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c-Abl mediates angiotensin II-induced apoptosis in podocytes

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Abstract

Background—Angiotensin II (Ang II) has been reported to cause podocyte apoptosis in rats both *in vivo* and *in vitro* studies. However, the underlying mechanisms are poorly understood. In the present study, we investigated the role of the nonreceptor tyrosine kinase c-Abl in Ang II-induced podocyte apoptosis.

Methods—Male Sprague-Dawley rats in groups of 12 were administered either Ang II (400 kg⁻¹·kg⁻¹·min⁻¹) or Ang II + STI-571 (50 mg·kg⁻¹·d⁻¹) by osmotic minipumps. In addition, 12 rats-receiving normal saline served as the control. Glomeruli c-Abl expression was carried out by real time PCR, Western blotting and immunolabeled, and occurrence of apoptosis was carried out by TUNEL staining and transmission electron microscopic analysis. *In vitro* studies, conditionally immortalized mouse podocytes were treated with Ang II (10⁻⁹-10⁻⁶ M) in the presence or absence of either c-Abl inhibitor, Src-I1, specific c-Abl siRNA, or c-Abl plasmid alone. Quantification of podocyte c-Abl expression and c-Abl phosphorylation at Y245 and Y412 was carried out by real time PCR, Western blotting and immunofluorescence imaging. The nuclear c-Abl and p53 were quantified by co-immunoprecipitation and Western blotting studies. Podocyte apoptosis was analysed by flow cytometry and Hoechst-33342 staining.

Results—c-Abl expression was demonstrated in rat kidney podocytes *in vivo* and cultured mouse podocytes *in vitro*. Ang II-receiving rats displayed enhanced podocyte c-Abl expression. And Ang II significantly stimulated c-Abl expression in cultured podocytes. Furthermore Ang II upregulated podocyte c-Abl phosphorylation at Y245 and Y412. Ang II also induced an increase of nuclear p53 protein and nuclear c-Abl-p53 complexes in podocytes and podocyte apoptosis. Down-regulation of c-Abl expression by c-Abl inhibitor (Src-I1) as well as specific siRNA inhibited Ang II-induced podocyte apoptosis; conversely, podocytes transfected with c-Abl plasmid displayed enhanced apoptosis.

Conclusions—These findings indicate that c-Abl may mediate Ang II-induced podocyte apoptosis, and inhibition of c-Abl expression can protect podocytes from Ang II-induced injury.

Keywords

c-Abl; Apoptosis; Angiotensin II, Podocyte; p53

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Competing interests

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

Introduction

Angiotensin II (Ang II) has been considered an important risk factor in the progression of kidney diseases in general and glomerular cell injury in particular (Zhuo and Li 2011). Previous studies have reported that Ang II can induce podocyte apoptosis both in *in vivo* and *in vitro* (Ding, et al. 2002; Jia, et al. 2008; Ren, et al. 2012; Sanchez-Nino, et al. 2012; Tuncdemir and Ozturk 2011). However, the molecular mechanisms of Ang II-induced podocyte apoptosis remain unknown. Accumulating evidences in recent years have confirmed that p53-mediated apoptosis is a key step for cell death of podocytes (Jung, et al. 2012; Zhou, et al. 2012; Eid, et al. 2010; Wada, et al. 2008; Wada, et al. 2005). Puromycin-induced podocyte apoptosis is associated with increased p53 expression. The p53 inhibitor pifithrin- α can protect puromycin-induced apoptosis (Wada, et al. 2008; Wada, et al. 2005). The accumulation of plasma oxidized protein products (AOPPs) results in podocyte apoptosis, which reveals the co-localization and interaction with the receptor of advanced glycation end products (RAGE) in podocytes. Blocking RAGE can significantly decrease the activation of the p53/Bax/caspase-dependent pro-apoptotic pathway induced by AOPPs (Zhou et al. 2012).

