

# The Role of Angiotensin II Type 1 Receptor-Activating Antibodies in Vascular Inflammation

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## Abstract

**Background:** Studies demonstrated the autoantibodies against angiotensin II type 1 receptor (AT1-AAAs) could induce vascular endothelial dysfunction. Our objective is to investigate the effect of AT1-AAAs on atherosclerosis. **Methods:** AT1-AAAs were purified from sera of patients with primary hypertension. Thirty-six New Zealand white rabbits were underwent balloon-induced abdominal aortic endothelial injury and fed an atherogenic diet for 6 weeks and were randomly divided into six groups with different drugs for 4 weeks. The levels of AT1-AAAs, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) in different stage were detected by ELISA. The abdominal aortas of rabbits were stained with hematoxylin and eosin. The expression of matrix metalloproteinases-2 (MMP-2) in aortic tissue was detected by Western blotting. **Results:** The levels of TNF- $\alpha$  in the eighth week ( $0.17 \pm 0.04$ ,  $0.34 \pm 0.08$ ) and in the tenth week ( $0.23 \pm 0.04$ ,  $0.54 \pm 0.11$ ) were significantly higher than that at the beginning of test ( $0.04 \pm 0.03$ ,  $0.08 \pm 0.02$ ) in the group of AT1-AAAs with low-dose and high-dose ( $P < 0.05$ ). The level of IL-6 stimulated by high dose AT1-AAAs was higher in the tenth week ( $0.42 \pm 0.08$ ) than that at the beginning of test ( $0.04 \pm 0.01$ ,  $P < 0.05$ ). The relative area of plaque was significantly ( $P < 0.05$ ) increased in group of AT1-AAAs with low-dose ( $46.99\% \pm 13.06\%$ ) and high-dose ( $66.11\% \pm 19.67\%$ ) compared with other groups. The expression of MMP-2 was significantly higher ( $P < 0.05$ ) in group AT1-AAAs with low dose ( $0.42 \pm 0.03$ ) and high dose ( $0.54 \pm 0.04$ ) than that in other groups. **Conclusion:** The results showed that the AT1-AAAs could aggravate the inflammatory reaction and the plaque formation.

## Keywords

AT1 Receptors, Autoantibody, Inflammation, Atherosclerosis

## 1. Introduction

Agonistic antibodies against the AT1 receptor (AT1-AAAs) were found in patients with preeclampsia [1], malignant hypertension [2], refractory hypertension [3] and humoral renal transplant rejection hypertension [4]. These studies showed that the AT1-AA has an agonist-like activity effect similar to Ang II, such as promoting the proliferation of VSMCs [5], activating NF- $\kappa$ B and reactive oxygen species (ROS) [6], increasing the serum level of inflammatory factor like TNF- $\alpha$  and IL-6 [7], and contributing to endothelial dysfunction in preeclampsia [8]. The endothelial injury and vascular inflammatory are important initiating factors in early atherogenesis [9], then these lipids activate endothelial cells and macrophages to produce adhesion molecules and chemokines, subendothelial depositions of lipids, macrophage foam cells loaded with cholesterol and atherosclerotic plaque formation. Furthermore, the AT1 receptors widely express in endothelial cells and vascular smooth muscle cells (VSMCs). Activating AT1 receptor can result in increased endothelial permeability [10] [11]. So, these studies suggest that AT1-AAAs may be a reason for excessive AT1 receptor activation in hypertension and play a major part in the etiology and pathophysiology of hypertension. However, it remains unknown whether the AT1-AA exacerbates atherosclerosis by endothelial injury and vascular inflammatory. Therefore, this study firstly intended to investigate whether AT1-AA could accelerate the plaque formation and promote inflammation in local plaque in rabbit atherosclerosis model.

## 2. Material and Methods

### 2.1. Human Subjects

This study was approved by the Ethics Committee of Zhongshan Hospital, Sun Yat-sen University. The patients with essential hypertension (at least 18 years of age) participated in the study after written permission. Exclusion criteria included renal disease, liver disease, chronic inflammatory disease, sleep apnea and secondary hypertension. Blood samples for the tests were obtained from each participant before drug therapy. We centrifuged blood samples at 18,000 g for 10 min and stored the serum samples at  $-80^{\circ}\text{C}$ .

