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Involvement of accumulated endogenous NOS inhibitors and decreased NOS activity in the impaired neurogenic relaxation of the rabbit proximal urethra with ischaemia

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1 We examined the effect of ischaemia on the neurogenic and nitric oxide (NO)-mediated urethral relaxation.

2 Rabbits were divided into control and urethral ischaemia (UI) groups, which was prepared by the partial occlusion of bilateral iliac arteries using blood vessel occluders.

3 Neurogenic and NO-mediated proximal urethral relaxation induced by electrical field stimulation (EFS) was greatly impaired in the UI group, while relaxation by sodium nitroprusside (SNP) as a NO donor showed no difference between the two groups. Pretreatment with L-arginine significantly improved but did not normalize the impaired relaxation in the UI group. Not only basal level, but also stimulated production of cyclic GMP with EFS, were significantly decreased in the UI group.

4 The tissue contents of N^G-methyl-L-arginine (L-NMA) and asymmetric N^G, N^G-dimethyl-L-arginine (ADMA) in the proximal urethra were increased following ischaemia. While L-arginine and symmetric N^G, N'^G-dimethyl-L-arginine (SDMA) contents remained unchanged. Exogenously applied authentic L-NMA and ADMA (1–100 μM) concentration-dependently inhibited the EFS-induced urethral relaxation in the control group. The inhibition with L-NMA and ADMA was undetectable in the presence of 3 mM L-arginine.

5 The Ca^{2+}-dependent NOS activity in the urethra from the UI group was significantly lower than that from the control group and was not restored by an addition of 3 mM L-arginine.

6 These results suggest that the impaired neurogenic and NO-mediated urethral relaxation with ischaemia is closely related to the increased accumulation of L-NMA and ADMA and decreased NOS activity, which would result in an accelerated reduction in NO production/release.
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Keywords: Ischaemia; proximal urethra; L-NMA; ADMA; L-arginine; NOS activity

Abbreviations: ADMA, asymmetric N^G, N^G-dimethyl-L-arginine; cyclic GMP, cyclic 3′:5′-guanosine monophosphate; IBMX, 3-isobutyl-l-methylxantine; L-NMA, N^G-methyl-L-arginine; L-NOARG, N^G-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; SDMA, symmetric N^G, N'^G-dimethyl-L-arginine; SNP, sodium nitroprusside

Introduction

Major extensive pelvic visceral surgery is known to frequently cause dysfunction in the lower urinary tract (Blaivas & Barbalias, 1983; Yalla & Andriole, 1984). The etiology of the dysfunction has been reported to be mainly caused by complete or incomplete neural ablation, or neural traction injury during the surgery (Mundy, 1982; Leveckis *et al*., 1995). It has also been suggested that bladder ischaemia due to reduction of blood flow by surgical trauma, could produce bladder dysfunction, such as decrease in bladder contraction, compliance and capacity with increased post-voiding residual volume (Lin *et al*., 1988).

Efficient micturition depends not only on bladder contraction but also urethral relaxation. It is well known that nitric oxide (NO) plays an important role in the proximal urethral relaxation during the voiding phase (Andersson *et al*., 1992; Bennett *et al*., 1995). Recently, it has been reported that cavernosal (Azadzoi *et al*., 1998) or prostatic (Kozlowski *et al*., 2000) relaxation through excitation of the neural NO pathway is impaired by the chronic ischaemia. These reports predispose the hypothesis that urethral ischaemia caused by surgical trauma may cause an impairment of NO-mediated urethral relaxation, which may bring about obstructive voiding and increased post-voided residual urine.

Vallance *et al*. (1992) have obtained evidence that N^G-methyl-L-arginine (L-NMA) and asymmetric N^G, N^G-dimethyl-L-arginine (ADMA) play a role as endogenous inhibitors for NO synthesis. Increased methylarginines within cells and tissues may be a mechanism for regulation of NOS. Recently, it has been reported that the accumulation of endogenous NOS inhibitors in regenerated endothelial cells is associated with decreased NO production/release and occur-

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rence of intimal hyperplasia following endothelial denudation of the rabbit carotid artery (Azuma *et al*., 1995), and that the concentration of these inhibitors was increased in plasma with peripheral arterial occlusive disease (Böger *et al*., 1997) and in endothelial cells with diabetes mellitius (Masuda *et al*., 1999). However, there is no study of whether endogenous NOS inhibitors are involved in the occurrence of disorders related with NO-mediated responses in the lower urinary tract.

Thus the present experiments were designed to investigate the effect of ischaemia by partial vessel occlusion on the urethral relaxation in connection with changes in NO production and contents of L-NMA and ADMA in the proximal urethra.

Methods

Experimental animals

Japanese White male rabbits weighing approximately 2.5 kg were divided into control ($n=7$) and urethral ischaemia (UI, $n=9$) groups. These rabbits were housed in a temperature ($23\pm1^{\circ}C$) and humidity ($50\pm20\%$) controlled room and were fed regular chow (RC4, Oriental Yeast) throughout the experimental periods. This study complied with the Animal Welfare Regulation of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Vessel occlusion

The animals were anaesthetized by intravenous injection of sodium pentobarbitone (25 mg kg^{-1}). An abdominal midline incision was made and the descending aorta and iliac arteries were exposed. Blood vessel occluders (4 mm ID, Unique Medical Co. Ltd, Tokyo, Japan) were placed around the bilateral common iliac arteries. Control rabbits underwent sham surgery without placing occluders. After achieving adequate haemostasis, the animals were allowed to recover and were placed on a short course of intramuscular penicillin. Two weeks later, the body weight of the animals was recorded and the following studies were performed.

Measurement of bladder and urethral wall blood flows

Bladder and urethral wall blood flows were measured with a laser Doppler probe connected to a laser Doppler flowmeter (TBF-LN1, Unique Medical Co. Ltd, Tokyo, Japan) under the sodium pentobarbitone anaesthesia. The flowmeter was calibrated against an internal standard reading flow in units of ml min^{-1} 100 g^{-1} tissue. For the recording of the blood flow, the laser probes were placed at three different sites of the bladder dome and anterior urethra, respectively, and average blood flow was recorded. Systemic blood pressure was monitored through a polyethylene catheter (Clay-Adams PE-90, Becton-Dickinson Co, Franklin Lakes, NJ, U.S.A.) inserted into the right carotid artery.

