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Is Nitric Oxide Involved in Relaxation of Urinary Bladder?

Ki Churl CHANG[1]* and Byung Ha CHUNG[2]

[1]Department of Pharmacology, Cardiovascular Research Institute, College of Medicine, Gyeongsang National University, Chinju 660-280 [2]Department of Urology College of Medicine, Yonsei University, Seoul, 120-752, Korea

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Abstract—We investigated whether nitric oxide (NO) may serve a role in bladder function by immunohistochemical analysis of the distribution of intrinsic NADPH-diaphorase and functional study of isometric tension recordings via a photo-induced adequate nitric oxide (PIANO) generating system using rat bladder. Results suggest that a small number of NADPH-diaphorase-positive perikarya are present within the bladder wall and within adjacent small ganglia. Furthermore, NADPH-diaphorase-positive nerve fibers were observed in the adventitial and muscular layers, subjacent to the urothelium and perivascular fibers. Rat bladder strips precontracted with 3 μM carbachol were reversibly relaxed upon NO generation by UV irradiation. PIANO-mediated relaxation was sensitive to oxygen free radicals. In addition, tissue cGMP levels were increased by the PIANO generating system and elevated cGMP levels were decreased by pretreatment of guanylate cyclase inhibitor, methylene blue. These results indicate that NO may serve a role in modulating bladder tone in the rat.

Keywords □ nitirc oxide, rat bladder, NADPH diaphorase, photorelaxation

L-ARG/NO pathway has now been recognized in various organs and is an important and ubiquitous effector system in the regulation of a diverse set of physiological processes (Moncada *et al.*, 1991; Ignarro, 1990; Stuehr and Griffith, 1992). Hope *et al.*, (1991) demonstrated that neuronal NADPH-diaphorase is identical to NO synthase and NOS is the enzyme responsible for synthesis of NO. Others have tested whether NO mediates relaxation in bladder, but so far the results are conflicting (Klarskov, 1987 ; James *et al.*, 1993; Persson and Andersson, 1992). Furthermore, the mechanism underlying the bladder relaxation during filling is not fully understood. We proposed the photo-induced adequate nitric oxide (PIANO) generating system as another investigational tool to research for the role of NO by exploiting the properties that the NO- and NO_2-carrying molecule are photoactivated to release NO (Chang *et al.*, 1993a; 1993b). The PIANO-mediated relaxation study is effective in the vascular smooth muscle (Chang *et al.*, 1993a, 1993b), trachea (Chang *et al.*, 1993a), and corpus cavernosum (Chung and Chang, 1994) as well as in the uterine smooth muscle (Lee and Chang, 1995). Thus, we investigated whether NO may serve a role in bladder function by characterizing the distribution of intrinsic NADPH-diaphorase and by isometric tension recording via PIANO generating system. An account of some of these results has been published in abstract form (Chung and Chang, 1993).

* To whom correspondence should be addressed.

Materials and Methods

Materials

N^w-nitro-L-arginine (L-NOARG), streptozotocin (STZ), sodium dihydrogen phosphate were purchased from Sigma Chemical Co (St. Louis MO).

General Procedures

Male Sprague-Dawley rats (15 to 18 weeks, 350~400 g) were anesthetized with ketamine (0.75 mg/kg, i.p) and xylazin (0.1 mg/kg), and perfused transcardinally with 300 ml of 0.9% saline followed by 300 ml of 2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.2) at room temperature. Following the perfusion, bladder was transacted at its junction with the external urethral sphincter, placed in fresh fixative for 4 hrs at 4℃.

Histochemical analysis

To demonstrate sites of NO synthase, we employed

the NADPH-diaphorase histochemical technique (Schmidt *et al.*, 1992), which strains NOS containing nerve cells and fibers. The specimens were cut into small blocks, washed in PB, and saturated overnight at 4℃ with 25% sucrose in PB. Blocks were frozen in liquid nitrogen and then serially sectioned (8 μM) using cryostat. Sections were thawed onto gelatinized slides and allowed to air dry. Sites of NO synthesis in neuronal perikarya and fibers were demonstrated with the NADPH-diaphorase histochemical technique. The slide-mounted sections of bladder were hydrated in 0.1 M PB, pH 7.4 for 10 min, then incubated in a solution containing 1.0 mg/ml β-NADPH, 0.1 mg/ml nitroblue tetrazolium, 0.2% Triton X-100 in PB for 30 min at 37℃. The reaction was discontinued by multiple washings in PB, the sections were counterstained with methyl green and coverslipped with buffered glycerol and viewerd using a microscope.

