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Effects of Nitric Oxide on Bladder Outlet Obstruction in Rats

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We investigated the changes in bladder function resulting from bladder outlet obstruction (BOO), with particular emphasis on the role of nitric oxide (NO) in bladder response. Twelve-week-old female Wistar rats ($n = 36$) were surgically treated in various ways by dividing them into 6 groups each with 6 animals: 2 sham-operated groups, 2 obstructed groups for 1 week or 6 weeks, and 2 groups obstructed and intraperitoneally injected with 150-mg/kg L-arginine once daily for 1 week or 6 weeks. We conducted bladder strip stimulation studies using carbachol and KCl. Then the bladder was used for measurement of NO synthase (NOS) activity and was also studied histologically using immunohistochemical staining. Bladders in the obstructed groups were significantly heavier than in the sham-operated groups. The mean weight of bladders in the groups obstructed and treated with L-arginine was almost the same as that in the sham-operated groups. Tissue bath studies demonstrated decreased contractility in response to cholinergic stimulation at obstruction, but contractility at obstruction was improved by L-arginine for 1 week or 6 weeks. NOS activity in bladder tissue was lower in the obstructed groups than in the sham groups, and higher in the L-arginine-combined groups than in the obstructed groups. These results indicate that bladder dysfunction may be improved by treatment with L-arginine. This study suggests that increased NO by treatment with L-arginine plays a role in improving bladder dysfunction caused by BOO.

key words: bladder outlet obstruction; nitric oxide; nitric oxide synthase activity; L-arginine

Bladder outlet obstruction (BOO) is one of the most common urological problems in elderly men. An increase in urinary frequency and urgency are typical symptoms in the early stages of outlet obstruction, appearing secondarily to benign prostatic hyperplasia. Despite the high prevalence of BOO, the mechanisms responsible for bladder dysfunction induced by BOO remain poorly understood. In an animal model, bladder weight was significantly increased (Saito et al., 1993a; Berggren and Uvelius, 1998; Kim et al., 2000), and contractile response was significantly decreased by BOO. Some authors have reported that detrusor mitochondrial damage may explain voiding dysfunction after BOO (Lu et al., 2000). Other reports have found that BOO induces reduction in blood flow (ische-

mia), and that ischemia caused by BOO induces bladder dysfunction (Lin et al., 1995; Buttyan et al., 1997).

Recently, several studies have revealed that nitric oxide (NO) is an important neurotransmitter and intracellular messenger that promotes homeostasis in many tissues (Saito and Miyagawa, 1999). One of the most important effects of NO is reported to be its role as a neurotransmitter in non-adrenergic non-cholinergic nerves, and the L-arginine-NO pathway has been shown to play a role in muscle relaxation and vasodilation (Arthur and Burnett, 1995). NO stimulates the formation of guanylate cyclase in smooth muscle cells, converting GTP to 3' 5'-cyclic GMP (cGMP) (Burnett, 1997). A cascade of cGMP-dependent intracellular events

Abbreviations: BOO, bladder outlet obstruction; bNOS, brain NOS; cGMP, 3' 5'-cyclic GMP; ED₅₀, median effective dose; E_{max}, maximum effective dose; eNOS, endothelial NOS; iNOS, inducible NOS; i.p., intraperitoneal; NO, nitric oxide; NOS, NO synthase

then leads to a decrease in intracellular calcium, ultimately causing smooth muscle relaxation and vasodilation, in part through changes in potassium conductance (Seftel et al., 1996; Burnett, 1997). Recently, it has been reported that NO is induced by ischemia injury (Saito and Miyagawa, 1999), and that NO synthase (NOS) isoform activity, that is, inducible NOS (iNOS) activity, was decreased in long-term obstruction for 5 weeks (Lemack et al., 1999).

On the other hand, NOS activity did not show significant changes in short-term obstruction (Shabsigh et al., 2000). These results may suggest that bladder dysfunction caused by BOO is associated with loss of physiological effects of NO.

In this study, we investigated the effect of NO on bladder dysfunction caused by BOO with organ bath techniques. Further, we measured NOS activity in bladder tissue and studied immunohistochemical staining. The aim of this study was to determine whether or not the bladder dysfunction was due to BOO by treatment with L-arginine.

