Autoantibody against AT1 receptor from preeclamptic patients induces vasoconstriction through angiotensin receptor activation

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Preeclampsia is a serious pathologic complication during pregnancy, and its pathogenesis remains poorly understood. Recent studies have demonstrated that autoantibodies against angiotensin II type 1A receptor (AT1-AA) are present in women with preeclampsia. However, their role in the development of hypertension in preeclamptic patients has never been previously investigated. The present study was designed to determine whether AT1-AA isolated from the sera of preeclamptic patients causes vascular constriction and, if so, to further investigate the cellular receptors that mediate their vasoactivity. Blood samples were collected from 49 pregnant women (preeclampsia = 31, control = 18) and AT1-AA was detected using enzyme-linked immunosorbent assay. Vasoconstrictive effect of purified IgG from the sera of either preeclamptic patients or normal pregnant women was determined in isolated rat thoracic aorta, arteriae cerebri media and coronary artery. Compared with normal pregnant women, frequency of AT1-AA positive samples was markedly increased in preeclamptic patients (80.7 vs. 5.6%, \( P < 0.01 \)). In isolated thoracic aortic rings, middle cerebral artery and coronary artery segments, AT1-AA induced vasoconstriction in a concentration-dependent fashion (\( P < 0.01 \)). The vasoconstrictive effect of AT1-AA was completely blocked by losartan, an AT1-receptor antagonist. These data demonstrate that the AT1-AA causes significant vascular constriction in large conduit vessel as well as small resistant vessels though activation of the AT1 receptor. These results suggest that overproduction of AT1-AA is a novel risk factor in pregnant women and may play a causative role in the development of hypertension and vascular injury in preeclamptic patients. J Hypertens 26:1629–1635 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Abbreviations: ACh, acetylcholine; Ang II, angiotensin II; AT1-AA, autoantibodies against angiotensin II type 1A receptor; CVD, cardiovascular disease; DBP, diastolic blood pressure; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin; LST, losartan; NADPH, nicotinamide adenine dinucleotide phosphate; ns IgG, nonspecific IgG; PBS, phosphate-buffered saline; KCl, potassium chloride; SBP, systolic blood pressure; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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Introduction

Preeclampsia, a serious pregnancy-specific disorder characterized by hypertension, proteinuria, abnormalities in coagulation and vascular tone, remains a leading cause of maternal and neonatal morbidity and mortality. It affects about 3–5% of pregnancies worldwide. Although substantial efforts and some progress have been made in the past, limited understanding in the etiology and pathophysiology of preeclampsia remains as a roadblock in identifying the best preventive and effective treatment methods for this life-threatening disease [1].

Emerging evidence indicates that vascular functional and structural damage caused by yet to be identified mediators present in the maternal circulation of preeclampsia patients plays a causative role in the development of hypertension and proteinuria, two of the major symptoms in these patients [2]. In 1999, Wallukat et al. [3] first reported that the autoimmune antibody against the second extracellular loop (165–191) of angiotensin II receptor type 1 (AT1-AA) is present in preeclamptic patients but not in healthy pregnancies or those with essential hypertension, and proposed that AT1-AA induces, similar to angiotensin II, an increase of the beating rate in spontaneously beating neonatal rat cardiomyocytes. Several recent in-vitro experimental studies further demonstrated that AT1-AA induces Ca\(^{2+}\) release [4] and activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [5] in vascular smooth muscle cells, indicating that AT1-AA increases oxidant stress and calcium mobilization. These clinical and experimental results strongly suggest that AT1-AA might be one of the molecules
mediating vascular injury in preeclamptic patients. However, whether AT1-AA isolated from preeclamptic patients can cause vasoconstriction, which would be the most critical evidence establishing a firm link between production of AT1-AA and development of hypertension in these patients, has not been previously investigated.

Therefore, the aims of the current study were to determine whether AT1-AA isolated from preeclamptic patients causes vascular constriction in conduit and resistant vessels and, if so, to further investigate the cellular receptors that mediate their vasoactivity.

