Rapamycin attenuated podocyte apoptosis via upregulation of nestin in Ang II-induced podocyte injury

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Abstract

Background: Angiotensin II (Ang II) contributes to the progression of glomerulosclerosis mainly by inducing podocyte injury. Convincing evidence indicates that the mTOR inhibitor rapamycin plays a fundamental role in the protection of podocyte injury. Nestin, a major cytoskeleton protein, is expressed stably in podocytes and correlates with podocyte damage. The purpose of this study was to investigate the effect of rapamycin in podocyte injury induced by Ang II, and clarify the role and mechanism of Nestin in the protection of podocyte injury.

Methods and Results: We established an Ang II perfusion animal model, and the effects of interfere treatment of rapamycin on podocyte were detected *in vivo*. *In vitro*, podocytes were stimulated with Ang II and rapamycin to observe podocyte injury, nestin-siRNA was transfected to investigate the mechanism in the process. We observed that Ang II induce podocyte injury both in vivo and in vitro, while rapamycin treatment relieved Ang II-induced podocyte injury. Also we found that nestin co-localized with p-mTOR in glomeruli, and the protection effect of rapamycin was reduced by nestin-siRNA in podocytes. Moreover, co-IP indicated the interaction between nestin and p-mTOR, and nestin could affect podocyte injury via mTOR/P70S6K signaling pathway.

Conclusion: Thus, here, we demonstrated that Rapamycin attenuated podocyte apoptosis via upregulation of nestin through mTOR/P70S6K signsling pathway in Ang II-induced podocyte injury.

Key Words: Nestin, Rapamycin, Ang II, Podocyte injury, Apoptosis

Introduction

Podocytes, which function to maintain the integrity of glomerular filtration barrier, are terminally highly differentiated in renal glomerulus [1]. Podocyte injury causes proteinuria and leads to the

progression of glomerular disease [2] . Angiotensin II (Ang II), an important active effector in renin-angiotensin system (RAS), contributes to podocyte injury, including podocyte apoptosis, cytoskeletal rearrangement and loss of the slit diaphragm [3-5].

Several researches have shown that podocyte injury is closely related to the activation of adaptive mammalian target-of-rapamycin (mTOR) [6-9], which plays an important role in glomerular disease. Rapamycin, which mediated by inhibition of mTOR, was shown to have immunosuppressive and antiproliferative efficiency [10-11]. It has been revealed that diabetic nephropathy rats treated by rapamycin were protected from kidney impairment [12].

Nestin is a class VI intermediate filament (IF) protein , which was characterized in neuroepithelial stem cells initially [13],and participate in the composition of cytoskeleton. Nestin is stably expressed in mature podocytes and plays a significant role in maintaining the normal morphology and function of podocytes [14-15]. However, the involved signaling mechanism of nestin function in glomerular podocytes has not yet been established.

In the current study, we hypothesized that rapamycin attenuate podocyte injury under Ang II conditions, and nestin could play an important role in the process. Given that, we explore the effects of rapamycin on podocytes with changes of nestin expression and elucidate the underlying mechanisms.

Materials and methods

Animals

Eight-week-old , 20-22-g male C57BL/6 mice (Vital River Laboratory Animal Technology, Beijing, China) were included in the experiments. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Mice underwent a sham operation under light anesthesia with 3% isoflurane. Two weeks after surgical intervention, an osmotic minipump (model 2004; Alzet, Cupertino, CA) was implanted subcutaneously in mice to infuse Ang II for 4 weeks. Mice were divided into three experiment groups randomly: 1. control group (sham operation, n=6); 2. Ang II group (Ang II, 400 ng/kg/min [16], subcutaneous embedded anosmotic minipump, n=6); 3. Ang II+RP group (Ang II, 400 ng/kg/min, subcutaneous embedded minipump, intraperitoneal injection 1 mg/kg/day RP, n=6); The drinking water of all groups contained 1% sodium chloride throughout the experimental period. Kidneys were collected for biochemical and renal pathological analysis.