A critical molecule associated with apoptosis in epithelial cells and neuron is the non-receptor protein tyrosine kinase, c-Abl, which is localized in both nucleus and cytoplasm. The molecular weight of c-Abl is approximately 140 kDa, which is conserved in the evolution and demonstrated in many tissues and cells, including kidney (Colicelli 2010; Jia, et al. 2008; Klein, et al. 2011; Qiu, et al. 2010; Wang, et al. 2010; Xu, et al. 2010). The amino terminus of c-Abl contains SH2 and SH3 and structural domain of tyrosine kinase for specific binding; cytoplasmic c-Abl interacts with actin cytoskeleton and can modulate its remodeling; moreover, interaction of structural domains with other proteins can regulate the activity of c-Abl. Since the carboxy terminus of c-Abl contains p53-binding domain (Colicelli 2010), the nuclear c-Abl is involved in the regulation of cell cycle in a p53-dependent manner (Colicelli 2010; Xu et al. 2010). Similarly, some reports suggested that c-Abl-induced apoptosis can be partially executed by p53-dependent manner (Jia et al. 2008; Westlund, et al. 2009).

Ang II can enhance the generation of reactive oxygen species (ROS) in podocytes in a time-dependent manner (Yadav, et al. 2010). As expected, the role of c-Abl in oxidative stress- and DNA-damage-induced apoptosis in cells is well understood (Jia et al. 2008; Klein et al. 2011). In the present study, we asked whether podocytes expressed c-Abl, if yes, whether it played a role in Ang II-induced apoptosis. In order to explore the involved mechanism, we studied the effect of Ang II on the interaction of c-Abl with major proapoptotic signal pathways such as p53.

Materials and Methods

Animals

Thirty-six male Specific pathogen Free (SPF) Sprague Dawley rats weighing between 110g and 140g were purchased from Research Center of Medical Experimental Animals of Wuhan University and were maintained at a controlled temperature and humidity under an artificial light cycle, with a free access to tap water and standard rat chow. Embedded with osmotic mini-pump (Alzet model 2002 or 2004, CA), rats were randomly subjected to normal saline infusion, or Ang II infusion at 400 ng/kg/min, or Ang II at 400 ng/kg/min + STI-571 (Glivec, Novartis, Switzerland) at 50 mg/kg/day by means of intragastric administration for 14 or 28 days. Animals were sacrificed at days 14 and 28. Kidneys were perfused with vanadate (a phosphatase inhibitor) before isolation. Part of the kidney was used for renal pathological and the remainder was stored at -80°C for biochemical analysis.

Detached glomeruli for Western blotting and Real time-PCR analysis. Glomeruli detachment method refers to reference (Sanwal, et al. 2001). In brief, 200 mg kidney tissue was collected, cortex and medulla were detached on ice, then glomeruli were isolated by differential sieving, loop through 150µm, 105µm, 75 µm screen cloth.

Cell culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York). Briefly, cells were maintained at 33°C in the presence of 10 units/ml interferon. The medium consists of RPMI 1640 (HyClone, USA) containing 10% fetal calf serum (Gibco, USA), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invitrogen, USA), and 10 units/ml recombinant mouse interferon- (Peprotech, USA). To induce differentiation, podocytes were cultured at 37°C for 10-14 days without interferon. Every experimental setup and result was confirmed in three different clones of podocytes.

Apoptosis assays

Apoptosis in kidney tissues was assessed by using TUNEL (Promega, USA) staining according to the manufacturer's instruction. In brief, paraffin dewaxed cortical sections (3µm) were incubated with 3% H₂O₂ for 30 min followed by 0.1% Triton X-100 in phosphate buffered saline (PBS) for 15 min at room temperature. Sections were washed and exposed to TdT buffer for 5 min and incubated in a moist chamber with a mixture of TdT and digoxigenin-11-dUTP in TdT buffer (R&D Systems, Minneapolis, Minn, USA) for 1 h at room temperature. Subsequently, sections were washed in PBS for 15 min and then incubated for 30 min with streptavidin-biotin-peroxidase-conjugated anti-digoxigenin-11-dUTP antibody, and antibody binding sites were visualized using diaminobenzidine. The slides were counterstained with hematoxylin. Negative control included the omission of TdT; positive control included the pretreatment of sections with 0.1U/µl deoxynuclease-1 before TdT staining. Apoptotic podocytes from single cross-section (through the glomerulus) were counted using the Weibel-Gomez method (Nicholas, et al. 2011). Percentage of apoptotic podocytes was recorded in 50 random fields (frontal sections of glomeruli) in 6 cortical sections for each variable.

