European Journal of Pharmacology 661 (2011) 86–91

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular Pharmacology

Down-regulation of DDAH2 and eNOS induces endothelial dysfunction in sinoaortic-denervated rats

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ARTICLE INFO

Article history:
Received 13 December 2010
Received in revised form 1 April 2011
Accepted 14 April 2011
Available online 28 April 2011

Keywords:
dimethylarginine dimethylaminohydrolase 2
Nitric oxide
Endothelial nitric oxide synthase
Endothelial dysfunction
Sinoartic-denervation
AngiotensinII

ABSTRACT

The aim of present study was to investigate whether downregulation of dimethylarginine dimethylaminohydrolase (DDAH2) and endothelial nitric oxide synthase (eNOS) induced endothelial dysfunction in sinoaortic-denervated (SAD) rats. SAD rats exhibited significantly higher blood pressure (BP) variability and markedly lower baroreflex sensitivity. However, there was no significant difference in BP between SAD rats and sham-operated rats. In SAD rats, ultrastructural analysis revealed that endothelial cells were degenerated and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) study showed that apoptotic aortic endothelial cells increased. Circulating angiotensinII (AngII), asymmetric dimethylarginine (ADMA) and malondialdehyde (MDA) levels in SAD rats were similar to sham-operated rats, but aortic AngII and MDA levels locally increased. Endothelium-mediated relaxation of thoracic aorta isolated from SAD rats was impaired compared to sham-operated rats, whereas the sodium nitroprusside-induced relaxation was quite similar. Western blotting results showed that DDAH2 and eNOS expressions decreased significantly in the aortae of SAD rats. Treatment of primary cultured rat aortic endothelial cells with AngII (1 μM) resulted in a marked reduction of DDAH2 and eNOS expressions, and coadministration of losartan (1 μM), an AT_1 receptor antagonist, abolished the effect. In conclusion, downregulation of DDAH2 and eNOS induced endothelial dysfunction in SAD rats. DDAH2 and eNOS may be the potential targets for treatment of endothelial dysfunction.

1. Introduction

Sinoaortic denervation is a classic model of arterial baroreflex (ABR) dysfunction, which results in various organ damages including cardiac hypertrophy, aortic remolding and renal lesions (Parati et al., 1987; Sega et al., 2002; Su and Miao, 2001). Sinoaortic denervation-induced aortic distensibility and structure changes were first reported by Lacolly et al. (1995); sinoaortic denervation-induced endothelial damage was first observed by Shen et al. (2006). Endothelial dysfunction is recognized as an independent risk factor for the development of cardiovascular diseases. However, the underlying molecular mechanisms by which sinoaortic denervation induces endothelial dysfunction are still little understood.

It is well known that nitric oxide (NO) plays a crucial role in the regulation of endothelial function. Endothelial NO is primarily produced by endothelial nitric oxide synthase (eNOS), which is the most important enzyme for NO formation in endothelium. In addition, there is increasing evidence which suggests an important role for dimethylarginine dimethylaminohydrolase 2 (DDAH2) in the pathogenesis of endothelial dysfunction (Boger et al., 1998; Hasegawa et al., 2007; Vallance et al., 1992; Zoccali et al., 2001). DDAH2 is located with eNOS in the cytosol of endothelial cells and responsible for metabolizing asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of eNOS (Anthony et al., 2005).

The renin-angiotensin system also plays a major role in vascular biology, and it is well established that angiotensinII (AngII) produces endothelial dysfunction in blood vessels in vitro (Didion et al., 2005, 2009; Dzau, 2001).

In current study, we found SAD rats exhibited diminished NO production and elevated angiotensinII (AngII) level in aortae locally. Therefore, we investigated the expression alteration of aortic eNOS and DDAH2 in SAD rats, and the relationship between AngII and those two proteins.

2. Materials and methods

2.1. Animals and study design

Male Sprague-Dawley (SD) rats (2 months old, 200–250 g) were purchased from the Sino-British SIPPR/BK Lab Animal Ltd. All the animals were entrained to controlled temperature (23–25 °C), 12-h light and 12-h dark cycles (light, 08:00–20:00 h; darkness, 20:00–08:00 h), and free access to food and tap water. All the animals used in this work received humane care in compliance with institutional

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doi:10.1016/j.ejphar.2011.04.041

animal care guidelines, and were approved by the Local Institutional Committee. All the surgical and experimental procedures were in accordance with institutional animal care guidelines.

