

Original Article

Autoantibody against angiotensin AT1 receptor from preeclamptic patients enhances collagen-induced human platelet aggregation

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Hypercoagulability, platelet activation, and thrombocytopenia are the chief characteristics of preeclampsia, but their responsible underlying molecular mechanisms remain obscure. Recent studies have demonstrated that the autoantibody against angiotensin II type 1 receptor (AT1-AA) constitutes a novel risk factor for preeclampsia. However, the role of AT1-AA in platelet activation and hypercoagulability in preeclampsia has never been investigated. In the present study, we determined whether AT1-AA promotes platelet aggregation *in vitro*, and dissected the potential underlying mechanisms. AT1-AA was detected by enzyme-linked immunosorbent assay. After immunoglobulin G fractions purified from the preeclamptic patient positive sera were added to platelets isolated from healthy volunteers, platelet aggregation and intracellular Ca²⁺ levels were detected. AT1-AA significantly enhanced *in vitro* collagen-induced platelet aggregation, an effect blocked by the AT1 receptor antagonist losartan. Additionally, AT1-AA increased and maintained collagen-induced cytosolic calcium concentration throughout the experiment. We demonstrated for the first time that AT1-AA significantly promotes collagen-induced platelet aggregation through angiotensin type 1 receptor activation *in vitro*, potentially via increased intracellular Ca²⁺ concentration, supporting AT1-AA as a potential contributor to the hypercoagulable state of preeclampsia.

Keywords autoantibody; angiotensin II type 1 receptor; platelet aggregation; calcium

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Introduction

With worldwide prevalence of 2%–5%, preeclampsia remains a major cause of both maternal and neonatal morbidity and mortality [1–3]. Although both platelet activation and thrombocytopenia are associated with preeclampsia, several studies have suggested that platelet aggregation augments hypercoagulability [4,5]. Enhanced platelet aggregation releases vasoconstrictors and cytotoxic factors which enhance hypercoagulability, completing a vicious positive feedback loop. Vascular endothelial injury releases thromboxane A₂ and endothelin which further promotes platelet aggregation. Moreover, studies have confirmed the involvement of the renin–angiotensin system in preeclampsia development, as evidenced by increased angiotensin type 1 receptor (AT1R) activation [6]. Experiments demonstrated that angiotensin II (Ang II) induces the aggregation and activation of platelets via activation of AT1R [7,8]. However, the over-activation of AT1R cannot be explained fully by Ang II stimulation alone. It remains unknown whether other factors contribute to platelet aggregation and activation in preeclampsia.

In 1999, Wallukat *et al.* [9] first reported that the auto-immune antibody against the second extracellular loop (165–191) of the angiotensin II receptor type 1 (AT1-AA) is an adverse factor in preeclamptic women, demonstrating receptor agonist-like effects (e.g. increased calcium mobilization) [10]. Previously, we demonstrated that rats which were immunized with the synthetic peptide corresponding to the sequence of the second extracellular loop of the human AT1R exhibited thrombocytopenia at the 28th week after

initial immunization (Supplementary Fig. S1). Platelet aggregation is a cause of thrombocytopenia, particularly in instances involving endothelial damage [11]. However, it remains unknown whether AT1-AA exacerbates platelet aggregation.

Therefore, in the present study we investigated whether AT1-AA isolated from preeclamptic patients augments platelet aggregation *in vitro*, and determined the characteristics of such aggregation, and further identified the responsible underlying mechanisms, which will establish a firm link between AT1-AA and hypercoagulability in preeclamptic patients.

Materials and Methods

Patient selection

This study was approved by the Institutional Committee for the Protection of Human Subjects of Shanxi Medical University Hospital. Women with a past medical history significant for endocrine or autoimmune diseases were excluded from the experiment. All pregnant women gave written informed consent prior to study participation. As per the guidelines of the International Society for the Study of Hypertension in Pregnancy [12], preeclampsia is defined by a blood pressure of $\geq 140/90$ mmHg post 20th gestational week in a previously normotensive woman, with proteinuria (at least 0.3 g per 24 h). The study includes 46 subjects divided into two groups. The studied group includes 30 preeclamptic patients (gestational age at delivery ranging from 35 to 39 weeks, average 37 weeks), and the control group includes 16 normal pregnant women (gestational age at delivery ranging 38–40 weeks, average 39 weeks). Thirty preeclamptic patients and 28 normal pregnant women were enrolled into the study in two teaching hospitals of Shanxi Medical University. Antecubital vein blood was collected and centrifuged at 4000 g for 10 min to obtain serum, which was further analyzed for the presence of AT1-AA.

