

ORIGINAL ARTICLE

Dimethylarginine dimethylaminohydrolase in rat penile tissue: reduced enzyme activity is responsible for erectile dysfunction in a rat model of atherosclerosis

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Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is mainly metabolized by N^G, N^G -dimethylarginine dimethylaminohydrolase (DDAH). We investigated whether altered cavernosal ADMA–DDAH metabolism might cause impairment of erection in rat model of atherosclerosis (AS). Male Sprague–Dawley rats (3 months old) were divided into an AS group and a normal control (Con) group ($n = 20$ in each group). The AS rats received AS-prone treatment (6 weeks of 1% cholesterol diet plus early 2 weeks of N^G -nitro-L-arginine methyl ester (3 mg ml^{-1} per day) treatment). After 6 weeks, rats underwent cavernosometry measuring the maximal intracavernosal pressure/mean arterial pressure (ICP/MAP) ratios as a surrogate marker of erectile function. The amount of cavernosal ADMA was assessed by immunoblot analysis and correlated with the ICP/MAP. Isoform-specific DDAH expression was compared by immunohistochemistry. Cavernosal DDAH and NOS activity were measured. Cavernosal malondialdehyde levels were assayed to determine the degree of lipid peroxidation. Compared to the controls, the AS rats had signs of impaired erectile function. Higher cavernosal ADMA was observed in the AS rats. The cavernosal ADMA had a moderately negative correlation with the ICP/MAP. Immunohistochemistry revealed the expression of both isoforms was not affected by the presence of AS. However, significantly diminished DDAH as well as NOS activity was observed in the AS group. In addition, elevated cavernosal malondialdehyde levels were noted in the AS rats. Our study showed that decreased cavernosal DDAH activity is the cause of cavernosal ADMA accumulation leading to reduced cavernosal NOS activity and impairment of erectile function.

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Introduction

Evidence-based data have indicated that erectile dysfunction (ED) is common in men with chronic coronary artery disease (CAD).^{1,2} The high frequency of ED in this population has suggested that something associated with the systemic vasculopathy is associated with ED. Damage to the endothelium from smoking, hypertension, hyperlipidemia and diabetes affects not only the coronary arteries

but also arteries throughout the body, including those of the corpora cavernosa of the penis.³ These conditions are frequently associated with a reduced endothelium-mediated vasodilation, where nitric oxide (NO) has a critical function.^{4–6} If the bioactivity of NO is significantly reduced, this could lead to endothelial dysfunction that contributes to ED as a result of the atherosclerotic vascular disease. Therefore, preservation or restoration of NO bioactivity could be a potential target for ED treatment, especially in the presence of systemic atherosclerosis (AS).

Potential contributing abnormality to decrease the NO bioactivity is increased levels of asymmetric N^G, N^G -dimethyl-L-arginine (ADMA), although the mechanisms of endothelial dysfunction are likely multifactorial. By competing with arginine, through the Y^+ transporter system, ADMA can inhibit all

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types of NO synthase (NOS) and has been associated with endothelial dysfunction.⁷ Elevation of ADMA has often been observed in patients with various cardiovascular diseases where endothelial dysfunction has been identified as the common etiology.⁷ ADMA is mainly metabolized by dimethylarginine dimethylaminohydrolase (DDAH).⁸ This enzyme has two types: type 1 is closely associated with neuronal NOS and type 2 with endothelial NOS.⁷ Recent study has identified both isoforms in rabbit penile tissue.⁹ It has been reported that either decreased expression of DDAH or reduced activity of DDAH is responsible for the elevation of ADMA.^{10,11}

Given its pharmacological function as a modulator of NO activity, and the relationship with endothelial dysfunction, it is likely that ADMA is associated with the pathogenesis of ED. Several lines of evidence have suggested the potential relationship between ADMA and ED, especially in patients with CADs.^{12,13} Intracavernosal injection of 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.¹⁴ Moreover, cavernosal accumulation of ADMA was observed in a rabbit model of cavernosal ischemia, which mimicked the ED observed in AS.¹⁵ Taken together, in ED patients with AS, elevated ADMA is likely to be found in penile tissues. However, the function of ADMA in penile erections has not been to date sufficiently investigated. Moreover, the reason for ADMA elevation has not been explored.

To clarify these issues, we designed a study to examine the relationship between penile ADMA and erectile function in a rat model of AS. In addition, the mechanism involved in ADMA elevation was explored in relationship to DDAH.