Apoptosis in kidney tissues was also assessed by using transmission electron microscopic analysis. Paraformaldehyde-glutaraldehyde-fixed 1-mm³ blocks of renal cortices were postfixated with 1% osmium in 0.1M cacodylate buffer for 1 h, dehydrated in graded ethanols, embedded in Epon, sectioned, stained with uranyl acetate and lead citrate, and examined and photographed with a Hitachi H600 transmission electron microscope (Hitachi, Tokyo, Japan).

Apoptosis in cultured podocytes was evaluated by flow cytometry of adherent cells to detect terminal deoxynucleotidyl transferase activity as incorporation of fluorescein isothiocyanate-deoxyuridine triphosphate (FITC-Dutp) compared with propidium iodide (PI) using the Apo-Direct kit (Invitrogen, USA) according to the manufacturer's instruction.

Apoptosis in cultured podocytes was also assessed by using Hoechst-33342 (Sigma, USA) staining. Cells were prepared under control and experimental conditions by staining with Hoechst-33342 (1µg/ml) at room temperature for 5 min, then washed by ice-cold PBS for three times. Podocytes were observed under fluorescence microscope, percentage of apoptotic cells was recorded in ten random fields in 3 wells for each variable. Three sets of experiments were carried out.

Immunofluorescence and immunohistochemistry

The cell climbing film was fixed in 4% paraformaldehyde with 0.1% Triton X-100 for 30 min at 4°C, and blocked by bovine serum albumin for 30 min at room temperature. c-Abl antibody (1:100; Santa Cruz, USA) was used as the primary antibody for overnight at 4°C. FITC-conjugated IgG was used as the secondary antibody at 37 °C for 45 min. Subsequently, the cell climbing film was flushed with PBS 5 min×3 times, and the cells were observed under fluorescence microscope.

Immunostaining for c-Abl expression was performed on paraffin-embedded sections. Slides were deparaffinized and treated with 3% H₂O₂ for 30 min at room temperature. Antigen retrieval for c-Abl was performed in high pressure citrate buffer (0.01 mol/L, pH 6.0) for 10 min. Endogenous peroxidase was blocked with 5% bovine serum albumin in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) for 30 min, and sections were incubated with rabbit anti-c-Abl antibody (1:100; Abbiotec, USA) overnight at 4°C. Sections were washed in PBS, followed by biotinylated anti-rabbit secondary antibody and avidin–biotin peroxidase complex (Dako) for 30 min. After rinsing, the peroxidase activity was visualized by DAB (Dako), and sections were counter-stained with haematoxylin. Negative controls were performed by omitting the primary antibody and replacing it with normal rabbit IgG. 50 randomly chosen glomeruli were analysed using Image-pro plus 5.10 software (Media Cybernetics) at a magnification ×400 and integrated optical density (IOD) was used as relative amount of glomerular positive staining.

Co-Immunoprecipitation and Western blotting

Co-immunoprecipitations were carried out as per the directions of co-immunoprecipitation kit (Beyotime, China). Podocytes were washed carefully with ice-cold PBS on ice. 500 µl IP/WB lysis buffer (Beyotime, China) was added to cells. The lysate was incubated for 30 min on ice and centrifuged at 15,000g for 20 minutes at 4°C. c-Abl rabbit antibody (CST, USA) was added to the supernatant and centrifuged at 4°C overnight. The samples were mixed with 30 µl agarose beads IgG + IgA and centrifuged at 4°C for 3 hours, followed by centrifugation of beads at 2500g for 1 minute at 4°C and washing of beads with IP lysis buffer, 5 times. Subsequently, the samples were mixed with 5×lane marker sample buffer and heated at 95–100°C for 5 min. For western blot, cells were lysed as described above. The protein samples were separated on 8% SDS-PAGE and then transferred to nitrocellulose membrane (GE Healthcare, USA) by semidry blotting. c-Abl rabbit polyclonal antibody (1:1000; CST, USA), c-Abl-phospho Y245 rabbit polyclonal antibody (1:1000; CST, USA), c-Abl-phospho Y412 rabbit polyclonal antibody (1:1000; CST, USA), -actin mouse monoclonal antibody (1:1000; Santa Cruz, USA), PCNA mouse monoclonal antibody (1:1000; Thermo, USA) were used as primary antibodies. Horseradish peroxidase-conjugated IgG (1:5000; CST, USA) was used as the secondary antibody. Blots were visualized by the enhanced chemiluminescence reaction (Santa Cruz, USA) and developed on the film.

