

Original Article

Roles and possible mechanisms of autoantibodies against the angiotensin AT1 receptor in vascular calcification of rats

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Received February 11, 2018; Accepted March 24, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: The aim of this study was to investigate the effect of AT1-AAs on vascular calcification. Wistar rats were immunized with synthetic peptides corresponding to the second extracellular loop of AT1 receptor. The titer of AT1-AAs in rat serum, SBP, and HR were detected weekly. Histological analysis and biochemical parameters were measured 4 months after initial immunization. The level of osteopontin and osteocalcin was measured by Western blot analysis. The results showed that the titer of AT1-AAs, SBP, and HR were all increased significantly 4 weeks after initial immunization. Compared with the control group, the contractile force of the aortic ring of the immunized group to phenylephrine was significantly higher, and relaxation function was significantly reduced. The ALP activity and protein levels of osteoporotin and osteocalcin were increased in the aortic tissue of the immunized group. Histological examination showed varying degrees of calcification within each cell layer with von Kossa staining in the immunized group. Losartan treatment not only significantly lowered SBP and HR to the similar level of the control group but inhibited the ALP activity and protein levels of osteoporotin and osteocalcin. Our study demonstrated that AT1-AAs contributed to the progression of vascular calcification, suggesting that AT1-AAs may play a vital role in the occurrence of cardiovascular disease in patients with severe hypertension.

Keywords: Autoantibodies against angiotensin AT1 receptor (AT1-AA), severe hypertension, vascular calcification, osteopontin, osteocalcin

Introduction

Vascular calcification is an important pathological process in atherosclerosis, hypertension, type 2 diabetes mellitus, and renal vascular disease [1]. Vascular calcification can decrease the vasodilator capacity, increase vascular stiffness increased, and trigger plaque rupture and thrombosis, leading to acute deterioration of cardiovascular disease. Therefore, vascular calcification is a strong prognostic marker of cardiovascular disease and mortality in chronic kidney disease, which has long been considered a passive, degenerative and end-stage process of atherosclerosis and inflammation [2, 3].

In recent years, evidence of molecular biology shows that vascular calcification is an active

process that is similar to physiological mineralization of skeletal tissues. Apoptosis and osteochondrogenic transdifferentiation of vascular smooth muscle cells play a role in the process, in addition to degeneration and degradation of elastin may play an important role in its developmental process [4]. There is no effective preventive measure or medical therapy for vascular calcification at present. A number of cardiovascular-active substances such as adreno-medullin, [5] endothelin, [6] and angiotensin II [7] are receiving increased attention for their role in the pathophysiology of vascular calcification. More evidence has shown that certain immune mechanisms are involved in the pathogenesis of cardiovascular disease. The autoantibodies against angiotensin AT1 receptor (AT1-AAs) were reported to be involved in the pathogenesis of cardiovascular diseases [8].

AT1-AA induce vascular calcification

It was found in our previous studies that AT1-AAs promoted collagen deposition, increased tissue fibrosis, and ultimately led to cardiac and vascular remodeling, and that enhanced vascular response to norepinephrine played an important role in the development of severe hypertension, heart failure, and other cardiovascular diseases [9, 10]. However, whether AT1-AAs are directly or indirectly involved in the pathogenesis of vascular calcification remains unknown. To answer this question, we used a synthetic peptide corresponding to the second extracellular loop of the AT1 receptor as an autoantigen to immunize rats for 8 months and observed the long-term effect of AT1-AAs on the development of vascular calcification in these rats.

Materials and methods

Animals

Female Wistar rats (Experimental Animal Center of Shanghai Jiao Tong University School of Medicine, Shanghai, China) were maintained in specific pathogen-free spaces and fed with normal rat chow and tap water ad libitum with a 12:12 h light-dark cycle (lights on at 07:00 h, lights off at 19:00 h) at a constant ambient temperature ($23 \pm 2^\circ\text{C}$) and humidity ($60\% \pm 5\%$). All animal procedures were performed according to the guidelines approved by the university.

