IJKD KIDNEY DISEASES

Upregulation of Cabin1 During Injury to Renal Tubular Epithelial Cells in Rats

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Original Paper

Introduction. Calcineurin-binding protein 1 (Cabin1) interacts with calcineurin and p53, but its function in renal tubular epithelial cell (RTEC) is unclear. We established 5/6 nephrectomized rats and angiotensin II-induced injury to the RTECs in vitro, to observe the expression of Cabin1 during RTEC injury.

Materials and Methods. Sprague-Dawley rats were sacrificed at 4 and 8 weeks after 5/6 nephrectomy. Renal pathology and mitochondrial damage were detected by light and electrical microscope. The distribution of E-cadherin and α-smad were detected by indirect immunofluorescence staining. Cabin1 protein expression was detected by Western blot.

Results. Obvious tubulointerstitial fibrosis was found in the nephrectomized rats at 8 weeks after 5/6 nephrectomy, accompanied by the increasing levels of creatinine, as well as the disruption of E-cadherin and overexpression of α-smad in RTECs. Moreover, the mitochondria became swollen and mitochondrial cristae were disrupted and poorly defined in the RTECs. Compared to the sham-operated rats, Cabin1 protein expression was significantly increased at 8 weeks after 5/6 nephrectomy, while angiotensin II-induced Cabin1 protein expression significantly increased 48 hours after stimulation in normal rat kidney epithelial cells.

Conclusions. Injury to the RTEC and Cabin1 protein overexpression occurred in a time-dependent manner both in vitro and in vivo. Cabin1 may become a potential molecular target in RTEC injury.

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INTRODUCTION

Chronic kidney disease (CKD) is a common and severe clinical problem. Plenty of studies have reported that the renin-angiotensin system plays an important role in the progression of CKD, while angiotensin II is the main effector.[1] Angiotensin II induces the damage to renal glomerular and tubular epithelial cells by upregulating transforming growth factor-β, which results in the development of renal fibrosis.[2] Progression of renal fibrosis contributes to the decreasing of kidney function.[3] However, the exact mechanism of angiotensin II in renal tubular epithelial cell (RTEC) injury has not been fully illuminated.

Calcineurin-binding protein 1 (Cabin1), which interacts with calcineurin and p53, is a key regulator of cell apoptosis.[4] Our previous study showed that Cabin1 was localized in RTECs and glomerular podocytes, and it underwent nuclear translocation during podocyte injury.[5] Moreover, knockdown of Cabin1 with small interfering RNA induced the decreasing expression of p53 protein in podocyte cell line.[6] While tacrolimus restored podocyte injury and stabilized the expression of Cabin1 in

5/6 nephrectomized rats.[7] To date, the function of Cabin1 in RTEC injury has not been reported. To demonstrate the function and mechanism of Cabin1 in RTEC injury, we established 5/6 nephrectomized rat model and angiotensin II-injured RTEC model in vitro.

MATERIALS AND METHODS

Establishment of Nephrectomized Rat Model

With the approval of the Animal Ethics Committee (Guangdong Province), experiments were conducted on Sprague-Dawley rats (Medical Laboratory Animal Center of Guangdong Province, Guangzhou, China). In this study, we strictly followed the principles of laboratory animal care. The 5/6 nephrectomy was performed under general anesthesia. Each 5/6 nephrectomy group included 5 rats and the control group included 6. By retroperitoneal approach, 2 poles of the left kidney were removed by polectomy. One week later, the right kidney was removed. Sham-operated rats underwent anesthesia, ventral laparotomy, and manipulation of the renal pedicles without removal of renal mass.[6] Rats were sacrificed at 4 and 8 weeks after the operation, and blood and urine samples were collected for serum creatinine, blood urea nitrogen (BUN), and 24-hour urinary protein excretion, and then remnant or control kidneys were removed. Kidney tissues were prepared for Western blot, Masson trichrome, electron microscopy, and indirect immunofluorescence staining.

Morphological Studies

Two-micrometer sections of kidney tissues were cut and stained with Masson trichrome. All slides were evaluated by an experienced pathologist using an Olympus CH20BIMF200 microscopy. Sections from each kidney cortex were graded for the presence of interstitial fibrosis.[8] The scale for each rat was reported as the mean of 20 random medium-power (× 200) fields per section.