Real time-PCR

Total RNA was extracted from cultured podocytes and isolated glomeruli using Trizol (Invitrogen, USA). The amount of RNA obtained was determined by spectrophotography at 260 nm, and 1 µg RNA was reverse transcribed to cDNA. For PCR amplification, different amounts of the synthesized cDNA (diluted 1:10 in water) were analyzed to evaluate the linearity of the reaction. The relative mRNA level was assessed by CT. The primer amplification was carried out as follows: c-Abl primer: forward, 5'-GTGAAGCCCAAACGAAA-3'; reverse, 5'-CAGCCAGAGTGTGAAGC-3'. -actin primer: forward, 5'-AGCCATGTACGTAGCCATCC-3'; reverse, 5'-TCTCAGCTGTGGTGGTGAAG-3'.

Transfection

The c-Abl plasmid (pEGFP-c-Abl) was kindly provided by Dr. Traci Bock (University of Kentucky, College of Medicine, USA) (Rafalska, et al. 2004). Transfection of c-Abl plasmid was carried out using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction.

Two small interfering RNAs (siRNA) for gene-specific silencing of c-Abl were designed and synthesized by QIAGEN (Germany) company. To select the effective siRNA, all the above siRNA duplexes and a nonspecific nonsilencing siRNA (AllStars Negative Control siRNA; QIAGEN, Germany) were transfected. Transfection of siRNA was carried out using Lipofectamine RNAiMax (Invitrogen, USA) according to the manufacturer's instruction.

Statistic analysis

The data was analyzed using SPSS 17.0. Quantitative data was presented as the mean $\pm$ SEM, at least from three independent experiments. Statistically significant differences in mean values were tested by Student's *t*-test or by one-way ANOVA using Dunnett's test in multiple comparisons. The difference at *p*-value < 0.05 was considered statistically significant.

Results

Effect of c-Abl inhibition on AngII-induced podocyte apoptosis

In a rat model of Ang II infusion, 12 rats were infused with Ang II (400 ng/kg/min) and other group of 12 rats were infused with Ang II (400 ng/kg/min) + STI-571 (50 mg/kg/day, gastric lavage) either for 14 or 28 days. Rats-receiving normal saline infusion served as the control group. Percentage of apoptotic podocytes was recorded in 50 random fields in 6 renal cortical sections for each variable. As shown in Fig.1A, electron microscopic analysis of glomeruli in the Ang II-infused rats on day 14 revealed podocytes with foot processes in closer apposition, narrowed slit diaphragms, and enriched nuclear euchromatin (podocyte apoptosis). And on day 28, the glomeruli developed broadening and segmental fusion of foot processes, devoid of slit diaphragms, accompanied by podocyte apoptosis. No obvious podocyte apoptosis was observed in STI-571 treatment groups. As shown in Fig.1B, the percentages of apoptotic podocytes in Ang II-infused rats were higher than those in control groups (12.62 ± 3.09 vs. 3.52 ± 1.17 at 14 days, 18.69 ± 3.86 vs. 5.05 ± 1.94 at 28 days) and in STI-571 treated groups (12.62 ± 3.09 vs. 6.69 ± 1.96 at 14 days, 18.69 ± 3.86 vs. 7.64 ± 2.23 at 28 days) at the same time points. Ang II-infused rats showed a 3.5 to 4.4-fold increase in percentages of the apoptotic podocytes.