Enzyme-linked immunosorbent assay

AT1-AA levels were measured as previously described [13]. Briefly, the synthetic 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) according to the second extracellular loop sequence of the AT1 receptor, was dissolved in a Na_2CO_3 solution (pH 11.0), and pipetted into 96-well plates overnight at 4°C (1 $\mu\text{g}/\text{ml}$, 50 $\mu\text{l}/\text{well}$). Each well was saturated with PBS Milk Tween (PMT) solution [0.1% (w/v) albumin bovine V, 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS-T), pH 7.4] for 1 h at 37°C. After three times wash with PBS-T, 50 μl of serum [dilutions in PMT, 1 : 10 (v/v)] was added into each well and incubated for 1 h at 37°C. After three times wash, biotinylated goat anti-human Immunoglobulin G (IgG) (1 :

1000 dilutions, Sigma, St Louis, USA) was added and incubated for 1 h at 37°C. After three times wash, streptavidin–peroxidase conjugates (1 : 2500 dilutions, Sigma) in the same buffer was added to each well and incubated for 1 h at 37°C. Finally, 2, 2-azino-di (3-ethylbenzothiazoline) sulfonic acid- H_2O_2 (Roche, Basel, Switzerland) substrate solution was added, and allowed to react for 30 min in dark at room temperature. Absorbance values at 405 nm were detected by a microplate reader (Spectra Max Plus, Molecular Devices Corp., Sunnyvale, USA). The positive/negative (P/N) ratio [(specimen optical density (OD) – blank control OD)/(negative control OD – blank control OD)] of each sample were also calculated. The positivity of the serum sample to AT1-AA was defined as $\text{P/N} \geq 2.1$; negativity was defined as $\text{P/N} \leq 1.5$.

Preparation of IgG

On the basis of enzyme-linked immunosorbent assay (ELISA) detection results, AT1-AA-positive preeclamptic patients were chosen. IgG fractions from preeclamptic patients were purified by IgG affinity column (MabTrap Kit, Amersham, Buckinghamshire, UK). As control, IgGs from AT1-AA-negative normal pregnant women (nIgG) were prepared by the same procedure.

Platelet and plasma preparation

Blood samples from 20 healthy adult volunteers (none having received steroids or anti-platelet aggregation therapy) were obtained. Blood was collected in tubes containing 3% acid citrate dextrose (1/9, v/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 10 min. Platelet-poor plasma (PPP) was obtained by further centrifugation at 1500 g for 10 min to remove remaining cells. Gel-filtered platelets were isolated from PRP by Sepharose 2B column in PIPES buffer (PIPES 5 mM, NaCl 1.37 mM, KCl 4 mM, glucose 0.1%, pH 7.0).

Platelet aggregation *in vitro*

Platelet aggregation was assessed by an aggregometer (Chrono-Log, Havertown, USA). Platelet concentration in PRP was adjusted to $3 \times 10^8/\text{ml}$ by autologous PPP. PPP was used to standardize aggregometer 100% light transmission. PRP (250 μl) was stirred at 1000 rpm, and warmed to 37°C in the aggregometer, 1–5 μl IgG was added and incubated for 20 min. Various agonists, such as 0.5 $\mu\text{g}/\text{ml}$ collagen (Nycomed Pharma, Ismaning, Germany), 2 μM adenosine 5'-diphosphate (Sigma), 5 $\mu\text{g}/\text{ml}$ adrenaline (Cayman Chemical, Ann Arbor, USA), and 1 mM arachidonic acid (final concentration, Cayman Chemical), were subsequently added. Platelet aggregation images were viewed by using a Zeiss Axiovert 135 inverted fluorescent

microscope (Zeiss, Oberkochen, Germany) and captured by a digital camera (DP70; Olympus, Tokyo, Japan).

