

Original Article

Agonistic antibody to angiotensin II type 1 receptor accelerates atherosclerosis in ApoE^{-/-} mice

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Abstract: This study aimed to investigate the effects of agonistic antibody to angiotensin II type 1 receptor (AT1-AA) on atherosclerosis in male ApoE^{-/-} mice which were employed to establish the animal models of AT1-AA in two ways. In the first group, mice were injected subcutaneously with conjugated AT1 peptide at multiple sites; in the second group, mice were infused with AT1-AA prepared from rabbits that were treated with AT1 peptide intraperitoneally. Mice in each group were further randomly divided into five subgroups and treated with AT1 peptide/AT1-AA, AT1 peptide/AT1-AA plus valsartan, AT1 peptide/AT1-AA plus fenofibrate, AT1 peptide/AT1-AA plus pyrrolidine dithiocarbamate (PDTC) and control vehicle, respectively. Antibodies were detected in mice (except for mice in control group). Aortic atherosclerotic lesions were assessed by oil red O staining, while plasma CRP, TNF- α , nuclear factor-kappa B (NF- κ B) and H₂O₂ were determined by ELISA. CCR2 (the receptor of MCP-1), macrophages, and smooth muscle cells were detected by immunohistochemistry. P47phox, MCP-1 and eNOS were detected by RT-PCR, while P47phox, NF- κ B and MCP-1 were detected by Western blot assay. The aortic atherosclerotic lesions were significantly increased in AT1 peptide/AT1-AA treated mice, along with simultaneous increases in inflammatory parameters. However, mice treated with valsartan, fenofibrate or PDTC showed alleviated progression of atherosclerosis and reductions in inflammatory parameters. Thus, AT1-AA may accelerate aortic atherosclerosis in ApoE^{-/-} mice, which is mediated, at least in part, by the inflammatory reaction involving nicotinamide-adenine dinucleotide phosphate oxidase, reactive oxygen species, and NF- κ B. In addition, valsartan, fenofibrate and PDTC may inhibit the AT1-AA induced atherosclerosis.

Keywords: Agonistic antibody to angiotensin II type 1 receptor, atherosclerosis, inflammation

Introduction

Atherosclerosis is a chronic inflammatory disease that involves the interactions among endothelial cells, macrophages, smooth muscle cells and cytokines [1-3]. Oxidative stress plays a key role in atherosclerosis associated with endothelial dysfunction due to the inactivation of nitric oxide (NO) by superoxide [4]. NADPH oxidase is an important source of ROS [4], including superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), and excess ROS contributes to various pathophysiological processes such as endothelial dysfunction and atherosclerosis [5]. NF- κ B plays a critical role in the regulation of gene expression of inflammatory molecules including adhesion molecules, chemokines and cytokines such as vascular cell adhesion mole-

cule (VCAM)-1, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)- α [6]. A variety of evidence has demonstrated that angiotensin II may cause atherosclerosis either through activating the synthesis of ROS, NF- κ B and NADPH oxidase, or by impairing NO activity and inducing oxidative stress [7, 8]. The presence of agonistic antibodies to angiotensin II type 1 receptor (AT1-AA) has been confirmed in the serum of patients with malignant hypertension, preeclampsia, refractory hypertensive, and renal-allograft rejection [9-12]. Accumulating evidence indicates that AT1-AA may contribute to ROS production, inflammatory response and NF- κ B activation by activating NADPH oxidase [12-14]. AT1-AA may also stimulate the surface adhesion molecule expression, tissue factor production and increase the endo-

thelial cell permeability [15-17]. These effects of AT1-AA may be involved in the pathogenesis of atherosclerosis *in vivo*. In postpartum women with a history of preeclampsia, AT1-AA was still detectable after delivery and AT1-AA may be used to predispose to and/or predict subsequent cardiovascular risk [18]. Moreover, Dechend et al [19] found an atherosclerosis-like lesion in the spiral arteries of the placenta in a rat model of preeclampsia in which AT1-AA was detectable. We therefore tested the hypothesis that AT1-AA could result in atherosclerosis *in vivo*. To determine whether AT1-AA is an active player for atherosclerosis *in vivo*, the animal models of AT1-AA induced atherosclerosis were established in two ways, and the role of AT1-AA in the atherosclerosis development was investigated in ApoE^{-/-} mice. In the first group, mice were injected subcutaneously with conjugated AT1 peptide at multiple sites; in the second group, mice were infused weekly with AT1-AA prepared from rabbits that were treated with AT1 peptide intraperitoneally. The aortic pathogenic lesion and the inflammatory parameters were detected and analyzed.

Materials and methods

Subjects

This study was approved by the Ethics Committee of Union Hospital and the patients with hypertension and/or coronary heart disease (CHD) were enrolled after informed consent was obtained. Patients with hepatic and renal disease (creatinine levels > 2.0 mg/dl), malignancies, systemic or local infection and receiving systemic immunosuppressants or glucocorticoids were excluded. Hypertension was diagnosed when the resting blood pressure was above 140/90 mmHg (measured at least twice). CHD was defined as a main complaint coronary heart disease and at least one coronary artery with stenosis of more than 50% by coronary angiography. A total of 108 patients with hypertension and/or CHD and 16 healthy controls were recruited. The plasma AT1-AA was detected by ELISA as described previously [11].

Animals

All the animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Animals were allowed to accommodate to the environment for one week. Male Japanese white rabbits (specific pathogen free) purchased from the Hubei Research Center of Laboratory Animal, China, were maintained in cages independently, and given *ad libitum* access to food and water. Eight-week-old male ApoE^{-/-} mice with C57BL/6J background were purchased from the Jackson Laboratory (Maine, NE, USA). They were bred in Peking University, China and raised in the Experimental Animal Center of Tongji Medical College. The mice were housed in a room with a 12-hour light/dark cycle and the temperature was maintained at 22°C. They were given *ad libitum* access to food and water for 16 weeks.