Cell culture

The immortalized mouse podocyte cell lines （MPCs） in the study were gifted from Dr Junwei Yang(Nanjing Medical University). Podocytes were cultured in RPMI-1640 medium (GIBCO, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin G (GIBCO, USA) and 10 U/ml recombinant mouse IFN-γ at 33 °C for proliferation firstly. Then, podocytes were cultured without IFN-γ at 37 °C to induce differentiation for 10-14 days. Differentiated podocytes were made quiescent and the cells were then exposed to Ang II (10^{-6} mol/L) and rapamycin (500 ng/ml) treatment for the indicated time periods.

Transfection of small interference RNA

MPCs were transfected with nestin siRNA (sense, 5'- GGAAGUGACUAGUGAGACATT-3' and antisense, 5'- UGUCUCACUAGUCACUUCCTT-3') (genecham, Shanghai, China) using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. In brief, siRNA for nestin at a concentration of 40nM was diluted into each 6-well plate with Transfection Medium (Opti-MEM, Invitrogen,USA) and incubated for 5 min. Diluted lipofectamine reagent with Opti-MEM and siRNA were mixed and incubated at room temperature for 20 min. After 6 h of transfection, cells were used for future experiment.

Immunohistochemistry

Immunohistochemical stains were performed on 4% paraformaldehyde-fixed, paraffin-embedded 3-μm renal tissue sections. After antigen recovery, the sections were incubated with primary antibodies against nephrin (1:200, mouse, sc-377246) overnight at 4˚C. The sections were then incubated with polyperoxidase anti-mouse IgG. Reactions were stained with a DAB substrate kit (MXB biotechnologies, DAB-0031, Fuzhou, China) and counterstaining was performed using hematoxylin. The sections were captured under a microscope (OLYMPUS, BX53, Tokyo, Japan), and then Image ProPlus was used to quantify the average optical density value.

Immunofluorescence staining

The kidney paraffin sections (3 μm) were prepared. Cells grown on glass cover slips were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After being blocked with 5% BSA for 1 h, the slides of kidney and cells were incubated with primary antibodies against WT1(1:200, rabbit, #83535, Cell Signaling Technology), p-mTOR (1:100, rabbit,

#5536, Cell Signaling Technology), nestin (1:200, mouse, sc-23927, Santa Cruz Biotechnology) and nephrin (1:200, mouse, sc-376522, Santa Cruz Biotechnology) in PBS containing 1% BSA at 4°C overnight. FITC/TRITC- conjugated IgG was used as a secondary antibodies for 1 h at room temperature. All samples were mounted with DAPI dye for 5 minutes and captured under the confocal microscope (OLYMPUS, FV1000, Tokyo, Japan).

Annexin V staining assay

Podocytes from different groups were quantified by Annexin V staining according to the manufacturer's instructions (KeyGEN Biotech, Jiangsu, China). Briefly, podocytes were harvested and then washed twice with phosphate-buffered saline (PBS). The cells were resuspended in 100 μl of ice-cold binding buffer and then incubated with 5 μl of Annexin V(conjugated with FITC) for 15 min in the dark. After resuspension in 400 μl of binding buffer, the cells were observed under a fluorescence microscope (OLYMPUS, BX53, Tokyo, Japan).

TUNEL assay

The tissue sections(3μm) were used to detect DNA fragmentation for apoptosis by using the One Step TUNEL Apoptosis Assay Kit (KeyGEN Biotech, Jiangsu, China) according to the manufacturer's instructions. Kidney cells with TRITC nuclear markers were considered TUNEL positive. The cells were counterstained with DAPI and fluorescent images were acquired with the confocal microscope.

Western blotting assay

Renal tissues and cultured cells were harvested after treatment, and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. BCA assay (KeyGEN Biotech, Jiangsu, China) were used to detect protein concentration. Detection of protein expression by western blotting was performed according to established protocols [17].The same amounts of protein were subjected to 10% SDS-PAGE and transferred to PVDF membrane (Millipore, HATF09025). The membrane were blocked with 5% BSA in TBST for 1 h. And the membrane were incubated overnight at 4 ℃ with the primary antibodies as followed: Nephrin (1:1000, mouse, sc-376522, Santa Cruz Biotechnology), Nestin (1:500, mouse, sc-23927, Santa Cruz Biotechnology), Bax (1:1000, rabbit, AF0120, Affinity Biosciences), p53（1:1000, mouse, BF8013, Affinity Biosciences）and GAPDH (1:1000, rabbit, #5174, Cell Signaling Technology)

were used. Secondary HRP-conjugated goat anti-mouse or anti-rabbit antibodies (Cell Signaling Technology) were used. Western ECL substrate (Biosharp, China) was used to visualize the proteins.

