Accumulated endogenous NOS inhibitors, decreased NOS activity, and impaired cavernosal relaxation with ischemia

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Received 16 May 2001; accepted in final form 18 January 2002

Masuda, Hitoshi, Toshihiko Tsuji, Tetsuo Okuno, Kazunori Kihara, Moritaka Goto, and Hiroshi Azuma. Accumulated endogenous NOS inhibitors, decreased NOS activity, and impaired cavernosal relaxation with ischemia. Am J Physiol Regulatory Integrative Comp Physiol 282: R1730–R1738, 2002; 10.1152/ajpregu.00277.2001.—We examined whether endogenous inhibitors of nitric oxide (NO) synthesis are involved in the impaired cavernosal relaxation with ischemia in rabbits. Two weeks after cavernosal ischemia caused by partial vessel occlusion, endothelium-dependent and electrical field stimulation (EFS)-induced neurogenic NO-mediated relaxations, but not sodium nitroprusside (SNP)-induced relaxation, were significantly impaired in the isolated corpus cavernosum. The Ca2+—dependent NO synthase (NOS) activity and the basal and stimulated cGMP productions with carbachol or EFS were significantly decreased (NOS) activity and the decreased NOS activity, and impaired cavernosal relaxation with ischemia. Two weeks after cavernosal ischemia caused by partial vessel occlusion, endothelium-dependent and electrical field stimulation (EFS)-induced neurogenic NO-mediated relaxations, but not sodium nitroprusside (SNP)-induced relaxation, were significantly impaired in the isolated corpus cavernosum. The Ca2+—dependent NO synthase (NOS) activity and the basal and stimulated cGMP productions with carbachol or EFS were significantly decreased after ischemia. Supplementation of excess L-arginine partially recovered both of the impaired relaxations. The contents of NO2—monomethyl-L-arginine (L-NMMA) and asymmetric NO2—N02—dimethyl-L-arginine (ADMA) but not L-arginine and symmetric NO2—N02—dimethyl-L-arginine (SDMA) were increased in the cavernosal tissues after ischemia. Authentic L-NMMA and ADMA but not SDMA concentration dependently inhibited both relaxations without affecting the relaxation produced by SNP in the control. Excess L-arginine abolished the inhibition with L-NMMA and ADMA. These results suggest that the impaired NO-mediated cavernosal relaxations after ischemia are closely related to the decreased NOS activity and the increased accumulation of L-NMMA and ADMA.

Asymmetric NO2,N02—dimethyl-L-arginine; L-arginine; NO2—monomethyl-L-arginine; corpus cavernosum

Major extensive pelvic visceral surgery is known to cause erectile dysfunction frequently. The etiology of the dysfunction has been reported to be mainly caused by damage of the pelvic autonomic nerves during the surgery (18, 27). It has also been suggested that cavernosal ischemia due to reduction of cavernosal arterial blood flow could produce erectile dysfunction (4, 5), and the severity of the arterial occlusion correlates with the degree of erectile dysfunction (2).

It is well known that nitric oxide (NO) plays an important role in the endothelium-dependent and neurogenic relaxations of cavernosal tissue during erectile state (21, 23). The impairment of endothelium-dependent and neurogenic cavernosal relaxations with chronic ischemia is reportedly known to cause the disruption of the NO formation due to an alteration in the expression or activity of NO synthase (NOS) and the increased output of constrictor eicosanoids in cavernosal tissue (3).

Valianche et al. (40) have obtained evidence that asymmetric NO2,N02—dimethyl-L-arginine (ADMA) plays a role as endogenous inhibitor 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 occurrence of intimal hyperplasia after endothelial denudation of the rabbit carotid artery (6) and that the concentration of these inhibitors was increased in plasma with peripheral arterial occlusive disease (11) and in endothelial cells with diabetes mellitus (31). In a previous report, it was found that intracavernously injected ADMA inhibits the pressor responses to pelvic nerve stimulation in the corpus cavernosum of anesthetized dogs, and the inhibitory effect is reversed by L-arginine (1). However, there is no study of whether endogenous NOS inhibitors are involved in the occurrence of disorders related to NO-mediated responses in the erectile dysfunction.