### 2.2. Preparation of Ig Fraction and Affinity Purification of AT1-AAAs

The peptide corresponding to the sequence of the second extracellular loop of the human AT1 receptor positions 165aa - 191aa (I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-HY-E-S-Q-N-S-T-L) [5] was synthesized by an automated, multiple solid-phase peptide synthesizer (PSSM-8 type; Shimadzu, Tokyo, Japan). The peptide was evaluated by HPLC analysis on a Vydac C-18 column and more than 95% purity was achieved. The level of AT1-AAAs was measured by ELISA as we used previously, and the results were expressed as optical density (OD) values [5]. The titer was shown by the value of P/N when the sera were diluted in 1:40

[(P/N = (the A value of test - the A value of blank)/(the A value of negative control - the A value of blank)]. Preparation of the Ig fractions was outlined in detail earlier. Briefly, the Ig fractions in the sera were isolated by ammonium sulfate precipitation at a saturation of 40%. The Ig fractions from positive AT1-AAs sera were loaded on a sepharose 4B CNBr-activated gel (Pharmacia, USA) to which the second extracellular loop of the human AT1 receptor peptide was covalently linked. The antibodies were eluted with 3 mol/L potassium thiocyanate (pH = 7.4) followed by immediate extensive dialysis against phosphate buffered saline; the purity was detected by SDS-PAGE. The elution fraction (affinity-purified AT1-AAs) was either used immediately or stored at  $-80^{\circ}\text{C}$ .

### 2.3. Synthetic Seven-Amino Acid Epitope Peptide

For the neutralization experiments, we used a synthetic peptide corresponding to a sequence on the second extracellular loop of the human AT1 receptor (A-F-H-Y-E-S-Q, 7aa) [12]. The 7aa is a special inhibitor peptide of AT1-AAs.

### 2.4. Building Atherosclerosis Animal Model and Introduction of AT1-AAs into Rabbit

Thirty-six New Zealand white rabbits underwent balloon-induced abdominal aortic endothelial injury and were fed an atherogenic diet containing 1% cholesterol and 4% coconut oil (120 - 140 g/day) for 10 weeks. Balloon-induced aortic wall injury was performed with a 4F balloon catheter introduced through the right femoral artery to the abdominal aorta after rabbits had been anesthetized with an intravenous injection of pentobarbital sodium (30 mg/kg). The balloon was inflated with saline at 8 atmosphere standard and the catheter was retracted down to the iliofemoral artery. This process was repeated three times in each rabbit to ensure denudation of the endothelium of the abdominal aorta. After 6 weeks, rabbits were randomly divided into six groups ( $n = 6$  each): group of control injected with PBS, group of low-dose AT1-AAs ( $20 \text{ ug}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) and group of high-dose AT1-AAs ( $40 \text{ ug}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ), group of high-dose AT1-AAs with oral losartan ( $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ), group of high-dose AT1-AAs with oral simvastatin ( $4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ), group of high-dose AT1-AAs with 7aa ( $1.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ), respectively on the basis of high-cholesterol diet for 4 weeks. We resuspended the affinity-purified AT1-AAs (100 ug) with 500 ul saline and then introduced them into rabbits by ear vein injection. We collected plasma and measured the level of AT1-AA by ELISA at each of different time point. All animal studies were reviewed and approved by the Animal Care Committee of Sun Yat-sen University.

### 2.5. ELISA of Lipids, TNF- $\alpha$ and IL-6

We centrifuged the plasma of rabbits on 4th, 6th, 8th and 10th week at 18,000 g for 10 min and stored the serum samples at  $-80^{\circ}\text{C}$ . Serum levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by enzymatic assays

using an automated biochemical analyzer (Roche-Hitachi 917, Block Scientific, Inc., New York, USA). Serum levels of interleukin-6 (IL-6), TNF- $\alpha$  were assayed on highly sensitive ELISA kit (R & D Laboratories, USA).

