Enhanced vasoconstriction to α_1 adrenoceptor autoantibody in spontaneously hypertensive rats

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Autoimmune activities have been implicated in the pathogenesis of hypertension. High levels of autoantibodies against the second extracellular loop of α_1 -adrenoceptor (α_1 -AR autoantibody, α_1 -AA) are found in patients with hypertension, and α_1 -AA could exert a α_1 -AR agonist-like vasoconstrictive effect. However, whether the vasoconstrictive effect of α_1 -AA is enhanced in hypertension is unknown. Using aortic rings of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats, we observed the vasoconstrictive responses to α_1 -AA with phenylephrine (α_1 -AR agonist) as a positive control drug. Aortic nitrotyrosine levels were also measured by ELISA and immunohistochemistry. The results showed that the aortic constrictive responses to α_1 -AA and phenylephrine (both 1 nmol L^{-1} – $10 \text{ } \mu\text{mol L}^{-1}$) were greater in SHR than in WKY rats. Endothelial denudation or L-NAME (a non-selective NOS inhibitor) ($100 \text{ } \mu\text{mol L}^{-1}$) increased α_1 -AA- or phenylephrine-induced vasoconstrictions both in SHR and WKY. However, selective iNOS inhibitor 1400W ($10 \text{ } \mu\text{mol L}^{-1}$) enhanced the α_1 -AA-induced aortic constriction in WKY, but not in SHR. The aortic nitrotyrosine level was significantly higher in SHR than WKY, as shown by both ELISA and immunohistochemistry. These results indicate that the vasoconstrictive response to α_1 -AA is enhanced in SHR, and this altered responsiveness is due to endothelial dysfunction and decreased NO bioavailability. The study suggests an important role of α_1 -AR autoimmunity in the pathogenesis and management of hypertension especially in those harboring high α_1 -AA levels.

autoimmunity, adrenergic receptor, blood vessel, endothelium, protein nitration, hypertension

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Hypertension, a “silent killer” in the worldwide population, affects nearly one billion people in the whole world, and constitutes a major risk factor for myocardial infarction, stroke, and both heart and renal failure [1]. Whereas secondary hypertension (accounting for only 5%–10% of all hypertension cases) always has an identifiable etiology, the

underlying mechanisms for primary hypertension (also known as essential hypertension) remain largely unknown.

Recently, increasing evidences from both animal models and humans indicate that primary or secondary alterations in immune function may play a role in both initiation and maintenance of the hypertensive state [2,3], and much of the patient population suffering primary or malignant hypertension harbors autoantibody against the second extracellular loop of α_1 -adrenergic receptor (α_1 -AR autoantibody, α_1 -AA)

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[4,5]. α_1 -AA exerts a positive chronotropic effect [5]. We and others have reported that α_1 -AA could constrict rat aorta rings by activating α_1 -AR [6,7]. These findings suggest that autoimmunity may serve as a potential etiology for hypertension. Unfortunately, these *in vitro* studies were performed mostly in normotensive rats, conclusions drawn from these experiments may only reflect the action of α_1 -AA in normal condition. Hypertension is associated with enhanced responsiveness to vasoconstrictive agents and decreased endothelium-dependent relaxation [8–11]. Up-regulation of α_1 -AR expression and hyperresponsiveness to α_1 -AR agonists in arteries of SHR are observed [12,13]. Therefore, to better understand the importance of elevated α_1 -AA level present in hypertensive patients, it is necessary to further identify the action of α_1 -AA, a potential endogenous sympathomimetic agent, on the vascular tone in hypertension.