Patients and methods

Materials
Losartan (LST) (Cozaar), human angiotensin II (Ang II) (catalog No. A9525), phenylephrine hydrochloride and potassium chloride (KCl) were obtained from Sigma (St Louis, Missouri, USA). Gamma bind G sepharose was purchased from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden).

Patient selection
The research protocol was approved by the Institutional Committee for the Protection of Human Subjects of Shanxi Medical University Hospital. All patients were informed about the purpose and protocol of the study, and written consent was obtained. The study adheres to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, revised 13 November 2001, effective from 13 December 2001. According to the guidelines of the International Society for the Study of Hypertension in Pregnancy [6], preeclampsia is defined by an increase in blood pressure (BP) to at least 140 systolic BP (SBP) or 90 mmHg diastolic BP (DBP), or both, after the 20th week of gestation in a previously normotensive woman, combined with proteinuria (protein excretion at least 0.3 g per 24 h, spot urine protein/creatinine ratio 30 mg/mmol or at least 2+ protein by dipstick). For patients with preeclampsia, the gestational age at delivery ranged from 37–40 weeks, with an average of 39 weeks. Normotensive pregnant individuals were characterized by uncomplicated pregnancies with normal-term deliveries. For normotensive patients, the gestational age at delivery ranged from 38–41 weeks, with an average of 39 weeks. Thirty-one preeclamptic patients and 18 normal pregnancy women were enrolled into the study between March 2006 and May 2007 in four urban teaching hospitals of Shanxi Medical University.

Streptavidin-enzyme-linked immunosorbent assay
A venous blood sample (10 ml) was drawn from each woman into tubes containing EDTA. Immediately after sampling, plasma was separated by centrifugation at 4000 g for 10 min and frozen at −80°C. The level of AT1-AA was measured by enzyme-linked immunosorbent assay (ELISA) as we used previously, and the results were expressed as optical density (OD) values [7]. Briefly, synthetical peptide 165–191 (I-H-R-N-V-F-F-I-I-N-T-N-I-T-V- C-A-F-H- Y-E-S-Q-N-S-T-L), which is the sequence of the second extracellular loop of AT1 receptor (5 μg/ml) in a 100 mmol/l Na2CO3 solution (pH 11.0), was coated on microtitre plates overnight at 4°C. The wells were then saturated with 0.1% PBT buffer [0.1% (w/v) albumin bovine V, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS), pH 7.4] for 1 h at 37°C. After washing three times with PBS-T, human sera dilutions were added to the saturated microtitre plates for 1 h at 37°C. After three washings, biotinylated goat anti-human IgG antibodies (Sigma) (1:1000 dilutions in PBT) were added for 1 h at 37°C. Following three washings, streptavidin–peroxidase conjugate (Sigma) at 1:2000 dilution in the same buffer was added to the wells and incubated under the same conditions. Finally, 2,2-azino-di(3-ethylbenzothiazoline) sulphonic acid (ABTS)-H2O2 (Roche, Basel, Switzerland) substrate buffer was added and reacted for 30 min in the dark at room temperature. The ODs were measured at 405 nm using an ELISA reader (Spectra Max Plus, Molecular Devices, Sunnyvale, California, USA). We also calculated positive/negative (P/N) ratio [(the OD of sample − the OD of empty control)/(the OD of negative control − the OD of empty control)] of each sample, and those samples with a P/N value at least 2.1 were considered as AT1-AA positive [7].

Preparation of the immunoglobulin G
On the basis of a sera-positive response in an ELISA to the peptide 165–191, immunoglobulin fractions G (IgG) from the positive sera of preeclampsia were prepared by MabTrap Kit (Amersham). The prepared IgG fraction was neutralized with synthetic peptide 165–191, and remaining IgG termed as ‘nonspecific’ IgG (nsIgG) was used as a negative control. The IgG from 17 healthy nonpreeclamptic pregnant women (nplIgG) whose AT1-AA was negative was prepared in an identical fashion and used as a control.

