peripheral nervous systems, as well as in cells of the diffuse neuroendocrine system.^{33,34} Its presence indicates the expression of γ gene which has been identified in all nerve cells.³³ NSE has therefore been used as a molecular marker for specific localization of neurons.^{35,36} With this labelling method, the present cell counting has shown that the total number of intramural ganglion cells exceeds that estimated by Gabella²⁹ using NADH diaphorase histochemistry also on the wholemount preparations of the bladder. It has been reported that neuronal population in the pelvic plexus between male and female differs and this, in part, may be attributed to sex difference in the function of the bladder neck and urethra.³⁷ The same explanation may be offered to explain the difference between the total neuronal population in the male guinea pig urinary bladder in the present study and that of Gabella²⁹ using female animals. Another possibility would be that the labelled cells stained by NADH-d histochemistry do not reflect a full neuronal population of the bladder.

2. Correlation Between NADPH-d and NOS

The present double labelling study has revealed that 70.9% of the NADPH-d reactive intramural ganglion cells co-expressed NOS immunoreactivity. The proportion of NOS/NADPH-d double-labelled neurons was higher than that obtained from separate staining for these two enzymes (NOS = 1470 ± 221 ; NADPH-d = 2750 ± 150 , NOS/NADPH-d = 54%) in the whole bladder. One possible explanation for this may be that prior labelling of NOS immunoreactivity had partially quenched the expression of NADPH-d reactivity in the double labelling study.

The lack of total coincidence of the two enzymes may be attributed to the fact that NOS may occur in different isoforms.³⁸ It has been generally accepted that NADPH-d demonstrated histochemically in fixed brain tissue is a marker for NOS.³⁹ This is supported by the colocalization of NADPH-d and NOS immunoreactivity in the peripheral nervous system.^{12,40} On the other hand, there are examples in some parts of the nervous system where the two enzymes do not co-exist.^{41,42} For example, NADPH-d has been demonstrated in L6 and S1 dorsal root ganglia innervating the bladder of the rat, but this is not paralleled by NOS immunoreactivity.⁴³ Lack of immunoreactivity to NOS has also been observed in some

NADPH-d positive intrinsic neurons of guinea pig heart⁴⁴ and in NADPH-d positive cultured myenteric neurons of newborn guinea pig caecum.⁴⁵ Dawson et al³⁹ described NADPH-d activity in the adrenal cortex without coincident localization of NOS-immunoreactivity. It is known that NOS contains the amino acid sequences and cofactor binding sites necessary to confer diaphorase activity.³⁹ There are, however, enzymes with diaphorase activity that do not synthesize NO,⁴⁶ an example being the cytochrome P-450 reductase. Matsumoto et al⁴⁶ have reported that there is a low correlation between brain areas rich in NADPH-d activity and NOS immunoreactivity in fresh brain tissue. Fixation reduces much of NADPH-d activity, resulting in a much greater correlation between the two enzymatic activities in different brain regions. Moreover, the type of fixative and the degree of fixation affect the magnitude of the histochemical staining.⁴⁷ It was speculated that neurons with NADPH-d activity that do not stain for NOS probably never produced NO and the diaphorase activity is associated with another enzyme that was not affected by fixation.⁴⁸ These results together with the present study indicate that while NADPH-d and NOS may be colocalized in some sites of nervous tissue, a one-to-one correlation between these two enzymes does not always exist. In other words, the NOS immunoreactive cells observed represent probably only a subpopulation of NADPH-d neurons. In the present study, since the majority of the NADPH-d reactive neurons co-expressed NOS, the former enzyme is still considered to be a useful marker for NOS.