Animals were sacrificed with an overdose of sodium pentobarbitone, lower urinary tracts were removed *en bloc* and kept in a Petri dish containing ice-cold modified Krebs solution and dissected free of adherent tissues. The proximal urethra was used for isometric tension experiments in the organ bath and for determinations of cyclic GMP, NOS activity and endogenous NOS inhibitors. For the measurement of NOS activity and endogenous NOS inhibitors, the urethra was frozen in liquid nitrogen immediately after the dissection.

Measurement of mechanical responses

Proximal urethra was made in transverse strips of approximately 4 mm width, 8 mm length and weighing 80 mg. When subjected to electrical field stimulation (EFS), the strips were suspended vertically between two parallel platinum electrodes in the 10 ml organ chambers filled with oxygenated Krebs solution maintained at a temperature of $37\pm0.5^{\circ}C$ and continuously bubbled with 95% O_2 and 5% CO_2. One end of each strip was connected to a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co. Ltd, Tokyo, Japan) in order to record the changes in isometric tension on a pen-writing oscillograph (R64, Rika Denki Kogyo Co., Tokyo, Japan). The length of the strips was adjusted several times until a stable tension of 1 g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution, and during this period the bathing solution was replaced every 20 min with fresh solution. Relaxations in response to EFS and sodium nitroprusside (SNP) during the contraction caused by phenylephrine (PE, 10 μM) were observed. EFS was performed with the aid of an electronic stimulator (SEN-3201, Nihon Koden Kogyo Co., Tokyo, Japan), which delivered trains of rectangular pulses (supramaximum voltage, 0.3 ms duration at frequencies of 0.5–20 Hz for 10 s). EFS was applied every 3 min in the presence of atropine (1 μM) and guanethidine (10 μM) in order to eliminate cholinergic and adrenergic components. Frequency-response curves to EFS were obtained before and after treatment with L-arginine (3 mM), L-NMA (1–100 μM), ADMA (1–100 μM) or SDMA (100 μM). Following a 60 min washout period, the strips were contracted again with PE (10 μM) and the responses to SNP were examined in the presence or absence of 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 10 μM), a novel and potent soluble guanylate cyclase inhibitor (Moro *et al*., 1996). Relaxations induced by EFS and SNP were expressed as a percentage of the PE (10 μM) contraction. The composition of the modified Krebs solution was as follows (mM): NaCl 118.0, KCl 4.7, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}2H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0.

Measurement of cyclic GMP

As described above, proximal urethral preparations were suspended in organ bath under the resting tension of 1 g. Cyclic GMP levels were measured in unstimulated preparations and in preparations subjected to 10 Hz EFS for 30 s under the PE (10 μM) contraction. The cyclic GMP level was determined according to the method described previously (Masuda *et al*., 1997). In brief, tissues were rapidly transferred into 10% trichloroacetic acid (TCA) with liquid nitrogen in order to stop the reaction. All experiments were performed in the presence of 10 μM 3-isobutyl-1-methylxantine (IBMX) as a non-selective phosphodiesterase inhibitor.

The net production of cyclin GMP was expressed as the difference between the production with EFS and that with EFS in the presence of 100 μM N^G-nitro-L-arginine (L-NOARG) as a NOS inhibitor (Kobayashi & Hattori, 1990). The basal cyclic GMP level was taken as the value without EFS. The protein concentration of the sample was determined by use of the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Determination of L-arginine and methylarginines

The contents of L-arginine, L-NMA, ADMA and SDMA in the proximal urethra of control and UI groups were determined by means of automated high-performance liquid chromatography according to the method described previously (Azuma *et al*., 1995; 1997). The contents of L-arginine and methylarginines are given as nmoles and pmoles g^{-1} wet weight, respectively. In order to estimate the apparent concentrations of L-arginine and methylarginines in the proximal urethra, tissue water content was determined. Urethral specimens were completely lyophilized with the aid of the centrifugal vaporizer (CVE-100D, Eyela, Tokyo, Japan). The difference between the wet weight and dry weight was assumed as the tissue water content (Azuma *et al*., 1997).

Measurement of nitric oxide synthase (NOS) activity

NOS activity in the proximal urethra was measured by determining the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline (Moore *et al*., 1993). Neuronally-derived NOS is primarily detectable in the cytosolic or soluble fraction (Knowles *et al*., 1990; Schmidt *et al*., 1991). The frozen tissue was homogenized in a Polytron (Kinematica, Luzern, Switzerland), at maximum speed for 15 s each $\times$ 4 to a 25% homogenate in the buffer consisting of (mM): sucrose 320, HEPES 10, EDTA 0.1, dithiothreitol (DTT) 1, pepstatin 1 μM and leupeptin 1 μM (pH 7.2). The homogenate was centrifuged at $1200 \times g$ for 20 min at 4°C and the pellet was discarded.

The supernatant was filtered through one layer of gauze and centrifuged at $10{,}000 \times g$ for 60 min at 4°C. The supernatant (cytosolic fraction) was decanted from the pellet (particulate fraction). Incubation mixtures consisted of 340 μl of the supernatant and 50 μl of the buffer described above containing NADPH 2 mM, $CaCl_2$ 2 mM, 30 U ml^{-1} calmodulin, 5 μM flavin adenine dinucleotide (FAD), 14 μM tetrahydrobiopterin (BH_4), 20 μM L-arginine and 0.1 μCi ml^{-1} [^{3}H]-L-arginine. The reaction mixture was incubated at 37°C for 45 min in a shaking water bath. Preliminary experiments revealed that the reaction was linear during this time. Incubation was terminated by the addition of 1 ml of ice-cold stop buffer (5 mM HEPES containing 2 mM EDTA). Samples were applied to a 1 ml column of Dowex AG50W-X8 (Na^+ form) to remove unmetabolized [^{3}H]-L-arginine. The columns were then washed with 1.5 ml of water, and [^{3}H]-L-citrulline was quantified in the flow-through fraction using a liquid scintillation counter (TRI-CARB 2750TR/LL, Packard Instrument Co., Meriden, CT, U.S.A.). To determine the effects of authentic L-NMA, ADMA and SDMA on the NOS activity, parallel samples were processed in the presence of various concentrations (0.1 – 100 μM) of methylarginines. Calcium-independent NOS activity was measured in the incubation mixture removing $CaCl_2$ and containing 2 mM EDTA. Enzyme activity was expressed as pmol citrulline mg^{-1} protein min^{-1}.