In experiment 1, animals were divided into two groups: (1) sham-operated group; (2) sinoaortic denervated group. 4 weeks later, measurements of hemodynamic parameters and plasma parameters including asymmetric dimethylarginine (AMDA), angiotensinII (AngII) and malondialdehyde (MDA) content were performed. Thoracic aortae were removed for ultrastructural analysis by electron microscopy, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) study and measurements of aortic relaxation in response to acetycholine (ACh) or sodium nitroprusside (SNP), MDA content, NOx ($NO_2^- + NO_3^-$) content, AngII content, ACE activity and AT_1R expresseion.

In experiment 2, primary rat aortic endothelial cells (RAECs) were cultured. Cell protein was collected for analysis by Western blotting on DDAH2 and eNOS expression after 24 h of stimulation with AngII (1 μM) or AngII plus losartan (1 μM), an AT_1 receptor antagonist.

2.2. Sinoaortic denervation

Sinoaortic denervation was performed according to the previously described method (Li et al., 2008; Miao et al., 2002). The rats after sinoaortic denervation were housed in individual cage with controlled temperature (23–25 °C), 12-h light–dark cycle, and free access to food and tap water.

2.3. Cell culture

RAECs were isolated from the thoracic aorta of 6-week-old male SD rats using a method essentially as described (Sugiyama et al., 2005) and cultured in Medium 199 (Gibco Laboratories Inc., Grand Island, NY) containing 15% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH) and 30 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA) on collagen-coated dishes (IWAKI, Chiba, Japan) at 37 °C in an atmosphere of 5% CO_2. Subcultured RAECs (passages 6) grown to confluence were starved with Medium199 containing 0.5% calf serum for 24 h and used for experiments 2.

To make sure that cells cultured from the aorta of rats were endothelial cells, cells were fixed in ice-cold 4% paraformaldehyde for 30 min. After incubation for 30 min in 5% albumin/Tris-buffered saline (TBS; 20 mM tris base, 137 mM NaCl, pH 7.6) 0.1% Tween, endothelial cells were incubated with a 1:100 dilution of a eNOS monoclonal antibody (BD Biosciences, San Diego, CA). Cells were washed twice for 5 min with TBS-T, incubated for 30 min with 1:500 FITC-conjugated goat anti-mouse antibody (Abcam, Cambridge, UK), then rewashed twice for 5 min with TBS-T. Cells were visualized under microscope. Most of cells were showed eNOS-positive, which indicated that most of cells cultured from the aorta of rats were endothelial cells.

2.4. BP measurement

Systolic BP, diastolic BP and heart period were recorded as previously described (Fu et al., 2004; Liu et al., 2003). Briefly, rats were anesthetized with a combination of diazepam (6 mg/kg, i.p.) and ketamine (40 mg/kg, i.p.). A floating polyethylene catheter was inserted into the lower abdominal aorta via the left femoral artery for BP measurement, and another catheter was indwelled in the left femoral vein for intravenous injection. The catheters were exteriorized through the interscapular skin. After a 48-h recovery period, the animals were placed for BP recording in individual cylindrical cages with food and water. The aortic catheter was connected to a BP transducer via a rotating swivel what allows the animals to move freely in the cage. After about 14-h habituation, the BP signals were digitized by a microcomputer, and beat-to-beat systolic BP, diastolic BP and heart period values were determined on line. The mean values of these parameters during the 24-h were calculated and served as systolic BP, diastolic BP and heart period for study. The mean standard deviation over the mean was calculated and defined as the quantitative parameter of BP variability and heart period variability.

