Angiotensin II type I receptor agonistic autoantibody-induced apoptosis in neonatal rat cardiomyocytes is dependent on the generation of tumor necrosis factor-α

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Angiotensin II type I receptor agonistic autoantibodies (AT1-AA) are related to pre-eclampsia and hypertension and have a direct effect of stimulating the production of tumor necrosis factor-alpha (TNF-α) in the placenta. TNF-α is a known mediator of apoptosis. However, few studies have reported the role of TNF-α and its relationship within AT1-AA-induced apoptosis of cardiomyocytes. In this study, neonatal rat cardiomyocytes were treated with various concentrations of AT1-AA. The apoptosis of neonatal rat cardiomyocytes was determined using TUNEL assay and flow cytometry. The level of secreted TNF-α was measured by enzyme-linked immunosorbent assay, and caspase-3 activity was measured by a fluorogenic protease assay kit. AT1 receptor blockade and TNF inhibitor were added to determine whether they could inhibit the apoptotic effect of AT1-AA. Results showed that AT1-AA induced the apoptosis of neonatal rat cardiomyocytes in a dose-dependent and time-dependent manner. AT1-AA increased TNF secretion and caspase-3 activities. AT1 receptor blockade completely abrogated AT1-AA-induced TNF-α secretion, caspase-3 activation, and cardiomyocyte apoptosis. TNF-α receptor inhibitor significantly attenuated AT1-AA-induced neonatal rat cardiomyocyte apoptosis. AT1-AA in the plasma of pre-eclamptic patients promoted neonatal rat cardiomyocyte apoptosis through a TNF-caspase signaling pathway.

Keywords angiotensin II type I receptor agonistic autoantibodies (AT1-AA); apoptosis; tumor necrosis factor-alpha; neonatal rat cardiomyocytes

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Introduction

Cardiovascular disease is a very common disease and is the leading cause of death and disability in the world. Each year, ~3 million people died of cardiovascular disease in China, accounting for about 20% of the world’s total deaths related to cardiovascular disease. Epidemiological investigations and animal experiments have shown a clear association between an adverse intrauterine environment and an increased risk of cardiovascular disease and hypertension in adult life [1]. This phenomenon is called fetal programming, or the fetal origins of adult diseases as described by Barker [2].

Pre-eclampsia is a systemic syndrome of pregnancy clinically characterized by new onset of proteinuria and hypertension. It is a major cause of fetal/neonatal morbidity and mortality, and is believed to be very strongly associated with fetal growth retardation [3]. Some studies indicated that the effect of pre-eclampsia may persist long after pregnancy and the offspring may have an increased risk for cardiovascular disease in adulthood [4,5]. It was also found that the offspring of pre-eclamptic women may have severe cardiac defects at birth, including increased cardiomyocyte hypertrophy and heart/body weight index, and elevated blood pressure in childhood and adolescence as well [6]. Further studies have revealed that the major cause of fetal demise and the occurrence of adult cardiovascular disease was cardiomyocyte apoptosis during the fetal period [7,8], although the exact mechanism remains unclear.

The renin–angiotensin system (RAS) plays a pivotal role in many cardiovascular diseases. Angiotensin II (Ang II) is the effector peptide of the RAS and mediates most of its
relevant biological effects via AT1 receptor activation. Many recent studies have indicated that Ang II type I receptor agonistic autoantibody (AT1-AA) is an additional risk factor associated with an increased incidence of pre-eclampsia [9–13]. AT1-AA exhibits an agonist-like activity similar to Ang II which has a stimulatory positive chronotropic effect [13], and stimulates the synthesis and release of tumor necrosis factor-alpha (TNF-α) by activating AT1 receptors, which is directly or indirectly involved in the pathogenesis of pre-eclampsia [10]. TNF-α is a prototypical inflammatory cytokine and is known as a mediator to induce apoptosis via activation of TNFRs [14]. As Ang II is a powerful inducer of cardiomyocyte apoptosis, it is closely related to TNF-α and its receptors [15]. AT1-AA belongs to IgG class immunoglobulin [16], and may pass through the placenta and enter fetal circulation. So we hypothesize that TNF-α is the downstream protein and plays a pivotal role in AT1-AA-induced apoptosis of fetal cardiomyocytes.