Chemicals

The chemicals used were L-phenylephrine hydrochloride (PE), guanethidine sulphate, atropine sulphate, L-citrulline, L-arginine hydrochloride, N^G-methyl-L-arginine (L-NMA), asymmetric N^G,N^G-dimethyl-L-arginine (ADMA), symmetric N^G, N^G-dimethyl-L-arginine (SDMA), 3-isobutyl-1-methylxanthine (IBMX), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), HEPES, sucrose, ethylenediaminetetraacetic acid (EDTA), DL-dithiothreitol (DTT), leupeptin, reduced nicotine amide dinucleotide phosphate (NADPH), calcium chloride ($CaCl_2$), calmodulin, magnesium chloride ($MgCl_2$), bovine serum albumin and Dowex AG50W-X8 cation exchange column (Na^+ form) (all from Sigma Chemical Co. St. Louis, MO, U.S.A.). Bradford reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.), N^G-nitro-L-arginine (L-NOARG, Research Biochemicals Inc., Natick, MA, U.S.A.), sodium nitroprusside (SNP, Wako Pure Chemicals, Tokyo, Japan), tetrodotoxin (TTX, Sankyo Co, Tokyo, Japan) and [^{3}H]-L-arginine (Amersham Pharmacia Biotech, U.K.).

Data analyses

Deviations from the mean regarding the frequency response curves were statistically analysed by use of a factorial two-way analysis of variance (ANOVA). Potencies of L-NOARG, L-NMA and ADMA on the NOS activity were compared in terms of IC_{50} values, which were concentrations producing 50% inhibition of NOS activity. The K_M and V_{max} values were estimated by non-linear analysis of the model. $V = (V_{max} \times [S])(K_M + [S])^{-1}$ in which V is the initial velocity (pmol citrulline mg^{-1} protein min^{-1}) and [S] is the L-arginine concentration (μM). Student's *t*-test (2-tailed) for unpaired data was used and statistical significance was determined at $P < 0.05$.

Results

Baseline data

Mean arterial pressure (MAP) and body weight of UI group were not different from those of control group (Table 1), while bladder and urethral tissue weights were significantly greater ($P < 0.05$) in the UI group compared with the control group (Table 1). Bladder and urethral wall blood flows in the UI group were significantly decreased ($P < 0.01$) compared with the control group under the empty bladder condition (Table 1).

Functional responses of the proximal urethra

The contractile responses to phenylephrine (PE) were not significantly different between the two groups. The contractile responses induced by 10 μM PE in the control and UI groups were 1.54 ± 0.23 g and 1.41 ± 0.17 g, respectively. The pre-treatment with L-arginine, L-NOARG and ODQ did not have a significant effect on the resting tension or on the contractile

response induced by 10 μM PE. In the presence of 1 μM atropine and 10 μM guanethidine, EFS at frequencies of 0.5–20 Hz produced transient relaxations of the urethral strips from the control and UI groups, which had been precontracted with 10 μM PE. The transient relaxations were abolished by the pretreatment with 1 μM TTX or 100 μM L-NOARG (Figures 1 and 2). The EFS-induced relaxations in the UI group were significantly reduced as compared with the control group (Figures 1 and 2). The maximum relaxation was determined to be 59.2±5.2% at 10 Hz in the control ($n=7$) and 29.5±5.2% at 10 Hz in the UI group ($n=7$) ($P<0.01$). Pretreatment with L-arginine (3 mM) for 30 min improved but did not normalize the impaired relaxation responses in the UI group and significantly ($P<0.05$) enhanced the relaxation responses in the control group (Figure 2). On the other hand, relaxation caused by SNP (0.01–100 μM) as a NO donor was not different from each other (Figures 1 and 3). The relaxation was significantly ($P<0.01$) inhibited by 10 μM ODQ as an inhibitor of guanylate cyclase (Figure 3).

Cyclic GMP production in the proximal urethra

EFS (10 Hz for 30 s) increased the cyclic GMP contents from basal level of 1.8±0.3 to 5.6±1.4 pmol mg^{-1} protein in the control group and from 0.8±0.2 to 2.1±0.4 pmol mg^{-1} protein in the UI group, respectively. Basal level as well as EFS-induced production of cyclic GMP were significantly lower in the UI group than those in the control. L-NOARG (100 μM) reduced the cyclic GMP production below the basal level in the two groups (Figure 4). Net production of cyclic GMP was also significantly ($P<0.05$) lower in the UI group.

L-arginine and methylarginines contents in the proximal urethra

The contents of L-arginine, L-NMA, ADMA and SDMA are summarized in Table 2. The contents of L-NMA and ADMA in the UI group were approximately 2 fold and 3 fold higher than those values in the control group, respectively ($P<0.01$)

Table 1 Mean arterial pressure (MAP), body weight (BW), wet weight and wall blood flow of proximal urethra and bladder in the control (C) and urethral ischaemia (UI) groups

Group	*MAP* (mmHg)	*BW* (Kg)	*Weight* (mg) *Urethra*	*Weight* (mg) *Bladder*	*Blood flow* (ml min^{-1} 100 g^{-1}) *Urethra*	*Blood flow* (ml min^{-1} 100 g^{-1}) *Bladder*
C	90.6±4.2	2.79±0.5	264±18	2756±185	17.2±2.8	14.6±3.2
UI	93.1±3.8	2.81±0.3	389±14[a]	3863±193[b]	7.9±1.9[b]	5.8±1.7[b]

Results are given as means±s.e.mean of 5–7 determinations from different animals. [a] and [b]; significantly different *vs* corresponding value in the control at $P<0.05$ and $P<0.01$, respectively.

