Angiotensin II type 1 receptor autoantibody as a novel regulator of aldosterone independent of preeclampsia

Jie Yang\(^a\), Li Li\(^b\), Jian-Yu Shang\(^a\), Lin Cai\(^c\), Li Song\(^a\), Su-Li Zhang\(^a\), Hao Li\(^a\), Xiao Li\(^a\), Wayne Bond Lau\(^b\), Xin-Liang Ma\(^d\,\(^e\), and Hui-Rong Liu\(^a\,\(^e\,\(^f\)

**Background:** Preeclamptic women and their infants have significant morbidity and mortality worldwide. Abnormal aldosterone signaling is involved in the pathogenesis of preeclampsia, and the presence of agonistic autoantibodies against the angiotensin II type 1 receptor (AT\(_1\)-AA) during disease has been observed. The role of AT\(_1\)-AA in aldosterone generation with or without disease and the long-term impact of AT\(_1\)-AA circulation in blood remain unclear.

**Method:** We therefore assessed circulating AT\(_1\)-AA and aldosterone levels in 76 patients with preeclampsia (35 severe and 41 mild), 26 patients with gestational hypertension, and 50 normotensive healthy pregnant women.

**Results:** First, the correlation of AT\(_1\)-AA levels was confirmed for preeclamptic patients. We report here that all AT\(_1\)-AA-positive hypertensive pregnant women exhibited decreased aldosterone levels, and early-onset preeclampsia patients with high proteinuria showed an inverse correlation of aldosterone levels with AT\(_1\)-AA. To study this effect in more detail, we confirmed that AT\(_1\)-AA decreased aldosterone levels in pregnant rats and then demonstrated that aldosterone levels decreased in response to the chronic administration of AT\(_1\)-AA into nonpregnant rats.

**Conclusion:** These results suggested that AT\(_1\)-AA regulates levels of aldosterone, which was tested with cell culture studies, revealing that activation of AT\(_1\) receptors by AT\(_1\)-AA directly led to abnormal aldosterone generation in a time and dose-dependent manner. We present here a mechanism for regulation of aldosterone production: AT\(_1\)-AA activates AT\(_1\) receptors on adrenocortical cells independent of pregnancy, in a time and dose-dependent manner.

**Keywords:** aldosterone, angiotensin II type 1 receptor, autoantibodies, preeclampsia

**Abbreviations:** ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ALD, aldosterone; ANG II, angiotensin II; AT\(_1\)-AA, agonistic autoantibodies against the angiotensin II type 1 receptor; AT\(_1\)R, angiotensin II type 1 receptor; IHC, immunohistochemical; nsIgG, nonspecific immunoglobulin; OGTT, oral glucose tolerance test; PRA, plasma renin activity; sFlt-1, soluble Fms-like tyrosine kinase-1; TUNEL, terminal deoxynucleotidyl nick-end labeling

**INTRODUCTION**

Preeclampsia, characterized by new-onset hypertension and proteinuria, affects 5–7% of worldwide pregnancies, causing significant maternal and infantile morbidity and mortality [1]. Preeclampsia appearing after the 20th week of gestation is classified into early (<34 weeks) or late (>34 weeks) type, dependent upon the onset time of clinical manifestation and causative mechanisms [2]. Shallow trophoblast invasion with subsequent placental ischemia and hypertension is thought to be a primary inciting event of preeclampsia [2]. However, there may be other critical events. Accumulating evidence has suggested that agonistic autoantibodies against the angiotensin II type 1 receptor (AT\(_1\)-AA) have a role in preeclampsia. The presence of autoantibody can result in pathophysiological alterations related to preeclampsia, including shallow trophoblast invasion, human placental blood vasculature constriction, mobilization of intracellular Ca\(^{2+}\), impaired uterine perfusion, increased soluble Fms-like tyrosine kinase-1 (sFlt-1) [3], and, as we show here, may be a cause of preeclampsia. A recent study has shown that the transfer of purified AT\(_1\)-AA from preeclamptic women into pregnant mice contributes to hypertension and proteinuria of the mice, both of which are hallmark features of preeclampsia [4]. Significantly, AT\(_1\)-AA is detectable before the 20th week of gestation in women with abnormal uterine perfusion [5] and...
Yang et al.

persists for 18 months after delivery in women with pre-eclampsia [6]. Furthermore, AT1-AA, not restricted to pregnancy, remains detectable for an extended period in renal transplant patients with severe vascular rejection or/and malignant hypertension [7]. However, the impact of long-term circulation of AT1-AA in nonpregnancy remains to be determined.