Endothelium plays an important role in the regulation of vascular tone by releasing vasorelaxing factors (including nitric oxide (NO), endothelium-derived hyperpolarizing factor, and prostacyclin) or vasoconstrictive factors (such as endoperoxides, thromboxane A₂ and endothelin) in response to vasoactive substances or physical stimuli [14–16]. Endothelial cells could attenuate the effects of vasoconstrictive substances [17–19] via NO release [20,21], but this vascular tone flexibility is diminished in hypertensive states, and this situation is associated with decreased NO bioavailability and endothelial dysfunction [22,23]. However, the responsiveness of arteries from SHR to α_1 -AA and the role of impaired endothelial function in this reaction are basically unknown.

The signaling molecule NO regulates vital functions such as neurotransmission or vascular tone, gene transcription and translation, and post-translational modifications of proteins [24]. On the other hand, NO can react with superoxide anion ($O_2^{\cdot-}$), forming potent cytotoxin peroxynitrite ($ONOO^-$), an important reactive nitrogen species which may cause oxidative damage and protein nitration [25]. There are three isoforms of NO synthase (NOS) in mammals, including neuronal 'n'NOS (constitutively expressed in neurons and some other cell types), inducible 'i'NOS (its expression can be induced by cytokines and other agents in almost cell types), and endothelial 'e'NOS (constitutively expressed in endothelial cells and few other cell types). The nNOS and eNOS are low-output enzymes and iNOS is a high-output enzyme. The eNOS-derived NO is a protective factor in the vasculature, and the large amounts of NO produced by iNOS are mainly responsible for vascular peroxynitrite formation [26,27]. However, which subtype of NOS contributes to the potential hyperresponsiveness of arteries to α_1 -AA stimulation and α_1 -AA-related hypertension is unclear.

Therefore, we designed this study aiming to investigate whether the vasoconstrictive action of α_1 -AA is enhanced at

least partially due to a decline of endothelium-derived NO, and further, to identify the subtype of nitric oxide synthases (eNOS or iNOS) which contributes to the arterial hyperresponsiveness to α_1 -AA stimulation in hypertension.

1 Materials and methods

1.1 Animals

For observing vascular reactivities to α_1 -AA, SHR and WKY rats were used. Sixteen-week-old male SHRs and age- and gender-matched WKY rats were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Rats were reared in the animal facilities under regular dark-light cycles, and fed ad libitum. Systolic blood pressure (SBP) was measured by tail cuff plethysmography. The SBP was significantly higher in SHR (197 ± 9 mmHg) than in WKY rats (121 ± 6 mmHg) ($P < 0.05$). For active immunization, Wistar rats were used. These Wistar rats underwent serial injections of the peptides corresponding to the second extracellular loop of α_1 -adrenoceptor (α_1 -AR-EC_{II}) to breed α_1 -AA (protocols described below). All animal procedures were conducted in accordance with "Guiding Principles in the Use and Care of Animals" (NIH Publication No. 85-23, revised 1996), and approved by the Life Ethics Committees of Peking Union Medical College.

1.2 α_1 -AR-EC_{II} immunization and α_1 -AA purification

The protocols for α_1 -AA generation and purification were described in our previous study [28]. Briefly, Wistar rats were immunized with α_1 -AR-EC_{II} (amino acid sequence 176–202) by subcutaneous injection. In the first immunization, Freund's complete adjuvant was used together with α_1 -AR-EC_{II}. Freund's incomplete adjuvant and α_1 -AR-EC_{II} were used in the subsequent immunizations. Sham rats received saline injection. Injections were performed every two weeks after the initial immunization for the first three months, and then one injection per month for the remaining five months. The immunization lasted for totally eight months. Before injection and four weeks after injection, blood samples were taken for identifying the appearance of serum α_1 -AA. Serum α_1 -AA levels of both immunized rats and sham rats were determined by enzyme linked immunosorbent assay (ELISA). Serum α_1 -AA positivity was defined as $P/N \geq 2.1$. Here, $P/N = (\text{specimen A} - \text{blank A}) / (\text{negative control A} - \text{blank control A})$. α_1 -AA titer was determined by continuous doubling dilution of the serum samples starting from 1:10 and expressed as the maximal dilution when $P/N \geq 2.1$ [29]. In this study, α_1 -AA refers to serum immunoglobulin G (IgG) against α_1 -AR-EC_{II}. The α_1 -AA was then purified using a MabTrap kit, and its level and specificity were determined by a Coomassie brilliant blue detec-

tion kit (Jiancheng Bioengineering Company, Nanjing, China) and by SDS-PAGE and ELISA, respectively.

