Angiotensin II down-regulates nephrin–Akt signaling and induces podocyte injury: role of c-Abl

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ABSTRACT Recent studies have shown that nephrin plays a vital role in angiotensin II (Ang II)–induced podocyte injury and thus contributes to the onset of proteinuria and the progression of renal diseases, but its specific mechanism remains unclear. c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase that is involved in cell survival and regulation of the cytoskeleton. Phosphorylated nephrin is able to interact with molecules containing SH2/SH3 domains, suggesting that c-Abl may be a downstream molecule of nephrin signaling. Here we report that Ang II–infused rats developed proteinuria and podocyte damage accompanied by nephrin dephosphorylation and minimal interaction between nephrin and c-Abl. In vitro, Ang II induced podocyte injury and nephrin and Akt dephosphorylation, which occurred in tandem with minimal interaction between nephrin and c-Abl. Moreover, Ang II promoted c-Abl phosphorylation and interaction between c-Abl and SH2 domain–containing 5′-inositol phosphatase 2 (SHIP2). c-Abl small interfering RNA (siRNA) and STI571 (c-Abl inhibitor) provided protection against Ang II–induced podocyte injury, suppressed the Ang II–induced c-Abl–SHIP2 interaction and SHIP2 phosphorylation, and maintained a stable level of nephrin phosphorylation. These results indicate that c-Abl is a molecular chaperone of nephrin signaling and the SHIP2-Akt pathway and that the released c-Abl contributes to Ang II–induced podocyte injury.

INTRODUCTION Podocytes and their foot processes interposed slit diaphragm (SD) play a crucial role in establishing the selective permeability of the glomerular filtration barrier (Greka and Mundel, 2012). Podocyte injury is associated with proteinuria and the progression of glomerular diseases. Angiotensin II (Ang II) is a well-known risk factor for the initiation and progression of kidney disease (Ruster and Wolf, 2006, 2013). In addition to its hemodynamic effects on renal tissue, the direct effect of Ang II on podocyte injury has been documented extensively (Yu et al., 2013; Shengyou and Li, 2014). Recent studies show that Ang II–induced cytoskeletal rearrangement and podocyte apoptosis contribute to the onset of proteinuria and the progression of renal diseases, but the exact molecular mechanism of Ang II–induced podocyte injury remains unknown.

Nephrin is a widely studied transmembrane protein in the SD region, and several SH2 or SH3 domain–containing proteins, such as podocin (Li et al., 2004), CD2AP (Huber et al., 2003), and Nck (Jones et al., 2006), bind the cytoplasmic domain of nephrin, which suggests that nephrin is a signaling molecule that transmits signals from the SD to the interior of podocytes. Previous studies showed that phosphorylated nephrin is crucial for promoting the survival and maintenance of the stress fibers in podocytes (Huber et al., 2003; Zhu et al., 2008). In concert with those reports, our previous studies demonstrated that Ang II is able to promote nephrin dephosphorylation and podocyte injury, but the nephrin signal transduction pathway deserves further study (Ren et al., 2012). Akt is the major component of the phosphoinositide 3-kinase (PI3K)/Akt pathway and plays an important role in regulating the cytoskeleton and the survival status of various cells.
FIGURE 1: Ang II–induced podocyte injury in vivo and in vitro. (A) Representative transmission electron microscopic images of the ultrastructure of capillary loops in each group (original magnification, ×10,000). The black arrows indicate foot process fusion. The white asterisks show chromatin agglutination in the podocyte nuclei. cap, capillary lumen; podo, podocyte. Scale bar, 1900 nm. (B, C) Representative images of double immunofluorescence staining of WT1 and TUNEL in the kidney sections from each group (original magnification, ×400) and quantification of apoptotic podocytes. AngII, Ang II-infused group; Normal, normal saline–infused group. Scale bar, 20 μm. *p < 0.05 compared with the...
c-Abl is a nonreceptor protein tyrosine kinase that is expressed in podocytes and contains several functional domains that interact with multiple signaling molecules (Panjarian et al., 2013). Among these functional domains, the SH2 and SH3 modules of c-Abl are regarded as interaction modules and allosteric inhibitors of the catalytic domain (Corbi-Verge et al., 2013). After activation, this protein participates in the regulation of diverse cellular events, including cytoskeletal rearrangement and apoptosis (Ba et al., 2005; Dudek et al., 2010; Mitra and Radha, 2010; Wang et al., 2013). In addition, our previous study showed that c-Abl mediates Ang II–induced podocyte injury (Chen et al., 2013). We hypothesize that c-Abl participates in nephrin–Akt signaling in podocytes. Therefore we examined the role of c-Abl in Ang II–induced podocyte injury and nephrin signal transduction.