In normal human pregnancy, there is an increase in cardiac output and an enlargement of circulatory volume with decreased peripheral vascular resistance [8]. A decreased level of aldosterone (ALD), one of the most important hemodynamic-regulatory hormones for plasma volume, and the resulting inadequate plasma volume expansion during pregnancy are involved in preeclampsia. Impaired ALD synthesis precedes manifestation of clinical preeclampsia [9], correlates with preeclampsia severity, and restricts circulatory volume expansion, resulting in increased blood pressure and protein excretion [10]. However, effects of AT1-AA on ALD production, independent of pregnancy, especially in terms of the impact of long-term circulation of AT1-AA, were not examined in the study, and the correlation of AT1-AA to ALD in preeclampsia patients remains unknown. The aim of the current study was to address the impact of AT1-AA on ALD production, independent of pregnancy, improving and building the working model underlying AT1-AA-regulated ALD production under both conditions. We demonstrate that AT1-AA-mediated ALD secretion is a time and dose-dependent process in which long-term presence of AT1-AA at a high level damages ALD production via activation of AT1R on the adrenocortical cells, yet with an increase in ALD upon short-term exposure.

METHODS

In addition to the methods described here, an expanded ‘Methods’ section is available as Supplementary Methods (http://links.lww.com/HJH/A462).

Patients

The study protocol was approved by the Institutional Committee for the Protection of Human Subjects of The Third People’s Hospital of Chengdu and conformed to the Declaration of Helsinki. The patients were enrolled in the study between November 2010 and 2012. As per the function \( n = 2P \times \frac{Q \times U_a + U_g^2}{(p_t - p_b)^2} \), where \( p_t = p_a \times \frac{P}{1 + p_a (OR - 1)} \), \( P = 0.5, Q = 1 - P \), where \( U_{al} = (0.05) \) and \( U_{g} = 0.1 \) are 1.960 and 1.282, respectively, the minimum sample size for the case-control study should be either 33 [the odds ratio (OR) is 16.04] [3] or 18 (OR is 7.74 [5]). All patients and 50 normotensive pregnant women characterized by an uncomplicated, normal-term delivery (total 152 enrolled women) were informed of the study’s purpose and protocol. Participants’ written consent was obtained. Preeclamptic women were diagnosed with severe preeclampsia, mild preeclampsia, and gestational hypertension on the basis of criteria set by the International Society for the Study of Hypertension in Pregnancy (ISSHP) [12] and the US National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [13]. Patients were excluded from the study for comorbidities of pre-existing hypertension, autoimmunity, diabetes, endocrine dysfunction, cancer, chronic wasting diseases, renal injury, or implant. The diagnosis of gestational diabetes mellitus was made when two or more values exceeded the criteria of Carpenter and Coustan (8 mmol/l) using the 100-g, 3-h oral glucose tolerance test (OGTT).

Blood samples for the study were obtained shortly after diagnosis and allowed to clot before centrifugation at 3000 r.p.m. for 15 min, and then stored at −80°C. Table 1 lists general clinical patient data.

Determination of aldosterone, renin, angiotensin II, and AT1-AA

Commercially available radioimmunoassay kits (Beijing North Institute of Biological Technology, Beijing, China) were utilized for determining plasma ALD, Ang II concentrations, and plasma renin activity (PRA) as per manufacturer’s protocols. AT1-AA levels were measured by streptavidin-ELISA as reported previously [4,14–16]. Briefly, a 96-well plate was coated with the synthetic peptide HRNVFFIINTNIVCAFHYESQNSTL corresponding to residues 165–191 of the second extracellular loop of human AT1R, and was incubated overnight at 4°C. After blocking, 50 μl of sample serum per well was added (final dilution 1 : 40 for clinical samples of patient serum, or 1 : 10 for in-vitro samples utilizing rat serum) to the plates and incubated at 37°C for 90 min, followed by washing, addition of biotinylated goat antihuman IgG (1 : 1000; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), streptavidin-horseradish peroxidase (1 : 2000; Santa Cruz Biotechnology, Inc.), and finally the substrate 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma-Aldrich Inc., St Louis, Missouri, USA). The optical density (OD) values were measured at 405 nm by ELISA (Spectra Max Plus, Molecular Devices, Sunnyvale, California, USA).