Preparation of the AT1 peptide and AT1-AA

A peptide corresponding to amino acids 165-191 of the second extracellular loop of human AT1 receptor was produced using an automated multiple solid-phase peptide synthesizer in our laboratory. The peptide purity was determined by high performance liquid chromatography (HPLC) and 95% purity was noted. The sequence of this peptide was as follow: IHRNVFFIENTNITVCAFHYEQNSTL. To prepare AT1-AA, male Japanese white rabbits weighing 2.0-2.5 kg (n = 6) were used, the AT1 peptide was conjugated and rabbits were handled as previously described and raised for 9 weeks [20]. At week 9, blood was collected from these rabbits for the determination of antibody response to this peptide by ELISA as previously described [20]. Five immunized rabbits had anti-peptide antibodies at a high titer (above 1:12000). Subsequently, the rabbits were anesthetized and sacrificed by exsanguination from the common carotid arteries, and IgG was prepared as described previously [21]. The AT1-AA activity was measured by a bioassay that is used to evaluate the beats per minute (bpm) of rat myocardial cells *in vitro*, as described previously [22]. Results indicated statistical significance (data not shown).

Animal models and treatments

The animal models of atherosclerosis were established with AT1-AA in mice in two ways. In the first group, mice were injected subcutaneously with the conjugated peptide, prepared as described previously and emulsified in Freund's complete adjuvant (FCA), at multiple sites. A

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Table 1. AT1-AA content in patients of different groups (OD value)

	ACS		SCAD		CAD-	
	HTN+	HTN-	HTN+	HTN-	HTN+	HTN-
OD	0.37 ± 0.11	0.28 ± 0.13	0.34 ± 0.1	0.25 ± 0.09	0.21 ± 0.1	0.12 ± 0.07
N	18	20	24	22	24	16

Footnotes: data are expressed as means ± SE; OD: optical density; ACS: acute coronary syndrome; SCAD: stable coronary artery disease; CAD-: non-coronary artery disease. HTN+: patients with hypertension; HTN-: patients without hypertension.

Table 2. Body weight and plasma lipid in different groups

Group	N	Body weight	Cholesterol (mol/L)	Triglycerides (mol/L)
AT1 peptide				
POS	6	33.1 ± 0.6	11.48 ± 1.95	0.69 ± 0.04
V	6	30.9 ± 2.1	10.66 ± 0.56	0.67 ± 0.03
F	6	33.4 ± 2.7	7.09 ± 0.14**	0.44 ± 0.02**
P	6	29.8 ± 0.7	11.06 ± 0.91	0.7 ± 0.1
NEG	6	33.6 ± 0.5	10.48 ± 1.17	0.68 ± 0.07
AT1-AA				
POS	6	31.8 ± 1.8	11.51 ± 1.64	0.71 ± 0.18
V	6	31.7 ± 0.7	11.2 ± 1.34	0.71 ± 0.1
F	6	31.5 ± 1.5	7.31 ± 0.34**	0.45 ± 0.05**
P	6	30.4 ± 1.1	11.74 ± 1.26	0.69 ± 0.09
NEG	6	30.9 ± 0.9	11.5 ± 1.28	0.76 ± 0.08

Footnotes: data are expressed as means ± SE. POS: positive group; V: valsartan; F: fenofibrate; P: PDTC; NEG: negative group. **P < 0.001 vs. positive group.

were infused weekly with antibodies prepared from rabbits, and even-aged male ApoE^{-/-} mice treated with BSA in the same manner served as a negative control group. In both groups, mice were randomly further divided into five subgroups. The number of mice and treatments used in each subgroup are shown in **Table 2**. Subsequently, these mice were given *ad libitum* access to food and water for 16 weeks. Valsartan at 1 mg/kg/d (Novartis Pharma AG, Switzerland) and fenofibrate at 30 mg/kg/d (Laboratories FOURNIER, France) were intragastrically administered once daily. Pyrrolidine dithiocarbamate (PDTC) (30 mg/kg) (Sigma, USA) was intraperitoneally administered once every other day. Blood was collected at weeks 0, 4, 8, 12 and 16 after overnight fasting and processed for the detection of antibody titer by ELISA. In negative control groups, blood was collected at week 0. The antibody titer in different groups is shown in **Figure 1**.

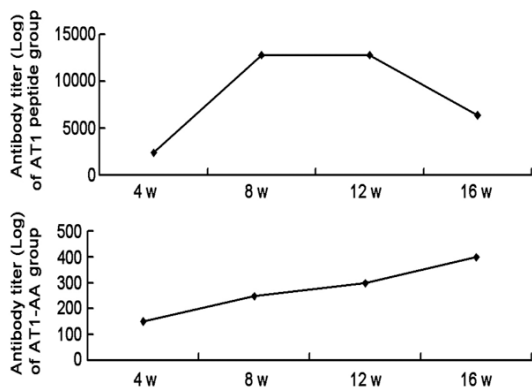


Figure 1. Antibody titer in AT1 peptide group and AT1-AA group. Blood was collected at the end of 4th, 8th, 12th, and 16th weeks, and at 4th, 8th and 12th weeks before booster injection.

booster injection (emulsified in Freund's incomplete adjuvant (FIA)) was performed at weeks 2, 4, 8, and 12. The even-aged male ApoE^{-/-} mice were treated with Freund's adjuvant alone in the same manner and served as a negative control group. In the second group, animals

Measurement of plasma lipids

At the end of study, mice were fasted overnight, intraperitoneally anesthetized with sodium pentobarbitone at 40 mg/kg and sacrificed by exsanguination from the right ventricle. The blood was collected and anti-coagulated with heparin. The plasma was collected through centrifugation at 5500 g for 15 min at 4°C, and then stored at -80°C until assay. Total serum cholesterol (TC) and triglyceride (TG) were determined by using an automated biochemical analyzer (Audicom-AC880A).

Measurement of CRP, TNF-α, NF-κB and H₂O₂ in the plasma

The plasma contents of CRP, TNF-α, NF-κB were measured in duplicate by ELISA with corresponding kits (R&D Systems, Minneapolis, USA) according to manufacturer's instructions. H₂O₂ was detected with a commercially available kit according to manufacturer's instructions (Beyotime Institute of Biotechnology, China).