RESULTS

Effects of Ang II on podocyte injury in vivo and in vitro

Ang II–infused rats developed severe proteinuria compared with saline-infused rats (Figure 1D). An electron microscopic analysis demonstrated the existence of diffuse foot process fusion (cytoskeletal rearrangement) and chromatin agglutination (podocyte apoptosis) in Ang II–treated rats (Figure 1A). A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to further confirm that Ang II can induce podocyte apoptosis in vivo. As shown in Figure 1, B and C, the Ang II–infused rats exhibited higher apoptotic rates than those observed in normal rats (10.52 ± 3.35 vs. 3.52 ± 1.06 on day 14; 15.26 ± 5.13 vs. 3.51 ± 1.14 on day 28). These findings are consistent with our previous observations.

To assess the effects of Ang II on cultured podocytes, we treated the cells with Ang II (10−7 M) at various time points (0, 0.25, 0.5, 3, 6, and 12 h). As shown in Figure 1, E and F, Ang II promoted cytoskeletal rearrangement in the podocytes in a time-dependent manner. The stress fibers in normal cells were disorganized after Ang II treatment. In addition, previous reports showed that reorganization of the podocyte cytoskeleton would affect cellular motility (Hsu et al., 2008), which would promote cell detachment and apoptosis. Therefore we further evaluated the effects of Ang II on podocyte migration and apoptosis. As shown in Figure 1, G–I, Ang II markedly provoked podocyte migration and apoptosis in a time-dependent manner.

Effects of Ang II on nephrin expression and Akt dephosphorylation in vivo and in vitro

Previous studies indicated that nephrin plays an important role in podocyte injury and that Akt signaling could participate in this process, but the signal transduction pattern has not been well characterized (Zhu et al., 2008; Hyvonen et al., 2010; Li et al., 2015). Phosphorylated Akt is regarded as the activated form. As shown in Figure 2A, the levels of nephrin and phosphorylated nephrin were significantly decreased in the glomeruli of Ang II–infused rats, and these decreases were accompanied by a reduction in the levels of phosphorylated Akt. Incubation of the cultured podocytes with Ang II significantly reduced the expression levels of nephrin and phosphorylated nephrin in a time-dependent manner (Figure 2B). Furthermore, the phosphorylated Akt level was also decreased after Ang II stimulation in vitro (Figure 2B). According to the quantitative results, the reduction in phosphorylated nephrin results from the combination of decreases in total nephrin expression and its dephosphorylation.

Effects of nephrin on Ang II–induced podocyte injury and Akt phosphorylation

To further verify that Akt signaling participates in nephrin signal transduction, we transfected pc-DNA3.1-NPHS1 into cultured podocytes. As shown in Figure 3A, pc-DNA3.1-NPHS1 transfection significantly reversed the nephrin down-regulation and dephosphorylation of nephrin and Akt observed in Ang II–treated podocytes compared with the effects observed in untransfected or pcDNA3.1-transfected podocytes. Nephrin overexpression partially weakened the F-actin disruption induced by Ang II (Figure 3B). Moreover, cell migration and apoptosis were partially rescued by the transfection of Ang II–stimulated podocytes with nephrin plasmid (Figure 3, C and D). These findings indicate that Akt serves as a downstream intermediate of nephrin signaling and contributes to Ang II–induced podocyte injury.

Effect of Ang II on the interaction of nephrin and c-Abl

Phosphorylated nephrin tends to interact with molecules containing SH2/SH3 domains (Barletta et al., 2003; Jones et al., 2006). Previous studies showed that c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase involved in cell survival and cytoskeletal regulation. Immunofluorescence assays and coimmunoprecipitation were performed to test whether c-Abl plays a role in nephrin signal transduction in Ang II–treated podocytes. Double immunofluorescence staining revealed that c-Abl and nephrin were colocalized in a linear pattern along the glomerular capillary loops in vivo (Figure 4A) and were coexpressed in the cell membranes and cytoplasm of cultured podocytes (Figure 4C) in the absence of Ang II. According to the quantitative results presented in Figure 2, Ang II stimulation induced not only a decrease in the total expression of nephrin but also its dephosphorylation. Thus we hypothesize that Ang II treatment results in less phosphorylated nephrin available to colocalize with normal group at the same time point. (D) Quantitative analysis of urinary protein excretion in the different groups (n = 6 for each group). *p < 0.05 compared with the normal group at the same time point. (E, F) FITC-phalloidin staining and quantification of cortical F-actin score of each group of differentiated mouse podocytes stimulated with 10−7 M Ang II for various time points (a–f, podocytes treated with Ang II at 10−7 M for 0, 0.25, 0.5, 3, 6, and 12 h, respectively). Scale bar, 10 μm. *p < 0.05 compared with the podocytes treated with Ang II for 0 h. (G, H) Representative migration results and quantification of podocytes treated with 10−7 M Ang II for various time points. Scale bar, 100 μm. *p < 0.05 compared with podocytes treated with Ang II for 0 h. (I) Flow cytometry analysis of the apoptotic rate of differentiated mouse podocytes treated with 10−7 M Ang II for various time points. *p < 0.05 compared with podocytes treated with Ang II for 0 h.
Effects of Ang II on the phosphorylation of c-Abl and SH2 domain–containing 5′-inositol phosphatase 2 and their interaction