3. Colocalization of NOS and Other Neurotransmitters

i) NOS and AChE

Specifically, anti-choline acetyltransferase (ChAT, the enzyme responsible for the synthesis of ACh) has been the antiserum of choice for localization of ACh.^{49,50} However, preliminary study by us in localizing ChAT immunoreactivity in the urinary bladder has not been successful thus confirming the finding of others.⁵¹ In view of this, AChE histochemistry as adopted by others^{29,32} instead has been used for present detection of sites of ACh activity. Although the reliability of AChE as a cholinergic marker has sometimes been questioned, it is nonetheless a useful marker for cholinergic neurons in the autonomic ganglia.⁵² Present results have revealed the wide occurrence of AChE positive neurons notably in the bladder base where the largest ganglia were distributed. The number of AChE positive neurons in the bladder base was comparable to NADPH-d positive cells, but in the bladder body and dome, it was significantly higher. This suggests that ACh is not only the major neurotransmitter in the bladder base, but it also plays a major role in the bladder body and dome. Present results also revealed that the total number of AChE

positive neurons in the whole bladder was about 3110, contributing to about 89% of the total neuronal population (3260 ± 421) as estimated earlier by using NSE as the neuronal marker. This is consistent with results of other studies^{29,32} which have shown over 80% of the intramural neurons expressed AChE reactivity. In view of their cholinergic nature, it is suggested that the majority of the intramural ganglion cells in the urinary bladder are parasympathetic postganglionic neurons.

ii) TH and NADPH-d

The present study has revealed the lack of colocalization of TH and NADPH-d suggesting that the TH positive neurons in the urinary bladder represent a separate neuronal population. This invites speculation that the sympathetic postganglionic neurons are not capable of producing NO. TH is an enzyme responsible for the synthesis of catecholamine (CA).⁵³ The latter is known to be a sympathetic nerve mediator that serves to maintain closure of the bladder outlet and internal urethral sphincter.³⁰ CA-containing nerves have been detected in the bladder base and some regions of the urethral smooth muscle in a variety of species including human.^{54,55} In agreement with previous studies, present results also showed that TH reactive neurons were distributed mainly in the bladder base especially in the trigone area. This supports that TH plays a role sympathetically in closure of the bladder neck and urethra.^{30,56}

iii) VIP and NADPH-d

VIP has been implicated to act as a co-transmitter with ACh in parasympathetic nerves.⁵² In the urinary bladder, VIP may function as a neuro-modulator as it causes relaxant responses to the detrusor, trigone and neck.^{57,58} The distribution of VIP immunoreactivity in the bladder of human is much the same as that in animals such as the guinea pig, cat, and pig with a denser innervation at the bladder base than that in the body.^{55,59,60} It has been shown that VIP immunoreactivity was detected in 10% to 15% of the bladder ganglion cells that are stained heavily for AChE.⁵² Thus, it was suggested that VIP was very likely colocalized with ACh in some bladder ganglion cells.^{52,61} Although NADPH-d and VIP were co-expressed in some of the neurons in pancreas,⁶² intestine,⁶³ gall bladder⁶⁴ and vas deferens⁶⁵ in various species, their functional relationship has remained uncertain in the urinary bladder. The present study has demonstrated that some of the VIP immunoreactive intramural neurons and fibres also expressed NADPH-d reactivity. Taken together with results of previous studies,^{52,61} it is suggested that VIP may be released together with NO from the parasympathetic postganglionic neurons to act as neuromodulator coordinating the relaxation of smooth muscle in the bladder base or urethra.

iv) CGRP, SP and NADPH-d

The present study has shown that in the bladder walls CGRP and SP immunoreactivities were confined exclusively to nerve fibres and some of them were closely associated with the intramural ganglion cells stained for NADPH-d. Both SP and CGRP are known to act as transmitters in sensory nerves.^{30,66} Indeed SP and CGRP immunoreactivities have been localized in the dorsal root ganglion cells identified as supplying the bladder and urethra by retrograde tracing technique.⁶⁷ Further evidence of the sensory origin of CGRP immunoreactive fibres has been gained from the fact that the immunoreactivity was depleted following the removal of dorsal root ganglia at L1, L2, L6 and S1 spinal segments.⁶⁸ The present study has shown the close association of some positively stained CGRP and SP fibres with neuronal somata that expressed NADPH-d reactivity. It is postulated that through such unique configuration NO released from the latter cells may act as a retrograde transmitter exerting its influence on the sensory afferent pathways. Furthermore, it has been described that afferent neurons can release neuropeptides from their peripheral terminals in the viscera.³⁰ Thus, the transmitters released from the sensory nerve endings in the bladder may play an efferent role by direct postjunctional effects on the detrusor muscle.⁶⁶ In the light of the above, it is tempting to speculate that CGRP and SP may also be released from the sensory terminals in the bladder walls to reciprocally influence the NADPH-d positive intramural ganglion cells in the urinary bladder.