Isometric tension study

The bladder was removed and cleaned of adhering fat and connective tissue, and strips (2×2×10 mm) were prepared. Each strip was mounted in a 10 ml water jacketed muscle chamber containing 37℃ modified Krebs-Ringer bicarbonate solution which was gassed with 95% O_2/1/5% CO_2 and had the following composition (mM): NaCl (136.9), KCl (5.4), $MgCl_2$ (1.0), $NaHCO_3$ (23.8), $CaCl_2$ (1.5), glucose (5.5) and EDTA (0.03). The tissues were equilibrated at 1 g tension for more than 90 min, with washing at 20 min intervals, prior to drug addition. Isometric tension was recorded on a Grass physiograph (model 7E) using a force displacement transducer (FT-03). After reaching a plateau of contraction, tissues were exposed to UV light (1~60 sec) using a long wavelength UV lamp (366 nm, Mineralight UV GL 58, San Gabriel, CA) in the presence or absence of test substances.

Nitrite determination

In a series of test tubes containing 10 mM (for UV lamp only) or 0.1 mM of STZ was exposed to UV light from 10 to 60 min with different light energy level sources. At the indicated time, nitrite production was quantified by the methods of Stuehr and Griffith (1992), using a Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4). Nitrite concentrations were calculated from a standard curve using sodium nitrite as the standard.

Cyclic GMP determination

Cyclic GMP levels were measured in detrusor strips that had been equilibrated under tension and subjected to precontraction with carbachol. Strips were quickly frozen with the aid of brass clamps precooled in liquid nitrogen after exposing UV light for 15 sec in the presence of STZ and/or methylene blue. Samples were extracted and assayed for cGMP by radioimmunoassay as described (Chang *et al.*, 1992).

Statistics

Data were expressed as mean ± SEM. Differences between two groups were determined by Student's t test and were considered significantly different if $P<0.05$. Drug concentrations were expressed as a final negative log molar concentration.

Results

Histological staining of NADPH-diaphorase positive cells.

In the cryostat sections of rat urinary bladder, a discrete population of NADPH-diaphorase positive perikarya were observed embedded within the detrusor muscle. In addition, some NADPH-diaphorase-positive perikarya were located within small ganglia subjacent to the bladder adventitia and perivascular plexus (Fig. 1). NADPH-diaphorase-positive nerve fibers were frequently observed in small nerves and as individual fibers within the bundles of detrusor muscle fibers (Fig. 1). When incubated in the absence of substrate, NADPH-diaphorase reactivity was not observed, and under positive control, rat brain was stained positively for NADPH-diaphorase (data not shown).

PIANO-mediated relaxation

Rat detrusor did not relax in response to UV light alone. However, after STZ (0.1 mM) and N-NOARG (0.3 mM) treatment, rat bladder strips precontracted

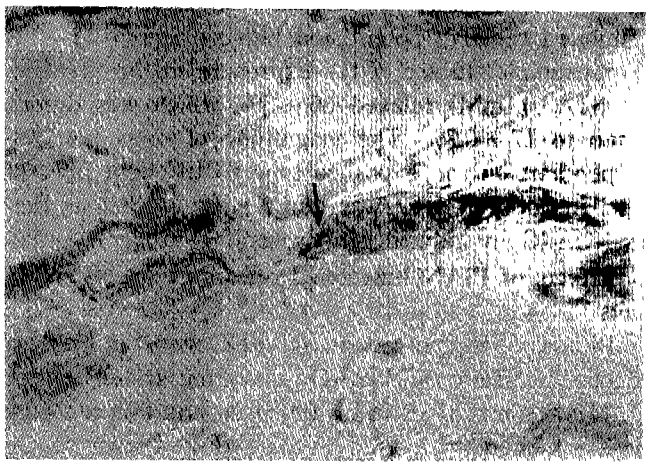

Fig. 1. Distribution of NADPH-diaphorase nerve fibers within the rat bladder. Several fine varicosities of NADPH-diaphorase-positive nerve fibers (arrowheads) are observed within the detrusor muscle (200).