Previous observations suggested that c-Abl phosphorylation at tyrosine 412 is necessary for its activation and signal transduction (Brasher and Van Etten, 2000; Tammer et al., 2007). Thus, to determine whether c-Abl is activated in response to Ang II, we stimulated podocytes with Ang II (10^{-7} M) at various time points. The results from a Western blot analysis showed that incubation of the podocytes with Ang II significantly enhanced c-Abl phosphorylation in a time-dependent manner, with maximal phosphorylation at ∼3 h (Figure 5A). Previous studies showed that lipid phosphate SH2 domain–containing 5′-inositol phosphatase 2 (SHIP2) down-regulates the PI3K/Akt pathway by hydrolyzing phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) to phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and that this enzymatic activity is controlled by phosphorylation at tyrosine 986/987 (Batty et al., 2007; Prasad et al., 2009). Phosphorylated c-Abl is able to interact with SHIP2 and regulate its activity (Mokhtari et al., 2013). Thus we examined the interaction between c-Abl and SHIP2 by coimmunoprecipitation and SHIP2 phosphorylation using a Western blot assay and found that c-Abl was coimmunoprecipitated with SHIP2 in podocytes and that its binding to SHIP2 was significantly promoted by Ang II treatment for 30 min (Figure 5B). As shown in Figure 5C, similar to the results obtained for c-Abl, the SHIP2 tyrosine phosphorylation was notably increased in the Ang II–exposed podocytes in a time-dependent manner.

Effects of nephrin on the nephrin–c-Abl and c-Abl–SHIP2 interactions and c-Abl phosphorylation

To further characterize the transduction of the nephrin signal, we further examined the nephrin–c-Abl and c-Abl–SHIP2 interactions and c-Abl phosphorylation in pc-DNA3.1-NPHS1–transfected podocytes. As shown in Figure 6A, the nephrin–c-Abl interaction was enhanced in the nephrin-overexpressing podocytes. In addition, the Ang II–induced phosphorylation of c-Abl and its interaction with SHIP2 were markedly decreased by nephrin overexpression (Figure 6, A and B).

Effect of c-Abl on nephrin–Akt signal transduction

A recombinant plasmid (pcDNA3-Abl-His6-FLAG) was transfected into cultured podocytes to overexpress c-Abl. As shown in Figure 7A, c-Abl transfection elevated c-Abl expression by ∼60% relative to the levels observed in the control cells. The expression of phosphorylated nephrin was not affected in the c-Abl plasmid–transfected cells with or without Ang II treatment (Figure 7A). c-Abl overexpression could mimic the effects of Ang II on SHIP2 phosphorylation and Akt dephosphorylation. Moreover, transfection with the c-Abl plasmid overtly enhanced the Ang II–induced interaction of c-Abl with SHIP2 and SHIP2 phosphorylation accompanied by a further induction of Akt dephosphorylation (Figure 7, A and B).

Effect of c-Abl on Ang II–induced podocyte injury

Related in vitro experiments showed that c-Abl overexpression had a similar effect on podocyte injury as Ang II (Figure 7, C–E). In

c-Abl. As shown in Figure 4, A and C, nephrin–c-Abl colocalization was suppressed after Ang II stimulation both in vivo and in vitro. The same results were obtained in the immunoprecipitation assay. As shown in Figure 4, B and D, the presence of Ang II decreased the level of nephrin–c-Abl complex in the glomeruli and cultured cells. Moreover, Ang II stimulation resulted in a greater reduction in nephrin–c-Abl complex expression compared with the reduction in nephrin expression; thus the reduction in the interaction between nephrin and c-Abl is partially due to a reduction in nephrin expression.

Effects of nephrin on the nephrin–c-Abl and c-Abl–SHIP2

interactions and c-Abl phosphorylation

To further characterize the transduction of the nephrin signal, we further examined the nephrin–c-Abl and c-Abl–SHIP2 interactions and c-Abl phosphorylation in pc-DNA3.1-NPHS1–transfected podocytes. As shown in Figure 6A, the nephrin–c-Abl interaction was enhanced in the nephrin-overexpressing podocytes. In addition, the Ang II–induced phosphorylation of c-Abl and its interaction with SHIP2 were markedly decreased by nephrin overexpression (Figure 6, A and B).