Assessment of aortic atherosclerotic lesions

The top half of the hearts with 1 mm of the proximal aorta attached were processed with a modified method described previously [23]. Mice were flushed with phosphate buffer saline (PBS), followed by rapid removal of the hearts and aortas. The top half of the hearts with 1 mm of the proximal aorta attached was snap-frozen in OCT for cryostat sectioning. Then, 10- μ m serial sections were obtained since the aortic valve leaflets and stained with oil red O for the detection of lipids. Five sections (sections were saved every 100 μ m) for each animal were used for analyzing the amount of lesions. The mean was calculated for each animal and subsequently for each group. The detection was performed by an observer blinded to this experiment.

Analysis of CCR2, macrophages and smooth muscle cells by immunohistochemistry

Frozen proximal aorta sections (10 μ m) were immunostained for a chemokine receptor (CCR2), the specific receptor of monocyte chemoattractant protein-1, using a primary antibody against CCR2 (Abcam, Hong Kong) according to the manufacturer's instructions. Macrophages and smooth muscle cells were detected in proximal aorta sections (10 μ m) using a primary antibody to CD68 (Abcam, Hong Kong) and a monoclonal antibody to α -Actin (Abcam, Hong Kong), respectively, according to the manufacturer's instructions.

Detection of mRNA expression of P47phox, MCP-1 and eNOS by RT-PCR

Total RNA was extracted from whole aortas using the Trizol reagent (Invitrogen, Carlsbad, Calif) and processed for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with a SYBR Green real-time PCR master mix kit (TaKaRa Biotechnology [Dalian] Co. Ltd, China) according to the manufacturer's instructions. The mRNA expression of target genes in each sample was detected in duplicate and normalized to that of GAPDH [24]. The primers used in this study were as follows: P47 phox: forward: 5-CGA-AGA-AGC-CTG-AGA-CAT-ACC-3; reverse: 5-ATA-TCC-CCT-TTC-CTC-ACC-ACC-3; MCP-1: forward: 5-TTC-CTC-CAC-CAC-CAT-GCA-G-3; reverse: 5-CCA-GCC-GGC-AAC-TGT-GA-3; eNOS: forward: 5-CTG GAC ATC ACT

TCC CCG-3; reverse: 5-GAG-CTG-GCT-CAT-CCA-CGT-3; GAPDH: forward: 5-ACC-ACA-GTC-CAT-GCC-ATC-AC-3; reverse: 5-TCC-ACC-ACC-CTG-TTGCTG-TA-3.

Detection of protein expression of P47phox, NF- κ B and MCP-1 by Western blot assay

Proteins were extracted from pooled arteries (n = 6 per group) and the protein concentration was detected in each group. The extracted proteins of equal amount were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk, the membrane was incubated overnight at 4°C with anti-P47phox (Abcam, Hong Kong), anti-NF- κ B/P65 (Santa, USA), anti-MCP-1 (Abcam, Hong Kong), or anti- β -actin (Abcam, Hong Kong) antibody. Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies and visualization was done with an enhanced chemiluminescence system. Protein bands were scanned into a computer and quantified using ImageLab System. The protein expression of each target gene was normalized to that of β -actin. Western blot assay was performed as previously described [25].

Statistical analysis

Data are expressed as mean \pm standard error (SE). The means were compared with Student's t-test (for plasma lipids, body weight of mice, CRP, TNF- α , NF- κ B and H₂O₂, and aortic atherosclerotic lesions) or Mann-Whitney U test (for P47phox, MCP-1, NF- κ B, and eNOS). A value of P < 0.05 was considered statistically significant.

Results

AT1-AA titer in patients with CHD

According to the clinical conditions, 124 participants were divided into six groups: 1) acute coronary syndrome (ACS) and hypertension: ACS with HTN+ group; 2) acute coronary syndrome without hypertension: ACS with HTN- group; 3) stable coronary artery disease (SCAD) with hypertension: SCAD with HTN+ group; 4) stable coronary artery disease without hypertension: SCAD with HTN- group; 5) hypertension: CAD- with HTN+ group; 6) controls: CAD- with HTN- group (**Table 1**).