Discrimination of AT1-AA positive and negative group

The cut-off value distinguishing positive and negative samples was determined using the BIOREBA AG protocol [17], by using the formula: Cut-off = (Mean of the mean values up to the ‘step’ + 3SD) × 1.1. Briefly, the means of the two readings of each sample were sorted in ascending order in a histogram in which data classified as the background were presented as a slight linear increase of OD values. But a clear increment called ‘step’ distinguished potential positive samples from the preceding background values. The calculation of the cut-off indicated that the next values on the right side of the ‘step’ were regarded as positive serum samples. The determinations of ‘step’ and the cut-off value had to be done for each processed plate.

Isolation and purification of AT1-AA from preeclamptic women and rats

Two steps isolated AT1-AA from the serum of preeclamptic patients or autoantibody-positive rats. Firstly, immunoglobulin G (IgG) from either serum of preeclamptic patients or
Autoantibody and aldosterone

| TABLE 1. Clinical features of patients enrolled in the study |
|---------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Patient group | Normotensive pregnant women (NT, n = 50) | Gestational hypertension (GH, n = 26) | Mild preeclampsia (mild PE, n = 41) | Severe preeclampsia (severe PE, n = 35) | P value |
| Age (years)   | 29 ± 3.2         | 30 ± 3.1         | 28 ± 3           | 29 ± 2.98        | NS               |
| Height (cm)   | 158.0 ± 0.5     | 156.0 ± 1.1     | 156.1 ± 2.3     | 157.3 ± 0.9     | NS               |
| Weight at term (kg) | 67.8 ± 0.5 | 66.3 ± 1.3 | 67.5 ± 0.6 | 66.0 ± 1.8 | NS           |
| Gestation weeks at sampling | 31 ± 3         | 32 ± 2          | 31 ± 1          | 31 ± 2          | NS               |
| Gestation weeks when delivery (mean) | 39.6         | 38.7            | 38.4            | 35.4            | <0.001            |
| Delivery (n) | 0/1             | 0/1             | 0/1             | 0/1             | NS               |
| Systolic pressure (mmHg) | 122 ± 7        | 136.03 ± 8     | 145.2 ± 6       | 160.8 ± 5       | <0.001            |
| Diastolic pressure (mmHg) | 73 ± 5         | 99.3 ± 7       | 100.2 ± 7       | 106.5 ± 6       | <0.001            |
| 24-h urinary protein (g/24h) | <0.1          | 0.208 ± 0.07   | 0.576 ± 0.02    | 7.03 ± 1.6      | <0.001            |
| Gestational diabetes mellitus (GDM) [n (%)] | 0             | 1 (3.8)         | 2 (4.9)         | 3 (8.6)         | <0.001            |
| Smoking [n (%)] | 0             | 0               | 1 (2.4)         | 0               | NS               |

Values are expressed as mean ± SD. *P < 0.001 vs. normotensive pregnant women.

rats was obtained via antihuman IgG or antirat IgG column as per manufacturer protocols (MaBTrap Kit; Amersham, Piscataway, New Jersey, USA). Secondly, AT1-AA was purified from total human IgG or rat IgG by the peptide corresponding to second extracellular loop of human AT1R (AT1R-EC II) sharing significant sequence homology of 92.6% with the corresponding domain of rat AT1R, linked to Sepharose 4B CNBr-activated gel [18]. Unbound IgG was washed and used as nonspecific IgG (nsIgG) control group. Bounded IgG was eluted and tested for biological activity by the responses to AT1R-mediated constriction of thoracic aorta in vitro (see Supplementary Fig. S3, http://links.lww.com/HJH/A462), as reported previously [19]. Rats actively immunized with sequence of human AT1R-EC II were used as the source of rat AT1-AA as previously described [19,20].

Chronic administration of rat AT1-AA into nonpregnant rats

All studies were performed upon female Wistar rats housed in a temperature-controlled room (25°C) with a 12 h:12 h light:dark condition (source of rats). All experimental procedures were in accordance with the National Institute of Health Guidelines for experimental use and care for animals. All protocols were approved by the Institute of Animal Care and Use center in Capital Medical University. Purified rat AT1-AA (8 μg/g, Supplementary Fig. S1, http://links.lww.com/HJH/A462), was resuspended in saline or nsIgG and introduced into nonpregnant rats via tail vein injection (n = 8, per group) [19,20]. A booster injection was applied every 14 days (a number derived from the AT1-AA half-life, see Supplementary Fig. S2, http://links.lww.com/HJH/A462). To minimize the effect of circadian rhythm on hormone measurements, all serum and plasma were collected between 0900 and 1100 h in resting condition, 1 day prior to the booster injection.