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FIGURE 3: Nephrin overexpression attenuated Ang II–induced Akt dephosphorylation and podocyte injury. The podocytes were transfected with no plasmid, pcDNA3.1, or pcDNA3.1-NPHS1 and then stimulated with Ang II (10^−7 M) for 1 h. Untreated and untransfected podocytes were defined as normal cells. (A) The phospho-nephrin and phospho-Akt expression levels were analyzed by Western blotting. *p < 0.05 compared with the normal cells; #p < 0.05 compared with cells treated with Ang II only. (B) FITC-phalloidin staining and quantification of the cortical F-actin score of each group. (a) Normal group, (b) Ang II–stimulated group, (c) pcDNA3.1+AngII–stimulated group, and (d) pcDNA3.1-NPHS1+Ang II–stimulated group. Scale bar, 10 μm. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only. (C) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only. (D) Flow cytometry analysis of apoptosis in the different groups. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only.
Ang II plays a vital role in the initiation and progression of proteinuric kidney diseases in general and podocyte injury in particular. Our previous studies demonstrated that Ang II exerts a direct effect on podocyte injury (Ding et al., 2002; Ren et al., 2012), but the mechanism remains elusive. In the present study, we found that c-Abl–mediated nephrin–Akt signaling contributes to Ang II–induced podocyte injury.

Nephrin signaling is important for maintaining podocyte structure and function. Verma et al. (2003) demonstrated that the Src-family kinase Fyn directly binds to and phosphorylates nephrin both in vivo and in vitro. Nephrin phosphorylation has been considered important for maintenance of the morphology and function of podocytes under physiological conditions. Fyn-knockout mice display abnormal foot processes and proteinuria (Jones et al., 2006). In addition, c-Abl plasmid transfection significantly augmented Ang II–induced F-actin disruption (Figure 7C). Moreover, c-Abl overexpression markedly enhanced Ang II–induced cell migration and apoptosis (Figure 7, D and E). In contrast, c-Abl siRNA or pretreatment with STI571 significantly ameliorated Ang II–induced podocyte injury (Figure 8, C–E).

**DISCUSSION**

Ang II plays a vital role in the initiation and progression of proteinuric kidney diseases in general and podocyte injury in particular. Our previous studies demonstrated that Ang II exerts a direct effect on podocyte injury (Ding et al., 2002; Ren et al., 2012), but the mechanism remains elusive. In the present study, we found that c-Abl–mediated nephrin–Akt signaling contributes to Ang II–induced podocyte injury.

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that Akt signaling is regulated by nephrin and that nephrin–Akt signaling contributes to sustaining the integrity of the actin cytoskeleton and podocyte survival. The difference between these alterations suggests that other mechanisms may be involved in the regulation of Akt signaling. Of interest, even though Akt phosphorylation was reversed, the podocyte injury appeared to be irreversible, which suggests that the Akt dephosphorylation-triggered downstream signal is persistent. Huber et al. (2003) demonstrated that nephrin expression facilitates the maintenance of Akt phosphorylation at least partially through the nephrin–CD2AP–PI3K pathway in detachment-induced podocyte apoptosis, which suggests that basal Akt activation exerts a protective effect on podocyte injury. Additional studies need to be performed to address the question of whether other mechanisms are involved in the regulation of nephrin–Akt signaling.

Previous studies showed that nephrin phosphorylation initiates molecular interactions with SH2 or SH3 domain–containing proteins, such as Nep1, podocin, and Nck, to thereby maintain the integrity of the actin cytoskeleton and ensure cell survival (Barletta et al., 2003; Huber et al., 2003; Jones et al., 2006). c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase involved in cell survival and cytoskeletal regulation (Hopkins et al., 2012; Kalwa et al., 2012; Wang et al., 2013). However, it is not known whether c-Abl binds to nephrin. In the present study, the interaction between nephrin and c-Abl was detected in glomeruli and podocytes under physiological conditions. Ang II treatment attenuated this association, and this effect was accompanied by c-Abl phosphorylation. Previous reports showed that SH2/SH3 domains are involved in the regulation of nephrin–Akt signaling. Of interest, c-Abl is present in two entirely distinct configurations under different conditions: an inactivated orbicular configuration with the tandem SH3/SH2 domains mechanically clamped to the N- and C-lobes of the catalytic domain, and an activated linear configuration with the catalytic domain exposed to tyrosine kinases (Nagar et al., 2003; de Oliveira et al., 2013; Panjarian et al., 2013). We propose that when phosphorylated nephrin recruits c-Abl through its SH2/SH3 domains, the inactivated configuration of c-Abl is in a stable state, and, when dissociated from nephrin, c-Abl shifts to its linear configuration and is phosphorylated by tyrosine kinases.

