Alterations of NOS, arginase, and DDAH protein expression in rabbit cavernous tissue after administration of cigarette smoke extract

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Imamura M, Waseda Y, Marinova GV, Ishibashi T, Obayashi S, Sasaki A, Nagai A, Azuma H. Alterations of NOS, arginase, and DDAH protein expression in rabbit cavernous tissue after administration of cigarette smoke extract. Am J Physiol Regul Integr Comp Physiol 293: R2081–R2089, 2007. First published September 19, 2007; doi:10.1152/ajpregu.00406.2007.—Cigarette smoking is an independent risk factor for vasculogenic erectile dysfunction (ED). Nitric oxide (NO) has been demonstrated to be the principal mediator of cavernous smooth muscle relaxation and penile erection. Therefore, we examined whether or not enzyme activities and factors involved in the NO generation pathway are affected in rabbit corpus cavernosum after administration of nicotine- and tar-free cigarette smoke extract (CSE). CSE was prepared by bubbling a stream of cigarette smoke into phosphate-buffered saline. CSE was injected subcutaneously into adult male rabbits once a day for 5 wk. In the CSE group, significantly decreased cyclic GMP production as a marker of NO generation was associated with attenuated overall nitric oxide synthase (NOS) activity, enhanced arginase activity, accumulation of endogenous NOS inhibitors such as monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA), and decreased dimethylarginine dimethylaminohydrolase (DDAH) activity as a metabolizing enzyme of endogenous NOS inhibitors. Neuronal NOS (nNOS) and DDAH I protein expression were decreased without altering endothelial NOS expression, while arginase I expression was upregulated. These results suggest that impaired NO production would result from blunted NOS activity, which is possibly brought about by the downregulation of nNOS protein, accumulation of endogenous NOS inhibitors, and enhanced arginase activity together with upregulation of arginase I protein in cavernous tissue. The impaired DDAH activity due to decreased expression of DDAH I protein would result in an accumulation of endogenous NOS inhibitors with CSE. These alterations may be relevant to induction of the erectile dysfunction following CSE.

erectile dysfunction; nitric oxide generation pathway; anti-asymmetric dimethylarginine antibody; immunohistochemistry; Western blot

ACCORDING TO FELDMAN ET AL. (18) and MCVARY ET AL. (39), THERE ARE STRONG PARALLELS AND SHARED RISKS AMONG SMOKING, CORONARY ARTERY DISEASE, ATHEROSCLEROSIS, AND ERECTILE DYSFUNCTION (ED). CLINICAL AND BASIC SCIENCE STUDIES PROVIDE STRONG INDIRECT EVIDENCE THAT SMOKING MAY AFFECT PENILE ERECTION BY IMPAIRMENT OF ENDOTHELIUM-DEPENDENT SMOOTH MUSCLE RELAXATION. NUMEROUS STUDIES HAVE SHOWN ENDOTHELIAL DYSFUNCTION IN ED IN SMOKERS AND EXPERIMENTAL MODELS. A RECENT REVIEW SUMMARIZED SOME PROPOSED MECHANISMS SUCH AS THE WAY IN WHICH FREE RADICALS AND AROMATIC COMPOUNDS DECREASE THE ENDOTHELIAL SYNTHESIS OF NITRIC OXIDE (NO), CAUSING IMPAIRED ENDOTHELIUM-DEPENDENT RELAXATION OF ARTERIES, WHICH IS THE EARLIEST CLINICAL SIGN OF ENDOTHELIAL DYSFUNCTION (45). NO HAS BEEN DEMONSTRATED TO BE THE PRINCIPAL MEDIATOR OF Cavernous smooth muscle relaxation and penile erection (4, 14, 24, 30, 46–48). HOWEVER, THE PRECISE MECHANISMS OF IMPAIRMENT OF NO PRODUCTION WITH CIGARETTE SMOKING HAVE NOT BEEN FULLY ELUCIDATED.