In this study, we attempted to clarify the mechanism by which AT1-AA induces neonatal rat cardiomyocyte apoptosis, and to see whether the pro-/anti-apoptotic effects of AT1-AA are related to the increase of TNF-α release from cardiomyocytes.

Materials and Methods

Preparation of IgG
Ten pre-eclamptic women were selected according to the previously described criteria of the International Society for the Study of Hypertension in Pregnancy and were prospectively matched with 10 normal pregnant women as control. The study was approved by the Ethics Committee of Shanghai Ninth People’s Hospital affiliated to Shanghai Jiao Tong University, and written informed consent forms were signed by all subjects. Total serum IgG from pre-eclamptic patients and control patients was purified with a MAbTrapTM Kit (Amersham, Little Chalfont, UK) according to the manufacturer’s instructions. AT1-AA activity was detected by enzyme-linked immunosorbent assay (ELISA) as described previously [17].

Isolation of neonatal rat cardiomyocytes and cell culture
Primary culture of neonatal rat cardiomyocytes was performed as described previously [18]. In brief, the neonatal rat heart was removed within 30 min after birth. Cardiomyocytes were dispersed by digestion with 0.08% trypsin and agitation for 10 min at 37°C. This digestion step was repeated six times, and cells were collected by centrifugation. Isolated cells were re-plated into a 100-mm petri dish for 2 h in Dulbecco’s modified Eagle’s medium (DMEM); ( Gibco-BRL, Bethesda, USA) with 10% fetal calf serum ( Gibco-BRL) in a 37°C incubator to reduce fibroblast contamination. Nonattached viable cells were collected and seeded into a flask or culture plate in DMEM supplemented with 10% fetal bovine serum (FBS) for 6 h, followed by incubation in 10% FBS and 10 µM cytosine arabinoside, which preferentially reduced the proportion of nonmyocytes. The flask or plate was placed in a humid cell incubator at 37°C with a mixed gas containing 5% CO2.

Myocytes were cultured with cytosine arabinoside for 24 h before treatment with AT1-AA in the presence or absence of losartan, and then DMEM with 10% FBS was replaced by DMEM with 1% FBS. In the subsequent signal transduction experiment, AT1 receptor blocker losartan (1 µM) and TNF inhibitor etanercept (1 µM) were added 6 h prior to the treatment with AT1-AA.

Determination of the apoptotic index by TUNEL assay
The TUNEL assay was performed on cells plated on glass coverslips with an in situ cell death detection kit (Roche, Philadelphia, PA, USA). The cells were washed with phosphate buffered saline (PBS) and fixed for 30 min with 4% paraformaldehyde. TUNEL reaction was performed according to the manufacturer’s instructions. For quantitative analysis, the apoptotic index was calculated by the number of TUNEL-positive cells in five different visual fields (∼200 cells/field).

Flow cytometry
Apoptotic neonatal myocytes were quantified by flow cytometric analysis as described previously [19]. In brief, ∼5000 cells per sample were measured. Floating and trypan-stained adherent cells were collected, washed with PBS, and fixed in ice-cold 70% ethanol overnight at 4°C. After fixation, cells were washed twice with PBS and stained with DNA-specific fluorochrome propidium iodide (PI) (50 µg/ml) containing 10 µg/ml RNase. After 30-min gentle agitation at room temperature, cells were analyzed by a FACS 440 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Red fluorescence was detected through a 563–607-nm bandpass filter.

Caspase activity assay
Caspase-3 or caspase-8 activities were measured by using a caspase-3 or caspase-8 colorimetric assay kits ( Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s recommendations. In brief, the cells were treated for 48 h with 0.01–10 µM of AT1-AA. The cell lysates (100 mg of protein) were mixed with the reaction buffer and 200 mM caspase-3 substrate DEVD-pNA or caspase-8 substrate IETD-pNA and incubated at 37°C for 4 h. Optical density was measured at 405 nm.
TNF measurement
Primarily cultured neonatal rat cardiomyocytes were treated with or without AT1-AA for the indicated time. After incubation, the medium was collected and TNF levels were quantitated according to the manufacturer’s instructions and guidelines using ELISA kit specific for rats (Shanghai Heng Yuan Biotechnology Co., Ltd, Shanghai, China).