Thus the present experiments were designed to investigate the effect of ischemia by partial vessel occlusion on endothelium-dependent and neurogenic cavernosal relaxations in connection with the impaired NO production by the accumulated endogenous NOS inhibitors and decreased NOS activity.

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MATERIALS AND METHODS

Experimental animals. Japanese White male rabbits weighing ~2.5 kg were used for all the experiments. These rabbits were housed in a temperature (23 ± 1°C)- and humidity (50 ± 20%)-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.

Protocol. The rabbits were divided into two weight-matched groups, control (n = 10) and corpus cavernosal ischemia (CCI, n = 9). The animals of control and CCI groups underwent sham and vessel occlusive operations under anesthesia, respectively. After 2 wk, measurements of iliac and intracavernosal blood flows were performed under anesthesia in all animals. Thirty minutes later, they were killed to provide specimens for the in vitro study.

Vessel occlusion. The animals were anesthetized by intravenous injection of pentobarbital sodium (25 mg/kg). The animals breathed spontaneously. An abdominal midline incision was made, and the descending aorta and iliac arteries were exposed. Blood vessel occluders (4-mm ID, Unique Medical, Tokyo, Japan) were placed around the bilateral common iliac arteries. Control rabbits underwent sham surgery without placing occluders. After achieving adequate hemostasis, 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.

Measurements of iliac and intracavernosal blood flows. The animals were again anesthetized by intravenous injection of pentobarbital sodium (25 mg/kg). The animals breathed spontaneously. Systemic blood pressure was monitored through a polyethylene catheter (Clay-Adams PE-90, Franklin Lakes, NJ) inserted into the right carotid artery. Blood flow through the iliac arteries was measured by placing a perivascular flow sensor connected to a laser-Doppler flowmeter (TBF-LN1, Unique Medical). The flowmeter was calibrated against an internal standard reading flow in units of milliliters per minute. Intracavernosal blood flow was measured with a laser-Doppler flow probe contained in a 23-gauge needle as reported by Azadzoi et al. (4). The probe was placed into the cavernosal tissue and was connected to the laser-Doppler flowmeter. The flowmeter was calibrated against an internal standard reading flow in units of milliliters per minute per 100 g. Intracavernosal blood flow was monitored before and after intracavernosal injection of 5 mg papaverine. Peak intracavernosal blood flow was defined as the maximum increase in the intracavernosal blood flow during erection.

Tissue procurement. Thirty minutes after measurements of blood flows, blood samples were withdrawn via the jugular vein, and animals were killed with an overdose of pentobarbital sodium; the penis was excised and kept in a Petri dish containing ice-cold modified Krebs solution and dissected free of the tunica albuginea and adherent tissues. In some experiments, the endothelium lining the lacunar spaces of the corpus cavernosum was disrupted and/or removed by detergent treatment (14, 36). The cavernosal tissues were used for isometric tension experiments, cGMP determination, and measurements of NOS activity, endogenous methylarginines, and l-arginine. For the measurements of NOS activity, endogenous methylarginines, and l-arginine, the cavernosal tissues were frozen in liquid nitrogen immediately after the dissection and stored at ~80°C.

Disruption of endothelium. To test whether endothelial function is involved in carbachol (CCh)-induced relaxation, the corpus cavernosum was treated with saponin to damage endothelial cells. Saponin has been shown to be a suitable concentration to cause deendothelialization without damaging vascular and corporal smooth muscle integrity or function (28, 36). The isolated penis from control rabbit was placed in the Petri dish containing modified Krebs solution, and 23-gauge needles were inserted into the proximal and distal ends of the right and left corpora, respectively. Five milliliters of saponin (100 μg/ml) was infused into the right corpora through the proximal needle. After 3 min, Krebs solution was infused through the proximal needle for washout. Using the same method, the left corpora were treated with saponin. The corpora cavernosa were then removed and tested for endothelial integrity.