For observing the influence of endogenously generated α_1 -AA on arterial active nitrogen species, the α_1 -AR-EC_{II}-immunized Wistar rats were killed eight months after the initial immunization, and their aortae were harvested for immunohistochemical staining of nitrotyrosine, a footprint of ONOO⁻ formation.

1.3 Preparation of aortic rings and recording of vasomotor activity

Rats were euthanized by cervical dislocation and exsanguinated. Thoracic aortae were carefully harvested, then transferred immediately into chilled (4°C) HEPES solution composed of (in mmol L⁻¹) NaCl 144, KCl 5.8, CaCl₂ 2.5, MgCl₂ 1.2, HEPES 5, and D-glucose 11, pH 7.4. The adherent connective tissues of each aorta were carefully trimmed and the aorta was cut into rings of 3 mm length. Care was taken not to stretch the vessel to avoid damage of the luminal surface. Endothelial denudation was achieved by gentle intimal friction with a wooden dowel, and confirmed by the disappearing of the relaxing response to graded acetylcholine (Ach, 1 nmol L⁻¹–1 μ mol L⁻¹) in phenylephrine-precontracted aortic rings.

Aortic rings were suspended with two wire hooks in water-jacketed tissue bath containing 10 ml of HEPES solution (bubbled with 95% O₂ and 5% CO₂, 37°C). The upper hook was connected to a force transducer and the lower hook was connected to the bottom of the tissue, and changes in isometric force were recorded with Chart 5.4 data acquisition system (PowerLab, ADInstruments Co., Ltd). Two-hour equilibration was allowed prior to any experimental intervention. During equilibration, the bath was flushed every 15 min with fresh HEPES solution. Resting tension was adjusted to 1.0 g (a preload found optimal for force development in preliminary studies) and all subsequent measurements represented forces above this baseline tension. After equilibration, the rings were activated twice with HEPES solution containing 60 mmol L⁻¹ KCl (obtained by equimolar replacement of NaCl with KCl in HEPES solution) or 10 μ mol L⁻¹ phenylephrine, at 45-min intervals.

Endothelial integrity was demonstrated by vasorelaxation exceeding 70% when stimulated by 10 μ mol L⁻¹ acetylcholine. When contraction induced by 60 mmol L⁻¹ KCl and relaxation induced by 10 μ mol L⁻¹ acetylcholine were reproducible (defined as <10% change between successive contraction and relaxation), the effect of α_1 -AA was measured. Aortic rings were allowed to rest for at least 30 min after each contraction.

For data presentation, vasoconstrictive responses induced by α_1 -AA or phenylephrine were expressed as a percentage of the tone (gram) invoked by 60 mmol L⁻¹ KCl. Vaso-relaxation responses were expressed as percentage of the

peak tone invoked by 1 μ mol L⁻¹ phenylephrine.

1.4 Vascular reactivity assays

Aortic concentration-response curves for α_1 -AA and phenylephrine (both 1 nmol L⁻¹–10 μ mol L⁻¹) were established at the conditions of endothelial intact or denudation. The role of endothelium in the reactivity of aorta rings to α_1 -AA and the underlying NO signaling were evaluated by analyzing the effect of N^ω-nitro-L-arginine methyl ester (L-NAME, 100 μ mol L⁻¹), a non-selective inhibitor of NOS. To further determine which subtype of NO synthases (eNOS or iNOS) is responsible for the altered vascular responsiveness to α_1 -AA in SHR, we established α_1 -AA concentration-response curves of aortic rings with or without endothelium denudation, and in the presence or absence of N-(3-(aminomethyl)benzyl)-ace-tamidine hydrochloride (1400W) (10 μ mol L⁻¹), a selective iNOS inhibitor.