To determine the effects of Ang II on cultured podocytes, the cells were treated with Ang II (10^{-8} mol/L) at several time points (0h, 1h, 3h, 6h, 12h and 24h). Cells were also treated with Ang II (10^{-8} mol/L) in the presence of 50 nmol/L c-Abl inhibitor, Src inhibitor-1 (Src-I1, Sigma, USA) for 6h. As shown in Fig. 1C and 1D, the Ang II promoted podocyte apoptosis in a time-dependent manner. During 3 h to 24 h, Ang II-treated podocytes displayed 3 to 10-fold increase in induction of apoptosis when compared to control group at the respective time points. Nonetheless, pretreatment with Src-I1 (50 nmol/L) significantly inhibited podocyte apoptosis.

Effect of Ang II on c-Abl expression in podocytes

To evaluate the effect of Ang II on podocyte c-Abl expression, Ang II-infused rats were sacrificed and kidney sections were immunolabeled for c-Abl, changes of glomerular c-Abl mRNA and protein level were examined. As shown in Fig.2A and 2B, podocytes displayed both cytosolic and nuclear expression of c-Abl. Ang II-receiving rats displayed upregulated

podocyte expression of c-Abl (Figs. 2Ab and 2Ae) when compared with the normal saline-receiving rats (Figs. 2Aa and 2Ad). However, podocyte c-Abl expression was down regulated in STI-571-treated rats (Figs. 2Ac and 2Af) when compared with Ang II-infused rats at the respective time points. As shown in Fig. 2C and 2D, glomerular c-Abl mRNA and protein expression level were enhanced by Ang II, but down regulated in STI-571 treated rats.

To confirm the effect of Ang II on podocyte c-Abl expression in vitro, we next examined the changes of c-Abl mRNA and protein level of cultured podocytes. Podocytes were treated with Ang II for 6h at different concentration (10^{-9} ~ 10^{-6} mol/L) and with Ang II (10^{-8} mol/L) for several time points (0h, 3h, 6h, 12h and 24h). As shown in Fig. 2E and 2F, Ang II enhanced podocyte c-Abl mRNA expression both in dose- and time-dependent manners. Ang II also stimulated c-Abl protein expression both in dose- and time-dependent manners. (Figs. 2G and 2H).

Effect of Ang II on c-Abl phosphorylation

Phosphorylation of the c-Abl protein at tyrosine 245 and at tyrosine 412 was indicative of an increase in the kinase activity (Westlund et al. 2009; Xu et al. 2010). Thus, to determine whether c-Abl was activated in response to Ang II, podocytes were stimulated by Ang II (10^{-8} mol/L) at different time points. Phosphorylation was determined by immunoblotting with c-Abl-phospho Y245 and Y412 antibodies. As shown in Fig. 3A and Fig. 3B, Ang II significantly stimulated phosphorylation at tyrosine 245 and tyrosine 412, respectively ($P < 0.05$).

Furthermore, the phosphorylated c-Abl was inhibited by c-Abl inhibitor Src-I1 (50 nmol/L) in the presence of Ang II as shown in Figs. 3C-D.

Effect of Ang II on nuclear c-Abl expression in podocytes

Because the nuclear pool of c-Abl has been implicated in proapoptotic effect (Jia et al. 2008; Xu et al. 2010), the subcellular location of c-Abl expression after Ang II stimulation in podocytes was analysed. c-Abl staining in the nucleus (by immunofluorescence) was dramatically increased in Ang II-stimulated podocytes (Figs. 4Ca-e). Western blot analysis of the nuclear fraction shown that the c-Abl protein was significantly increased in the podocytes exposed to Ang II (Fig. 4B); however, the cytoplasmic c-Abl levels did not show any changes (Figs. 4A and 4D).

Furthermore, c-Abl inhibitor, Src-I1 (50 nmol/L) inhibited Ang II-induced nuclear increase of c-Abl in podocytes (Fig. 4Df).

Effect of Ang II on nuclear p53 expression in podocytes

Previous studies have shown that p53 can mediate apoptosis (Liu, et al. 2009; Westlund et al. 2009), and the c-Abl has p53-binding domain (Colicelli 2010). We tested whether c-Abl played a role in p53 expression in Ang II-treated podocytes. As shown in Fig. 5A, After Ang II treatment of podocytes, there was a significant increase in the nuclear expression of p53 in a time-dependent manner. c-Abl inhibitor, Src-I1 significant inhibited Ang II-stimulated ($P < 0.05$) p53 expression in the nucleus of podocytes (Fig. 5B).