Renal samples were processed through a primary and a secondary fixation, acetone dehydration, and then EponSpurr resin infiltration as described previously.[7] Samples were rocked overnight and then embedded and polymerized at 60°C for 24 hours. Thin sections were collected and stained with uranyl acetate and lead citrate. A transmission electron microscopy (Philip CM 10, Holland) was used to observe the samples.

Renal Tubular Epithelial Cell Culture and Treatment

Normal rat kidney epithelial cells (NRK-52E), a gift from professor Jiang Zongpei (the 6th affiliated hospital of Sun Yat-sen University) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), containing 10% fetal bovine serum (Gibco, USA), 100 U/mL of penicillin-streptomycin (BioWhittaker, USA). The cells were stimulated with different concentrations of angiotensin II (Sigma, USA) dissolved in culture medium, control cells were cultured in medium without angiotensin II. In our preliminary experiment, different doses of angiotensin II (10^{-6} M, 10^{-7} M, and 10^{-8}M) were used to establish NRK-52E cell injury model in vitro. We found 10^{-6}-M angiotensin II-induced significant NRK-52E cells damage. Cells were harvested at 24 and 48 hours for Western blot.

Western Blotting

Renal samples or cultured NRK-52E cells were collected and lysed in lysis buffer (Cell Signaling Technology; Danvers, MA, USA). Equal amounts of protein loadings were separated by SDS-PAGE on 8% gels and electrophoretically transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat milk powder in phosphate-buffered saline for 1 hour. Membranes were incubated in primary antibody (Cabin1, Novus, Littleton, CO, USA; GAPDH, Kangchen, Shanghai, China) overnight at 4°C. Finally, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or mouse immunoglobulin G antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. An enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the bound antibodies. The specific protein bands were scanned and quantified by use of a densitometer (Alpha Fluorchem 8900, AlphaInnotech, Witec; Littau, Switzerland).

Immunofluorescence Staining

The sections of paraffin-embedded renal tissues were rehydrated and subjected to antigen repairing pot for antigen retrieval overnight. Then, renal sections were permeabilized with 0.3% Triton-100 for 5 minutes, followed by becoming blocked with 5% bovine serum albumin for 60 minutes. To observe the distribution of E-cadherin and α-smad, the sections were incubated with primary

antibody (E-cadherin, BD Biosciences, San Jose, CA, USA; α-smad, Millipore, Boston, MA, USA) overnight at 4°C, followed by incubation with goat anti-mouse immunoglobulin G (Cell Signaling Technology, Boston, MA, USA) for 1 hour. Finally, the renal sections were mounted on glass slides with Mounting Medium (R&D Systems; Minneapolis, MN, USA) and viewed by using confocal fluorescence microscope (Zeiss LSM510, Oberkochen, Germany).

Statistical Analysis

For comparisons between more than 2 groups, statistical analysis was performed using the 1-way analysis of variance. Pairwise comparisons were evaluated by the Student-Newman-Keuls procedure or Dunnett T3 procedure when the assumption of equal variances did not hold. *P* values less than .05 were considered significant. Statistical analyses were performed with the SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Biochemical Parameters and Tubulointerstitial Fibrosis

Proteinuria began to rise from 4 weeks after nephrectomy, and obviously increased at 8 weeks after the operation. Serum creatinine and BUN also significantly increased at 8 weeks after 5/6 nephrectomy (Figure 1). By 4 weeks after the operation, minor interstitial fibrosis and tubular atrophy were shown in model group. By 8 weeks after the operation, tubulointerstitial fibrosis were found in the nephrectomized rats. Moreover, tubulointerstitial fibrosis score was significantly higher in the rats at 8 weeks after 5/6 nephrectomy than the sham-operated rats (Figure 2).