CO-Immunoprecipitation (CO-IP) and immunoblotting

Cells were lysed with lysis buffer, and proteins were immunoprecipitated from the cell lysates with the indicated primary antibodies overnight at 4 °C according to the manufacturer's instructions (abs955, Absin, Shanghai, China). Immunoprecipitates were mixed with protein G agarose beads for 1 h at 4 °C, collected, and washed with lysis buffer. After incubation, the immunoprecipitates complexes were rinsed and analyzed content of the sample by immunoblotting.

Statistical analysis

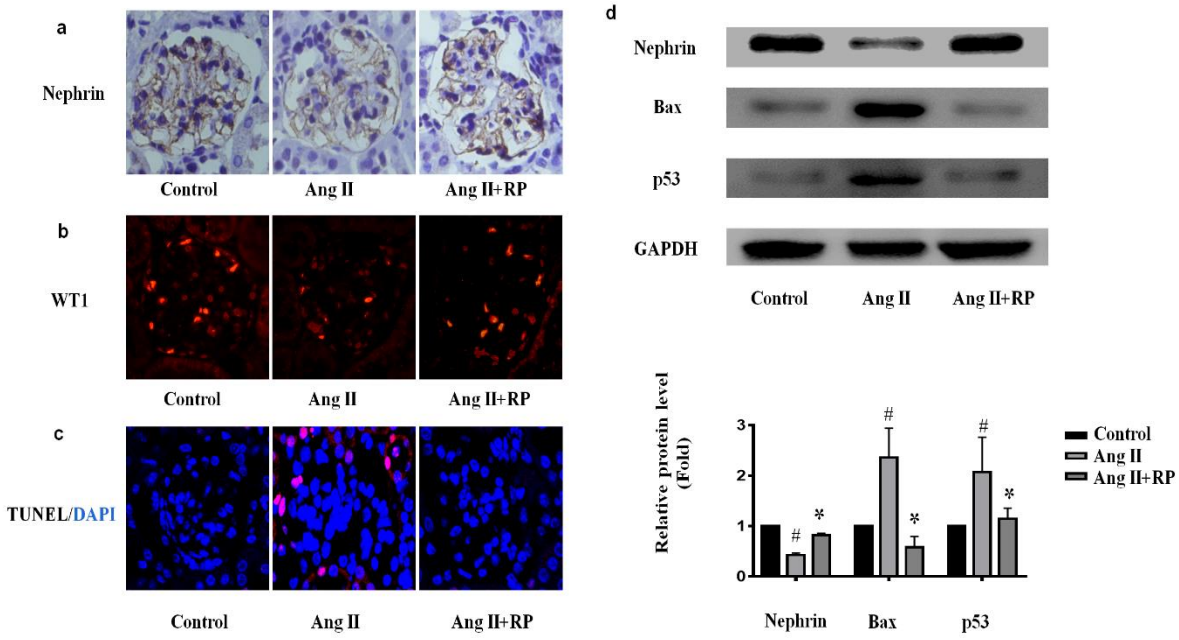
Results are expressed as mean±SEM from at least three independent experiments. Statistical analysis was performed using Prism 6.0 for Windows (GraphPad Software, Inc., California, U.S.A.). Inter-group comparisons were assessed by ordinary ANOVA followed by Bonferroni's multiple comparison post-test. A two-sided P-value of <0.05 was considered to indicate statistically significant differences.

