Nitric Oxide 87 (2019) 10–22

Contents lists available at ScienceDirect

Nitric Oxide

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

The role of NO-cGMP pathway inhibition in vascular endothelial-dependent smooth muscle relaxation disorder of AT1-AA positive rats: protective effects of adiponectin

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ARTICLE INFO

Keywords:
Angiotensin II type 1 receptor
Autoantibody
Adiponectin
Endothelial-dependent smooth muscle relaxation disorder
Nitric oxide

ABSTRACT

Angiotensin II type 1 receptor autoantibodies (AT1-AA) cause endothelial-dependent smooth muscle relaxation disorder. It is well understood that impairment of the NO–cGMP signaling pathway is one of the mechanisms of endothelial-dependent smooth muscle relaxation disorder. However, it is still unclear whether AT1-AA induces endothelial-dependent smooth muscle relaxation disorder via the impairment of the NO–cGMP signaling pathway. In addition, adiponectin exerts vascular endothelial protection through the NO–cGMP signaling pathway. Therefore, the purpose of this investigation was to assess the mechanism of vascular endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA and the role of adiponectin in attenuating this dysregulation.

Serum endothelin-1 (ET-1), adiponectin and AT1-AA were detected by enzyme-linked immunosorbent assay. In preeclamptic patients, there was an increased level of AT1-AA, which was positively correlated with ET-1 and negatively correlated with adiponectin, as elevated levels of ET-1 suggested endothelial injury. AT1-AA-positive animal models were actively immunized with the second extracellular loop of the angiotensin II type 1 receptor (AT1R-ECII) for eight weeks. In thoracic aortas of AT1-AA positive rats, ET-1 was elevated, endothelium-dependent vasodilation was decreased. Paradoxically, as the upstream element of the NO-cGMP signaling pathway, NO production was not decreased but increased, and the ratio of p-VASP/VASP, an established biochemical endpoint of NO-cGMP signaling pathway, was reduced. Moreover, the levels of nitrotyrosine and gp91phox which indicate the presence of peroxynitrite ($ONOO^-$) and superoxide anion ($O_2^{\cdot -}$) were increased. Pretreatment with the $ONOO^-$ scavenger FeTMPyP or $O_2^{\cdot -}$scavenger Tempol normalized vasorelaxation. Key enzymes responsible for NO synthesis were also assessed. iNOS protein expression was increased, but p-eNOS(Ser1177)/eNOS was reduced. Preincubation with the iNOS inhibitor 1400 W or eNOS agonist nebivolol restored vasorelaxation. Further experiments showed levels of p-AMPKα (Thr172)/AMPKα, which controls iNOS expression and eNOS activity, to have been reduced. Furthermore, levels of the upstream component of AMPK, adiponectin, was reduced in sera of AT1-AA positive rats and supplementation of adiponectin significantly decreased ET-1 contents, improved endothelial-dependent vasodilation, reduced NO production, elevated p-VASP/VASP, inhibited protein expression of nitrotyrosine and gp91phox, reduced iNOS overexpression, and increased eNOS phosphorylation at Ser1177 in the thoracic aorta of AT1-AA positive rats.

These results established that impairment NO–cGMP pathway may aggravate the endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats and adiponectin improved endothelial-dependent

Abbreviations: AT1-AA, angiotensin II type 1 receptor autoantibodies; NO-cGMP, nitric oxide-cyclic guanosine monophosphate; ET-1, endothelin-1; $ONOO^-$, peroxynitrite; $O_2^{\cdot -}$, superoxide anion; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; AICAR, Acadesine; gAPN, globular adiponectin

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https://doi.org/10.1016/j.niox.2019.02.006
Received 30 August 2018; Received in revised form 16 January 2019; Accepted 19 February 2019
Available online 01 March 2019

smooth muscle relaxation disorder by enhancing NO–cGMP pathway. This discovery may shed a novel light on clinical treatment of vascular diseases associated with AT1-AA.

1. Introduction

Vascular dysfunction caused by endothelial injury is an essential early event in the pathogenesis of many cardiovascular diseases. With regard to the impairment of vascular structure and function caused by abnormal activation of the renin-angiotensin-system (RAS), the treatment effects of angiotensin-converting enzyme inhibitors (ACEI) are inferior to the effects that have been observed with AT1 receptor blockers (ARBs) [1]. This suggests that angiotensin II (Ang II) is not the only substance that activates the AT1 receptor, which implies that some other unknown substances are involved.

AT1R autoantibodies (AT1-AA) are detected in a variety of vascular diseases (such as preeclampsia [2], primary hypertension [3], malignant hypertension [4] and systemic sclerosis [5]). These autoantibodies exert a receptor agonist-like effect, but unlike Ang II, they lead to sustained activation of AT1R. In a previous report from our laboratory, endothelium-dependent vasorelaxation was reduced in AT1-AA positive rats, and AT1-AA caused a significant increase of lactate dehydrogenase (LDH) activity in cocultured HUVECs after 48 h [6], which suggested that AT1-AA led to endothelial-dependent smooth muscle relaxation disorder. However, the underlying mechanism involved in the endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA remains unknown. It has been well established that the impaired NO–cGMP signaling cascade is one of the classical mechanisms of endothelial-dependent smooth muscle relaxation disorder [7]. However, whether endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive patients and animals is associated with impaired NO–cGMP signaling cascade is unclear.

Serum levels of adiponectin are significantly lower in patients and animals with endothelial-dependent smooth muscle relaxation disorder [8–10], and adiponectin supplementation has been shown to improve endothelial function through the NO-cGMP signaling cascade [11,12]. Moreover, our previous work has demonstrated that physiological adiponectin doses enhance endothelial-dependent smooth muscle relaxation disorder in a NO-mediated manner [13]. However, whether supplementation of adiponectin attenuates endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA has not been previously investigated.

Therefore, the aims of this investigation were: (1) to establish an AT1-AA positive animal model and to determine if an impaired NO–cGMP signal pathway is a candidate mechanism for endothelial-dependent smooth muscle relaxation disorder; and (2) determine whether supplementation of adiponectin improves endothelial-dependent smooth muscle relaxation disorder by enhancing NO–cGMP signal pathway. We tested the hypothesis that supplementation with adiponectin would significantly attenuate markers of endothelial-dependent smooth muscle relaxation disorder in a preclinical animal model with the NO-cGMP pathway impaired. Results from this investigation would provide valuable insight into the potential therapeutic benefits of adiponectin supplementation and may lead to further development of this treatment strategy.

2. Materials and methods

2.1. Ethics and clinical experiment

All protocols used herein were approved by the Institutional Committee for the Protection of Human Subjects in Chengde Medical College Affiliated Hospital. All patients were informed of the purpose and protocol of the study, and written consent was obtained. The clinical trial was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Inclusion criteria: According to the guidelines of the International Society for the Study of Hypertension in Pregnancy [14], preeclampsia is defined by an increase in blood pressure (BP) to at least 140 mmHg systolic BP (SBP) or 90 mmHg diastolic BP (DBP), or both, after the 20th week of gestation in a previously normotensive woman and in the presence of proteinuria (+). In our present study, for preeclamptic patients, the gestational age at delivery ranged from 38 to 42 weeks, with an average of 40 weeks. Uncomplicated pregnancies characterized healthy pregnant women with normal-term deliveries. For healthy pregnant women, the gestational age at delivery ranged from 36 to 40 weeks, with an average of 38 weeks. Twenty-five preeclamptic patients and 20 healthy pregnant women were enrolled in the study between October 2016 and March 2017 in Chengde Medical College Affiliated Hospital. All serum samples were collected from the 8-h fasted research subjects. Adiponectin and ET-1 levels were measured with Total Adiponectin/Acrp30 Quantikine ELISA Kit (R&D, USA) and ET-1 using a Quantikine ELISA Kit (R&D, USA).

Exclusion criteria included autoimmune disease or endocrine disease, acute myocardial infarction, malignant tumor, liver and renal failure, chronic gastritis, outflow tract obstruction, pregnancy and lactating women, and infectious diseases.

2.2. Animals

All preclinical animal procedures utilized herein conformed to the "Guiding Principles in the Use and Care of Animals" published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Animals were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. China. Before the experiments, rats were maintained in 12-h light-dark cycles and food and water were available *ad libitum*. Healthy AT1-AA-negative male Sprague-Dawley rats weighing 0.18–0.20 kg, 6–8 weeks old (SPF grade, 6 rats in each group) were randomly divided into 3 groups: (1) vehicle; (2) AT1-AA immunized; (3) AT1-AA immunized + globular adiponectin (gAPN). The peptide corresponding to the sequence of the second extracellular loop of the human AT1 receptor (165–191, I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T) was synthesized by GL Biochem (Shanghai) Ltd. At first, the immunized group rats were actively immunized using 0.4 μg per g body weight of the corresponding peptide, which was emulsified in the same volume of complete Freund's adjuvant and injected subcutaneously at multiple sites. Two weeks later, a booster injection of a mixture of 0.4 μg per g body weight of the corresponding peptide in the same volume of incomplete Freund's adjuvant was injected subcutaneously at one location, once every two weeks until the eighth week. The vehicle group rats were injected using adjuvant and saline without peptides following the same procedure as the immunized. Animals were euthanized by a physical method (decapitation, a suggested method for rodents by AVMA Guidelines on Euthanasia). All animals were euthanized with sodium pentobarbital (50 mg/kg, i.p.) to reduce animal anxiety on the guillotine and ensure the euthanasia was rapidly accomplished to lessen the animal suffering. The blood samples of all rats were collected for detection of serum AT1-AA, adiponectin, and ET-1 levels.