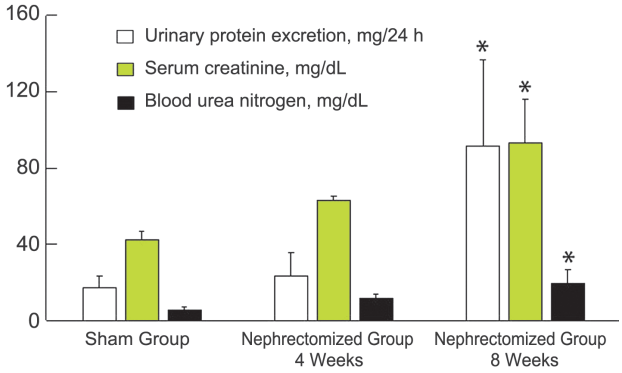

Figure 1. Biochemical parameters at different time points in 5/6 nephrectomized rats and the sham-operated group.
*$P < .05$ compared to the sham group

Ultrastructural alteration in Renal Tubular Epithelial Cell Mitochondria

In the sham-operated rats, RTECs showed normal mitochondria with preserved membranes and cristae. In contrast, mitochondrial damage was found in RTECs of 5/6 nephrectomized rats. By 4 weeks after the operation, swollen and irregular-shaped mitochondria were noted in the model group. By 8 weeks, the swollen of mitochondria became more severe, and mitochondria cristae were disrupted and poorly defined (Figure 3).

Distribution of E-Cadherin and α-Smad

E-cadherin and α-smad belong to the indicator of RETCs injury. In the sham-operated rats, E-cadherin was evenly linearly distributed along the renal tubular lumen, while α-smad merely located at afferent arteriole. In 5/6 nephrectomized rats, the distribution of E-cadherin had a dot-like pattern, yet the expression of α-smad was much more significant (Figure 4).

Cabin1 Protein in the Remnant Kidney

Renal samples of rats were collected for Western blot at 4 and 8 weeks after 5/6 nephrectomy. Compared to the sham-operated rats, Cabin1 protein expression started to increase at 4 weeks and reached a significant level at 8 weeks after 5/6 nephrectomy (Figure 5).

Cabin1 protein in Angiotensin II-injured Cells

The RTECs were collected for Western blot at 24 and 48 hours after stimulating by angiotensin II. Angiotensin II-induced Cabin1 protein expression obviously increased in the cultured RTECs at 48 hours after stimulation (Figure 6).

DISCUSSION

The 5/6 nephrectomized rat is the classic model of CKD by which renal fibrosis can be detected under microscopy as early as 4 weeks after the operation.[9] Our results showed that obvious tubulointerstitial fibrosis was found in nephrectomized rats at 8 weeks after 5/6 nephrectomy, accompanied by the disruption of E-cadherin and overexpression of α-smad in the RTECs. Moreover, the mitochondria became swollen and mitochondrial cristae were

