Angiotensin-(1-7) attenuates damage to podocytes induced by preeclamptic serum through MAPK pathways

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Received April 6, 2014; Accepted July 17, 2014

DOI: 10.3892/ijmm.2014.1870

Abstract. The underlying mechanisms of proteinuria, a main characteristic of preeclampsia (PE), have not yet been fully elucidated. Evidence indicates that the renin-angiotensin system (RAS) is involved in the pathogenesis of this disease, including decreased angiotensin-(1-7) [Ang-(1-7)] levels in the circulation and urine. In the present study, we examined the damage to podocytes induced by preeclamptic serum and the effects of Ang-(1-7) on podocytes treated with preeclamptic serum, as well as the underlying mechanisms. The podocytes were incubated with serum obtained from women with PE or with serum from women with normal pregnancies for different periods of time. Cell viability was determined by CCK-8 assay. The cells were treated with various concentrations of Ang-(1-7) and A779 [an (Ang-(1-7) antagonist]. The effects of Ang-(1-7) on the expression of podocyte-specific proteins [nephrin, Wilms tumor-1 (WT-1) and podocin] and the phosphorylation of mitogen-activated protein kinases (MAPKs) were investigated by western blot analysis. Changes in F-actin rearrangement were determined by immunofluorescence. Podocyte apoptosis was determined by flow cytometry. The results revealed that in the cultured podocytes incubated with preeclamptic serum, there was a decrease in the expression of podocyte-specific proteins (nephrin and WT-1 but not podocin), a rearrangement of F-actin and apoptosis compared with the control group. However, treatment with Ang-(1-7) attenuated podocyte injury in the preeclamptic group, which may be mediated through the downregulation of MAPK (p38, ERK1/2 and JNK) phosphorylation. Thus, our data suggest that Ang-(1-7) plays a protective role in PE through the downregulation of MAPK phosphorylation.

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Key words: preeclampsia, podocyte, angiotensin-(1-7), mitogen-activated protein kinase phosphorylation

Introduction

Preeclampsia (PE) is one of the leading causes of maternal and neonatal morbidity and mortality worldwide and is characterized by a new onset of hypertension and proteinuria after 20 weeks gestation (1). Kidney damage plays an important role in the pathogenesis of PE. The rates of complications due to worsening renal function and hypertension are increased among pregnant women with moderate or severe renal insufficiency (2). Great advances have been made in recent years in understanding the pathogenesis of PE (3). However, further studies are required in order to fully elucidate the underlying mechanisms of PE and the development of acute kidney injury and chronic kidney disease in mothers.

Studies have demonstrated that proteinuria in pregnant women with PE is due to direct damage to podocytes (4). Adverse agents that act on podocytes can induce the proliferation, apoptosis and necrosis of podocytes, podocyte detachment from the glomerular basement membrane, as well as a loss of podocyte differentiation markers (5).

The renin-angiotensin-aldosterone system (RAAS), particularly renal RAAS, plays an important role in the development of chronic kidney disease. In recent years, RAS has also been shown to be involved in the pathogenesis of PE in a series of studies and has been shown to induce various characteristics of this disease (6,7). In normal pregnancy (NP), women are normotensive despite the upregulation of RAS components, suggesting that there is a counter-regulatory mechanism to reverse the vasoconstriction and sodium retention induced by angiotensin (Ang) II (8). In this regard, Valdés *et al* (9) found that plasma levels of angiotensin-(1-7) [Ang-(1-7)] are elevated in normotensive pregnant women, and that concentrations of urinary Ang-(1-7) are also markedly increased throughout pregnancy, indicating that Ang-(1-7) may play a vasodilator role. The heptapeptide, Ang-(1-7), is an important component of RAS and acts as a counter-regulatory peptide in RAS, often balancing the physiological actions of Ang II. It has been shown that Ang-(1-7) binds to the Mas G protein-coupled receptor to exert many of its biological effects (10). Merrill *et al* (11) found that Ang-(1-7) was significantly decreased in pregnant women with PE compared with women NP. Although the levels of renin, Ang I and Ang II are decreased in pregnant women

with PE (12), pregnant women with PE have an increased sensitivity to Ang II (13), indicating an exaggerated pressor response. Thus, we hypothesized that the development of proteinuria in PE may be attributed to the imbalance between the angiotensin-converting enzyme (ACE)-Ang II-AT1R axis and the ACE2-Ang-(1-7)-Mas axis and the decreased Ang-(1-7) expression in the circulation and renal system.

Studies have indicated that Ang-(1-7) plays a protective role possibly through the downregulation of mitogen-activated protein kinase (MAPK) phosphorylation in the kidneys. As previously demonstrated, in cultured rat proximal tubular cells, Ang-(1-7) potently inhibits the Ang II-stimulated phosphorylation of ERK1/2, p38 MAPK and JNK (c-Jun N-terminal kinase), an effect that is reversed by pre-treatment with A779, a potent and selective Ang-(1-7) antagonist (14). In renal epithelial LLC-PK cells, high glucose-stimulated protein synthesis and the phosphorylation of p38 MAPK are also inhibited by Ang-(1-7) (15). Although it appears that podocytes express ACE2 and are able to generate Ang-(1-7) in culture (16), to the best of our knowledge, there is currently no information on the effects of Ang-(1-7) on podocyte signalling (17).

