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Decreased Ang-(1-7) and Downregulated Intrarenal RAS May Contribute to the Direct Podocyte Injury With Proteinuria in Preeclampsia

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Abstract

The mechanisms of proteinuria development in preeclampsia (PE) are still enigmatic. Renin–angiotensin system (RAS) components may play a role. Maternal serum and urinary concentrations of angiotensin-(1-7) [Ang-(1-7)], angiotensin II (Ang II), and angiotensinogen in women with PE (n = 14), gestational hypertension (n = 14), and normal pregnancy were quantified. The alteration in these concentrations was used to evaluate their relationships with podocyturia and proteinuria in PE. In addition, the podocytes cultured in vitro were interfered in serum of preeclamptic and normotensive pregnant women, with or without Ang-(1-7). The morphologic change in podocyte was observed using a microscope. The changes in podocyte-specific proteins (nephrin, CD2-associated protein [CD2AP]), the cytoskeletal protein F-actin, the tight junction protein (ZO-1), and Mas receptor (MasR) were examined by immunofluorescence. Western blot was used to examine the expression and variation of MasR. We found that the concentrations of RAS components were associated with prepartal urinary podocyte number, random urine albumin/creatinine ratio, blood pressure, and renal function. The expression of nephrin, F-actin, ZO-1, and MasR on podocytes interfered in serum of PE was significantly decreased compared to normal control and normal pregnant serum group in vitro, yet their expression was significantly increased after coculture by 10^{-6} mol/L Ang-(1-7) and the preeclamptic serum. The expression of CD2AP had no significant difference. We concluded that decreased Ang-(1-7) and downregulated intrarenal RAS contributed to the direct podocyte injury with proteinuria in PE.

Keywords

renin–angiotensin system (RAS), Ang-(1-7), Mas, podocyte, preeclampsia

Introduction

Preeclampsia (PE) is a severe hypertensive complication of pregnancy, which is significantly related to maternal perinatal mortality and morbidity.[1] Proteinuria is a clinical feature and late maker of PE, the mechanism of which is incompletely defined.

Previous research had indicated that the pathological characterisic of PE is known as glomerular endotheliosis. Nevertheless, recent evidence supported that the derangements of slit diaphragm components resulted in foot process effacement and proteinuria.[2] The podocyte injury may occur prior to proteinuria and play an important role in the pathophysiology of PE.[2,3] The downregulation of podocyte-specific proteins (nephrin, CD2-associated protein [CD2AP]), and the cytoskeletal protein F-actin, ZO-1 [tight junction protein-1]) indicated podocyte injury. Our previous studies had also suggested that the urinary podocyte excretion, which reflected the podocyte injury, was correlated with random urine albumin/creatinine ratio (ACR) and blood pressure.[4] Podocyturia was useful for the identification of PE and normal pregnancy (NP).[4]

Renin–angiotensin system (RAS) may be a vital link in the mechanisms of albuminuria and hypertension development in PE.[5,6] A newer RAS component angiotensin-(1-7) [Ang-(1-7)] as the antagonist of Ang II was especially interesting because it

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was functionally associated with the overall mechanisms that intrinsically regulated the functions of RAS,[7-11] including on the renal system.[7] Angiotensin-(1-7) counterregulated the Ang II through combining with its specific Mas receptor (MasR), one of the G-protein-coupled receptors.[11] Accordingly, Ang-(1–7) –Mas axis may play an important role in the pathogenesis of PE.[12] Furthermore, the relationship between circulating and intrarenal RAS is very complicated in PE. Some investigations have documented that the changes in angiotensinogen (AGT) levels can modulate the activity of the RAS and urinary AGT levels reflect the activity of the intrarenal RAS[13-15] rather than ultrafiltration of the circulating RAS components.[15] The aim of this study was to investigate the relationship between podocyte injury and RAS regulation as well as their effects on proteinuria development in PE.

Materials and Methods

Specimen

The serum and urinary samples were obtained from 14 women with PE, 14 with gestational hypertension, and 13 gestational age-matched normotensive pregnant women prior to any medication for this assay. Maternal fasting blood samples were all collected before labor. The fresh first-voided morning urine samples were collected before or after labor, respectively. The enrolling and eliminant criteria of these participants and the pretreatment of the clear serum and urinary supernatants were the same as our previous study.[4] The process of sample collection was aseptic for follow-up in vitro. The study was approved by the local ethics committee before initiation and consistent with ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Enzyme-Linked Immunosorbent Assay for Serum and Urinary RAS Components

Serum and urinary Ang-(1-7) (RapidBio Inc, USA), Ang II (ASSAYPRO, USA), and AGT (IBL, Japan) concentrations were measured in duplicate with commercially available enzyme-linked immunosorbent assay kits according to the manufacturers' instructions. The minimal detectable doses of Ang-(1-7), Ang II, and AGT were 5, 30, and 0.31 ng/mL, respectively. The interassay and intra-assay coefficients of variation were less than 11% and 9% for Ang-(1-7), 7.1% and 5.0% for Ang II, and range from 4.3% to 7.0% and 4.4% to 5.5% for AGT, respectively. The correlation coefficient of standard curves was >0.99.

Immunofluorescence for Quantification of Postpartum Urinary Podocytes

Fresh first-voided morning urine (50-100 mL) 1 week after labor was collected for 3 consecutive days, and the urinary podocytes number was counted daily as in the previous description.[4] The urinary sediment was incubated with 10 μg/mL antihuman podocalyxin monoclonal antibody (R&D) for 60 minutes at 37°C and with Cy2-labeled anti-mouse Immunoglobulin G (IgG; United Chemicon) at a dilution of 1:100 for 30 minutes at 37°C. Next, cell nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich) for 5 minutes at 5 μg/mL. Immunofluorescence microscopy was used for inspection. The cells with both podocalyxin and nuclei positive were defined as entire podocyte. The podocyte number was shown as the average number of podocytes from 3 times counting for each individual with cells/mL of urine. Quantitative analysis was performed by 2 of the authors in a blind fashion. Quantitation of postpartum 24-hour urinary protein was also estimated by standard methods.