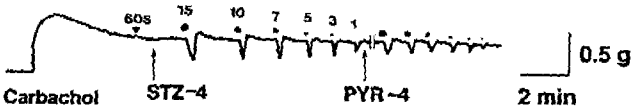

Fig. 2. Typical tracings of NO-mediated relaxation in rat detrusor strips that was precontracted with carbachol (3 μM). NO was generated by photoactivating of streptozotocin (STZ). Oxygen free radical generating agents (PYR) inhibted NO-mediated relaxation. Arabic numbers indicated the time of UV irradiation (second). // indicates time laps for 20 min.

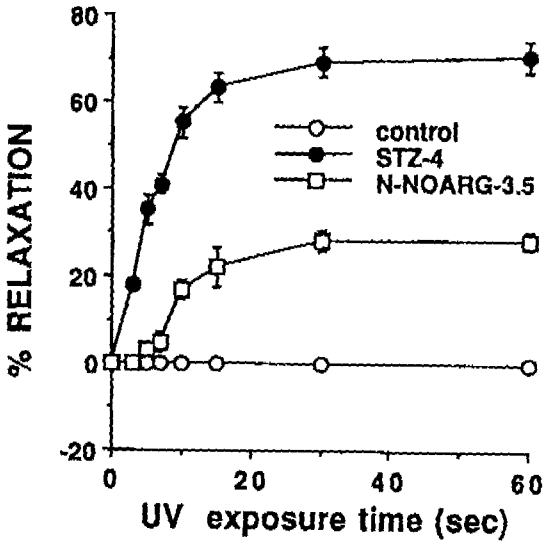

Fig. 3. Effects of PIANO-mediated relaxation by different photosensitizers (STZ, L-NOARG) in rat detrusor. Each point represents mean ± SEM of 6 different experiments.

with 3 μM carbachol were reversibly relaxed upon UV irradiation. Therefore, STZ and N-NOARG sensitized UV light-induced relaxation of rat bladder and pyrogallol (PYR, 0.1 mM), O_2 generator, significantly ($P<0.05$) inhibited the STZ-potentiated photorelaxation (Fig. 2). For example, 15 sec of UV exprosure in the presence of STZ (0.1mM) relaxed 56 ± 3%, which was diminished to 17 ± 0.7%. The magnitude of relaxation was dependent on the exposure time to UV light until 20 sec. In addition, STZ was much more efficacious than N-NOARG in inducing the relaxation (Fig. 3).

Effects of PIANO-mediated relaxation on cGMP levels

Different concentrations of STZ were utilized as photosensitizers, the PIANO generating system caused cGMP accumulations in rat detrusor, and this response was inhibited by methylene blue (Fig. 4), which also inhibited the relaxant response (data not shown).

Effect of different types of light energy on the accumulation of nitrite from STZ

As shown in Fig. 5, nitrite, a spontaneous oxidation

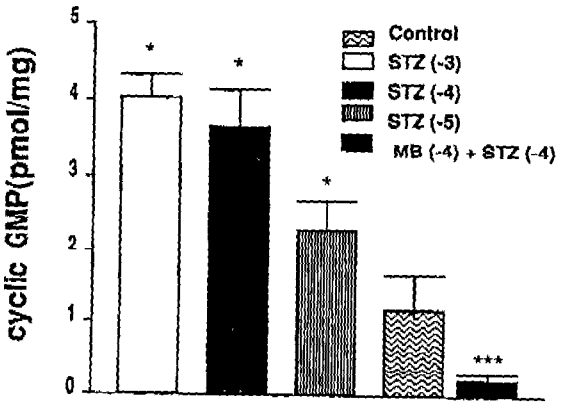

Fig. 4. Effect of photolysis of different concentrations of STZ for 15 sec on cGMP levels in rat detrusor strips and inhibitory effect of MB. *indicates significantly different ($P<0.05$) from control and ***represents significantly different ($P<0.001$) from corresponding control, STZ (-4). The numbers in the parenthesis indicate the log molar concentrations of drug.