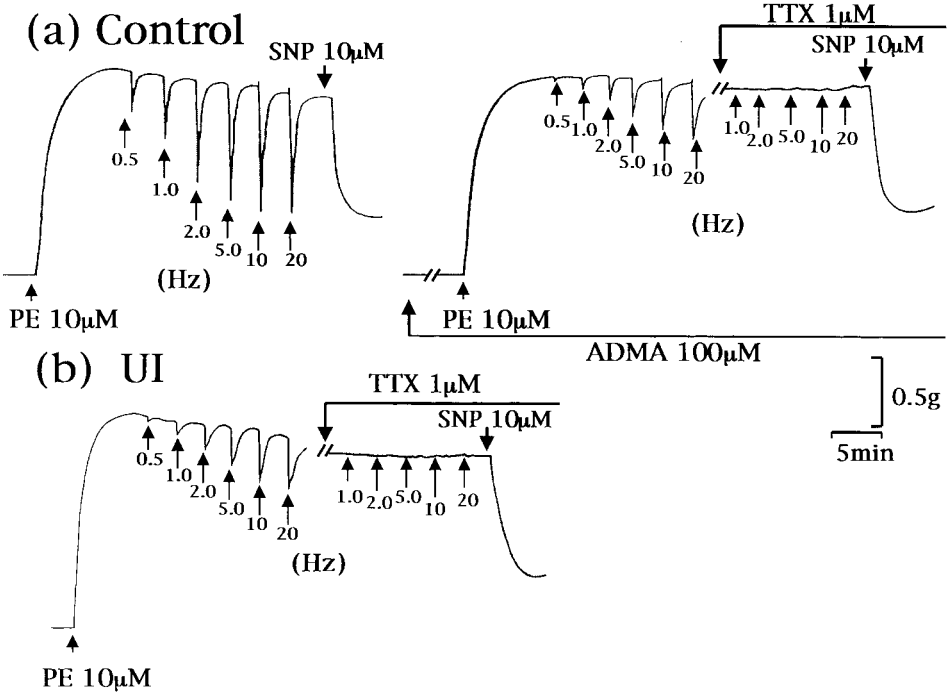

Figure 1 Representative tracings showing relaxation responses to electrical field stimulation (EFS) and sodium nitroprusside (SNP) in rabbit urethral strips from control (a) and urethral ischaemia (UI, b) groups and the effects of ADMA and tetrodotoxin (TTX). EFS were delivered to strips precontracted with phenylephrine (PE, 10 μM) in the presence of atropine (1 μM) and guanethidine (10 μM). In the control group (a), 100 μM ADMA partially inhibited the EFS induced relaxation. Addition of tetrodotoxin (TTX, 1 μM) abolished the responses to EFS in both groups.

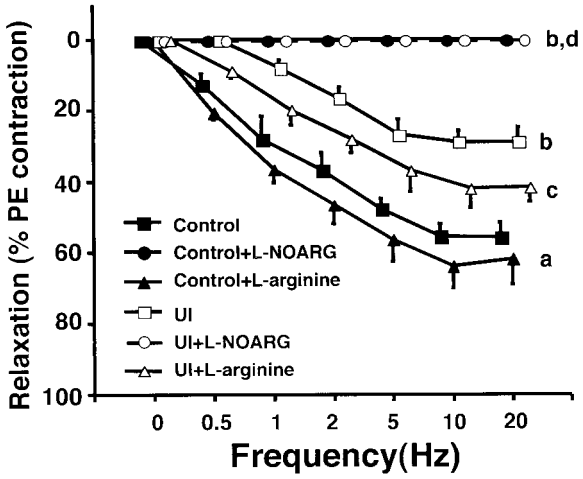

Figure 2 Electrical field stimulation (EFS)-induced relaxation during the contraction caused by phenylephrine (PE, 10 μM) in rabbit urethral strips of control and urethral ischaemia (UI) groups and effects of N^G-nitro-L-arginine (L-NOARG, 100 μM) and L-arginine (3 mM). Atropine (1 μM) and guanethidine (10 μM) were present throughout the experiments. Results are expressed as a percentage of the PE contraction. Data points represent mean$\pm$s.e.mean of measurements in 5–7 strips from different animals. Vertical bars show s.e.mean. [a]$P<0.05$; [b]$P<0.01$ (ANOVA) *vs* frequency-response curve obtained in the untreated control group. [c]$P<0.05$; [d]$P<0.01$ (ANOVA) *vs* the curve obtained in the untreated UI group.

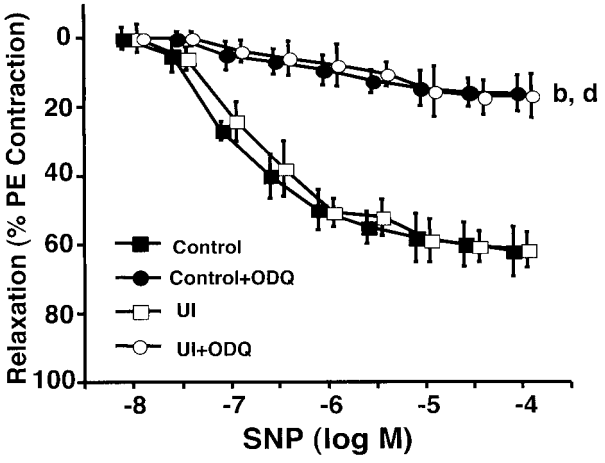

Figure 3 Sodium nitroprusside (SNP)-induced relaxation during the contraction caused by PE (10 μM) in rabbit urethral strips of control and UI groups and effect of ODQ (10 μM). Atropine (1 μM) and guanethidine (10 μM) were present throughout the experiments. SNP was added cumulatively after the PE contraction had reached a steady-state level. Results are expressed as a percentage of the PE contraction. Data points represent mean$\pm$s.e.mean of measurements in 5–7 strips from different animals. Vertical bars show s.e.mean. [b]$P<0.01$ (ANOVA) *vs* concentration-response curve obtained in the untreated control group. [d]$P<0.01$ (ANOVA) *vs* the curve obtained in the untreated UI group.