Materials and methods

Animal groups and treatment protocol

This study was reviewed and approved by the committee on the ethics of animal experiments, Seoul National University, College of Medicine, and was conducted according to the guidelines for animal experiments of Seoul National University.

A total of 20 male 12-week-old Sprague–Dawley rats were divided into a normal control (Con) group and an AS group ($n = 10$ in each group). While the Con group was given standard rat chow and drinking water, the AS group received a 1% cholesterol diet (CRF-1; Oriental Yeast Co., Osaka, Japan) for 6 weeks. To elicit intimal changes associated with AS, we gave the rats N^G -nitro-L-arginine methyl ester (3 mg ml^{-1}) in the drinking water, initially for 2 weeks as previously described.¹⁶ Despite the popularity in ED research, rats have seldom been used to investigate the ED associated with AS due to inherent tolerability to

high-cholesterol diet. This intimal priming method caused to elicit multiple pelvic AS within 6 weeks, facilitating the use of rats as a potential animal model for AS. According to our previous studies, these rats showed evidence of mild to moderate pelvic AS and ED.¹⁶

In vivo assessment of erectile function and tissue procurement

After 6 weeks, all rats in both groups underwent cavernosometry during cavernous nerve stimulation, as described previously.^{16,17} The stimulation parameters were 3 V at varying frequencies of 2.5–20 Hz with square wave duration of 0.2 ms for 50 s. Both mean arterial pressure (MAP) and intracavernosal pressure (ICP) were continuously monitored during the electrical stimulation. Comparisons of the (1) ICP/MAP and (2) area under curve (AUC) corresponding to the duration of electrical stimulation were carried out.

At the end of the experiment, blood samples were collected from the jugular vein, centrifuged at 1500 g for 20 min at 4 °C, and plasma was stored at –70 °C for biochemical assays. The penis was excised, the penile shaft was separated from the crura and a 2–3 mm transversal slice was cut just distal to the point of contact of both crura. The segments were maintained in 4% formaldehyde solution for immunohistochemistry. The remaining proximal crural segment was dissected out and then immediately frozen in liquid nitrogen and stored at –80 °C.

Immunoblot for cavernous ADMA

Homogenized cavernosal strips containing ice-cold buffer (20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_3VO_4 and 1% phenylmethylsulfonyl fluoride) were centrifuged at 10 000 g for 20 min to remove insoluble material. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

An equal volume (50 μg) of samples was then loaded and run on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, which was subsequently probed with mouse monoclonal antibody against ADMA (Upstate Biotechnologies, Lake Placid, NY, USA); this was followed by addition of horseradish peroxidase-conjugated secondary antibodies. Peroxidase-conjugated anti-rabbit IgG antibodies using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) detected the bound antibodies. To adjust for loading differences, we reprobbed the membranes with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The tissue amount of ADMA was quantified by densitometry (Image-Pro plus; Media Cybernetics, Bethesda, MD, USA).

Immunohistochemistry for cavernosal DDAH isoforms

Tissue sections (5 μm) were deparaffinized, rehydrated and fixed with methanol –0.3% hydrogen peroxide at room temperature for 30 min. Tissue sections were blocked with 100 \times diluted antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 3 min and incubated with rodent block (Lab Vision, Fremont, CA, USA) for 1 h at room temperature, followed by overnight incubation with a primary goat polyclonal antibody against DDAH 1 or a mouse monoclonal antibody against DDAH 2 (1:50 dilution) overnight. Sections were incubated sequentially with biotinylated antibody (goat anti-rabbit IgG) and horseradish streptavidin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature at each step. The samples were then incubated with diaminobenzidine using the DAB Substrate System (Lab Vision) and counterstained with hematoxylin, which stained all cell nuclei bluish, whereas DDAH 1- and DDAH 2-positive cells stained brown.

Four sections ($\times 400$) from each animal were assessed, and the mean immunostaining score in endothelial, smooth muscle and nerve was calculated. Each section was scored blind as follows: 0, no staining; 1, slightly stained; 2, moderately stained and 3, strongly stained.

Measurement of cavernous NOS and DDAH activity

To determine the constitutive NOS activity, we used a commercial kit for the NOS assay (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) for assaying the conversion of L-arginine to L-citrulline in the cavernous homogenates. To determine the iNOS activity in the penile samples, we repeated the above-mentioned experiments under calcium-free conditions.