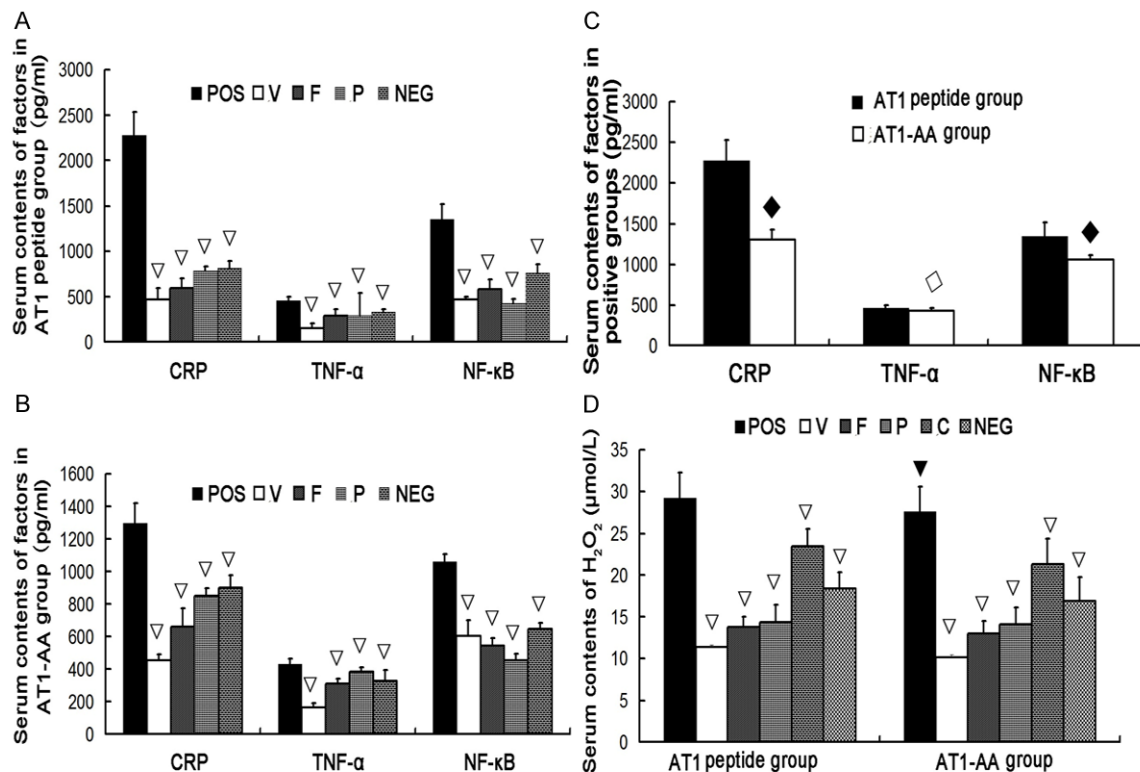


Figure 2. Plasma contents of different inflammation-related factors in different groups. AT1-AA increased the plasma contents of inflammation-related factors of ApoE^{-/-} mice, and valsartan, fenofibrate and PDTc reduced CRP, TNF- α , NF- κ B and H₂O₂ when compared with positive groups (ELISA). Moreover, AT1-AA increased the plasma contents of these factors in AT1 peptide group as compared to AT1-AA group. POS: positive group; V: valsartan; F: fenofibrate; P: PDTc; NEG: negative group. ∇ P < 0.001 vs. positive group in AT1 peptide group and AT1-AA group, \diamond P < 0.05, *P < 0.001 vs. positive group of AT1 peptide group.

ACS group (ACS with HTN+ group and ACS with HTN- group) (n = 38); SCAD group (SCAD with HTN+ group and SCAD with HTN- group) (n = 46); non-CAD group (CAD- group) (CAD- with HTN+ group and CAD- with HTN- group) (n = 40). Statistical analysis revealed that there was significant difference in the AT1-AA titer among ACS group, SCAD group and CAD- group (P = $7.48 \times 10^{-8} < 0.01$). The AT1-AA titer was comparable between ACS group and SCAD group (P = 0.37 > 0.05). Significant difference in AT1-AA titer was also observed between HTN+ group (n = 66) and HTN- group (n = 58) (P = 0.0006 < 0.01). In addition, marked difference was also noted in the AT1-AA titer (P = 0.027 < 0.05) among CHD group (ACS group and SCAD group) (n = 84) (0.31 ± 0.12), hypertension group (n = 66) (0.30 ± 0.12), and CHD combined with hypertension group (n = 42) (0.35 ± 0.11). The highest AT1-AA titer was observed in CHD with HTN+ group, but there was no remarkable difference between CHD group and HTN+ group (P = 0.35 > 0.05). These findings demonstrated

that both HTN+ patients and CHD patients have elevated plasma AT1-AA.

Body weight and plasma lipid profile

All the ApoE^{-/-} mice in both groups had comparable body weight and displayed no differences in the activities and behaviors. Body weight was lower in treated PDTc mice of AT1 peptide group, but this reduction was not significant in AT1-AA group and the difference may have resulted from the separation of littermates. In addition, fenofibrate significantly reduced serum TG (by 34.33%) and TC (by 33.49%) in AT1 peptide group, and TG (by 36.62%) and TC (by 34.73%) in AT1-AA group. Notably, there were no significant differences in the plasma TG and TC in other groups.

AT1-AA titer in mice

Figure 1 showed the changes in AT1-AA titer. In AT1 peptide group, the AT1-AA titer rose from

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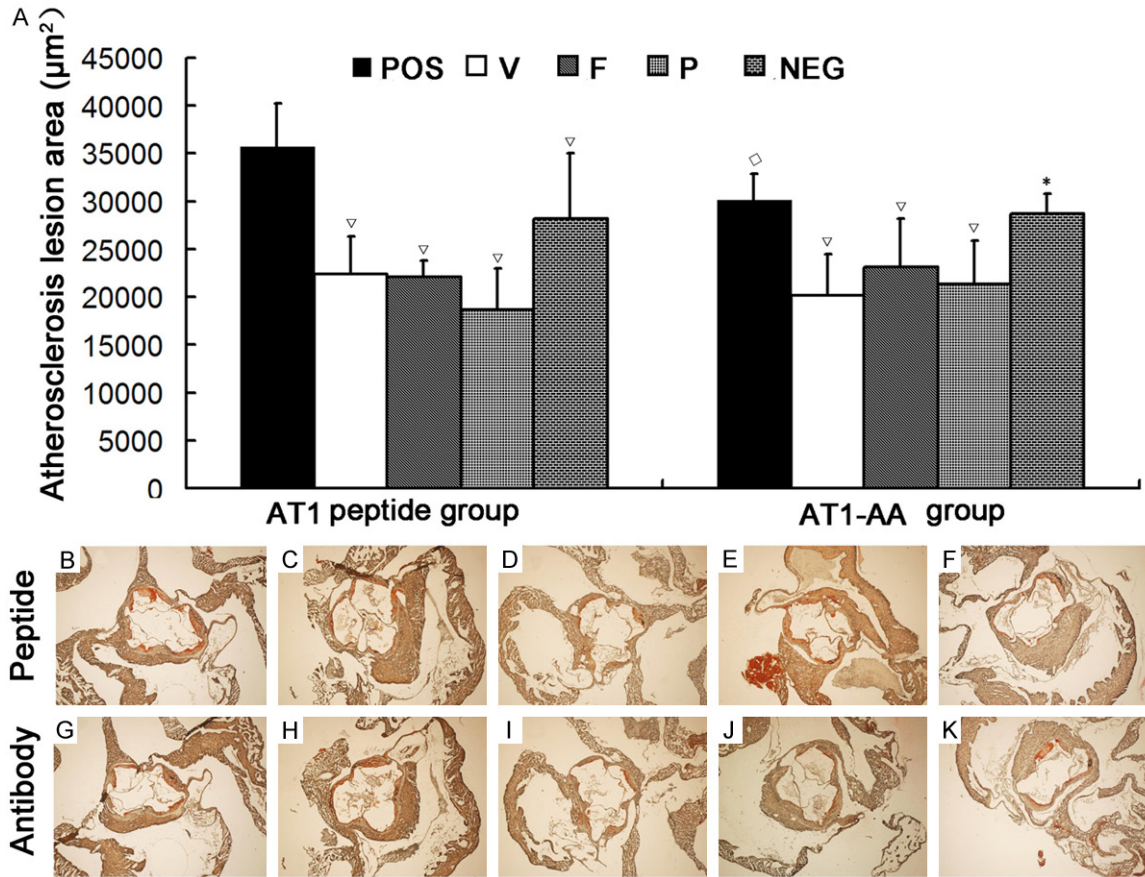