## 2.6. Histopathologic Analysis

Rabbits were euthanized by overdose of intravenous pentobarbital. The abdominal aorta was dissected and excised to observe plaque. A two centimeter-size tissue was taken from the abdominal aorta in all rabbits and fixed in 4% formaldehyde. Tissues were embedded in paraffin and cut 5  $\mu$ m serial sections and then stained them with HE. The relative area of abdominal aortic plaques was compared in different groups.

## 2.7. Western Blot Analysis of MMP-2

Total protein of aortas was extracted following the instruction of the Mem-PER<sup>®</sup> Eukaryotic Membrane Protein Extraction Reagent Kit, and the protein concentration was quantified with a BCA protein quantity assay kit. The protein was separated by 12% SDS-polyacrylamide (SDS-PAGE) gel electrophoresis under 4°C, and then the protein was transferred onto microporous polyvinylidene fluoride membranes in running buffer with 20% methanol. After nonspecific sites were blocking with 5% milk-Tris Buffered Saline-tween 20 (TBST), membranes were incubated with anti-MMP-2 antibody and anti-GAPDH antibody overnight, washed in TBST and a horseradish peroxidase-linked antibody was employed as a secondary antibody. The bands of interest were detected using an enhanced chemiluminescent technique. Western blot experiment was done 5 or more times. Quantification of the protein bands was carried out by laser densitometry.

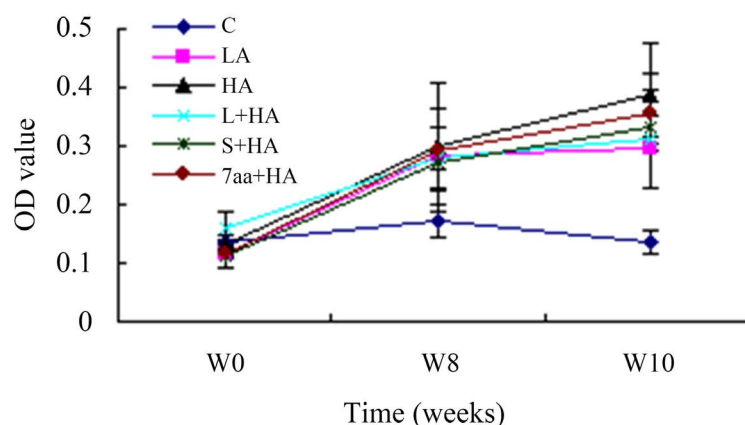
## 2.8. Statistical Analysis

Data were expressed as mean  $\pm$  standard error of the mean (SEM). Comparison of continuous variables among multiple groups was performed by analysis of variance with ANOVA and *post hoc* comparisons were made using LSD test. Categorical data such as the proportion of plaque area was analyzed by chi-square test. Values of  $p < 0.05$  were considered to denote statistical significance.

# 3. Results

## 3.1. The Levels of AT1-AAs and Lipids in the Different Time Period

The levels of AT1-AAs in the eighth and tenth week were significantly higher than at the beginning of test in all groups excluding control one (all  $P < 0.05$ , **Figure 1**). In addition, the values of TC and LDL were obviously increased after four weeks. However, the level of lipids in group of high-dose AT1-AAs with simvastatin was lower in the tenth week than that in the fourth week. The levels of lipids were not significantly different among other groups at different time periods (data not shown).



**Figure 1.** The levels of AT1-AAs in different groups and periods. C: control group (stimulated by negative Ig-AT1-AAs); LA: low-dose Ig-AT1-AAs; HA: high-dose Ig-AT1-AAs; L: losartan; S: simvastatin; 7aa: an inhibitor peptide of AT1-AAs. W0: the beginning of test before balloon-induced abdominal aortic endothelial injury, W8: the eighth week, W10: the tenth week. The levels of AT1-AAs in the eighth and tenth week were significantly higher than at the beginning of test in all groups excluding control one, all  $P < 0.05$ .

