Changes in cardiac structure and function in rats immunized by angiotensin type 1 receptor peptides

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Angiotensin II (Ang II) is known to induce cardiomyocyte hypertrophy by activating the Ang II type 1 (AT1) receptor. Some studies have demonstrated that the autoantibodies against angiotensin AT1 receptor (AT1–AAs) cause functional effects, which is similar to those observed for the natural agonist Ang II. In this study, we investigated the effects of AT1–AAs on cardiomyocytes’ structure and function. Male Wistar rats were immunized with synthetic peptides corresponding to the second extracellular loop of AT1 receptor and Freund’s adjuvant. The titers of AT1–AAs in rat serum were detected by enzyme-linked immunosorbent assay every week. Hemodynamic analysis and heart weight (HW) indices were measured on the 4th and 8th months after initial immunization, respectively. Cultured neonatal rat cardiomyocytes were used to observe the hypertrophic effects of AT1–AAs. Results showed that systolic blood pressure and heart rate were significantly increased, the titers of AT1–AAs were also increased after 4 weeks of initial immunization. Compared with control group, the HW/body weight (BW) and left ventricular weight/BW of immunized rats were increased significantly and cardiac function was enhanced compensatively. The cultured neonatal rat cardiomyocytes respond to AT1–AAs stimulation with increased 3H-leucine incorporation and cell surface area in a dose-dependent manner. These results suggest that the AT1–AAs have an agonist effect similar to Ang II in hypertrophy of cardiomyocytes in vivo and in vitro. AT1–AAs are involved in the pathogenesis of cardiovascular diseases and hypertension.

Keywords cardiac hypertrophy; hemodynamics; autoantibodies against the angiotensin AT1 receptor; active immunization

Introduction

The renin–angiotensin system (RAS) is integrally involved in maintaining hemodynamic status. Angiotensin II (Ang II) is the primary effector hormone of the RAS [1]. Ang II plays a major pathophysiological role in the genesis of cardiac hypertrophy. It stimulates several signaling molecules, and subsequently activates their target genes in the nucleus and leads to cell hypertrophy. Most of the physiological and pathophysiological cardiovascular actions of Ang II are mediated through the AT1 receptors [2]. Losartan and other AT1 antagonists reduce left ventricular hypertrophy in spontaneously hypertensive rats [3].

More and more evidences have shown that immune mechanisms involved in the pathogenesis of cardiovascular disease including atherosclerosis [4], malignant hypertension [5], etc. In the past 20 years, compelling evidence has presented regarding agonistic autoantibodies directed against the G-protein-coupled receptors. The autoantibodies against angiotensin AT1 receptor (AT1–AAs) were first described by Wallukat et al. [6]. Their results showed that AT1–AAs increased cultured cardiomyocytes contraction rates from 20 to 40 b.p.m., which was blocked by AT1 receptor antagonists but not by AT2 receptor antagonists. Zhou et al. [7] reported that pregnant mice showed a pre-eclampsia-like syndrome when exposed to the extracted AT1–AAs from the serum of pre-eclamptic patients, including hypertension, proteinuria, and so on. Clinical studies have shown that AT1–AAs could play a pathogenic role in various cardiovascular diseases, including pre-eclampsia [6,8], malignant essential hypertension [9], renal-allograft rejection [10], and systemic sclerosis [11]. These patients have changes in cardiac structure and function and are seropositive for AT1–AAs. However, it is still unknown as to whether the AT1–AAs are directly or indirectly involved in the pathogenesis of the cardiovascular disease and hypertension.
In order to address this question, a synthetic peptide corresponding to the second extracellular loop of the human AT1 receptor was used as an autoantigen to immunize rats for 8 months, and the long-term effect of AT1–AAs on cardiac structural and function changes of rats were observed. At the same time, we also observed the changes of cultured neonatal rat ventricular cardiomyocytes with hypertrophy induced by AT1–AAs.

Materials and Methods

Animals
Male Wistar rats were obtained from the experimental animal center, Shanghai Jiao Tong university school of medicine (Shanghai, China) and maintained in specific pathogen-free spaces. Rats were fed 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 ± 2°C) and humidity (60% ± 5%). All animal procedures were performed according to the guidelines approved by the Shanghai Jiao Tong university school of medicine.