Measurement of platelet intracellular calcium

Gel-filtered platelets were incubated with 5 μM Fluo-3/AM (Sigma) in the absence of light at 37°C for 30 min, and centrifuged at 1500 g for 15 min. Modified Tyrode's buffer was added, and the platelet concentration was adjusted to $2 \times 10^7/\text{ml}$. The platelet suspension was separated into two groups. Each group was incubated for 30 min with one of two treatments: AT1-AA (10^{-7} mol/l) or nIgG (10^{-7} mol/l). Calcium mobilization within platelets was monitored by a flow cytometer (BD Biosciences, San Diego, USA). Fluo-3/AM has an excitation light wavelength of 506 nm, and an emission wavelength of 526 nm. Each sample was activated with collagen (0.5 $\mu\text{g}/\text{ml}$) after 20 s. Fluorescence was detected for 10 min.

Statistical analysis

All values are expressed as mean \pm SD. Statistical analysis was performed with SPSS 16.0 software (IBM, USA). The

Table 1 Clinical data of patients with preeclampsia and healthy groups

Groups	Normal pregnancy ($n = 16$)	Preeclampsia ($n = 30$)
Maternal age (years)	28 (24–33)	30 (25–34)
Gestational age at sampling (weeks)	39 (38–40)	37 (35–39)
SBP (mmHg)	116 ± 14	$162 \pm 10^{**}$
DBP (mmHg)	74 ± 6.2	$102 \pm 8.1^{**}$
Proteinuria (mg/day)	63.4 ± 8.2	$476 \pm 32.3^{**}$
Platelet ($\times 10^9/\text{l}$)	231.8 ± 48.7	$159.7 \pm 65.2^{**}$

SBP, systolic blood pressure; DBP, diastolic blood pressure.

$^{**}P < 0.01$ vs. normal pregnancy.

t -test was used for comparing two independent samples. $P < 0.05$ were considered statistically significant.

Results

Sera levels of AT1-AA were markedly increased in preeclamptic patients compared with control pregnant women

The clinical data of preeclamptic patients and normal pregnant women are summarized in **Table 1**. Compared with normal pregnant women, the titers of AT1-AA detected by ELISA were markedly increased in the sera of preeclamptic patients (OD value: 0.54 ± 0.13 vs. 0.27 ± 0.12 , $^{**}P < 0.01$) [**Fig. 1(A)**]. As illustrated in **Fig. 1(B)**, only 2 of 28 normal pregnant women were AT1-AA positive (7.14%). According to the P/N value, the positive rate of the autoantibody in the preeclamptic patients was 63.33% [19/30, **Fig. 1(B)**]. These data demonstrated that AT1-AA sera levels in preeclamptic patients were significantly increased compared with those in normal pregnant women.

IgG fractions isolated from AT1-AA-positive sera of preeclamptic patients had no direct effect upon platelet aggregation

Figure 2 shows that IgG fractions isolated from AT1-AA-positive sera of preeclamptic patients (concentrations varying at 10^{-8} – 10^{-6} mol/l) had no direct effect upon platelet aggregation. Similar results were obtained at each concentration tested ($n = 6$ – 8 per concentration).

IgG fractions from AT1-AA-positive sera of preeclamptic patients enhanced collagen-induced platelet aggregation, but had no effect upon adrenaline, ADP, or arachidonic acid-induced platelet aggregation

As summarized in **Fig. 3**, IgG fractions (10^{-7} and 10^{-6} mol/l) from AT1-AA-positive sera of preeclamptic patients significantly enhanced collagen (0.5 $\mu\text{g}/\text{ml}$)-induced platelet