### 3.2. The Expression of TNF- $\alpha$ and IL-6 in Different Groups

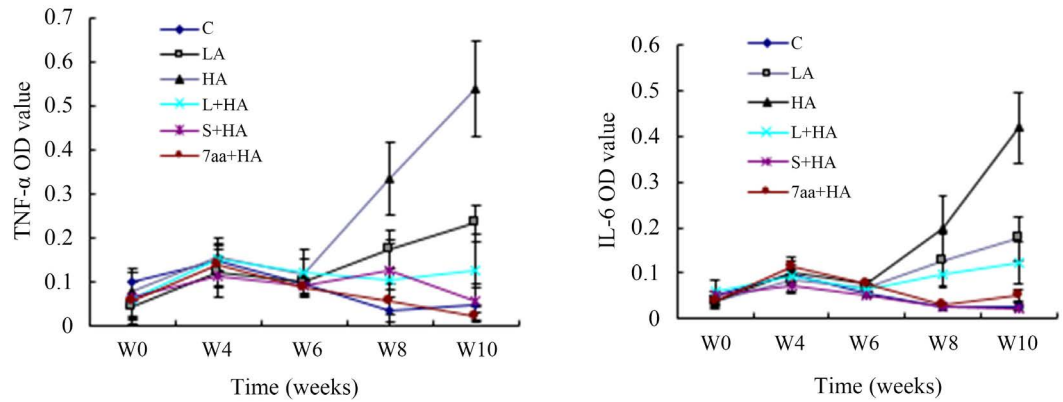
The levels of TNF- $\alpha$  in the eighth week (respectively,  $0.17 \pm 0.04$ ,  $0.34 \pm 0.08$ ) and the tenth week (respectively,  $0.23 \pm 0.04$ ,  $0.54 \pm 0.11$ ) were significantly higher than that at the beginning of test (respectively,  $0.04 \pm 0.03$ ,  $0.08 \pm 0.02$ ) either in the group of low-dose AT1-AAs or high-dose ones ( $P < 0.05$ ). The level of IL-6 stimulated by high-dose AT1-AAs was higher in the 10th week ( $0.42 \pm 0.08$ ) than that at the beginning of test ( $0.04 \pm 0.01$ ) ( $P < 0.05$ ). The expression of TNF- $\alpha$  and IL-6 was significantly positive relative to prevalence of AT1-AAs both in group of low-dose and high-dose AT1-AAs. The expression of TNF- $\alpha$  and IL-6 in else groups did not show significant difference in different period (Figure 2).

### 3.3. The Relative Plaque Area in Different Groups

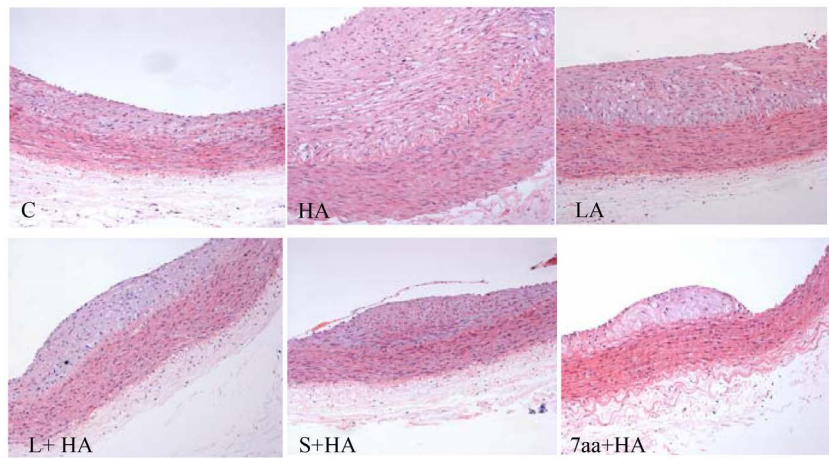
The relative area of plaque is the plaque area percentage of arterial lumen cross section. Intimal thickening, VSMC proliferation and plaque formation in group of low-dose AT1-AAs and in group of high-dose AT1-AAs were more obvious than that in other groups (Figure 3). The relative plaque area was significantly increased in group of low-dose AT1-AAs ( $46.99\% \pm 13.06\%$ ) and high-dose ones ( $66.11\% \pm 19.67\%$ ) compared with that in group of control ( $27.71\% \pm 7.46\%$ ), in group of AT1-AAs with losartan ( $34.27\% \pm 12.38\%$ ), in group of AT1-AAs with simvastatin ( $24.03\% \pm 8.56\%$ ) and in group of AT1-AAs with 7aa ( $28.54\% \pm 12.50\%$ ) ( $P < 0.05$ ).