SHIP2 is a well-known phosphatase that hydrolyzes PI(3,4,5)P3 to PI(3,4)P2, resulting in the down-regulation of Akt signaling (Pesesse et al., 2001). Wisniewski et al. (1999) demonstrated that c-Abl binds directly to SHIP2 via its SH3 domain. In our studies, we observed that Ang II enhances the interaction between c-Abl and SHIP2. The inhibition of c-Abl activity by ST1571 not only dissociated SHIP2 from c-Abl, but it also suppressed SHIP2 phosphorylation. Mokhtari et al. (2013) demonstrated that imatinib inhibits c-Abl–induced dephosphorylation; in particular, we observed a transient alteration of phospho-Akt and a persistent dephosphorylation of nephrin. To determine whether Akt is a downstream intermediate of nephrin signaling, we evaluated the levels of Akt phosphorylation in podocytes with altered nephrin expression. Nephrin overexpression attenuated the aforementioned Ang II–induced events, indicating that Akt signaling is regulated by nephrin and that nephrin–Akt signaling contributes to sustaining the integrity of the actin cytoskeleton and podocyte survival. The difference between these alterations suggests that other mechanisms may be involved in the regulation of Akt signaling. Of interest, even though Akt phosphorylation was reversed, the podocyte injury appeared to be irreversible, which suggests that the Akt dephosphorylation-triggered downstream signal is persistent. Huber et al. (2003) demonstrated that nephrin expression facilitates the maintenance of Akt phosphorylation at least partially through the nephrin–CD2AP–PI3K pathway in detachment-induced podocyte apoptosis, which suggests that basal Akt activation exerts a protective effect on podocyte injury. Additional studies need to be performed to address the question of whether other mechanisms are involved in the regulation of nephrin–Akt signaling.
FIGURE 7: Effects of c-Abl plasmid transfection on nephrin–Akt signal transduction and Ang II–induced podocyte injury. The podocytes were transfected with no plasmid, pcDNA3, or pcDNA3-Abl-His6-FLAG and then stimulated with 10^−7 M Ang II for 1 h. Untreated and untransfected podocytes were defined as the normal group. (A) Representative Western blots of phospho-nephrin, phospho–c-Abl, phospho-SHIP2, and phospho-Akt expression in the different groups. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only. (B) Representative coimmunoprecipitation results of the interaction between c-Abl and SHIP2 in the different groups. *p < 0.05 compared with the normal cells. #p < 0.05 compared with the cells treated with Ang II only. (C) FITC-phalloidin staining and...
SHIP2 activation, which supports the hypothesis that SHIP2 activation is a downstream event of c-Abl signaling. Several studies found that SHIP2 overexpression can reduce Akt phosphorylation and promote cell injury in various cell lines, including podocytes (Hyvonen et al., 2010). An increasing number of reports support the notion that c-Abl promotes SHIP2 phosphorylation and contributes to Akt dephosphorylation and podocyte injury.

In conclusion, the present study shows that c-Abl is a molecular chaperone for nephrin signaling and the SHIP2–Akt pathway and that c-Abl released from nephrin is involved in Ang II–induced podocyte injury (Figure 9). These findings provide a basis for testing new therapeutic strategies for renin/angiotensin/aldosterone system–associated podocyte injury.

**MATERIALS AND METHODS**

**Animals**
Twenty-four male specific-pathogen-free Wistar rats weighing between 140 and 160 g were supplied by the Hubei Research Center of Experimental Animals (Hubei, China) and raised in a temperature- and humidity-controlled laminar flow room under an artificial light cycle with free access to tap water and standard rat chow. Rats embedded with an osmotic minipump (Model 2002 or 2004; Alzet, Cupertino, CA) were randomly assigned to the normal saline infusion group or the Ang II (Sigma-Aldrich, St. Louis, MO) infusion group, with Ang II administered at 400 ng/kg per minute for 14 or 28 d. We collected 24-h urine samples in metabolic cages and measured urinary proteins on days 7, 14, 21, and 28. The animals were killed on days 14 and 28. The kidneys were perfused with vanadate (a phosphatase inhibitor) before isolation and stored at −80°C for biochemical and renal pathological analyses.

**Cell culture**
Conditionally immortalized mouse podocytes were kindly provided by Peter Mundel (Massachusetts General Hospital, Boston, MA) and cultured under standard conditions. The medium consisted of RPMI 1640 (HyClone, Logan, UT) with 10% heat-inactivated fetal calf serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10 U/ml recombinant mouse interferon-γ (Pepro Tech, Rocky Hill, NJ), and the cells were maintained at 33°C. To induce differentiation, the podocytes were cultured at 37°C for 10–14 d in the absence of interferon. The resulting differentiated podocytes were used in all subsequent experiments. The differentiated cells were stimulated with Ang II (10−7 M) for various times (0–12 h) or for 1 h. c-Abl was inhibited by the addition of STI571 (10 μM, 30 min; Enzo Life Sciences, Farmingdale, NY) before exposure to Ang II. All of the experimental results were verified in three separate cultures of podocytes.