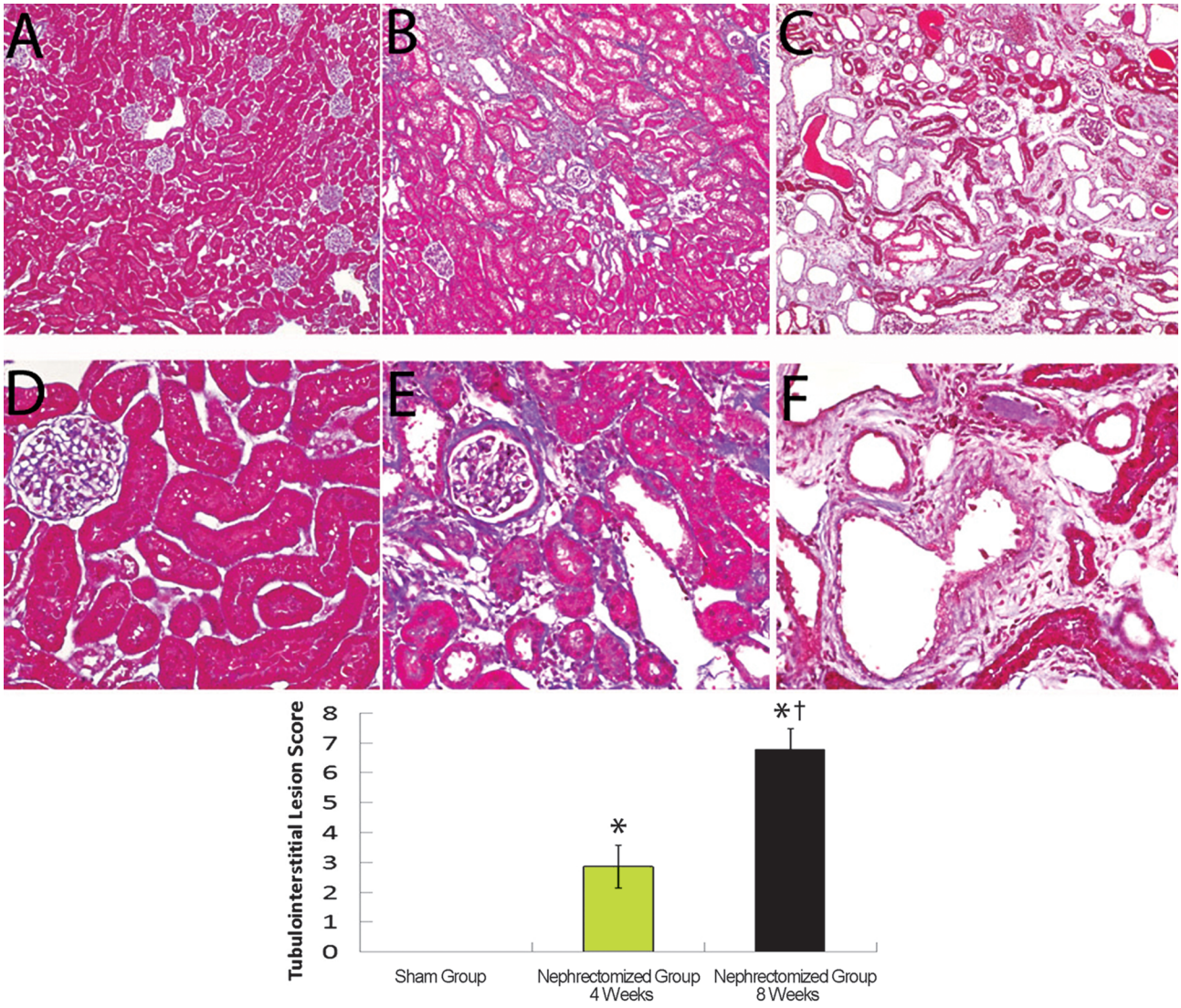

Figure 2. Tubulointerstitial fibrosis at different time points in 5/6 nephrectomized rats. The renal sections were detected by light microscopy after Masson staining. A and D, Normal renal cortex. B and E, Minor interstitial fibrosis and tubular atrophy were showed in model group weeks after operation. C and F, Obvious tubulointerstitial fibrosis were found in nephrectomized rats 8 weeks after operation. Moreover, tubulointerstitial fibrosis score was significantly higher in rats at 4 and 8 weeks after 5/6 nephrectomy (× 100 in A to C; × 400 in D to F).
*P < .05 compared to the sham group
†P < .05 compared to 4 weeks after the operation

disrupted and poorly defined in the RTECs. At the same time, Cabin1 protein expression was significantly increased in 5/6 nephrectomized rats and angiotensin II-injured NRK-52E cells. These results indicated Cabin1 might play an important role in RTEC injury.

Angiotensin II is a key inducer of renal fibrosis, which promotes mesangial cell proliferation and tubular cell apoptosis, extracellular matrix accumulation, and epithelial-mesenchymal transition.[10] Moreover, angiotensin II plays an important role in renal profibrotic factors, by regulating transforming growth factor-β and nuclear factor-κB.[11,12] Our previous study has indicated that angiotensin II-induced Cabin1 is overexpressed and undergone nuclear translocation in cultured podocytes.[5] In this study, we also found angiotensin II-induced Cabin1 upregulation during RTEC injury. Angiotensin II combines with cell surface G protein-coupled receptors, which couple to heterotrimeric G proteins of the Gq/11 family.[13] While studies showed that Gq promoted renal cells damage via calcineurin-nuclear factor of activated T cells pathway.[14] Calcineurin-specific inhibitors,