We assayed DDAH activity by determining L-citrulline formation in cavernous tissue homogenates, as previously described in pulmonary tissues.¹⁸ The cavernosal samples were homogenized in a medium containing 0.1 M sodium phosphate buffer (pH 6.5), 1 mM PMSF, 1 μM pepstatin A and 2 μM leupeptin. The homogenate was centrifuged and the supernatant was collected. The reaction mixture consisted of 90 μl of supernatant, 1 mmol l^{-1} ADMA, 20 mM EDTA and 0.1 M sodium phosphate buffer (pH 6.5). The samples were incubated for 2 h and the reaction was stopped by 10% trichloroacetic acid. For the negative controls, homogenates were boiled for 10 min to inactivate the enzyme, and the resultant background values were subtracted from the experimental data to determine the genuine DDAH activity. Then the supernatant was incubated with diacetyl monoxime (0.8%) and antipyrine (0.5%) at 60 $^{\circ}\text{C}$ for 110 min. The amount of L-citrulline was determined by spectrophotometric analysis at 466 nm. One unit of DDAH activity was expressed as the amount that catalyzed the formation of 1 μmol citrulline from ADMA per minute at 37 $^{\circ}\text{C}$.

Both the NOS and DDAH activities are normalized by the protein contents of the cavernosal tissue and expressed as citrulline production in pmol per min mg protein.

Determination of lipid peroxidation of cavernosal tissue with the malondialdehyde assay

To measure the lipid peroxidation in cavernosal tissue, we used an malondialdehyde (MDA)-586 colorimetric assay kit (OXIS International, Portland, OR, USA) as previously described.¹⁹ The degree of lipid peroxidation was determined by an MDA standard curve as the expressed MDA equivalent content (nmol MDA per ml).

Statistical analysis

All data are means \pm s.e. Spearman's correlation coefficients were obtained to evaluate the relationship between surrogate markers of erectile function (ICP/MAP and AUC) and cavernosal ADMA level. Differences between the groups were assessed by the Student's *t*-test with a *P*-value < 0.05 regarded to be significant.

Results

Comparison of erectile function

Before electrostimulation, measurement of MAP revealed higher MAP in AS group than Con group (120 ± 6 vs 102 ± 4 , $P = 0.02$) but comparison of baseline ICP/MAP was not different between them (12.1 ± 4.6 in AS group vs 14.7 ± 4.2 in Con group, $P = 0.16$). During cavernous nerve stimulation, the AS group had lower ICP/MAP and AUC than the Con group (Figure 1). Comparison of both indices between the two groups revealed a significant difference at 5–20 Hz ($P < 0.05$).

Immunoblots for the measurement of cavernosal ADMA

Tissue levels of ADMA were semiquantitatively measured by western blot assay. As shown in Figure 2, the penile tissue of the AS group had greater amount of ADMA than the Con group. Densitometry revealed a more than three times higher density of ADMA in the AS group. In AS group, the Spearman's correlation coefficient indicated a moderate but significant negative correlation between surrogate markers of erectile function and the ADMA expression ($\rho = -0.43$ for ICP/MAP,

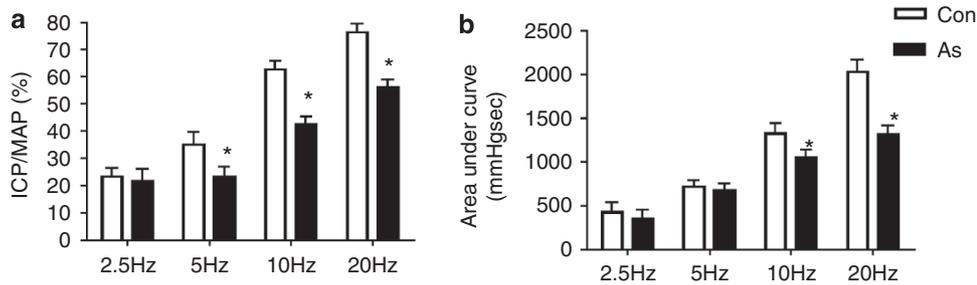


Figure 1 Results of cavernosometry (3 V, 0.2 ms, 50 s). Comparison of intracavernosal pressure/mean arterial pressure (ICP/MAP) (a) and area under curve (b) between normal control (Con) and atherosclerosis (AS) groups showed significant impairment of erectile response in the AS group at higher frequencies. Asterisk (*) denotes statistical significance ($P < 0.05$).