In the present study, we hypothesized that serum from pregnant women with PE induces podocyte injury and apoptosis and that this represents an important mechanism to maintain and aggravate proteinuria. In addition, we corrected Ang-(1-7) deficiency in serum from pregnant women with PE *in vitro* to explore the protective effects of Ang-(1-7) on podocytes under these conditions and to elucidate the underlying mechanisms.

Materials and methods

Study participants. From March 2011 to January 2012, pregnant women with PE (n=20) and gestational age-matched normotensive pregnant women (n=20) were recruited from the Department of Gynecology and Obstetrics, The Fifth People's Hospital of Shanghai, Fudan University, Shanghai, China. PE was diagnosed by increased blood pressure (≥140/90 mmHg) and proteinuria (300 mg in one 24-h urine collection or >1+ by dipstick in a random urine analysis) after 20 weeks of gestation in a previously normotensive and non-proteinuric woman. Patients with chronic hypertension, pre-existing proteinuria or renal disease were excluded from this study. The patients enrolled in the present study were all newly admitted inpatients and all samples were collected prior to any medication being administered. The biological study was approved by the Ethics Committee of the Fifth People's Hospital of Shanghai. After obtaining ethical approval and written informed consent from all participants, blood samples were collected from the pregnant women with PE and women with NP. Maternal age, parity, gestational age at delivery, birth weight, blood pressure values, urinary protein excretion, serum creatinine and serum uric acid, serum urea levels and estimated glomerular filtration rate (eGFR) were recorded for each study participant (Table I).

Table I. Clinical characteristics of study participants.

	PE (n=20)	NP (n=20)
Maternal age, years	30.3±5.4[a]	27.2±2.6
Body mass index (BMI), Kg/m^2	27.7±4.3	26.7±2.3
Blood pressure, mmHg		
Systolic	156.8±18.1[b]	119.3±8.4
Diastolic	97.4±11.8[a]	73.7±7.7
Gestational age delivery, weeks	36.7±2.5[b]	40.0±1.0
Birth weight of child, g	2713±793.5[b]	3526±360.4
Primigravida	14	12
Urinary protein	1+-3+[b]	Negative
Serum creatinine, μmol/l	56.9±9.8[b]	45.2±9.6
Serum uric acid, μmol/l	361.5±92.0[a]	289.0±82.4
Serum urea, mmol/l	4.5±2.0[a]	2.9±1.2
eGFR (MDRD), ml/min/1.73 m^2	162.3±42.8	120.0±27.4

eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease. Values are the means ± SD of 20 pregnant women with preeclampsia (PE) and 20 women with normal pregnancies (NP). [a]P<0.05, [b]P<0.005 compared to women with NP.

Cell culture. The conditionally immortalized human podocytes that were used for the experiments were kindly provided by academician Zhihong Liu (Research Institute of Nephrology of the Jinling Hospital of Nanjing University School of Medicine, Nanjing, China), and the podocyte cell line of Zhihong Liu was provided by Professor Peter Mundel (Department of Medicine and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, New York, NY, USA). The human podocytes were cultured as previously described (18). The podocytes were propagated and seeded at 33˚C in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1X insulin, transferrin and selenium solution (ITS) (Invitrogen, Carlsbad, CA, USA). Type I collagen (Invitrogen) is always used to coat the culture dishes to promote podocyte proliferation. Under these permissive conditions, podocytes are small in size, exhibit a polygonal or 'cobblestone' appearance and have a relatively small cytoplasmic volume. Subsequently, the cells were incubated at 37.0˚C for 10-14 days where they grew to 80% confluence. Under these growth restrictive conditions, the podocytes undergo growth arrest, increase in size, stop replicating, display a more complex arborized pattern of foot process extensions, and express markers of mature podocytic differentiation comparable with filtration slits *in vivo*.

Cell viability assay. Cell viability was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan). The podocytes were seeded at a concentration of 10^5/ml in 96-well plates. After adherence, the cells were incubated with serum from pregnant women with PE, or serum from women with NP (1:10 dilution) for different periods of time (6, 12 and 24 h). When the effects of preeclamptic serum and serum from women with NP on podocyte cell viability were investigated, various concentrations of Ang-(1-7) (10^{-5}, 10^{-6} and 10^{-7} mol/l) (Sigma-Aldrich, St. Louis, MO, USA) and A779 (10^{-5}, 10^{-6} and 10^{-7} mol/l) (Bachem, Bubendorf, Switzerland) were added to the cells for 12 h. At the end of the treatment period, 10 μl of CCK-8 were added to each well, and the cells were further incubated at 37˚C for 2 h. The absorbance of CCK-8 was detected at 450 nm using a microplate reader (680 Enzyme-linked Immunosorbent Monitor; Bio-Rad, Tokyo, Japan).