Measurement of mechanical responses. Strips of corpus cavernosum tissue approximately 3 × 3 × 10 mm were submerged in the 10-ml organ chambers filled with oxygenated Krebs solution maintained at a temperature of 37 ± 0.5°C and continuously bubbled with 95% O2-5% CO2. One end of each strip was connected to a force-displacement transducer (TB-612T, Nihon Kohden Kogyo, Tokyo, Japan) to record the changes in isometric tension on a pen-writing oscillograph (R64, Rika Denki Kogyo, 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. All experiments were performed in the presence of guanethidine (10 μM) and indomethacin (1 μM). Tissues receiving electrical field stimulation (EFS) were treated with atropine (1 μM) in combination with guanethidine and indomethacin. EFS was performed with the aid of an electronic stimulator (SEN-3201, Nihon Kohden Kogyo), which delivered trains of rectangular pulses (supramaximum voltage, 0.3-ms duration at frequencies of 0.5–20 Hz for 10 s). Frequency-response curves to EFS or cumulative concentration-response curves to CCh were obtained during the contraction caused by phenylephrine (PE; 10 μM). After this, the tissues were washed repeatedly until the basal tension was regained (for 60 min) and treated with l-arginine (3 mM), Nω-monomethyl-l-arginine (l-NMMA; 1–100 μM), ADMA (1–100 μM), or symmetrical Nω,Nω-dimethyl-l-arginine (SDMA) (100 μM). Frequency-response curves to EFS or cumulative concentration-response curves to CCh were repeated during the contraction with PE (10 μM). After a 60-min washout period, the strips were contracted again with PE (10 μM), and the responses to sodium nitroprusside (SNP) were examined in the presence or absence of authentic NOS inhibitors or 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ, 10 μM), a novel and potent soluble guanylate cyclase inhibitor (34). Relaxations were expressed as a percentage of the PE (10 μM) contraction. The maximum response (Emax) and the concentration producing 50% of Emax (EC50) were obtained from the log concentration-response curves. The composition of the modified Krebs solution was as follows (in mM): 118.0 NaCl, 4.7 KCl, 1.2 MgSO4·7H2O, 2.5 CaCl2·2H2O, 1.2 KH2PO4, 25.0 NaHCO3, and 10.0 glucose.

Measurement of cGMP. The cGMP level was determined according to the method described previously (31, 32). Cavernosal preparations weighing ~20 mg that were not stretched or subjected to active tension were preincubated in modified Krebs solution for 20 min at 37°C in the organ chamber. After being washed with fresh Krebs, tissues were subjected to a second 30-min incubation. During this
incubation period, some tissues were treated with N\textsuperscript{G}-nitro-L-arginine (L-NNA; 100 \mu M). All tissues were then exposed to 10 \mu M PE for 20 min. After that, some tissues were subjected to 10-Hz EFS for 30 s and rapidly transferred into 10% TCA with liquid nitrogen to stop the reaction. In the other tissues, CCh (10 \mu M) was added for 5 min, and immediately after termination of the incubation the tissues were transferred into TCA with liquid nitrogen. All experiments were performed in the presence of 10 \mu M IBMX as a nonselective phosphodiesterase inhibitor. The net production of cGMP was expressed as the difference between the production with EFS or CCh (10 \mu M) and that with EFS or CCh (10 \mu M) plus 100 \mu M L-NNA as a NOS inhibitor (25). The basal cGMP level was taken as the value without EFS or CCh (10 \mu M) except for IBMX. The protein concentration of the sample was determined by use of the protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

**Measurement of NOS activity.** NOS activity in the corpus cavernosum was measured by determining the conversion of L-[\textsuperscript{3H}]arginine to L-[\textsuperscript{3H}]citrulline (32). The tissue was homogenized in a Polytron (Kinematica, Lucerne, Switzerland), four times at maximum speed for 15 s each, to a 25% homogenate in the buffer consisting of 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM diethiothreitol (DTT), 1 \mu M pepstatin, and 1 \mu M leupeptin (pH 7.2). The homogenate was centrifuged at 10,000 \texttimes g for 20 min at 4°C, and the supernatant was decanted from the pellet. Incubation mixtures consisted of 340 \mu l of the supernatant and 50 \mu l of the buffer described above containing 2 mM NADPH, 2 mM CaCl\textsubscript{2}, 30 U/ml calmodulin, 5 \mu M flavin adenine dinucleotide (FAD), 14 \mu M tetrahydrobipterin (BHi), 40 \mu M L-arginine, and 0.2 \mu Ci/ml L-[\textsuperscript{3H}]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\textsuperscript{+}) cation-exchange column (Wako Pure Chemicals, Tokyo, Japan). The difference between the wet weight and dry weight was assumed as the tissue water content (7, 32).