Figure 3. Detection of atherosclerosis lesion area of the aortic root of ApoE^{-/-} mice in AT1 peptide group and AT1-AA group. A: Average lesion area of different groups. Data are expressed as means \pm SE (n = 6 per group). B-K: Detection of lipid deposition by oil red O-staining of the aortic root. B-F: AT1 peptide group treated with AT1 peptide (POS), AT1 peptide plus valsartan (V), AT1 peptide plus fenofibrate (F), AT1 peptide plus PDTC (P), Freund's adjuvant (NEG); G-K: AT1-AA group treated with AT1-AA (POS), AT1-AA plus valsartan (V), AT1-AA plus fenofibrate (F), AT1-AA plus PDTC (P), BSA (NEG). Magnification: $\times 40$. POS: positive group; V: valsartan; F: fenofibrate; P: PDTC; NEG: negative group. *P < 0.05, ∇ P < 0.001 vs. positive group, \diamond P < 0.05 vs. positive group of AT1 peptide group.

1:2400 at week 4 to 1:12800 at weeks 8 and 12, and maintained stable at 1:12800 between weeks 8 and 12. At the end of week 16, the AT1-AA titer dropped to 1:6480, but still remained higher than that at week 4. In AT1-AA group, the AT1-AA titer was 1:150 at week 4, 1:250 at week 8, 1:300 at week 12 and 1:400 at week 16. The AT1-AA titer in control groups was comparable to that before study (data not shown).

Plasma levels of CRP, TNF- α , NF- κ B and H₂O₂ in mice

When compared with negative group, the concentrations of CRP, TNF- α , NF- κ B and H₂O₂ were increased by 147.44%, 41.21%, 80.77%, and 65.46%, respectively, in the plasma of AT1 peptide group, and 59.74%, 28.53%, 41.33%, and

51.69% in the plasma of AT1-AA group. Valsartan, fenofibrate, and PDTC could reduce these increases (**Figure 2**). Valsartan reduced these inflammation-related factors by 73.12%, 53.85%, 62.52%, and 62.64% in AT1 peptide group, and 61.5%, 49.42%, 57.16%, and 60.62% in AT1-AA group. Fenofibrate reduced these factors by 69.72%, 37.77%, 56.71%, and 54.97% in AT1 peptide group, and 61.16%, 46.75%, 48.75%, and 49.12% in AT1-AA group. PDTC caused a 62%, 34.64%, 68.08%, and 52.97% decrease in the factors of AT1 peptide group, and a 54.22%, 28.63%, 61.67%, and 45.03% decrease in AT1-AA group. Lastly, when compared with AT1-AA group, the concentrations of CRP, TNF- α , NF- κ B and H₂O₂ were increased by 41.07%, 16.37%, 27.33%, and 18.95%, respectively in AT1 peptide group.

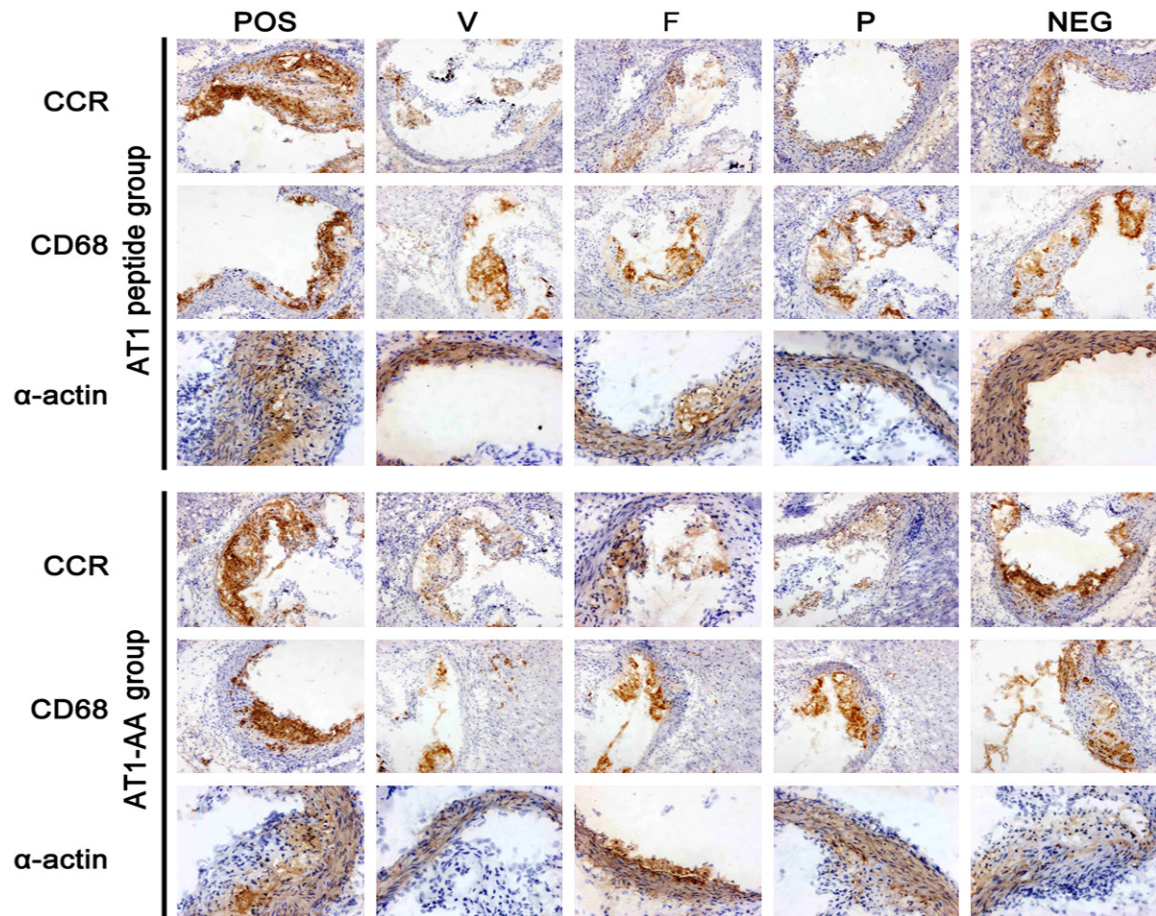


Figure 4. Immunohistochemistry for CCR2, α -actin and CD68 in the aortic root. Immunohistochemistry for CCR2, smooth muscle cells and macrophages in the aortic root of ApoE^{-/-} mice with anti-CCR2, anti- α -actin and anti-CD68 antibody. AT1 peptide group treated with AT1 peptide (POS), AT1 peptide plus valsartan (V), AT1 peptide plus fenofibrate (F), AT1 peptide plus PDTTC (P), Freund's adjuvant (NEG); AT1-AA group treated with AT1-AA (POS), AT1-AA plus valsartan (V), AT1-AA plus fenofibrate (F), AT1-AA plus PDTTC (P), BSA (NEG). CCR2 and CD68: $\times 100$; α -actin: $\times 200$.