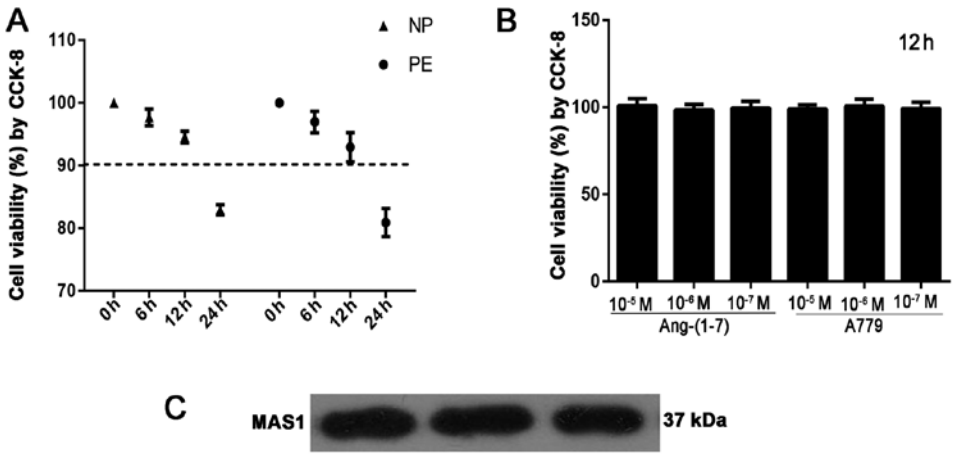

Figure 1. (A) Effects treatment with serum from pregnant women with preeclampsia (PE) and serum from women with normal pregnancies (NP) on podocyte viability. The viability of podocytes cultured with serum from pregnant women with PE and women with NP were assessed by CCK-8 assay. The podocytes were incubated for 0-24 h with preeclamptic serum and serum from women with NP. (B) Effects of angiotensin-(1-7) [Ang-(1-7)] and A779 [an Ang-(1-7) antagonist] on podocyte cell viability. The viability of podocytes incubated with Ang-(1-7) and A779 was examined by CCK-8 assay. Podocytes were incubated with Ang-(1-7) and A779 at the concentration of 10^{-5}, 10^{-6} and 10^{-7} mol/l for 12 h. The results are expressed as the means ± SD values of 3 independent experiments. (C) Expression of Mas receptor on podocytes. Mas receptor expression was detected by western blot analysis using an antibody specific for the Mas receptor in podocytes.

Western blot analysis. The cells were incubated for 12 h with serum derived from pregnant women with PE or serum from women with NP (1:10 dilution). To verify the concentration of Ang-(1-7) required to exert protective effects on podocytes incubated with serum pregnant women with PE, the cells were stimulated with Ang-(1-7) at concentrations of 10^{-5}, 10^{-6} and 10^{-7} mol/l. In some experiments, the cells were pre-exposed to A779 (10^{-5} mol/l, 30 min). Serum from women with NP with A779 [a potent and selective Ang-(1-7) antagonist (10^{-5} mol/l)] was used as a control. The cells were washed twice with ice-cold PBS and harvested in a lysis buffer containing a protease inhibitor cocktail. Equal amounts of proteins were run on 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked at room temperature with 5% milk in Tris-buffered saline Tween-20 (TBS-T) for 1 h and then probed at 4˚C overnight with the following primary antibodies: anti-podocin and anti-Wilms' Tumor-1 (WT-1) were from Abcam (Burlingame, CA, USA); anti-nephrin antibody was from Sigma-Aldrich; anti-MAS1 antibody was from Novus Biologicals (Oakville, ON, Canada); anti-GAPDH and anti-p-JNK were from Cell Signaling Technology (Beverly, MA, USA); and anti-p-p38 and anti-p-ERK1/2 were purchased from Epitomics (Burlingame, CA, USA). The membranes were washed with TBS-T and then incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence detection system. Exposures were recorded on X-ray film.

Immunofluorescence. The cells were incubated with FBS or serum derived from pregnant women with PE, serum from women with NP (1:10 dilution), or serum derived from pregnant women with PE in the presence of Ang-(1-7) (10^{-6} mol/l), and the podocytes pre-incubated with A779 (10^{-5} mol/l, 30 min) were stimulated with serum derived from pregnant women with PE in the presence of Ang-(1-7) (10^{-6} mol/l). The cells were grown on coverslips, washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized using 0.1% Triton X-100 for 15 min and incubated in a blocking buffer (5% BSA in PBS, pH 7.4). The cells were then probed with anti-F-actin antibody (1:400)(Abcam) at room temperature for 2 h. The cells were washed with PBS followed by the addition of secondary antibody (Cell Signaling Technology) for 2 h at room temperature. Coverslips were then mounted onto glass microscope slides with DAPI. The cells were observed and photographed using a laser scanning confocal fluorescence microscope (Leica DM5000; Leica Microsystems, Wetzlar, Germany).