NO PRODUCTION, ON ONE HAND, DEPENDS ON NITRIC OXIDE SYNTHASE (NOS) ACTIVITY AND NOS PROTEIN EXPRESSION. THERE ARE REPORTS DESCRIBING THAT NOS ACTIVITY WAS DECREASED IN THE HUMAN PENIS WITH DIABETES (8) AND IN THE AGED RAT AORTA (7). XIE ET AL. (56) DEMONSTRATED THAT SMOKING REDUCES PENILE NOS ACTIVITY AND NEURONAL NOS EXPRESSION WITHOUT CHANGES IN ENDOTHELIAL NOS EXPRESSION IN THE RAT. ON THE OTHER HAND, NO PRODUCTION ABSOLUTELY DEPENDS ON THE AVAILABILITY OF L-ARGININE TO NOS, SINCE NOS SHARED L-ARGININE AS A COMMON SUBSTRATE WITH ARGINASE (11, 42). IN THIS REGARD, L-ARGININE CATABOLISM VIA THE ARGINASE PATHWAY CAN ACT AS AN ENDGENOUS NEGATIVE CONTROL SYSTEM TO REGULATE OVERALL NO PRODUCTION. RECENT STUDIES HAVE DEMONSTRATED THAT ARGINASE ACTIVITY IN THE CORPUS CAVERNOSUM IS INCREASED BY HYPERGLYCEMIA AND AGING (8, 48). IN ADDITION, ELEVATED ENDGENOUS NOS INHIBITORS SUCH AS MONOMETHYLARGININE (MMA) AND ASYMMETRIC DIMETHYLARGININE (ADMA) MAY BE ANOTHER IMPORTANT FACTOR IN IMPAIRMENT OF NO PRODUCTION (5, 54). THESE Methylarginines COMPETITIVELY INHIBIT NOS ACTIVITY AND IMPAIR NO PRODUCTION IN ENDOTHELIAL CELLS. AN ELEVATED LEVEL OF ADMA HAS BEEN FOUND IN MULTIPLE DISORDERS IN WHICH NOS DYSFUNCTION HAS BEEN IMPLICATED, SUCH AS HYPERCHOLESTEROLEMIA (8), RENAL FAILURE (54), HYPERTENSION (53), AND HYPERGLYCEMIA (56). WE HAVE DEMONSTRATED (37) THAT THE CONTENTS OF MMA AND ADMA WERE INCREASED IN THE CORPUS CAVERNOSUM AND NO PRODUCTION WAS IMPAIRED AFTER ISCHEMIA IN THE RABBIT. MMA AND ADMA ARE METABOLIZED BY DIMETHYLARGININE DIMETHYLAMINOHYDROLASE (DDAH) TO L-CITRULLINE AND METHYLAMINES (43). THEREFORE, IMPAIRED DDAH ACTIVITY RESULTS IN THE ACCUMULATION OF ENDGENOUS NOS INHIBITORS (34, 35), THEREBY IMPAIRING NO PRODUCTION.

ON THE BASIS OF THE FINDINGS DESCRIBED ABOVE, THE PRESENT EXPERIMENTS WERE DESIGNED TO INVESTIGATE WHETHER OR NOT ENZYME ACTIVITIES AND FACTORS INVOLVED IN THE NO GENERATION PATHWAY ARE INRECTED IN RABBIT CORPUS CAVERNOSUM AFTER ADMINISTRATION OF CIGARETTE SMOKE EXTRACT (CSE) (22, 29, 58, 59) FOR A BETTER UNDERSTANDING OF THE MECHANISMS CAUSING ED WITH CIGARETTE SMOKING.

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MATERIALS AND METHODS

Experimental protocol. Nine-week-old Japanese White male rabbits were used for the present experiments and randomly divided into two groups. Sixteen rabbits were injected subcutaneously with nicotine- and tar-free CSE (22, 58, 59) once a day for 5 wk, and 15 rabbits injected with phosphate-buffered saline (PBS) served as control. The rabbits were killed by exsanguination from femoral arteries under anesthesia with intravenous pentobarbital sodium (25 mg/kg). The penis was excised, and the cavernous tissues were dissected free from the surrounding tunica albuginea. Approximately 5 mg of fresh cavernous specimen was used for the determination of cyclic GMP production. For measurement of NOS, arginase, and DDAH activities, expression of endothelial NOS, neuronal NOS, arginase I and II, and DDAH I and II proteins and tissue content of endogenous methyl-arginines and l-arginine, the remaining cavernous tissues were stored at −80°C until assayed.

This study complied with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. The experimental protocol was approved by the Animal Welfare Regulation Committee of the Tokyo Medical and Dental University.

Preparation of CSE. CSE was prepared according to the method described by Yokode et al. (59), Yamaguchi et al. (58), and Hoshino et al. (22). Briefly, a stream of cigarette smoke was bubbled into PBS (3 Frontier Light cigarettes/ml; Japan Tobacco). To remove tar and nicotine, the stream of smoke was passed through a Cambridge filter before being bubbled under gentle aspiration. It took ~5 min to consume one cigarette. The CSE solution (pH 7.0) was sterilized through a 0.22-μm pore filter (Millipore).