Results

Rapamycin attenuated podocyte injury in Ang II -infused mice

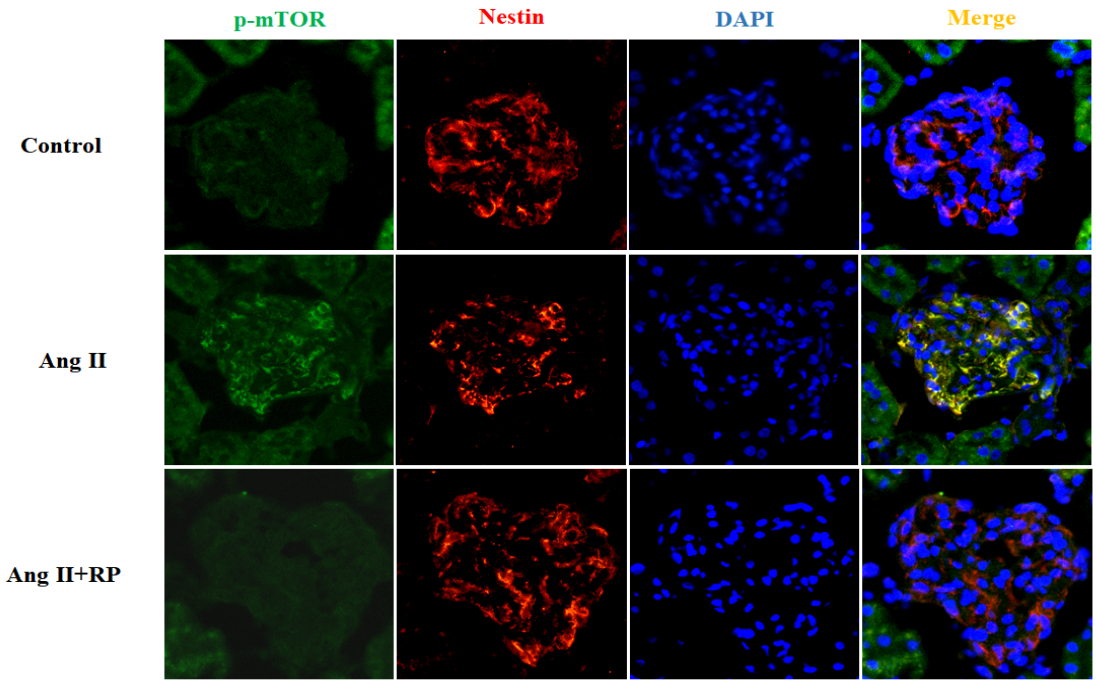
We established an Ang II infused mice model to investigate the effects of rapamycin in glomerular and podocyte injury. The dysregulation or loss of nephrin, an important structural molecule in silt diaphragm, has been suggested to precede podocyte loss. As shown in Fig.1, deletion of nephrin was confirmed via immunohistochemistry and western blotting assays of kidney sections after Ang II stimulation compared with control group in our study. In the presence of rapamycin, podocyte injury induced by Ang II was attenuated. A higher expression level of nephrin was noticed after injection of rapamycin compared with Ang II group (Fig.1a and 1d). Additionally, immunochemical staining for Wilms's tumor protein (WT1), a surrogate maker for podocyte number, suggested that the number of podocytes was decreased in Ang II group compared with the control group. The number of WT-1 immunopositive podocytes was higher in glomeruli of the rapamycin-treated group than in glomeruli of Ang II group (Fig.1b). TUNEL assay showed that Ang II induced podocyte apoptosis, which was decreased by rapamycin treatment (Fig.1c). The expression of glomerular apoptosis-related proteins including Bax and p53 were determined to investigate the effects of rapamycin on podocytes apoptosis. In Ang II group, remarkable up-regulation

of Bax and p53 were found, whereas significant inhibition of Bax and p53 expression were noticed after interference of rapamycin (Fig.1d). All these suggest that rapamycin attenuated podocyte injury in Ang II -infused mice.



Inhibition of mTOR signaling activation by rapamycin restored nestin expression in glomeruli of Ang II-infused mice