1.5 ELISA to measure aortic nitrotyrosine level

Nitrotyrosine is a footprint of peroxynitrite (ONOO⁻) formation. A higher level of peroxynitrite in the vascular bed reflects an increase of reactive nitrogen species and protein nitration. Nitrotyrosine level in the aortic rings was estimated by double-antibody sandwich ELISA method described previously [30] and expressed as nmol/g protein.

1.6 Immunohistochemical staining of aortic nitrotyrosine

Thoracic aortae from both sham Wistar rats and α_1 -AR-EC_{II} actively immunized Wistar rats were fixed with formalin, developed and imbedded in paraffin wax. Sections of 5 μ m were cut with a microtome. A standard immunohistochemical ABC method was used for the staining. The sections were incubated in turn with the primary antibody (monoclonal rabbit anti-rat nitrotyrosine), biotinylated secondary antibodies and ABComplex/HRP, and the positive signals were visualized with liquid DAB substrate-chromogen system. The sections were then counterstained with hematoxylin, dehydrated and mounted.

1.7 Western blotting

To observe and compare the difference of iNOS expression in the vascular tissue between WKY and SHR, iNOS expression was analyzed in aortic segments from WKY and SHR under either basal conditions (immediately after dissection of the artery from the animal) or after reactivity experiments. These aortic segments were homogenized in ice-cold Tris-EDTA buffer (in mmol L⁻¹: Tris 50, EDTA 1.0, pH 7.4). Homogenates (30 mg protein per lane) and prestained molecular SDS-PAGE standards (Bio-Rad La-

laboratories, Hercules, CA, USA) were electrophoretically separated on a 7.5% SDS-PAGE and then transferred onto polyvinyl difluoride membranes overnight at 4°C, using a Mini Trans-Blot Cell System (Bio-Rad) containing 25 mmol L⁻¹ Tris, 190 mmol L⁻¹ glycine, 20% methanol and 0.05% SDS. Then, the membrane was blocked for 60 min at room temperature in Tris-buffered solution (10 mmol L⁻¹ Tris, 100 mmol L⁻¹ NaCl, 0.1% Tween 20) with 5% powdered non-fat milk. The membrane was then incubated for 1 h at room temperature with mouse monoclonal antibody for iNOS (1:1000; Upstate Biotechnology, Lake Placid, NY, USA). After washing, the membrane was incubated with anti-mouse IgG antibody (1:2000) conjugated to horseradish peroxidase (MBL, Nagoya, Japan). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, GE Healthcare, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, GE Healthcare). Signals on the immunoblot were quantified with an NIH image computer program. The same membrane was used to determine β -actin expression, and the content of the latter was used to correct iNOS expression in each sample by means of a monoclonal antibody anti- β actin (1:40000; Sigma Chemical). Results were expressed as the ratio between signals on the immunoblot corresponding to iNOS and to β -actin. To compare the results of protein expression within the same experiment and with other experiments, we assigned a value of one to the ratio in arteries from WKY and used that value to calculate the relative density of the other bands in the same gel.

1.8 Reagents

Phenylephrine, N^ω-nitro-L-arginine methyl ester (L-NAME), N-(3-(aminomethyl) ben-zyl) acetamide hydrochloride (1400W), and acetylcholine were purchased from Sigma Inc. (St. Louis, USA). The peptides corresponding to the amino acid sequence of α_1 -AR-EC_{II} (amino acid residues 176-202: G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-A-G-Y-A-V-F-S-

S-V) [5] was synthesized via solid-phase synthesis process by an automated peptide synthesizer. A 95% purity of the peptide was verified by high performance liquid chromatography (HPLC) on an automated amino acid analyzer. This work was completed by GL Biochem LTD (Shanghai, China). Monoclonal rabbit anti-rat nitrotyrosine were purchased from Upstate Technology Co., and biotinylated secondary antibody, ABCComplex/HRP and DAB were from DAKO Co.