Materials and Methods

Animal model

All animal experiments were performed in accordance with the guidelines set out by the Tottori University Committee for Animal Experimentation. Twelve-week-old female Wistar rats, mean body weight 218 g, were used ($n = 36$) (SLC, Shizuoka, Japan). The 36 rats were anesthetized with intraperitoneal (i.p.) administration of 50-mg/kg sodium pentobarbital. Out of the 36 rats, 24 were used as models for partial obstruction as follows. With the rat in the spinal position after a midline suprapubic incision, the urethra was exposed without damaging the bladder neck and urethra. A 2-0 silk suture was passed behind the urethra and the catheter was put on the urethra. Subsequently, the urethra and catheter were ligated and the catheter was removed. The presence of the catheter ensured that the ligature did not significantly compress the urethra. The outside diameter of each cath-

eter was approximately 1.70 mm for obstruction (mild obstruction) (Saito et al., 1993b). Finally, the incision was closed. The remaining 12 rats were used for the sham operation. Sham-operated rats underwent a similar procedure with a dissection around the urethra, but without a urethral ligature. After the operation, the animals were divided into 6 groups each with 6 rats: Group C-1, 1 week after sham operation; Group C-6, 6 weeks after sham operation; Group OB-1, 1-week obstruction; Group OB-6, 6-week obstruction; Group Ar-1, 1-week obstruction with i.p. administration of 150-mg/kg L-arginine once daily for 1 week and Group Ar-6, 6-week obstruction with i.p. administration of 150-mg/kg L-arginine once daily for 6 weeks. The rats who underwent the operation were anesthetized, and in 1-week-treated rats (Groups C-1, OB-1 and Ar-1), bladders were removed on the 8th day from induction of the operation. In 6-week-treated rats (Groups C-6, OB-6 and Ar-6), bladders were removed on the 43rd day from induction of the operation. The removed bladder specimens were weighed and immediately used for the strip study, measurement of NOS activity and histological examination.

Measurement of contractile response

Each rat was anesthetized with an i.p. injection of 50-mg/kg sodium pentobarbital, and the urinary bladder was removed as low on the urethra as possible. After measuring the bladder weight, the bladder dome was immediately separated from the bladder base at the level of the ureteral orifices, and was separated into longitudinal strips. The muscle strips (5×1.5 mm) were suspended in organ baths containing a 3.0-mL Krebs-Henseleit's solution at 37°C. The tissues were equilibrated with a mixture of 95% O₂ and 5% CO₂. Isometric force was monitored with a TB-612T transducer (Nihon Koden, Tokyo, Japan) and processed by a MacLab multiport controller (AD Instruments Japan, Tokyo). Contractile response to carbachol was expressed as force per cross-sectional area of muscle (grams per square millimeter). Cumulative dose-response curves were constructed in a stepwise manner after the

response to the previous concentration had reached a plateau. Contractile response of the same muscle strips to 100-mmol/L KCl was also monitored.

Muscle NOS activity

Bladder dome tissues were homogenized in homogenization buffer containing phosphate-buffered saline, pH 7.4. Homogenates were centrifuged at 4°C for 20 min at 10,000 × *g*, and the next supernatant solutions were ultracentrifuged at 4°C for 15 min at 100,000 × *g*. Filtration of the 10,000 × *g* supernatant through a 0.45-μ filter prior to ultrafiltration can increase the ultrafiltration rate. Up to 40 μL of a sample was added to each sample well for the microtiter plate, then 10 μL of freshly NADPH solution (1 mmol/L) and 10 μL of nitrate reductase solution were added to each well. After incubating at room temperature for 60 min, 10 μL of the co-factor solution and 10 μL of the lactate dehydrogenase solution were added to each well. This was followed by incubation at room temperature for 20 min, and then a 100-μL Griess reagent was added to each well. Finally, a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to read the absorbance at 540 nm. The values were estimated per tissue weight and per amount of protein in the tissue. Protein was determined using a commercial kit (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL).