(Table 2). There was no significant difference in the L-arginine and SDMA contents between the two groups. Since the tissue water content was determined to be 0.814 ± 0.007 ml g^{-1} wet weight in the control group ($n=7$) and 0.801 ± 0.004 ml g^{-1} wet weight in the UI group ($n=7$),

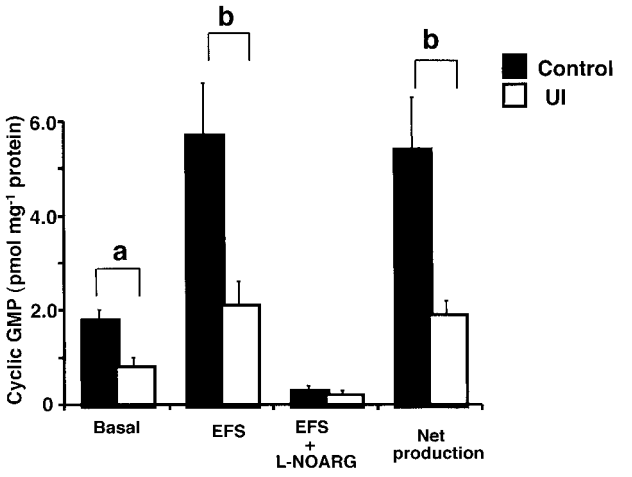

Figure 4 Cyclic GMP levels measured under basal condition and electrical field stimulation (EFS) in the control and urethral ischaemia (UI) rabbits. Results are given as mean$\pm$s.e.mean of 5–7 determinations from different animals. Vertical bars show s.e.mean. [a] and [b]; significantly different from the control at $P<0.05$ and $P<0.01$, respectively. All experiments were performed in the presence of 10 μM 3-isobutyl-l-methylxanthine (IBMX) as a non-selective phosphodiesterase inhibitor. The net production of cyclic GMP was expressed as the difference between the production stimulated by EFS and that stimulated by EFS in the presence of N^G-nitro-L-arginine (L-NOARG, 100 μM) as an inhibitor of nitric oxide synthase. The basal level was given as the value without EFS.

Table 2 Contents of L-arginine, L-NMA, ADMA and SDMA in the proximal urethra in the control (C) and urethral ischaemia (UI) groups

	Contents			
	L-arginine	*L-NMA*	*ADMA*	*SDMA*
Group	(nmoles g^{-1} w. w.)	(pmoles g^{-1} w. w.)		
C	275 ± 29	1258 ± 122	1398 ± 120	451 ± 61
UI	294 ± 35	2385 ± 169[b]	3237 ± 278[b]	510 ± 115

Results (nmoles in L-arginine or pmoles in methylarginines g^{-1} wet weight) are given as mean$\pm$s.e.mean of 6–7 determinations from different animals. [b]; significantly different *vs* corresponding value in the control at $P<0.01$.

respectively, the apparent concentrations (μM) of L-arginine, L-NMA, ADMA and SDMA at lowest limit were calculated to be 337.5 ± 36.2, 1.55 ± 0.15, 1.72 ± 0.15 and 0.55 ± 0.08 in the control group and 366.7 ± 43.7, 2.98 ± 0.21, 4.04 ± 0.35 and 0.64 ± 0.14 in the UI group, respectively.

Effects of authentic L-NMA, ADMA and SDMA on relaxation responses to EFS and SNP

The pretreatment with all three methylarginines had no significant effect on the resting tension or on the contractile response induced by 10 μM PE. Exogenously applied L-NMA (1–100 μM) and ADMA (1–100 μM), but not SDMA (100 μM) concentration-dependently inhibited the EFS-induced relaxations in the proximal urethra of the control (Figures 1 and 5). L-NMA and ADMA did not produce inhibition of the urethral relaxation in the presence of 3 mM L-arginine. In the UI group, authentic L-NMA at only 100 μM significantly ($P<0.05$) inhibited the EFS-induced