Immunization

Eighteen female Wistar rats aged 8 weeks were randomly divided into three groups: a control group, an immunization group, and an immunization plus losartan treatment group. Rat immunization was performed as described previously [14]. Briefly, free peptide (0.4 mg/kg) was emulsified in complete Freund's adjuvant and injected subcutaneously at multiple points in the rats. Two weeks later, a booster injection (0.4 mg/kg, in incomplete Freund's adjuvant) was injected subcutaneously at one point. Rats continued receiving booster injections at the same dose as the first booster injection biweekly for a total of 5 times. After three episodes of immunization, some animals in the treatment group were administered with losartan (20 mg/kg/day, DuPont Merck, Wilmington, USA) until the end of immunization. As controls, the rats received adjuvant without peptides fol-

lowing the same procedure as the immunized group. Systolic blood pressure (SBP) was measured before each immunization by the tail cuff method, and sera were collected by tail bleeding for detection of serum AT1-AAs.

Echocardiography

Echocardiography was performed by a blinded examiner one day before tissue harvest in rats under 1.5% isoflurane anesthesia using the VisualSonics Vevo 2100 system (Visualsonics) equipped with a 30 MHz center frequency ultrasound transducer. The left ventricular fractional shortening (LVFS), left ventricular end-diastolic dimension (LVDd), and interventricular septal (IVS) thickness were obtained.

Thoracic aortic ring relaxation assay

Rats were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Sigma), and sacrificed by cervical dislocation and bleeding. The descending aorta was rapidly dissected out and immersed in chilled Krebs's solution, following the composition (mmol/L) of: 118 NaCl, 5 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 2 CaCl_2 , 25 NaHCO_3 and 11 glucose; and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). After removing the excessive fat and connective tissue, the aorta was determined for the following indicators.

Endothelium-dependent vasodilation was detected as previously described [9]. The aortic ring about 4 mm in length was cut for the tension experiment. Two stainless-steel triangles were inserted through each vessel ring with care to preserve the endothelial layer. Each aortic ring was suspended in 10-ml organ baths filled with Krebs's solution, at $37 \pm 0.5^\circ\text{C}$, and bubbled with 95% O_2 and 5% CO_2 . One triangle was attached to the bottom of the organ bath and the other was connected to an isometric force transducer (Kent Scientific, Torrington, CT, USA), which was connected to a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia) and recorded on a PC using Chart 5.0 software. Each ring was stretched to a resting tension of 2 g and allowed to equilibrate for 60-90 min. After equilibration, the vessel rings were precontracted with KCl (final concentration: 60 mmol/L), washed, and normalized to the baseline level. Then the rings were soaked in Krebs's

AT1-AA induce vascular calcification

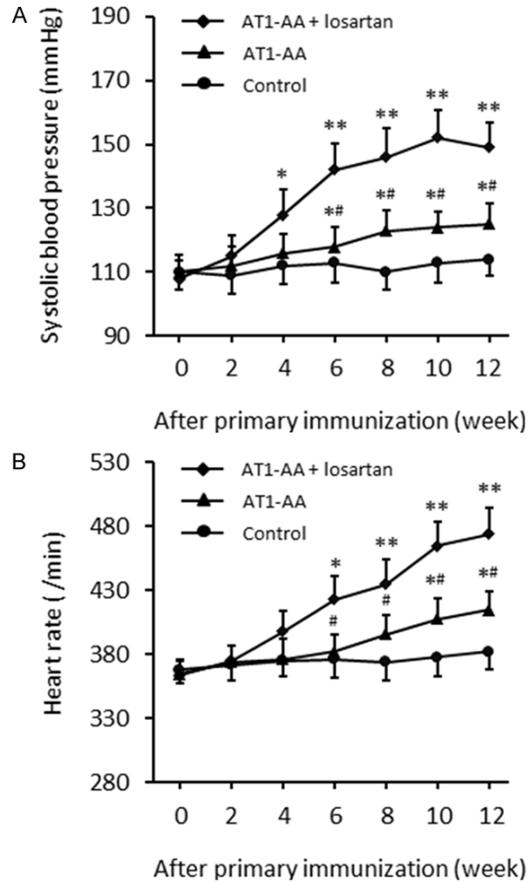


Figure 1. Changes in systolic blood pressure (A) and heart rate (B) in the three groups. *P < 0.05, **p < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. immunization group.

solution supplemented with phenylephrine (final concentration: 10^{-6} mol/L). After the contraction reaction reached a plateau, concentration-response curves to the acetylcholine (10^{-10} to 10^{-4} mol/L) were constructed by cumulative administration. Simultaneously, the vasodilator response to sodium nitroprusside (10^{-10} to 10^{-6} mol/L) was evaluated in the endothelium-denuded aorta.