**Western immunoblotting**
The total proteins from the glomeruli and podocytes were extracted with radiocommunoprecipitation assay (RIPA) buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 12,000 rpm and 4°C for 20 min. The supernatants were then mixed with loading buffer and boiled at 95–100°C for 5 min. Equal amounts of protein were separated through 10% SDS–PAGE and then transferred to nitrocellulose membranes (GE Healthcare, Fairfield, CT). The membranes were incubated overnight at 4°C with a primary antibody (nephrin guinea pig polyclonal antibody, 1:500 [PROGEN Biotechnik, Heidelberg, Germany]; p-nephrin (Y1217) rabbit monoclonal antibody, 1:1000 [Epitomics, Burlingame, CA]; c-Abl rabbit polyclonal antibody, 1:500 [Cell Signaling Technology, Boston, MA]; p-c-Abl(Y412) rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; SHIP2 mouse monoclonal antibody, 1:100 [Santa Cruz Biotechnology, Dallas, TX]; p-SHIP2(Tyr986/987) rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; (p-)Akt rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; β-actin mouse monoclonal antibody, 1:2000 [Antigen, Hubei, China]; and glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody, 1:1000 [Antigenec]). An Alexa Fluor 680/790-labeled goat anti-rabbit/goat anti-mouse immunoglobulin G (IgG) antibody (1:10,000; Li-COR Biosciences, Lincoln, NE) was used as the secondary antibody, and the blots were visualized using a LI-COR Odyssey Infrared Imaging System.

**Immunofluorescence assay**
The frozen kidney sections were blocked with 5% bovine serum albumin for 30 min at room temperature. The cell-climbing film (cell growing on a glass slide) was fixed in 4% paraformaldehyde with 0.1% Triton X-100 for 30 min at 4°C. The sections were incubated with a mixture of guinea pig anti-nephrin polyclonal antibody (1:50; PROGEN Biotechnik) and rabbit anti–c-Abl polyclonal antibody (1:50; Cell Signaling Technology) or with fluorescein isothiocyanate (FITC)–phalloidin (2.5 μg/ml; Sigma-Aldrich) overnight at 4°C and then with FITC/tetramethylrhodamine–conjugated IgG as the secondary antibody at 37°C for 90 min in the dark. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Antogene) for 5 min. All microscopic images were recorded using a confocal microscope (Olympus, Japan). The F-actin cytoskeletal reorganization of each cell was scored using a scale ranging from 0 to 3 based on the degree of cortical F-actin ring formation (0, no cortical F-actin, normal stress fibers; 1, cortical F-actin deposits under half of the cell border; 2, cortical F-actin deposits exceeding half of the cell border; 3, complete cortical ring formation and/or total absence of central stress fiber; Hsu et al., 2008).

**Coimmunoprecipitation**
Coimmunoprecipitation experiments were performed according to the manufacturer’s instructions (P2012; Beyotime, China). The total proteins from the cultured podocytes were extracted using lysis buffer (20 mM Tris, 150 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). A c-Abl rabbit polyclonal antibody (1:200; Cell Signaling Technology) was added to the protein samples, and these were then rotated overnight at 4°C. The mixture was then loaded with 40 μl of protein

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**quantification of the cortical F-actin score of each group.** (a) Normal group, (b) Ang II–stimulated group, (c) pcDNA3+Ang II–stimulated group, (d) pcDNA3-c-Abl+Ang II–stimulated group, and (e) pcDNA3-c-Abl group. Scale bar, 10 μm. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only. (D) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only. (E) Flow cytometry analysis of apoptosis in the different groups. *p < 0.05 compared with the normal cells; #p < 0.05 compared with the cells treated with Ang II only.
FIGURE 8: Effects of the knockdown or inhibition of c-Abl on nephrin–Akt signal transduction and Ang II–induced podocyte injury. Podocytes were transfected with no siRNA, scrambled siRNA, or c-Abl siRNA or pretreated with STI571 and then stimulated with $10^{-7}$ M Ang II for 1 h. Untreated and untransfected podocytes were defined as the normal group. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only.

(A) Representative Western blots of phospho-nephrin, phospho-c-Abl, phospho-SHIP2, and phospho-Akt expression in the different groups. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only.

(B) Representative coimmunoprecipitation results of the interaction between c-Abl and SHIP2 in the different groups. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only.

(C) FITC-phalloidin staining and quantification of the cortical F-actin score of each group. (a) Normal group,
was a gift from Benjamin Turk (Addgene plasmid #52684). The transfection of the c-Abl/nephrin plasmid was performed using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, 2 × 10^5 cells were seeded in a six-well plate and transfected with the complexes containing 2 μg of either c-Abl or nephrin plasmid (or a negative control with pcDNA3.1/pcDNA3) and 6 μl of the X-tremeGENE transfection reagent under normal growth conditions for 72 h. G418 (Sigma-Aldrich) was used to select the stably transfected cell lines.

Cell migration assay
Podocytes were seeded into six-well culture plates precoated with type 1 collagen (C3867; Sigma-Aldrich) and cultured under standard conditions. After the cells were prepared under control or experimental conditions, two wounds were made to each well using a sterile pipet tip. The detached cells were subsequently removed by gently washing with PBS. The cells were then cultured at 37°C for another 18 h. Images of the gaps were photographed 0 and 18 h after scraping with an inverted phase-contrast microscope. The number of cells crossing the 1-mm wound border was calculated. Three independent experiments were performed.