Then we examined the effects of Ang II on c-Abl-p53 interactions. This association was observed in both directions when either c-Abl or p53 proteins were immunoprecipitated. As shown in Fig. 5C-D, the c-Abl-p53 complex was increased in Ang II-treated podocytes.

Effect of transfection of c-Abl siRNA on Ang II-induced c-Abl expression and apoptosis in podocytes

To silence c-Abl expression, podocytes were transfected with c-Abl siRNA in the presence or absence of Ang II. The c-Abl mRNA was inhibited by about 65% after transfection with c-Abl siRNA by real-time PCR analysis (Fig. 6A). The c-Abl protein was also decreased after transfection with c-Abl siRNA (Fig. 6B). As shown in Fig. 6C and 6D, c-Abl siRNA transfection inhibited Ang II-induced podocyte apoptosis ($P < 0.05$).

Effect of transfection of c-Abl plasmid on Ang II-induced podocyte apoptosis

To increase c-Abl expression, podocytes were transfected with c-Abl plasmid (pEGFP-c-Abl) in the presence or absence of Ang II stimulation. Subsequently, podocyte apoptosis was evaluated by Hoechst-33342 staining and flow cytometry, respectively. As shown in Fig. 7B and Fig. 7C, c-Abl plasmid transfection exacerbated apoptosis in Ang II treated podocytes.

Discussion

In the present study, we have found that Ang II can induce podocyte apoptosis both *in vivo* and *in vitro*, and c-Abl inhibitor, STI-571, Src-I1 or transfection with siRNA specific to c-Abl can inhibit Ang II-induced podocyte apoptosis. In contrast, transfection with c-Abl plasmid can exacerbate Ang II-induced podocyte apoptosis. These findings indicated Ang II-induced podocyte apoptosis was mediated through c-Abl.

In the present study, c-Abl is expressed in podocytes. Ang II not only upregulated c-Abl in podocytes but also induced podocyte apoptosis. The up-regulated expression of c-Abl may be associated with podocyte apoptosis. The c-Abl is automatically inhibited under normal conditions, and can be activated in other ways such as phosphorylation with the change of the molecular structure, or interaction with other proteins (Bradley and Koleske 2009). A variety of stimuli such as growth factors, cell-matrix and cell-cell adhesion, DNA damage, oxidative stress, and bacterial invasion can activate c-Abl kinases, thus causing autophosphorylation of c-Abl (Bradley and Koleske 2009). Autophosphorylation occurs in a stepwise fashion with the phosphorylation of Y412 followed by the phosphorylation of Y245, or the simultaneous phosphorylation of Y412 and Y245 (Bradley and Koleske 2009). Herein, we have demonstrated that Ang II can activate c-Abl through the phosphorylation at Y412 and Y245. The c-Abl inhibitor Src-I1 can decrease the phosphorylation of c-Abl kinases. Src-I1 is a potent inhibitor of Src, which can also inhibit other Src family members, such as Lck and Csk. However, in contrast with PP1 and PP2 (other Src inhibitors), it did not inhibit p38 /p38 MAPKs or CK1 (Bain, et al. 2007), which suggests that c-Abl is activated by Ang II primarily, and then performs the modulation of podocyte apoptosis.

Latrunculin B and an $\alpha 3 / \alpha 5$ -integrin function-blocking arginine-glycine-aspartic acid (RGD) peptide RGDfV can induce transient phosphorylation of c-Abl and nuclear localization in human brain microvascular endothelial cells. In turn, c-Abl mediates the endothelial apoptosis (Xu et al. 2010). High glucose can induce neural progenitor cell apoptosis, which is associated with the nuclear localization of c-Abl (Jia et al. 2008). Adriamycin-induced DNA damage stimulates the translocation of c-Abl into the nucleus and induces chromatin structural change through histone modifications (Aoyama, et al. 2011). The carboxy terminus of c-Abl contains three nuclear localization sequences (NLS) and a nuclear export sequence (NES). These domains can maintain c-Abl shuttling from cytoplasm to nuclei (Bradley and Koleske 2009). The overexpression of neuronal c-Abl can lead to neuronal loss and neuroinflammation in the mouse forebrain (Schlatterer, et al.