Analysis of apoptosis by flow cytometry. Treatment of the podocytes was carried out as described above. The FITC Annexin V Apoptosis Detection kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The cells were washed twice with cold PBS and then suspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. Subsquently, 100 μl of the solution were transferred to a 5-ml culture tube, and 5 μl of Annexin V and 5 μl propidium iodide (PI) were added; the mixture was gently vortex and incubated for 15 min at room temperture (25˚C) in the dark. Subsquently, 400 μl of 1X binding buffer were added to each tube. Flow cytometric analysis was performed within 1 h.

Statistical analyses. Values are shown as the the means ± SD. SPSS (version 16.0) software was used to analyze the data using the Student's t-test or one-way ANOVA or the rank sum test. P-values <0.05 were considered to indicate a statistically significant difference.

Results

Analysis of podocyte viability. To determine the effects of serum from pregnant women with PE and serum from women with NP on podocytes, cell viability was examined by CCK-8 assay. The results revealed that exposure to serum from pregnant women with PE and from women with NP induced a decrease in cell viability in a time-dependent manner (Fig. 1A). At the same time, we did not observe any significant toxic effects of Ang-(1-7) (10^{-5}, 10^{-6} and 10^{-7} mol/l) and A779 (10^{-5}, 10^{-6} and

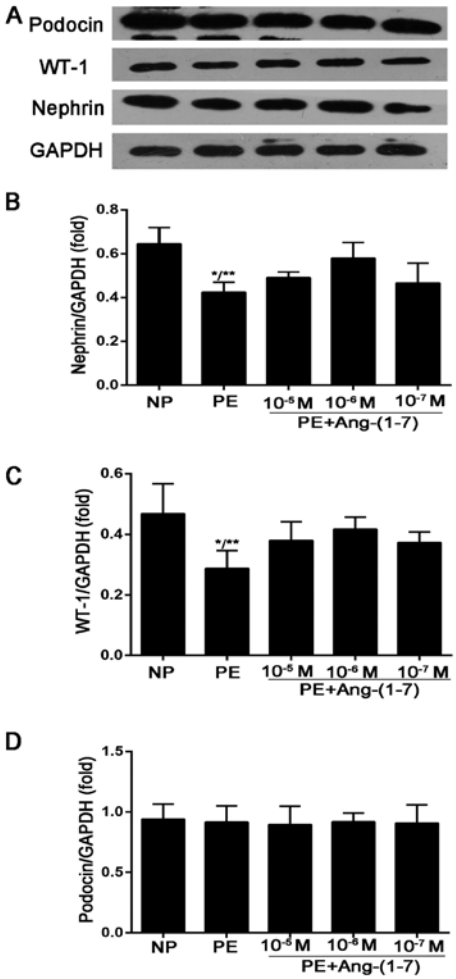

Figure 2. Effects of angiotensin-(1-7) [Ang-(1-7)] on the expression of nephrin, podocin and Wilms tumor-1 (WT-1) in podocytes treated with preeclamptic serum. Podocytes were treated with serum from women with preeclampsia (PE), serum from women with normal pregnancies (NP), or serum from women with PE plus Ang-(1-7) (10^{-5}, 10^{-6} and 10^{-7} mol/l) for 12 h. Bar graphs (B-D) demonstrate the means ± SD of 3 independent experiments. *P<0.05 vs. NP, **P<0.05 vs. PE + Ang-(1-7) (10^{-6} mol/l). (A) Representative blot is depicted above the graphs, showing nephrin, podocin, WT-1 and GAPDH.

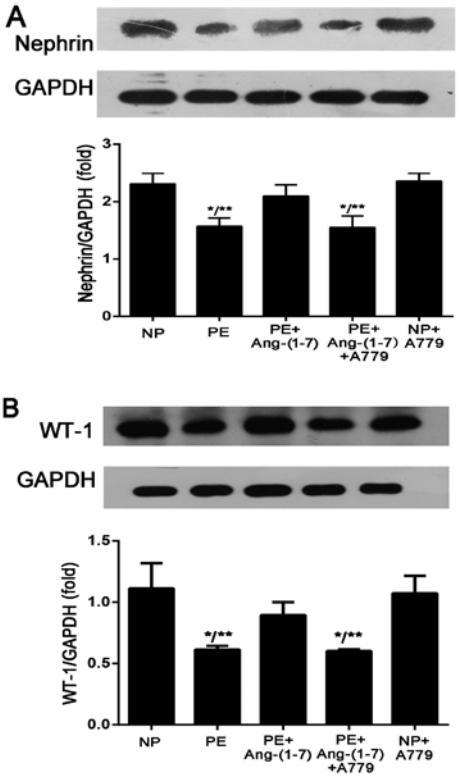

Figure 3. A779, an antagonist of angiotensin-(1-7) [Ang-(1-7)], reversed the protective effects of Ang-(1-7) on podocyte injury. Graph depicts the effects of treatment with serum from pregnant women with preeclampsia (PE) and serum from women with normal pregnancy (NP) for 12 h with or without Ang-(1-7) (10^{-6} mol/l) or A779 (10^{-5} mol/l), on nephrin and Wilms tumor-1 (WT-1) expression. Top panels were representative western blots. Bottom panels are the summary data from 3 independent experiments. *P<0.01 vs. NP, **P<0.05 vs. PE + Ang-(1-7) (10^{-6} mol/l).