2.3. Measurement of vascular function

The thoracic aortic segments and small mesenteric arteries were excised and placed in ice-cold oxygenated Hepes buffer (mM: NaCl, 144; KCl, 5.8; $MgCl_2{\cdot}6H_2O$, 1.2; $CaCl_2$, 2.5; Glucose, 11.1; Hepes, 5; pH

7.38–7.40). The thoracic aortic segments and mesenteric arteries were cleaned of adhering tissues, cut into rings (2 mm length), and incubated with vehicle or globular adiponectin (gAPN, purchased from PeproTech Company) (Rocky, Hill, NJ). 2 μg/mL was selected as the optimal dose and used in the rest of the experiments in a cell culture incubator [15]. After 4 h of incubation, endothelial function was performed as described below. After the equilibration period, the artery segments were exposed to Hepes buffer containing 60 mM potassium (mM: NaCl, 144; KCl, 60; $MgCl_2{\cdot}6H_2O$, 1.2; $CaCl_2$, 2.5; Glucose, 11.1; Hepes, 5; pH 7.38–7.40) until reproducible contractile responses were obtained. After washing with HEPES buffer, segments of thoracic aortas and mesenteric arteries were precontracted with norepinephrine (NE, 10^{-6} mol/L) and phenylephrine (PE, 10^{-6} mol/L) respectively. Once a stable contraction was achieved, increasing concentrations of vasodilators (10^{-9}-10^{-5} mol/L) were added to the chamber to obtain cumulative concentration-response curves. Endothelium-dependent dilation was measured by acetylcholine (ACh), and endothelium-independent dilation was measured by sodium nitroprusside (SNP). FeTMPyP ($ONOO^-$ scavenger; 10^{-5} mol/L; Sigma Aldrich) [16], Tempol (superoxide anion scavenger; 10^{-4} mol/L; Torcis) [17]$^{\cdot}$, 1400 W (inducible nitric oxide synthase (iNOS) inhibiter; 10^{-6} mol/L; Sigma Aldrich) [18], Nebivolol (endothelial nitric oxide synthase (eNOS) agonist; 10^{-4} mol/L; Torcis) [19], L-NAME (NOS inhibitor, 10^{-1} mol/L; Torcis) [15], AICAR (AMPK agonist; 10^{-3} mol/L; Torcis) and Compound C (AMPK inhibitor; 10^{-5} mol/L; Torcis) [20] was pre-incubated in the chamber for 30 min in order to inhibit $ONOO^-$ or its sources in vehicle and AT1-AA immunized group rats' arteries.

2.4. Streptavidin-enzyme-linked immunosorbent assay

Venous blood samples were collected in vials without the use of an anticoagulant agent. After centrifugation at 4 °C, the sera were immediately separated and stored at −80 °C until assay. The levels of AT1-AA in patients were measured using enzyme-linked immunosorbent assay (ELISA), and the results were expressed as optical density (OD) values. Briefly, synthetical peptide 165–191 (I-H-R-N-V-F-F-I-I-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T-L), which is the sequence of the second extracellular loop of the AT1 receptor (5 mg/mL) in a 100 mmol/L Na_2CO_3 solution (pH 11.0), was coated on microtiter plates overnight at 4 °C. The wells were then saturated with 0.1% PMT buffer [0.1%(w/v) albumin bovine V, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS), pH = 7.4] for 1 h at 37 °C. After washing three times with PBS-T, human serum dilutions were added to the saturated microtiter plates for 1 h at 37 °C. After three washings, biotinylated goat anti-human IgG antibodies (Sigma) (1:1000 dilutions in PMT) were added for 1 h at 37 °C. Following three washings, streptavidin-peroxidase conjugate (Sigma) at 1:2000 dilution in the same buffer was added into the wells and incubated under the same conditions. Finally, 2,2-azino-di (3-ethylbenzothiazoline) sulfonic acid (ABTS) -H_2O_2 (Roche, Basel, Switzerland) substrate buffer was added and reacted for 30 min in the dark at room temperature. The ODs were measured at 405 nm using an ELISA reader. We also calculated positive/negative (P/N) ratio [(the OD of sample-the OD of empty control)/(the OD of negative control-the OD of empty control)] of each sample, and those samples with a P/N value at least 2.1 were considered as AT1-AA positive [21]. A modified ELISA was used to detect the levels of AT1-AA in rats' serum [22].

Adiponectin and ET-1 contents were determined by the corresponding Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (R& D, USA), Rat Total Adiponectin/Acrp30 Quantikine ELISA Kit (R&D, USA) and ET-1 Quantikine ELISA Kit (R&D, USA) following the manufacturer's instructions, respectively.

2.5. Nitrite and nitrate measurement

The serum levels of nitrite plus nitrate (NOx) were assessed as nitrite concentration as described previously [23]. Briefly, sera from the vehicle and AT1-AA immunized group rats were diluted in three times, and ultrafiltered through a 10 kDa cutoff filter (BioVision) to remove sera proteins. NOx was measured by a commercial kit (BioVision) based on the Griess reaction. The nitrate was converted to nitrite with nitrate reductase. The Griess Reagent was added to the total nitrite, and the color was developed for 10 min at room temperature. After the reaction was completed, NOx concentrations were determined at an optical density of 540 nm by comparison with standard solutions of sodium nitrite.

2.6. Immunohistochemical staining

Immunohistochemical staining was determined using the method as described previously [24]. Briefly, thoracic aortic segments were removed and stored in 4% paraformaldehyde for 48 h. Fixed segments were dehydrated and embedded in paraffin and sections were cut into 6 mm thickness and mounted onto glass slides. Antigen was retrieved by using a microwave method (citric acid buffer, pH 6.0). Endogenous catalase was inactivated with 3% hydrogen peroxide for 10 min at room temperature. The sections were stained with primary antibody anti-nitrotyrosine (NT), Abcam; anti-vasodilator-stimulated phosphoprotein (VASP), Abcam; anti-phosphorylated VASP (Ser239) p-VASP (Ser239), Abcam; anti-gp91phox, Abcam; anti-inducible nitric oxide synthase (iNOS), Abcam; anti-endothelial nitric oxide synthase (eNOS), Santa Cruz; anti-phosphorylated eNOS (Ser1177) p-eNOS (Ser1177), Santa Cruz; anti-neuronal nitric oxide synthase (nNOS), Abcam; at 4 °C overnight and peroxidase-conjugated affinipure secondary antibody (Santa Cruz) at 37 °C for 30 min, successively. Target proteins were detected with diaminobenzidine (DAB). Protein quantification was performed by mean density of staining of the vessel tissues using Image-Pro Plus (version 6.0).

2.7. DHE staining

Briefly, the freshly thoracic aortic arteries were frozen at optimal cutting temperature compound and transverse sections were produced using a cryostat. Sections were incubated with dihydroethidine (2 mmol/L, Invitrogen, Carlsbad, CA, US) at 37 °C for 30 min in the dark. The slides were briefly rinsed with PBS and then mounted with VECTASHIELD HardSet Mounting Medium with DAPI (Vector). DHE fluorescence was quantified by automated image analysis using Image Pro Plus 6.0 software.

2.8. Western blot analysis

The thoracic aortic segments were pulverized in liquid nitrogen and resolubilized in lysis buffer. Equal amounts of protein (80 μg protein/lane) were electrophoresed on a 10% SDS-polyacrylamide gel and electrophoretically transferred to a poly (vinylidene difluoride) membrane (Millipore, Billerica, MA). After blocking with 5% skim milk in Tris-buffered saline at room temperature for 1 h, we incubated the membrane with an antibody against NT, p-VASP (Ser239), VASP, gp91phox, iNOS, eNOS, p-eNOS (Ser1177), nNOS, p-AMPKα (Thr172), AMPKα, HSP90α, HSP90β, AKT, caveolin-1 and endothelin A2 overnight at 4 °C. The membrane was then washed with PBS and incubated with horseradish peroxidase-conjugated IgG antibody (Cell Signaling) for 1 h at 37 °C. The blots were developed with an enhanced chemiluminescence detection kit (Applygen Technologies Inc, Beijing). The immunoblotting was visualized with ChemiDocXRS (Bio-Rad Laboratory, Hercules, CA), and the blot densities were analyzed with Image Lab software.

2.9. Statistics analysis

The ratio of P/N was used to represent the patients' AT1-AA levels

and is presented as a median ± interquartile range. The relationship among serum levels of AT1-AA, adiponectin, and ET-1 was analyzed using linear regression. All other data are presented as mean ± the standard error of the mean (SEM). Data were analyzed using SPSS19.0 and PRISM 5.0 statistical software. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Differences were considered statistically significant at a value of $P < 0.05$.

3. Results

3.1. Increased AT1-AA levels were positively correlated with ET-1 contents and negatively correlated with adiponectin contents in pregnant women serum

20 healthy pregnant women and 25 preeclamptic patients were included in the present investigation. Maternal medical records were reviewed, and blood samples were obtained from each participant. The age of the pregnant women ranged from 23 to 37 years, and the average age was 30.67 ± 6.08 years. Compared with healthy pregnant women, systolic and diastolic blood pressure increased (Table 1, $P < 0.05$ *vs.* Healthy pregnancy), proteinuria was positive (Table 1) and there were no significant changes in glucose and lipid metabolism (Table S1) in preeclamptic patients.

Serum samples from healthy pregnant women and preeclamptic patients were collected to study the correlation between the levels of AT1-AA, ET-1, and adiponectin. As illustrated in this study, serum levels of AT1-AA ($P < 0.01$ *vs.* Healthy pregnancy, Fig. S1) and ET-1 ($P < 0.01$ *vs.* Healthy pregnancy, Fig. 1C) were higher, but serum levels of adiponectin were lower in the preeclampsia group than those in healthy pregnancy group ($P < 0.01$ *vs.* Healthy pregnancy, Fig. 1A). In addition, the levels of AT1-AA were positively correlated with levels of ET-1 (n = 45, Fig. 1D), but negatively correlated with levels of adiponectin (n = 45, Fig. 1B). Moreover, there was no significant correlation between the serum levels of ET-1 and adiponectin (n = 45, Fig. S2).

3.2. Endothelial injury and vasorelaxation disorders in AT1-AA positive rats

To further investigate the mechanisms of endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA, an AT1-AA positive rat model was successfully established (Fig. S3). After collection of rat serum and isolation of thoracic aortas and mesenteric arteries, as shown in Fig. 2, ET-1 levels in sera and thoracic aortas of AT1-AA positive rats were elevated ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. Vehicle, n = 6, Fig. 2A and E). Ach is involved in the regulation of vasodilation by binding to the acetylcholine M receptor on the surface of endothelial cells, which promotes the release of NO from endothelial cells to relax the smooth muscle cells. In this way, Ach-induced endothelium-dependent relaxation can indicate the ability of the endothelium to regulate smooth muscle relaxation [25]. Moreover, SNP as a NO donor, not only directly releases nitric oxide without generating oxygen radicals [26], but also can reduce the level of superoxide anion concentration, thereby improving and attenuating endothelial-dependent smooth muscle relaxation disorder [27]. In this way, SNPs are used to detect endothelium-independent relaxation responses of blood vessels. In our study, the concentration-dependent relaxation responses to acetylcholine in thoracic aortas and mesenteric arteries were decreased in AT1-AA positive rats ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Vehicle, n = 6, Fig. 2B, Fig. S4A), and the concentration-dependent relaxation responses to sodium nitroprusside remained unchanged in AT1-AA positive rats ($P > 0.05$ *vs.* Vehicle, n = 6, Fig. 2C, Fig. S4B).