Thoracic aorta preparations and in-vitro vasoconstriction
Male Wistar rats (220–240 g) were anesthetized and killed by cervical dislocation, the thoracic aorta was carefully removed and immediately placed in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (in mmol/l: NaCl, 144; KCl, 5.8; CaCl2, 2.5; MgCl2 1.2; HEPES 5; and D-glucose, 11.0, pH 7.4). The aortas were cut into rings of 3 mm in length. The rings were suspended on two wire hooks in water-jacketed tissue baths containing 10.0 ml of HEPES solution bubbled with 100% O2 and maintained at 37°C. The upper hook was connected to a force transducer, and changes in isometric force were recorded by a PowerLab system (AD Instruments Co., Ltd, Sydney, Australia). Passive tension was adjusted to
2.0 g, and all subsequent measurements represent force generated above this baseline. A 2-h equilibration period was allowed before any experimental intervention, and the bath was flushed every 15 min with the fresh HEPES solution during equilibration. After equilibration, the rings were maximally constricted with 120 mmol/l KCl followed by extensive washing, and the procedure was repeated three to five times until a stable vasoconstriction was observed. The integrity of endothelium was ensured by observation that the relaxation induced by 10 μmol/l acetylcholine (ACh) on the contraction generated by 30 mmol/l KCl was greater than 70%. Those vascular segments with an abnormal vasoconstriction (>10% variation between successive contractions) or relaxation (<70% relaxation to 10 μmol/l ACh) were excluded from further experiments. Those rings with a constant vasoconstriction and relaxation to ACh were exposed to different concentrations of AT1 autoantibody IgG, and the vasoconstrictive effect was observed.

Small resistance artery preparation
To examine the vasoactive effects of AT1-AA in the small resistance artery, the third-order branches of the superior middle cerebral arteries and coronary arteries were isolated from adult rats. The rings (2 mm in length) were mounted on a wire myograph (Multi Myograph System-610M, Danish Myo Technology A/S, Aarhus, Denmark) using two tungsten wires (40 μm in diameter) using two tungsten wires (40 μm in diameter). The rings were normalized according to standard procedures provided by the manufacturer, stretched to a state equal to 80 mmHg and equilibrated for at least 1 h. The rings were constricted with KCl and relaxed with ACh repeatedly until responses were reproducible as mentioned above. Vasoconstrictive response to AT1-AA was then determined.

Statistical analysis
All of the values are expressed as mean ± SD. The statistical analysis was performed by paired t-test and unpaired t-test (for clinical information only). P < 0.05 was considered statistically significant.

Results
Sera levels of AT1-AA were markedly increased in preeclamptic patients compared with the normal pregnant women
Clinical data are summarized in Table 1. There was no difference in maternal age or gestational age at sampling. Of 49 pregnant women enrolled, 31 exhibited significant hypertension and proteinuria that qualifies the diagnosis of preeclampsia. Compared with normotensive pregnant individuals, sera levels of AT1-AA were markedly increased in preeclamptic patients (0.59 ± 0.1 vs. 0.30 ± 0.11, P < 0.01) (Fig. 1a). As illustrated in Fig. 1b, only one of 18 normotensive pregnant women was AT1-AA positive (5.6%) whereas 25 of 31 preeclamptic patients had a P/N value greater than 2.1 (80.6% positive). These data demonstrated that sera levels of AT1-AA are markedly increased in preeclamptic patients compared with the normal pregnant women.

Immunoglobulin G fractions from AT1-AA positive sera of preeclamptic patients constricted rat thoracic aorta rings in a dose-dependent fashion
As illustrated in Fig. 2b, IgG fraction from AT1-AA positive sera of preeclamptic patients caused significant vasoconstriction that is similar to that observed with Ang II (Fig. 2a). Interestingly, a vasoconstriction that was comparable to that achieved by 0.01 μmol/l of Ang II was observed when the vessels were exposed to 0.1 μmol/l of IgG fraction, a concentration that is pathologically relevant. Moreover, the vasoconstrictive response to 0.1 μmol/l of immunoglobulin was completely blocked by 1 μmol/l LST (P < 0.01). These results provide clear evidence that IgG fraction isolated from the AT1-AA positive sera of preeclamptic patients causes significant vasoconstriction in conduit vessel by stimulating AT-1 receptor.