Histopathology and immunohistochemistry

Tissue samples were collected, fixed in 10% (v/v) formalin, embedded in paraffin, and processed for immunohistochemical (IHC) staining as per standard methods [4]. AT1-AA was detected by horseradish peroxidase-conjugated rabbit antibody to rat IgG (1:200, Santa Cruz Biotechnology, Inc.).

Cell culture and treatments

NCI-H295R human adrenocortical carcinoma cells were cultured in DMEM/Ham's F12 (1:1) medium and supplemented with 5% fetal calf serum (FBS), insulin-transferrin-selenium A, streptomycin (100 μg/ml), and penicillin (100 U/ml) (all from BD Bioscience, Bedford, Massachusetts, USA). Cells were maintained in a 37°C humidified atmosphere (5% CO2) and subcultured onto 24-well dishes at 1×104 cells/well for subsequent treatments, including cellular viability assay using Cell Counting Kit-8 (Dojindo Molecular Technology, Japan); determination of cellular injury by lactate dehydrogenase (LDH) release (Supplementary Methods, http://links.lww.com/HJH/A462); and cellular apoptosis assays via caspase-3 activity, the measurement of which were shown as fold-change (the ratio between an initial and final value) (CaspACE Assay System; Promega, Madison, Wisconsin, USA). To assay cell viability, representative photomicrographs were recorded of cells stained with terminal deoxynucleotidyl nick-end labeling (TUNEL) (Deadend fluorometric TUNEL System; Promega) [21]. The culture medium was assayed for ALD by radio-immunoassay as described previously [21]; measurements were normalized via protein concentration of cell lysates and shown as fold-change. Prior to treatments, cells were maintained overnight in low-serum (1–2% FBS) complete medium. Measurements to determine LDH leakage were expressed as the change in absorption per minute (K, 10−3). All measurements were performed in triplicate.

Statistical analysis

Results are expressed as mean ± SEM or mean ± SD. All data were subjected to statistical analysis by SPSS version 17.0 (SPSS Inc., Chicago, Illinois, USA). The chi-square test or the Fisher’s exact test was used to analyze categorical variables. One-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test and the Mann–Whitney test, was used to compare the mean or median of different groups. Correlation and regression analysis were performed to explore the relativity between two variables. P values less than 0.05 were considered to be statistically significant.
Yang et al.

**RESULT**

**AT**<sub>1</sub>-**AA levels are significantly greater in early-onset preeclamptic patients**

To investigate whether AT<sub>1</sub>-AA is more elevated in early-onset vs. late-onset preeclampsia, blood samples were collected from pregnant women and serum were analyzed for AT<sub>1</sub>-AA levels. Results of our study confirmed that patients with aberrantly elevated blood pressure had substantially increased AT<sub>1</sub>-AA levels compared to normotensive pregnant patients (0.669 ± 0.133 vs. 0.315 ± 0.093; P < 0.001), and significantly greater levels of AT<sub>1</sub>-AA in severe preeclamptic patients compared to either mild preeclamptic patients (0.780 ± 0.104 vs. 0.598 ± 0.102; P < 0.05) or women with gestational hypertension (0.632 ± 0.116; P < 0.05, Fig. 1a), which is in good agreement with a previous study [14]. In addition, we showed that AT<sub>1</sub>-AA levels were significantly greater in early-onset compared to late-onset preeclampsia (0.703 ± 0.132 vs. 0.567 ± 0.111; P < 0.05, Fig. 1b). As shown previously, for a small cohort of severe preeclampsia patients [22], we observed that all preeclamptic women in our larger study (mild and severe preeclampsia) showed a positive correlation between the levels of AT<sub>1</sub>-AA and both increased SBP (r = 0.556, P < 0.05; Fig. 1c) and proteinuria (r = 0.565, P < 0.001; Fig. 1d).