Quantification of calcium content in aortas

The aorta segments were first dried at 70°C and weighed with a precision balance, and subsequently digested in 65% nitric acid at 65°C overnight, dried at 180°C and re-dissolved with a blank solution (27 nmol/L KCl and 27 $\mu\text{mol/L}$ LaCl_3). Serum and tissue calcium content was measured by flame atomic absorption spectrometry (Model 3110, Perkin-Elmer, Norwalk, CT, USA).

Alkaline phosphatase (ALP) activity assay

ALP activity in plasma and aortic tissues was measured as described previously [7]. Abdominal aortic blood was collected, and 2 mL sample was mixed with heparin (50 U/mL). Plasma was separated after centrifugation at 3000 rpm for 15 min at 4°C . Approximately 10 mg aortic tissue was homogenized in ice-cold buffer (20 mmol/L HEPES, 0.2% NP-40, and 20 mmol/L MgCl_2 , pH 7.4), and centrifuged at 8000 rpm for 10 min. The supernatant was collected to measure the plasma and tissue ALP activity by using an ALP assay kit. The results of ALP activity in the aorta were normalized to total protein, as determined by the Coomassie brilliant blue.

Histological observation

As described previously, the aortic segments obtained from the same location of different groups were fixed in 10% formaldehyde [9]. Tissue samples were dehydrated, paraffin embedded, and cut into 6- μm -thick sections. Some of the slides were stained with hematoxylin-eosin (H&E), and the others were dehydrated before being incubated in 1.5% silver nitrate solution for 1 h in the sunlight, then immersed in 5% sodium thiosulfate for 2 min, and finally counterstained with safranin (red staining). Slides were observed for the vascular structure under an optical microscope by two investigators who were blind to the treatment conditions.

Western blot analysis of osteopontin and osteocalcin

Western blot analysis was performed as described previously [12]. Briefly, the rat aortic tissues were homogenized by sonication at 4°C , and the protein concentration of the supernatant was measured with a protein assay reagent kit (Bio-Rad Laboratories). Soluble protein (80 μg) from each sample was separated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore) using standard electroblotting procedures. The blots were then blocked and incubated overnight at 4°C with the primary antibodies (1:500 anti-OPN and 1:500 anti-OCN, Abcam, Cambridge, MA, USA). Immunolabeling was detected using an enhanced chemiluminescence kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's instructions.

AT1-AA induce vascular calcification

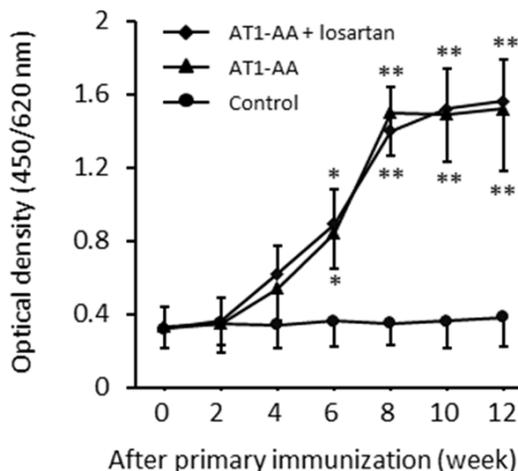


Figure 2. Changes in AT1-AAs titer (represented by optical density values). Values are means + SD. * $P < 0.05$, ** $P < 0.01$ vs. control group.