Mouse Podocytes Cell culture

The conditionally immortalized mouse podocyte cell line[16] was provided by Chuanming Hao (Division of Nephrology, Huashan Hospital, Fudan University, Shanghai, China) and was originally a gift from Peter Mundel (Department of Medicine and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, New York, New York). The mouse podocytes were cultured as previously described.[17] The culture dishes were precoated with type I collagen (Sigma-Aldrich) at 37°C for 1 hour and then the podocytes were inoculated and cultured at 33°C in Roswell Park Memorial Institute-1640 medium (fetal bovine serum [FBS]; Gibco BRL, Gaithersburg, Maryland) containing 10% FBS (Gibco BRL), 100 U/mL penicillin (Sigma-Aldrich), 100 U/mL streptomycin (Sigma-Aldrich), and 50 U/mL recombinant interferon γ (IFN-γ; Sigma-Aldrich) in a humidified incubator with 5% CO_2 to promote proliferation. The culture medium was replaced twice or thrice a week with decreasing IFN-γ until at a level of 10 U/mL. Subsequently, the proliferative podocytes were transferred at 37°C without IFN-γ for 10 to 14 days to induce differentiation. The podocytes' shape/structure was observed under the inverted phase contrast microscope and the differentiated podocytes at 37°C were used for follow-up experiments.

Mouse Podocytes Cell Treatment and Grouping

The mouse podocytes were incubated with 10% FBS or serum of NP women, serum of women with PE, or coincubated with 10^{-6} mol/L Ang-(1-7) and 10% serum of women with PE for 48 hours, respectively. Accordingly, they were separated into 4 groups: normal control (NC), NP, PE, and Ang-(1-7) + PE. They were starved for 12 hours before experiments.

Immunofluorescence for Alteration of Mouse Podocyte's Specific Proteins Nephrin, CD2AP, and Cytoskeletal Proteins F-Actin, ZO-1

The differentiated mouse podocytes culturing on sterile coverslips were washed thrice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton X-100 for 15 minutes, washed thrice again and naturally dried, and then blocked with 5% bovine serum

antigen for 30 minutes at room temperature. The cells were incubated with podocyte-specific protein antinephrin (1:50; Santa Cruz Biotechnology), anti-CD2AP (1:400; Abcam), cytoskeletal protein anti-F-actin (1:600; Sigma-Aldrich), anti-ZO-1 (1:50; Santa Cruz Biotechnology), and anti-MAS1 (1:50; Santa Cruz Biotechnology) polyclonal antibody, respectively, at 37°C for 60 minutes. After washing with PBS, cells were stained with Cy2 (or Cy3)-conjugated anti-rabbit IgG (1:200; United Chemicon) at 37°C for 30 minutes. Mounting was done with 4,6-diamino-2-phenyl indole (Sigma-Aldrich) to stain nuclei. Podocytes were observed and photographed by fluorescence microscopy (Olympus IX70 and NIKON TE2000E, Tokyo, Japan). Each group podocytes were provided with 3 duplicates and the experiments were all repeated 3 times.

Western Blot Analysis for Expression of MasR on Podocyte

The mouse podocytes were washed twice with precooling PBS and collected in a lysate containing a proteinase inhibitor cocktail. The lysate adding with loading buffer was boiled to denaturation at 100°C for 5 minutes. Protein samples (60 μg) of each group were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked at room temperature with 5% skimmed milk in Tris-buffered saline Tween-20 (TBS-T) for 1 hour and then incubated at 4°C overnight with the MasR (anti-MAS1, 1:200; Santa Cruz Biotechnology) and β-actin (1:3000; Sigma-Aldrich) primary antibodies. The membranes were washed thrice for 10 minutes every time with TBS-T, followed by incubation with horseradish peroxidase–conjugated secondary antibodies (1:3000; Santa Cruz Biotechnology) for 1 hour at room temperature. The intensities of the immunoreactivity were detected with the enhanced chemiluminescence kit (Bio-Rad). The images were developed on X-ray film and normalized to β-actin with computerized analysis. The experiments were repeated at least 3 times.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (parametric data) or median (interquartile range; nonparametric data). One-way analysis of variance, Student-Newman-Keuls, and Pearson correlation were used for parametric data, yet the Mann-Whitney U test and Spearman correlation were used for nonparametric data. All P values were 2 tailed, and $P < .05$ was considered statistically significant. SPSS 13.0 was used for all statistical analyses.

Results

Serum and Urinary Ang-(1-7), Ang II, and AGT Levels of the Participants

Serum and urinary Ang-(1-7) concentrations in women with PE (serum: 53.88 $\pm$ 17.97 pg/mL; urine: 69.99 $\pm$ 19.09 pg/mL)

were all significantly lower than in gestational hypertension (serum: 70.96 $\pm$ 19.83 pg/mL, $P < .05$; urine: 98.78 $\pm$ 23.99 pg/mL, $P = .001$) and NP groups (serum: 72.49 $\pm$ 20.59 pg/mL, $P < .05$; urine: 92.91 $\pm$ 18.04 pg/mL, $P < .01$). There was no significant difference between gestational hypertension and NP (Figure 1A). Compared with the gestational hypertension (serum: 44.20 $\pm$ 41.41 pg/mL, $P > .05$; urine: 68.95 $\pm$ 26.54 pg/mL, $P > .05$) and the normotensive groups (serum: 39.89 $\pm$ 40.38 pg/mL, $P > .05$; urine: 48.77 $\pm$ 22.95 pg/mL, $P > .05$), there were no significant differences in serum and urinary Ang II concentrations in PE (serum: 52.53 $\pm$ 31.69 pg/mL; urine: 67.53 $\pm$ 25.49 pg/mL; Figure 1B). Serum and urinary AGT concentrations in women with PE (serum: 69.47 $\pm$ 18.68 μg/mL; urine: 41.01 $\pm$ 64.29 ng/mL) were all lower than in gestational hypertension (serum: 82.93 $\pm$ 12.34 μg/mL, $P < .05$; urine: 86.60 $\pm$ 62.75 ng/mL, $P > .05$) and NP groups (serum 90.94 $\pm$ 14.92 μg/mL, $P = .001$; urine 116.21 $\pm$ 65.59 ng/mL, $P < .01$; Figure 1C). Furthermore, the differences in clinical profiles including shorter gestational weeks, lower infant weights at birth, higher serum urea nitrogen, serum creatinine, uric acid, and lower estimated glomerular filtration rate and creatinine clearance had confirmed the existence of PE in our previous study.[4]

Comparison of Antepartum and Postpartum Urinary Podocyte, Protein Excretion, and Blood Pressure

It had been demonstrated that antepartum podocyturia, the random urine ACR, and proteinuria were significantly higher in the PE group than other 2 groups.[4] In the current study, we found that the postpartum podocyturia decreased most obviously ($P < .001$) in the PE group, although it all decreased and there was no significant difference among the 3 groups. The postpartum 24-hour urine protein excretion concomitant gradually decreased. The postpartum blood pressure was also significantly decreased in PE and the gestational hypertension groups but was still higher than that of postpartum normotensive control. There was no significant difference between antepartum and postpartum normotensive control (Table 1).