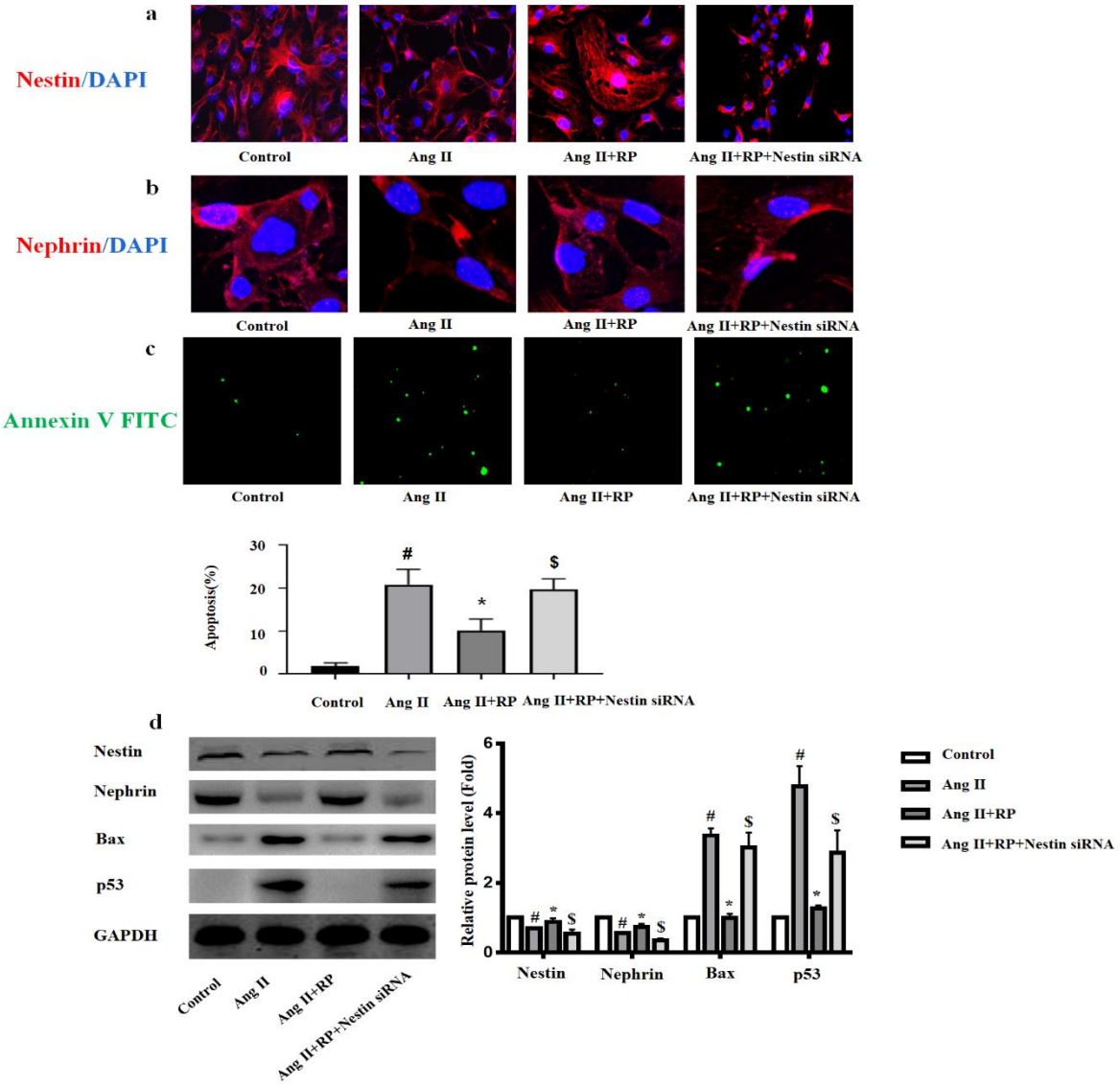
We evaluated the expression level of signal transduction in renal tissue samples by p-mTOR immunofluorescence. As demonstrated in Fig.2, the expression of p-mTOR in glomeruli increased significantly, suggesting the activation of mTOR signaling. And it was showed that Ang II+RP group exhibited a lower p-mTOR expression than Ang II group. Interesting， nestin, a cytoskeleton disruption, was co-localization with p-mTOR in glomeruli. The expression of nestin was weakly observed in glomeruli of Ang II group. In the rapamycin-treated group, nestin expression was higher in contrast (Fig.2).These results indicated that rapamycin attenuated mTOR signaling activation by restored nestin expression in glomeruli of Ang II-infused mice.