**Potencies of L-NNA, L-NMMA, and ADMA on the NOS activity.** Potencies of L-NNA, L-NMMA, and ADMA on the NOS activity were compared in terms of IC\textsubscript{50} values, which were determined by use of the protein assay reagent (Bio-Rad Laboratories), L-NNA (Research Biochemicals, Natick, MA), papaverine hydrochloride (Dainippon, Osaka, Japan), SNP (Wako Pure Chemicals, Tokyo, Japan), TTX (Sankyo, Tokyo, Japan), and L-[\textsuperscript{3H}]arginine (Amersham Pharmacia Biotech).

**Calculations and statistical analyses.** Deviations from the mean regarding the frequency-response curves to EFS or cumulative concentration-response curves to CCh were statistically analyzed by use of a factorial two-way ANOVA. Potencies of L-NNA, L-NMMA, and ADMA on the NOS activity were compared in terms of IC\textsubscript{50} values, which were concentrations 50% inhibition of NOS activity. The Michaelis-Menten constant (K\textsubscript{m}) and maximal velocity (V\textsubscript{max}) values were estimated by nonlinear analysis of the model V = (V\textsubscript{max} \times [S])/(K\textsubscript{m} + [S]), in which V is the initial velocity (pmol citrulline·mg protein\textsuperscript{−1}·min\textsuperscript{−1}) and [S] is the L-arginine concentration (in \mu M). 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 the CCI group were not different from those of the control group (Table 1). Arterial blood flows (ml/min) in the right and left iliac arteries of the CCI group were significantly lower (P < 0.05) compared with the control group (Table 1). Under the basal state, intracavernosal blood flow in the CCI group was not different from that in the control group. Intracavernosal injection of 5 mg papaverine increased intracavernosal blood flow from basal values in both groups. The peak intracavernosal blood flow value in the CCI

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>Body wt, kg</th>
<th>Iliac Artery Blood Flow, ml/min</th>
<th>Peak Intracavernosal Blood Flow, ml·min\textsuperscript{−1}·100 g\textsuperscript{−1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Papaverine</td>
<td>Basal</td>
<td>Papaverine</td>
</tr>
<tr>
<td>Control</td>
<td>90.6 ± 4.2</td>
<td>2.79 ± 0.5</td>
<td>25.3 ± 4.1</td>
<td>9.2 ± 2.8</td>
</tr>
<tr>
<td>CCI</td>
<td>93.1 ± 3.8</td>
<td>2.81 ± 0.3</td>
<td>26.2 ± 6.3</td>
<td>14.8 ± 3.6</td>
</tr>
</tbody>
</table>

Results are means ± SE of 5–7 determinations from different animals. MAP, mean arterial pressure; CCI, corpus cavernosal ischemia.

**Table 1. Mean arterial pressure, body weight, iliac artery blood flow, and intracavernosal blood flow in the control and CCI groups**

*Significantly different from corresponding value in the control (C) at P < 0.01. Papaverine, papaverine at a dose of 5 mg was injected into the corpus cavernosum.
group was significantly lower ($P < 0.01$) compared with the control group (Table 1).