Apoptosis assay
Podocyte apoptosis in kidney tissue was assessed by double IF staining with WT-1 and TUNEL according to the manufacturer’s instructions (Roche Applied Science, Basel, Germany). Briefly, antigen retrieval from the dewaxed paraffin sections (3 μm thick) was performed in high-pressure citrate buffer (0.01 mol/l, pH 6.0) for 10 min, and the retrieved antigen was blocked with 10% goat serum for 30 min at room temperature. The sections were then incubated with polyclonal anti–WT-1 antibody (1:100; Santa Cruz Biotechnology) overnight. After washing, Cy3-conjugated anti-rabbit IgG antibody (Boster, Hubei, China) was added for 60 min. The sections were then incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP for 60 min at room temperature. The omission of TdT was used as a negative control. The number of apoptotic podocytes in each glomerular cross-section was counted using the Weibel–Gomez method (Nicholas et al., 2011).

The degree of apoptosis in the cultured podocytes was also evaluated by flow cytometry with annexin V-FITC and 7-aminoactinomycin D (7-ADD) double staining according to the manufacturer’s instructions (FITC Annexin V Apoptosis Detection Kit with 7-AAD; BioLegend, San Diego, CA).

Statistical analyses
The quantitative data are presented as the means ± SEM, and the statistical analyses were performed using SPSS, version 17.0. Statistical comparisons of the groups were conducted with one-way analysis of variance, and the least significant difference test was used for multiple comparisons. Differences with p < 0.05 were considered statistically significant.

(b) Ang II–stimulated group, (c) Scramble siRNA+Ang II–stimulated group, (d) c-Abl siRNA+Ang II–stimulated group, and (e) STI571+Ang II–stimulated group. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only. (D) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only. (E) Flow cytometry analysis of apoptosis in the different groups. *p < 0.05 compared with the normal cells; †p < 0.05 compared with the cells treated with Ang II only.
The control of phosphatidylinositol 3,4,5-trisphosphate concentrations by activation of the Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2, SHIP2. Biochem J 407, 255–266.

REFERENCES

Ba X, Chen C, Gao Y, Zeng X (2005). Signaling function of PSGL-1 in neutrophil: tyrosine-phosphorylation-dependent and c-AbI-involved alteration in the F-actin-based cytoketoskeleton. J Cell Biochem 94, 365–373.

Barletta GM, Kovan IA, Verma RK, Kerjaschki D, Holzman LB (2003). Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers. J Biol Chem 278, 19266–19271.

Batty IH, van der Kaay J, Gray A, Telfer JF, Dixon MJ, Downes CP (2007). Batty I, van der Kaay J, Gray A, Telfer JF, Dixon MJ, Downes CP (2007). The control of phosphatidylinositol 3,4-bisphosphate concentrations by activation of the Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2, SHIP2. Biochem J 407, 255–266.

Brasher BB, Van Etten RA (2000). c-AbI has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. J Biol Chem 275, 35631–35637.

Chen C, Liang W, Jia J, van Goor H, Singhal PC, Ding G (2009). Aldosterone induces apoptosis in rat podocytes: role of PI3-K/Akt and p38MAP kinase signaling pathways. Am J Physiol Renal Physiol 283, F173–F180.

Chen XH, Ren ZL, Liang W, Zhao DQ, Liu YP, Chen C, Singhal PC, Ding GH (2013). c-AbI mediates angiotensin II-induced apoptosis in podocytes. J Mol Histol 44, 597–608.

Corbi-Verge C, Marinelli F, Zafra-Ruano A, Ruiz-Sanz J, Luque I, Faraldo-Gomez JD (2013). Two-state dynamics of the SH3-SH2 tandem of AbI kinase and the allosteric role of the N-cap. Proc Natl Acad Sci USA 110, E1372–E1380.

de Oliveira GA, Pereira EG, Ferretti GD, Valente AP, Cordeiro Y, Silva JL (2013). Intramolecular dynamics within the N-Cap-SH3-SH2 regulatory unit of the c-AbI tyrosine kinase reveal targeting to the cellular membrane. J Biol Chem 288, 28331–28345.

Ding G, Reddy K, Kapasi AA, Franki N, Gibbons N, Kasisnah BS, Singhal PC (2002). Angiotensin II induces apoptosis in rat glomerular epithelial cells. Am J Physiol Renal Physiol 283, F173–F180.

Dudek SM, Chiang ET, Camp SM, Guo Y, Zhao J, Brown ME, Singleton PA, Ding G, Reddy K, Kapasi AA, Franki N, Gibbons N, Kasinath BS, Singhal PC (2002). Angiotensin II induces apoptosis in rat glomerular epithelial cells. Am J Physiol Renal Physiol 283, F173–F180.