Univariate Correlation Among Clinical Profiles, Podocyturia, and RAS Components

Serum Ang-(1-7) has significant negative correlation with podocyturia ($P < .05$). Serum and urinary Ang-(1-7) all correlated inversely with ACR (serum $P < .01$; urinary $P = .006$). Moreover, serum AGT was also inversely associated with ACR ($P < .05$), BUN ($P < .05$), creatinine ($P < .01$), systolic blood pressure (SBP), and diastolic blood pressure ($P = .001$) but positively correlated with estimated glomerular filtration rate ($P < .001$) and endogenous creatinine clearance rate (Ccr; $P < .05$). Urinary AGT was inversely associated with podocyturia ($P < .05$) and SBP ($P < .05$) but positively correlated with Ccr ($P < .05$; Table 2). Additionally, the positive correlations were found between serum Ang II and urinary Ang II (r/P: 0.632/ <.001; Figure 2A), serum AGT, and urinary AGT (r/P: 0.386/
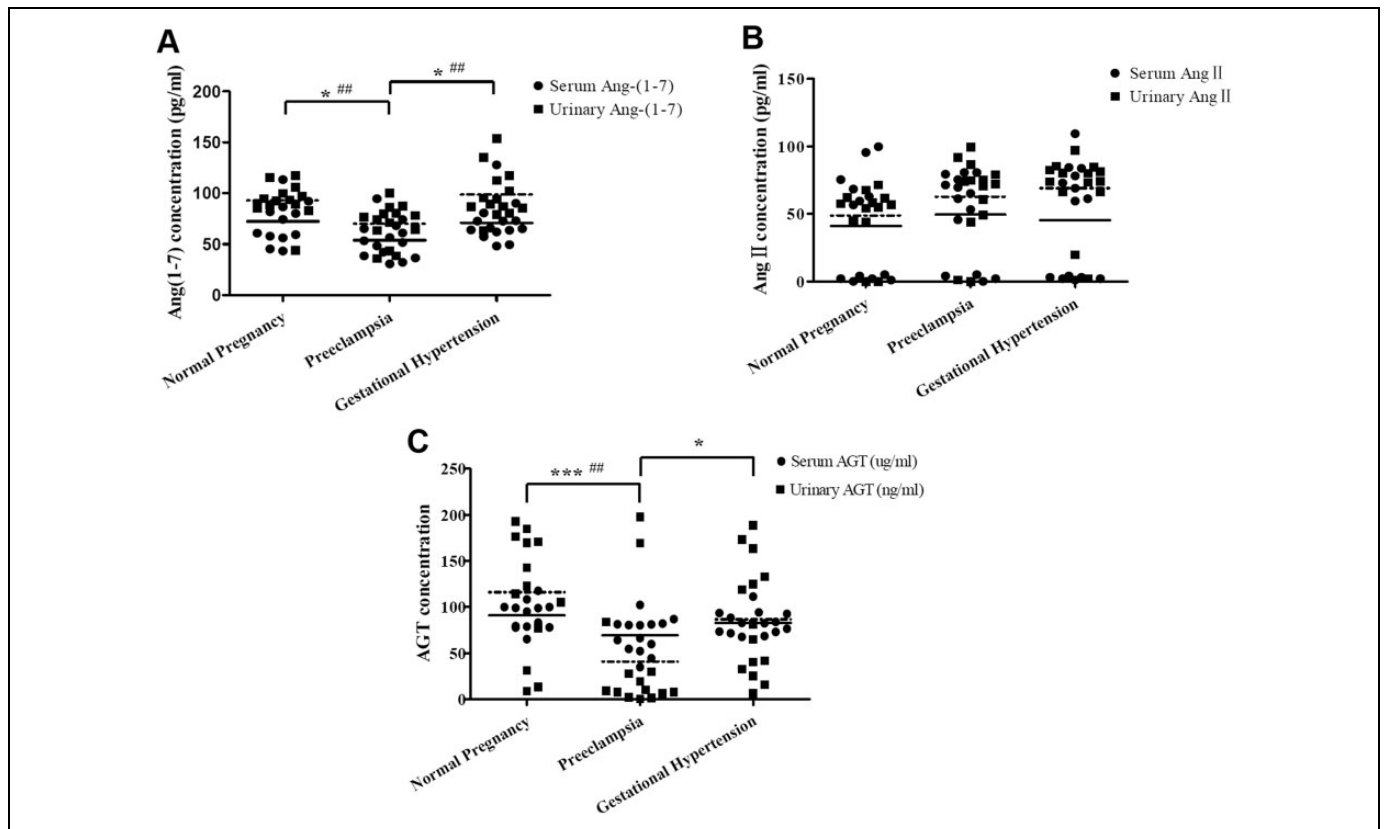
Figure 1. The concentrations of serum and urinary angiotensin-(1-7) (Ang-[1-7]) (A), angiotensin II (Ang II; B), and angiotensinogen (AGT; C). *P < .05, **P < .01, ***P ≤ .01 compared in serum; ##P < .01 compared in urine. The solid lines represent mean in serum and the dashed lines represent mean in urine.

Table 1. Comparison of Antepartum and Postpartum Urinary Podocytes Number, Proteinuria, and Blood Pressure.[a,b]

	Normal Pregnancy (n = 13)	P	Preeclampsia (n = 14)	P	Gestational Hypertension (n = 14)
Podocyturia, cells/mL					
Antepartum[4]	0.52 (0.01-1.5)	<.001	3.10 (2.37-5.66)[c]	.02	1.41 (0.71-3.73)[d,e]
Postpartum	0.08 (0-0.34)	.07	0.45 (0-1.76)[f]	.6	0.19 (0-0.53)
Proteinuria (g/24 h)					
Antepartum[4]	No data		2.39 (0.82-7.33)	<.001	0.18 (0.16-0.25)[f]
Postpartum	No data		1.37 (0.84-2.91)		No data
Blood pressure, mm Hg					
Antepartum SBP[4]	117.31 ± 9.04	<.001	150.71 ± 19.30[c]	.5	146.21 ± 9.36[c]
DBP[4]	74.23 ± 5.72	<.001	98.21 ± 8.68[c]	.4	96.29 ± 7.78[c]
Postpartum SBP	113.08 ± 4.80	<.001	125.42 ± 11.52[c,f]	.9	124.86 ± 8.47[c,f]
DBP	71.54 ± 5.55	.003	80.75 ± 8.85[f,g]	.7	79.57 ± 6.98[f,g]

Abbreviations: DBP, diastolic blood pressure, SBP, systolic blood pressure.
[a]All antepartum data were cited by our previous study.[4]
[b]Values are expressed as mean ± standard deviation (SD; parametric data) or median (interquartile range; nonparametric data).
[c]P < .001 compared to antepartum normotensive pregnancy.
[d]P < .05 compared to antepartum normotensive pregnancy.
[e]P < .05 compared to antepartum preeclampsia.
[f]P < .001 compared to antepartum preeclampsia.
[g]P < .01 compared to antepartum normotensive pregnancy.