Atherosclerotic lesions in mice

The atherosclerotic lesion area was measured at a cross section of ApoE^{-/-} mice (**Figure 3**). Results showed AT1-AAs accelerated the atherosclerosis in positive groups (AT1 peptide and AT1-AA groups vs the negative groups: 24.62% and 12.19%, respectively). Valsartan, fenofibrate and PDTTC decreased the atherosclerotic lesion area by 48.91%, 44.98%, and 44.37%, respectively, in AT1 peptide group and 48.34%, 43.19%, and 37.57%, respectively, in AT1-AA group. When compared with AT1-AA group, the atherosclerotic lesion area increased by 9.17% in AT1 peptide group.

CCR2, macrophages and smooth muscle cells (SMC) in atherosclerotic lesions

Figure 4 showed the representative photographs from immunohistochemistry of proximal

aorta sections (10 μ m) for CCR2, CD68 and α -Actin. Results showed AT1-AA enhanced macrophages and smooth muscle cells (SMCs) in all positive groups of in both AT1 peptide and AT1-AA groups, when compared with negative group. In addition, the CCR2 expression was higher in positive groups versus negative groups. Valsartan, fenofibrate and PDTTC reduced the accumulation of macrophages and SMCs and inhibited CCR2 expression.

mRNA expressions of p47phox, MCP-1 and eNOS in aortic tissues

The mRNA expressions of p47phox, MCP-1, eNOS were detected (**Figure 5**), and normalized to that of GAPDH. In positive groups, the mRNA expressions of p47phox increased by 53% and 85%, and MCP-1 increased by 28% and 41%, in AT1 peptide and AT1-AA groups, respectively, when compared with negative control group.

AT1-AA accelerates atherosclerosis

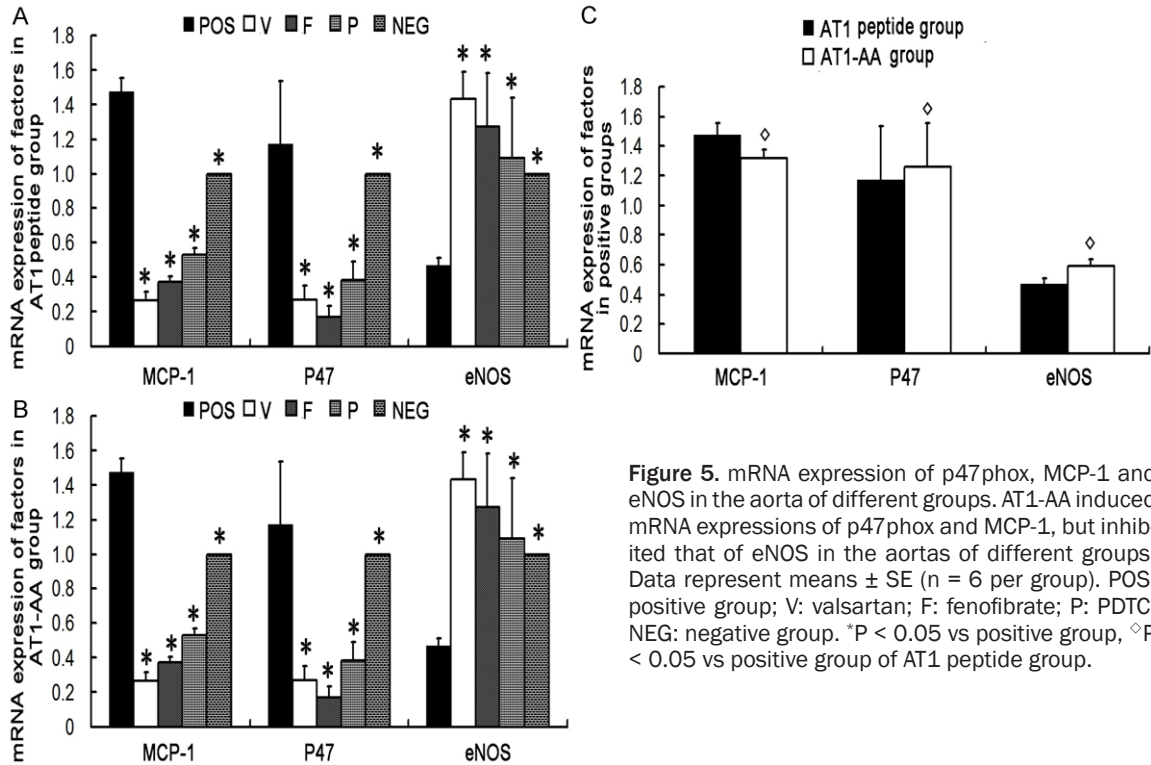


Figure 5. mRNA expression of p47phox, MCP-1 and eNOS in the aorta of different groups. AT1-AA induced mRNA expressions of p47phox and MCP-1, but inhibited that of eNOS in the aortas of different groups. Data represent means \pm SE (n = 6 per group). POS: positive group; V: valsartan; F: fenofibrate; P: PDTC; NEG: negative group. *P < 0.05 vs positive group, \diamond P < 0.05 vs positive group of AT1 peptide group.