Statistical analysis
Results are expressed as mean ± SEM. Data were analyzed using SigmaStat statistical software. For each experiment, statistical treatment included a one-way analysis of variance followed by a Student–Newman–Keuls test for pairwise comparison. A value of $P < 0.05$ was considered statistically significant.

Results
AT1-AA induces cardiomyocyte apoptosis in a dose-dependent and time-dependent manner
To study the dose–response effect of AT1-AA on cardiomyocyte apoptosis, neonatal rat cardiomyocytes were exposed to various concentrations of AT1-AA, and apoptosis was estimated 48 h after culture by TUNEL assay [Fig. 1(A)]. The results showed that the pro-apoptotic effect of AT1-AA on cardiomyocyte viability was gradually enhanced between 0.1 and 10 μM, and AT1-AA at 1 μM appeared to have the strongest action. The concentration of 1 μM was therefore chosen for AT1-AA for the subsequent experiments.

To study the time-dependent cell apoptosis by AT1-AA treatment, the effect of AT1-AA (1 μM) at different incubation times (12, 24, 36, and 48 h) was observed. It was found that AT1-AA exerted the most significant action in inducing cell apoptosis at an incubation time of 48 h [Fig. 1(B)]. Therefore, the data obtained from 1 μM AT1-AA and 48 h of incubation were presented in this study.

AT1-AA induces TNF-α secretion from cardiomyocytes
Previous studies reported that AT1-AA could directly stimulate TNF-α production in the placenta, and TNF-α-mediated apoptosis was increased due to AT1-receptor activation in both mouse placenta and human villous explants [10]. To determine whether the production of TNF was regulated by AT1-AA in cardiomyocytes, primarily cultured cells were
exposed to a series of concentrations (0.01, 0.1, 1, and 10 μM) of AT1-AA for 6 h. Then, the culture media were collected to measure the levels of secreted TNF by ELISA assay. Data showed that TNF-α production in cardiomyocytes increased in a dose-dependent manner in response to AT1-AA. AT1-AA significantly increased TNF-α production in cardiomyocytes [Fig. 2(A)]. The time course of TNF-α production in cardiomyocytes treated with AT1-AA was slightly different. TNF-α bioactivity in the supernatant reached the peak level at the 6 h time point, decreased thereafter, and finally returned to the baseline level at the 16 h time point [Fig. 2(B)].

**Activation of caspase-3 and caspase-8 are required for AT1-AA-induced apoptosis**

Previous studies have found that caspases were activated by Ang II through AT1 receptor in cardiomyocytes [20]. To confirm whether caspase-3 and caspase-8 could mediate AT1-AA-induced apoptosis of cardiomyocytes, caspase-3 and caspase-8 activities were detected in the culture medium. As shown in Fig. 3A, AT1-AA stimulated the promotion of serum-deprived caspase-3 and caspase-8 activation in cardiomyocytes in a dose-dependent manner. AT1-AA (1 μM) stimulated the promotion of caspase-3 and caspase-8 activity to ~305% and 252% compared with the control (P < 0.05) [Fig. 3(A)]. AT1 receptor blocker losartan and soluble TNF-α inhibitor etanercept completely abolished the promoting effect of AT1-AA on caspase-3 and caspase-8 activation [Fig. 3(B)]. These data suggested that AT1-AA stimulated the promotion of caspase-3 caspase-8 activity and exerted its pro-apoptotic effect through AT1 receptor and TNF-α receptor.

**AT1-AA increases the percentage of apoptotic cells**

To confirm whether AT1-AA induced cardiomyocyte apoptosis, the percentage of apoptosis was calculated by the percentage of cells containing subdiploid quantities of DNA as revealed by PI staining with a flow cytometer. The experimental results showed that 4.4 ± 1.4% of the control cells had hypodiploid DNA content typical of apoptosis. Treatment with AT1-AA (1 μM) for 24 h increased the percentage of apoptotic cells to 24.7 ± 5.3% (P < 0.05 vs. control group, n = 6), and this effect was reversed with losartan (10 μM) and etanercept (1 μM) [Fig. 4E]. Neither losartan nor etanercept alone, i.e. in the absence of AT1-AA, had any effect on cardiomyocyte apoptosis (data not shown).