Table 2. Univariate Correlation Among Clinical Profiles, Podocyturia, and Ang-(1-7) Concentrations as Assessed by Pearson or Spearman Correlation Coefficient.[a,b]

Parameter	*r/P* Value (n = 41)								
	Podocyturia	ACR	BUN	SCr	UA	eGFR	Ccr	SBP	DBP
Serum Ang-(1-7)	−0.358/.032	−0.410/.008	−0.198/.221	−0.291/.069	−0.440/.004	0.249/.126	0.182/.262	−0.196/.218	−0.287/.069
Urinary Ang−(1−7)	−0.292/.084	−0.423/.006	−0.272/.089	−0.296/.063	−0.195/.228	0.277/.088	0.226/.161	−0.310/.049	−0.140/.381
Serum AGT	−0.167/.331	−0.358/.022	−0.338/.033	−0.405/.009	−0.302/.058	0.540/<.001	0.367/.020	−0.509/.001	−0.493/.001
Urinary AGT	−0.399/.016	−0.140/.384	−0.306/.055	−0.282/.078	−0.245/.128	0.226/.167	0.313/.049	−0.345/.027	−0.314/.045

Abbreviations: ACR, albumin/creatinine ratio; AGT, angiotensinogen; Ang (1-7), angiotensin-(1-7); BUN, blood urea nitrogen; Ccr, endogenous creatinine clearance rate; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; SCr, serum creatinine; UA, uric acid.
[a]*r* and *P* values were given.
[b]The data of antepartum podocyturia and clinical profiles (BUN, SCr, UA, eGFR, Ccr, SBP, and DBP) were cited by our previous study[4] for present analysis.
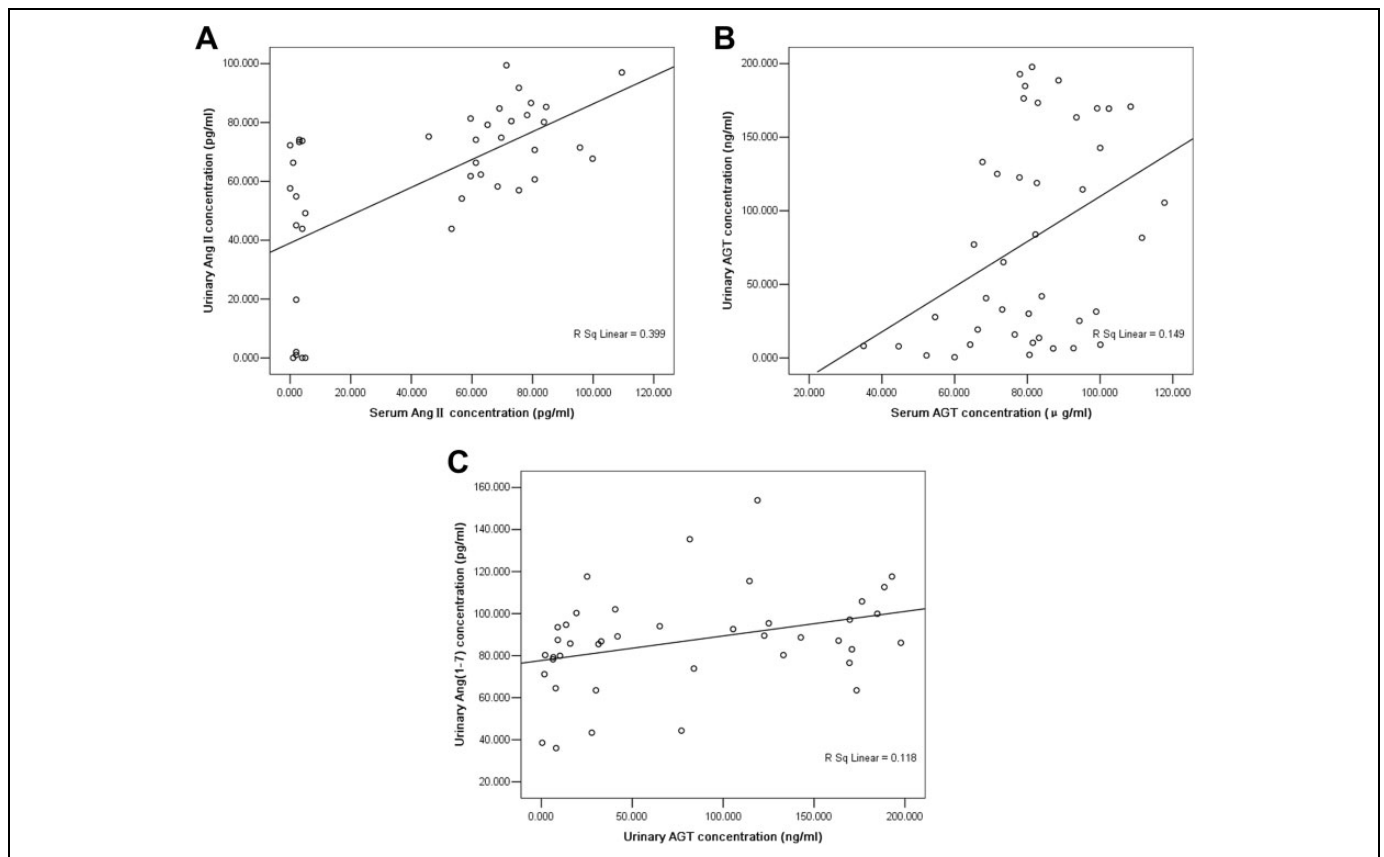
Figure 2. The correlation between serum and urinary angiotensin II (Ang II; A); serum and urinary angiotensinogen (AGT; B); urinary angiotensin-(1-7) (Ang-[1-7]) and urinary AGT (C).

.013; Figure 2B), as well as urinary Ang-(1-7) and urinary AGT (*r/P*: 0.343/.028; Figure 2C).

The Role of Serum and Urinary Ang-(1-7) and AGT in Identifying PE

The sensitivity, specificity, and other indices of different cutoff values of serum and urinary Ang-(1-7) were evaluated. The cutoff value with the highest Youden index was defined as the optimization. So, the cutoffs of serum Ang-(1-7) ≤55 pg/mL and urinary Ang-(1-7) ≤80 pg/mL were considered to be very accurate predictors of PE (Table 3). Different combinations of serum and urinary Ang-(1-7) in parallel or in series may reinforce the identification of PE (Table 4). These findings revealed that Ang-(1-7) and AGT may be participants in podocyte injury and valuable for identifying PE.