Elloul S, Kedrin D, Knoblach NW, Beck AH, Toker A (2014). The adherens junction protein α-catenin associates with phosphoinositide 3-OH kinase and stimulates AKT-dependent signaling. Mol Cell Biol 23, 4917–4928.

Eppert R, Huber TB, Kartl D, Tarpley J, Charo IF, Thapa P, Meltzer PS, Wang C, Wang H, McDonald JR, Warshauer D, Albrecht M, Wang Y, Liu L, Shen Y, He P, Li Q (2010). The control of phosphatidylinositol 3,4,5-trisphosphate concentrations by activation of the Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2, SHIP2. J Biol Chem 285, 21184–21192.

Fred RG, Ravassard P, Scharfmann R, et al. (2013). Imatinib mesilate-induced phosphatidylinositol 3-kinase signaling and improved survival in insulin-producing cells: role of Src homology 2-containing inositol 5-phosphatase interaction with c-AbI. Diabetologia 56, 1327–1338.

Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bommann W, Clarkston B, Superti-Furga G, Kuriyan J (2003). Structural basis for the autoinhibition of c-AbI tyrosine kinase. Cell 112, 859–871.

Nicholas SB, Basgen JM, Sinha S (2011). Using stereotactic techniques for podocyte counting in the mouse: shifting the paradigm. Am J Nephrol 33(Suppl 1), 1–7.

Pavlovic J, Lacedo RE, Chen S, Engen JR, Smithgall TE (2013). Structure and dynamic regulation of abl kinases. J Biol Chem 288, 5443–5450.

Pesesse X, Dewaste V, De Smedt F, Laffargue M, Giuriato S, Moreau C, Payrastre B, Erneux C (2001). The Src homology 2 domain containing inositol 5-phosphatase SHIP2 is recruited to the epidermal growth factor (EGF) receptor and dephosphorylates phosphatidylinositol 3,4,5-trisphosphate in EGF-stimulated COS-7 cells. J Biol Chem 276, 28348–28355.

Prasad NK, Werner ME, Decker SJ (2009). Specific tyrosine phosphorylations mediate signal-dependent stimulation of SHIP2 inositol phosphatase activity, while the SH2 domain confers an inhibitory effect to maintain the basal activity. Biochemistry 48, 6285–6287.

Ren Z, Liang W, Chen C, Yang H, Singhal PC, Ding G (2012). Angiotensin II induces nephrin dephosphorylation and podocyte injury: role of caveolin-1. Cell Signal 24, 443–450.

Ruster C, Wolf G (2006). Renin-angiotensin-aldosterone system and progression of renal disease. J Am Soc Nephrol 17, 2985–2991.

Ruster C, Wolf G (2013). The role of the renin-angiotensin-aldosterone system in obesity-related renal diseases. Semin Nephrol 33, 44–53.

Shengyu, Li Y (2014). The effects of siRNA-silenced TRPC6 on podocyte apoptosis and apolipoprotein E(H) receptor and dephosphorylates phosphatidylinositol 3,4,5-trisphosphate in EGF-stimulated COS-7 cells. J Biol Chem 276, 28348–28355.

Shen L, Yang A, Yao P, Sun X, Chen C, Mo C, Shi L, Chen Y, Liu Q (2014). Gadolinium promoted proliferation in mouse embryo fibroblast NIH3T3 cells through Rac and PI3K/Akt signaling pathways. BioMetals 27, 753–762.

Tammer I, Brandt S, Hartig R, Konig W, Backert S (2007). Activation of AbI by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. Gastroenterology 132, 1309–1319.

Venkatadreddy M, Cook L, Abuharb Q, Verma R, Garg P (2011). Nephrin regulates lamellipodia formation by assembling a protein complex that includes SH2pi, filamin and lamellipodin. PLoS One 6, e28701.

Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, Wiggins RC, Killen P, Holzman LB (2003). Fyn binds to and phosphorylates the kidney cytoskeletal protein nephrin. J Biol Chem 278, 20716–20723.

Wang R, Mercats OP, Jia L, Panettieri RA, Tang DD (2013). Raf-1, actin dynamics, and abelson tyrosine kinase in human airway smooth muscle cells. Am J Respir Cell Mol Biol 48, 172–178.

Wisniewski D, Strife A, Swedeman S, Ejdurjen-Brommage H, Geronamos 5, Kavanagh WM, Tempst P, Clarkson B (1999). A novel SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP2) is constitutively tyrosine phosphorylated and associated with src homologous and collagen gene (SHC) in chronic myelogenous leukemia progenitor cells.Blood 93, 2707–2720.

Yu SY, Qi R, Zhao H (2013). Losartan reverses glomerular podocytes injury induced by AngII via stabilizing the expression of GLUT1. Mol Biol Rep 40, 6295–6301.

Zhong L, Song N, Aoudjit L, Li H, Kawachi H, Lemay S, Takano T (2008). Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podoocytes. Kidney Int 73, 556–566.