Measurement of mechanical responses. Changes in isometric tension of the ~3 × 3 × 10-mm cavernous specimens in response to electrical field stimulation (EFS) were examined according to a method described previously (37, 38, 48). Under contraction caused by 10 μM phenylephrine, EFS was performed with the aid of an electronic stimulator (SEN-3201, Nihon Kohden Kogyo, Tokyo, Japan), which delivered trains of rectangular pulses (supramaximum voltage, 0.3-ms duration at frequencies of 0.1–2.0 Hz for 10 s). The effects of 0.3% and 1.0% CSE, 100 μM nitroarginine, and 1 μM tetrodotoxin were examined after control responses were obtained. The composition of 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 glucose.

Measurement of cyclic GMP. Cyclic GMP production was determined according to a method described previously (38). In brief, cavernous specimens weighing ~5 mg were preincubated in modified Krebs solution for 35 min at 37°C and transferred into a fresh modified Krebs solution, followed by a further 30-min incubation. Specimens were then rapidly transferred into 10% trichloroacetic acid (TCA) with liquid nitrogen to stop the reaction. Phenylenediamine at a concentration of 1 μM and 1 μM carbachol were added immediately and 15 min after transfer of the specimens into the fresh Krebs solution. All experiments were performed in the presence of 10 μM 3-isobutyl-1-methylxanthine (IBMX) as a nonselective inhibitor of phosphodiesterases (PDEs). Cyclic GMP levels were determined with a radioimmunoassay kit (Yamasa Shoyu, Tokyo, Japan). Nitroarginine (100 μM) was used to assess the involvement of activation of NOS in generation of cyclic GMP. The net production of cyclic GMP was expressed as the difference between production with phenylephrine plus carbachol and that with phenylephrine plus carbachol plus nitroarginine. The basal cyclic GMP level was taken as the value without any agonist and antagonist except for IBMX. Cyclic GMP is expressed as picomoles per milligram of protein. The amount of protein was determined with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Preparation of crude NOS and arginine. After thawing, cavernous specimens were homogenized in a Polytron (Kinematica, Lucerne, Switzerland) at maximum speed three times for 20 s each to a 20% homogenate in a buffer consisting of 50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 μM peptide A, and 2 μM leupeptin (pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was decanted from the pellet.

NOS activity. NOS activity was measured by determining the conversion of L-[14C(U)]arginine (specific activity: 11,581 MBq/μmol) to L-[14C(U)]citrulline as described previously (38). In brief, incubation mixtures consisted of 100 μl of the supernatant obtained according to the method described above and 20 μl of buffer containing 1 mM NADPH, 4 μM flavin adenine dinucleotide (FAD), 4 μM flavin mononucleotide (FMN), 10 μM tetrahydrobiotin (BHL), 1 mg/l calmodulin, 2.5 mM CaCl₂, and 0.1 μM/l l-[14C(U)]arginine. The reaction mixture was incubated at 37°C for 1 h in a shaking water bath. The incubation was terminated by keeping the tubes on ice for 5 min. Samples were then applied to 1-ml columns of Dowex AG50W-X8 (Na⁺ form) to remove unmetabolized l-[14C(U)]arginine. The columns were then washed with 1.5 ml of distilled water, and L-[14C(U)]citrulline was quantified in the flow-through fraction with a liquid scintillation counter (Tri-Carb 2750TR/LL, Packard Instrument, Meriden, CT). NOS activity is expressed as picomoles of L-citrulline per milligram of protein per 60 min. The activity was also measured in the presence of 100 μM nitroarginine. The net activity was expressed as the difference between activities in the absence and presence of nitroarginine.

Arginase activity. Arginase activity was measured by determining the conversion of L-[14C(U)]guanidoarginine to L-[14C(U)]urea as previously described (38). In brief, aliquots of tissue extract were incubated for 120 min at 37°C in buffer containing 10 mM Tris·HCl, 1 mM MnCl₂, and 0.08 μCi/ml of L-[14C(U)]guanidoarginine (pH 9.6). Reactions were terminated by adding 400 μl of ice-cold stop buffer containing 250 mM sodium acetate and 100 mM urea (pH 4.5). After addition of water, samples were passed through a column containing Dowex 50W-X8 resin (Dow Chemical, Midland, MI) to remove unmetabolized L-[14C(U)]guanidoarginine. The columns were then washed with water, and 100 μl of buffer containing 10 mM Hepes, pH 7.5, containing 1 mg/l pepstatin A, and 2 μM leupeptin. The enzyme was determined in the presence and absence of 10 μM N³-hydroxy-nor-l-arginine (nor-NOHA) as an arginase inhibitor (12). Results are given as the net activity calculated from the difference of the activities in the presence and absence of nor-NOHA.