3.3. NO overproduction, reduced the ratio of p-VASP/VASP, elevated levels of $ONOO^-$ and its source ($O_2^{\cdot-}$) in thoracic aortas of AT1-AA positive rats

Under normal conditions, vascular endothelial cells can be continuously active and produce NO to maintain basal vasodilator tone. In order to detect the production of NO in arteries, the level of NOx is highly correlated with NO level in serum [28,29]. Compared with vehicle group rats, NOx levels in sera and thoracic aortas of AT1-AA positive rats were increased ($^{*}P < 0.05$ vs. Vehicle, n = 6, Fig. 3A and B). This suggested that the levels of vascular NO in AT1-AA positive rats increased, rather than decreased. The large amount of NO and superoxide anion ($O_2^{\cdot-}$) react at a rate similar to that of biological diffusion [30]. This group's endothelial-dependent smooth muscle relaxation disorder may not have been caused by the reduction of NO production, but from the outlet obstruction of NO, which was the NO–cGMP signaling pathway impairment.

In all cases, according to previous reports and publications [31,32] the activity of vascular smooth muscle cGMP is based on the level of p-VASP/VASP, which can serve as a reference indicator or surrogate parameter. In our study, the ratio of p-VASP/VASP in AT1-AA positive rat thoracic aorta was lower than in rats in the vehicle group ($^{*}P < 0.05$ *vs.* Vehicle, n = 6, Fig. 3C and D), which implied that the NO–cGMP signaling pathway was impaired.

To further examine the contradiction between the increased production of NO and impaired NO–cGMP signaling in AT1-AA positive rats, we measured the amount of peroxynitrite ($ONOO^-$), a downstream product of NO, in rat thoracic aortas. $ONOO^-$ nitrated tyrosine on proteins to form NT (nitrotyrosine) and NT is generally considered as the biomarker of the $ONOO^-$ in tissues and cells [29]. In our study, the levels of NT were increased in AT1-AA positive rat thoracic aorta ($^{**}P < 0.05$ *vs.* Vehicle, n = 6, Fig. 3C and D). To identify the role of $ONOO^-$ in AT1-AA positive rat vasorelaxation disorders, the $ONOO^-$ scavenger (FeTMPyP, 10^{-5} mol/L) was used to inhibit $ONOO^-$ in thoracic aortas. Vasorelaxation responses to Ach were normalized after $ONOO^-$ scavenger preincubation in AT1-AA positive thoracic aorta ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 3E), which suggested that $ONOO^-$ was involved in endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats.

Moreover, the gp91phox protein expression, a major component of NADPH oxidase and ROS generation in the thoracic aorta was determined. As illustrated in Fig. 3C, 3D, S5, compared with vehicle group rats, increased gp91phox content and DHE fluorescence staining in thoracic aortas of AT1-AA positive rats were observed ($^{*}P < 0.05$ *vs.* Vehicle, n = 6, Fig. 3C and D, Fig. S5). There were gp91phox positive staining both in endothelial tissue and smooth muscle cells of the thoracic aorta. And there were also literature reported [33,34], that after digesting primary cultured vascular smooth muscle cells (VSMC) isolated from rat aorta, the gp91phox protein expression were successfully detected in VSMC by western blot technique. Pretreatment with the $O_2^{\cdot-}$ scavenger Tempol ($10-4$ mol/L) ameliorated the

Table 1
Basic information and clinical profile in preeclampsia and healthy pregnant women.

Characteristics	Healthy pregnancy (n = 25)	Preeclampsia (n = 20)
Maternal age (years)	31 ± 5.3	30 ± 6.8
Gestational age at sampling (weeks)	40 ± 2.5	38 ± 2.8
BMI	29.7 ± 2.7	31.6 ± 4.6
SBP (mmHg)	117.6 ± 9.4	152.1 ± 18.1*
DBP (mmHg)	79.0 ± 8.8	100.7 ± 10.6*
Proteinuria	(-)	(+ … + + +)

$^{*}P < 0.05$ *vs.* Healthy pregnancy, n = 45. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

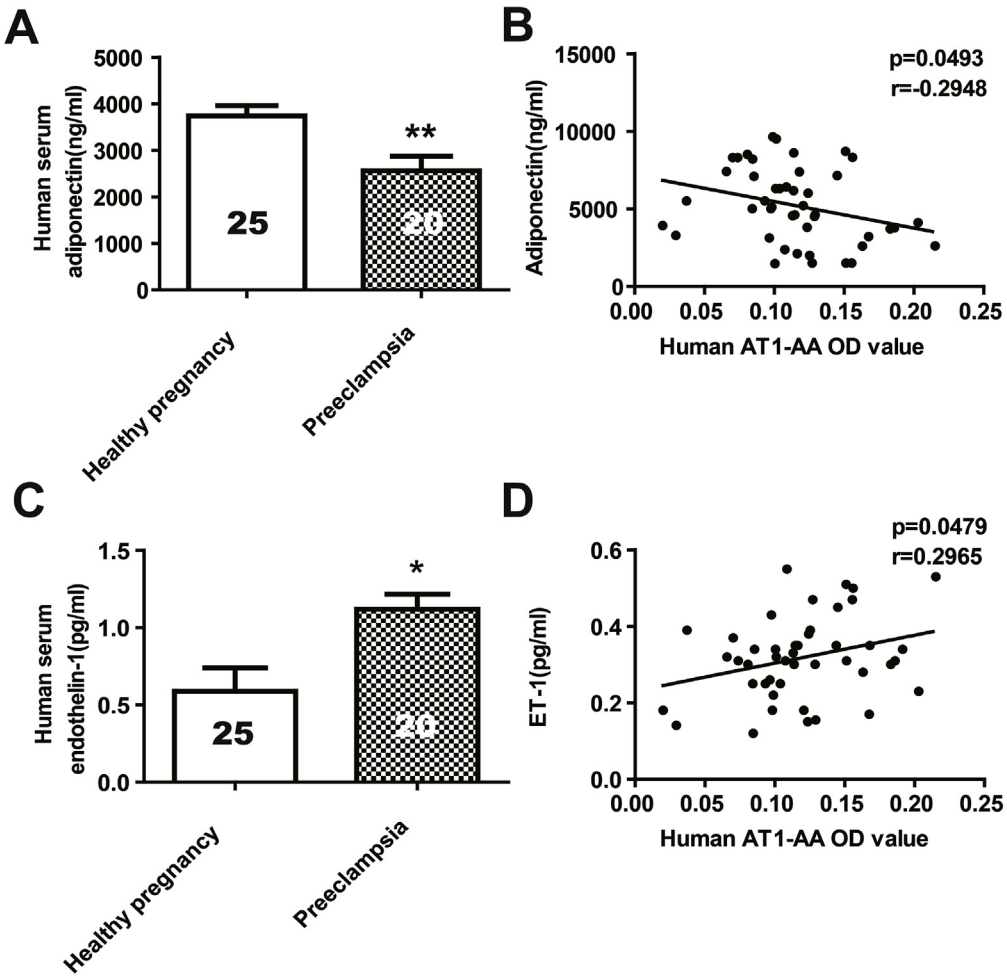

Fig. 1. Changes of Adiponectin, ET-1, and AT1-AA in the serum of pregnant women. (A) The levels of serum adiponectin were decreased in the preeclampsia group compared with healthy pregnancy group, $^{**}P < 0.01$ *vs.* healthy pregnancy. (B) The levels of serum AT1-AA were negatively correlated with the levels of serum adiponectin, $P < 0.05$, r = −0.2946. (C) The levels of endothelin-1 were elevated in the preeclampsia group compared with the healthy pregnancy group,$^{**}P < 0.01$ *vs.* healthy pregnancy. (D) The levels of serum AT1-AA were positively correlated with the levels of serum endothelin-1, $P < 0.05$, r = 0.2965, n = 45.

vasorelaxation dysfunction in AT1-AA positive rat thoracic aorta ($^{\#}P < 0.05$ *vs.* AT1-AA immunized, n = 6, Fig. 3E), which implied that $O_2^{\cdot -}$ was involved in endothelial-dependent smooth muscle relaxation disorder of AT1-AA positive rats.

3.4. Elevated expression of iNOS, reduced eNOS phosphorylation at Ser1177 in thoracic aortas of AT1-AA positive rats

Based on the demonstration of increased vascular NO production of AT1-AA positive rats, further investigation was focused on the NO synthase (NOS) that catalyzed NO production.

Compared with the vehicle group, iNOS protein expression was increased, the levels of phosphorylation of eNOS at Ser1177 were reduced, and the expression of nNOS protein was constant in thoracic aortas of AT1-AA positive rats ($^{**}P < 0.01$ *vs.* Vehicle, n = 6, Fig. 4A and B). Pretreatment with the iNOS inhibitor 1400 W (10^{-4} mol/L) attenuated considerably but not completely the normalized vasorelaxation of thoracic aortas in AT1-AA positive rats ($^{\#}P < 0.05$ *vs.* AT1-AA immunized, n = 6, Fig. 4C), which implied that iNOS inhibition only partially improved vasorelaxation disorders in AT1-AA positive rats. Therefore, compared with incubating 1400 W, the ACh-induced vasorelaxation in AT1-AA positive rats' thoracic aortas were increased when they were incubated with nebivolol (eNOS agonist, 10^{-6} mol/L) ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 4C), but it was still a partial improvement. Interestingly, co-incubation with 1400 W and nebivolol, vasorelaxation responses to Ach in thoracic aortas were normalized in AT1-AA positive rats ($^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 4D).