Histological examination of the rat bladder

Morphological changes in the rat bladder were studied by light microscopy. The rat bladder dome of each group was immediately removed and fixed with 3.7% formaldehyde-saline. After fixation, the tissues were embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. The serial sections were also subjected to immunohistochemical staining for endothelial NOS (eNOS) and iNOS. In brief, deparaffinized sections were retrieved using DAKO Target Retrieval Solution and a DAKO LSAB Kit (Carpenteria, CA). They were then re-

acted with rabbit anti-eNOS or -iNOS polyclonal antibody (Chemicon, Temecula, CA) at a 1:200 dilution overnight at 4°C. The specifically bound 1st antibodies were visualized by biotinylated horse anti-rabbit secondary antibody (1:200 dilution) with a Histofine SAB-PO (G) Kit (Nichirei Co., Tokyo). Mayer's hematoxylin was used for counterstaining. Negative controls were established for each group according to the procedure mentioned above, but without 2nd antibodies.

Data analysis

Contractile data were calculated as grams of active force per cross-sectional area in millimeters. The cross-sectional area was calculated using the following equation:

$$\text{cross-sectional area} = \text{weight}/(\text{length} \times 1.05),$$

where 1.05 is the assumed density of the muscle. The dose ratio was obtained from the ratio of median effective dose (ED₅₀) values (the concentration of agonist that produces a half-maximal contractile response) for carbachol. ED₅₀ values were calculated as geometric means, whereas maximum effective dose (E_{max}) values were calculated as arithmetic means. NOS activity was calculated as follows:

$$\text{NOS activity} = \{(A540 - b)/m\}(200/40)/60,$$

where *A540* is the sample absorbance at 540 nm, *b* is the y-intercept, *m* is the slope, 200 is the final volume, 40 is the sample volume, and 60 is the NOS reaction incubation time in minutes. Statistical comparison of differences between groups was performed using analysis of variance and Fisher's test for the multiple comparison; *P* < 0.05 was regarded as the level of significance.

Drugs and chemicals

L-arginine, carbachol and pentobarbital were purchased from Sigma (St. Louis, MO). DAKO Target Retrieval Solution and the DAKO LSAB Kit were from DAKO. Polyclonal antibodies against eNOS and iNOS were from Chemicon. The Histofine SAB-PO (G) Kits were from

Nichirei. The NOS activity assay kit was from OXIS International, Inc. (Portland, OR). The BCA Protein Assay Reagent Kit was from Pierce. All other chemicals were of reagent grade.

Results

Bladder weight

Bladder weight was significantly increased by obstruction. For the long-term groups, the mean bladder weight at 6 weeks of obstruction was increased 2-fold compared to that in the sham-operated group. However, the mean bladder weight at 6 weeks of obstruction with treatment by L-arginine was almost the same as that in the sham-operated group. The tendency in the 1-week groups was similar to that in the 6-week groups (Table 1).

Contractile response to carbachol and KCl

Concentration-dependent contractile responses of bladder strips to carbachol are shown in Table 2, together with their E_{\max} and ED_{50} values summarized. At both 1 week and 6 weeks, the obstructed groups showed significantly lower E_{\max} and ED_{50} values than the sham-operated groups. Contractility of bladder muscles of rats receiving L-arginine significantly recovered compared to that in the obstructed groups, but was not equal to that in the sham-

Table 1. Bladder weight

Group [number of animals]	Bladder weight (g)
C-1 [6]	0.109 ± 0.005
OB-1 [6]	0.174 ± 0.024
Ar-1 [6]	0.136 ± 0.012
C-6 [6]	0.114 ± 0.006
OB-6 [6]	0.242 ± 0.035
Ar-6 [6]	0.149 ± 0.012

Bladder weight data are shown as mean ± SEM in each group.

Ar, L-arginine-dosed; C, sham-operated; OB, obstructed.

* $P < 0.05$.

operated groups. Contractile response induced by 100-mmol/L KCl showed a pattern parallel to those of E_{\max} values of carbachol in each group.

Muscle NOS activity

At 1 week and 6 weeks, NOS activity was reduced in the obstructed groups as compared to the sham-operated groups, and was increased in the L-arginine groups as compared to the obstructed groups. But there was no significant difference among the groups (Table 3).