4. Alteration Of NADPH-d Reactivity in the Intramural Ganglia Following Complete Urethral Obstruction

Acute urinary retention is a common problem in urologic patients.⁶⁹ For example, it has been reported that in 212 cases with prostatic obstruction 42% of the patients presented with acute urinary retention.⁶⁹ Experimental study has shown that urinary retention induced by acute urethral ligation could lead to a 47% reduction in muscarinic receptor density and 75% decrease in the contractile responses to muscarinic stimulation and field stimulation in rabbits.²⁴ It was speculated that the neural alterations elicited by acute urinary retention might be attributed to hypoxia as a result of reduced blood flow as well as mechanical damage due to high intravesical pressure.²⁴ Moreover, the presence of inflammation, ischaemic damage and edema in the overdistended bladder might facilitate such neural alterations during the initial period of the urethral obstruction.²⁴ Although this and the present animal model, which conceivably would result in urinary retention, are different from the human bladder outlet obstruction which is a slow and gradual process, it has been reported that neural alterations could occur in the initial period of urinary retention.²⁴ Clinically, Gosling et al²² have shown 56 per cent reduction in the autonomic innervation of the

urinary bladder in patients with bladder outlet obstruction. The significant denervation following outlet obstruction may markedly impair the neuromuscular control of the bladder²² and is usually associated with the detrusor instability.²¹ The latter which is manifested by frequency and urgency of micturition is often seen in patients with bladder outlet obstruction.^{20,21,30} It may be alleviated once the urethral obstruction is relieved but persists in approximately one-third of the patients.⁷⁰

The present experimental study has revealed that a sizeable number of the intramural neurons displayed signs of degenerative changes in the early stage after urethral obstruction in the guinea pig. Many neurons underwent complete disintegration and total lysis as demonstrated by the wholemount preparations. The earliest degenerative changes seemed to affect the mitochondria which appeared swollen as detected ultrastructurally at 12 hours after urethral obstruction. The degenerative alteration was progressive so that by 48 hours, almost all mitochondria underwent total vacuolation and lysis, a characteristic feature in neuronal degeneration.⁷¹

A striking feature in the present study was the increased NADPH-d expression in the intramural ganglion cells within 12 hours after urethral obstruction. This was sustained until 24 hours accompanied by neuronal degenerative changes and consequent cell death. At 48 hours, the total number NADPH-d positive neurons in the urinary bladder was significantly decreased (32%). One possible explanation for the marked reduction may be that some neurons had ceased to express NADPH-d reactivity after urethral obstruction. On the other hand, present morphological results seem to suggest that extensive and irreversible neuronal damage would have accounted for the major cell loss. In regard to the alterations in the NADPH-d expression, recent studies have shown the considerable plasticity of neuronal NADPH-d in different pathological events. For example, increased NADPH-d activity was observed in the lumbar spinal cord neurons after inflammation of the rat hindpaw,¹³ in the cortical pyramidal neurons after stab lesions in the rat,⁷² in rat vagal motor neurons after axotomy⁷³ and in the lumbar spinal cord in rats following rhizotomy.⁷⁴ It has been suggested that the upregulated NADPH-d expression after nerve lesion was related to both neurodegenerative and neuroregenerative effects.^{72,75} In this connection it has been reported that small amounts of NO might be beneficial, whereas larger amounts of NO might be detrimental as it is known to mediate neuronal death, e.g. in excitotoxic and hypoxic insults.⁷⁵ Present results suggest that the upregulated NADPH-d expression after urethral ligation may be attributed to hypoxia and inflammation in the overdistended bladder. To this end, the increased NADPH-d expression in the intramural gan-

glion cells after acute urethral obstruction may be an harbinger to consequent neuronal degeneration and cell death as this might indicate elevated NOS expression and, hence, greater NO production. Activation of neuronal NOS, however, is calcium dependent.⁷⁶ In the absence of evidence of Ca⁺⁺ influx into the intramural neurons after urethral ligation, the above remains purely speculative as the cells may produce normal levels of NO.

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