Table 3. The Cutoff Values of Serum and Urinary Ang-(1-7) With the Highest Youden Index for Preeclampsia Diagnosis.

Cutoff Value, pg/mL	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Agreement Rate (%)	Youden Index, r	Positive LR	Positive LR
Serum Ang-(1-7)								
55	57.14	84.62	80	64.71	70.37	0.42	3.71	0.51
Urinary Ang-(1-7)								
80	71.43	92.31	90.91	75.00	81.48	0.64	9.29	0.31

Abbreviations: Ang (1-7), angiotensin-(1-7); LR, likelihood ratio.

Table 4. Different Sensitivity and Specificity Using Serum and Urinary Ang-(1-7) alone, in Parallel or in Series.

	Test Method	Sensitivity (%)	Specificity (%)
1	Urinary Ang-(1-7) $\leq$ 80 pg/mL	71.43	92.31
2	Serum Ang-(1-7) $\leq$ 55 pg/mL	57.14	84.62
1 and 2	In parallel	87.75	78.11
1 and 2	In series	40.81	98.82

Abbreviation: Ang (1-7), angiotensin-(1-7).

Observation of Podocyte Morphology In Vitro

The proliferative mouse podocytes at 33°C were shown to have a cobblestone appearance or short clostridial form with small cell body and less cytoplasm, with little or no foot process (Figure 3A). The differentiated mouse podocytes at 37°C for 10 to 14 days stretch out several long fusiform foot processes with gradually enlarged cell body and abundant cytoplasm. The multiple-stagefoot processes extended to stellate or complexly scrambled arborization and interlaced each other to form a network (Figure 3B). After normal culture for 48 hours with 10% FBS, although cells grew closely to 90% confluence and were arranged tightly, there was no evident change in morphology (Figure 3C). The morphous quality of mouse podocytes incubated with 10% serum of NP women for 48 hours still showed no evident change other than intensive growth (Figure 3D). Cells incubated for 48 hours with 10% serum of women with PE showed shrinking volume, attenuated tension, and disordered arrangement; cell body shrinkage to clumping; foot process shortens, decreases, or disappears; and some cells go through apoptosis and shedding (Figure 3E). The mouse podocytes cocultured for 48 hours with 10^{-6} mol/L Ang-(1-7), and 10% serum of women with PE (Ang-(1-7) + PE) showed similar morphous characteristics as those in the PE group but the changed extent was less than that of the PE group (Figure 3F).

Protective Effect of Ang-(1-7) on the Expression of Mouse Podocyte's Specific Proteins Nephrin, CD2AP, and Cytoskeletal Proteins F-actin, ZO-1, in Podocytes Induced by PE Serum In Vitro

Nephrin and CD2AP are capital podocyte-specific proteins that play an important role in maintaining the integrity of slit diaphragm in podocyte. The downregulation of these podocyte-specific proteins indicated podocyte injury and they were associated with proteinuria. The expression of nephrin was evident in mouse podocytes with NC and with serum of NP (Figure 4A1 and B1). The nephrin expressed most weak even no expression in mouse podocytes cultured with PE serum (Figure 4C1). However, nephrin was still expressed more intensely in mouse podocytes cocultured with 10^{-6} mol/L Ang-(1-7) and 10% serum of women with PE (Ang-(1-7) + PE) than in those with PE only (Figure 4D1), although the fluorescence intensity was slightly weaker than in the NC and NP groups. It means that Ang-(1-7) could rescue the downregulation of nephrin induced by PE serum. However, the CD2AP was expressed in mouse podocytes of 4 groups and their fluorescence intensity showed no evident differences, although the tensile force of podocytes was weaker and foot process was shorter in PE than in other groups (Figure 4A2-D2).

The distribution and rearrangement of cytoskeletal proteins may induce the ordered stress fibers to a reticular formation and then lead to destruction of filtration barrier and proteinuria. As shown in Figure 4A3-D3, the expression of F-actins in mouse podocytes in NC and NP groups was clearly filamentous and tightly arranged. The myofilaments were polar contribution (Figure 4A3 and B3). In contrast, the cytoskeleton was destroyed and the F-actins were partly or completely depolymerized in the PE group. Its filamentous structure was unclear, interrupted, or had disappeared (white arrow in Figure 4C3). However, these changes were reversed by treatment with Ang-(1-7). The filamentous structures were still seen clearly and the expression of F-actins was ameliorated in Ang-(1-7) + PE group (Figure 4D3).

ZO-1 was fairly continuous with a linear distribution along the cell membrane in NC and NP groups (white arrow in Figure 4A4 and B4). In contrast, the expression of ZO-1 was weakly positive and some cells were not expressed in PE (Figure 4C4). However, the linear distribution of ZO-1 and ameliorative expression was seen in mouse podocytes in the Ang-(1-7) + PE group, although partly discontinuous (Figure 4D4).