**Discussion**

The RAS plays an important role in normal pregnancy and pre-eclampsia, but changes in RAS components are different between them. Normal pregnancy is associated with increased activation of RAS components and reduced vascular responsiveness to Ang II, while pre-eclampsia is associated with suppressed RAS components and enhanced vascular responsiveness to Ang II. In 1999, Wallukat et al. [16] reported their remarkable finding that sera from women with pre-eclampsia contained AT1-AA. Previous studies have reported that AT1-AA acted like the natural agonist Ang II which exerts a positive chronotropic effect in cultured spontaneously beating rat cardiomyocytes, and stimulated the AT1 receptor-mediated system without desensitization [16]. Recently, Wenzel et al. [21] reported that AT1-AA could increase vascular sensitivity to Ang II in pregnant rats. The biological properties of AT1-AA could be blocked by losartan (an AT1 receptor antagonist) and AFHYESQ peptide (corresponding to a specific epitope associated with the second extracellular loop of the AT1 receptor, 181–187) [9]. Dechend et al. [22] reported that sera from pre-eclamptic women contained an IgG (type 3) autoantibody that reacted with the AT1 receptor.
During the past decade, many studies have confirmed that AT1-AA activates AT1 receptors on a variety of cell types and provokes biological responses that are relevant to the pathophysiology of pre-eclampsia [22]. AT1-AA arises approximately at the time when symptoms develop after about 20 weeks of gestation, and subsides within 6 weeks after delivery. It was also found that treatment of gestation day 13 pregnant mice with IgG from pre-eclamptic patients containing AT1-AA produced symptoms similar to pre-eclampsia, including elevated blood pressure, proteinuria, and fetal growth retardation [12].

Cardiomyocyte apoptosis during the fetal and early postnatal periods may play a central role in determining the number of myocytes in the adult heart. Previous in vitro studies suggested that stimulating activation of AT1 receptors had diverse effects on the induction of apoptosis of neonatal rat cardiomyocytes [20]. Ang II, at a concentration of 0.1 μM, induced apoptosis in neonatal rat cardiomyocytes by 15%–25%. Wallukat et al. [16] suggested that AT1-AA displayed an ‘agonist-like’ activity without desensitization, and had a longer effect, which is very different from the natural agonist of AT1 receptor. It is known that excessive stimulation using Ang II leads to apoptosis via AT1 receptor. To further confirm whether overstimulation of AT1-AA could exactly induce myocardial apoptosis, we used cultured neonatal cardiomyocytes as the model to see whether overstimulation of AT1-AA could induce fetal rat cardiomyocyte apoptosis. The results of our study showed that AT1-AA, at the concentration of 0.01, 0.1, 1, and 10 μM, caused 7.2 ± 2.6%, 13.5 ± 3.2%, 24.6 ± 4.8%, and 27.5 ± 5.8% apoptosis in cultured neonatal cardiomyocytes, respectively, which could be attenuated by AT1 receptor blocker losartan.

TNF-α is a multifunctional cytokine that plays an important role in inflammation and immunity, as well as in the control of cell proliferation, differentiation and apoptosis. Several studies have demonstrated that there is a close relationship between the RAS and TNF-α [15], for instance, Ang II treatment induced the production of TNF-α in cultured cardiomyocytes and fibroblasts. Administration of the AT1 receptor antagonist significantly lowered the plasma TNF-α concentration in patients with hypertension or heart failure. Kalra et al. [23] reported that Ang II induced TNF-α biosynthesis in the adult mammalian heart through a protein kinase C -dependent pathway. Irani et al. [10] confirmed that the effect of AT1-AA on pathophysiology of pre-eclampsia was mediated by a paracrine pathway by increasing synthesis and release of TNF-α via AT1 receptor activation. This paracrine release of TNF-α was correlated with the pathogenetic condition of pre-eclampsia. Zhou et al. [12] confirmed that AT1-AA-mediated TNF-α induction was responsible for both sEng and sFlt-1 induction and contributed to the decreased placental angiogenesis, suggesting that increased TNF-α may contribute to the pathogenesis of pre-eclampsia. These implications are strongly supported by recent studies demonstrating that TNF-α is an important factor contributing to pathological changes seen in an experimental model of pre-eclampsia in rats based on reduced uterine perfusion pressure [10].