Peptide synthesis
Peptide corresponding to the sequence of the second extracellular loop of the human AT1 receptor [6] (197–222, IHRNVFIENTNTVCAPHYESQNST) was synthesized with solid-phase synthetic methods and was purified with high-performance liquid chromatography.

Immunization
Forty-eight male Wistar rats at 8 weeks of age were randomly divided into two groups. Rats immunization was performed as described previously [12]. Briefly, free peptide (0.4 mg/kg) was emulsified in complete Freund’s adjuvant and injected subcutaneously at multiple points in rats. Two weeks later, a booster injection (0.4 mg/kg, incomplete Freund’s adjuvant) was injected subcutaneously at one point. Rats continued receiving booster injections which are the same as the first booster injection every 2 weeks during a period of 4 months. As controls, the rats received adjuvant without peptides following the same procedure as the immunized group. Systolic blood pressures were measured at baseline and at intervals of 1 week for 32 weeks by the tail cuff method, and serum were collected by tail bleeding for detection of serum AT1–AAs and Ang II concentration.

AT1–AAs detection and affinity purification
Serum AT1–AAs were detected by enzyme-linked immunosorbent assay (ELISA) as previously described [13]. Briefly, the peptides were coated (10 mg/ml in 100 mM Na2CO3) on 96-well plates. The wells were then saturated with PMT [phosphate-buffered saline supplemented with 5% (w/v) cow sera, 0.1% (V/V) Tween 20, and 0.01% (W/V) Thimerosal (Sigma, St Louis, USA)]. Fifty microliters of serial dilutions (doubling dilutions from 1:40 to 1:1280 in PMT) were added to the saturated wells overnight at 4°C. An affinity-purified biotinylated goat anti-rat IgG (H + L) was allowed to react for 1 h, followed by detection using streptavidin–peroxidase (1 mg/ml) (Sigma), and substrates H2O2 (2.5 mM) and 2,2′-azino-di (ethylbenzothiazoline) sulfonic acid (2 mM) (Sigma). Optical densities at 405 nm were measured after 30 min by a microplate reader (Molecular Devices, Sunnyvale, USA). Total IgG from immunized group and control group sera were purified with MAbTrapTM Kit (Amersham, Little Chalfont, UK), according to the manufacturer’s instructions.

Measurement of plasma Ang II concentration
Plasma Ang II concentration was determined with commercially available ELISA kits (Shanghai runwell technology Co., Ltd, Shanghai, China) according to the manufacturer’s recommendations. Briefly, the plasma and tissue homogenates samples were incubated with antiserum and [125I]-radiolabeled Ang II for 48 h at 4°C. Bound and free Ang II were separated by dextran-coated charcoal. The supernatants was decanted and counted by automated gamma counter.

Morphologic and hemodynamic analysis
Hemodynamic analysis was performed on the 4th and 8th months from the initial immunization. The closed chest method was performed to assess cardiac function [14]. Animals were placed on controlled heating pads, and core temperature measured by a rectal probe was maintained at 37°C. Polyethylene cannula filled with heparinized saline was palced into the left ventricle (LV) via the right carotid artery to record of left ventricular hemodynamic parameters by a digital acquisition and analysis system (PowerLab/4SP, ADInstruments, Pty. Ltd, Castle Hill, Australia). The indices of hemodynamic parameters, such as left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), the maximal rates of pressure rise (+dp/dt), and of pressure fall (−dp/dt) were recorded.

After the hemodynamic analysis, the rats were sacrificed with pentobarbital sodium, and hearts were quickly harvested. The LV was separated from the atrium and the right ventricle. Then, their weights were determined with a precision balance. The myocardial hypertrophy index was expressed as heart weight/body weight (HW/BW) and left ventricular weight/body weight (LVW/BW). The ventricles were cut into four slices from apex to base, after removal of atria and large vessels. The slices were fixed in 10% formaldehyde and dehydrated with graded concentrations of alcohol for embedding in paraffin. Left ventricular
thickness and cavity area were measured as described previously in [15]. The heart tissue was cut into 5 μm sections, and stained with hematoxylin and eosin (HE). Cardiomyocytes cross-sectional area was determined using the method described by Chen et al. [16]. Cardiomyocytes size from each group was evaluated by measuring the cross-sectional area of cells using the image analysis system (CIAS-1000, China Daheng Group, Inc., Beijing, China). Mean cardiomyocytes area was calculated by measuring 100 cells from sections stained with HE.