3.5. Reduced p-AMPKα (Thr172)/AMPKα in thoracic aortas of AT1-AA positive rats

The results mentioned above suggested that reducing iNOS expression and enhancing eNOS catalytic activity played a key role in improving endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats. The signaling pathway proteins that regulate iNOS expression and eNOS catalytic activity were observed. As illustrated in Fig. 5A, compared with the vehicle group, the ratio of p-AMPKα (Thr172)/AMPKα was decreased, the expression of HSP90α was decreased, the expression of HSP90β was elevated and the expressions of EndophilinA2, Caveolin-1 and AKT remained unchanged in thoracic aortas of AT1-AA positive rats ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Vehicle, n = 6, Fig. 5A). To confirm the role of AMPK in AT1-AA positive rats' endothelial-dependent smooth muscle relaxation disorder, AICAR (10^{-3} mol/L, AMPK agonist) was pre-incubated with AT1-AA positive rats' thoracic aortas. As illustrated in Fig. 5B, the iNOS protein expression was reduced, the phosphorylation of eNOS Ser1177 site was elevated ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6,

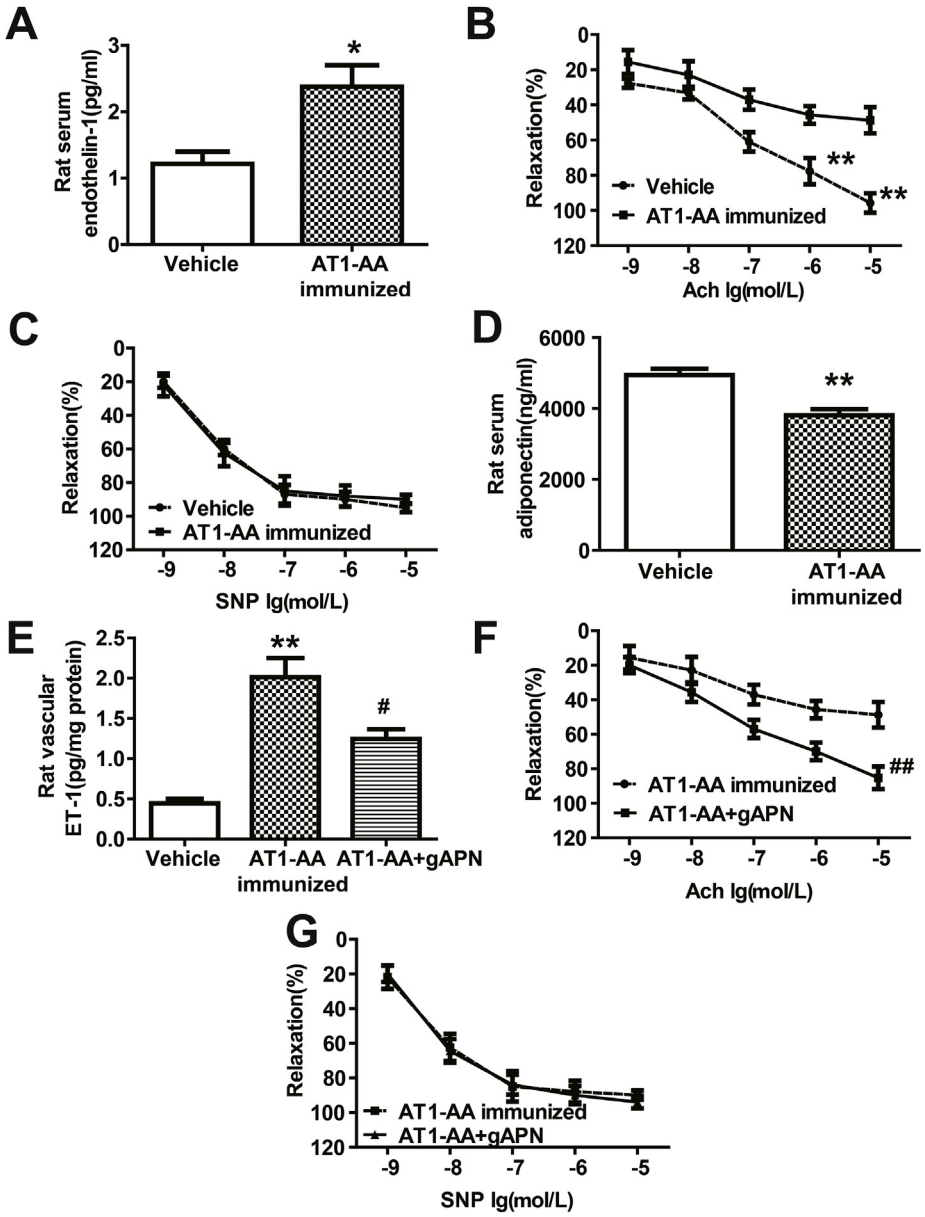

Fig. 2. Endothelial-dependent smooth muscle relaxation disorder were observed in thoracic aortic arteries of AT1-AA-positive rats and improved effects of adiponectin. (A) Levels of serum ET-1 were higher in AT1-AA positive rats than that in vehicle rats, $^{*}P < 0.05$ *vs.* Vehicle. (B–C) Ach-induced vasorelaxation was reduced, and SNP-induced vasorelaxation remained unchanged in thoracic aortic arteries of AT1-AA positive rats compared with vehicle rats, $^{**}P < 0.01$ *vs.* Vehicle. (D) Levels of serum adiponectin were lower in AT1-AA positive rats than that in vehicle rats, $^{**}P < 0.05$ *vs.* Vehicle. (E) Compared with vehicle rats, the contents of ET-1 were increased, and supplementation of gAPN reduced the contents of ET-1 in thoracic aortic arteries of AT1-AA positive rats, $^{**}P < 0.01$ *vs.* Vehicle, $^{\#}P < 0.01$ *vs.* AT1-AA immunized. (F–G) Supplementation of gAPN increased Ach-induced vasorelaxation and did not change SNP-induced vasorelaxation in thoracic aortic arteries of AT1-AA positive rats, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6.

Fig. 5B) and vasorelaxation was increased in AT1-AA positive rats' thoracic aortas ($^{**}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 5C). This suggested that AMPK was involved in the regulation of iNOS protein expression, eNOS (Ser1177) phosphorylation and endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats' thoracic aortas.

3.6. Supplementation of gAPN in vitro improved endothelial injury and vasorelaxation disorder in AT1-AA positive rats

In endothelial cells, AMPK was activated by adiponectin to increase NO bioavailability and preserve endothelial function [35]. The serum adiponectin deficiency, NOS abnormalities, oxidative/nitrative stress and impaired the NO–cGMP pathway shown in previous studies are all closely related to endothelial-dependent smooth muscle relaxation disorder. It is well known that preeclampsia is characterized by endothelial-dependent smooth muscle relaxation disorder and several clinical observations have demonstrated that serum adiponectin levels in preeclampsia are significantly lower than that in control groups [36,37]. Moreover, our results indicated that reduced serum adiponectin levels in the preeclampsia group and levels of AT1-AA were negatively correlated with adiponectin contents (Fig. 1A and B). Based on the above-mentioned negative correlation, further studies on animals showed that serum adiponectin levels were reduced in AT1-AA positive rats ($^{**}P < 0.01$ *vs.* Vehicle, n = 6, Fig. 2D). In addition, a previous study from our laboratory showed that a truncated and biologically active form of adiponectin called gAPN is protective against endothelial-dependent smooth muscle relaxation disorder [15]. Thus, when incubated with gAPN in vitro for 4 h, this mixture decreased ET-1 contents in AT1-AA positive rats' thoracic aortas ($^{\#}P < 0.05$ *vs.* AT1-AA immunized, n = 6, Fig. 2E), improved endothelial-dependent smooth muscle relaxation disorder in thoracic aortas and mesenteric arteries of AT1-AA positive rats, as evidenced by a significant improvement of the dose-response curve to ACh ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 2F, Fig. S4C) and a constant of the dose-response curve to SNP ($P > 0.05$ *vs.* AT1-AA immunized, Fig. 2G, Fig. S4D).