As summarized in Fig. 3 (last bar), addition of npIgG at concentrations as high as 5 μmol/l failed to cause any significant vasoconstriction. In addition, AT1-AA in preeclamptic sera was neutralized by a procedure described in the Methods section, and the vasoactivity of remaining IgG (nsIgG) was determined. As shown in Fig. 3 (second last bar), nsIgG (5 μmol/l) had no vasoconstrictive effect in aortic rings. Taken together, the results presented in Figs 2 and 3 demonstrated that it is the AT1-AA present in preeclamptic patients that causes AT1 receptor-dependent vasoconstriction in the conduit artery.

Immunoglobulin G fractions from AT1-AA positive sera of preeclamptic patients constricted rat small resistant vascular rings in a dose-dependent fashion
As summarized in Fig. 4, addition of IgG fraction isolated from AT1-AA positive sera of preeclampsia patients at a concentration of 0.01–1 μmol/l (right panel) caused significant vasoconstriction in middle cerebral artery and coronary artery segments that were almost identical to that observed with Ang II at 0.01–1 μmol/l (left panel). Moreover, the vasoconstrictive effect of IgG fraction in these small resistant vessels was completely blocked.
when the vessels were pretreated with 1 μmol/l LST (Fig. 5). It is noteworthy to indicate that coronary artery segments are far more sensitive than middle cerebral artery segments to the IgG fraction from AT1-AA positive sera of preeclampsia patients in their vasoconstrictive response. Finally, addition of nslIgG (IgG fractions

![Fig. 1](image)

(a) Concentration of AT1-AA from each of 31 preeclamptic patients (open circles) and 18 normotensive pregnant individuals (filled circles). Scatterplot represents levels of AT1-AA from each patient in each group. Experiments were repeated twice per sample. (b) Percentage of AT1-AA positive sera from two different groups. **P < 0.01; N, normotensive; OD, optical density; PE, preeclamptic.

![Fig. 2](image)

Vasoconstrictive effects of Ang II (a, positive control) and AT1-AA (b) in rat thoracic aortic rings incubated in Ca²⁺-free buffer. Thoracic aortic rings were treated with different concentrations (0.01, 0.1 and 1.0 μmol/l) of Ang II or IgG from AT1-AA positive sera of preeclampsia patients and constriction of aortic rings was measured. Concentration of LST is 1.0 μmol/l. The contraction of thoracic aorta rings is defined by g. *P < 0.05; **P < 0.01 vs. losartan treated group. *P < 0.05; **P < 0.05 vs. 1 μmol/l Ang II group or 1 μmol/l preeclamptic IgG group. n = 6–10. Ang II, angiotensin II; LST, losartan.
neutralized with the peptides corresponding to the second extracellular loop of AT1 receptor) or npIgG (IgG fractions from normotensive pregnant individuals) had no vasoconstrictive effect on either middle cerebral artery or coronary artery segments (Fig. 6).

Discussion

The syndrome of preeclampsia has been ascribed previously to generalized maternal, vascular lesion, poor placentation and excessive maternal inflammatory response. Nevertheless, vascular damage is a hallmark of preeclampsia, leading to decreased vasodilatation, vascular smooth muscle cell proliferation and increased interaction between leukocytes and the vessel wall [8].