10^{-7} mol/l) on podocytes at 12 h (Fig. 1B). Thus, we opted to use serum from pregnant women with PE and from women with NP with 12 h of incubation in the subsequent experiments to determine the underlying mechanisms.

Expression of Mas G protein-coupled receptor in podocytes. As is already known, there is no evidence of the expression of Mas in human podocytes. Thus, western blot analysis was performed to determine whether human podocytes express Mas, the putative receptor for Ang-(1-7). In 3 separate experiments, the results revealed a single band corresponding to the expected 37-kD product for Mas in human podocytes (Fig. 1C).

Effects of Ang-(1-7) on the expression of nephrin, podocin and WT-1 in podocytes treated with preeclamptic serum. The downregulation of podocyte-specific proteins may be involved in the appearance and development of proteinuria in women with PE. Thus, in this study, the expression of nephrin, podocin and WT-1 was assessed in human cultured podocytes treated with serum from pregnant women with PE and from women with NP. As shown in Fig. 2, the expression of nephrin and WT-1 decreased in the podocytes stimulated with preeclamptic serum compared with the cells incubated with serum from women with NP (nephrin, 0.42±0.04-fold vs. 0.64±0.07-fold of GAPDH, P<0.005; WT-1, 0.28±0.05-fold vs. 0.46±0.09-fold of GAPDH, P<0.05). However, the expression of podocin showed no significant difference among the groups. After the podocytes were treated with preeclamptic serum in the presence of Ang-(1-7) (10^{-5}, 10^{-6} and 10^{-7} mol/l) for 12 h, the results revealed that Ang-(1-7) exerted the maximum protective effect at a concentration of 10^{-6} mol/l, and the expression of nephrin and WT-1 was significantly increased (nephrin, 0.57±0.07-fold vs. 0.42±0.04-fold of GAPDH, P<0.05; WT-1, 0.41±0.04-fold vs. 0.28±0.05-fold of GAPDH, P<0.05) compared with the podocytes incubated only with preeclamptic serum (Fig. 2).

A779 reverses the protective effects of Ang-(1-7) on podocyte injury induced by preeclamptic serum. The selective Mas receptor antagonist, A779 [D-Ala7-Ang-(1-7)] (10^{-5} mol/l), was used to verify the protective role of Ang-(1-7) in another aspect. The results demonstrated that the addition of A779 reversed the protective effects of Ang-(1-7) on podocyte injury induced by preeclamptic serum. The podocytes were pre-treated with A779 for 30 min and then incubated with preeclamptic serum in the presence of Ang-(1-7) (10^{-6} mol/l) for 12 h. As shown in Fig. 3, after the addition of A779, the expression of nephrin and

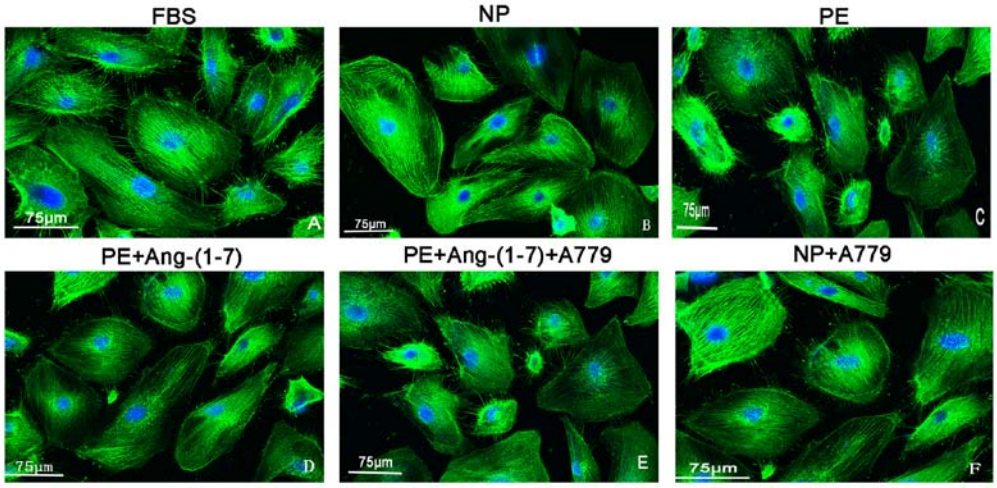

Figure 4. Effects of angiotensin-(1-7) [Ang-(1-7)] on changes in F-actin in podocytes incubated with preeclamptic serum. Immunofluorescence staining for F-actin (green) with a blue nuclear counterstain (DAPI) is shown. NP, serum from women with normal pregnancy; PE, serum from women with preeclampsia. Controls were treated with FBS.