### 3.4. The Expression of MMP-2

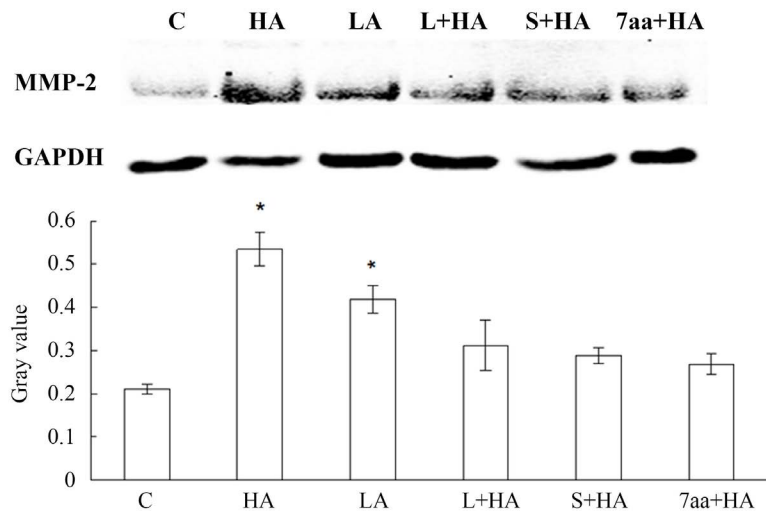
The protein level of MMP-2 was significantly higher in group of AT1-AAs with low-dose and high-dose than in other groups (all  $P < 0.05$ , Figure 4) but not



**Figure 2.** The level of TNF- $\alpha$  and IL-6 in different stage with different groups by ELISA. AT1-AAs stimulated the increase of TNF- $\alpha$  and IL-6 in a dose-dependent manner in 8th and 10th week. The increasing responses triggered by AT1-AAs were inhibited by losartan, simvastatin and 7aa.



**Figure 3.** Staining the abdominal aortas by hematoxylin-eosin in different groups. The intimal thickening and VSMC proliferation in HA group and LA group is more significant than that in other groups ( $P < 0.05$ ).



**Figure 4.** The expression of MMP-2 in different groups by Western blotting. The AT1-AAs increase the expression of MMP-2. \*HA or LA vs other groups, all  $P < 0.05$ .



significantly different among other groups. There was no obvious difference between group of low-dose AT1-AAs and group of high-dose ones. The gray levels were higher in group of low-dose AT1-AAs ( $0.42 \pm 0.03$ ) and in group of high-dose AT1-AAs ( $0.54 \pm 0.04$ ) than in group of control ( $0.21 \pm 0.01$ ), in group of high-dose AT1-AAs with losartan ( $0.31 \pm 0.06$ ), in group of high AT1-AAs with simvastatin ( $0.29 \pm 0.02$ ) and in group of high AT1-AAs with 7aa ( $0.27 \pm 0.02$ ). The results indicated that the AT1-AAs could markedly increase the expression of MMP-2 in the artery atheromatous plaque and the effects were inhibited by losartan, simvastatin and 7aa.