1.9 Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical differences were analyzed using Student's *t*-test and ANOVA followed by Bonferroni test. *P*-values less than 0.05 were considered statistically significant.

2 Results

2.1 α_1 -AA induces greater vasoconstriction in SHR than in WKY

We used phenylephrine (a selective α_1 -AR agonist) as a positive control drug. Phenylephrine elicited concentration-dependent constriction of the thoracic aorta rings. This effect was greater in SHR than WKY (Figure 1A), a result which is in concordance with previous reports [22,31,32]. Similar with phenylephrine, α_1 -AA also induced concentration-dependent vasoconstriction, and this action was greater in SHR than in WKY (Figure 1B). The mean values for 60 mmol/L KCl-induced tension increase of aortic rings were 2.34 $\pm$ 0.19 g for WKY (*n*=24) and 2.15 $\pm$ 0.23 g for SHR (*n*=24).

2.2 The vasoconstrictive action of α_1 -AA is augmented by endothelial denudation

Endothelial denudation enhanced the contractile responses

Figure 1 The concentration-response curves showing the constrictive actions of phenylephrine (A) and α_1 -AA (B) on isolated rat thoracic aortic rings with intact endothelium. When resting tension was stable, phenylephrine or α_1 -AA were added to the bath solution and reached serial cumulative concentrations (in mol L⁻¹) of 10^{-9} , 3×10^{-8} , 10^{-8} , 3×10^{-7} , 10^{-7} , 3×10^{-6} , 10^{-6} , 3×10^{-5} and 10^{-5} , respectively. The vasoconstrictive response to each concentration of phenylephrine or α_1 -AA achieved a stable plateau 5–10 min after administration. Note that the vasoconstrictive action of α_1 -AA showed a concentration (10^{-9} – 10^{-5} mol L⁻¹)-dependent fashion, and the maximal tension was about 85.48% $\pm$ 4.56% of that induced by 60 mmol L⁻¹ KCl in WKY and 92.40% $\pm$ 4.82% in SHR, respectively. Each value represents the mean $\pm$ SEM for 8–10 aortic rings obtained from separate rats. *, *P*<0.05 vs. WKY.

Figure 2 Concentration-response curves for phenylephrine- and α_1 -AA-induced contractions of isolated rat thoracic aortic rings from WKY (A and C) and SHR (B and D), with or without endothelial denudation. Note that vasoconstrictive responses to both agents were enhanced after endothelial denudation, and constrictions induced by the two agents were greater in SHR than WKY at endothelium intact. *, $P < 0.05$ vs. WKY with intact endothelium. #, $P < 0.05$ vs. SHR with intact endothelium.

Figure 3 Relaxation response to acetylcholine (ACh) in aortic rings pre-contracted with phenylephrine ($1 \mu\text{mol L}^{-1}$) in WKY and SHR. The relaxation to each concentration of acetylcholine (10^{-9} – $10^{-4} \text{ mol L}^{-1}$) achieved a stable plateau after 5–10 min. The concentration-dependent vasorelaxation in response to acetylcholine stimulation was observed in both WKY and SHR, but was greater in WKY than in SHR. $n=8$ – 10 . *, $P < 0.05$ vs. WKY.

of aorta rings to phenylephrine both in WKY (Figure 2A) and SHR (Figure 2B). Endothelial denudation also enhanced the contractile responses to α_1 -AA both in WKY (Figure 2C) and SHR (Figure 2D), indicating that endothelium plays a compensatory/inhibitory effect on the vasoconstrictive effects of both α_1 -AA and phenylephrine. However, this compensation of endothelium to the vasoconstrictive

action of α_1 -AA was significantly reduced in SHR compared with the normotensive WKY, as demonstrated by the significant difference in EC₅₀ ratio. In WKY, the ratio for EC₅₀ (endothelium intact)/EC₅₀ (endothelium denuded) was 2.05 ± 0.39 ; while for SHR, this EC₅₀ ratio was 1.14 ± 0.18 ($P < 0.01$).