2011). Our data have demonstrated that Ang II can induce the localization of nuclear c-Abl in podocytes. All of these findings have confirmed that c-Abl can mediate podocyte apoptosis by the activation of phosphorylation and nuclear localization through the interaction with nuclear proteins.

Some reports have demonstrated that c-Abl-induced apoptosis is executed partially by p53-dependent manner (Furlan, et al. 2011; Wang, et al. 2011), and the carboxy terminus of c-Abl contains p53-binding domain (Colicelli 2010). Therefore, p53 plays a critical role in tumor suppression, and represents a pivotal mediator of cellular responses to both intrinsic and extrinsic stress signals. The stress-mediated p53 activation generally results in growth arrest or cell apoptosis. As a nuclear transcription factor, p53 has the capability to activate or suppress the expression a large number of genes such as p21, Bax, p53INP1, Bid and Puma (Megyeri, et al. 2007; Skirnisdottir and Seidal 2012; Zhou, et al. 2012). Some evidences have suggested that p53 also has a non-transcriptional function with a direct role at mitochondria in promoting apoptosis (Saha, et al. 2010), suggesting that p53 is one of the most important proteins during podocyte apoptosis. In the present study, nuclear p53 can be upregulated in podocytes treated by Ang II, and the apoptotic podocytes reveal a simultaneous increase. These results suggest that Ang II induce podocyte apoptosis by increasing nuclear c-Abl and p53 expression. On the other hand, this study has also confirmed that c-Abl can bind p53 *in vitro* by immunoprecipitation assay. In our study, c-Abl-p53 complex in podocytes revealed an increase induced by Ang II, which suggests that c-Abl is important for the apoptotic signaling by the activation p53 pathway in podocytes under the stimulation of Ang II.

In summary, c-Abl can be expressed in rat kidney podocytes *in vivo* and cultured mouse podocytes *in vitro*. Treatment of podocytes with Ang II reveals an increase in c-Abl expression. Ang II can induce podocyte apoptosis, and importantly, c-Abl inhibitor or specific siRNA to c-Abl can inhibit pro-apoptotic effect of Ang II. In contrast, the overexpression of c-Abl by podocytes (through plasmid transfection) can increase the apoptosis in podocytes, demonstrating the role of c-Abl in mediating Ang II-induced podocyte apoptosis. This study provides a novel insight into the mechanism by which c-Abl mediates podocyte apoptosis under the stimulation of Ang II. Similarly, c-Abl inhibitor may protect podocytes against the insult from Ang II via the modulation of podocytes apoptosis. These findings maybe provide a basis to test new therapeutic strategies for decreasing podocyte apoptosis in kidney diseases.

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Fig. 1.

Ang II induced podocyte apoptosis *in vivo* and *in vitro*. (A) Apoptotic podocytes of rat kidneys were assessed by transmission electron microscopic analysis in different groups. (a) normal saline infused group on day 14; (b) Ang II infused group on day 14; (c) Ang II + STI-571 group on day 14; (d) normal saline infused group on day 28; (e) Ang II infused group on day 28; (f) Ang II + STI-571 group on day 28. Enriched nuclear euchromatin indicate apoptotic podocytes. Original magnification $\times 12\,000$, scale bars, $1\mu\text{m}$. ($n = 6$). (B) Apoptotic podocytes of rat kidneys were assessed by TUNEL staining in different groups. (a) normal saline infused group on day 14; (b) Ang II infused group on day 14; (c) Ang II + STI-571 group on day 14; (d) normal saline infused group on day 28; (e) Ang II infused