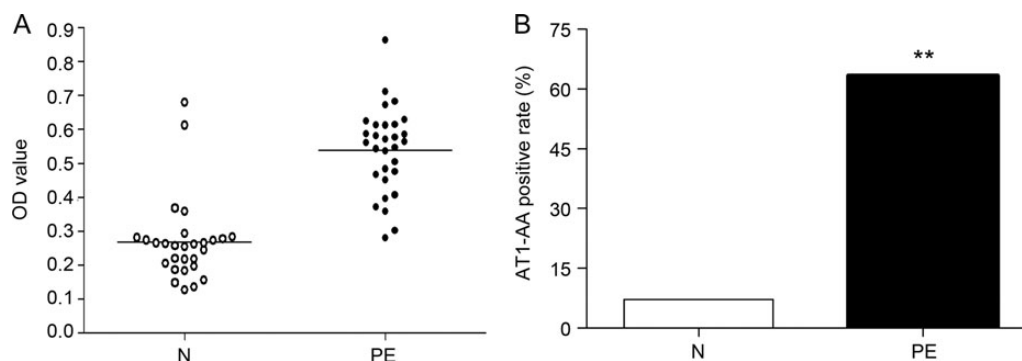


Figure 1 Sera levels of AT1-AA in the preeclamptic group and normal pregnancy group (A) The titers of AT1-AA were detected in 30 preeclamptic patients and 28 normal pregnant women using ELISA. $^{**}P < 0.01$ vs. N. (B) Percentage of AT1-AA-positive sera from two different groups was calculated. $^{**}P < 0.01$ vs. N. N, normal pregnancy group; PE, preeclamptic group; OD, optical density.

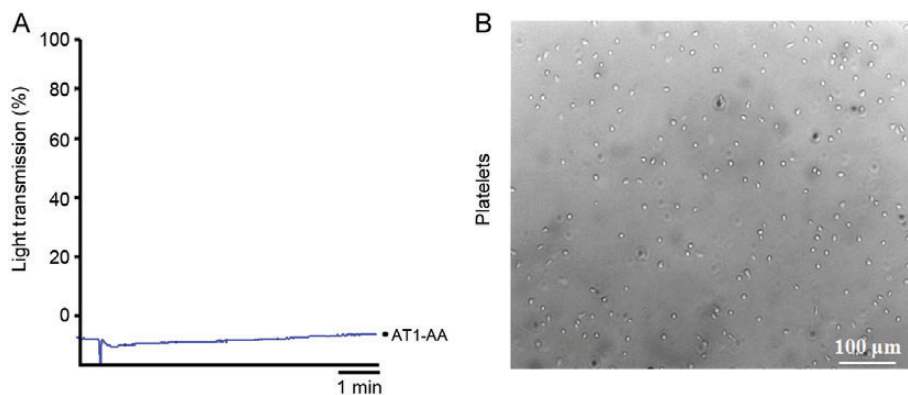


Figure 2 Effects of IgG fractions isolated from AT1-AA-positive sera of preeclamptic patients upon platelet aggregation (A) Isolated AT1-AA (10^{-8} – 10^{-6} mol/l) alone exerted no direct effect upon platelet aggregation ($n = 6$ – 8 for each concentration). (B) No platelet aggregation was found during microscopy. Pictures of aggregates were taken under a $\times 10/0.30$ NA objective lens.