The endothelium-dependent vasorelaxation is usually damaged in hypertension. To clarify if endothelial function works normally at the present experimental condition, we tested the effect of acetylcholine, a well-known endothelium-dependent vasodilator, on the aorta rings of both WKY and SHR. As expected, acetylcholine elicited concentration-dependent relaxation of the phenylephrine-stimulated aortic constriction both in SHR and WKY rats. However, this vasodilatory action of acetylcholine was greater in WKY than SHR (Figure 3).

2.3 eNOS, but not iNOS, is involved in the enhanced vasoconstrictive action of α_1 -AA in SHR

The non-selective NOS inhibitor L-NAME ($100 \mu\text{mol L}^{-1}$) did not affect the basal aortic tone, but increased the vasoconstrictive response to α_1 -AA and phenylephrine in both in SHR and WKY (Figure 4), indicating that endothelium-released NO partially compensated the vasoconstrictive action of α_1 -AA and phenylephrine in both SHR and WKY. To our surprise, although selective inhibition of iNOS by 1400W ($10 \mu\text{mol L}^{-1}$) increased the vasoconstrictive re-

Figure 4 Effects of L-NAME and 1400W on phenylephrine- and α_1 -AA-induced constrictions in isolated rat thoracic aortic rings from WKY (A and B) and SHR (C and D). Aortic rings were preincubated with L-NAME ($10^{-4} \text{ mol L}^{-1}$) or 1400W ($10^{-5} \text{ mol L}^{-1}$) for 10 min, then graded phenylephrine or α_1 -AA were added to the bath solution. Each value represents the mean $\pm$ SEM for 8–10 arterial rings obtained from separate rats. *, $P<0.05$ vs. phenylephrine or α_1 -AA alone (without L-NAME or 1400W preincubation).

sponse to α_1 -AA and phenylephrine in WKY (Figures 4A and B), 1400W did not affect the SHR (Figures 4C and D). This result strongly suggests that it is the eNOS, but not iNOS, that dominantly responsible for the negative compensation of endothelium to the enhanced vasoconstrictive effect of α_1 -AA in SHR, and a reduction of eNOS activity may potentially contribute to arterial hyperreactivity to α_1 -AA in SHR.

In basal conditions, iNOS protein expression could not be detected in aortic segments from WKY and SHR (results not shown). However, iNOS protein expressions were detected in aortic segments from both strains under similar conditions of reactivity experiments, and the iNOS expressions were greater in arteries from SHR than from WKY (Figure 5).

2.4 Reactive nitrogen species is increased in the aorta of SHR

As the half-life of reactive nitrogen species (such as ONOO^-) is very short and difficult to measure, we examined the level of nitrotyrosine, an accepted footprint of peroxynitrite (ONOO^-), in the aortic tissues of SHR and WKY rats using double-antibody sandwich ELISA, in an attempt to estimate if iNOS-derived NO is involved in ONOO^- formation in the artery of SHR. The result turned out that nitrotyrosine level was significantly higher in SHR than in WKY (Figure 6).

Figure 5 Representative Western blot (A) and densitometric analysis (B) for the inducible isoform of nitric oxide synthase (iNOS) in aortic segments from WKY and SHR. Results are expressed as the relative expression of protein in SHR compared with WKY. *, $P<0.05$ vs. WKY ($n=8$ vessels), SHR ($n=5$ vessels).

Figure 6 Nitrotyrosine level in isolated rat thoracic aortae from WKY and SHR measured by ELISA. **, $P<0.01$ vs. WKY ($n=12$ –14 vessels per group).