Inhibition of nestin attenuated the protective effect of rapamycin in Ang II-induced podocyte injury

To futher explore the effect of nestin in the protection of rapamycin, podocytes were cultured and differentiated as described. We employed siRNA interference to assess the effect of nestin depletion prior to subjecting the podocytes to Ang II stimulation. The expression of nestin was decreased in podocyte under Ang II condition, while rapamycin treatment upregulated nestin expression. Following transfection with nestin siRNA, we observed that, the morphology of podocytes changed significantly, and the podocyte mutation was short or disappeared by immunofluorescence (Fig.3a). In addition, Ang II treatment led to a decrease in nephrin expression, and a higher nephrin protein level was detected in Ang II+RP group compared with Ang II group. Meanwhile, the expression of nephrin was down-regulated when infected with nestin-siRNA (Fig.3b). The protein expression of nestin and nephrin were also detected by western blotting, and the results were consistent with the changes shown in immunofluorescence (Fig.3d). To determine the effect of nestin on podocyte apoptosis, the cells were subjected to FITC-Annexin V staining. It was observed that podocyte apoptosis increased significantly when treated with Ang II, as evidenced by the number of Annexin V-positive cells (green), and RP treatment reduced podocyte apoptosis. And we found that the number of apoptotic podocytes were enhanced in cells co-treated with nestin siRNA in comparison to Ang II+RP group (Fig.3c). We also

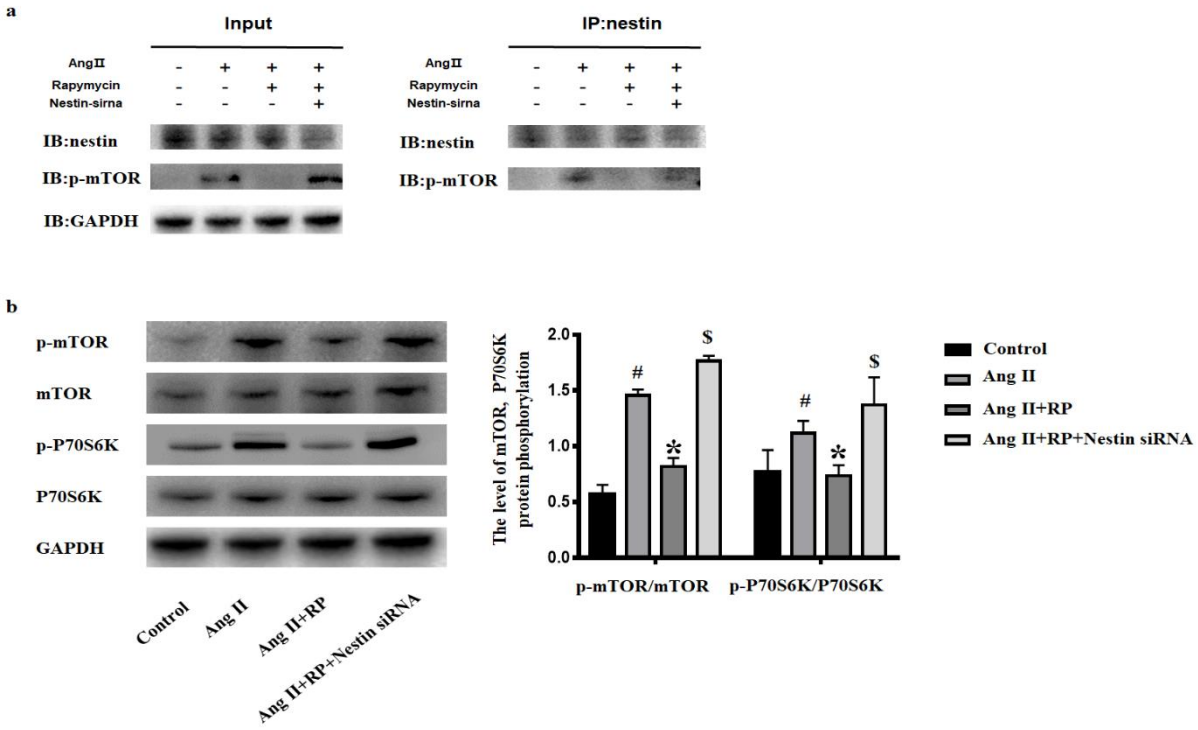
investigated the expression of Bax and p53. The results of western blotting analysis revealed that Bax and p53 protein level were increased in Ang II stimulation, while their expression was decreased when co-treated with Ang II and RP than treated with Ang II alone. Corresponding to cellular apoptosis, the expression of Bax and p53 were higher when knocked down with nestin siRNA compared with Ang II+RP group (Fig.3d).



Nestin combines with p-mTOR and inhibition of nestin enhances mTOR/P70S6K activation .