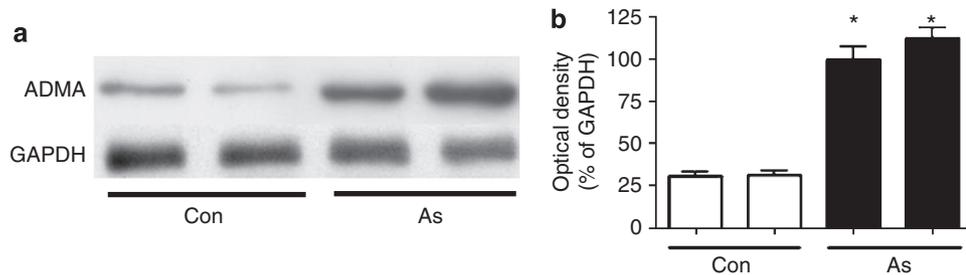


Figure 2 Expression of cavernosal asymmetric dimethylarginine (ADMA) in control (Con) and atherosclerosis (AS) rats (a) representative immunoblot (b) relative expression of ADMA after adjusted by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Asterisk (*) denotes statistical significance ($P < 0.05$).

P (one-tailed) = 0.002; $\rho = -0.32$ for AUC, P (one-tailed) = 0.03).

group compared to the Con group (28.6 ± 5.7 vs 55.8 ± 4.9 , $P < 0.001$).

Immunohistochemical staining for DDAH isoforms
Immunohistochemical localization of DDAH isoforms within the rat penile tissue is shown in Figure 3. Immunoreactivity for DDAH 1 was seen in the cytoplasm of trabecular smooth muscle, endothelium and neural tissue, but only the modest endothelial staining was observed for DDAH 2. The expression of DDAHs was not affected by the presence of pelvic AS. Comparison of the mean immunostaining score revealed no significant difference in the isoforms was noted between the AS and Con groups.

Cavernous NOS/DDAH activity

Comparatively lower constitutive NOS and DDAH activity was found in the AS group (Figure 4). Compared to Con group, the constitutive NOS and DDAH activity was reduced up to 54 and 37% in AS group, respectively.

ELISA for cavernosal MDA

The enzyme-linked immunosorbent assay (ELISA) revealed a higher cavernosal level of MDA in AS

Discussion

The salient findings from the present study on rats with pelvic AS are the following: (1) elevation of cavernosal ADMA expression in AS group and demonstration of a negative correlation between erectile function in the AS group and cavernosal ADMA expression; (2) histological confirmation of the expression of both isoforms of DDAH, responsible for the metabolism of ADMA in rat cavernosal tissue and (3) the association of reduced DDAH activity in the AS group with decreased NOS activity, which may lead to decreased erectile function. These results highlight the negative function of ADMA on erectile function in the presence of systemic AS, and confirmed the presence of a functional ADMA clearance system in rat penile tissue.

Along with the inhibitory pressure response to pelvic nerve stimulation following intracavernosal ADMA administration, the demonstration of a more than three times higher cavernosal accumulation of ADMA, in rats with AS compared to the controls, supports a claim of possible anti-erectile function of cavernosal ADMA in the presence of pelvic AS.