**Endothelium-dependent relaxation.** CCh at a concentration of 0.3 μM produced a relaxation in the control cavernosal strip that was contracted with 10 μM PE. The relaxation in response to CCh was significantly ($P < 0.001$) decreased after the treatment with saponin. The degree of relaxation was determined to be 79.8 ± 5.3% ($n = 6$) in the control strips without saponin and 10.2 ± 4.3% ($n = 6$) after the saponin treatment. These results indicate that CCh produces an endothelium-dependent relaxation (EDR) in cavernosal strips. CCh produced an EDR in a concentration-dependent manner, which was abolished by L-NNA as an inhibitor of NOS and significantly ($P < 0.01$) decreased in the CCI group (Fig. 1A). The $E_{\text{max}}$ and $EC_{50}$ values were determined to be 80.2 ± 5.9% ($n = 7$) and 7.69 ± 0.09 ($n = 7$), respectively, in the control, and 49.7 ± 4.6% ($P < 0.01$ vs. control, $n = 6$) and 7.12 ± 0.06 ($P < 0.05$ vs. control, $n = 6$), respectively, in the CCI group.

**EFS-induced neurogenic relaxation.** The EFS at frequencies of 0.5–20 Hz produced transient relaxations in the presence of 1 μM atropine, 10 μM guanethidine, and 1 μM indomethacin, which were abolished by the pretreatment with 1 μM TTX or 100 μM L-NNA (Fig. 1B). The EFS-induced relaxations were significantly ($P < 0.01$) reduced in the CCI group compared with those in the control group (Fig. 1B). The $E_{\text{max}}$ value was determined to be 78.3 ± 7.2% at 10 Hz in the control ($n = 7$) and 43.2 ± 5.2% at 10 Hz in the CCI group ($P < 0.01$ vs. control, $n = 7$).

**Relaxation response to SNP.** SNP (0.01–100 μM) as a NO donor produced a concentration-dependent relaxation of the cavernosal strips. Degree of the relaxation was not different between two groups and greatly ($P < 0.01$) reduced by the pretreatment with 10 μM ODQ as an inhibitor of guanylate cyclase (34) (Fig. 2).

**cGMP production by the cavernosal specimens.** The basal cGMP level and the stimulated production of the nucleotide with CCh or EFS were significantly ($P < 0.05$ or $P < 0.01$) decreased in the CCI group. L-NNA at a concentration of 100 μM reduced the stimulated cGMP productions below the basal level in two groups. Net production was also significantly ($P < 0.01$) lower in the CCI group (Fig. 3).

**NOS activity in the corpus cavernosum.** NOS activity was greatly reduced by removing Ca$^{2+}$ and including 2 mM EDTA in the medium and in the presence of 100 μM L-NNA. NOS activity was mainly Ca$^{2+}$ dependent in both groups and significantly ($P < 0.01$) decreased in the CCI group. On the other hand, there was no significant change in the Ca$^{2+}$-independent NOS activity between the two groups (Fig. 4). In the experiments conducted at different concentrations of L-arginine (ranging from 0 to 3,000 μM), L-[3H]citrulline was generated according to the Michaelis-Menten kinetics (Fig. 5). The apparent $K_m$ and $V_{\text{max}}$ values were estimated to be $6.4 ± 1.2$ μM and $20.3 ± 2.1$ pmol citrulline·mg protein$^{-1}·$min$^{-1}$ ($n = 4$), respectively, in the control, and $9.8 ± 1.9$ μM and $13.7 ± 1.9$ pmol citrulline·mg protein$^{-1}·$min$^{-1}$ ($n = 4$), respectively, in the CCI group. The NOS in the CCI group exhibited significantly ($P < 0.05$) lower $V_{\text{max}}$ and higher $K_m$ values.

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**Fig. 1.** Carbachol (CCh)-induced (A) and electrical field stimulation (EFS)-induced cavernosal relaxations (B) during the contraction caused by phenylephrine (PE; 10 μM) in the control and corpus cavernosal ischemia (CCI) groups and effects of $N^G$-nitro-L-arginine (L-NNA; 100 μM) and L-arginine (3 mM). Results are expressed as a percentage of the PE contraction. Data points represent means ± SE of measurements in 5–7 strips from different animals. Vertical bars show SEs. **$P < 0.01$ (ANOVA) vs. corresponding curves in the untreated control and untreated CCI group.