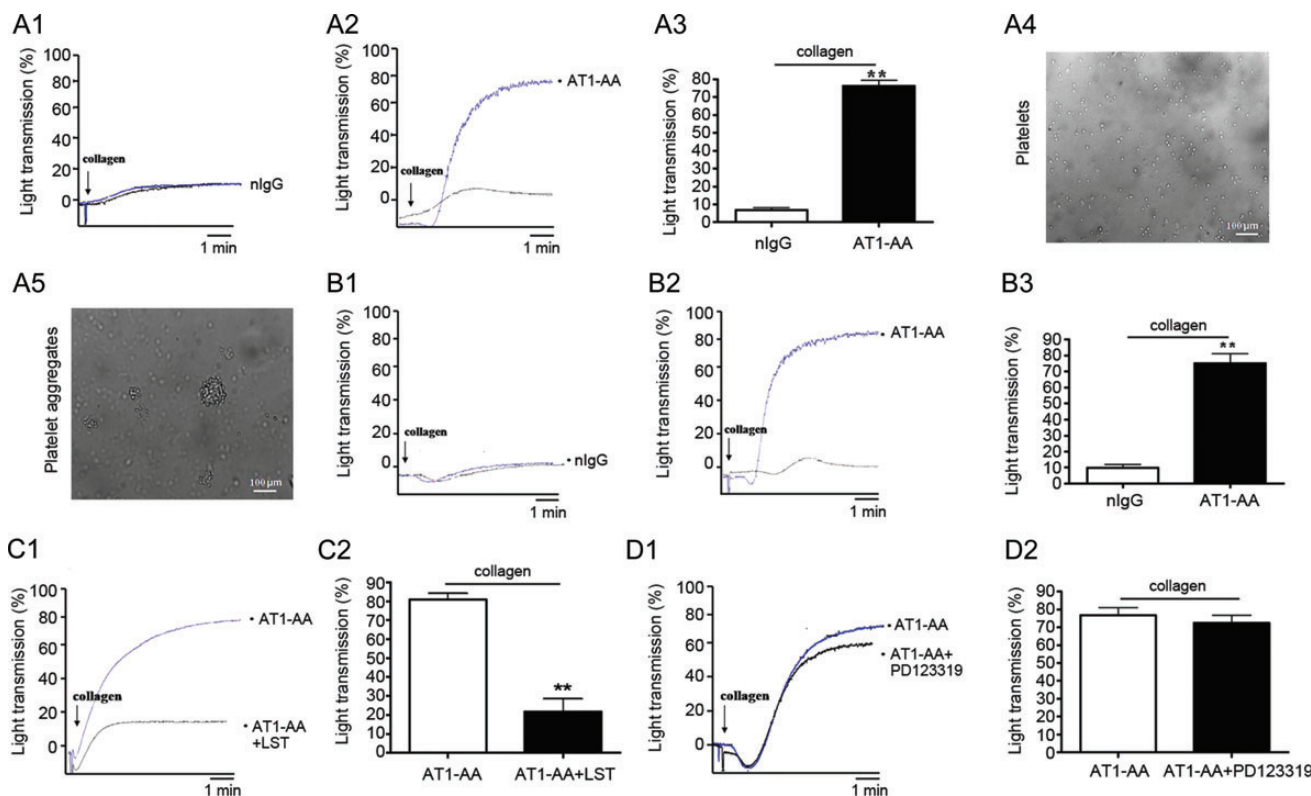


Figure 3 Effects of AT1-AA positive/negative IgG fractions upon collagen-induced platelet aggregation, with and without angiotensin 1 and 2 receptor blockade Aggregometer data tracings quantify level of platelet aggregation. AT1-AA-negative IgGs (nIgG, A1: 10^{-7} mol/l and B1: 10^{-6} mol/l) isolated from normal pregnant women sera had no effects upon collagen (0.5 μ g/ml)-induced platelet aggregation. AT1-AA-positive IgGs (A2: 10^{-7} mol/l and B2: 10^{-6} mol/l) isolated from preeclamptic patient sera significantly enhanced collagen (0.5 μ g/ml)-induced platelet aggregation. Data expressed as mean \pm SD. $n = 8$ /group. $**P < 0.01$ vs. nIgG group (A3 and B3). No platelet aggregates were induced by nIgG (10^{-7} mol/l) when examined under a microscope (A4, $\times 30/0.40$ NA objective). The enhancement of platelet aggregation was induced by AT1-AA-positive IgGs (10^{-7} mol/l) as observed by microscopy (A5, $\times 30/0.40$ NA objective). The effects of angiotensin 1 receptor blocker losartan (C1) were evaluated. Losartan (1 μ mol/l) inhibited platelet aggregation induced by AT1-AA positive IgGs (C2, $**P < 0.01$ vs. AT1-AA). The effects of angiotensin 2 receptor blocker PD123319 were evaluated (D1). PD123319 (1 μ mol/l) failed to inhibit platelet aggregation caused by AT1-AA (D2, $P > 0.05$ vs. AT1-AA). Data expressed as mean \pm SD. $n = 6$ – 8 /group. Aggregation percentages are expressed as a percentage of maximum aggregation.