2.5. Baroreflex sensitivity measurement

Under the above-mentioned BP recording conditions, baroreflex sensitivity was measured in conscious rat as previously described (Miao et al., 2004). A bolus injection of phenylephrine was given to induce a BP elevation. The dose of phenylephrine was adjusted to raise systolic BP by 20–40 mm Hg. There was a delay (about 1 s) between the elevation of BP (stimulus) and the prolongation of heart period (response) for arterial baroreflex. In rat, the heart rate is about 5 or 6/s. So heart period was plotted against systolic BP for linear regression analysis for 2–8 shifts (calculated by computer); the slope with the largest correlation coefficient of heart period and systolic BP was defined as baroreflex sensitivity. The mean of the 2 measurements served as the final result.

2.6. Electron microscopy

Freshly isolated thoracic aortas were fixed with 2.5% glutaraldehyde and suspended in agar. Tissues were postfixed with OsO4, dehydrated with a series of ethanol washes, rinsed with propylene oxide and embedded in LX-112 (Ladd Research Industries, Burlington, VT, USA), which polymerized at 65 °C. Samples were thin sectioned (80 nm), counterstained with uranyl acetate and lead citrate and examined with a JEM-2000EX transmission electron microscope (Jeol, Tokyo, Japan). Structures of endothelial cells were observed.

2.7. TUNEL study

Apoptotic activity on paraffin sections of the lower descending thoracic aorta was analyzed by a TUNEL method with a commercial kit (Boehringer Mannheim, Mannheim, Germany). Omission of the TdT enzyme in the TUNEL reaction was used as a negative control and resulted in no staining. Tonsil tissue was used as a positive control. Apoptosis number was measured by computer-assisted image analysis system (LEICA QUIPS, LEICA Imaging Systems LTD, England) and the results were expressed as an apoptotic index, calculated as the number of positive-staining nuclei per 1000 cells. For these counts, 2000 cells were randomly selected from each specimen.

2.8. Determination of ADMA

For the determination of plasma ADMA, we adopted the HPLC-mass spectrometry method as previously described (Scalera et al., 2004).

2.9. Aortic relaxation in response to acetylcholine and sodium nitroprusside

Thoracic aortas were removed, cleared of adhering connecting tissue, cut into rings 2 mm in length and placed in Krebs buffer. Protocols were performed on rings beginning at their optimum resting tone, previously determined to be 3 g for rat aorta. This resting tone was reached by stretching rings in 500 mg increments separated by 10-min intervals. Data were collected using a MacLab system and analyzed using Dose Response Software (AD Instruments, Colorado Springs, CO, USA). Vessel rings were preconstricted with phenylephrine (1 μmol/l) (Sigma, St. Louis, MO, USA), and their vasorelaxant dose responses to acetylcholine (1 nmol/l to 10 μmol/l) (Sigma) and sodium nitroprusside (1 nmol/l to 10 μmol/l) were recorded.

Relaxation to acetylcholine and sodium nitroprusside is expressed as a percent relaxation to phenylephrine-induced contraction.

2.10. AngiotensinII measurements

Blood samples were collected into polypropylene tubes containing 1 mmol/l p-hydroxymercury benzoate, 30 mmol/l 1,10-phenanthroline, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1 mmol/l pepstatin A and 7.5% EDTA. After centrifugation at 250 *g* for 10 min, plasma samples were stored at −80 °C.

Aortae were homogenized with 0.1 mol/l 0.045 n HCl in ethanol (10 ml/g tissue) containing 0.90 μmol/l p-hydroxymercury benzoate, 131.50 μmol/l 1,10-phenanthroline, 0.90 μmol/l PMSF, 1.75 μmol/l pepstatin A, 0.032% EDTA and 0.0043% protease-free bovine serum albumin and evaporated. After evaporation, the samples were dissolved in 0.003% trifluoroacetic acid.

Plasma and aortic samples were used for the measurement of AngII using a radioimmunoassay kit (China Institute of Atomic Energy, Beijing, China). The aortic results were corrected for protein content.

2.11. Angiotensin converting enzyme (ACE) activity

ACE activity was determined according to the previously described method (Marin-Castano et al., 2002). Aortic ACE activity was calculated as nmol/min/mg protein.