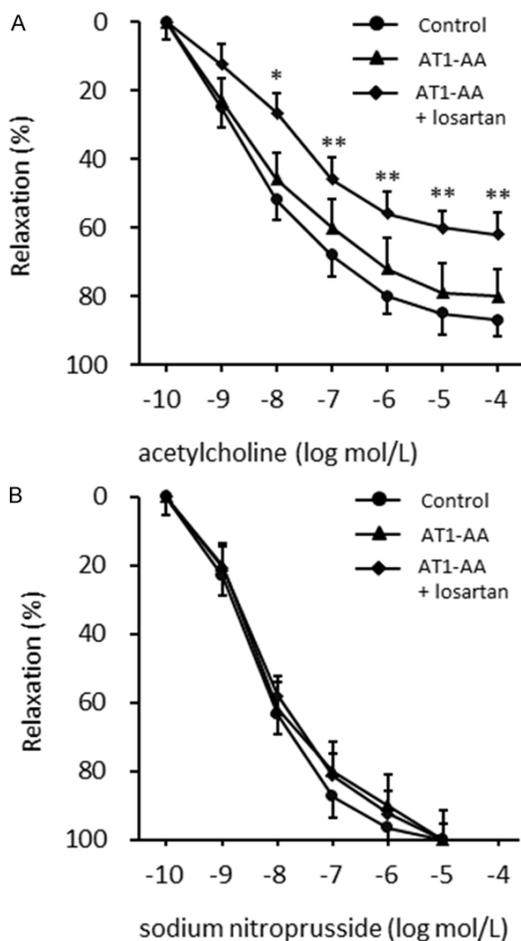


Figure 3. Concentration-response curves to acetylcholine in endothelium-intact and sodium nitroprusside in endothelium-denuded aortic rings. Vessels were stimulated with noradrenaline (10^6 M), when

the contraction reached a plateau, cumulative concentrations of acetylcholine and sodium nitroprusside were added to the bath. Data are expressed as percentage of noradrenaline-induced contraction. Each point represents mean \pm SD, $n = 6$. * $P < 0.05$ vs. control.

Statistical analysis

All data were calculated as mean \pm SD. Positivity was defined as a ratio of (sample A - blank A)/(negative control A - blank A) ≥ 2.1 . Antibody titer was reported as geometric mean. Comparisons between two groups involved the unpaired Student's *t* test and more than two groups, one-way ANOVA, followed by Newman-Keuls multiple comparison test. *P* values < 0.05 were considered statistically significant.

Results

SBP and heart rate (HR) changes in rats

Baseline SBP and HR were not significantly different between the three groups before initial immunization. As shown in **Figure 1A**, SBP began increasing 3 weeks after the initial immunization in the immunization group. At the 4th week, SBP of the immunization group was significantly elevated compared with that before initial immunization ($P < 0.01$). The maximum value (148.2 ± 10.9 mmHg) of the SBP was at 10 weeks and remained constant until the 4th month. HR in the immunization group was significantly greater than that in the control group 4 weeks after initial immunization (**Figure 1B**).

AT1-AA production after immunization

Four weeks after initial immunization, the titer of AT1-AAs increased with the increase in SBP, reached the peak at 8 weeks, and remained at this high level. AT1-AA change in losartan treatment group was similar to that in immunization group, while it remained unchanged significantly in the control group (**Figure 2**).

Changes in aortic ring systolic and relaxation function

The contractile force of the aortic ring in response to PE in the immunization group was significantly higher than that in the control group, and the mean maximum contractile force was 1.64 fold that of the control group (*P*