**Aldosterone levels are decreased in all AT**<sub>1</sub>-**AA-positive hypertensive women and are inversely correlated with AT**<sub>1</sub>-**AA in early-onset preeclamptic patients with high proteinuria**

In order to determine the relationship between AT<sub>1</sub>-AA and ALD in pregnant women, we analyzed the blood samples for ALD. We first confirmed that circulating ALD concentrations were greater in normotensive pregnancies compared to preeclamptic patients (0.5532 ± 0.07 vs. 0.254 ± 0.072 ng/ml; P < 0.001, Fig. 2a) [9]. To further understand the role of ALD in preeclampsia, we compared early-onset to late-onset preeclamptic women, and showed that early-onset preeclamptic patients displayed increased ALD concentrations (0.3108 ± 0.047 vs. 0.243 ± 0.071 ng/ml; P < 0.05, Fig. 2b). Additionally, patients without proteinuria had greater ALD levels compared to hypertensive women with proteinuria (0.3027 ± 0.082 vs. 0.254 ± 0.072 ng/ml; P < 0.05, Fig. 2c).

To stratify the distinctive features of patients with elevated AT<sub>1</sub>-AA, all hypertensive women were divided into AT<sub>1</sub>-AA-positive and AT<sub>1</sub>-AA-negative groups. Compared to the AT<sub>1</sub>-AA-negative group, the AT<sub>1</sub>-AA-positive group had a decreased concentration of circulating ALD (0.257 ± 0.075 vs. 0.296 ± 0.056 ng/ml; P < 0.05, Fig. 2d). To better assess the correlation between ALD and AT<sub>1</sub>-AA, we analyzed their levels via simple linear regression. No correlation between circulating ALD and AT<sub>1</sub>-AA was seen, suggesting that there might be some other external factors needed to be considered in the linear regression analysis. On the basis of the results that lower ALD and higher AT<sub>1</sub>-AA levels were associated with both early-onset preeclampsia and high proteinuria, we further stratified the patients according to the gestational week of preeclampsia onset and proteinuria. A clear inverse correlation between AT<sub>1</sub>-AA level and circulating ALD concentration was observed among the 50 early-onset preeclamptic patients with high proteinuria (>5.0 g/24 h) (r = −0.573; P < 0.001, Fig. 2e). No correlation was observed between serum AT<sub>1</sub>-AA and ALD among late-onset preeclamptic women with or without high proteinuria.
As plasma AT$_1$-AA levels increased in AT$_1$-AA-treated pregnant rats over 5 days of treatment (Fig. 3a), plasma ALD concentrations decreased ($0.345\pm0.07$ vs. $0.203\pm0.0887$ ng/ml; Fig. 3b). This decrease in ALD was accompanied by cellular atrophy and a looser arrangement in the zona glomerulosa of the adrenal cortex than in control rats (Fig. 3c). In contrast, these abnormalities were absent in the nslG-injected pregnant rats.

**Aldosterone levels decreased in response to increased AT$_1$-AA levels in nonpregnant rats**

To explore the impact of chronic administration of AT$_1$-AA on ALD generation without pregnancy, a passively immunized rat model was established, harboring clinically relevant AT$_1$-AA range in preeclamptic women (0.37–1.00 ng/ml) (Fig. 4a). Four weeks of rat AT$_1$-AA, but not nslG, administration decreased ALD concentrations ($0.273\pm0.03$ to $0.123\pm0.080$ ng/ml; $P<0.05$, Fig. 4b). After ceasing AT$_1$-AA passive immunization on week 8, ALD

**Confirmation that AT$_1$-AA decreased aldosterone levels in pregnant rats**

To validate use of a rat model for preeclampsia, purified AT$_1$-AA from preeclamptic women was administered to pregnant rats on gestation day 13 (analogous to early-onset preeclamptic timing in humans) for 5 days. As shown previously [4] and confirmed in results shown in supplementary data (http://links.lww.com/HJH/A462), we confirmed that AT$_1$-AA was readily detectable and remained biologically active (Fig. 3a, Supplementary Fig. S3, http://links.lww.com/HJH/A462); the AT$_1$-AA-positive pregnant rats exhibited hypertension, impaired renal functions, and marked pathological alterations in renal histology (Supplementary Figs. S4, S5, and S6, http://links.lww.com/HJH/A462); and the fetuses experienced intrauterine growth retardation and increased heart rates (Supplementary Fig. S7, http://links.lww.com/HJH/A462). These results demonstrate that AT$_1$-AA can induce the hallmarks of preeclampsia when injected into pregnant rats.
suppression reversed and recovered to normal levels 28 days after immunization. However, reinjection of AT₃-AA on week 12 decreased ALD levels again (Fig. 4c).