2.12. Determination of Nitrite/nitrate (NOx) Content

The levels of NOx, the stable end products of NO, in aortae were measured using a Total Nitrite/Nitrate Assay kit (Dojindo, Kumamoto, Japan) which employed the Griess method. NOx concentration was calculated as μmol/g of protein.

2.13. Measurement of aortic MDA

MDA concentration is a presumptive marker of oxidant-mediated lipid peroxidation. Aortic homogenates were used for the determination of MDA using a kit (Cayman, Ann Arbor, USA). MDA content was calculated as μmol/g of protein.

2.14. Western blotting

Equal amount of protein preparations (10 μg in 10 μl buffer) was run on SDS-polyacrylamide gels, electrotransferred to polyvinylidine difluoride membranes, and blotted with a primary antibody against AT_1R (1:500, Santa Cruz, CA, USA), DDAH2 (1:1000, Abcam, Cambridge, UK) and eNOS (1:500) overnight at 4 °C using slow rocking. Then, they were blotted with HRP-conjugated secondary antibody (1:5000, Sigma, MO, USA) and HRP-conjugated monoclonal antibody against β-actin (1:10000, Sigma) or GAPDH (1:10000, Sigma). Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, Amersham Pharmacia), and results were expressed as the ratio of the density of specific bands to the corresponding β-actin or GAPDH.

2.15. Statistical analysis

All data are presented as mean ± S.D. To compare the relaxation–response curves, a four parametric logistic model was fit to the dose–response curves. Comparisons of aortic relaxation between groups were made using analysis of variance followed by Student-Newman–Keuls post hoc test. Differences in hemodynamic parameters, plasma parameters, aortic AngII level, aortic MDA level, aortic NOx level and expressions of DDAH2 and eNOS in aortae and RAECs between groups were examined using paired t-tests. A probability level of less than 0.05 was considered significant.

3. Results

3.1. Change of hemodynamic parameters and plasma parameters after sinoaortic denervation

Hemodynamic parameters and plasma parameters (ADMA, AngII and MDA) were calculated 4 weeks after sinoaortic denervation. Compared to sham-operated rats, systolic BP availability and diastolic BP availability were higher; baroreflex sensitivity was lower in SAD rats. However, there was no difference in systolic BP, diastolic BP, heart period, and heart period availability between two groups (Table 1). The results showed that sinoaortic denervation induced dysfunction of arterial baroreflex, but did not alter BP. The levels of plasma ADMA, AngII and MDA were similar between sham-operated rats and SAD rats (Table 1).

3.2. Change of endothelial cells under electron microscopy after sinoaortic denervation

Transmission electron micrographs of the aortic endothelial cells of rats 4 weeks after sinoaortic denervation were shown in Fig. 1. The normal endothelial cells were ovoid shaped, being connected with neighboring endothelial cells and internal elastic lamina. The nucleus was also ovoid shaped and possessed central euchromatin and peripheral heterochromatin. Intracellular organelles were normal. No obvious condensation of chromatin or blebbing of cell membrane could be observed. Endothelial cells of SAD rats showed degenerative and swelling with blebbing of the cell membrane and increased condensation of peripheral nuclear chromatin.

3.3. Endotheliocyte apoptosis after sinoaortic denervation

To test whether sinoaortic denervation promotes apoptosis of endotheliocyte, the TUNEL method was carried out. Apoptotic cells were TUNEL-positive and exhibited an obvious darkbrown nucleus and unstained cytoplasm (Fig. 2). Apoptotic endothelial cells were found significantly higher in SAD rats than in sham-operated rats (5.1 ± 1.3 *vs.* 2.3 ± 0.9, $P<0.05$).

3.4. Change of aortic relaxation

Endothelium-mediated vascular relaxations of aortae in response to acetylcholine were markedly impaired in SAD rats compared with sham-operated rats, but the endothelium-independent relaxations to sodium nitroprusside were similar in both groups (Fig. 3A and B).

Table 1
Hemodynamic parameters and plasma parameters in sham-operated rats and SAD rats.