AT1-AA induce vascular calcification

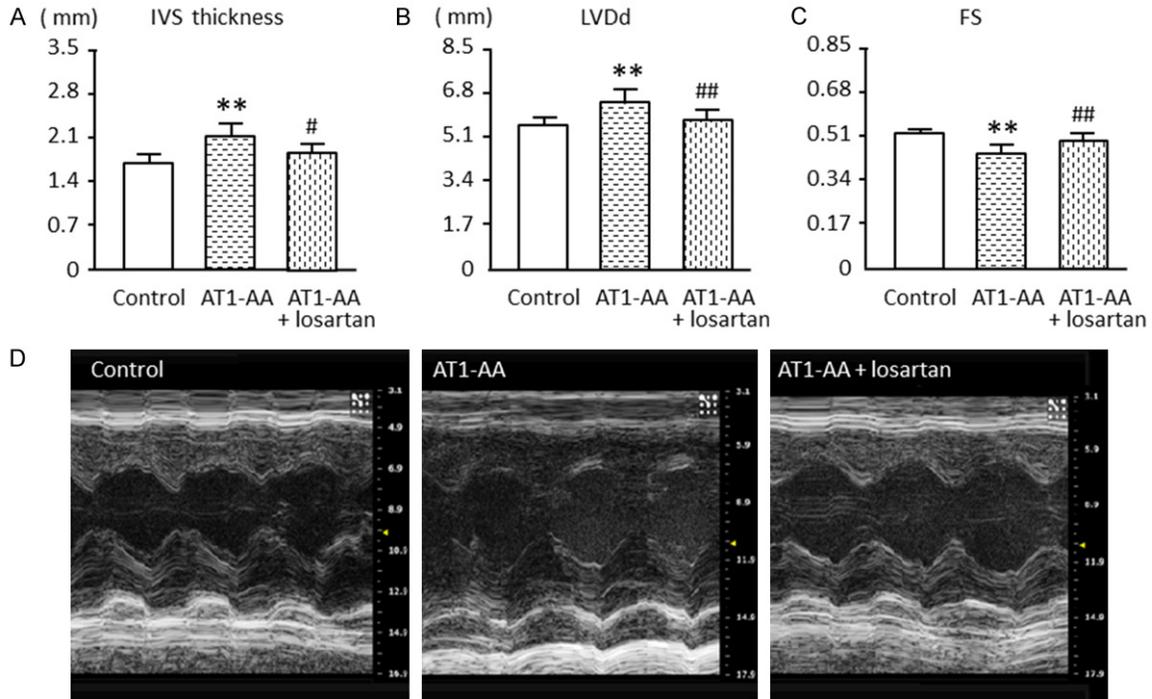


Figure 4. Echocardiographic findings. (A) IVS thickness, (B) LVDD, and (C) FS in the control, immunization and losartan treatment groups. Data represent the mean \pm SD (n = 6). *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. immunization group. (D) Representative M-mode echo cardiograms at left ventricular levels.

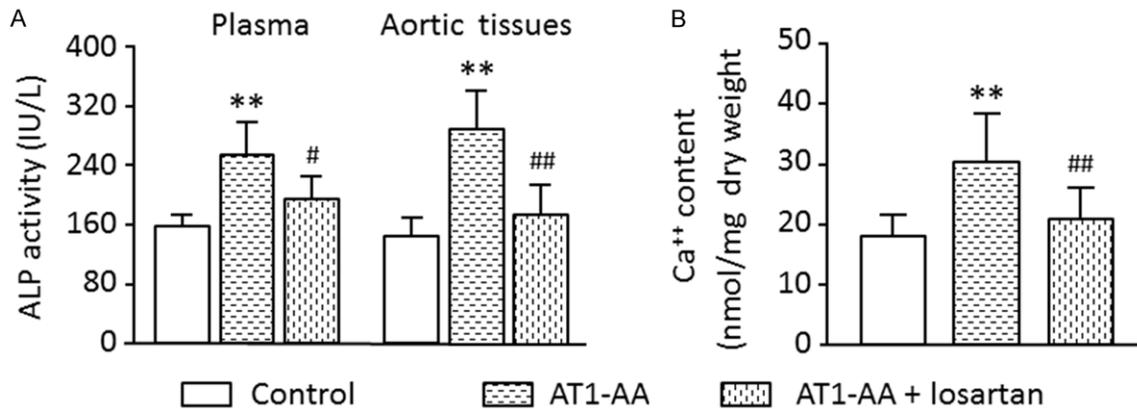


Figure 5. Alkaline phosphatase (ALP) activity in plasma or aorta and calcium content in aorta. The effect of losartan on ALP activity in plasma and aortas (A), and calcium content in aorta (B). Data are mean \pm SD. n = 6 in each group. *P < 0.05, **P < 0.01 vs. control, #P < 0.05, ##P < 0.01 vs. immunization group.

< 0.05). The maximum contractile force in the losartan treatment group was slightly attenuated compared with that in the immunization group, and the mean maximum contractile force was 1.13 fold that in the control group.

The effect of AT1-AAs on relaxing responses to acetylcholine and sodium nitroprusside in the three groups are shown in **Figure 3**. Com-

pared with the control group, the endothelium-dependent relaxation function was significantly reduced in the immunization group (P < 0.01). Compared with the immunization group, the percentage of aortic ring relaxation was significantly increased in losartan treatment group (P < 0.01). The acetylcholine (ACh) pD₂ values were not significantly different between the three groups. Observation of the non-endothe-

AT1-AA induce vascular calcification



Figure 6. Calcium deposition and vascular remodeling in the rat aorta. von Kossa staining of vascular calcium deposition (black/brown areas) of vascular structure. Magnification in 200 \times .