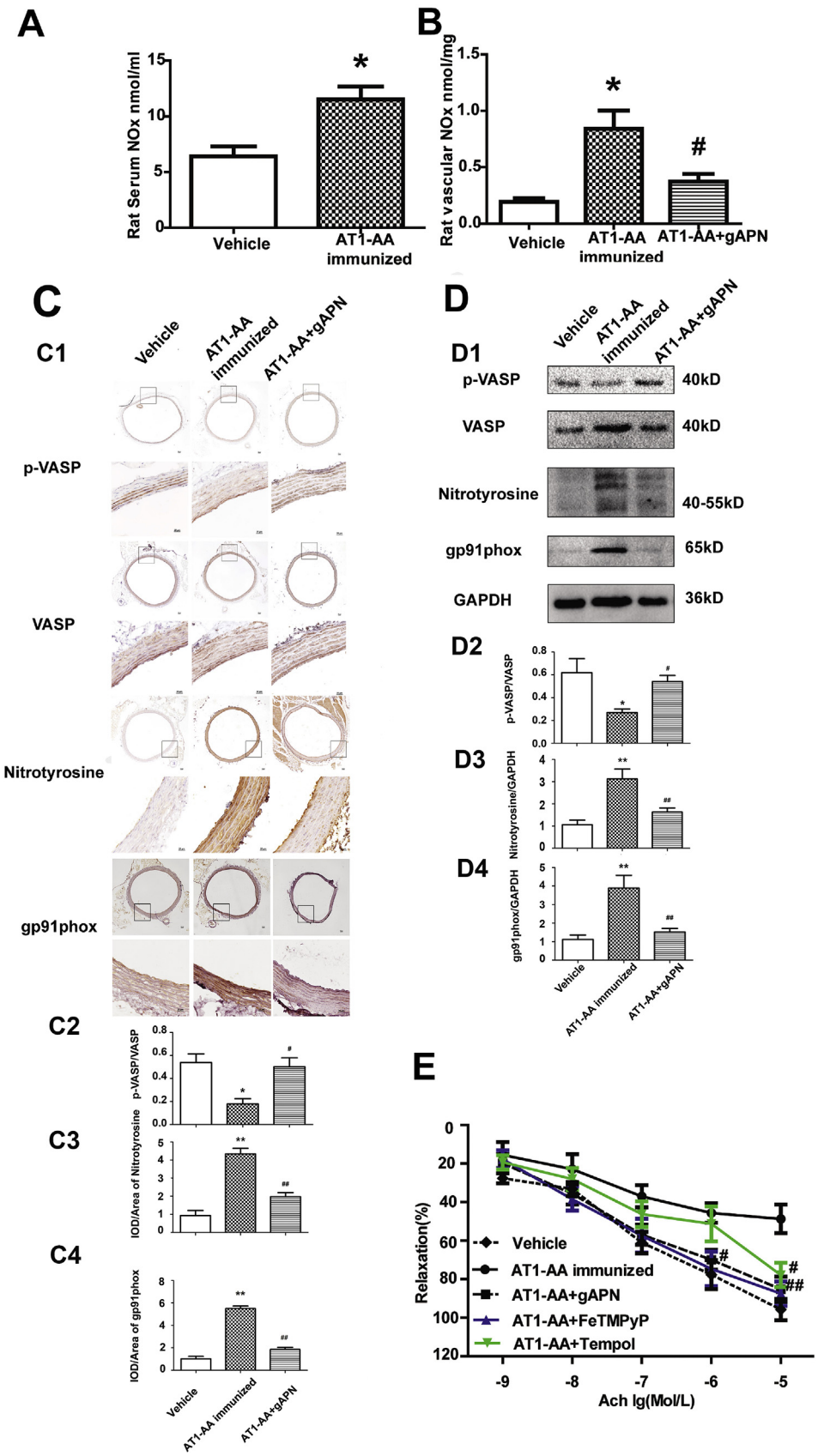

Fig. 3. NO-cGMP signal pathway impairment were observed in thoracic aortic arteries of AT1-AA positive rats and improved effects of Adiponectin. (A–B) Compared with vehicle group rats, the contents of NOx in sera and thoracic aortic arteries of AT1-AA positive rats were increased, and supplementation of gAPN decreased the contents of NOx in thoracic aortic arteries of AT1-AA positive rats, $^{*}P < 0.05$ *vs.* Vehicle, $^{\#}P < 0.05$ *vs.* AT1-AA immunized. (C–D) In thoracic aortic arteries of AT1-AA positive rats, the ratio of (C1, C2, D1, D2) p-VASP/VASP was reduced, the expression of (C1, C3, D1, D3) NT and (C1, C4, D1, D4) and gp91phox were elevated. Supplementation of gAPN reversed the indicators mentioned above. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized. (E) $ONOO^{-}$ scavenger FeTMPyP and $O_2^{\cdot-}$ inhibitor Tempol pretreatment increased Ach-induced vasorelaxation in AT1-AA positive rats, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6.

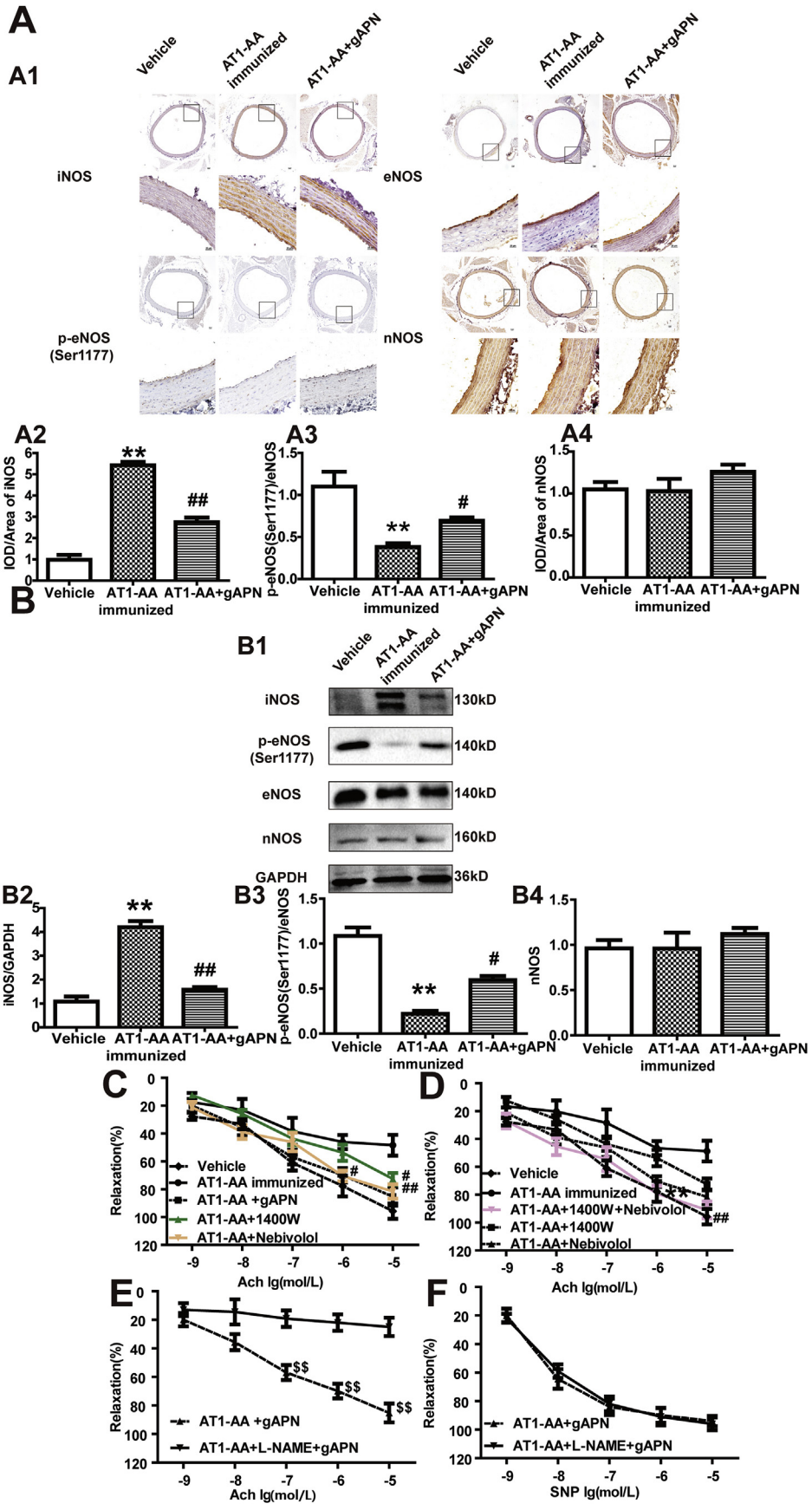

Fig. 4. Changes of iNOS and eNOS were observed in thoracic aortic arteries of AT1-AA positive rats and improved effects of Adiponectin. (A–B) Compared with vehicle rats, (A1, A2, B1, B2) increased iNOS expression, (A1, A3, B1, B3) reduced eNOS phosphorylation at Ser1177 in thoracic aortic arteries were observed in AT1-AA positive rats. And supplementation of gAPN reversed the indicators mentioned above. $^{**}P < 0.01$ *vs.* Vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized. (C) iNOS inhibitor 1400 W and eNOS agonist Nebivolol pretreatment partially increased Ach-induced vasorelaxation in AT1-AA positive rats. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized. (D) Combined utilization of 1400 W and Nebivolol effectively increased Ach-induced vasorelaxation in AT1-AA positive rats which were better than single utilization. $^{\#\#}P < 0.01$ vs. AT1-AA immunized. (E–F) L-NAME (NO synthesis inhibitor) completely blocked vasorelaxation in response to Ach but did not change vasorelaxation in response to SNP in thoracic aortic arteries pretreated with gAPN, $^{\$\$}P < 0.01$ *vs.* AT1-AA immunized + gAPN, n = 6.