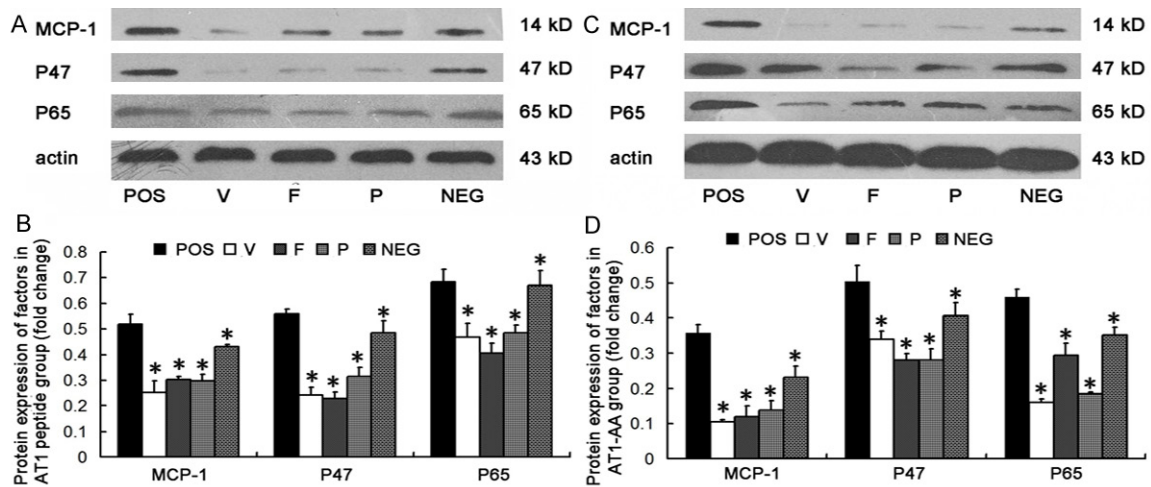


Figure 6. Protein expression of MCP-1, P47phox and P65 in the aorta of different groups. A, B: AT1 peptide group; C, D: AT1-AA group. A, C: representative protein bands; B, D: Densitometric measurement. Values are expressed as mean \pm SE (n = 6 per group). POS: positive group, V: valsartan, F: fenofibrate, P: PDTC, NEG: negative group. *P < 0.05 vs positive group.

However, eNOS was inhibited by 73% and 44% in AT1 peptide and AT1-AA groups, respectively. Valsartan markedly inhibited the mRNA expressions of these genes by 82.35% and 82.81% (p47phox), 82.16% and 78.72% (MCP-1), but increased that of eNOS by 529.63% and 251.79% in AT1 peptide and AT1-AA groups,

respectively. Fenofibrate reduced the mRNA expressions of these genes by 80.39% and 75% (p47phox), 78.38% and 74.47% (MCP-1) but increased that of eNOS by 492.59% and 250% in AT1 peptide and AT1-AA groups, respectively. Furthermore, PDTC reduced the mRNA expressions of these genes by 78.43%

and 75% (p47phox), 72.97% and 64.54% (MCP-1) but increased that of eNOS by 466.67% and 226.79% in AT1 peptide and AT1-AA groups, respectively. When compared with positive groups of AT1 peptide and AT1-AA groups, the mRNA expressions of P47phox and MCP-1 were increased by 19.53% and 31.21%, respectively, while eNOS was reduced by 51.79% in negative groups.

Protein expression of MCP-1, NADPH and NF- κ B in aortic tissues

Western blot assay demonstrated that the protein expressions of MCP-1, P47phox and P65 were increased in positive groups of AT1 peptide and AT1-AA groups when compared with negative groups (**Figure 6**). The protein expressions were detected by using proteins extracted from the nuclei. As expected, valsartan, fenofibrate and PDTC reduced the protein expression of all these proteins.

Discussion

Our results showed that not only patients with hypertension had AT1-AA, but patients with CHD had a high plasma AT1-AA. Thus, there might be an independent association between AT1-AA and CHD. Moreover, male ApoE^{-/-} mice were intraperitoneally injected with AT1 peptide or AT1-AA to establish animal models of atherosclerosis. Results showed AT1-AA was detectable in mice of both groups (**Figure 1**), accompanied by significant increase (24.62% and 12.19%) in the atherosclerotic lesion area in positive groups of both AT1 peptide and AT1-AA groups when compared with negative groups (**Figure 3**). Additionally, in positive groups, when compared with AT1-AA group, atherosclerosis lesion area was increased by 9.17% in AT1 peptide group (**Figure 3A**), suggesting that a high plasma AT1-AA accelerates the aortic atherosclerosis, as well as induces more severe atherosclerosis lesion. Our study for the first time confirms that AT1-AA may exert an atherogenic effect *in vivo*, which further extends the mechanisms underlying the pathogenesis of atherosclerosis.

In our study, AT1-AA accelerated atherosclerosis progression, accompanied by increased generation of inflammatory factors and ROS. *in vitro* experiments have shown the proatherogenic actions of AT1-AA [13, 19]. Currently, the

proatherogenic effect of AT1-AA may be closely related to the inflammatory responses and ROS. Among the consequences associated with AT1-AA, our results showed AT1-AA increased expression of CRP, TNF- α and NF- κ B in the plasma of mice (**Figure 2**). As is known, they are all inflammatory factors and NF- κ B can up-regulate the expression of inflammatory factors [26]. Both CRP and TNF- α have been shown to increase during atherosclerosis [23, 27, 28], and CRP is not only a risk factor for, but an active participant in the atherogenesis *in vivo* by stimulating the activation of complements in the lesions and perhaps down-regulating eNOS expression [23]. In addition, TNF- α has been shown to be associated with increased production of AT1-AA in certain conditions [29], suggesting that the proatherogenic effect of AT1-AA may be mediated via TNF- α . Meanwhile, a higher H₂O₂ was noted (**Figure 2**) in positive groups compared with negative groups, and an increase in H₂O₂ has been linked to the atheroaction in the atherosclerosis and endothelial dysfunction [5]. This suggests that AT1-AA-induced H₂O₂ production may contribute to atherosclerosis. Furthermore, our results demonstrated that the mRNA expression of eNOS (**Figure 5**) decreased in positive groups, indicative of endothelial dysfunction [30, 31], while valsartan, fenofibrate or PDTC reduced the amount of H₂O₂ and elevate eNOS. Although only the mRNA expression of eNOS was detected, it suggests that AT1-AA may lead to endothelial dysfunction. Moreover, Western blot assay (**Figure 6**) and ELISA (**Figure 2**) showed the increased expression of P65, a subunit of NF- κ B, which is an important participant in the development and progression of chronic inflammatory diseases such as atherosclerosis [32], in part by stimulating oxidative stress. Oxidative stress plays an crucial role in mediating vascular endothelial dysfunction in overweight and obese humans [33] and the inhibition of NF- κ B activity has been demonstrated as an alternative to conventional treatments for atherosclerosis [34, 35]. Consistent with this, our results showed valsartan, fenofibrate and PDTC were able to decrease the expressions of CRP, TNF- α , and NF- κ B, accompanied by reduced atherosclerotic lesion area when compared with mice in positive groups. Interestingly, these parameters increased in positive groups of AT1 peptide group when compared with AT1-AA group, which suggests that the effects of AT1-AA are some-