2.5 Long-term active immunization with α_1 -AR-EC_{II} increases nitrotyrosine expression in the aorta of Wistar rats

To further determine if endogenous α_1 -AA could also increase reactive nitrogen species in the vascular bed *in vivo*, Wistar rats were subjected to active immunization with α_1 -AR-EC_{II} for eight months as described above. Then, aortic arteries were harvested and nitrotyrosine expression was determined by immunohistochemistry. The nitrotyrosine-positive signals were observed in the aorta of immunized rat, but not in the sham rat (Figure 7). Nitrotyrosine expression was shown both in endothelium and medial smooth muscle layer, but was stronger in the endothelium (Figure 7). This result, combined with that of SHR (Figure 6), indicating that vascular reactive nitrogen species were increased in SHR, and also in Wistar rats with long-term endogenous α_1 -AA stimulation.

3 Discussion

This study linked α_1 -AR autoimmunity and hypertension and found that arterial constriction to α_1 -AA was significantly enhanced in SHR compared with the WKY control, and the impairment of endothelial function and subsequent decrease of NO release in SHR partially contributed to this augmented responsiveness to α_1 -AA. The results further suggest that abnormal activity of eNOS, but not iNOS, is responsible for the vascular hyperreactivity to α_1 -AA in SHR. In addition, we demonstrated that aortic nitrotyrosine level was significantly higher in SHR than WKY, suggesting that the arterial level of reactive nitrogen species is increased both in SHR and α_1 -AR-EC_{II}-immunized Wistar rats, and iNOS-derived NO appears not taking a role in vascular tone compensation, but mainly acts in the process of reactive nitrogen species and potential protein nitration. Taken together, the present study suggests that α_1 -AR autoimmunity plays an important role in vascular hyperreactivity

and protein nitration in hypertension, and strategies that suppress this autoimmunity may have a clinical perspective for treating hypertension.

The finding of adrenoceptor autoimmunity in hypertension greatly impacted the understanding of hypertension mechanisms. In 1994, Fu et al. [33] first reported that the circulatory α_1 -AA level was increased in patients with malignant hypertension compared with the normotensive subjects. α_1 -AA was subsequently demonstrated to manifest α_1 -AR agonist-like effects [5,6,34]. Wenzel and colleagues and our recent study demonstrated that α_1 -AA elicits remarkable vasoconstricting action in a concentration-dependent fashion in large conduit vessels as well as small resistance vessels via α_1 -AR activation [6,7]. However, as these previous results were obtained in normotensive animals, conclusions regarding the role and significance of α_1 -AA can only be made in the normotensive condition. Hypertension is a complicated pathologic state, associated with increased responsiveness to vasoconstrictive agents and decreased endothelium-dependent relaxation [8–11], and also related with upregulation of α_1 -AR expression and hyperreactivity to α_1 -AR agonists in arteries from SHR [12,13]. We for the first time observed that α_1 -AA exerts differential vasoconstrictive actions in hypertensive and normotensive animal models. The vasoconstrictive properties of α_1 -AA are similar with phenylephrine, a standard α_1 -AR agonist. Our data demonstrated an augmented vasoconstrictive response to α_1 -AA in SHR, suggesting that circulating α_1 -AA in patients with hypertension may elicit a more potent vasoconstrictive effect than in the normotensive subjects.

It has been reported that vasoconstriction, either induced by sympathetic stimulation or by sympathomimetic agents, is attenuated significantly in the presence of functional vascular endothelium. Several explanations have been proposed for the vasorelaxing properties of endothelium, including the release of vasodilator substances which oppose the vasoconstrictor effects of sympathomimetic agents

Figure 7 Immunohistochemical stains of aortic nitrotyrosine (400 $\times$). The aortic tissues were obtained from Wistar rats eight months after α_1 -AR-EC_{II} immunization. Note that nitrotyrosine (brown color) was negative in the sham rat, but was abundant in the immunized rat, and positive stains were observed both in the endothelium and the medial smooth muscle layer, but the signals were even stronger in the endothelium.