Histological changes in rat bladders by chemicals

A representative light microscopic photograph of the bladder from an L-arginine-dosed rat is shown in Fig. 1 and another from an obstructed rat in Fig. 2. Figure 1 shows eNOS positive in

Table 2. Functional data of obstructed rat bladders

Group [number of animals]	E_{\max} (g/mm ²)	ED_{50} (μmol/L)	100-mmol/L KCl (g/mm ²)
C-1 [6]	9.89 ± 0.88	1.60 ± 0.15	6.96 ± 0.91
OB-1 [6]	4.27 ± 0.52	0.89 ± 0.14	2.12 ± 0.26
Ar-1 [6]	8.93 ± 0.84	1.82 ± 0.26	5.93 ± 0.60
C-6 [6]	11.38 ± 0.57	2.11 ± 0.54	8.53 ± 0.58
OB-6 [6]	4.61 ± 0.38	1.30 ± 0.21	3.09 ± 0.29
Ar-6 [6]	6.79 ± 0.32	1.71 ± 0.43	4.85 ± 0.30

E_{\max} and ED_{50} are for carbachol. Data are shown as mean ± SEM in each group.

Ar, L-arginine-dosed; C, sham-operated; OB, obstructed.

* $P < 0.05$.

the mucosal region, vessels and smooth muscle region. In Fig. 2, iNOS is detected in the mucosal region, and positive in the infiltrated leukocytes. eNOS and iNOS were detected in all groups in the same manner.

Table 3. NOS activity in the rat bladder

Group [number of animals]	NOS activity (nmol/ng protein/min)
C-1 [6]	0.063 ± 0.004
OB-1 [6]	0.049 ± 0.005
Ar-1 [6]	0.063 ± 0.012
C-6 [6]	0.105 ± 0.016
OB-6 [6]	0.068 ± 0.013
Ar-6 [6]	0.079 ± 0.012

NOS activity data are shown as mean ± SEM in each group. Ar, L-arginine-dosed; C, sham-operated; OB, obstructed.



Fig. 1. Sections of rat bladder tissue from the L-arginine-dosed group in 6-week-immunostained by anti-endothelial nitric oxide synthase (eNOS) antibodies ($\times 100$).

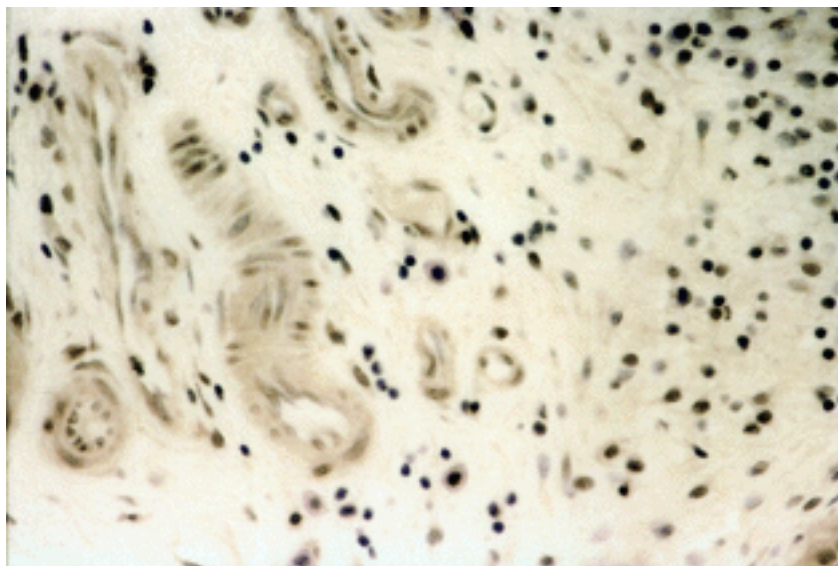


Fig. 2. Sections of rat bladder tissue from the obstructed group in 6-week-immunostained by anti-inducible nitric oxide synthase (iNOS) antibodies ($\times 200$).

Discussion

Several authors have postulated that obstruction-induced ischemia is responsible for the bladder dysfunction seen with BOO (Lin et al., 1995; Buttyan et al., 1997).