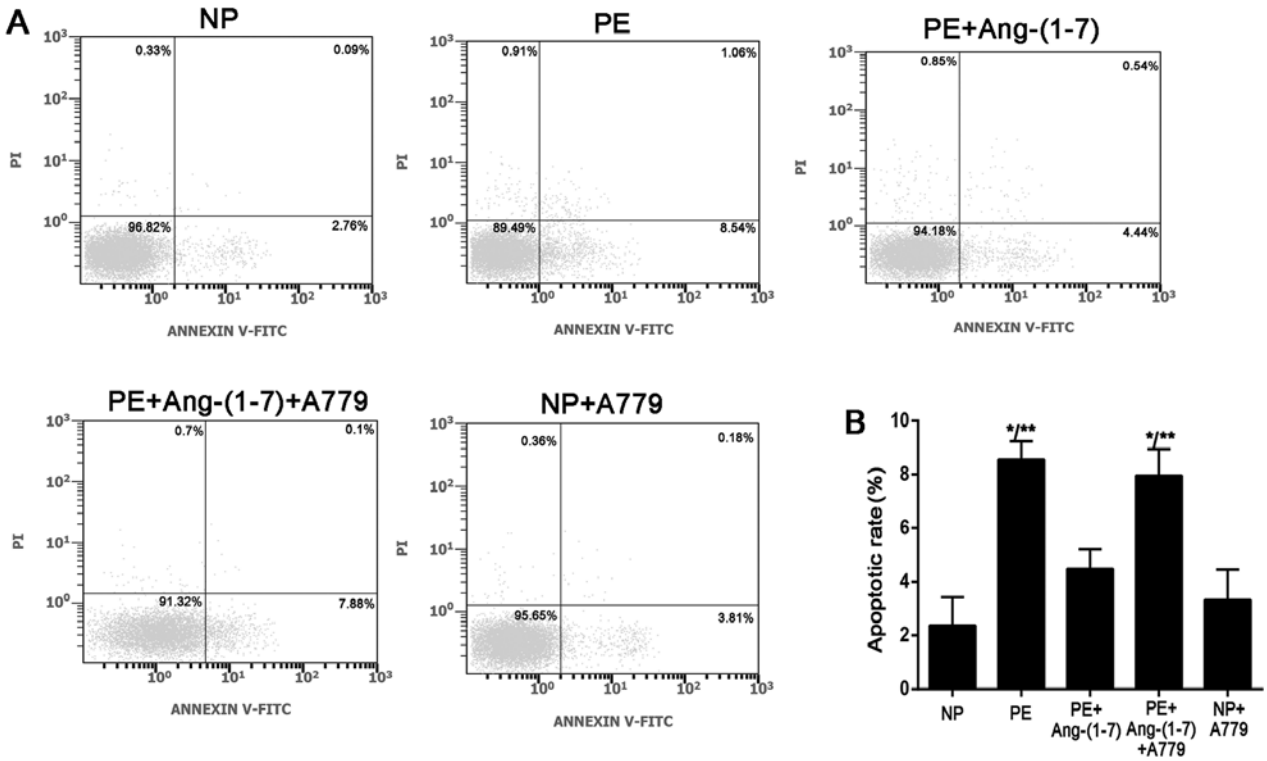

Figure 5. Effects of angiotensin-(1-7) [Ang-(1-7)] (10^{-6} mol/l) on the apoptosis of podocytes induced by preeclamptic serum. (A) Flow cytometric dot plots of Annexin V and propidium iodide (PI) staining. (B) Bar graph shows the percentage of cells with early apoptosis. Data are presented as the means ± SD. *P<0.001 vs. NP, **P<0.01 vs PE + Ang-(1-7) (10^{-6} mol/l). NP, serum from women with normal pregnancy; PE, serum from women with preeclampsia

WT-1 decreased compared with the addition of Ang-(1-7) only (nephrin, 1.55±0.20-fold vs. 2.1±0.19-fold of GAPDH, P<0.01; WT-1, 0.60±0.01-fold vs. 0.89±0.10-fold of GAPDH, P>0.05). Podocytes incubated with serum from NP in the presence of A779 (10^{-5} mol/l) served as a control and there was no significant difference compared with the podocytes incubated with serum from NP only; this finding suggests that Ang-(1-7) is required to maintain the increased expression of nephrin and WT-1 in PE.

Effects of Ang-(1-7) on the changes in F-actin in podocytes incubated with preeclamptic serum. Cytoskeletal rearrangement is a crucial early event in the pathophysiology of proteinuria and has been reported to result in foot process effacement (19). As shown in Fig. 4, the expression and orientation of F-actin was changed, including cortical F-actin ring formation and stress fibre attenuation in the podocytes that were treated with preeclamptic serum compared with those of the control group. In addition, this change was attenuated by