DDAH activity. DDAH activity was measured by monitoring the formation of L-[14C(U)]citrulline from N⁶-monomethyl-L-[14C(U)]arginine (L-[14C(U)]NMMA) according to a method described previously (34, 42, 44). After thawing, the tissue was homogenized in medium consisting of 0.1 M sodium phosphate buffer (pH 6.5), 1 mM PMSF, 1 μM pepstatin A, and 2 μM leupeptin. The homogenate was then centrifuged, and supernatant was collected. The reaction mixtures consisted of 90 μl of supernatant, 0.01 μCi L-[14C](H)NMMA, 0.1 μM MMA, and 20 mM EDTA. DDAH activity was assayed in the same manner as the determination of NOS and arginase activities after the reaction mixture was incubated at 37°C for 120 min.

Western blot analyses of endothelial and neuronal NOS, arginase I and II, and DDAH I and II. The cavernous tissue was homogenized in a buffer containing 250 mM sucrose, 25 mM imidazole, 1 mM EDTA, and one-tenth volume of a protease inhibitor cocktail (Sigma, St. Louis, MO). After homogenization, samples were sonicated, centrifuged at 10,000 g for 10 min to clear debris, and frozen at −80°C until assay for protein content (16). The protein concentration was determined for each sample with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). Proteins were separated on sodium dodecyl sulfate (SDS)-PAGE and electrotransferred to poly-
vinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membranes were probed with primary antibodies against endothelial NOS (BD Biosciences, San Jose, CA), neuronal NOS (BD Biosciences, arginase I (Santa Cruz Biotechnology, Santa Cruz, CA), arginase II (Santa Cruz Biotechnology), DDAH I (Orbigen, San Diego, CA), or DDAH II (Abcam, Cambridge, MA), followed by addition of horseradish peroxidase (HRP)-conjugated secondary antibodies. In all immunoblottings except for endothelial NOS, an immunoreaction enhancer solution (Can Get Signal, TOYOBO, Osaka, Japan) was used. After stripping, all membranes were probed with primary antibody against β-actin (Sigma) to obtain signals of internal standard. Antigenic detection was visualized by enhanced chemiluminescence (ECL) or ECL plus Western blotting detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ) with exposure to X-ray film. Densitometry was performed with a charge-coupled device camera and Dolphin 1D software (Wealtec, Sparks, NV).

Immunohistochemical localization of ADMA. Cavernous specimens were fixed in 10% paraformaldehyde in PBS (pH 7.4). The specimens were embedded in regular paraffin wax and cut into 3-μm-thick sections. Tissue sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by autoclave at 121°C for 15 min in the citrate buffer. Endogenous peroxidase was blocked by 3% H2O2 in methanol. Then 2% goat serum was applied for 30 min, followed by incubation at 4°C overnight with primary mouse anti-ADMA monoclonal antibody (MAb) (dilution 1:50 in PBS). Sections were then incubated in peroxidase-labeled antibody [Dako Envision kit, peroxidase/diaminobenzidine (DAB); Dako, Carpinteria, CA] at room temperature for 30 min. We used anti-von Willebrand factor antibody (rabbit polyclonal antibody) to confirm the endothelial cells. Antigen retrieval was performed by incubation at 90°C for 5 min in the citrate buffer. The sensitivity of anti-ADMA MAb was enhanced by a tyramine signal amplification technique (1). Sections were incubated with HRP-conjugated streptavidin (dilution 1:100) for 30 min, followed by a 10-min reaction with biotinylated tyramine (dilution of 1:50) and a secondary 30-min incubation with HRP-conjugated streptavidin according to the manufacturer’s instructions (NEN, Boston, MA). Immunoreactivity was visualized by simple staining with DAB solution (Nichirei, Tokyo, Japan) and Mayer’s hematoxylin (Wako, Osaka, Japan). Mayer’s hematoxylin (Wako, Osaka, Japan) was used for counterstaining. The sections were dehydrated and mounted with malinol (Muto Pure Chemicals, Tokyo, Japan). After stripping, all membranes were probed with anti-ADMA MAb enhanced by a tyramine signal amplification technique (1). Sections were incubated with HRP-conjugated streptavidin according to the manufacturer’s instructions (NEN, Boston, MA). Immunoreactivity was visualized by simple staining with DAB solution (Nichirei, Tokyo, Japan) and Mayer’s hematoxylin (Wako, Osaka, Japan). Mayer’s hematoxylin (Wako, Osaka, Japan) was used for counterstaining. The sections were dehydrated and mounted with malinol (Muto Pure Chemicals, Tokyo, Japan). The omission of the primary antibodies for von Willebrand factor and ADMA served as a negative control; no specific color was observed in either one. Determination of the number of positive cells was performed by one investigator, who was blinded to the experimental group. Anti-ADMA MAb was prepared according to a method described previously (25). Brown-staining cells were defined as positive cells and counted under the microscope (×400). The percentage of positive cells was calculated.