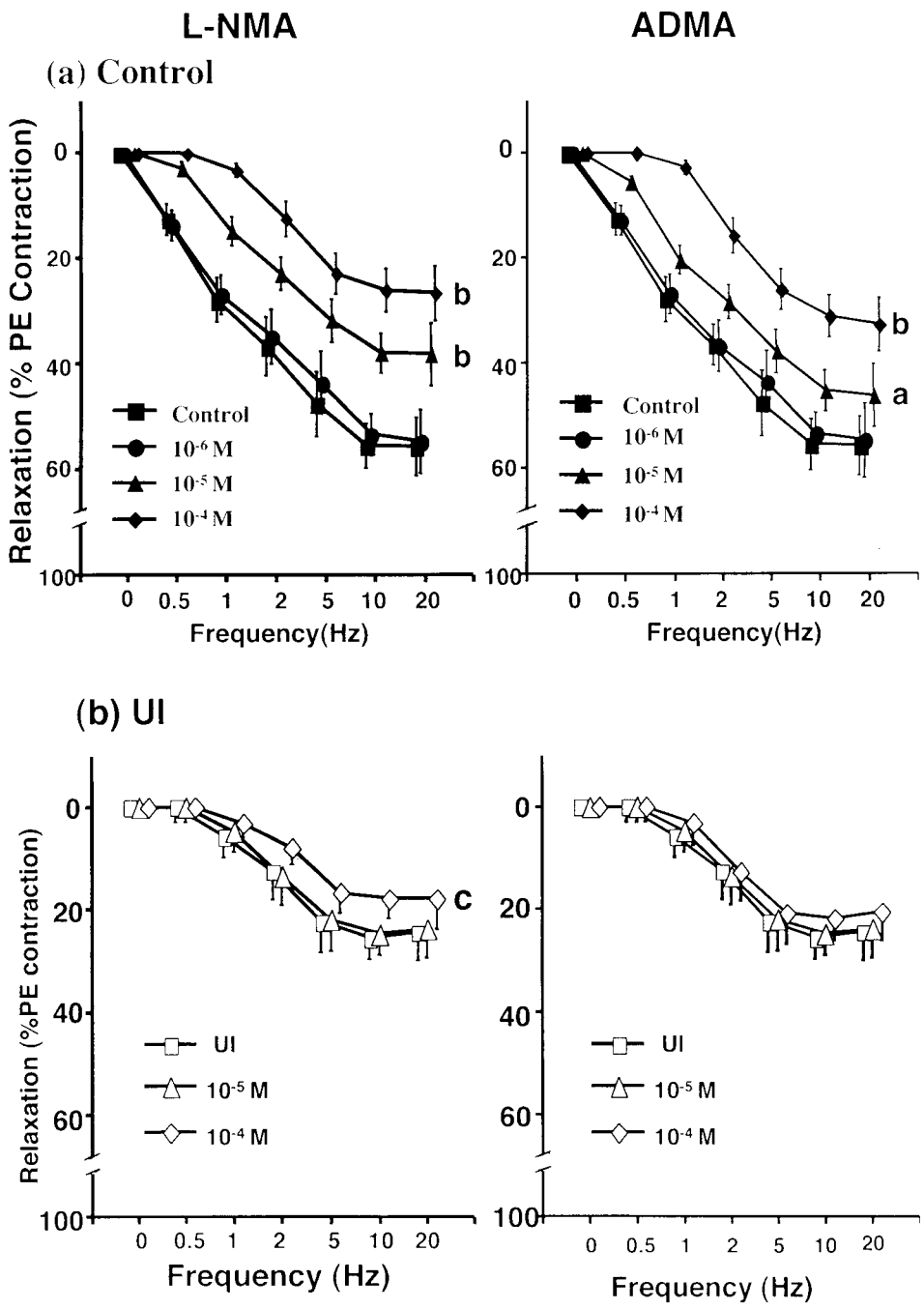

Figure 5 Inhibitory effects of L-NMA and ADMA on the urethral relaxations induced by EFS in the control (a) and urethral ischaemia (UI) (b) groups. The strips were pretreated with L-NMA or ADMA for 30 min. Atropine (1 μM) and guanethidine (10 μM) were present throughout the experiments. Results are expressed as a percentage of the PE (10 μM) contraction. Data points represent mean $\pm$ s.e.mean of measurements in 5–7 strips from different animals. Vertical bars show s.e.mean. $^{a}P<0.05$; $^{b}P<0.01$ (ANOVA) *vs* frequency-response curve obtained in the untreated control group. $^{c}P<0.05$ (ANOVA) *vs* curve obtained in the untreated UI group.

relaxation (Figure 5). On the other hand all three methylarginines failed to modify the SNP-induced urethral relaxations in both groups (data not shown).

NOS activity in the proximal urethra

NOS activity was exclusively detectable in the cytosolic fractions and mainly Ca^{2+}-dependent in both groups. Results refer to the cytosolic fraction unless otherwise stated. Ca^{2+}-dependent NOS activity in the UI group was significantly ($P<0.01$) reduced compared with the control group (Figure 6). In contrast, there was no significant change in the Ca^{2+}-independent NOS activity between the two groups. In experiments conducted at different L-arginine concentrations (ranging from 0 to 3000 μM), [^{3}H]-L-citrulline was generated according to the Michaelis–Menten kinetics (Figure 7). The

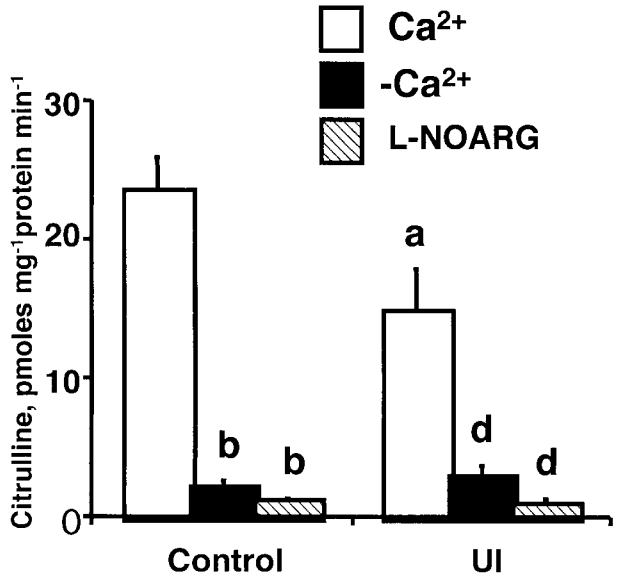

Figure 6 NOS activity in the proximal urethra in the control and UI groups. NOS activity was measured in the presence of 2 mM $CaCl_2$ (Ca^{2+}), in the absence of $CaCl_2$ and in the presence of 2 mM EGTA (-Ca^{2+}) and in the presence of $CaCl_2$ and 100 μM N^G-nitro-L-arginine (L-NOARG). All reaction mixtures contained 2 mM NADPH, 30 U ml^{-1} calmodulin, 5 μM FAD, 14 μM BH_4 and 20 μM L-arginine. Data represent mean $\pm$ s.e.mean of five measurements from different animals. Vertical bars show s.e.mean. [a]$P<0.05$, [b]$P<0.01$ *vs* untreated control, [d]$P<0.01$ *vs* untreated UI.