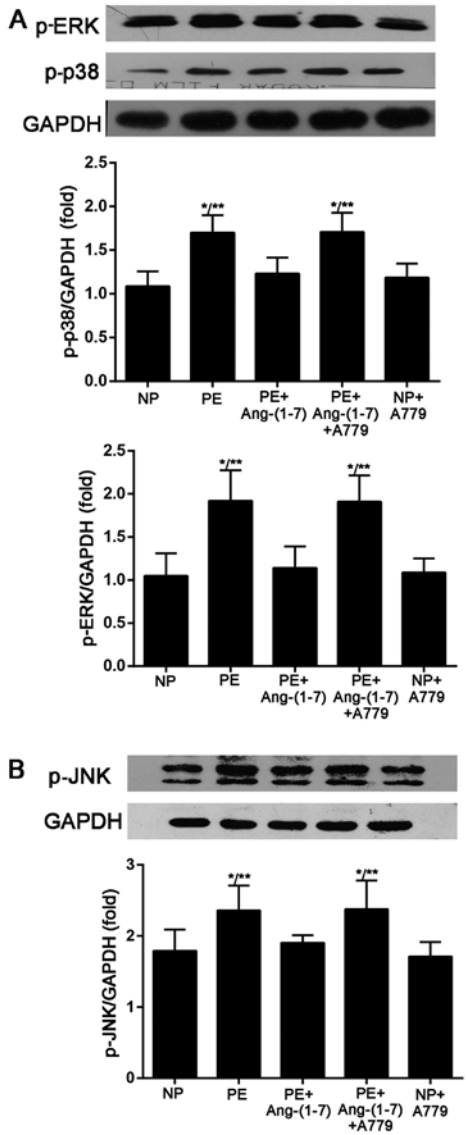

Figure 6. Effects of angiotensin-(1-7) [Ang-(1-7)] on mitogen-activated protein kinase (MAPK) (p38, ERK 1/2 and JNK) phosphorylation. Podocytes were treated with serum from pregnant women with preeclampsia (PE) or serum from women with normal pregnancies (NP) with or without Ang-(1-7) (10^{-6} mol/l) or A779 [an Ang-(1-7) antagonist] (10^{-5} mol/l) for 12 h. (A and B) Top panels are representative western blots. Bottom panels were the summary data from 3 independent experiments. *P<0.05 vs. NP, **P<0.05 vs. PE + Ang-(1-7) (10^{-6} mol/l).

treatment with Ang-(1-7) (10^{-6} mol/l) and this protective role was reversed by pre-treatment with A779 (Fig 4).

Effects of Ang-(1-7) (10^{-6} mol/l) on the apoptosis of podocytes induced by preeclamptic serum. To determine the effects of preeclamptic serum on the apoptosis of podocytes, we examined podocyte apoptosis by flow cytometry. The cells were treated with preeclamptic serum in the presence or absence of Ang-(1-7) (10^{-6} mol/l) and A779 (10^{-5} mol/l). Cells incubated with serum from women with NP served as a control. As shown in Fig. 5, the number of Annexin V^{+}/PI^{-} (apoptotic) cells was significantly increased in the PE group; there were 8.55±0.68% apoptotic cells in the PE group and 2.36±1.05% apoptotic cells in the NP group (P<0.001). As expected, after the addition of Ang-(1-7) (10^{-6} mol/l) in the PE group [PE plus Ang-(1-7)], the number of apoptotic cells (4.47±0.73%) was significantly decreased compared with the PE group, and this protective role was reversed by pre-treatment with A779 (7.95±0.99%, P<0.001). A779 had no effect on podocyte apoptosis, as shown in the NP plus A779 group (3.59±0.74%); this finding suggests that Ang-(1-7) is required to prevent the apoptosis of podocytes in PE and that the increased apoptosis in the group treated with serum from women with PE plus Ang-(1-7) and A779 did not result from A779 alone.

Effect of Ang-(1-7) on MAPK (p38, ERK 1/2 and JNK) phosphorylation. As illstrated in Fig. 6, the results from western blot analysis showed that the p38, ERK and JNK phosphorylation levels in the cultured podocytes that were stimulated with preeclamptic serum increased compared with those stimulated with serum from NP (p-p38, 1.69±0.20-fold vs. 1.08±0.16-fold of GAPDH, P<0.005; p-ERK, 1.92±0.35-fold vs. 1.04±0.26-fold of GAPDH, P<0.005; p-JNK, 2.35±0.35-fold vs. 1.79±0.30-fold of GAPDH, P<0.05). The addition of Ang-(1-7) to the cells prevented the preeclamptic serum-induced increase in p-p38, ERK and JNK phosphorylation (p-p38, 1.23±0.18-fold vs. 1.69±0.20-fold of GAPDH, P<0.05; p-ERK, 1.14±0.25-fold vs. 0.87±0.18-fold of GAPDH, P<0.01; p-JNK, 1.90±0.10-fold vs. 2.35±0.35-fold of GAPDH, P<0.05) and the effects of Ang-(1-7) were blocked by the Mas receptor inhibitor, A-779.

Discussion

In the present study, we used a conditionally immortalized differentiated human podocyte cell line to explore the damage sustained by glomerular epithelial cells from preeclamptic serum and found that cultured podocytes underwent functional and structural morphologic changes that resulted from the downregulation of podocyte-specific proteins (nephrin and WT-1), cytoskeletal rearrangement (specialized arrangement of F-actin) and apoptosis. In addition, we determined the role of Ang-(1-7) deficiency under these conditions (PE), and the results revealed that Ang-(1-7) attenuated podocyte injury induced by preeclamptic serum through the downregulation of MAPK phosphorylation.