Contents of L-arginine and methylarginines. Contents of L-arginine, MMA, ADMA, and symmetric dimethylarginine (SDMA) in cavernous tissue were determined by means of high-performance liquid chromatography (HPLC) as reported previously (6, 36). Cavernous tissue (100 mg wet wt) was minced with scissors, homogenized, and sonicated at 50 W, 28 KHz for 15 s (3 times at 1-min intervals) in ice-cold 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) and centrifuged at 10,000 g for 20 min at 4°C to separate the supernatant. After addition of TCA in a final concentration of 5%, the supernatant was centrifuged at 1,600 g for 15 min to obtain the test solution for determination of L-arginine and methylarginines with the aid of HPLC. Results are given as nanomoles per gram of wet weight for L-arginine and picomoles per gram of wet weight for methylarginines.

To determine the concentrations of MMA and ADMA to inhibit cavernous NOS activity by 50%, concentration-inhibition curves were obtained at different concentrations of these inhibitors (0.3–30 μM at 0.5 log unit increasing concentration).

**Statistical analysis.** All results are expressed as means ± SE. Statistical analyses were carried out by one-way ANOVA or Student’s t-test. Difference was regarded as significant at *P* < 0.05.

**RESULTS**

**Baseline data.** Subcutaneous injection of CSE in a volume of 3 ml/animal once a day for 5 wk did not affect the body weight changes of the rabbits. The mean body weight at the beginning of the experiments (9 wk of age) was determined to be 1.46 ± 0.06 kg in the PBS group (*n* = 15) and 1.57 ± 0.05 kg in the CSE group (*n* = 16). Body weight increased to 2.14 ± 0.04 kg in the PBS group (*n* = 15) and 2.19 ± 0.02 kg in the CSE group (*n* = 16) 5 wk later. The corresponding values were not significantly different between the two groups. The behavior of rabbits after injection of CSE appeared to be usual and was not different from the control group given PBS.

**Inhibitory effect of CSE on relaxation in response to EFS.** Frequency-dependent relaxation of the cavernous specimen was caused by EFS during the contraction in response to 10 μM phenylephrine and abolished in the presence of 100 μM nitroarginine or 1 μM tetrodotoxin, indicating that the relaxation was neurogenic and NO mediated. CSE at final concentrations of 0.3% and 1.0% definitely attenuated the relaxation in a concentration-dependent and reversible manner (Fig. 1).

**Cyclic GMP production.** Net cyclic GMP production under stimulation with carbachol was significantly (*P* < 0.005) decreased in the CSE group (Fig. 2), although the reduction in the basal level was slight (*n* = 4 each). The cyclic GMP production stimulated with carbachol was almost completely inhibited in the presence of 100 μM nitroarginine as an inhibitor of NOS.

**NOS activity and NOS protein expression.** NOS activity in the cavernous specimens of the CSE group was determined to be 123.0 ± 12.7 nmol citrulline/mg protein (*n* = 5), which was significantly (*P* < 0.005) lower than that in control specimens.
Western blot analyses revealed that expression of neuronal NOS protein expression was decreased in specimens of the CSE group without change in endothelial NOS expression (Fig. 3B).

**Arginase activity and protein expression.** The arginase activity of 65.7 ± 3.1 pmol urea/mg protein in the control group (n = 5) was significantly (P < 0.05) increased to 137.4 ± 25.1 pmol urea/mg protein (n = 5) in the CSE group (Fig. 4A). Nor-NOHA at a concentration of 10 μM produced almost complete inhibition of arginase activity in the two groups. Arginase I protein expression was significantly (P < 0.005) upregulated in the CSE group (Fig. 4B). In contrast to arginase I, arginase II protein expression was indistinctly detectable and remained unaffected even in the CSE group (data not shown).

**DDAH activity and protein expression.** DDAH activity of 2,093 ± 54.2 fmol citrulline/mg protein (n = 4) in the control group was significantly (P < 0.005) decreased in cavernous specimens of the CSE group (1,003 ± 163 fmol citrulline/mg protein, n = 4) (Fig. 5A). In addition to changes in the enzyme activity, only DDAH I was significantly (P < 0.05) downregulated in the CSE group (Fig. 5B), although DDAH I and II protein expression was detectable in the two groups.