To further explore the basis for decreased ALD production in AT₃-AA-positive nonpregnant rats, we isolated the adrenal glands from immunized and control rats when autoantibody levels peaked (after 8 weeks of AT₃-AA immunization). In order to evaluate AT₃-AA presence, adrenal cortical zona tissues were analyzed by IHC staining using the antibody against rat IgG, and demonstrated a strongly positive result compared to controls, AT₃-AA levels of which were undetectable all the time (Fig. 4e). These data suggested that AT₃-AA may directly affect ALD synthesis, as evidenced by its accumulation around the zona glomerulosa layer. The presence of substantial cellular atrophy and disruption in cellular morphology in the outermost zone of the cortex of adrenal glands were observed by H&E staining in nonpregnant rats chronically injected with purified rat AT₁-AA (Fig. 4d). These results demonstrated that the presence of AT₁-AA leads to cellular injury and tissue impairment in adrenal glands. In contrast, none of the nsgG-injected rats showed significant pathological alterations of adrenal glands (Fig. 4d). These studies demonstrated that the long-term presence of AT₁-AA results in reduced circulating ALD levels, independent of pregnancy.

**Activation of angiotensin II type 1 receptor by AT₁-AA directly led to abnormal aldosterone generation in cultured cells in a time- and dose-dependent manner**

To explore the mechanistic basis for abnormal ALD generation, we determined the time and dose-dependent effects of AT₁-AA on cultured NCI-H295R human adrenocortical tumor cells. These cells of human origin were chosen as they are reliably and widely used to study ALD production responsive to AT₁R activation [21]. The autoantibody distribution was visualized by immunofluorescence imaging and found to co-localize with AT₁R on the plasma membrane in AT₁-AA-treated H295R cells (Fig. 5a, Supplementary Fig. S8, http://links.lww.com/JHH/A462). As illustrated in Fig. 5b, longer treatments (≥24 h) of AT₁-AA at higher concentrations (≥1 μg/ml) inhibited cell growth (0.71 ± 0.069 vs. 1.66 ± 0.037; *P < 0.001; AT₁-AA treatment at 1 μg/ml after 48 h), accompanied by a 3.92 ± 0.49-fold increase in LDH release and a 2.11 ± 0.17-fold increase in caspase-3 activity (Fig. 5c and d). Likewise, TUNEL assays showed that the weak fluorescent labeling observed in the nuclei of control cells was greatly increased in AT₁-AA-treated cells (Fig. 5e). These findings indicate an increased cellular death (necrosis and apoptosis) in AT₁-AA-treated cells. Chronic administration of AT₁-AA (1 μg/ml after 48 h) led to an almost two-fold decrease in secreted ALD in culture supernatant (Fig. 6a). Consistent with earlier in-vivo animal studies [11], the short-term presence (24 h) of AT₁-AA at a low level (0.5 μg/ml) stimulated both cellular ALD release and cell growth (Fig. 6a and Fig. 5b, respectively), mimicking the pressor function of Ang II.

Of significance, the AT₁R antagonist, losartan, protected AT₁-AA-treated H295R cells from cellular apoptosis, as demonstrated by a greater than 1.5-fold decrease in caspase-3 activity compared to AT₁-AA-treated group (Fig. 6c) and a weaker positive-TUNEL staining (Fig. 5e). Losartan was also protective against necrosis, as demonstrated by a decrease in LDH leakage and improved ALD production in
the AT$_{1}$-AA-treated group as compared to the losartan-treated group (Fig. 6d and b). These data indicate that autoantibody-induced cellular death and abnormal ALD secretion are mediated by AT$_{1}$R activation. Lastly, increased ALD secretion that is mediated by the short-term presence of AT$_{1}$-AA (0.5 mg/ml after 24 h) was also blocked by the receptor antagonist losartan (Fig. 6e). Together, these findings indicate that AT$_{1}$-AA directly affected the cellular viability and generation of ALD in a time and dose-dependent manner via AT$_{1}$R activation in NCI-H295R cells, suggesting a mechanism by which ALD is regulated.

**DISCUSSION**

In this study, we show that there is a time/dose–response relationship between circulating ALD levels and the presence of AT$_{1}$-AA levels in the blood, which is independent of pregnancy. Upon chronic infusion of AT$_{1}$-AA into nonpregnant rats, circulating ALD concentrations dropped dramatically, which was consistent with in-vitro observations of the decrease in ALD production and increase in cellular death of adrenocortical cells after long-term exposure. In contrast, low doses of AT$_{1}$-AA stimulated both cellular growth and ALD production upon short-term exposure. These AT$_{1}$-AA-induced features were blocked by AT$_{1}$R antagonist losartan, indicating that the changes in ALD production and cell viability resulted from AT$_{1}$-AA-mediated AT$_{1}$R activation. Overall, our present findings demonstrate a direct time and dose-dependent regulatory role of AT$_{1}$-AA in ALD secretion, independent of pregnancy, with a short-term increase in ALD and a long-term decrease.