	Sham-operated rats	SAD rats
Hemodynamic parameters		
Systolic BP (mm Hg)	137 ± 6.8	134 ± 9.4
Diastolic BP (mm Hg)	88 ± 8.5	90 ± 7.6
Heart period (ms)	147 ± 8.6	146 ± 9.8
Systolic BP variability (mm Hg)	8.5 ± 1.6	15.6 ± 3.1[b]
Diastolic BP variability (mm Hg)	6.2 ± 1.8	11.5 ± 2.3[b]
Heart period variability (ms)	21.2 ± 4.3	22.5 ± 5.2
Baroreflex sensitivity (ms/mm Hg)	0.66 ± 0.10	0.24 ± 0.07[b]
Plasma parameters		
ADMA (μM)	0.53 ± 0.11	0.59 ± 0.14
AngII (pg/ml)	187 ± 33.5	199 ± 40.8
MDA (ng/ml)	2.76 ± 0.58	3.06 ± 0.73

Values are represented as mean ± S.D.; ADMA: asymmetric dimethylarginine; AngII: angiotensinII; MDA: malondialdehyde; [b]$P<0.01$ *vs.* sham-operated rats. n = 12 in each group.

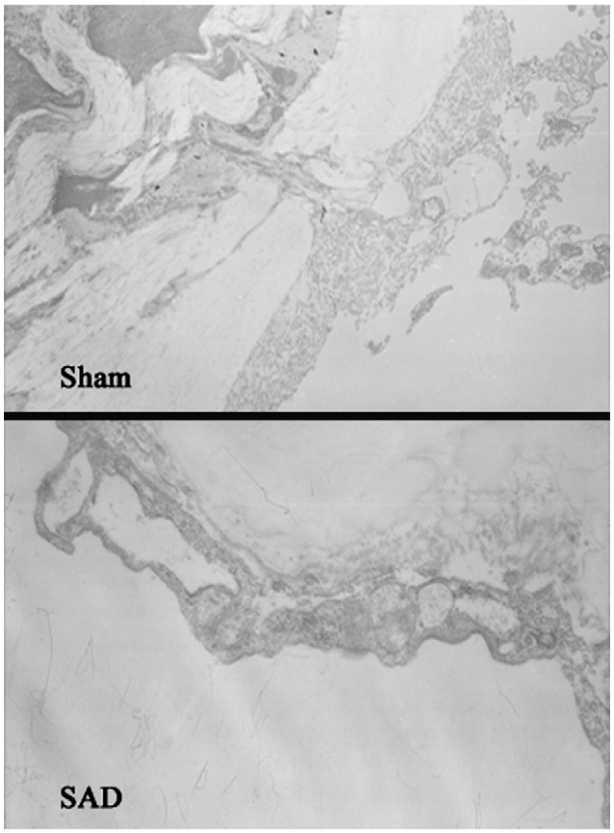

Fig. 1. Transmission electron micrographs of aortic wall endothelial cells of sham-operated rats (Sham) and sinoaortic denervated (SAD) rats 4 weeks after operation (×8000).