**Fig. 2.** Sodium nitroprusside (SNP)-induced relaxation during the contraction caused by PE (10 μM) in rabbit cavernosal strips of control and CCI groups and effect of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM). Data points represent means ± SE of measurements in 5–7 strips from different animals. Vertical bars show SEs. **$P < 0.01$ (ANOVA) vs. corresponding curves in the untreated control and untreated CCI groups.
Effect of L-arginine on the CCh- and EFS-induced relaxations. The pretreatment with 3 mM L-arginine for 30 min significantly (P < 0.05) improved but did not normalize the impaired CCh- and EFS-induced relaxations in the CCI group. However, both the CCh- and EFS-induced relaxations in the control were unaffected by the excess L-arginine supplementation (Fig. 1, A and B).

Contents of L-arginine and methylarginines in the cavernosal specimens and plasma. Contents of L-arginine, L-NMMA, ADMA, and SDMA in the corpus cavernosum are summarized in Table 2. Contents of L-NMMA and ADMA in the CCI group were approximately twofold and threefold higher than those values in the control group, respectively (P < 0.01). There were no significant differences in the L-arginine and SDMA contents between the two groups. Because the tissue water content was determined to be 0.819 ± 0.006 ml/g wet weight in the control group (n = 7) and 0.803 ± 0.004 ml/g wet weight in the CCI group (n = 7), respectively, the apparent concentrations (in μM) of L-arginine, L-NMMA, ADMA, and SDMA at the lowest limit were calculated to be 328.4 ± 58.6, 0.40 ± 0.04, 0.52 ± 0.08, and 0.23 ± 0.04 in the control group and 352.4 ± 53.3, 0.84 ± 0.12, 1.70 ± 0.14, and 0.24 ± 0.08 in the CCI group, respectively. On the other hand, plasma concentrations of L-arginine and three methylarginine derivatives remained unchanged after the ischemia. These results are shown in Table 3.

Effects of authentic L-NMMA, ADMA, and SDMA on the CCh- and EFS-induced relaxations. As shown in Fig. 6, A and B, authentic L-NMMA (10 and 100 μM) and ADMA (10 and 100 μM), but not SDMA (100 μM; data not shown), inhibited the EDR produced by CCh and the relaxations produced by EFS in the control cavernosal strips. The inhibitory effects were undetectable in the presence of 3 mM L-arginine. Three authentic methylarginines at a concentration of 100 μM each failed to modify the SNP-induced relaxations in the two groups (data not shown).

Effects of authentic L-NMMA, ADMA, and SDMA on NOS. NOS prepared from the corpus cavernosum of both groups was inhibited by authentic L-NMMA and

Table 2. Contents of L-arginine, L-NMMA, ADMA, and SDMA in the corpus cavernosum in the control and CCI groups

<table>
<thead>
<tr>
<th>Group</th>
<th>L-Arginine, pmol/g wet wt</th>
<th>L-NMMA, pmol/g wet wt</th>
<th>ADMA, pmol/g wet wt</th>
<th>SDMA, pmol/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>269 ± 48</td>
<td>325 ± 36</td>
<td>428 ± 62</td>
<td>185 ± 29</td>
</tr>
<tr>
<td>CCI</td>
<td>283 ± 51</td>
<td>675 ± 98</td>
<td>1,362 ± 112</td>
<td>193 ± 68</td>
</tr>
</tbody>
</table>

Results are given as means ± SE of 6–7 determinations from different animals. L-NMMA, N\textsuperscript{G}-monomethyl-L-arginine; ADMA, asymmetric N\textsuperscript{G}, N\textsuperscript{G}-dimethyl-L-arginine; SDMA, symmetric N\textsuperscript{G}, N\textsuperscript{G}-dimethyl-L-arginine. *Significantly different from corresponding value in the control at P < 0.01.
ADMA in a concentration-dependent manner but not by SDMA even in a high concentration of 100 μM (Fig. 7). The inhibitory potencies of L-NMMA and ADMA were compared with that of L-NNA in terms of IC50 (in μM), which were determined to be 7.76 for L-NMMA, 12.7 for ADMA, and 0.79 for L-NNA in the control group. The rank order of inhibitory potency was L-NNA > L-NMMA > ADMA (P < 0.01, L-NNA vs. L-NMMA, L-NNA vs. ADMA; P < 0.05, L-NMMA vs. ADMA). The IC50 values (in μM) of L-NMMA, ADMA, and L-NNA were 8.53, 14.6, and 0.93, respectively, in the CCI group. These values were not significantly different from the corresponding value in the control.