TNF-α has been implicated in many pathogenic conditions including cardiovascular injury and disease states. The chronic overexpression of TNF-α may be involved in

Figure 4 Flow cytometric analysis of DNA contents in different groups
Cardiomyocytes were exposed to 1 μM AT1-AA for 24 h, stained with PI, and sorted for DNA content by fluorescence-activated flow cytometry. (A–D) Showed the DNA profiles of control cells (A), AT1-AA-treated cells (B), cells treated with AT1-AA and losartan or etanercept (C, D). (E) Percentage of apoptotic cells (%). aP < 0.01 vs. control; bP < 0.01 vs. AT1-AA.
the promotion of apoptosis and many other pathological processes induced by Ang II [15,24]. Since AT1 receptor can be involved in TNF-α production, we have investigated whether early treatment with AT1 receptor blocker losartan could limit TNF-α production within cardiomyocytes.

Our study has shown that AT1-AA treatment altered TNF-α expression in a time-dependent manner: it was first detected at 2 h, rapidly increased between 2–6 h, reached the peak at 6 h, and then restored to the control level after 16 h. We speculated that AT1-AA may promote AT1 receptor internalization; cardiomyocytes may induce the degradation of AT1-AA, and TNF-α rapid degradation may contribute to rapid decline of TNF-α levels in culture medium. Apoptosis is a programmed process, once apoptosis starts, it proceeds rapidly. Cells undergoing apoptosis may completely disappear in a short time. It is well established that TNF-α can induce apoptosis in a variety of cell types. Shen et al. [25] found that addition of high-dose TNF-α into the culture medium markedly increased the activation of caspase-3 in a time-dependent manner, and induced apoptosis of the cultured neonatal rat cardiomyocytes, which began at 6 h and reached the peak at 24 h after exposure. The results of our experiment showed that after treatment with AT1-AA for 6 h, the TNF-α level in the culture medium was 24 folds as high as that in the control group. Thus, we believe that the significantly increased level of TNF-α was involved in neonatal rat cardiomyocytes apoptosis induced by AT1-AA, although the high level of TNF-α only lasted for several hours from 4 to 12 h. In addition, our findings suggested that TNF inhibitor etanercept could significantly abrogate the apoptotic effect of AT1-AA on neonatal rat cardiomyocytes.

Previous studies have confirmed that TNF-α-induced apoptosis is through multiple signaling pathways [24]. Caspase activation is a cellular event associated with the onset of apoptosis-induced TNF-α. Caspase-3 activation via TNF family receptors (e.g. Fas), Fas-activated death domain protein and caspase-8 represents the extrinsic pathway. Therefore, we also investigated whether caspase-8 and caspase-3 activation was an important mechanism underlying AT1-AA-induced cardiomyocyte apoptosis. Our findings suggested that etanercept pre-treatment reduced not only apoptosis but caspase-3 and caspase-8 activity induced by AT1-AA in cultured neonatal rat cardiomyocytes, indicating that TNF-α may play a central role in apoptosis induced by AT1-AA. Therefore, we hypothesized that pre-eclampsia pregnancy could induce fetal cardiomyocyte apoptosis, and increase the expression of apoptosis-related proteins such as TNF-α. Treatment of pre-eclampsia with AT1 receptor antagonists has a protective effect on the apoptosis of fetal cardiomyocytes.

In conclusion, our results indicated that AT1-AA could induce the apoptosis of cultured cardiomyocytes by hyperstimulating AT1 receptor. In addition, the release of TNF-α from cardiomyocytes and its subsequent activation of caspase-8 and caspase-3 play a key role in AT1-AA-induced apoptosis. Losartan and etanercept can block the cardiomyocyte apoptosis induced by AT1-AA very effectively, suggesting that AT1 receptor blocker and TNF inhibitor therapy for pre-eclampsia may prevent fetal cardiomyocyte from apoptosis.

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References