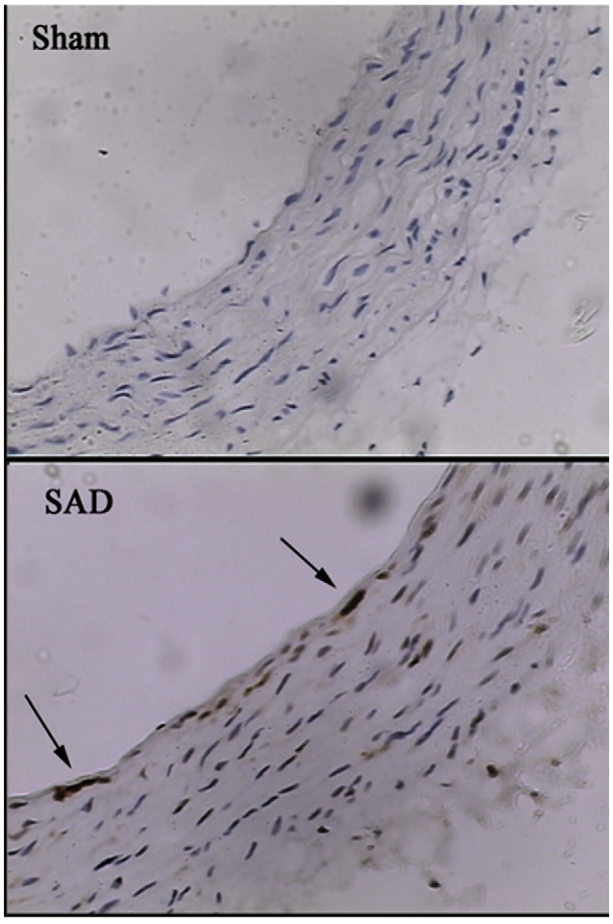

Fig. 2. Apoptotic endothelial cells (TUNEL-positive cells, with dark-brown nucleus and unstained cytoplasm) in sham-operated rats (Sham) and sinoaortic denervated rats (SAD) (TUNEL×400).

3.5. Change of aortic MDA and NOx levels after sinoaortic denervation

As shown in Fig. 4A, compared to sham-operated rats, aortic MDA content in SAD rats was elevated, indicating that sinoaortic denervation resulted in aggravated oxidative stress in aortae. As shown in Fig. 4B, aortic NOx content in SAD rats was lower than that in sham-operated rats, indicating that NO formation in aortae was decreased after sinoaortic denervation.

3.6. Change of aortic AngII content, ACE activity and AT_1R expression after sinoaortic denervation

As shown in Fig. 5, both aortic AngII content (Fig. 5A) and ACE (Fig. 5B) activity in SAD rats were higher than sham-operated rats. But, AT_1R (Fig. 5C and D) expression in the aortae was similar between two groups.

3.7. Western blot analysis of aortic DDAH2 and eNOS expression after sinoaortic denervation

Aortae were removed for the detection of protein expression of DDAH2 and eNOS. Western blotting result showed that protein expression of aortic DDAH2 and eNOS was significantly lower in SAD rats compared to sham-operated rats (Fig. 6).

3.8. Effect of AngII and AT_1R antagonist-losartan on the expression of DDAH2 and eNOS protein in cultured RAECs

Primary cultured RAECs were used to investigate the influence of AngII and losartan on the expression of DDAH2 and eNOS (Fig. 7). It was showed that incubation of AngII resulted in a marked reduction of DDAH2 and eNOS protein, and coincubation of losartan, an AT_1R antagonist, abolished its effect on DDAH2, and partly abolished its effect on eNOS.

4. Discussion

The main new findings of the current study were summarized as follows: (1) Sinoaortic denervaton induced endothelial dysfunction in aortae of rats, supported by degenerative and apoptotic endothelial cells, elevated aortic oxidative levels, and impaired vasorelaxation to the endothelium-dependent dilator acetylcholine. (2) Though plasma AngII showed no significant alteration, aortic AngII level exhibited a

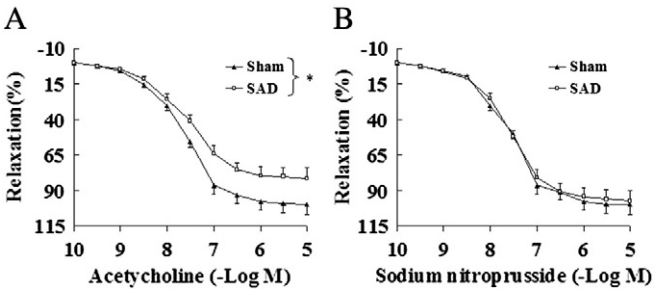

Fig. 3. Relaxations to acetycholine (A) and to sodium nitroprusside (B) of isolated aortae in sham-operated rats and SAD rats. *$P<0.05$ *vs.* sham-operated rats. n = 7 in each group.