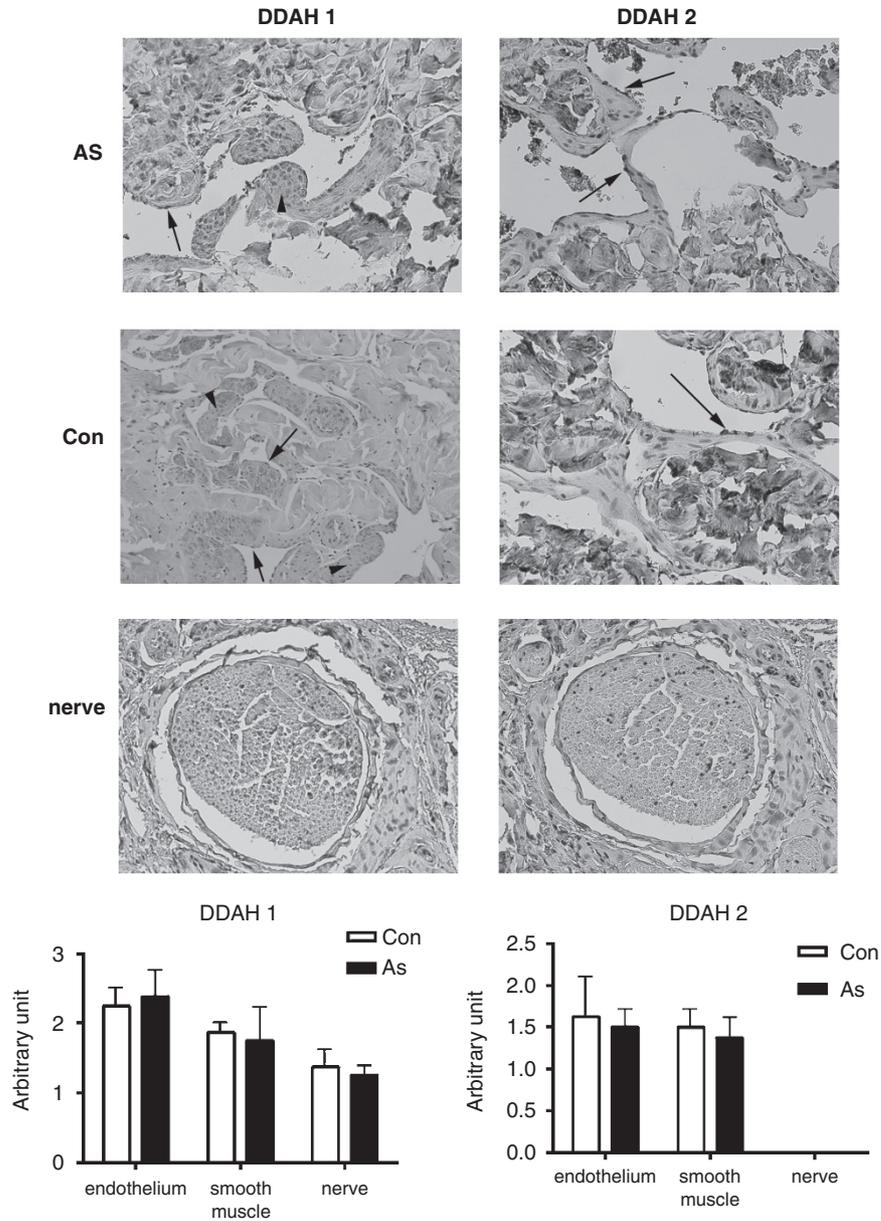


Figure 3 Immunohistochemical evaluation of both dimethylarginine dimethylaminohydrolase (DDAH) isoforms in rat cavernosal tissues. Immunoreactivities are seen in brown. Upper two rows revealed representative pictures from normal control (Con) and atherosclerosis (AS) groups. Compared to DDAH 2, which showed only modest endothelial staining (arrow), DDAH 1 showed a greater staining intensity and was found in both endothelial (arrow) and trabecular smooth muscle (arrowhead). Representative neural picture in third row was from Con group. Similar findings were observed in AS group, so only pictures from Con group were displayed. Abundant staining for DDAH 1 was identified in neural cells, whereas no immunostaining was observed for DDAH 2. Comparison of staining scores showed similar staining intensity of both isoforms in the Con and AS groups, as shown in densitometry.

Several human studies have associated plasma ADMA levels with ED in patients with CAD.^{12,13} Elevated plasma ADMA reduces systemic NO bioactivity, leading to systemic as well as coronary endothelial dysfunction in patients with ED.²⁰ On the basis of human data, it is reasonable to speculate that the elevated plasma ADMA levels interfere with the erectile response due to systemic endothelial dysfunction. However, the demonstration of cavernosal ADMA accumulation, after bilateral clamping

of the internal iliac arteries, has suggested the possibility that increased levels of local ADMA might directly cause alteration of the erectile function by inhibiting the local NOS system.¹⁵ Our data on rats with AS supports the potential function of cavernosal ADMA accumulation in the development of ED. Future study is needed to determine the potential contribution of the local accumulation of ADMA to erectile function and to investigate whether the treatment aimed at lowering ADMA,