**DISCUSSION**

Inhibitory effects of saponin treatment or L-NNA on the CCh-induced relaxation and of TTX or L-NNA on the EFS-induced relaxation in both groups suggest that the former relaxation is characterized to be endothelium and NO dependent and the latter relaxation is characterized to be neurogenic and NO dependent.

The current experiments demonstrated that the cavernosal relaxations caused by CCh and EFS, but not by SNP, were impaired in the CCI group, assuming that the ischemia results in a decreased endothelium-dependent and neurogenic NO production and/or release. This assumption is partially supported by the finding that the basal and stimulated cGMP productions with CCh or EFS were significantly reduced in the CCI group. It is well established that NO binds the heme group of soluble guanylate cyclase (13, 19), thereby stimulating the production of cGMP. Consequently, the generation of cGMP is widely used as an index of NO biosynthesis (20, 29).

In the current study, the NOS activity was significantly lower in the ischemic cavernosum. The findings 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) suggest that the reduced NOS activity after ischemia would be a reflection of the decreased NOS protein. This speculation may be supported by the demonstration that the pulmonary hypertension induced after chronic ischemia is associated with a decreased endothelial NOS (eNOS) activity resulting from diminished eNOS protein and mRNA expressions (8, 16). However, the current experiments demonstrated that L-arginine supplementation significantly restored the impaired endothelium-dependent and neurogenic relaxations in the CCI group. It is reportedly known that L-arginine supplementation re-

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**Table 3. Plasma concentrations of L-arginine, L-NMMA, ADMA, and SDMA in the control and CCI groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>L-Arginine, nmol/ml</th>
<th>L-NMMA, pmol/ml</th>
<th>ADMA, pmol/ml</th>
<th>SDMA, pmol/ml</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>158 ± 19</td>
<td>110 ± 13</td>
<td>642 ± 49</td>
<td>363 ± 43</td>
</tr>
<tr>
<td>CCI</td>
<td>163 ± 24</td>
<td>136 ± 23</td>
<td>675 ± 56</td>
<td>298 ± 31</td>
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</table>

Results are given as means ± SE of 6–7 determinations from different animals.
stores the impaired endothelium-dependent pulmonary vasodilation in chronically hypoxic rats (15) and induces NO-dependent vasodilation in patients with critical limb ischemia (9). Also, L-arginine content in the corpus cavernosum remained unchanged after ischemia, and the apparent concentration of L-arginine, which was estimated on the basis of tissue water content, was in the 300- to 400-μM range in both groups. Furthermore, the determined $K_m$ value for the NOS activity was $\sim 10$ μM in the CCI group. That is, although an apparent L-arginine level is enough to saturate NOS in the CCI group, the capability of the cavernosum to produce and/or release NO was significantly enhanced by L-arginine, which has been in line with the finding reported as the “arginine paradox” (17). Two possibilities are considered to explain the discrepancy: 1) accumulation of endogenous NOS inhibitors (39) 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-NMMA and ADMA in plasma (10, 11) and tissue (6, 31, 32) might explain in part the mechanism decreasing NO production. The current experiments demonstrated that contents of L-NMMA and ADMA in the cavernosal tissue were significantly increased after ischemia without changing the plasma concentration of methylarginines. Also, authentic L-NMMA and ADMA displayed a concentration-dependent inhibition of endothelium-dependent and neurogenic relaxations in the control group, and this inhibition was undetectable in the presence of 3 mM L-arginine. The above findings suggest that the reduced production and/or release of NO after ischemia is at least partly due to the local accumulation of the endogenous NOS inhibitors, and L-arginine supplementation would act by competing with these inhibitors and, in turn, restore the NO production.