**Immunohistochemical localization of ADMA.** Immunohistochemical analyses revealed that the number of arteriolar endothelial cells positive to ADMA antibody in the cavernous tissue was significantly increased in the CSE group (Fig. 6).

**L-Arginine and methylarginine content in cavernous tissue.** L-Arginine, MMA, ADMA, and SDMA contents in cavernous tissue were determined with the aid of HPLC. The contents of MMA and ADMA were significantly (P < 0.005) increased in the CSE group, as shown in Table 1. In contrast, the contents of L-arginine and SDMA remained unchanged even in the CSE group.
ADMA, which had been obtained from the IC50 experiments, were determined to be 2.7 and 15.4 M in the CSE group (n = 3) and 1.04 ± 0.06 and 3.06 ± 0.28 M in the PBS group (n = 3), respectively. To estimate the inhibitory effect on cavernous NOS activity at determined apparent concentrations of MMA and ADMA, we used the equations y = 36.3x + 34.3 for MMA and y = 40.2x + 2.3 for ADMA, which had been obtained from the IC50 experiments for cavernous NOS, where y is inhibition % for NOS activity and x is log concentrations of inhibitors. The inhibition with MMA was 12.1 ± 1.4% at 0.25 ± 0.02 M in the PBS group and 34.8 ± 0.9% at 1.04 ± 0.06 M in the CSE group (n = 3 each). Inhibition with ADMA was not found at 0.59 ± 0.05 M in the PBS group and 21.7 ± 1.7% at 3.06 ± 0.28 M in the CSE group (n = 3 each). The inhibitory effects of these NOS inhibitors became significantly higher (P < 0.005) in the CSE group. The IC50 values of MMA and ADMA for cavernous NOS were determined to be 2.7 and 15.4 M, respectively.

**DISCUSSION**

It is well established that NO binds the heme group of soluble guanylyl cyclase to produce cyclic GMP, which is widely used as an index of NO biosynthesis (17, 23). The present experiments demonstrated a significantly impaired cyclic GMP production in the cavernous specimens of the CSE group. Furthermore, cyclic GMP production under stimulation with carbacbol in the control and CSE groups was almost abolished in the presence of nitroarginine as an inhibitor of NOS, leading us to assume that the cyclic GMP production in the cavernous tissue depends on NO that had been generated through activation of NOS. Regarding the mechanisms of impairment of NO production by CSE, we examined whether or not alterations of NOS, arginase, and DDAH activities, expression of these enzyme proteins, and contents of endogenous NOS inhibitors are involved (see Fig. 7).

The impaired cyclic GMP production following CSE exposure was associated with decreased overall NOS activity and neuronal NOS protein expression, whereas endothelial NOS protein expression remained unaffected. Similar results have been reported previously, that is, Xie et al. (56) demonstrated that smoking attenuated penile NOS activity and neuronal NOS expression without altering endothelial NOS expression in the rat. Therefore, it appears likely that the impaired NO production partially results from attenuated overall NOS activity, which would be brought about by the downregulation of neuronal NOS protein expression. However, it is still possible that free radical(s) rather than NO is produced as a result of “NOS uncoupling” (19) due to accumulation of endogenous NOS inhibitors as described below. In this study, we used detergent-free buffer and sonication to prepare samples for endothelial NO expression (16), which is mainly localized in caveolar membrane (49, 51). Although the membrane fraction including endothelial NOS is expected to be obtainable by this method (16, 50, 51), investigation with samples obtained by different methods would be of great interest, since the components of samples could be variable depending on the methods used to prepare samples (51). The NOS isoform responsible for impaired NOS activity by administration of CSE might be still controversial and requires more detailed investigation.

Arginase has been reported to be responsible for regulating NO synthesis by modulating intracellular L-arginine availability. Arginase was found in human corpus cavernosum, and the inhibition of this enzyme enhanced NO-dependent relaxation of cavernous smooth muscle (31, 48). Accelerated arginase activity was reported in aged cavernous tissues (48) and human cavernosum with diabetes mellitus (8). Therefore, we investigated whether accelerated arginase activity is implicated in impairment of NO production in CSE-injected cavernous tissues. Arginase activity was significantly enhanced in the CSE group. These results lead us to assume that the accelerated arginase activity, which results in part from the upregulation of arginase I protein expression, decreases L-arginine availability to NOS, thereby impairing NO production. This assumption seems to be supported by the demonstration of Berkowitz et al. (7) that arginase reciprocally regulated NOS activity and contributed to endothelial dysfunction in aging blood vessels. It has been reported that N\(^2\)-hydroxy-L-arginine (NOHA) as an intermediate of NO production from L-arginine is an arginase inhibitor (12). Thus the arginase may be in an inactive state when NO/NOHA production is sufficient, while the enzyme activity may be enhanced when NO/NOHA production is impaired.