As demonstrated before, Immunofluorescence staining showed that nestin and p-mTOR were co-localization in glomeruli. Co-IP assay was carried out to verify the interaction between nestin and p-mTOR. Co-IP demonstrated that nestin could interact with p-mTOR (Fig.4a). And then we detected the phosphorylation levels of mTOR and P70S6K protein by western blotting (Fig.4b).We analyzed the

relative levels of mTOR, p-mTOR, P70S6K, and p-P70S6K. The levels of mTOR, and P70S6K protein were approximately equal in each group. Western blotting assay also exhibited a significant increase in the p-mTOR and p-P70S6K protein levels in Ang II group compared with control group. Furthermore, the levels of p-mTOR, and p-P70S6K proteins were decreased when treated with rapamycin. Interestingly, compared with the Ang II+RP group, mTOR and P70S6K phosphorylation was up-regulated when knock down the expression of nestin .



Discussion

A multitude of clinical and experimental studies suggested that podocyte injury is a frequent pathological phenomenon due to various stresses and pathological stimuli, and it causes proteinuria formation and detachment from the glomerular basement membrane in kidney diseases [1]. Previous studies have confirmed that Ang II, an important active effector in the RAS, plays a critical role in CKD progression and podocyte injury [18]. It is reported that Ang II infusion contributes to proteinuria and hypertension in vivo and leds to podocytes injury especially apoptosis subsequently [19]. We found that Ang II infusion induced podocyte injury through the study *in vivo*, following with decreased of nephrin expression and loss of podocytes, as revealed in the previous study.

The mTOR pathway, which plays a central role in cellular growth, metabolism, apoptosis and

proliferation, is essential for kidney growth during physiological body growth[20]. A growing number of studies have shown that mTOR signaling pathway is an important member of maintaining podocyte homeostasis and tubelar transportation in renal diseases [21-22]. In podocytes, abnormal integrated mTOR signals can be activated by numerous growth factors and cytokines. Excessive activity of mTOR complexes can result in severe pathologic effects, including the mislocalization of slit diaphragm proteins, thickening of glomerular basement membrane, loss of podocyte and effacement of podocyte foot-press [23]. Rapamycin, as the inhibitor of mTOR signal, has been proven to be an effective therapeutic approach in animal models of glomerular disease and in clinical studies [24].Our data showed that mTOR signaling was activated after stimulation with Ang II *in vivo*, and podocyte damage was involved in the activation of the mTOR pathway. Also we found that rapamycin could attenuated podocyte apoptosis in Ang II induced podocyte injury.

Nestin, a cytoskeleton-associated class VI intermediate filament (IF) protein, is expressed in glomerular endothelial cells and tubuloepithelial cells transiently during renal development. In addition, nestin is highly expressed in the mature glomerular podocyte [25]. Recent findings indicate that the disruption of the cytoskeleton is related to podocyte injury [26]. It was demonstrated that nestin plays an important role in maintaining the stability of podocytes and normal podocyte function [14]. It has been proved that nestin expression in podocytes is closely related to proteinuria in kidney diseases, and alterations of nestin may occur to enable the podocytes to undergo morphological changes [27]. In the present study, decreased nestin expression and increased p-mTOR expression were observed in Ang II induced glomerulus. In addition, we found that rapamycin inhibited mTOR activation followed with upregulation of nestin. We surmised that nestin plays an important role in the protection of rapamycin in Ang II induced podocyte injury. To probe into the role of nestin in the process, we employed siRNA interference to assess the effect of nestin depletion prior to subjecting the podocytes. Our results showed that the restored effect of rapamycin on nephrin expression was attenuated by nestin inhibition in Ang II induced podocyte injury. The numbers of apoptotic podocytes were increased when infected with nestin-siRNA. These results suggested that nestin is not only a maker of podocyte injury, but also a damage promoting factor in the process.