Podocyte injury is usually characterized by the disappearance or effacement of foot processes leading to proteinuria. The foot processes of neighbouring podocytes form an interdigitating pattern with slits that are bridged by a protein complex known as the slit diaphragm (SD) (20). The downregulation of specialized proteins associated with slit-pores (e.g., nephrin and podocin) and podocyte-specific transcriptional factors (e.g., WT-1) may be involved in the initiation and development of proteinuria in pregnant women with PE. Our data indicated that the incubation of podocytes with preeclamptic serum resulted in the decreased expression of nephrin and WT-1 in podocytes and had no effect on podocin expression; these results are consistent with those of other studies (4). The unique shape of podocytes and the maintenance of the processes characterize the well-developed cytoskeleton (20). Cytoskeletal rearrangement has been suggested to underlie foot process effacement, which is a crucial early event in the pathophysiology of proteinuria. The F-actin cytoskeleton in

podocyte foot processes is believed to be dynamically maintained at a steady-state with a low turnover (21). In the present study, the expression and orientation of F-actin was altered in the podocytes that were treated with preeclamptic serum compared with the control group (treated with serum from women with NP), which may contribute to podocyte injury in PE. Podocyte depletion has long been shown to be a typical characteristic of glomerulosclerosis and is now considered a key factor in the progression of renal diseases (22). In this study, we found that the apoptosis of podocytes in the PE group was increased compared to the NP group. Our previous study indicated that the number of urinary podocytes in pregnant women with PE was significantly higher than that in women with gestational hypertension without proteinuria and women with NP (23). These results indicate that the progressive depletion of podocytes in PE is associated with an increase in podocyte apoptosis and the detachment from the glomerular basement membrane.

Treatment with Ang-(1-7) has been shown to reduce proteinuria in stroke-prone spontaneously hypertensive rats (SHRSP) (24), and Ang-(1-7) has been shown to have therapeutic potential for reversing glomerulosclerosis by counteracting the effects of Ang II in a rat model of experimental glomerulonephritis (25). The data from the present study indicate that correcting the Ang-(1-7) deficiency in PE can partly reverse the downregulation of podocyte-specific proteins, cytoskeletal rearrangement and the apoptosis of podocytes.

However, the effects of Ang-(1-7) on the kidneys remain controversial. Shao *et al* (26) found that exogenous Ang-(1-7) injection did not ameliorate diabetic rat renal injury induced by streptozotocin (STZ), and it accelerated progressive diabetic nephropathy. Many factors result in these inconsistent results, such as different experimental designs and different doses of Ang-(1-7). In this study, the doses of Ang-(1-7) used in the experiments ranged from 10^{-7} to 10^{-5} mol/l, and we found that Ang-(1-7) exerted a maximum protective effect on podocyte injury in PE at a concentration of 10^{-6} mol/l.

In the present study, we elucidated the underlying mechanisms of the protective role of Ang-(1-7) in PE. The results revealed that Ang-(1-7) partly inhibited the phosphorylation of p38, ERK1/2 and JNK in cultured podocytes treated with preeclamptic serum, which was reversed by pre-treatment with A779, suggesting an effect mediated by the binding of Ang-(1-7) to the receptor, Mas. In primary cultures of mouse mesangial cells, Moon *et al* (27) showed that Ang-(1-7) attenuated AngII-induced MAPK phosphorylation, as well as the expression of transforming growth factor (TGF)-β1, fibronectin and collagen IV. However, Zimpelmann and Burns (28) demonstrated that treatment of a human mesangial cell line with Ang-(1-7) caused a rapid and significant increase in arachidonic acid release, as well as the phosphorylation of MAPKs. Overall, Ang-(1-7)/Mas signaling in the kidneys is complex and more detailed studies at the cellular level are required.

In this study, we determined the role of Ang-(1-7) in the pathogenesis of PE and found that Ang-(1-7) reversed podocyte injury in PE through the activation of the podocyte Mas receptor and inhibition of the MAPK pathway. However, a role for correcting Ang-(1-7) deficiency in this condition as a therapeutic strategy requires further investigation.

Acknowledgements

The present study was supported by grants from the Major State Basic Research Development Program of China (973 Program) (no. 2012CB517700), Phase III of the Nephropathy Discipline Construction of 211 Project of State Education Commission, the Shanghai Biopharmaceutics Major Project (08dz1900603), the Fudan University '985' Hospital Key Discipline Construction Project (2012FDYSXK02), the Shanghai Municipal Science and Technology Project (124119a7802), the Shanghai City Health Bureau Project (20114311) and Minhang District Key Disciplines Construction. We thank academician Zhihong Liu for the donation of the podocyte cells. We thank Professor Zhaoxia Wang for providing technical advice and Peng Fang for technical support.

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