Measurement of cardiomyocytes hypertrophy
To determine cell hypertrophy, we measured [3H]-leucine incorporation and cardiomyocytes surface area. After digested, centrifuged, and re-suspended in Dulbecco’s modified Eagle medium (DMEM, Gibco, Invitrogen, USA), cells from at least three dishes were counted at each time point, by use of phase-contrast microscopy. To examine the effect of AT1–AAs on cell hypertrophy, non-stretched cultured cardiaomyocytes were treated with AT1–AAs (1 μM) in the presence or absence of either the AT1 antagonist losartan (10 μM, DuPont Merck, Wilmington, USA), or the AT2 antagonist PD 123319 (1 μM, Sigma), or the a1 adrenoceptor antagonist prazosin (10 μM, Sigma).

Protein synthesis was measured by [3H]-leucine incorporation as described previously in [17]. Cardiomyocytes plated on 24-well plates were treated with AT1–AAs in the presence or in the absence of losartan for 24 h. [3H]-leucine (1 mCi/well) was added 4 h before harvest. The radioactivity of incorporated was determined by use of a scintillation counter. Cardiomyocytes surface area was determined by use of image analysis system (CIAS-1000). The cells were randomly selected in two or three dishes for each experimental group. A total of 60 cells were examined in each experimental group.

Statistical analysis
Results were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance. The null hypothesis was rejected when the P-value was <0.05.

Results
AT1 receptor peptides cause increased blood pressure and heart rate in rats
Basal systolic blood pressure (SBP) levels were not significantly different in immunized group and control group before initial immunization. As shown in Fig. 1(A), SBP began to increase 3 weeks after the initial immunization in the immunized group, but the data did not show the statistical significance compared with the baseline [P > 0.05, Fig. 1(A)], while SBP remained unchanged in the control group. At the 4th week, SBP of the immunized group was significantly elevated compared with that before initial immunization (P < 0.01). The maximum value (142.5 ± 4.2 mmHg) of the SBP was at 6 weeks and remained constant until the 4th month. After the 4th month it gradually declined. However, by the 8th month after initial immunization, the value of the SBP of immunized group was still greater than the control group. The heart rate in the control group and immunized group before initial immunization was similar. However, the heart rate in the immunized group was significantly greater than the control group at 3 weeks after initial immunization [Fig. 1(B)].

AT1–AAs production after immunization
After 3 weeks from initial immunization when the blood pressure increased, the titers of AT1–AAs also increased. The titers increased to peak at 14 weeks and then decreased. The controls did not show any significant change (Fig. 2).

Effects of AT1–AAs on plasma Ang II levels
There was no difference for plasma Ang II levels in immunized group (236 ± 105 ng/L, n = 7) and control group (208 ± 95 ng/L, n = 8) 4 months after initial immunization. Plasma Ang II levels of immunized group (308 ± 184 ng/L) 8 months after initial immunization was slightly
higher than that of the control group (246 ± 192 ng/L), but this difference was no significant ($P > 0.05$) (Table 1).

**AT1 receptor peptides cause changes of cardiac tissue weight and histopathology**

There was no significant difference between the original and final BW in the two groups. HW, LVW, HW/BW ratio, and LVW/BW ratio also significantly increased in immunized rats compared with the control rats (Table 2). Compared with those of the control rats, the immunized rats showed significant changes in the LV 8 months after initial immunization. The wall thickness and cavity dimensions of LV were enlarged in the immunized group [Fig. 3(A,B)]. Immunized rats showed a marked and progressive increase in cardiomyocytes size at the 4th and 8th months after initial immunization [Fig. 3(C,D)]. The results of histopathological examination showed that cardiomyocyte cross-sectional area and volume fractions of interstitial fibrosis were both significantly higher in immunized rats than in the control rats 4th and 8th months after initial immunization (Fig. 4). No significant histopathological change was detected in control cardiomyocytes.