what dose-dependent. Previous findings have demonstrated that angiotensin II receptor antagonist valsartan can decrease CRP, inhibit the inflammatory cells from infiltrating the vascular wall [36], and significantly decrease the NF- κ B activity [37]. Fenofibrate, a peroxisome proliferator-activated receptor- α (PPAR- α) agonist, has been reported to suppress NF- κ B [38], inhibit macrophage recruitment by activating of PPAR- α [39], and also act as a negative regulator of pro-inflammatory genes by antagonizing the activity of inflammatory transcription factors [40]. PDTC also decreased the expression of TNF- α and interleukin-6 by inhibiting NF- κ B activity [41], and perhaps also decreased CRP in the serum. We speculate that, through the mechanisms mentioned above, these three drugs can also reduce the plasma content of H₂O₂ and improve the pathological changes during the atherosclerosis.

In addition, the increased expression of NADPH oxidase component p47phox was observed at both mRNA (**Figure 5**) and protein levels (**Figure 6**). It has been indicated that AT1-AA can increase ROS production and inflammatory responses by activating NADPH oxidase [13]. AT1-AA also stimulates NADPH oxidase, resulting in increases in both ROS production and NF- κ B activation [26]. Recent reports reveal that the increased NADPH oxidase complex in the vascular cells plays an important role in the increased production of vascular superoxide in atherosclerosis [42, 43] and may result in coronary endothelial dysfunction [44]. Previous studies have demonstrated that deletion of p47phox significantly depresses ROS generation *in vitro* and markedly inhibits atherosclerosis *in vivo* [45]. In our study, results showed that the high NADPH oxidase expression and NF- κ B activity are accompanied by more atherosclerotic lesions in positive groups when compared with negative groups. After administration of valsartan, fenofibrate and PDTC, there was a reduction in NADPH oxidase expression, NF- κ B activity and atherosclerotic lesion area as compared to positive groups. Furthermore, the expressions of p47phox and NF- κ B were higher in positive group of AT1 peptide group versus AT1-AA group. Recent reports reveal that the activation of NADPH oxidase through AT1 receptor increases H₂O₂ production [46], while valsartan represses this effect. Moreover, fenofibrate improves the endothelial

function, oxidative stress, and vascular inflammation by inhibiting the activity of NADPH oxidase [47] and PDTC has been suggested to inhibit the NADPH oxidase activation and TNF- α production in rats [48]. It suggests that NADPH oxidase and NF- κ B, at least in part, mediate AT1-AA induced acceleration of atherosclerosis. Further studies are necessary to elucidate how AT1-AA affects the NADPH oxidase activity, NF- κ B expression and other molecules involved in the AT1-AA-induced atherosclerosis.

The expression of MCP-1 was detected by RT-PCR (**Figure 5**) and Western blot assay (**Figure 6**), and that of CCR2 by immunohistochemistry (**Figure 4**). Atherosclerosis is a chronic inflammatory disease involving the accumulation of mononuclear cells in the blood vessel wall, and chemokines play an important role in the inflammatory response of the arterial wall [2, 3]. MCP-1 is an important regulator of the inflammatory reaction in the blood vessel wall [49, 50], and MCP-1 and CCR2 have been implicated as critical players in the recruitment of monocytes and macrophages [51]. Genetic deletions of MCP-1 or CCR2 have been shown to decrease monocyte accumulation and lesion formation in mice susceptible to atherosclerosis [52, 53]. Inhibiting CC-CK activity is also shown to lessen the monocyte recruitment and vascular remodeling in both native atherosclerosis and vein graft accelerated atherosclerosis [54, 55]. In the present study, results showed the increased expression of MCP-1 and CCR2 was also accompanied by accumulation of more macrophages and smooth muscle cells in the blood vessel wall and severe atherosclerotic lesions in positive groups. Moreover, the positive groups of AT1 peptide group had higher expression of MCP-1 and CCR2, and more macrophages and smooth muscle cells when compared with AT1-AA group. Furthermore, reductions in macrophages, smooth muscle cells, and atherosclerosis lesions in the blood vessels were observed in mice treated with valsartan, fenofibrate and PDTC. These mice also had reduced MCP-1 and CCR2 expression, which suggests that MCP-1/CCR2 is involved in the improvement of atherosclerotic lesions. Monocytes and macrophages have been identified as a major source of superoxide anions, resulting in damage to the vasculature and further contributing to atherosclerosis due to the activation of NADPH oxidase [56].

In conclusion, our findings demonstrate that increased serum AT1-AA in ApoE^{-/-} mice accelerates atherosclerosis, supporting the potentially atherogenic effects of AT1-AA in previous *in vitro* studies. In addition, the effects of AT1-AA are dose-dependent in positive groups of AT1 peptide group and AT1-AA group. AT1-AA has been reported to mediate hypertension [57], and further studies are warranted to investigate the atherosclerotic effect of AT1-AA-mediated hypertension. We speculate that AT1-AA, through certain mechanisms, activates NADPH oxidase and NF- κ B, and furthermore increases the expression of TNF- α , CRP, MCP-1, CCR2 and H₂O₂, while decreases the expression of eNOS, all of which aid to accelerate atherosclerosis. However, the specific mechanism need to be further studied. We conclude that, for patients with atherosclerosis, it is necessary to not only reduce traditional risk factors, but perform therapies targeting AT1-AA.

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