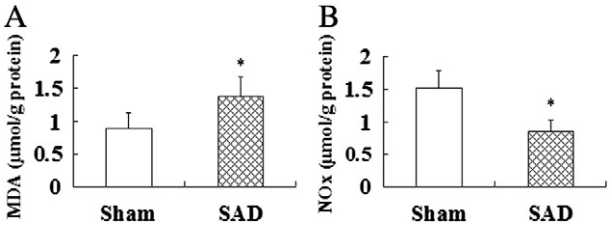

Fig. 4. Aortic MDA and NOx ($NO_2^- + NO_3^-$) content in sham-operated rats and sinoaortic-denervated rats. MDA: malondialdehyde; $^*P<0.05$ *vs.* sham-operated rats. n = 12 in each group.

dramatic elevation in SAD rats compared to sham-operated rats. (3) There was a significant reduction in protein expression of aortic DDAH2 and eNOS in SAD rats compared to sham-operated rats. (4) In vitro, AngII lowered the expression of DDAH2 and eNOS in cultured RAECs, and this effect could be abolished via coincubation with losartan.

When the reflex arc is interrupted by sinoaortic denervation, arterial baroreflex function is markedly impaired (Buchholtz and Nathan, 1984; Norman et al., 1981; Saito et al., 1986). Arterial baroreflex is one of the most important mechanisms for cardiovascular regulation, especially in maintaining the stability of BP (Van-Vliet and Montani, 1999). Baroreflex dysfunction promotes severe vascular disease such as atherosclerosis (Cai et al., 2005), in which endothelial dysfunction may play an important role. In present study, sinoaortic denervation-induced endothelial dysfunction was first confirmed in rats.

To investigate the underlying molecular mechanisms, the role of NO and NO formation-related enzymes was examined in our study. Aortic NOx content was shown decreased in SAD rats, which might account for the impaired endothelium-dependent vasorelaxation. Reduction of NO formation and elevated oxidative stress in aortae in SAD rats might account for the endothelial degeneration and apoptosis. Two interesting enzymes including eNOS and DDAH2 associated with NO production were shown downregulated in SAD rats. Downregulation of eNOS had been observed by immunohistochemistry reported by Shen et al. (2006). In the present study, we reconfirmed it with a more quantitative method of Western blotting. The eNOS plays an important role in endothelium dependent vasodilatation along with other vasoactive substances in blood vasculature (Hermann et al., 2006; Spieker et al., 2000). The impairment of endothelium-dependent dilatation, which is accompanied by the alteration in the expression and/or activity of the eNOS, has consistently been observed in conduit arteries and resistance

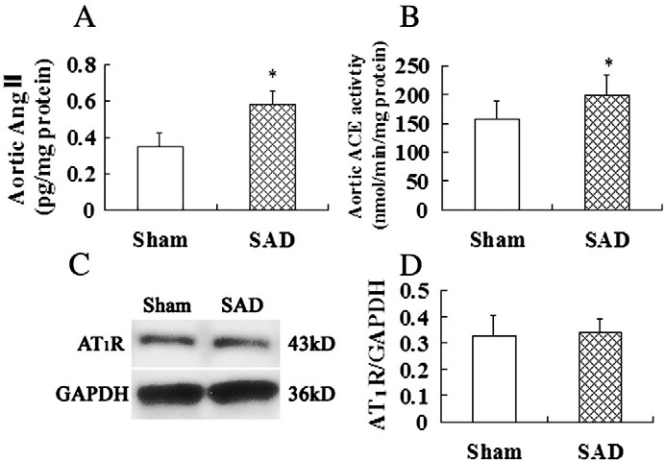

Fig. 5. Aortic AngII levels (A), ACE activities (B) and Western blotting results (C) and responding quantification (D) of AT_1R expression in sham-operated rats and SAD rats. AngII: angiotensinII; ACE: angiotensin converting enzyme; $^*P<0.05$ *vs.* sham-operated rats. n = 12 in each group.

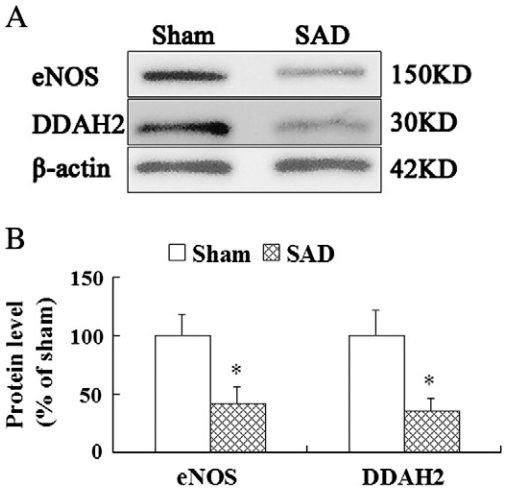

Fig. 6. Expression of eNOS and DDAH2 in the aortae of sham-operated rats and sinoaortic-denervated (SAD) rats. Western blotting results (A) and responding quantification (B) of eNOS and DDAH2 in sham-operated rats and SAD rats was shown. $^*P<0.05$ *vs.* sham-operated rats.