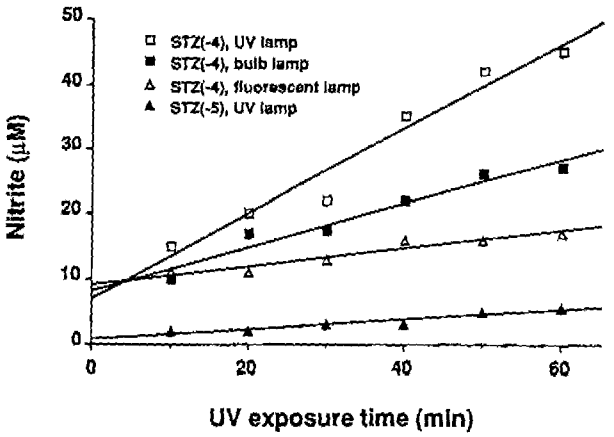

Fig. 5. Light energy- and exposure time-dependent accumulation of nitrite by photolysis of STZ.

product of NO, has been accumulated by photoactivation of STZ. A greater amount of nitrite was accumulated by high energy levels of UV exposure rather than lower energy levels. Even though the same energy level of light used, nitrite was accumulated more by the exposure time dependently.

Discussion

While numerous studies have examined the effects of cholinergic, adrenergic, purinergic and peptidergic agents on bladder contraction (De Groat and Steers, 1972), recent studies implicate NO as an important transmitter or messenger molecule in autonomic neurotransmission (Buga and Ignarro, 1992; Busch *et al.*, 1992). The NANC-nerve mediated relaxation, involving the L-ARG/NO pathway, can be demonstrated (Klars-

kov, 1987; James *et al.*, 1993) but not (Persson and Andersson, 1992) in detrusor smooth muscle. The present experiments were performed to discover whether NO is involved in urinary bladder function in the rat. In order to discover this, we used morphological and physiological methods. Our results indicate there is a small population of NADPH-diaphorase neuronal perikarya that are intrinsic for the detrusor muscle and located within small ganglia subjacent to the bladder adventitia. Individual NADPH-diaphorase-positive perikarya did not demonstrate a prediction for a particular region of the bladder, but were evenly distributed throughout the detrusor muscle. In recent years it has been demonstrated that NANC-nerve mediated relaxation in gastrointestinal smooth muscle and penile erection is through a L-ARG/NO pathway, which is identical to NO (Busch *et al.*, 1992; Li and Rand, 1990; Ignarro, 1992). Further, neuronal NADPH-diaphorase is identical to NO synthase and NOS is the enzyme responsible for synthesis of NO (Hope, 1991). NADPH-diaphorase-positive nerve fibers were evident throughout the bladder, but were generally more abundant near the bladder base. If NO serves a physiological role in the rat bladder, it relaxes the detrusor smooth muscle, and the relaxation pattern, i.e., rate of relaxation, duration etc. are similar to the pattern of authentic NO in other smooth muscles. To test the effect of NO, a PIANO-mediated relaxation was used. Since NO is liable and has very short half life, PIANO method may good to study muscle kinetics with regards to NO. This method is indeed effective to study NO-mediated relaxation in various kinds of smooth muscles such as vascular (Chang et al., 1993a, 1993b), trachea (Chang *et al*,., 1993a), uterus (Lee and Chang, in press) and corpus cavernosum (Chung and Chang, 1994). As shown in Fig. 5, a greater amount of nitrite, stable NO product, was accumulated by high energy levels of light exposure. This finding is consistent with others that released NO is depending on the distance from the light source (Chen and Gillis, 1993). Thus, NO generated from the photolysis of STZ may be responsible for the bladder relaxation in the present experiment. Since L-ARG/NO/cGMP system is an effective regulatory function in muscle relaxation (Moncada *et al.*, 1991; Ignarro, 1990; Stuehr and Griffith, 1992), we further investigated if PIANO generating system activates guanylate cyclase. Tissue cGMP levels were increased by PIANO which was inhibited by the presence of guanylate cyclase inhibitor. It seems likely, therefore, that NO is responsible for relaxation of rat detrusor by increasing cGMP levels. We found that photolysis of STZ (10 mM) for 15 sec coused about 2 folds increase in cGMP contents in bladder tissues, but not nitrite contents in test tubes. At the present time, reason for this different results cant not be adequately explained. However, tissue by itself may have some influence on cGMP levels by light. Because UV light can relax vascular smooth muscles by increasing cGMP levels without photosensitizers (Furchgott *et al.*, 1984).

In conclusion, the present data show that NO synthase-staining cells exists in rat detrusor by demonstrating for NADPH-diaphorase-positive neurons. Transient, rapid and reversible relaxation was observed in the rat bladder strips by PIANO system. This relaxation was accompanied by increments of cGMP and was inhibited by the presence of a guanylate cyclase inhibitor, indicating that NO may serve a functional role in detrusor relaxation.

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