#### 4. Discussions

AngII increases NAD(P)H oxidase-derived ROS by AT1 receptor. ROS and particularly superoxide have become an important part of vascular biology, which can promote the endothelial dysfunction and the expression of adhesion molecules, and increase the infiltration of inflammatory cells and the oxidation of LDL and the accumulation of lipid-laden macrophages [6]. Activated macrophages in the plaque produce inflammatory cytokines and promote the development of atherosclerosis [13]. Previous studies showed that activated immune cells in the atherosclerotic plaque produced inflammatory cytokines TNF- $\alpha$  which induced the production of substantial amounts of IL-6. Then, IL-6 stimulated the production of large amounts of acute-phase reactants such as C-reactive protein (CRP). These cytokines at all steps had important biologic effects and their amplification at each step of the cascade made the measurement of downstream mediators such as CRP particularly useful for clinical diagnosis. The AT1-AAs have some similar effects as AngII. Some studies showed that AT1-AAs contributed to elevated ET-1 production and increased TNF- $\alpha$ /IL-6 signaling is a key mechanism underlying increased ET-1 production and subsequent maternal features of preeclampsia [7]. Our results confirmed the positive correlation between the AT1-AAs and the inflammatory factors such as TNF- $\alpha$  and IL-6 in atherosclerosis animal models. The AT1-AAs could activate the immune cells to produce TNF- $\alpha$  and IL-6, and aggravate the local endothelial dysfunction and injury, which need be further studied.

We have previously described that AT1-AAs promoted VSMC proliferation through JAK-STAT and NF- $\kappa$ B pathway *in vitro* [5]. At the same time, this study indicated that the intimal hyperplasia and VSMC proliferation were more obvious in AT1-AAs group than other groups in animal models. In addition, the matrix metalloproteinases (MMPs) have been thought to contribute to the development and progression of atherosclerosis [14] [15]. Growth of atherosclerotic plaques, also known as negative remodeling, requires the proliferation and migration of VSMCs within the arterial wall, and this progression is largely dependent on MMPs, particularly MMP-2 and MMP-9. The activation of MMP-2 plays a critical role in AngII/AT1-induced vascular inflammation and cardiac fibrosis [16] [17]. Our studies concluded that the AT1-AAs induced obviously increased the level of MMP-2 in artery tissue, which was partially compensated by

Losartan and 7aa. These studies indicated that AT1-AAAs played important roles in the vascular inflammation and the development of atherosclerosis, and these effects were positively correlated to MMP-2. Whether the AT1-AAAs increase the adhesion and migration of VSMC through MMP-2 need to be confirmed in further studies.

This study demonstrated that the AT1-AAAs could accelerate intimal thickening, VSMC proliferation, plaque formation and inflammatory reaction in vascular wall. Moreover, the activity effects triggered by AT1-AAAs were significantly inhibited by losartan and 7aa. In addition, simvastatin could inhibit the expression of inflammatory factors induced by AT1-AAAs. These results show that the local inflammation on the vascular wall stimulated by AT1-AAAs could be a result of activation of the AT1 receptor. Previous studies showed that angiotensin II type 1 receptor antagonism (ARB) improves endothelial function and reduces atherogenesis and the effects were beyond the control of blood pressure [18] [19] [20]. In addition, the latest clinic research has shown the higher AT1-AAAs levels were associated with inflammation and hypertension, and ARB treatment may blunt the harm associated with high levels of AT1-AAAs [21]. So, ARB may potentially have beneficial effects on patients with coronary atherosclerosis disease (CAD), especially in patients with positive AT1-AAAs. Further studies will be needed to evaluate the use of immune-directed therapies in atherosclerotic cardiovascular disease with high titre AT1-AAAs such as immunoadsorption.

Our study confirms that the AT1-AAAs accelerate the inflammatory and atherosclerosis of vascular wall by binding the AT1 receptor. Measurement of AT1-AAAs in hypertensive patients with the coronary artery diseases can provide additional useful information, especially indicating subjects at a higher risk for acute coronary disease.

### Limitations

This study preliminary showed that the activities of AT1-AAAs in vascular inflammation and the development of atherosclerosis. But the atherosclerosis plaque is not obvious in animal models because of short-time experiment. We should require longer time animal models to assess studies. In addition, further studies about the molecular mechanism of TNF- $\alpha$ /IL-6 increasing by AT1-AAAs should be studied. The comparison of the ARB in CAD patients with positive AT1-AAAs and negative ones should be further researched.

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### Conflicts of Interest

There are no conflicts of interests to declare.



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