![Graph](image-url)  
Fig. 5. Effect of CSE on dimethylarginine dimethylaminohydrolase (DDAH) activity (A) and expression of DDAH I and II proteins (B) in rabbit cavernous specimens. Three milliliters/animal of nicotine- and tar-free CSE or PBS as a control was injected subcutaneously once a day for 5 wk. Results are means ± SE for 4 different specimens from 4 different rabbits. Significant difference vs. corresponding value in the control (PBS): *P < 0.05, **P < 0.005.
impaired, which in turn results in a vicious circle of NO-generating pathway.

To explain the impaired NO production with CSE, we should consider whether endogenous NOS inhibitors are involved in impairing NOS activity (Fig. 7). We have reported (37) that the impaired NO-mediated cavernous relaxation after ischemia is partly due to the increased accumulation of endogenous NOS inhibitors such as MMA and ADMA. Furthermore, elevation of serum ADMA levels has been demonstrated to be associated with impaired endothelium-dependent relaxation in animals and patients with diabetes mellitus (33, 36), atherosclerosis (9, 60), and hyperhomocysteinemia (52) as well as in aging (28, 57). In the present experiment, immunohistochemical analyses revealed that the number of endothelial cells positive to ADMA antibody in the cavernous arterioles was significantly increased in the CSE group. Increased tissue contents of MMA and ADMA were also observed by HPLC analyses. However, it is important to consider whether concentrations of accumulated MMA and ADMA are effective to inhibit NOS. The inhibition of cavernous NOS activity by MMA and ADMA at apparent concentrations of 1.04 and 3.06 μM in the CSE group was assessed to be 34.8% and 21.7%, respectively. These values were significantly (P < 0.005) greater than corresponding values of 12.1% and 0% in the control group. Thus the increased concentrations of MMA and ADMA in the CSE group seem to be effective to inhibit NO production. Meanwhile, it seems plausible that the ratio of L-arginine to methylarginines also contributes to NO production. In the present experiments, the significantly decreased L-arginine-to-methylarginines ratio in cavernous tissue (slightly decreased L-arginine and significantly increased MMA and ADMA in CSE group) was associated with impaired cyclic GMP production as a marker of NO production, suggesting that the accumulation of endogenous NOS inhibitors in cavernous tissue would, at least partly, result in the impaired NO production following CSE.

Furthermore, DDAH activity and expression of DDAH I protein were significantly decreased in the cavernous specimens of the CSE group. Since DDAH is the enzyme that metabolizes MMA and ADMA to L-citrulline and methylamines (43), it seems possible to assume that the decreased DDAH activity is one of the causes of the accumulation of MMA and ADMA. This seems to be partly supported by the finding that SDMA, which is not a substrate for DDAH, remained unchanged in the present experiment. Another possible mechanism for the accumulation of methylarginines is activation of the cationic amino acid transporter known as system y+ (10). MMA, ADMA, and SDMA enter cells through this transporter (10). However, it is unlikely that CSE treatment affects transporter activity in the cavernous tissue, because SDMA content remained unchanged in the present experiment.

It is widely accepted that both enzymes neuronal NOS and endothelial NOS play significant roles in the mechanism of erectile function. Burnett (13) has reported that neuronal NOS is possibly involved in initiating cavernous relaxation, while the activation of endothelial NOS may facilitate the attainment and maintenance of full erection. If so, it appears likely that downregulation of neuronal NOS protein in concert with accelerated arginase activity and accumulation of endogenous NOS inhibitors after CSE is implicated in impairing the initiation of cavernous relaxation and that the attainment and maintenance of full erection are compromised.

Table 1. Content of L-arginine and methylarginines in rabbit corpus cavernosum of control and CSE groups

<table>
<thead>
<tr>
<th>Group</th>
<th>L-Arginine, mmol/g wet wt</th>
<th>MMA, 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>213.7 ± 9.1</td>
<td>207.7 ± 17.2</td>
<td>483.2 ± 42.9</td>
<td>182.8 ± 9.7</td>
</tr>
<tr>
<td>CSE</td>
<td>182.0 ± 15.9</td>
<td>854.0 ± 48.4</td>
<td>2,503.4 ± 228.9</td>
<td>183.0 ± 19.4</td>
</tr>
</tbody>
</table>

Results are means ± SE of 3 determinations. Three milliliters per animal of nicotine- and tar-free cigarette smoke extract (CSE) or phosphate-buffered saline as a control was injected subcutaneously once a day for 5 wk. *Significant difference at P < 0.005 vs. corresponding value in control. MMA, mono-methylarginine; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.
maintenance of full erection mediated by activation of endothelial NOS is possibly attenuated by the accelerated arginase activity and accumulation of endogenous NOS inhibitors.