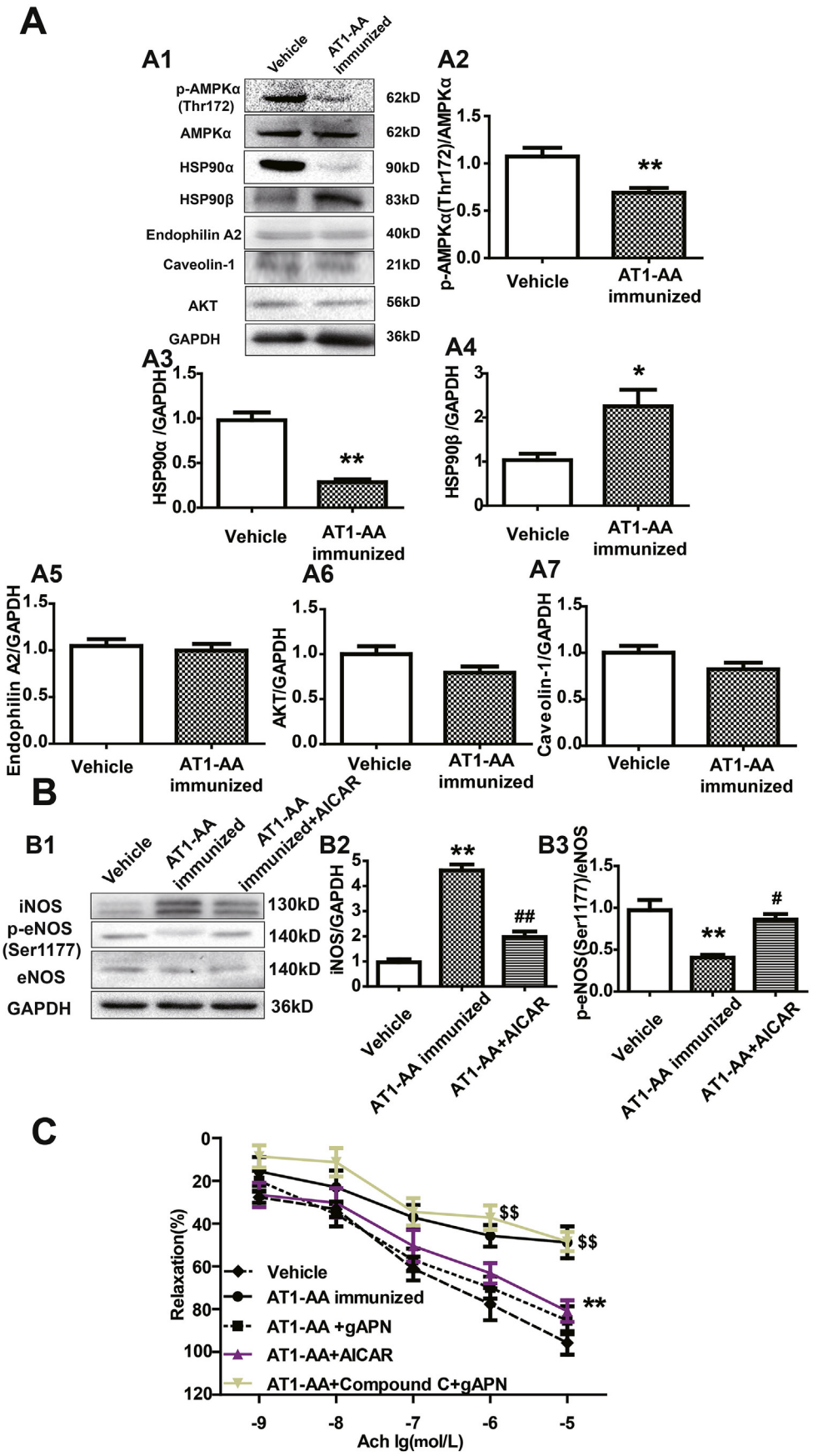

Fig. 5. Reduced p-AMPKα(Thr172)/AMPKα was observed in thoracic aortic arteries of AT1-AA positive rats. (A) Compared with vehicle rats, (A1, A2) p-AMPKα (Thr172)/AMPKα was decreased; (A1, A3) the expression of HSP90α was decreased; (A1, A4) the expression of HSP90β was elevated, and the expressions of (A1, A5) endophilinA2, (A1, A6) caveolin-1, and (A1, A7) AKT remained unchanged in thoracic aortic arteries of AT1-AA positive rats. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Vehicle. (B) After preincubating AICAR (AMPK agonist) with AT1-AA positive rats' thoracic aortic arteries, the iNOS protein expression was reduced and the phosphorylation of the eNOS Ser1177 site was elevated. $^{**}P < 0.01$ *vs.* Vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized. (C) AICAR increased Ach-induced vasorelaxation response in AT1-AA positive rats' thoracic aortic arteries and the addition of Compound C (AMPK inhibitor) partially blocked vasorelaxation in response to Ach in thoracic aortic arteries pretreated with gAPN$^{**}P < 0.01$ *vs.* AT1-AA immunized, $^{\$\$}P < 0.01$ *vs.* AT1-AA immunized + gAPN, n = 6.

3.7. Supplementation of gAPN in vitro reduced NO overproduction, increased p-VASP/VASP, decreased the contents of $ONOO^-$ and its source ($O_2^{\cdot -}$) in thoracic aortas of AT1-AA positive rats

Incubated with gAPN markedly reduced NOx level ($^{\#}P < 0.05$ *vs.* AT1-AA immunized, n = 6, Fig. 3B); increased p-VASP/VASP; reduced the protein expression of NT, gp91phox and ROS generation in AT1-AA positive rats' thoracic aortas ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 3C and D, Fig. S5).

3.8. Supplementation of gAPN in vitro reduced iNOS overexpression, increased eNOS phosphorylation at Ser1177 in thoracic aortas of AT1-AA positive rats

Incubation with gAPN markedly reduced iNOS protein expression, increased phosphorylation of eNOS at Ser1177 in AT1-AA positive rats' thoracic aortas ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* AT1-AA immunized, n = 6, Fig. 4C and D). To further determine whether gAPN increased Ach-induced vasorelaxation by regulating NOS catalytic activity, a portion of gAPN -treated thoracic aortic segments was pretreated with L-NAME (10^{-1} mol/L). As illustrated in Fig. 4E, the addition of L-NAME completely blocked ACh induced vasorelaxation but did not change the vasorelaxation in response to SNP in thoracic aortas treated with gAPN ($^{\$\$}P < 0.01$ *vs.* AT1-AA immunized + gAPN, n = 6, Fig. 4E and F). Interestingly, in mesenteric arteries treated with gAPN, the endothelium-dependent relaxation induced by ACh and endothelium-independent relaxation induced by SNP were not affected by L-NAME ($P > 0.05$ *vs.* AT1-AA immunized + gAPN, n = 6, Figs. S4E and S4F).

3.9. Supplementation of gAPN in vitro improved endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats via AMPK

To further determine whether gAPN attenuated endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats through AMPK, a portion of gAPN-treated thoracic aortic segments was pretreated with Compound C (AMPK inhibitor, 10^{-5} mol/L). As illustrated in Fig. 5C, the addition of Compound C partially blocked vasorelaxation in response to ACh in those thoracic aortic arteries treated with gAPN ($^{\$\$}P < 0.01$ *vs.* AT1-AA immunized + gAPN, n = 6, Fig. 5C).

4. Discussion

In this study, we demonstrated that AT1-AA was negatively correlated with adiponectin in sera of pregnant women. In addition, using AT1-AA positive rats, we indicated that AT1-AA-induced damage to endothelial function was caused by impairment of the NO-cGMP pathway. Finally, we demonstrated that serum adiponectin was decreased in AT1-AA positive rats, and supplementation of adiponectin improved endothelial vasorelaxation via the NO-cGMP pathway.

As shown in this study, the level of ET-1 was significantly elevated in preeclamptic patients. Given that ET-1 is a polypeptide secreted by endothelial cells, the rise in serum level of ET-1 is likely due to endothelial cell damage. Therefore, an elevated level of ET-1 is one of the specific indicators reflecting endothelial injury [38]. Importantly, there was a positive linear correlation between AT1-AA and ET-1 levels in maternal serum. Ample evidence showed that AT1-AA extracted from the serum of preeclamptic patients decreased the endothelium-dependent vasorelaxation in rat arteries [22]. The above results suggest that AT1-AA is associated with vascular endothelial injury and vasorelaxation disorders in preeclamptic patients. Perhaps the most novel aspect is investigating the exact mechanism involved in endothelial injury and vasorelaxation disorder induced by AT1-AA.

From a clinical perspective, the above scientific question should be examined in preclinical models of preeclampsia, while focusing on the mechanism of endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA. This will be a challenging research question to address given that preeclampsia is affected by factors such as estrogen and progestin, which may complicate the experimental design. To eliminate the influence of these factors. AT1-AA positive rat models were established by active immunization with the synthetic human AT1R-EC_{II} peptide. Previous studies have reported that LDH activity in the supernatant of human umbilical vein endothelial cells incubated with AT1-AA extracted from preeclampsia women was increased [22]. Elevated levels of serum ET-1, vascular endothelial damage, vasorelaxation disorder and cardiac capillary endothelial injury were all observed in AT1-AA positive rats [6]. As shown in the present investigation, high levels of ET-1 were observed in sera and thoracic aortas in AT1-AA positive rats. Relaxation responses to ACh were reduced in thoracic aortas and mesenteric arteries of AT1-AA positive rats. However, little is known about the potential mechanism of vasorelaxation disorder induced by AT1-AA.

NO release from endothelial cells can directly relax vascular smooth muscle cells. Although in vitro clinical studies have indicated that AT1-AA significantly reduced endothelial NO production by promoting endothelial microparticles generation in HUVECs [39], the existence of a relationship between the reduced endothelial NO production and endothelial-dependent smooth muscle relaxation disorder induced by AT1-AA remains unclear. Moreover, the degree of NO production in the vasculature of AT1-AA positive rats has not been clarified. NO is a highly reactive signaling molecule, with the major constituents of NO being NO_2^- and NO_3^-. Both NO_2^- and NO_3^- have been used as surrogate markers of NO production, and both are collectively referred to as NOx. As illustrated in our study, levels of NOx in serum and thoracic aortic arteries of AT1-AA positive rats were not reduced, but instead elevated, which suggests that the production of NO was increased in the vascular system of AT1-AA positive rats. The mechanism behind the increased NO production and vasodilation disorder in AT1-AA positive rats remains unclear.

NO-induced relaxation is associated with increased levels of cGMP in vascular smooth muscle cells, an interaction known as the NO–cGMP signaling pathway. In all cases, p-VASP (Ser239)/VASP serves as a sensitive monitor of the NO–cGMP signaling pathway. In the present study, relative to vehicle group rats, p-VASP/VASP was reduced in AT1-AA positive rats' thoracic aortas. Also, the improvement of impaired NO–cGMP pathway reversed endothelial-dependent vasorelaxation in AT1-AA positive rats. This suggests that an impaired NO–cGMP pathway was involved in endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats. The NO–cGMP pathway was impeded, but NO production was increased, which suggests that NO may influence more than the regulation of vascular tone. Therefore, it is critical to further explore the reactions that may involve NO.

NO reacts with $O_2^{\cdot -}$ to form $ONOO^-$ at an almost near-diffusion-limited rate of $6.7 \times 10^9\ M^{-1}s^{-1}$. This reaction rate is approximately ten times greater than that between the superoxide anion and superoxide dismutase [30]. $ONOO^-$ is a key element in resolving the contrasting roles of NO in physiology and pathology. This reaction rapidly deactivates NO, which prevents NO from activating soluble guanylate cyclase (sGC) and increasing levels of cyclic guanosine monophosphate (cGMP). Ultimately, cGMP-mediated vasodilation is limited.

Conversely, $ONOO^-$ induces lipid peroxidation and endothelial-dependent smooth muscle relaxation disorder [40]. Since $ONOO^-$ is a product of tyrosine nitration mediated by reactive species such as $ONOO^-$, the levels of the $ONOO^-$ in tissues are usually reflected by the content of NT [41]. In our study, the protein expression of nitrotyrosine was increased in AT1-AA positive rats' thoracic aortic arteries. Vasorelaxation responses to ACh were normalized after incubation with the $ONOO^-$ scavenger FeTMPyP, (10^{-5} mol/L), which suggested that $ONOO^-$ was involved in vascular dysfunction in AT1-AA positive rats.