Effect of Ang-(1-7) on the Expression of MasR in Podocytes Induced by PE Serum

Mas receptor was significantly positively expressed in mouse podocytes in NC and NP groups (Figure 4A5 and B5). No evident difference was seen between NC and NP groups. In contrast, the expression of MasR was extremely weak, even
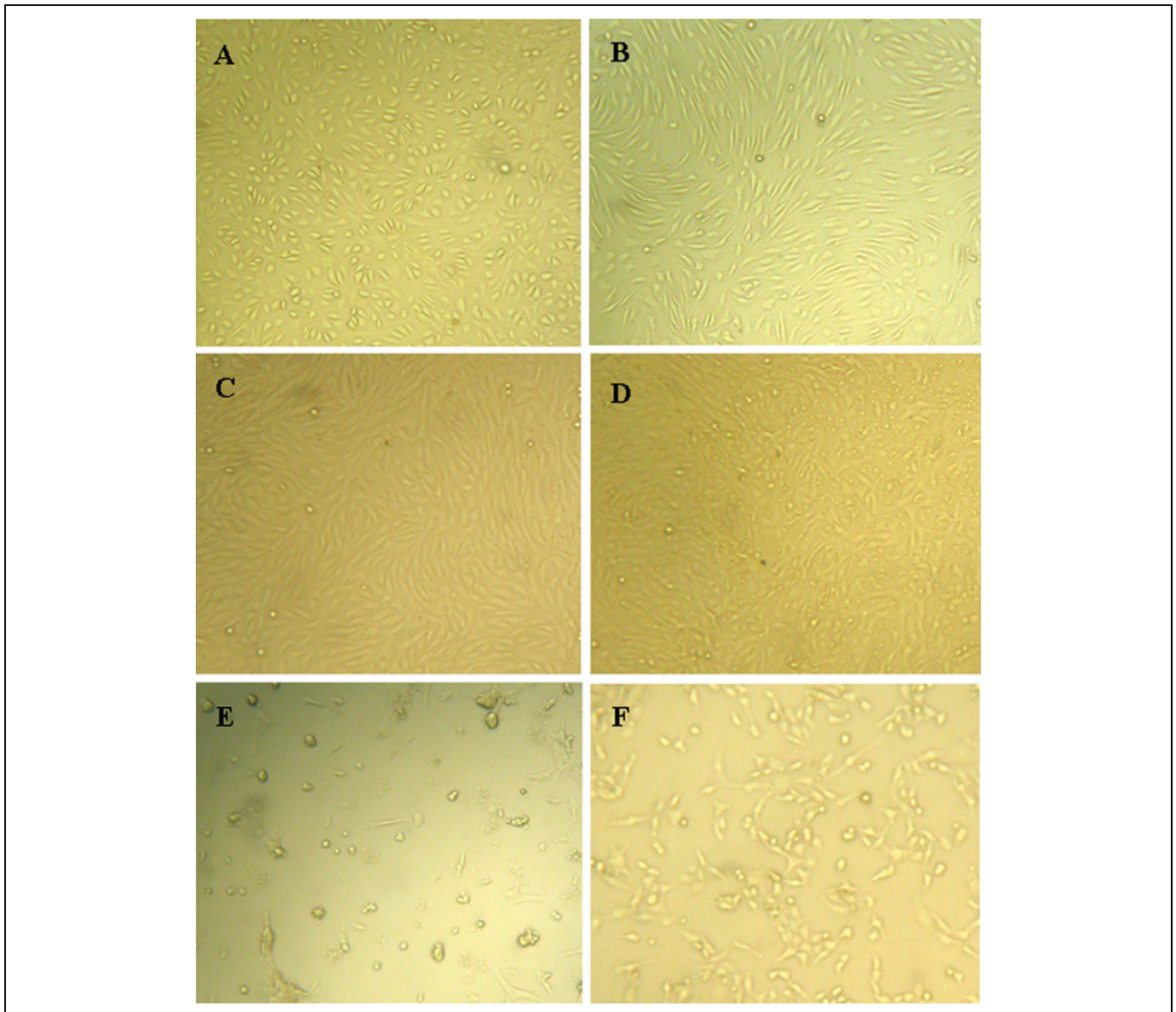
Figure 3. The morphologic change in podocytes in vitro under the inverted phase contrast microscope (×200): (A) proliferative podocytes at 33°C; (B) differentiated podocytes at 37°C; (C) normal control (NC): cultured with 10% fetal bovine serum (FBS) for 48 hours; (D) normal pregnancy (NP): cultured with 10% serum of NP women for 48 hours; (E) preeclampsia (PE): cultured with 10% serum of women with PE for 48 hours; (F) Ang-(1-7) + PE: cocultured with 10^{-6} mol/L Ang-(1-7) and 10% serum of women with PE for 48 hours.

though there was no expression in mouse podocytes in PE (Figure 4C5). However, the MasR was still positively expressed in Ang-(1-7) + PE, although cell body grew downward and secondary foot process decreased or disappeared (Figure 4D5).

In addition, we also detected the expression of MasR by Western blot analysis (Figure 5). Its changing tendency among 4 groups was coincident with immunofluorescence detection. The MasR expressed in mouse podocytes in NC (MAS1/β-actin: 1.11 $\pm$ 0.02) and NP groups (MAS1/β-actin: 1.12 $\pm$ 0.03) showed no significant difference. The expression of MasR was significantly decreased in mouse podocytes in PE (MAS1/β-actin: 0.53 $\pm$ 0.03, $P < .05$) than in NC and in NP

groups. However, it was reversed by Ang-(1-7) (Ang-(1-7) + PE: MAS1/β-actin: 1.15 $\pm$ 0.05, $P < .05$ vs PE). These results meant that the treatment with Ang-(1-7) could upregulate the expression of MasR in mouse podocytes induced by PE serum.

Discussion

The onset of hypertension and proteinuria, which is associated with renal injury, is critical for the diagnosis of PE.[18] Glomerular endotheliosis, a typical pathological change in PE, also exists in women with gestational hypertension without proteinuria and in healthy pregnant women. Therefore, a look from podocyte is a novel renal perspective of PE recently.[19] Active
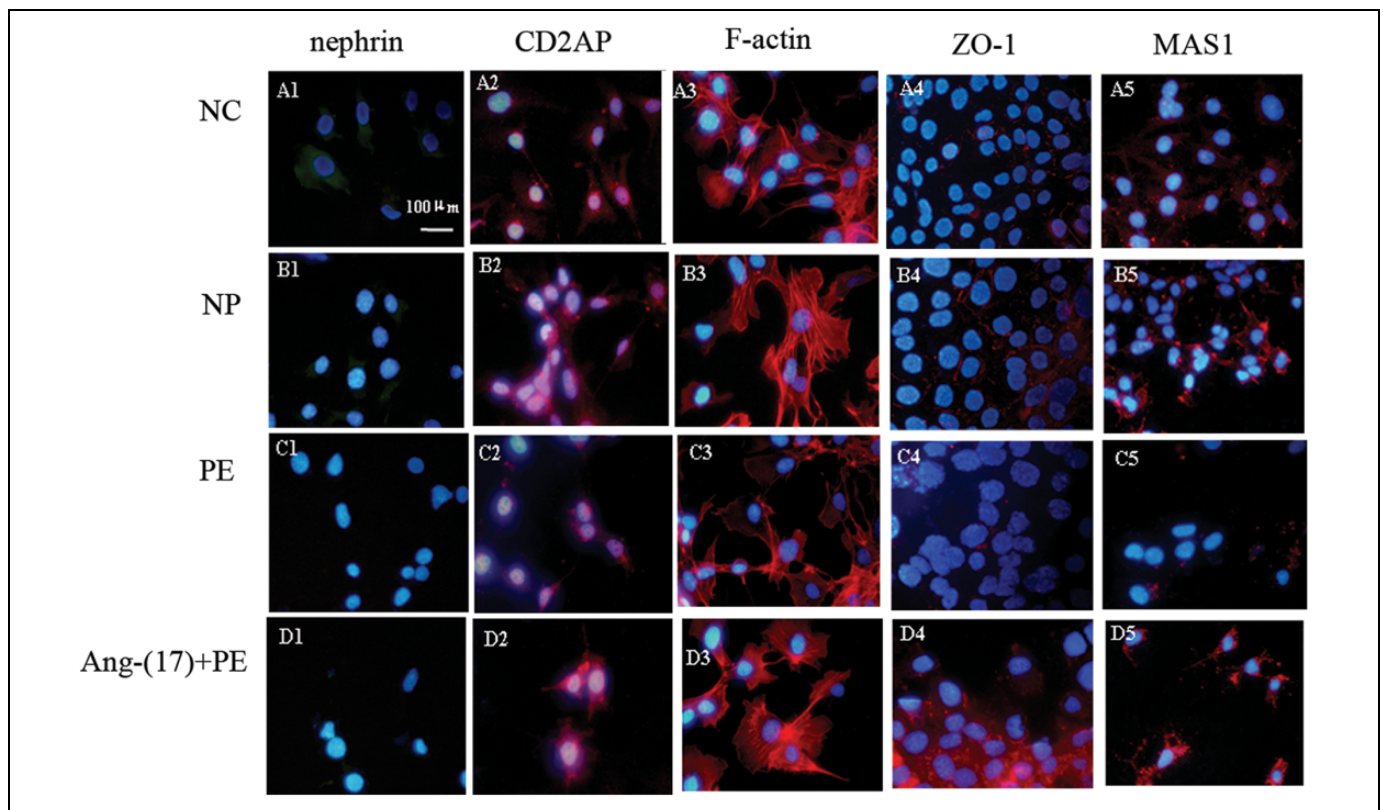
Figure 4. Immunofluorescence for the expression of nephrin, CD2-associated protein (CD2AP), F-actin, ZO-1, Mas receptor (MasR) in podocytes induced by preeclampsia (PE) serum (×400). 1, Nephrin was displayed as green; 2, was displayed as red; 3, F-actin was displayed as red; 4, ZO-1 was displayed as red; 5, Mas receptor was displayed as red. Note: (A) normal control (NC): cultured with 10% fetal bovine serum (FBS); (B) normal pregnancy (NP): cultured with 10% serum of NP women; (C) preeclampsia (PE): cultured with 10% serum of PE women; (D) angiotensin-(1-7) (Ang-[1-7]) + PE: cocultured with 10⁻⁶ mol/L Ang-(1-7) and 10% serum of PE women.