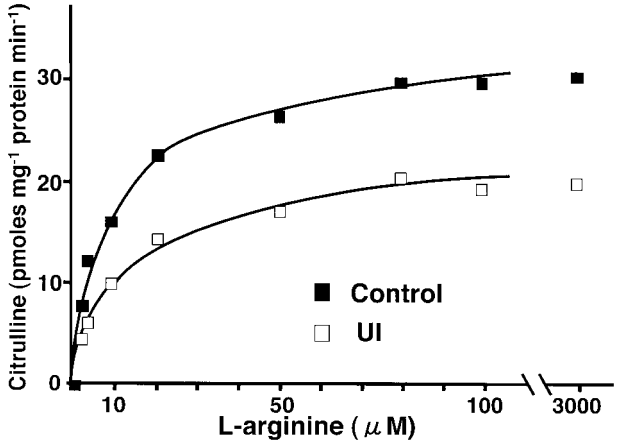

Figure 7 Representative experiment showing the plot of velocity for NOS activity (pmol citrulline mg^{-1} protein min^{-1}) *vs* concentration of L-arginine in the proximal urethra in the control and UI groups. NOS activity was measured in the presence of 2 mM $CaCl_2$, 2 mM NADPH, 30 U ml^{-1} calmodulin, 5 μM FAD, 14 μM BH_4 at different L-arginine concentrations (0–3000 μM). The K_M and V_{max} values were estimated by non-linear regression, fitting the data to a rectangular hyperbola.

apparent K_M and V_{max} values of NOS were estimated to be 9.5 ± 1.2 μM and 29.1 ± 3.6 pmol citrulline mg^{-1} protein min^{-1} ($n=4$) in the control, respectively; and 10.2 ± 1.9 μM and 19.3 ± 3.6 pmol citrulline mg^{-1} protein min^{-1} ($n=4$) in the UI group, respectively (Figure 7). V_{max} but not K_M of the control was significantly ($P<0.01$) higher than that of the UI group.

Inhibitory effects of authentic L-NMA, ADMA and SDMA on the NOS activity

NOS activity prepared from the proximal urethra of both groups was inhibited by authentic L-NMA and ADMA in a concentration-dependent manner (Figure 8), but not by SDMA even in a high concentration of 100 μM ($n=4$ in each group, data not shown). The inhibitory potencies of L-NMA and ADMA were compared with that of L-NOARG in terms of IC_{50} (μM), which were determined to be 7.82 for L-NMA, 12.1 for ADMA and 0.84 for L-NOARG, in the control group. The rank order of inhibitory potency was L-NOARG > L-NMA > ADMA ($P<0.01$; L-NOARG *vs* L-NMA, L-NOARG *vs* ADMA, $P<0.05$; L-NMA *vs* ADMA). On the other hand, IC_{50} (μM) of L-NMA, ADMA and L-NOARG in the UI group as 8.42, 13.9 and 0.96, respectively. These values were not significantly different from control values.

Discussion

EFS-induced transient urethral relaxations in both groups were abolished by the pretreatment with TTX or L-NOARG in the presence of atropine and guanethidine, suggesting that the relaxations are characterized to be neurogenic, non-adrenergic, non-cholinergic and NO-dependent.

The current experiments demonstrated that urethral relaxations caused by EFS, but not by SNP were impaired in the UI group, assuming that the ischaemia results in a decreased NO production and/or release. This assumption is partly supported by the finding that the basal level and the stimulated cyclic GMP production with EFS were significantly reduced in the UI group. It is well established that NO binds the haem group of soluble guanylate cyclase (Ignarro, 1990a; Chinkers & Garbers, 1991), thereby stimulating the production of cyclic GMP. Consequently, the generation of cyclic GMP is widely used as an index of NO biosynthesis (Ignarro, 1990b; Lüscher *et al*., 1990).

In the current study, L-arginine supplementation partially but not completely restored the impaired relaxation responses in the UI group and significantly enhanced the relaxation responses in the control group. It is reportedly known that L-arginine supplementation restores the impaired endothelium-dependent pulmonary vasodilation in chronically hypoxic rats (Eddahibi *et al*., 1992) and induces NO-dependent vasodilation in patients with critical limb ischaemia (Bode-Böger *et al*., 1996a). Also, it has been reported that intravenous infusion of L-arginine induces vasorelaxation in healthy humans (Kanno *et al*., 1992; Bode-Böger *et al*., 1994). L-arginine content in the urethra remained unchanged following ischaemia and apparent concentration of L-arginine, which was estimated on the basis of tissue water content, was 300–400 μM range in both groups. Furthermore, the determined K_M value for the cytosolic NOS activity was approximately 10 μM in both groups, which value was similar to that reported by the other investigators (Garcia-Pascual *et al*., 1996). That is, although an apparent L-arginine level is enough to saturate NOS in both groups, the capability of the urethra to produce and/or release NO was significantly enhanced by L-arginine in both groups, which was in line with the finding reported as the 'Arginine Paradox'

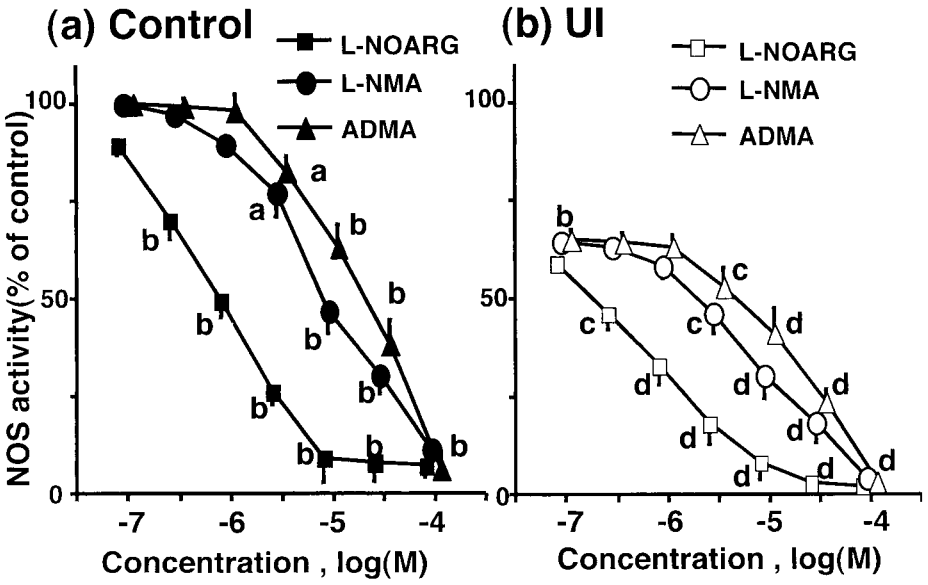

Figure 8 Inhibitory effects of L-NOARG, L-NMA and ADMA on the NOS activity in the control (a) and UI (b) groups. Values are expressed as percentage of control NOS activity. Data points represent mean ± s.e.mean of five measurements from different animals. Vertical bars show s.e.mean. [a]$P<0.05$, [b]$P<0.01$ *vs* untreated control NOS activity. [c]$P<0.05$, [d]$P<0.01$ *vs* untreated UI NOS activity.