Figure 6f details the working model of AT$_{1}$-AA in regulating ALD production. It has been well documented that AT$_{1}$-AA increases sFlt-1 in pregnant mice, which blocks vascular endothelial growth factor (VEGF)-mediated signaling that is important for normal endothelial function and thereby results in hypertension and renal dysfunction [4,23]. Notably, Siddiqui et al. [11] reported that AT$_{1}$-AA-mediated excessive sFlt-1 levels were also responsible for the adrenal...
Yang et al.

![Image](https://www.jhypertension.com/)

**FIGURE 5** Marked cell injury responsive to long-term presence of AT1-α in a high concentration. After the treatment of AT1-α from patients' serum (1 μg/ml, 12 h), NCI-H295R cells were fixed with 4% formaldehyde and immunolabeled with an antibody specific against AT1R (Rhodamine; red, middle column) and co-stained with fluorescein isothiocyanate (FITC)-tagged IgG specific for human AT1-α (green, middle column). Merged images are presented in the right column (a). Magnification: upper panels, 125×; lower panels, 256×. AT1-α treatment (0.5 μg/ml) for 24 h stimulated cellular growth (b), whereas increased cellular death after long-term AT1-α presence (>1 μg/ml) in NCI-H295R cells was evidenced by increased LDH leakage (c) and caspase-3 activity (d), together with obvious TUNEL staining shown in the representative images (e left, green fluorescence indicates TUNEL-positive apoptotic nuclei and the blue one indicates total NCI-H295R cell nuclei; upper and middle panels, magnification: 20×; e right, quantification of apoptotic nuclei: mean of the ratio of positive nuclei to total NCI-H295R cell nuclei). (a) P < 0.05, (++) P < 0.001 vs. vehicle group. Values are expressed as mean ± SEM. AT1-α, agonistic autoantibodies against the angiotensin II type 1 receptor; IgG, immunoglobulin G; TUNEL, terminal deoxynucleotidyl nick-end labeling.

Gland vascular impairment and consequent ALD reduction in a pregnancy-dependent manner, which were attenuated by injection of VEGF121 into pregnant mice. Data from our clinical study complement these animal studies, showing an inverse correlation between AT1-α and ALD in women with early-onset pre-eclampsia, accompanied by high proteinuria. After confirming AT1-α-induced ALD reduction in pregnant rats, we further explored the long-term effect of AT1-α on ALD production. Independent of pregnancy, a long-lasting presence of AT1-α induced a dramatic decrease in ALD levels, accompanied by morphological changes in adrenal glands of non-pregnant rats. Interestingly, such levels and time of exposure are similar to the presence of AT1-α in the sera of essential hypertension patients [24,25]. We also observed an obvious antibody accumulation around the zona glomerulosa layer after 8 weeks of AT1-α exposure, suggesting that instead of via stimulating sFlt-1 generation during pregnancy, AT1-α may directly regulate ALD production by activating AT1Rs of zona glomerulosa cells. Supporting this possibility, we provided in-vitro evidences that upon long-term exposure, a relatively high dose of AT1-α resulted in cell death and impaired ALD production via activation of AT1Rs on NCI-H295R adrenocortical cells. It is noteworthy that the short-term AT1-α treatment was determined to stimulate cellular proliferation and increase ALD levels through activating adrenal AT1R, consistent with the previous in-vivo animal studies by Siddiqui et al. [11]. That is because AT1-α can act like a functional mimic of Ang II, and Ang II is known to stimulate ALD generation. However, accumulating evidences show that autoantibodies as larger proteins possess their chemical and physical features distinctive from corresponding receptor agonists normally comprised of less than 10 amino acids [26,27]. Thus, one would expect that AT1-α would affect ALD levels differently. On the basis of this expectation, it was not surprising to see that AT1-α worked as a novel dual regulator for ALD production. Previous results came up a working model that AT1-α-mediated increase in sFlt-1 is a pregnancy-specific factor responsible for reduced ALD production [11]. Here, we improve this model by showing that AT1-α via activating...
AT$_1$Rs of zona glomerulosa cells regulates ALD production in a time and dose-dependent, yet pregnancy-independent, manner. Although we did not determine the level of sFlt-1 through the whole process, it is unlikely that sFlt-1-induced adrenal gland vascular impairment may contribute to AT$_1$-AA-induced ALD reduction in nonpregnant rats because the placenta, especially preeclamptic placenta, has been proved to produce sFlt-1 [3]. Moreover, we previously observed that long-term AT$_1$-AA exposure did not increase endothelin-1 (ET-1) levels and damage capillary endothelium until after 9 months of immunization [28]. In contrast, ALD levels dropped significantly after simply 8 weeks of exposure. Thus, AT$_1$-AA may regulate ALD through other mechanisms. It is known that some autoantibodies can regulate endocytosis of their receptors, resulting in increased and non-desensitizing receptor activation [29]. Earlier studies have shown that AT$_1$-AA induces excessive Ca$^{2+}$ influx in the vascular smooth muscle cells [30]. Since Ca$^{2+}$ overload inhibits biosynthesis of ALD [31,32], it is possible that sustained activation of adrenal AT$_1$R induced by long-term presence of AT$_1$-AA attenuates AT$_1$R internalization, resulting in Ca$^{2+}$ overload and consequent impairment of ALD synthesis. The role of AT$_1$-AA-triggered Ca$^{2+}$ overload in ALD regulation has not been established, and further studies on autoantibody-mediated changes in adrenal AT$_1$R internalization can help to clarify the underlying mechanism. Taken together, we believe that activation of adrenal AT$_1$R by AT$_1$-AA is not directly responsible for reduced ALD levels in pregnant rats, but participates in chronic impairment of ALD production in a pregnancy-independent manner.