An important question is whether the concentrations of L-NMMA and ADMA are high enough to inhibit the NOS activity. Apparent concentrations (in μM) of L-NMMA and ADMA, which were calculated on the basis of tissue water content, were 0.4 and 0.5 in the control and 0.8 and 1.7 in the CCI group, respectively. NOS that had been prepared from control cavernosal tissue was inhibited by authentic L-NMMA (0.8 μM) plus ADMA (1.7 μM) by $\sim 28\%$, while the enzyme activity was inhibited by authentic L-NMMA (0.4 μM) plus ADMA (0.5 μM) by $\sim 4\%$ (Masuda, unpublished observation). Furthermore, because methylarginines are concentrated within cells (30), the intracellular concentrations of these inhibitors would be possibly higher than the estimated concentrations based on the tissue water content. However, it should be noted that even in the cavernosum after ischemia, the ratio of endogenous inhibitors to L-arginine was small, suggesting endogenous L-arginine would overcome any competitive inhibition of NOS by L-NMMA and ADMA. Nonetheless, L-arginine supplementation augmented the cavernosal relaxations in the CCI group. One possible explanation for this discrepancy may be based on the cellular compartmentalization of L-arginine. 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 cavernosal function. On the other hand, if extracellular L-arginine transported into the cell mediated via system $\gamma^+$ transporter (12) 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 $\gamma^+$ arginine transporter and suggest that L-arginine may be directly delivered from the extracellular pool to eNOS via the $\gamma^+$ transporter (33). Further studies of the intracellular localization of transporter, methylarginines, neuronal NOS (nNOS), and eNOS will be required to elucidate the detailed mechanism of NOS inhibition with methylarginines in the corpus cavernosum.

The mechanisms increasing the contents of L-NMMA and ADMA are not clarified in a detailed manner in the present experiments. L-NMMA, ADMA, and SDMA enter cells through the cationic amino acid transporters known collectively as system $\gamma^+$, and the three methylarginines compete with each other and with L-arginine for transport (12). The system $\gamma^+$ transport has been located in vascular endothelial cells (33) and neuronal cells (37). Dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes L-NMMA and ADMA, is widely distributed in tissues (24) probably including the corpus cavernosum. Recently, it has been reported that two DDAH isoforms (DDAH I and DDAH II) were identified in human tissues, in which DDAH I predominates in tissues expressing nNOS and DDAH II predominates in tissues expressing eNOS (26). In the corpus cavernosum, DDAH I and DDAH II may be coexpressed and regulate endogenous NOS inhibitors in affecting eNOS and nNOS, respectively. This possibility may 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. Although SDMA does not inhibit all three isoforms of NOS (6, 32, 40) and is not a substrate for DDAH (30, 35), SDMA as well as L-NMMA and ADMA are all substrates for $\gamma^+$ transporter (12, 38) and are equally potent inhibitors of L-arginine uptake (38), suggesting that SDMA may affect the NO pathway via regulating L-arginine transport.

It has been reported that the $\gamma^+$ transporter is inducible and is upregulated by certain proinflammatory cytokines including tumor necrosis factor-$\alpha$ and interleukin-1β (12), and lipoproteins and cytokines increase ADMA by reducing DDAH activity in the human umbilical vein endothelial cells (22). Several cytokines in the ischemic state would modify the methylarginine levels in the corpus cavernosum.

In conclusion, this study suggests that impairment of endothelium-dependent and neurogenic cavernosal relaxation with ischemia possibly produces disruptions in the integrity of the NO pathway. Accumulation of L-NMMA and ADMA as endogenous NOS inhibitors and decreased NO activity are closely related to the
reduction of NO production/release in the corpus cavernosum with ischemia.

Perspectives

L-NMMA and ADMA are endogenously produced inhibitors of NOS. In the plasma, ADMA and SDMA are the major circulating forms of methylarginines, whereas the L-NMMA level is considerably lower. An elevated level of ADMA has been found in multiple disorders where NOS dysfunction has been implicated, such as hypercholesterolemia, atherosclerosis, congestive heart failure, renal failure, hypertension, thrombotic microangiopathy, peripheral arterial occlusive disease, and preeclampsia. Therefore, many researchers pay little attention to L-NMMA as an endogenous disease, and preeclampsia. Therefore, many research in Japan, and by the New Drug Research Foundation in Japan.

REFERENCES