(Förstermann *et al*., 1994). Two possibilities are considered to explain the discrepancy: (1) the existence of endogenous NOS inhibitors and (2) cellular L-arginine uptake or intracellular compartmentalization affecting L-arginine levels in the vicinity of the NOS.

Recently, it has been reported that accumulation of endogenous NOS inhibitors such as L-NMA and ADMA in plasma (Bode-Böger *et al*., 1996b; Böger *et al*., 1997) and tissue (Azuma *et al*., 1995; Masuda *et al*., 1999) might explain in part the mechanism decreasing NO production. In our current studies, relative figures of L-NMA : ADMA were about 1 : 1, and estimated minimum concentration of L-NMA and ADMA on the basis of tissue water content were 3–4 μM in the control urethra. Also, contents of L-NMA and ADMA were significantly increased following ischaemia and estimated concentration of L-NMA and ADMA was 7 μM. Indeed, authentic L-NMA and ADMA displayed a concentration-dependent inhibition of urethral relaxation caused by EFS in the control and this inhibition was undetectable in the presence of 3 mM L-arginine. The above findings suggested that accumulation of endogenous NOS inhibitors may modulate NO-mediated urethral function. An important question is whether the concentrations of L-NMA and ADMA are high enough to inhibit the NOS activity in spite of the saturating intracellular L-arginine concentration. 1 : 1 mixture of authentic L-NMA and ADMA at 3 μM inhibited by approximately 18% the NOS activity which had been partially purified from the control urethra (H Masuda, unpublished observation). According to Faraci *et al*. (1995), brain NOS activity was inhibited by 50% in the presence of 2 μM ADMA. However, since methylarginines are concentrated within cells (Macallister *et al*., 1994), the intracellular concentrations of these inhibitors would be possibly higher than the estimated concentrations, and the increased intracellular L-NMA and ADMA concentrations following ischaemia may induce more reduction of NOS activity compared with control. Endogenous L-arginine at 300–400 μM range, estimated on the basis of tissue water content, would overcome any competitive inhibition of NOS by L-NMA and ADMA. Nonetheless, L-arginine supplementation augmented the urethral relaxation in both groups. One possible explanation for this discrepancy may be based upon the compartmentalization of L-arginine in the cells. If the intracellular L-arginine is sequestered and, in turn, poorly accessible to NOS, endogenous NOS inhibitors in the vicinity of NOS may modulate NO-mediated urethral function even in the control tissues. On the other hand, if extracellular L-arginine transported into the cell mediated *via* system y^+ transporter (Bogle *et al*., 1995) is preferentially delivered to NOS when stimulated, pretreatment with high concentration of L-arginine may act by competing with endogenous NOS inhibitors to increase the NO production. More recent reports indicate that caveolae also contain the y^+ arginine transporter and suggest that L-arginine may be directly delivered from the extracellular pool to endothelial NOS (eNOS) *via* the y^+ transporter (McDonald *et al*., 1997). Further studies of the intracellular localization of transporter, methylarginines and neuronal NOS (nNOS) will be required to elucidate the detailed mechanism of NOS inhibition with methylarginines in the urethra.

The mechanisms increasing the contents of L-NMA and ADMA are not clarified accurately in the present experiments. Dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes L-NMA and ADMA, is widely distributed in tissues (Kimoto *et al*., 1995) probably including the urethra. It has recently been reported that two DDAH isoforms (DDAH I and DDAH II) were identified in the human tissues, in which DDAH I predominates in tissues expressing nNOS and DDAH II predominates in tissues expressing eNOS (Leiper *et al*., 1999). In the proximal urethra, DDAH I as well as nNOS may be mainly expressed, and ischaemic conditions may modify the DDAH I

expression and activity. This possibility seems to be supported partly by findings that the concentration of SDMA, which is not a substrate for the metabolizing enzyme, was similar between two groups examined in the current study. On the other hand, L-NMA, ADMA and SDMA enter cells through the cationic amino acid transporters known collectively as system y^+ and the three methylarginines compete with each other and with L-arginine for their transport (Bogle *et al*., 1995). In the current studies, L-NMA and ADMA almost completely inhibited the Ca^{2+}-dependent NOS activity but further impairment of the neurogenic relaxation in the UI group was hardly noticeable. Even in the control group, in contrast to enzyme activities, L-NMA and ADMA had a weak inhibitory effect on nitrergic urethral relaxations as compared with L-NOARG. The above findings suggest that L-NMA and ADMA do not easily enter NOS containing cells, as compared with L-NOARG, and the transport of L-NMA and ADMA may be reduced at 2 weeks after ischaemia. Intracellular concentration and transmembrane transport of these inhibitors will have to be determined simultaneously to clarify this problem.

Alternatively, a decrease in NOS gene and/or protein expression could account for altered NO production. The current experiments demonstrated that the NOS activity was significantly lower in the ischaemic urethra. The findings demonstrating that the reduced NOS activity remained unaffected even in the presence of 3 mM L-arginine, and that methylarginines and L-arginine were undetectable in the partially purified NOS preparation (M. Goto, unpublished observation) suggests that the reduced NOS activity following ischaemia would be a reflection of the decreased NOS protein. This speculation may be supported by the demonstration that the pulmonary hypertension induced after chronic ischaemia is associated with a decreased eNOS activity resulting from diminished eNOS protein and mRNA expressions (Fike *et al*., 1998; Berkenbosch *et al*., 2000). These results may explain the reason why L-arginine supplementation did not completely normalize the impaired urethral relaxation following ischaemia.

In conclusion, this study suggests that impairment of neurogenic urethral relaxation with ischaemia possibly produces disruptions in the integrity of the NO pathway. Accumulation of L-NMA and ADMA as endogenous NOS inhibitors and decreased NOS activity are closely related to the reduction of NO production/release in the proximal urethra with ischaemia.

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