aggregation [maximal platelet aggregation rate was $80.6 \pm 3.7\%$ and $82.4 \pm 4.3\%$, respectively, both $P < 0.01$, Fig. 3(A1–5 and B1–3)]. These findings suggest that

AT1-AA at concentrations $\geq 10^{-7}$ mol/l promotes collagen (0.5 μ g/ml)-induced platelet aggregation. Pretreatment with losartan (1 μ mol/l, AT1 receptor antagonist) abolished the

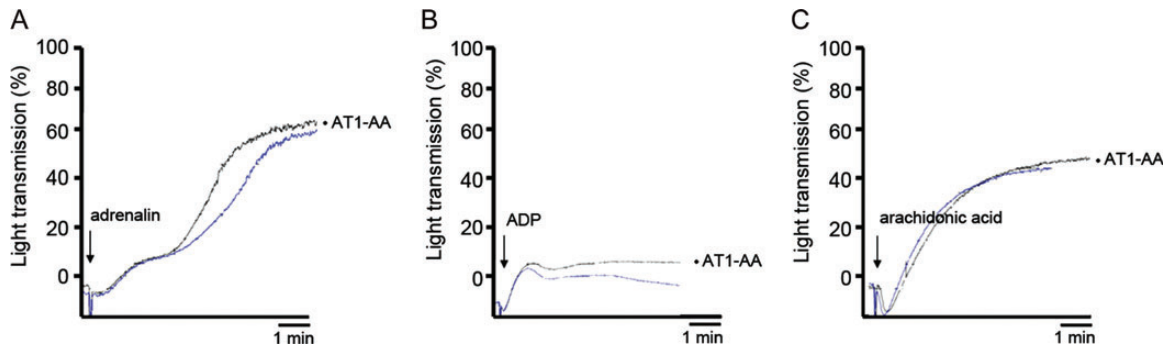


Figure 4 Effects of IgG fractions from AT1-AA-positive sera of preeclamptic patients upon adrenaline, ADP, and arachidonic acid-induced platelet aggregation. Platelet aggregation induced by (A) adrenaline (5 $\mu\text{g/ml}$), (B) ADP (2 μM), and (C) arachidonic acid (1 mM). Platelet aggregation failed to be induced by adrenaline, ADP, or arachidonic acid at AT1-AA concentrations ranging 10^{-8} – 10^{-6} mol/l. ($n = 6$ – 9 per concentration).

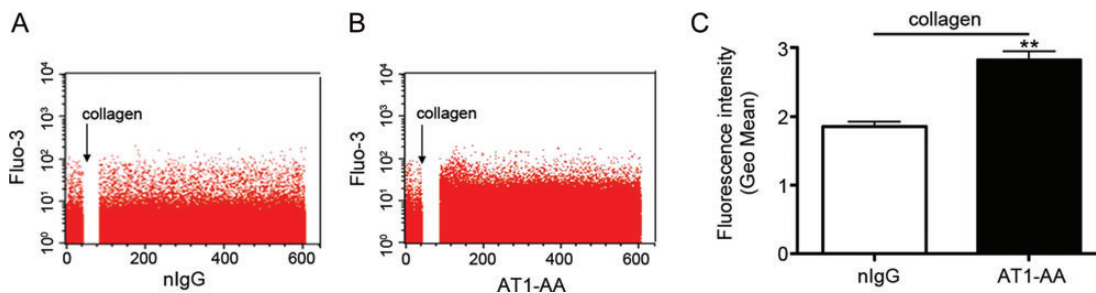


Figure 5 Effects of IgG fractions from AT1-AA-positive sera of preeclamptic patients upon collagen-induced cytosolic calcium mobilization. Collagen (0.5 $\mu\text{g/ml}$) was added 20 s later. A tracer from one representative experiment is shown on the left (A and B). There was no significant change in intracellular calcium levels when platelets were incubated with IgGs of AT1-AA-negative pregnant patients (nIgG, A). Intracellular calcium levels were markedly increased in platelets treated with IgG of AT1-AA-positive pregnant patients (B). Geo mean fluorescence intensity values were recorded. (C) Data are expressed as mean \pm SD ($n = 3$, shown on the right). ** $P < 0.01$ vs. nIgG group.