vessels as an effect of hypertension (Forstermann and Munzel, 2006; Münzel et al., 2008). Downregulation of DDAH2 expression in aortae in SAD rats was first observed. Since DDAH metabolizes ADMA and regulates levels of ADMA, it can determine bioavailable NO (Dayoub et al., 2003; Wang et al., 2007). Thus far, elevated ADMA levels have been associated with many established cardiovascular risk factors (Vallance and Leiper, 2004). In our present study, though circulating ADMA level showed no significant alteration, local downregulation of DDAH2 in aortae might lead to a local elevation of ADMA level in aortae after sinoaortic denervation. It was consistent with that plasma ADMA was regulated by DDAH1, which was expressed at sites of ADMA metabolism in the kidney cortex and liver, whereas aortic NO was regulated primarily by DDAH2, which was expressed strongly in blood vessels (Wang et al., 2007).

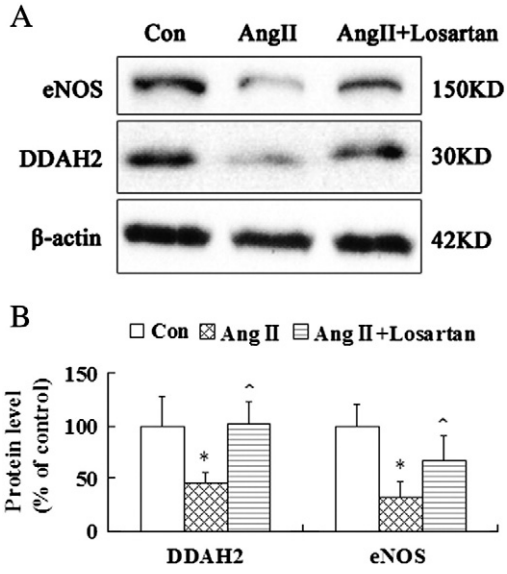

Fig. 7. Stimulation of RAECs with AngII or AngII plus losartan. Western blotting results (A) and responding quantification (B) of eNOS and DDAH2 expression in primary cultured RAECs after 24-h of stimulation with AngII (1 μM) or AngII plus losartan (1 μM). All experiments were performed triplicate. Values are present as mean ± S.D.; RAECs, rat aortic endothelial cells; Con, control; AngII, angiotensinII; $^*P<0.05$ *vs.* control; $^{\wedge}P<0.05$ *vs.* AngII-treated group.

In this study, we found that AngII levels did not show significant alteration in plasma, but increased markedly in aortae locally after sinoaortic denervation, which might contributed to the downregulation of DDAH2 and eNOS. It was reported by many studies that AT_1R blockade significantly augmented NOS activity and eNOS expression in cardiovascular system (Cavanagh et al., 2010; Costa et al., 2010; Loot et al., 2009; Ratliff et al., 2010). In addition it was also reported in human and animals that AngII could result in an elevation of ADMA, and AT_1 blockers could abolish the effect (Aslam et al., 2006; Chen et al., 2007; De-Gennaro et al., 2007; Ito et al., 2001; Jacobi et al., 2008). In our vitro study, we demonstrated that short-term incubation of RAECs with AngII resulted in a reduction of the expression of DDAH2 and eNOS, whereas coincubation with losartan almost completely abolished the effect, indicating that downregulation of DDAH2 and eNOS was, at least in part, due to the local elevation of aortic AngII level in SAD rats.

In conclusion, downregulation of DDAH2 and eNOS induced endothelial dysfunction in SAD rats. DDAH2 and eNOS may be the potential targets for treatment of endothelial dysfunction.

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