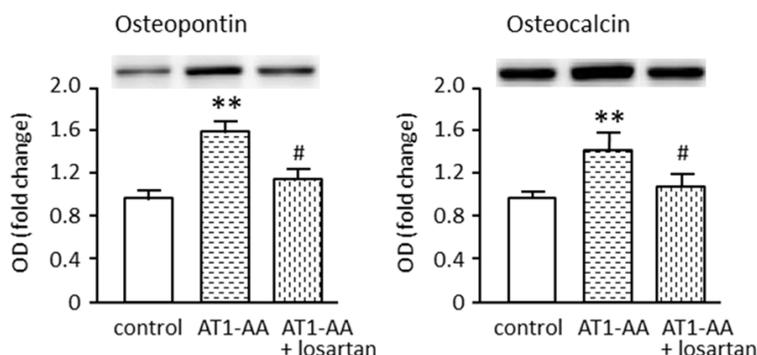


Figure 7. The effect of AT1-AAs on retinal level of OPN and OCN expression in the rat aortic tissue. Values are mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.05$ vs. immunization group.

lial dependent diastolic function by using sodium nitroprusside (SNP) after phenylephrine (PE) contraction showed no significant difference in E_{max} and pD_2 between the three groups.

Electrocardiographic changes

As shown in **Figure 4**, IVS thickness as an indicator of left ventricular hypertrophy was significantly greater in the immunization group than that in the control group (2.14 ± 0.06 mm vs. 1.78 ± 0.07 mm, $P < 0.001$). Treatment with losartan attenuated the effect of AT1-AA on IVS thickness (1.92 ± 0.03 mm, $P < 0.05$). Left ventricular end-diastolic dimension (LVDD) in the immunization group was significantly greater than that in the control group and losartan treatment group (6.88 ± 0.19 mm vs. 5.65 ± 0.10 , and 5.84 ± 0.13 mm). FS in the immunization group was significantly smaller than that in the other two groups. (0.43 ± 0.01 vs. 0.46 ± 0.01 and 0.49 ± 0.01 , $P < 0.01$).

Change of ALP activity and calcium content

Compared with the control group, ALP activity in the plasma and aorta increased by 63.2% and 113.2% in the immunization group respec-

tively (both $P < 0.05$). After losartan treatment, ALP activity in the plasma and aorta was reduced by 35.9% and 78.4% respectively (both $P < 0.05$), as compared with the immunization group (**Figure 5A** and **5B**). The calcium content in the immunization group was approximately 1.87-fold higher than that in the control group ($P < 0.01$). As compared with the immunization group, losartan treatment decreased the calcium content significantly by 52.3% ($P < 0.01$).

Histological observations

AT1-AA induced calcium deposition in the aortic tissue was assessed by the silver nitrate method (von Kossa staining), where black/brown areas in the elastic lamina of the tunica media represent strong positive staining for calcification. von Kossa staining demonstrates mountains of hydroxyapatite crystal deposition in the rat aorta of the immunization group (**Figure 6B**), and no von Kossa staining represents calcium deposition in the control group (**Figure 6A**). Positive staining in the medial layer of the aorta was reduced significantly in the losartan treatment group as compared with that in the immunization group (**Figure 6C**).

H&E staining revealed that the vascular walls were thickened and the elastic fibers were deranged in the aorta of the immunization group as compared with the control group. However, the vascular structures were recovered after losartan treatment.

Expression of OPN and OCN

Expression of OPN and OCN in rat aortic tissues was assayed by Western blot. The results showed that the protein levels of OPN and OCN were increased by 63.5% and 45.5% in the aortic tissue of the immunization group (both $P < 0.05$), and losartan had an inhibitory effect on OPN and OCN expression, with a 42.3% ($P < 0.05$) and 32.8% ($P < 0.01$) decrease in protein level respectively (**Figure 7A** and **7B**).

Discussion

Vascular calcification occurs in many diseases such as atherosclerosis, diabetes, and uremia as a pathologic response to defective calcium and phosphate homeostasis. It is the combined effect of multiple factors and multiple channels, and strongly associated with cardiovascular mortality and morbidity. Recent studies have found that vascular calcification is also involved in the pathogenesis of severe hypertension. Coronary artery calcification is often accompanied with changes in vascular function such as increased stiffness and reduced compliance, which may predict the future risk of developing cardiovascular diseases, such as myocardial ischemia, plaque rupture, and coronary thrombosis. Positive coronary artery calcification can prompt primary prevention interventions for patients with severe hypertension, including effective dietary modification, exercise, weight management, and lipid-lowering medications to reduce the risk.