**AT1 receptor peptides cause changes of cardiac function**

Along with the elevation of blood pressure in each group, the compensatory increase in cardiac function concomitantly occurred as shown in Table 2. Four months after initial immunization, the LVSP, LVEDP, and maximum ascending and declining rate of left ventricular pressure ($\pm \text{d}p/\text{d}t_{\text{max}}$) were markedly elevated in immunized rats. At the 8th month, the increase of LVEDP and $-\text{d}P/\text{d}t_{\text{max}}$, also was observed in the immunized group, which is an indicator of modified diastolic function (Table 3).

**AT1–AAs induced cultured neonatal rats cardiomyocytes hypertrophy**

Cardiomyocytes were exposed to different concentrations of AT1–AA (10 nM, 100 nM, 1 μM, and 10 μM) to determine the effect of AT1–AA on cardiomyocytes hypertrophy. The results showed that chronic exposure of cultured neonatal rats cardiomyocytes to antibody dose-dependently increased surface area [Fig. 5(A)] and $[^3\text{H}]-\text{leucine incorporation}$ [Fig. 5(B)]. Then we incubate the cardiomyocytes with 1 μM AT1–AAs for 24 h, the levels of both cell surface area and $[^3\text{H}]-\text{leucine incorporation}$ increased by 84.2% ($P < 0.05$) and 64.6% ($P < 0.01$), respectively. This effect could be abolished by losartan (an AT1 receptor antagonist), but not by PD123319 (a AT2 receptor antagonist) (Fig. 6).

**Discussion**

The AT1–AAs were first described in pre-eclampsia by Wallukat et al. [6]. This antibody has also been associated

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**Table 1 Change in plasma Ang II level 4 and 8 months after initial immunization ($n = 6–10$, mean ± SD)**

<table>
<thead>
<tr>
<th>Time (month)</th>
<th>Control group (ng/L)</th>
<th>Immunized group (ng/L)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>196 ± 87</td>
<td>193 ± 85</td>
</tr>
<tr>
<td>4</td>
<td>208 ± 95</td>
<td>236 ± 105</td>
</tr>
<tr>
<td>8</td>
<td>246 ± 192</td>
<td>308 ± 184</td>
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**Table 2 Changes of BW, HW, LVW and their ratio ($n = 6–10$, mean ± SD)**

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>4 months</th>
<th>8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>242.40 ± 12.70</td>
<td>318.70 ± 18.90</td>
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<tr>
<td>HW (g)</td>
<td>7.37 ± 0.17</td>
<td>9.94 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>LVW (g)</td>
<td>5.36 ± 0.15</td>
<td>7.23 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.04 ± 0.09</td>
<td>3.12 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>2.21 ± 0.08</td>
<td>2.26 ± 0.14</td>
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<table>
<thead>
<tr>
<th></th>
<th>Immunized group</th>
<th>4 months</th>
<th>8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>248.50 ± 20.80</td>
<td>305.10 ± 25.60</td>
<td></td>
</tr>
<tr>
<td>HW (g)</td>
<td>8.16 ± 0.27*</td>
<td>11.16 ± 0.32**</td>
<td></td>
</tr>
<tr>
<td>LVW (g)</td>
<td>5.87 ± 0.25*</td>
<td>8.48 ± 0.26**</td>
<td></td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.28 ± 0.16*</td>
<td>3.66 ± 0.21***</td>
<td></td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>2.37 ± 0.18*</td>
<td>2.64 ± 0.20***</td>
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</tbody>
</table>

* $P < 0.05$ vs. control group at the corresponding month; ** $P < 0.05$ vs. 4-month immunized group.
Figure 3 Histologically measured at the 4th and 8th months after initial immunization (A) LV wall thickness; (B) LV cavity area; (C) cardiomyocytes diameter; (D) cardiomyocytes cross-sectional area. Data are shown as mean ± SD, (n = 6–10). *P < 0.05 vs. control group; **P < 0.05 vs. 4-month immunized group.

Figure 4 Representative photomicrographs of cardiomyocyte size stained with HE (×200). (A and B): control group (4 and 8 month); (C and D): immunized group (4 and 8 month).