research found that podocyte pathophysiology in PE provides unique and exciting possibilities for improved diagnostic accuracy. The detection of urinary podocytes or their markers may promote the prediction and diagnosis of PE.[2] Moreover, the trigger mechanism of podocyte injury is still unclear.

Angiotensin-(1–7), as a vasodilator, could counteract the vasoconstrictive effect of Ang II, which is a key heptapeptide of RAS. It binds to the specific G-protein-coupled MasR and then plays an important role in counterregulation. The final effect of RAS may reflect a precise balance between these 2 arms.[20,21] Interestingly, the vasodilator response, kidney concentration, immunocytochemical expression, and urinary excretion of Ang-(1-7) were all increased during NP compared to normotensive no pregnancy.[22-24] However, the levels of plasma Ang-(1-7) in PE decreased significantly.[25] This information provided important evidence that Ang-(1-7) may play a critical role in the pathogenesis of PE. The increased Ang-(1-7) may be a protective factor for avoiding PE in NP. The present results suggest that serum and urinary Ang-(1-7) concentration all significantly decreased in PE, and we made a novel observation that the serum Ang-(1-7) was inversely correlated with podocyturia and ACR. The urinary Ang-(1-7) was also inversely correlated with ACR. It may be explained that the decreased Ang-(1-7) reduced the vasodilator response and then

increased the vasoconstrictor response and sensitivity of vessel to Ang II.[26] Although there was no significant difference in Ang II levels among the 3 groups, it still resulted in hypertension, podocyturia, and proteinuria in PE.

In addition, this study also found that serum and urinary AGT concentrations were all lower in women with PE. Serum AGT was inversely associated with ACR, renal function, and blood pressure. Urinary AGT was inversely associated with podocyturia. Studies confirmed that urinary AGT is a potential novel biomarker of the intrarenal RAS, which accurately reflects intrarenal RAS activity.[13,27,28] Urinary AGT has been used to study the intrarenal RAS in some hypertensive and renal diseases.[27,29-32] The excretion of urinary AGT might provide a specific index of intrarenal Ang II activity.[33] However, the levels of Ang II and renin in preeclamptic uteroplacental or decidual structures were significantly higher than in NP women.[6,34] According to these studies, we supposed that in PE, the circulating and intrarenal RAS might be downregulated, which was to accommodate the upregulated local uteroplacental RAS, and only the imbalance of this delicate equilibrium resulted in podocyte injury and proteinuria. The interaction of various RAS components and the relationship between circulating and local RAS may play a causal role in the etiology of PE. In our study, proteinuria is the manifestation
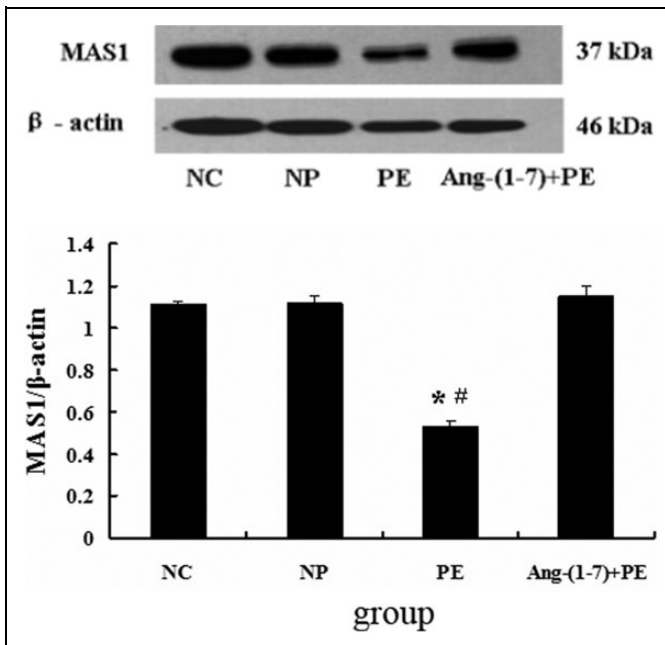
Figure 5. Western blot detection for the expression of Mas receptor (MasR) in podocytes induced by preeclampsia (PE) serum. Note: (A) normal control (NC): cultured with 10% fetal bovine serum (FBS); (B) normal pregnancy (NP): cultured with 10% serum of NP women; (C) preeclampsia (PE): cultured with 10% serum of PE women; (D) angiotensin-(1-7) (Ang-[1-7]) + PE: cocultured with 10^{-6} mol/L Ang-(1-7) and 10% serum of PE women. *P < .05 compared with NC and NP; #P < .05 compared with Ang-(1-7) + PE.

and result of renal injury in PE, and RAS component (including AGT) is the cause of proteinuria that we explored. So we measured urinary Angiotensinogen in patients with PE who have proteinuria into critical examination, and the assumption that this reflects renal production is imperative.