Renin-angiotensin system (RAS) plays a key role in the pathogenesis in a variety of cardiovascular diseases, mediated by its effector peptide, angiotensin II (Ang II). Ang II has been linked with a variety of different functions ranging from the initiation of severe vascular pathologies, such as atherosclerosis and hypertension. The role of Ang II in the pathogenesis of vascular calcification has become a hot research topic in recent years. Numerous studies have demonstrated that Ang II plays an important role in promoting development of vascular calcification, and therefore many researchers believe that it could become a new therapeutic strategy for vascular calcification [8, 9].

Recent animal experiments and clinical studies have demonstrated that AT1-AA is closely associated with the incidence of severe hypertension, not only in the presence of high titers of plasma AT1-AA in patients with severe hypertension, but also in animal models of hypertension established by immunized with second extracellular loop of the AT1 receptor, suggesting that AT1-AA plays a potential role in the pathogenesis of severe hypertension [14]. The exact binding site of AT1-AA to the seven amino acid stretches of the second extracellular loop of the AT1 receptor (the peptide AFHYESQ) exhibits an agonist-like activity similar to Ang II,

which has a stimulatory positive chronotropic effect [12]. Our previous studies [9, 11] reported that AT1-AA could induce pathological changes in experimental animals, such as myocardial fibrosis, myocardial apoptosis, endothelial damage, and vascular remodeling, all of which may contribute to the development of hypertension.

In these experiments, we used a synthetic peptide corresponding to the second extracellular loop of the human cardiac AT1 receptor to observe the long-lasting effect of antibodies on vascular calcification. The results showed that the titer of AT1-AAs in immunized rats increased rapidly one month after immunization, and at the same time, SBP and HR were increased significantly. HE staining showed that the aortic intima was thickened in varying degrees in the immunization group. The nuclei of medial smooth muscle cells were densely populated and the number of smooth muscle cell layers between the elastic plates was apparently increased, with more stroma and disordered arrangement of collagen fibers presenting as larger gaps and disordered layers. Von Kossa staining showed that there were large numbers of brown-black particles on and within vascular smooth muscle cells. Compared with the control group, the Ca^{2+} level of the arterial tissue in the immunization group was significantly increased as compared with that in the control group. The above results indicate that high-titer AT1-AA can cause arterial calcification. The extent of vascular calcification and the Ca^{2+} level of the arterial tissue were significantly reduced after losartan treatment.

Our results suggest that vascular calcification induced by AT1-AA may be an important pathophysiological mechanism in the development of cardiovascular disease in patients with severe hypertension. Tumor necrosis factor (TNF- α) as an important endogenous substance not only can cause vascular calcification but is one of the important downstream signaling molecules in the pathological role of AT1-AA. Anti-tumor necrosis factor antibodies are powerful therapeutic agents for the treatment of vascular calcification in atherosclerotic rat models [15].

ALP is a marker of early-stage osteoblast differentiation [16]. ALP level was increased in osteoblasts and preosteoblasts and expressed by osteoblast-like cells *in vitro*. The serum lev-

els of both OPN and OCN were found to correlate with the bone formation rate as determined histomorphometrically and therefore could serve as biochemical indices of osteoblastic activity. It was found in our study that ALP activity and protein expressions of OPN and OCN in the arterial tissue were significantly increased in the immunization group. However, these indicators were obviously decreased in rats treated with losartan ($P < 0.05$). These results suggest that AT1-AA may play a central role in maternal vascular calcification and endothelial dysfunction in severe hypertension, which may be associated with activation of AT1 receptor.

In conclusion, evidence from a number of sources indicates that AT1-AA contributes to the development of severe hypertension by multiple mechanisms. In this study, we found that AT1-AA could promote vascular calcification, and early treatment with AT1-receptor blockers can prevent or reduce the development of vascular calcification by inhibiting these mechanisms, thus reducing the risk of cardiovascular disease in patients with severe hypertension.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81670232).

Disclosure of conflict of interest

None.

Abbreviations

T1-AAs, autoantibodies against angiotensin AT1 receptor; HR, heart rate; Ach, acetylcholine; SNP, sodium nitroprusside; PE, Phenylephrine; SBP, systolic blood pressure; OPN, osteopontin; OCN, osteocalcin; LVFS, left ventricular fractional shortening; LVDd, left ventricular end-diastolic dimension; IVS, interventricular septal.

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AT1-AA induce vascular calcification

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