Our data demonstrated that BOO increased bladder weight and decreased contractility in response to carbachol and KCl. Bladder weight significantly increased in a 1- or 6-week outlet obstruction. But by treatment with L-arginine, bladder weight did not significantly increase compared to that in the sham-operated groups. The carbachol-induced developed tension was decreased by BOO. The contractile response recovered by treatment with L-arginine. In addition, we measured NOS activity in the experimentally obstructed bladders. At 6 weeks of obstruction, NOS activity was decreased by the obstruction, and was increased by treatment with L-arginine. But there was no significant difference among the groups. In the histological examination, there were no distinct differences in immunohistochemical staining among the groups. eNOS was positive in the mucosal region, vessels and smooth muscle region, and iNOS was detected in the mucosal region and in the infiltrated leukocytes in the bladder dome. These data indicate that bladder function may not to be associated with NOS activity. But treatment with L-arginine may increase NO, which may improve bladder function.

Bladder distention caused by BOO induces ischemic changes in the bladder (Lin et al., 1995). The ischemia caused by BOO temporarily increases NO concentration in the bladder for short-term obstruction (Saito and Miyagawa, 2001); however, NO is gradually decreased for long-term obstruction, and the decrease in NO induces bladder dysfunction. It is reported that NO is a mediator of relaxation of the urethra in rats (Mumtaz et al., 2000), and that, as a result, the load of the bladder is reduced. The rat detrusor is reported to be capable of responding to NO (Chung et al., 1996). NO induces vasodilation, and the released NO maintains the necessary blood supply via the circulatory system by dilating arteries (Saito and Miyagawa, 1999),

which may play a role in relaxing of the detrusor. Increased levels of NO treated with L-arginine may relax the urethra and decrease the load of the bladder, and recover the blood flow.

Histochemical examination revealed that a brain subtype of NOS (bNOS) was distributed in the proximal urethra of the dog (Takeda and Lepor, 1995), and in the rat bladder neck and urethra (Tamaki et al., 1995). Upon distribution of NOS in the bladder dome, our data demonstrated that eNOS was positive in the mucosal region, vessels and smooth muscle region, and that iNOS was detected in the mucosal region and the infiltrated leukocytes in the bladder dome, as was reported before (Saito and Miyagawa, 1999; Persson et al., 1999). These observations indicate that vasodilation and detrusor relaxation may be induced by treatment with L-arginine. As there is no significant difference in NOS activity in these groups, treatment with L-arginine increases NO in the bladder.

As a result, L-arginine prevents an increase in bladder weight and a decrease in the contractile response: that is, L-arginine prevents the bladder dysfunction caused by BOO. Because of this, the reasons for the results obtained in this experiment are speculated as follows: i) Experimentally obstructed bladder outlets in the rats induced bladder overload and ischemia, and resulted in an increase in bladder weight and bladder dysfunction; ii) Treatment by L-arginine increased NO in the urethra and bladder, decreased bladder overload and increased the blood flow of the bladder by vasodilation. These changes resulted in a decrease in bladder weight and recovery from bladder dysfunction.

The present findings reveal the possibility that bladder dysfunction caused by BOO is improved by treatment with L-arginine, which may increase NO concentration.

However, Saito et al. (1998) reported that reperfusion following ischemia causes more severe dysfunction than that of ischemia alone in the rat bladder. Reactive oxygen species, such as the superoxide radical, hydroxyl radical and hydrogen peroxide, are known to induce injury in several tissues. The generation of these reactive oxygen species is attributed to ische-

mia-reperfusion injury. These reactive oxygen species damage many biological molecules. Peroxynitrate, reacting with NO and superoxide radicals, is also reported to be a powerful oxidative product (Muijsers et al., 1997). Saito and Miyagawa (1999) offered the idea that peroxy-nitrate reacting with NO and superoxide radicals may be involved in ischemia-reperfusion injury in the bladder. In their reports, NO and peroxy-nitrate induce bladder dysfunction, a finding which indicates that NO has a cytotoxic effect in the experimentally obstructed bladder. However, our presented data demonstrated that NO has a cytoprotective effect in the bladder. Therefore, NO has both damaging and protective effects in many tissues.

In summary, i) bladder weight was increased by BOO, and the increase in bladder weight could be restrained by treatment with L-arginine; ii) the carbachol-induced developed tension was decreased by BOO, and was recovered with treatment by L-arginine; and iii) in the histological studies, eNOS was positive in the mucosal region, vessels and smooth muscle region, and iNOS was positive in the mucosal region and in the infiltrated leukocytes. It is suggested that treatment with L-arginine may improve the bladder dysfunction caused by BOO.

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