Another interesting finding of this study is that AT$_1$-AA was elevated in early-onset preeclampsia. Constituting approximately 5–20% of all preeclamptic cases, the early-onset subtype is a severe form of preeclampsia characterized by placental ischemia and intrauterine growth restriction due to impaired trophoblast invasion and increased placental vessel peripheral resistance [1,2]. Furthermore, early-onset preeclamptic mothers are predisposed to a higher rate of small-for-gestational-age infants who are at an increased risk for future short and long-term complications such as metabolic disorders and cardiovascular diseases [33]. We recently reported that middle-aged offspring of AT$_1$-AA-positive mother rats are prone to metabolic disorders even under a normal diet [16]. Similarly, other complications [34] observed in early-onset patients, such as small fetuses with organ growth retardation [35], can also be induced by the presence of AT$_1$-AA, yet the role of AT$_1$-AA during early-onset preeclampsia remains unclear. On the basis of the statistic analysis of gestational age-matched cases with a larger sample size than previous studies [23,36], our data showed that AT$_1$-AA was much more prevalent in early-onset preeclampsia. This supports the idea that AT$_1$-AA may play a crucial role in the cause of early-onset preeclampsia, but not late-onset preeclampsia [33,35]. We expect that blocking the action of AT$_1$-AA may provide more significant therapeutic benefit for patients with early-onset preeclampsia.
Yang et al.

ACKNOWLEDGEMENTS

A special thank you goes to Dr Esther Bullitt and Dr Chelsea Eppler for their valuable comments of this research work and assistance in writing the original manuscript.

This study has been supported by the grants from the Natural Sciences Foundation of China (NSFC) 81070263, 81300694; KZ201110025023 from Science and Technology Plan Project of Beijing Municipal Education Commission, Beijing China, 20131107120021 from 2013 Specialized Research Fund for Doctoral Program of Higher Education, Beijing China.

Conflicts of interest

Disclosures: All the authors gave their informed consents and reported no potential conflicts of interest and relationship with and/or ownership in industry.

REFERENCES


**Reviewer’s Summary Evaluation**

**Referee 3**

In this interesting study Yang and coworkers report that circulating AT1 autoantibody levels are increased in women with preeclampsia (particularly in those with early-onset preeclampsia) as compared to normotensive healthy pregnant women; an opposite, significant trend was found for plasma aldosterone levels. On the whole, these findings reinforce the view that impaired aldosterone synthesis may have a pivotal role in the pathogenesis of preeclampsia by altering the physiologic plasma volume expansion. Further investigations are needed to confirm and refine the observations by Yang and coworkers and, more importantly, to address the therapeutic implications of AT1 autoantibody blockade.