[35,36]. In addition, α_1 -AR activation in vascular endothelium is conducive to NO synthesis, thereby contributing to countering norepinephrine- and/or phenylephrine-induced vasoconstrictions [35,37]. The present study showed that endothelium removal or pretreatment with the non-selective NOS inhibitor L-NAME enhanced α_1 -AA-induced vasoconstriction in both WKY and SHR, indicating that eNOS and its product endothelium-derived NO is involved in the negative compensation of endothelium on the vasoconstrictive action of α_1 -AA both in SHR and WKY rats.

It is known that NOS promotes the production of NO in endothelium, but the subtype of NOS responsible for this process in hypertension has not yet been identified. eNOS localizes leadingly in the endothelium and is a low-output enzyme; while iNOS is expressed in many types of cells and is a high-output enzyme, and its expression could also be increased under the induction of certain stimuli. This expression profile suggests that eNOS is mainly responsible for vascular tone regulation, but iNOS may contribute to excessive NO production and underlie oxidative stress and protein nitration in response to pathological stimuli. Alvarez et al. [22] reported that iNOS mRNA and protein levels were higher in the aortae of SHR than WKY rats, but selective iNOS inhibitor 1400W did not affect phenylephrine-induced aortic constriction in SHR, suggesting that iNOS-derived NO in SHR is not involved in vascular tone regulation, but may take a role in the production of reactive nitrogen species and protein nitration, if compared with the normotensive rats.

We observed here that selective inhibition of iNOS by 1400W increased the vasoconstrictive response to α_1 -AA in WKY, but not in SHR, suggesting that iNOS affects vasoconstriction to α_1 -AA and phenylephrine in WKY but not in SHR, although the iNOS expression level was higher in the perfused aortic tissues of SHR than that of WKY. Other investigators also showed that the expressions of iNOS protein and mRNA of aortic tissues were higher in SHR than in WKY, but their experiments were conducted in perfused aortic tissues, not in fresh tissues [38,39]. We also showed here that the iNOS protein expression of perfused aortic segments was higher in the aortic segments of SHR than that of WKY. Some investigators suggested that endotoxin may induce iNOS expression under the *in vitro* perfusion condition compared with the *in vivo* condition (for example, examinations on fresh tissues), as endotoxin was usually present in the tissue bath, although with low level [40,41]. This may explain why iNOS expression was detectable in perfused tissues but undetectable in fresh tissues. The discrepancy between iNOS expression and vasomotor function (high expression level but no vasomotor function) in SHR compared with WKY can be explained as follows. The lower level iNOS-derived NO in WKY may be involved in vascular tone compensation under normotensive state, but higher level iNOS-derived NO in SHR may react with in-

creased superoxide anion to promote ONOO⁻ formation and protein nitration due to elevated oxidative stress in SHR, thereby decreasing NO bioavailability and increasing protein nitration, as evidenced by the high expression of nitrotyrosine (Figure 6) in the aorta of SHR. This result is consistent with the reported literature [22] and supports our hypothesis that during hypertension, decreased eNOS partially contributes arterial hyperreactivity to α_1 -AA, while increased iNOS does not contribute to vascular tone regulation, but is mainly involved in the production of reactive nitrogen species and protein nitration.

In conclusion, we demonstrate that the vasoconstrictive response to α_1 -AA is augmented in SHR compared with that of WKY rats. This phenomenon is associated with enhanced oxidative stress and increased reactive nitrogen species in SHR. These pathological changes may subsequently lead to reduction of NO bioavailability and impairment of endothelial compensation to α_1 -AA stimulation in hypertension. The study suggests a potential therapeutic modality in hypertension management, such as α_1 -AA removal or neutralization.

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