Table 3 Hemodynamic analysis at the 4th and 8th months after initial immunization (n = 6–10, mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Immunized group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 months</td>
<td>8 months</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>143.50 ± 5.20</td>
<td>146.50 ± 5.04</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>3.85 ± 1.24</td>
<td>3.91 ± 1.08</td>
</tr>
<tr>
<td>+ dp/dt (mmHg/ms)</td>
<td>4.53 ± 0.68</td>
<td>4.61 ± 0.74</td>
</tr>
<tr>
<td>− dp/dt (mmHg/ms)</td>
<td>4.03 ± 0.61</td>
<td>4.12 ± 0.85</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. control group at the corresponding month; **P < 0.05 vs. 4-month immunized group.
with malignant hypertension [9] and renal allograft rejection [10]. Our previous studies have shown that the frequency of occurrence and titers of AT1–AAs increased significantly during the development of hypertension in four different types of hypertensive models [18]. However, the role of AT1–AAs in the pathogenesis of the hypertension and pre-eclampsia is not clear. Therefore, we used a synthetic peptide, corresponding to the second extracellular loop of the human cardiac AT1 receptor, to observed long-lasting effects of antibody on cardiac function and structure. The results showed that the titers of AT1–AAs in immunized rats increased rapidly after 1 month from immunization (Fig. 2). LVSP and \( +\frac{dp}{dt}_{\text{max}} \) of immunized rats were significantly higher than those of control rats at the 4th and 8th months, suggesting AT1–AAs had a AT1 receptor agonist-like effect on the heart. The long-lasting positive effect on hearts may lead to damage in cardiac structure and function. LVSP of immunized rats during the later stage of the experiment increased steadily and significant increase of \( +\frac{dp}{dt}_{\text{max}} \) also prompted cardiac damage. Morphological observation showed that cardiomyopathies changes in cardiac structure not only at the anatomical level, but also at the single cell level, which was observed in all immunized rats but not in control rats. Hemodynamic, structural, and functional changes in the heart induced by AT1–AAs were similar to renal hypertension model rats. These experimental findings suggest that AT1–AAs may be involved in the pathogenesis of the development of hypertension and cardiac hypertrophy.

The previous studies have reported that AT1–AAs act like the natural agonist Ang II and exert a positive chronotropic effect in cultured spontaneously beating rat cardiomyocytes [9] and AT1–AAs stimulate the AT1 receptor mediated system without desensitization [19]. Therefore, in this study, we cultured neonatal rat cardiomyocytes to examine the hypertrophy effects of AT1–AAs extracted from serum of immune rats. The results showed that stimulation with AT1–AAs increased cell volume and protein synthesis, and these effects could be prevented by the selective AT1 receptor antagonist losartan (Fig. 6). Consistent with previous studies [6,9], AT1–AAs exerted their agonist-like effect via AT1 receptors, and stimulation of the AT1 receptor led to the functional and structural changes, including cardiac hypertrophy and remodeling.
We hypothesized that antibodies may be found in patients and in experimental animals with cardiac structural changes. The findings of our study, consistent with previous reports [9,20], showed that the α1 adrenoceptor antagonist and the AT2 receptor antagonist could not block access of AT1–AAs to the AT1 receptor.

Fu et al. [20] have reported that the changes of blood pressure and cardiac structure were not observed in rats immunized by AT1 receptor peptides. But Sun et al. [21] have reported that the AT1–AAs have an agonist effect similar to Ang II in proliferation of vascular smooth muscle cells. Comparing with our present data, we propose that less time of immunization, long-time interval and short observation period may contribute to negative results in Fu’s experiments. Thus, we used AT1 receptor peptide as an antigen to immunize rats once bi-weeks for 4 months, with a longer-term observation period up to 8 months. Our experimental results showed that AT1–AAs not only can induce harmful changes in the structure and function of the heart, but also cause of hypertrophy of cultured neonatal rat cardio myocytes. The exact mechanism of AT1–AAs action is not clear. Therefore, the function of antibody on the biological processes involved in the development of cardiovascular diseases will be studied in the future, especially the molecular mechanisms of signal transduction is an important focus point.

Funding

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