Potential involvement of the renin–angiotensin system in the pathogenesis of preeclampsia has been noted for decades [9]. However, the precise role of renin–angiotensin system remains unclear, and some reported results are controversial. Cooper et al. [10] demonstrated that active tissue renin concentrations are elevated, and renin mRNA expression is increased in placentas from preeclamptic patients compared with placentas from women with normal pregnancies. Gant et al. [11] reported that the sensitivity to Ang II is markedly increased in preeclamptic patients. However, a later clinical study showed that the
Ang sensitivity is not uniformly increased in patients with preeclampisia [12]. The autoimmune antibody against the second extracellular loop of angiotensin II receptor type 1 (AT1-AA) has been intensively discussed as a marker and possible inductor of the disease for more than 10 years [13]. In 1999, Wallukat et al. [3] first documented that immunoglobulin extracted from the serum of preeclamptic patients contains an antibody that binds to the AT1 receptor and has agonist activity. They also provided evidence that this autoantibody belongs to IgG antibody and binds to the peptides of the second extracellular loop of the AT1 receptor. Several studies about the function of AT1-AA were based on their results.

Wallukat et al. [3] first reported that this autoimmune antibody is detectable in preeclamptic patients but not in healthy pregnancies or those with essential hypertension. This result confirmed previous studies indicating that a high prevalence of AT1-AA is associated with preeclampsia. Second, we have demonstrated for the first time that AT1-AA isolated from preeclampsia patients causes significant vasoconstriction in conduit as well as small resistance arteries. Finally, we have provided the first direct evidence that AT1-AA induces vasoconstriction via activation of angiotensin type 1 receptor. Taken together, these results suggest that this agonistic autoantibody binds to the AT1 receptor and may cause characteristic of preeclampsia, such as increased peripheral vascular resistance, elevated cardiac afterload, promoted cardiac hypertrophy [19] and decreased blood supply [20].

The results obtained in the present study have demonstrated for the first time that AT1-AA increases coronary artery constriction in a dose-dependent fashion. Clinical studies demonstrated that preeclampsia is associated with a significantly increased risk for long-term cardiovascular disease (CVD) [21–23]. Our results suggest that AT1-AA may contribute to the cardiac complication associated with preeclampsia by its vasoconstrictive action on coronary artery. Whether the level of AT1-AA may be used to predict the future cardiac complications years after delivery warrants further study.

We also evaluated the vasoconstrictive effect of AT1-AA in the middle cerebral artery and demonstrated that AT1-AA caused the most significant vasoconstriction in this particular vascular system. It is well documented that severe preeclampsia is accompanied with a nervous system disorder manifested by dizziness, nausea and hyperspasmsia without clear explanation. It is possible that these symptoms are a consequence of cerebral ischemia caused by AT1-AA.
It is worth noting that, although over 80% preeclamptic patients are AT1-AA positive, a small portion of preeclamptic patients (approximately 20%) are AT1-AA negative yet hypertensive. This result indicates that AT1-AA/AT1-R axis is not the only mechanism that is responsible for hypertension in preeclamptic patients, and additional yet to be identified factors must exist. On the contrary, in our study, one patient in the nonpreeclampsia patient group is AT1-AA positive yet her BP is normal, suggesting that other factors exist in this patient that may counteract the effect of AT1-AA. Although identifying these factors is certainly beyond the scope of the present study, future experiments focusing on this important topic may yield not only scientifically, but also clinically important data.

**Study limitations**

Our work leaves some unanswered questions and paths for future work. Our data do not distinguish whether AT1-AA production is a primary or secondary event in preeclampsia. Future studies in which AT1-AA will be consecutively detected throughout the whole duration of pregnancy should clarify this issue, and the role of AT1-AA in vascular damage should continue to be explored. There are other limitations to our study. Although our result demonstrating that AT1-AA constricted middle cerebral artery and coronary artery segments strongly suggests that AT1-AA plays an important role in vascular lesion, future studies (e.g. measuring the effects of AT1-AA on uterine and renal arteries) are needed to support this conclusion. In our in-vitro experiment, we used normal rats, whose AT1-R distribution may be different compared with those rats immunized with the peptides corresponding to the second extracellular loop of AT1-R. Therefore, further study using immunized rats should yield more conclusive results.

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