However, the molecular mechanisms of cytoprotective role remain unclear. To further study, we investigated the relationship with mTOR and nestin. Co-IP method in podocyte verified the authenticity of the interaction between the two. Our studies revealed that mTOR interacted with nestin. Then, we

detected the phosphorylation levels of mTOR and P70S6K protein. We found that nestin affected the protective effect of rapamycin via mTOR/P70S6K signaling pathway. The mTOR is the catalytic subunit of two distinct protein complexes, mTORC1 and mTORC2, which can be distinguished by their unique composition and different substrates [28]. The main function of mTORC1 is the activation of anabolic processes, whereas mTORC2 has major roles in cytoskeleton organization and cell survival [29]. mTORC1 promotes protein synthesis largely through the phosphorylation of p70S6 kinase1 (P70S6K) effectors. The activation of (macro)autophagy is the consequences of rapamycin inhibition of mTORC1. Previous findings indicated that rapamycin disrupted the mTOR–autophagy balance by suppressing the phosphorylation of 4EBP1 and P70S6K in podocytes, and resulted in the restoration of podocyte damage [30]. mTORC2 plays an important role in the regulation of cytoskeleton structure [31]. These two mTOR complexes with different functions may cross-talk with each other to balance their signaling selfly. Moreover, either S6K1 or Akt has also been shown to control mTORC2 activity through a discovered negative-feedback loop recently [32]. It was found that mTOR/P70S6K interacted with Rac1 to recombine the actin cytoskeleton [33]. However, the underlying mechanism remains unclear and needs to be investigated further.

In conclusion, our study demonstrates that rapamycin can promote alleviate Ang II-induced podocyte injury by up-regulating of nestin *in vivo and vitro.* It was evaluated that nestin played a crucial role in podocyte via mTOR/P70S6K signaling pathway through RNA interference technology and other experiments. This finding not only helps us to elucidate the mechanism of podocyte injury and the protection of rapamycin, but also provides a new potential therapeutic strategy for the treatment of CKD.

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Availability of data and materials

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

W.G. and A.Z. designed the study. H.S. and Y.Z. performed the experiments and discussed the results. H.S. wrote the manuscript. T.H., G.Q. and S.L. performed the experiments and analyzed the data.

Ethics approval and consent to participate

The study was approved by Institutional Animal Care and Use Committee at Nanjing Medical University.

Conflict of interest

The authors declare no conflict of interest.

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Fig.1. Rapamycin attenuated podocyte injury in Ang II-infused mice.

(a) As indicated, Immunohistochemical staining for nephrin in kidney tissue from each group. Magnification, x400. (b) Representative images of WT-1 immunofluorescent staining in kidney tissues from the above group. Magnification, x400.(c) TUNEL staining (Red) in kidney sections after the indicated treatment. The sections were counterstained with DAPI (Blue). Magnification, x400. (d) Western blotting analysis of nephrin, Bax and p53 expression in the kidney. The levels of GAPDH were used as standard loading controls. The data are presented as mean ± SEM . #P<0.05 vs. control group, *P<0.05 vs. Ang II group.

Fig.2. Inhibition of mTOR signaling activation by rapamycin restored nestin expression in glomeruli of Ang II-infused mice.

Indirect immunofluorescent staining showed the expression and localization of p-mTOR (green), nestin (red), and DAPI (blue) in glomeruli from various group, as indicated. Magnification, x400.

Fig.3. Inhibition of nestin attenuated the protective effect of rapamycin in Ang II-induced podocyte injury. (a) Immunofluorescence staining showed the expression of nestin(Red) in podocyte after the indicated treatment. Magnification, x200.(b) Immunofluorescence staining showed the expression of nephrin(Red) in podocyte after the indicated treatment. Magnification, x400.(c) Annexin V FITC staining (Green) in podocyte after the indicated treatment. Magnification, x200. (d)Western blotting for nestin, nephrin, Bax and p53 after the indicated treatment. The data are presented as mean ± SEM. $^{\#}P<0.05$ vs. control group, $^{*}P<0.05$ vs. Ang II group.$^{\$}$ $P<0.05$ vs. Ang II +RP group.

Fig.4. Nestin combines with p-mTOR and inhibition of nestin enhances mTOR/P70S6K activation.

(a)Co-immunoprecipitation of nestin and p-mTOR in podocytes. Cell lysate was then extracted for co-immunoprecipitation with anti-nestin followed by probing with anti-nestin and anti-p-mTOR after the indicated treatment.(b) Western blotting for mTOR, p-mTOR, P70S6K, and p-P70S6K after the indicated treatment. The data are presented as mean ± SEM. $^{\#}P<0.05$ vs. control group, $^{*}P<0.05$ vs. Ang II group.$^{\$}$ $P<0.05$ vs. Ang II +RP group.