In addition to NO, $ONOO^-$ production is also closely related to the $O_2^{\cdot -}$. It has been reported previously that an increase of vascular $O_2^{\cdot -}$ in rats fed a high-fat diet [42] or injected with iron dextran [43] is due to the high expression of gp91phox which is the NADPH subunit

protein. Therefore, the expression of gp91phox was observed in the thoracic aorta of AT1-AA positive rats. Interestingly, we found that gp91phox was increased in these animals. Following the administration of the superoxide anion scavenger (Tempol, 10^{-4} mol/L), the vasorelaxation disorders of AT1-AA positive rat thoracic aortas were improved, which suggests that $O_2^{\cdot -}$ was involved in vasodilation disorders in AT1-AA positive rats.

Excessive NO still combines with $O_2^{\cdot -}$ at the rate of biological diffusion to produce $ONOO^-$. Thus, it is necessary to study the factors that catalyze NO synthesis. iNOS and eNOS are the enzymes that catalyze the production of NO from L-arginine and oxygen. Under normal physiological conditions, iNOS is not expressed to a large degree, leaving eNOS to catalyze the production of NO at pMol levels to exert vasodilation, inhibit platelet aggregation and adhesion, and prevent thrombus formation [44]. However, during the inflammatory response [45], high homocysteine [46], heavy metal [47] and lipopolysaccharide [48] and other factors stimulate iNOS expression in blood vessels. This effect leads to iNOS producing NO at a greater rate than eNOS, (at levels as high as the nmol range). In this study iNOS expression was increased in thoracic aortas from AT1-AA positive rats. Because 1400 W is a specific inhibitor of iNOS [49], after adding the iNOS inhibitor 1400 W (10^{-6} mol/L), the vasorelaxation disorder of AT1-AA rats' thoracic aortas was not completely reversed, which indicates this disorder cannot be completely attributed to the excessive level of iNOS expression. Alternatively, whether catalytic activity of eNOS was changed in thoracic aortas of AT1-AA positive rats, and if so, the effect of changes in catalytic activity of eNOS on vasorelaxation disorder in AT1-AA positive rats has never been demonstrated in AT1-AA positive rats.

eNOS is of particular importance in the vasculature, and its signaling capacity is due, in part, to its ability to interact with multiple protein partners. Post-translational modifications allow for eNOS modulation through the actions of several signaling cascades [50]. Phosphorylation appears to be a major factor in the regulation of eNOS's catalytic activity. Among the eNOS phosphorylation sites, preferential phosphorylation of eNOS on stimulatory Ser1177 has been shown to contribute to higher NO availability [51]. Phosphorylation of Ser1177 increases the Ca^{2+} sensitivity of eNOS and elevates NO production catalyzed by eNOS. Therefore, recent studies have found that many stimuli such as bradykinin [52], fluid shear stress [53] and hydrogen sulfide [54] all influence NO generation by regulating phosphorylation of Ser1177. In our study, reduced p-eNOS (Ser1177)/eNOS was observed in AT1-AA positive rats' thoracic aortic arteries.

Other investigations have reported that an eNOS agonist increases the level of vascular NO production [55]. The ACh-induced vasorelaxation in AT1-AA positive rat thoracic aorta was higher after administration of an eNOS agonist (nebivolol, 10^{-4} mol/L) than when a iNOS inhibitor was used. Nebivolol can increase the catalytic activity of eNOS and thereby promote eNOS-induced production of NO at the pmol level [56,57]. In our study, we found p-eNOS (Ser1177)/eNOS in the thoracic aorta of AT1-AA positive rats was decreased, suggesting that eNOS catalytic activity was reduced, so we applied nebivolol. However, this only yielded partial improvement in vascular function. This suggests that low eNOS catalytic activity is involved in endothelial-dependent smooth muscle relaxation disorder in AT1-AA positive rats. Interestingly, the combined utilization of eNOS agonists and iNOS inhibitors effectively increased the ACh-induced relaxation in AT1-AA positive rat thoracic aortic arteries which was better than single utilization. This suggests that combined utilization of eNOS agonists and iNOS inhibitors yield better performance. However, when faced with a vascular disease mediated by AT1-AA, it is difficult to remove autoantibody by routine blood purification, and drug combination increases the patient's burden, which can cause serious adverse events. Therefore, it remains imperative to identify drugs that have the effects of both eNOS agonist and iNOS inhibitor to ameliorate the vascular disease induced by AT1-AA.

Fortunately, some studies have reported that AMPK is a common upstream regulatory protein of iNOS and eNOS [58], and the AMPK agonist AICAR simultaneously changes iNOS protein expression and eNOS phosphorylation [59]. Our results suggest that p-AMPKα (Thr172)/AMPKα is reduced in AT1-AA positive rat thoracic aortic arteries. Preincubating AMPK agonists reduced iNOS protein expression, increased p-eNOS (Ser1177)/eNOS, elevated ACh-induced vasorelaxation in thoracic aortas of AT1-AA positive rats. It is necessary to find a drug to exert a vascular protective effect by effectively activating AMPK to have the dual effect of iNOS inhibitor and eNOS agonist.

Adiponectin is an endogenous polypeptide secreted by adipocytes and circulates at high concentrations (0.5–30 μg/mL) in plasma under normal physiological conditions [60]. Adiponectin can activate AMPK to reduce the expression of iNOS, increase the catalytic activity of eNOS, and play an essential role in the protection of vascular endothelium [61]. Additional research has shown that reduced levels of serum adiponectin and microvascular endothelial damage (such as leukocyte adhesion/platelet aggregation) are observed in early-onset sepsis mice [62]. Both preclinical and clinical investigations have shown that direct injection of recombinant adiponectin or elevating fat adiponectin concentrations by using transgenic technology improves vascular injury [63]. In our study, which was based on the existing clinical data, we conducted a correlation analysis and found that the maternal serum AT1-AA and adiponectin were negatively correlated. Based on this, an experimental animal model was established, serum levels of adiponectin in AT1-AA positive rats were reduced, and gAPN supplementation reversed vasorelaxation disorders in thoracic aortic and mesenteric arteries, reduced ET-1 contents, improved the impairment of NO–cGMP signaling pathway and abnormal iNOS expression/eNOS catalytic activity. Given that adiponectin is an endogenous protein hormone with little side-effects, it serves as an ideal candidate to effectively minimize adverse effects on mother and child.

5. Limitations and future directions

During data collection, clinical data that directly demonstrated patients had vascular endothelial-dependent smooth muscle relaxation disorder were not obtained (i.e., measuring brachial artery diameter changes after an increase in shear stress induced by reactive hyperemia).

In our animal experiments, the phenomenon of serum adiponectin reduction in AT1-AA-positive rats has been observed. It may be that AT1-AA affects the transcriptional function of PPAR-γ by binding the AT1R on adipocytes, thereby changing the content of adiponectin. But the specific mechanism underlying the relationship between AT1-AA and adiponectin remains to be fully established. p-AMPKα(Thr172)/AMPK α was reduced in AT1-AA positive rat thoracic aortas. AMPK agonist supplementation partially reversed elevated iNOS expression and abnormal eNOS catalytic activities in thoracic aortas of AT1-AA positive rats. However, the reason that the ratio of p-AMPKα(Thr172)/AMPKα in AT1-AA positive rats' thoracic aortas was reduced, and the relationship between the reduction in p-AMPKα(Thr172)/AMPKα and abnormal catalytic activities of iNOS and eNOS in AT1-AA positive rats' thoracic aortas, remains unclear.

6. Conclusions

The mechanism of endothelial-dependent smooth muscle relaxation disorder caused by AT1-AA was the impaired NO–cGMP signaling pathway. Supplementation of gAPN improved the NO–cGMP signaling pathway, which was a key target for treating endothelial-dependent smooth muscle relaxation disorder and vascular disease in AT1-AA positive patients, especially during pregnancy.

Conflicts of interest

The authors declare that there is no conflict of interest that could be

perceived as prejudicing the impartiality of the reported research.

Acknowledgments

This work was supported by the Major Research Plan of the National Natural Science Foundation of China (No.91539205, to Huirong Liu) and Postdoctoral Science Foundation of Beijing (2015ZZ-59, to Ye Wu). We would like to thank LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.02.006.