group on day 28; (f) Ang II + STI-571 group on day 28. Black arrows indicate apoptotic podocytes in Ang II-infused rats. $*P < 0.05$ versus control group at the same time point, $^{\#}P < 0.05$ versus Ang II infused group at the same time point, scale bars, $100\mu\text{m}$. (n = 6). (C) Apoptotic cells were assessed by Hoechst-33342 staining in cultured podocyte stimulated by Ang II (10^{-8}mol/L) with or without c-Abl inhibitor (Src-I1) at various time points. Original magnification $\times 400$. $*P < 0.05$ versus 0h of Ang II, $^{\#}P < 0.05$ versus Ang II-treated podocytes at 6h, scale bars, $10\mu\text{m}$. (n=3) (D) One representative experiment (percentage of apoptotic podocytes in the right 2 quadrants) was indicated by flow cytometry.

same time point, [#] $P < 0.05$ versus Ang II infused group at the same time point. (E) and (F): Real-time PCR detection of c-Abl mRNA expression in cultured podocytes treated by Ang II at different doses and time points. (G) and (H): Western blot detection of c-Abl protein expression in cultured podocytes. * $P < 0.05$ versus control group or oh group.

Fig. 3.

Phosphorylated c-Abl expression was increased in podocytes induced by Ang II. A and B: representative western blot for c-Abl and its phosphorylation at Y245 and Y412. * $P < 0.05$ versus 0h group, # $P < 0.05$ versus phosphorylation c-Abl of 0h group. C and D: effects of c-Abl inhibitor (Src-I1) on c-Abl phosphorylation at Y245 and Y412 in podocytes induced by AngII.

Fig. 4.

Nuclear c-Abl expression was increased in podocytes induced by Ang II. (A) Representative Western blot for c-Abl level in the cytoplasm. (B) Representative Western blot for c-Abl level in the nucleus. β -actin or PCNA were used as the cytoplasmic and nuclear markers respectively. * $P < 0.05$ versus 0h group. (C) Representative Western blot for c-Abl level in the nucleus after Ang II and Src-I1 treatment for 6h. * $P < 0.05$. (D) Immunofluorescence staining of podocyte c-Abl expression. Original magnification $\times 400$. a-e: podocytes treated by Ang II for 0h, 3h, 6h, 12h and 24h; f: podocytes treated with Src-I1 and Ang II for 6h.

Fig. 5.

Nuclear p53 expression in podocytes was increased by AngII. (A) Representative Western blot for p53 level in the nucleus of podocytes induced by Ang II (10^{-8} mol/L) for several time points. $*P < 0.05$ versus 0h group. (B) Representative western blot for p53 level in the nucleus induced by AngII and Src-11. $*P < 0.05$ compared with control group, $\#P < 0.05$ compared with AngII group. (C) p53 was immunoprecipitated at 0h, 6h and 12h in the presence of Ang II. (D) c-Abl was immunoprecipitated at 0h, 6h and 12h in the presence of Ang II. The results were representative of three separate experiments.

Fig. 6.

Podocyte apoptosis was decreased by c-Abl siRNA. (A) Real-time PCR detection of c-Abl mRNA expression in podocytes transfected with c-Abl siRNA in the presence of Ang II. Neg siRN representative a nonspecific nonsilencing siRNA. * $P < 0.05$ compared with control group. # $P < 0.05$ versus Ang II-induced group. (B) Western blot detection of c-Abl protein expression in podocytes transfected with c-Abl siRNA in the presence or absence of Ang II. (C) Apoptotic podocytes were detected by Hoechst-33342 staining in different groups. * $P < 0.05$ compared with control group. # $P < 0.05$ versus Ang II-induced group. (D) One representative experiment (percentage of apoptotic podocytes in the right 2 quadrants) was indicated by flow cytometry.

Fig. 7.

Podocyte apoptosis was increased by c-Abl plasmid. (A) Representative Western blot for podocyte c-Abl level in the presence or absence of Ang II. * $P < 0.05$ versus control group, # $P < 0.05$ versus Ang II-induced group. (B) Apoptotic podocytes were detected by Hoechst-33342 staining in different groups. * $P < 0.05$ versus control group, # $P < 0.05$ versus Ang II-induced group. (C) One representative experiment (percentage of apoptotic podocytes in the right 2 quadrants) was indicated by flow cytometry.