increase of collagen-induced platelet aggregation by AT1-AA [$21.73 \pm 6.80\%$ vs. $81.03 \pm 8.08\%$, $P < 0.01$, vs. AT1-AA, **Fig. 3(C1–2)**], but this effect was not abolished by PD123319 (1 μM , AT2 receptor antagonist) [$72.42 \pm 10.27\%$ vs. $76.62 \pm 11.00\%$, $P > 0.05$, vs. AT1-AA, **Fig. 3(D1–2)**]. These results provide clear evidence that IgG fractions isolated from AT1-AA-positive sera of preeclamptic patients causes significant platelet aggregation via AT1R stimulation. Importantly, as shown in **Fig. 4**, different concentrations of preeclamptic patient AT1-AA IgG fractions had no effect upon platelet aggregation induced by adrenaline [5 $\mu\text{g/ml}$, **Fig. 4(A)**], ADP [2 μM , **Fig. 4(B)**], or arachidonic acid [1 mM, **Fig. 4(C)**].

IgG fractions from AT1-AA-positive sera of preeclamptic patients augmented collagen-induced cytosolic calcium concentration

Intracellular platelet calcium levels are not markedly altered before or after platelet incubation with AT1-AA without collagen (data not shown). Pretreatment of platelets with 10^{-7} mol/l IgG fractions isolated from preeclamptic patient AT1-AA-positive sera resulted in collagen-induced elevation of free intracellular Ca^{2+} levels (**Fig. 5**). Intracellular

calcium levels were markedly increased in platelets treated with IgG fractions of preeclamptic patients, compared with those treated with control IgG fractions [$P < 0.01$, vs. nIgG group, **Fig. 5(C)**]. Platelets treated with IgG fractions of preeclamptic patients exhibited cytosolic Ca^{2+} concentrations persisted at a constant level until the termination of the experiment [**Fig. 5(B)**]. The intracellular calcium in platelets treated with IgG fractions from AT1-AA-negative pregnant women (nIgG) did not show any significant changes [**Fig. 5(A)**].

Discussion

In recent years, several studies have reported elevated AT1-AA levels in patients with preeclampsia [9], malignant hypertension [14], refractory hypertension [15], and renal transplantation hypertension [16]. We previously revealed that the persistence of AT1-AA induced the damage of vascular endothelial cell structure and function in rats [17]. As is known to all, endothelial injury promotes platelet activation and aggregation, and platelet aggregation is a fundamental step in thrombotic events. Preeclampsia is characterized by hypercoagulability. Determination of platelet aggregation

tendencies plays a critical role in the early detection of pre-eclampsia [5]. Therefore, the current study focused on the relationship between AT1-AA and platelet aggregation.

Our results demonstrated for the first time that AT1-AA isolated from preeclamptic patients enhanced collagen-induced platelet aggregation *in vitro*, similar to Ang II. However, Kappelle [7] and Utsugisawa *et al.* [8] demonstrated that Ang II promoted both independent and agonist (adrenaline, collagen, and ADP)-stimulated platelet aggregation at concentrations of 10 nM or less, with effects lost at 100 nM. In contrast, AT1-AA had no effect upon platelet aggregation independently, or with agonist at concentrations $\leq 10^{-8}$ mol/l. However, higher concentrations of AT1-AA (10^{-7} , 10^{-6} mol/l) enhanced collagen-induced platelet aggregation, while IgGs from normotensive pregnant patients exhibited no influence upon platelet aggregation. Therefore, as Ang II does not promote collagen-induced platelet aggregation at higher concentrations (Supplementary Fig. S2), we suspect that AT1-AA and Ang II exhibit different binding affinities to platelet receptors.