Our previous research demonstrated that the number of urinary podocytes in women with PE is significantly higher than in women with gestational hypertension or NPs before labor.[4] The present study contrasted the antepartum and postpartum changes including podocyturia, proteinuria (g/24 hours), and blood pressure, which suggested that the postpartum podocyturia decreased most obviously in the PE group and was accompanied by decreased proteinuria and blood pressure when this disorder has begun to recuperate due to placenta and fetus removing, yet not fully recovered. These results are essentially consistent with the findings that podocyturia in patients with PE is transient and almost paralleled with heavy proteinuria.[34] In addition, because of the complicated pathogenesis of PE, the effects of angiogenic factors, antagonists, and podocyte injury on development of proteinuria in PE had been explored[4]; the present study investigated the effect of decreased RAS components contributed to the direct podocyte injury with proteinuria in PE.

The ROC analysis demonstrated that the serum and urinary Ang-(1-7) were useful for identifying PE. They were highly sensitive and specific markers, especially in combination with

each other. Angiotensinogen might also discriminate PE from NP. Although our study was unable to evaluate the levels of Ang-(1-7) and AGT in early stages of pregnancy before the onset of PE, and the urinary AGT may be affected by state of proteinuria, these findings might provide an innovative view for the prediction and treated strategies of PE. Large longitudinal prospective cohort studies will be needed to confirm.

Nephrin and CD2AP are principal components of the podocyte's slit diaphragm to keep the normal structure and function of podocytes. They could form the slit diaphragm complex through their interaction. Their decreased expressions indicate podocyte injury. Nephrin is a transmembrane protein of the immunoglobulin superfamily that is especially capital and specifically expressed in podocyte and slit diaphragm. It has been demonstrated that nephrin could regulate actin dynamics in a phosphorylation-dependent manner and participate in the podocyte's signal transduction.[35] The abnormal expression of nephrin plays an important role in the mechanisms of proteinuria. CD2-associated protein, as a regulatory molecule, could anchor nephrin to the cytoskeleton.[36] In various types of renal diseases with acquired proteinuria, the expression of nephrin was downregulated and Ang II was known as an important reason of the nephrin's downregulated mechanisms.[37] Angiotensin II could affect the generation and expression of multiple molecules in podocytes and then cause the decreased expression of nephrin. Angiotensin II also could activate the cytokines to induce podocyte apoptosis and shedding.[38] Moreover, podocytes have a local RAS that includes Ang II type 1 receptors (AT1Rs) so that they contribute to intrarenal Ang II levels.[39] The present in vitro study showed that when the podocytes were incubated with serum of women with PE, the expression of CD2AP had no significant change; yet, it remains to be confirmed whether the messenger RNA and protein level of CD2AP changes. However, the expression of nephrin was obviously decreased. This meant that nephrin is the prime target protein leading to slit diaphragm injury in PE. After adding cocultured Ang-(1-7), the expression of nephrin was increased. These results of in vitro experiments were basically consistent with those of the other part of our research group, although the CD2AP and podocin did not show significant changes in our studies.[40] These findings suggested that Ang-(1-7) protected the podocytes from injury. We presumed that it may be ascribing to the effect of Ang-(1-7) antagonist Ang II.

In addition, slit diaphragm complex was also closely connected with cytoskeletal protein. Either disorder of cytoskeletal protein would induce the destruction of the filtration barrier and proteinuria. F-actin, the most important component of the podocyte cytoskeletal system, is a highly dynamic structure with a polar direction. It plays a crucial role in regulating the movement of cells and provides the internal mechanical support that drives cell motility.[41,42] ZO-1 as a member of the membrane-associated guanylate kinases superfamily of proteins is a key regulator of the cytoskeleton and concerned with nephrin. It had been confirmed that ZO-1 is responsible for sealing and organizing components of tight junctions by connecting them to the actin cytoskeleton and associating with

F-actin.[43,44] Furthermore, ZO-1 could interact with nephrin or impair the combination of nephrin and CD2AP, so that the cytoskeleton was deranged or foot process structure was destroyed and the glomerular filtration membrane was injured. All these actions eventually lead to proteinuria.[45] The in vitro experiments found that the expression of F-actin and ZO-1 in podocytes cultured by serum of PE was also significantly attenuated and the ordered filamentous structure of actin was destroyed. It may be because of decreased nephrin influencing the interaction of ZO-1 and F-actin, which leads to cytoskeleton rearrangement and foot process effacement. Simultaneously, the abnormal expression of ZO-1 and F-actin also affected the expression of nephrin, eventually leading to the destruction of glomerular filtration barrier and proteinuria.[46] However, the downregulation of F-actin and ZO-1 in podocytes was also rescued by Ang-(1-7); this may occur by binding the specific MasR to develop the protective effect. On the one hand, MasR could antagonist the AT1Rs' physiological action; on the other hand, MasR also could interact with AT1R through the oligomerization of receptors, thus producing a new function and property different from any receptor alone. The oligomerization may trigger the enhancement of signal way.[47] Angiotensin-converting enzyme (ACE) and ACE2 are important enzymes that regulate the production of Ang II and other RAS peptides. It is evident that ACE2-Ang-(1-7)-Mas axis has negative accommodation in the RAS. It may be a protective factor in the pathogenesis of PE. The clinical part of this study had confirmed that the serum and urinary Ang-(1-7) were significantly decreased in PE so that its protective factor was decreased. The in vitro experiment also showed that the expression of MasR in PE serum intervention group was significantly reduced, after treatment with Ang-(1-7) in podocyte cultured by serum of PE, and the expression of MasR was significantly increased accompanied by the significant increase in podocyte-associated proteins. These results from the examination of clinical specimens and in vitro experiments were consistent. It showed that MasR plays an important role in the protective effect of Ang-(1-7) to podocyte injury. In a word, the nature of this research is to emphasize the effect of RAS on podocyte injury and proteinuria in PE.

Certainly, one limitation of the study is that the involved signal transduction access, the concrete mechanisms about how to regulate the Ang-(1-7) and MasR, and the interaction of receptors in RAS are still unclear, although a part of the relevant signal transduction access had been investigated in our previous study.[40] Furthermore, this study provided preliminary confirmation about the effect of RAS components and MasR in podocyte injury in PE. The accordant results in clinical and in vitro experiments were obtained. In fact, both the previous and this study's vitro experiments were all stronger confirmation of the clinical results.

In summary, decreased Ang-(1-7) and downregulated intrarenal RAS may contribute to the podocyte injury and loss, which is a possible mechanism of proteinuria development in PE. The treatment with Ang-(1-7) may be a potential therapeutic tool in PE. Ang-(1-7) and podocyturia will be powerful factors for predicting PE.

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