References

[1] A.B. Sozen, et al., Drugs with blocking effects on the renin-angiotensin-aldosterone system do not improve endothelial dysfunction long-term in hypertensive patients, J. Int. Med. Res. 37 (4) (2009) 996–1002.
[2] G. Wallukat, et al., Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor, J. Clin. Invest. 103 (7) (1999) 945–952.
[3] F. Zhu, et al., Correlation between HLA-DRB1, HLA-DQB1 polymorphism and autoantibodies against angiotensin AT(1) receptors in Chinese patients with essential hypertension, Clin. Cardiol. 34 (5) (2011) 302–308.
[4] M.J. Ansari, et al., Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection, N. Engl. J. Med. 352 (19) (2005) 2027–2028.
[5] G. Riemekasten, et al., Involvement of functional autoantibodies against vascular receptors in systemic sclerosis, Ann. Rheum. Dis. 70 (3) (2011) 530–536.
[6] S.L. Zhang, et al., Endothelial dysfunction induced by antibodies against angiotensin AT1 receptor in immunized rats, Acta Pharmacol. Sin. 31 (10) (2010) 1381–1388.
[7] M. Thieme, et al., Phosphodiesterase 5 inhibition ameliorates angiotensin II-dependent hypertension and renal vascular dysfunction, Am. J. Physiol. Renal. Physiol. 312 (3) (2017) 474–481.
[8] J. Zhang, et al., A novel mechanism of diabetic vascular endothelialdysfunction: hypoadiponectinemia-induced NLRP3 inflammasome activation, Biochim. Biophys. Acta 1863 (6) (2017) 1556–1567.
[9] M. Beltrami-Moreira, et al., Association between plasma adiponectin and arteriolar vessel caliber among elderly hypertensive subjects, J. Am. Soc. Hypertens 9 (8) (2015) 620–627.
[10] B.D. Medoff, et al., Adiponectin deficiency increases allergic airway inflammation and pulmonary vascular remodeling, Am. J. Respir. Cell Mol. Biol. 41 (4) (2009) 397–406.
[11] R. Guo, et al., Adiponectin and its receptors are involved in hypertensive vascular injury, Mol. Med. Rep. 17 (1) (2018) 209–215.
[12] W. Zhao, et al., Adiponectin protects palmitic acid induced endothelial inflammation and insulin resistance via regulating ROS/IKKbeta pathways, Cytokine 88 (2016) 167–176.
[13] Y. Du, et al., Adiponectin at physiologically relevant concentrations enhances the vasorelaxative effect of acetylcholine via cav-1/AdipoR-1 signaling, PLoS One 11 (3) (2016) e0152247.
[14] M.A. Brown, et al., The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP), Hypertens. Pregnancy 20 (1) (2001) Ix–xiv.
[15] R. Li, et al., Adiponectin improves endothelial function in hyperlipidemic rats by reducing oxidative/nitrative stress and differential regulation of eNOS/iNOS activity, Am. J. Physiol. Endocrinol. Metab. 293 (6) (2007) E1703–E1708.
[16] S.M. Palomares, et al., Peroxynitrite decomposition with FeTMPyP improves plasma-induced vascular dysfunction and infarction during mild but not severe hyperglycemic stroke, J. Cerebr. Blood Flow Metabol. 32 (6) (2012) 1035–1045.
[17] L.A. Lesniewski, et al., Aging compounds western diet-associated large artery endothelial dysfunction in mice: prevention by voluntary aerobic exercise, Exp. Gerontol. 48 (11) (2013) 1218–1225.
[18] M. Staehr, et al., Disruption of COX-2 and eNOS does not confer protection from cardiovascular failure in lipopolysaccharide-treated conscious mice and isolated vascular rings, Am. J. Physiol. Regul. Integr. Comp. Physiol. 301 (2) (2011) R412–R420.
[19] Y. Wang, et al., Nebivolol alleviates aortic remodeling through eNOS upregulation and inhibition of oxidative stress in l-NAME-induced hypertensive rats, Clin. Exp. Hypertens. 39 (7) (2017) 628–639.
[20] S. Turkseven, et al., Prolonged AMP-activated protein kinase induction impairs vascular functions, Can. J. Physiol. Pharmacol. 91 (12) (2013) 1025–1030.
[21] H.R. Liu, et al., Screening of serum autoantibodies to cardiac beta1-adrenoceptors and M2-muscarinic acetylcholine receptors in 408 healthy subjects of varying ages, Autoimmunity 29 (1) (1999) 43–51.
[22] X. Yang, et al., Autoantibodies isolated from preeclamptic patients induce endothelial dysfunction via interaction with the angiotensin II AT1 receptor, Cardiovasc. Toxicol. 14 (1) (2014) 21–29.
[23] L. Tao, et al., Antioxidative, antinitrative, and vasculoprotective effects of a peroxisome proliferator-activated receptor-gamma agonist in hypercholesterolemia, Circulation 108 (22) (2003) 2805–2811.
[24] Z. Yan, et al., Myeloperoxidase increased cardiomyocyte protein nitration in mice subjected to nonlethal mechanical trauma, Biochem. Biophys. Res. Commun. 393 (3) (2010) 531–535.
[25] R.F. Furchgott, et al., The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, Nature 288 (1980) 373–376.
[26] P.G. Wang, et al., Nitric oxide donors: chemical activities and biological applications, Chem. Rev. 102 (4) (2002) 1091–1134.
[27] T.C. Buzinari, et al., Treatment with sodium nitroprusside improves the endothelial function in aortic rings with endothelial dysfunction, Eur. J. Pharm. Sci. 105 (2017) 144–149.
[28] K.V. Sastry, et al., Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy, Anal. Biochem. 306 (1) (2002) 79–82.
[29] M. Seimetz, et al., Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice, Cell 147 (2) (2011) 293–305.
[30] R.J. Mailloux, Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species, Redox Biol. 4 (2015) 381–398.
[31] U. Hink, et al., Role for peroxynitrite in the Inhibition of prostacyclin synthase in nitrate tolerance, J. Am. Coll. Cardiol. 42 (10) (2003) 1826–1834.
[32] A.L. Kleschyov, et al., Does nitric oxide mediate the vasodilator activity of nitroglycerin? Circ. Res. 93 (9) (2003) 104–112.
[33] S. Almajdoob, et al., Resveratrol attenuates hyperproliferation of vascular smooth muscle cells from spontaneously hypertensive rats: role of ROS and ROS-mediated cell signaling, Vasc. Pharmacol. 101 (2018) 48–56.
[34] H.J. Sun, et al., Salusin-β promotes vascular smooth muscle cell migration and intimal hyperplasia after vascular injury via ROS/NFκB/MMP-9 pathway, Antioxidants Redox Signal. 24 (18) (2016) 1045–1057.
[35] Z.V. Wang, et al., Adiponectin, cardiovascular function, and hypertension, Hypertension 51 (1) (2008) 8–14.
[36] R. D'Anna, et al., Adiponectin and insulin resistance in early- and late-onset preeclampsia, Bjog 113 (11) (2006) 1264–1269.
[37] A. Khosrowbeygi, et al., Leptin to adiponectin ratio in preeclampsia, Bangladesh Med. Res. Counc. Bull. 39 (1) (2013) 18–21.
[38] D. Gopalakrishna, et al., ET-1 stimulates superoxide production by eNOS following exposure of vascular endothelial cells to endotoxin, Shock 46 (1) (2016) 60–66.
[39] S. Yang, et al., Angiotensin II receptor type 1 autoantibodies promote endothelial microparticles formation through activating p38 MAPK pathway, J. Hypertens. 32 (4) (2014) 762–770.
[40] J.Y. Chen, et al., Nitric oxide bioavailability dysfunction involves in atherosclerosis, Biomed. Pharmacother. 97 (2018) 423–428.
[41] L. Thomson, 3-nitrotyrosine modified proteins in atherosclerosis, Dis. Markers 2015 (2015) 708282.
[42] G.J. Wetscher, et al., Free radical production in nicotine treated pancreatic tissue, Free Radic. Biol. Med. 18 (5) (1995) 877–882.
[43] R.F. Ribeiro Junior, et al., Chronic iron overload induces functional and structural vascular changes in small resistance arteries via NADPH oxidase-dependent O2(-) production, Toxicol. Lett. 279 (2017) 43–52.
[44] E. Gkaliagkousi, et al., Nitric oxide signalling in the regulation of cardiovascular and platelet function, Front. Biosci. (Landmark Ed) 16 (2011) 1873–1897.
[45] S. Becerril, et al., Targeted disruption of the iNOS gene improves adipose tissue inflammation and fibrosis in leptin-deficient ob/ob mice: role of tenascin C, Int. J. Obes. 42 (8) (2018) 1458–1470.
[46] S. Dayal, et al., Protective vascular and cardiac effects of inducible nitric oxide synthase in mice with hyperhomocysteinemia, PLoS One 9 (9) (2014) e107734.
[47] T.O. Faria, et al., Xanthine oxidase activation modulates the endothelial (vascular) dysfunction related to HgCl2 exposure plus myocardial infarction in rats, Cardiovasc. Toxicol. 18 (2) (2018) 161–174.
[48] K.W. Choy, et al., Paeonol attenuates LPS-induced endothelial dysfunction and apoptosis by inhibiting BMP4 and TLR4 signaling simultaneously but independently, J. Pharmacol. Exp. Therapeut. 364 (3) (2018) 420–432.
[49] S. Puttachary, et al., 1400 W, a highly selective inducible nitric oxide synthase inhibitor is a potential disease modifier in the rat kainate model of temporal lobe epilepsy, Neurobiol. Dis. 93 (2016) 184–200.
[50] S.M. Mutchler, et al., Compartmentalized nitric oxide signaling in the resistance vasculature, Nitric Oxide 49 (2015) 8–15.
[51] Pooja, et al., Post-translational modifications of eNOS augment nitric oxide availability and facilitates hypoxia adaptation in Ladakhi women, Nitric Oxide 78 (2018) 103–112.
[52] M.B. Harris, et al., Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation, J. Biol. Chem. 276 (19) (2001) 16587–16591.
[53] Y. Chao, et al., Low shear stress induces endothelial reactive oxygen species via the AT1R/eNOS/NO pathway, J. Cell. Physiol. 233 (2) (2018) 1384–1395.
[54] L. Kram, et al., The anti-thrombotic effect of hydrogen sulfide is partly mediated by an upregulation of nitric oxide synthases, Thromb. Res. 132 (2) (2013) e112–117.
[55] M.A. Aladag, et al., Nebivolol attenuates cerebral vasospasm both by increasing endothelial nitric oxide and by decreasing oxidative stress in an experimental subarachnoid haemorrhage, Br. J. Neurosurg. 31 (4) (2017) 439–445.
[56] L.J. Ignarro, et al., Nebivolol: a selective beta(1)-adrenergic receptor antagonist that relaxes Vascular smooth muscle by nitric oxide- and cyclic GMP-dependent mechanisms, Nitric Oxide 7 (2) (2002) 75–82.
[57] Y. Wang, et al., Nebivolol alleviates aortic remodeling through eNOS upregulation and inhibition of oxidative stress in l-NAME-induced hypertensive rats, Clin. Exp. Hypertens. 39 (7) (2017) 628–639.

[58] M.J. Shin, et al., Transduced PEP-1-AMPK inhibits the LPS-induced expression of COX-2 and iNOS in Raw264.7 cells, BMB Rep. 43 (1) (2010) 40–45.
[59] Y.Q. Zhang, et al., New progress in roles of nitric oxide during hepatic ischemia reperfusion injury, World J. Gastroenterol. 23 (14) (2017) 2505–2510.
[60] Y. Wang, et al., Restoring diabetes-induced autophagic flux arrest in ischemic/reperfused heart by ADIPOR (adiponectin receptor) activation involves both AMPK-dependent and AMPK-independent signaling, Autophagy 13 (11) (2017) 1855–1869.
[61] A.E. Achari, et al., Adiponectin, a therapeutic target for obesity, diabetes, and endothelial dysfunction, Int. J. Mol. Sci. 18 (6) (2017) e1321.
[62] X. Wang, et al., Adiponectin treatment attenuates inflammatory response during early sepsis in obese mice, J. Inflamm. Res. 9 (2016) 167–174.
[63] N. Ouchi, et al., Targeting adiponectin for cardioprotection, Expert Opin. Ther. Targets 10 (4) (2006) 57.