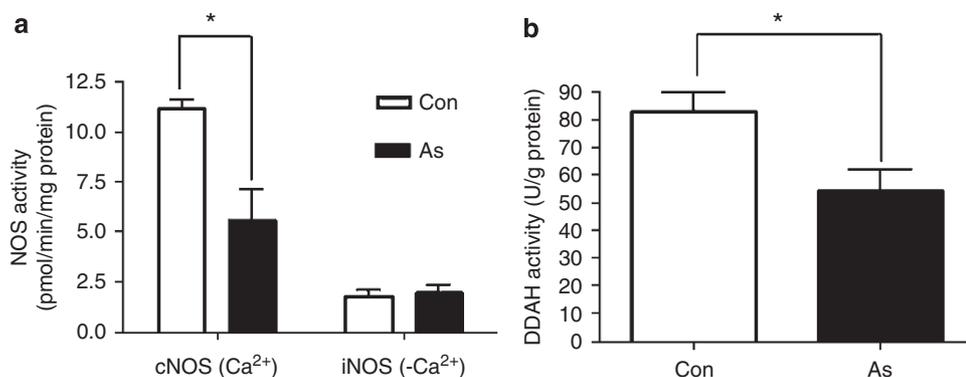


Figure 4 Comparison of nitric oxide synthase (NOS; both constitutive and inducible types) (a) and dimethylarginine dimethylaminohydrolase (DDAH) activities (b) between normal control (Con) and atherosclerosis (AS) groups. Asterisk (*) denotes statistical significance ($P < 0.05$).

either systemically or locally, could improve erectile function.

Despite the presence of several reports linking ADMA with ED, the mechanism associated with ADMA elevation has not been sufficiently explored. In other parts of the body, DDAH has been identified as the main metabolic pathway of ADMA. By western blot analysis, Japanese researchers have previously found both isoforms in the penile tissue of rabbits.⁹ Our immunohistochemical experiments confirmed this in the rat. In addition, although both isoforms were identified, type 1 showed a higher and wider immunoreactivity than did type 2 in the cavernosum of the rat. This does not necessarily mean that type 1 has a more significant functional function than type 2. However, the modest immunoreactivity of type 2 in the endothelial cells may indicate a limited function of type 2 DDAH in the penile erections of rats. Further study using transgenic animals is needed to clarify this.

Our study revealed no change in DDAH expression by immunohistochemistry. The DDAH activity was significantly attenuated in the AS rats. Moreover, the decreased DDAH activity was associated with the NOS activity, which has been shown to be a functional correlate of erectile function.²¹ Thus, diminished cavernosal DDAH activity might be one of the potential causes of impaired erectile function. The finding of decreased DDAH activity has been consistently found in models of diabetes, hyperhomocysteinemia and AS, where endothelial dysfunction has a critical pathological function.^{11,22,23} Because decreased bioactivity of NO is the most crucial feature of endothelial dysfunction, as well as ED, lowered DDAH activity might result in ADMA accumulation, leading to diminished NO production and subsequent endothelial and ED in AS rats.

Reactive oxygen species has been regarded as a possible cause of endothelial and ED; increased oxidative stress is often regarded as one of the initiating events of the development of AS.²⁴ In this

study, we observed increased MDA levels in the cavernosal tissue of AS rats. Interestingly, increased oxidative stress is known to be a strong inhibitor of DDAH. A recent study has identified highly reactive cysteine residues (Cys 249) at the active site of DDAH; its sulfhydryl group predisposes DDAH to easy oxidation or nitrosation after which it loses its activity.²⁵ The antioxidants polyethylene glycol-conjugated superoxide dismutase and pyrrolidine dithiocarbamate have been recently reported to preserve DDAH activity.^{11,22} These studies indicate that DDAH activity could be modulated by cardiovascular risk factors in a reactive oxygen species-sensitive manner.

Although we have demonstrated the presence of both isoforms, we have not clarified the functional function of each isoform in rat penile erections. Use of isoform-specific inhibitor or transgenic animals might elucidate their individual functions. The change of expression, location and activity, for various causes of ED, should be further investigated to identify its pathophysiology.

In conclusion, the results of this study demonstrated that increased cavernosal ADMA levels were associated with ED in a rat model of AS. Furthermore, suppression of DDAH activity was associated with increased cavernosal ADMA accumulation leading to decreased NOS activity and impairment of rat penile erections. Increased oxidative stress in the cavernosal tissue of rats might contribute to the diminished DDAH activity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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