To further determine the active receptor, we employed losartan and PD123319, specific AT1 and AT2 receptor antagonists, respectively. Similar to Ang II, PD123319 failed to inhibit platelet aggregation caused by AT1-AA, whereas losartan completely abolished platelet aggregation, confirming the involvement of AT1 but not AT2 receptor. Pretreatment with AT₁R-EC_{II} (1 μ mol/l) completely abolished enhanced collagen-induced platelet aggregation caused by AT1-AA (Supplementary Fig. S3).

Our results showed that platelet aggregation failed to be induced by ADP (2 μ M), adrenaline (5 μ g/ml), or arachidonic acid (1 mM) at three tested AT1-AA concentrations (ranging 10^{-8} – 10^{-6} mol/l), suggesting that AT1-AA and Ang II have dissimilar mechanisms and characteristics regarding platelet aggregation. In addition, AT1-AA had no promotion agonists (ADP, adrenaline, and arachidonic acid) at higher concentration-stimulated platelet aggregation (Supplementary Fig. S4).

Collagen, ADP, adrenaline, and arachidonic acid are the typical agonists used during platelet aggregation testing. Collagen supports platelet adhesion at injury sites via von Willebrand factor, glycoprotein VI, and integrin $\alpha 2\beta 1$, and induces platelet activation via a tyrosine kinase-based signaling pathway involving the kinase Syk and phospholipase C $\gamma 2$ (PLC $\gamma 2$) [18]. Glycoprotein VI (GPVI) is one of the important members of the immunoglobulin-receptor family protein on platelets, and is a critical collagen receptor. GPVI, complexed with Fc-receptor γ -chain in the platelet membrane, interacts with sub-endothelial collagen exposed upon vessel injury to initiate platelet activation. Collagen binding to GPVI induces calcium release and activating protein kinase C (PKC) in platelets. Therefore, our results suggested that AT1-AA promoted collagen-induced platelet

aggregation through the PKC pathway (Supplementary Fig. S5). AT1-AA has been reported to exhibit agonist-like effect via that AT1R activation mediated by PKC [9]. Therefore, GPVI may be involved in the selective AT1-AA-augmented platelet aggregation response noted in the current study, suggesting a collagen-related signal transduction pathway.

Preeclampsia is associated with abnormalities in calcium metabolism, and various cell types (platelets and lymphocytes) showed increased intracellular calcium levels. [19,20]. Thway *et al.* demonstrated that AT1-AA increased intracellular free Ca²⁺ concentrations and downstream activation of Ca²⁺ signaling pathways *in vitro* [10]. To our best knowledge, we are the first to investigate platelet calcium mobilization in response to AT1-AA. We demonstrated that AT1-AA significantly increased platelet cytosolic Ca²⁺ levels. Ca²⁺ plays a crucial role in many physiological and pathological processes such as platelet activation and vascular disorders [21,22]. Many signaling mechanisms increase platelet intracellular Ca²⁺ concentration [23]. Cytosolic calcium mobilization is the major signal transduction pathway in platelet activation and stimulates enzymatic systems [24]. In the present study, AT1-AA was found not to affect basal platelet aggregation, suggesting resting intracellular platelet calcium concentrations were not altered by AT1-AA. In resting platelets, low cytosolic calcium levels are present. During collagen stimulation, calcium stored within dense granules is released into platelet cytoplasm, increasing cytosolic calcium levels. AT1-AA may augment collagen-induced platelet aggregation by increasing cytosolic Ca²⁺ concentration. Activation of PLC $\gamma 2$ to yield inositol-1, 4, 5-trisphosphate has been reported to be involved with collagen-mediated cytosolic Ca²⁺ mobilization [18]. Future investigations are necessary to provide evidence for or against PLC $\gamma 2$ activation as a responsible mechanism within this particular context.

Finally, in the current study, we demonstrate sustained AT1-AA-induced cytosolic calcium mobilization for 10 min at the conclusion of the experiment. A significant time course difference of efficacy exists between AT1-AA and Ang II. Failure of AT1-AA to induce receptor desensitization leads to the maintenance of an elevated level of cytosolic calcium concentration. However, the mechanism is still poorly understood and requires further exploration.

Supplementary Data

Supplementary data are available at *ABBS* online.

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