It is well established that cyclic GMP is an important second messenger to modulate endothelin-1 production (26) and that endothelin-1 is one of the potent vasoconstrictors believed to play an important role in regulating penile blood flow (3, 40) and to contribute to some forms of ED (55). Therefore, increased endothelin-1 possibly resulting from impaired NO production (21, 41) may be involved in causing ED with CSE. However, a goal for subsequent investigations is determination of endothelin-1 concentration and clarification of the molecular mechanisms of modulation of endothelin-1 production in the cavernous tissue.

In 1996, Green and Rodgman (20) reported that 4,800 compounds had been identified in cigarette smoke. Cigarette smoke is composed of a vapor phase and a particulate phase. The former is arbitrarily defined as that portion of smoke aerosol which passes through a Cambridge glass fiber filter (20). In the present experiment, we used CSE that had been prepared by bubbling the cigarette smoke into PBS after filtration through a Cambridge filter and thus might contain water-soluble compounds derived from the vapor phase. CSE has been used for numerous in vitro and in vivo studies and has been shown to have a wide range of effects (22, 29, 58, 59). CSEs are used as a model for studying the effects of water-soluble cigarette smoke-derived components that are present in the bloodstream of individuals exposed to cigarette smoke. However, it must be remembered that the identity and concentration of all the water-soluble smoke-derived compounds in the bloodstream are not known. As is the case with any other animal model, there are some discrepancies between the CSE-exposed model and smoking in humans. The most obvious point is that CSE contains only water-soluble ingredients of cigarette smoking. On the other hand, there are initiatives that the health impact of active smoking rather than passive smoking could be assessed, because CSE was prepared by vacuuming cigarette smoke from the filter side just like human smoking, and that the subcutaneous injection of CSE could result in complete absorption and give us certainty as an experimental model. Taken together, a comparable study using a different model of smoking would be helpful for further understanding.

The relaxation of rabbit cavernous specimens caused by EFS was definitely inhibited by CSE at concentrations of 0.3% and 1.0%. This was the reason why we designed the present experiments. In addition to this observation, we have reported (37) that the impaired NO production following ischemia of bilateral common iliac arteries of the rabbit is accompanied by significant decreases in the relaxation of cavernous specimens caused by EFS. Furthermore, NO has been demonstrated to be the principal mediator of cavernous smooth muscle relaxation and penile erection (4, 14, 24, 30, 46–48). Thus the impaired NO production following CSE for 5 wk possibly results in the attenuated cavernous relaxation. However, we should perform additional experiments to ensure whether chronic CSE causes erectile dysfunction in vivo and in vitro, that is, experiments on the histological, immunohistochemical, and biochemical aspects as well as functional experiments would provide much information to improve our understanding of the cause of ED.

In conclusion, the impaired NO production could result from blunted NOS activity, which is possibly brought about by the downregulation of neuronal NOS protein, accumulation of endogenous NOS inhibitors, and accelerated arginase activity resulting from upregulation of arginase I protein in cavernous tissue. The impaired DDAH activity due to downregulation of DDAH I protein would result in an accumulation of endogenous NOS inhibitors with CSE (Fig. 7). These alterations would be relevant to reduction of the relaxation of corpus cavernosum smooth muscle following CSE.

**Perspectives and Significance**

To our knowledge, this is the first comprehensive study to elucidate the mechanisms of impairment of NO production in cavernous tissue after administration of CSE as a model of cigarette smoking. Recently, phosphodiesterase (PDE) V inhibitor has been highlighted as an effective therapy for ED; while around one-half of patients with moderate to severe ED achieved improvement through treatment with vardenafil, one of the PDE V inhibitors, to treat ED (15). Here, based on our results revealing the impaired NO production due to multiple sites of action with CSE, strategies focusing on the inhibition of arginase and activation of DDAH could be useful as an alternative approach and, furthermore, endothelin-1 might also
be a therapeutic target as suggested by us and others (2, 27, 32). In addition, it is possible that combination of these therapies could generate effective interaction. Although there are some limitations as discussed above, this study hopefully could be a kind of cornerstone for new approaches and combined therapy against ED caused by cigarette smoking.

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