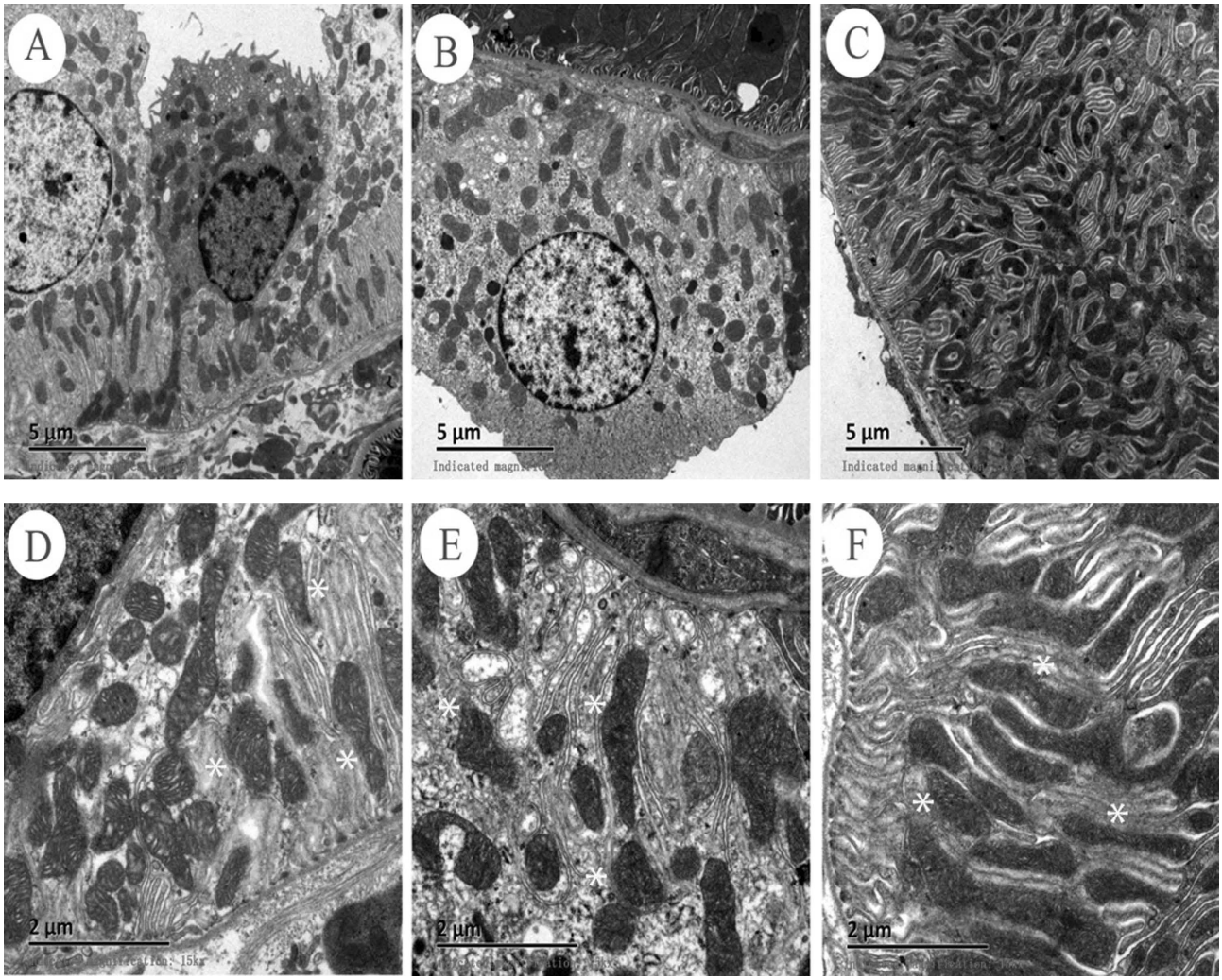

Figure 3. Ultrastructural alteration in renal tubular epithelial cells mitochondria in 5/6 nephrectomized rats. A and D, Renal tubular epithelial cells showed normal mitochondria with preserved membranes and cristae (asterisk) in the sham-operated rats. B and E, By 4 weeks, swollen and irregular-shaped mitochondria were noted in the model group. C and F, By 8 weeks, the swollen mitochondria became more severe, and mitochondria cristae were disrupted and poorly defined (× 5000 in A to C; × 15000 in D to F).

such as cyclosporine A and tacrolimus, alleviated renal cell damage by inhibiting the activation of calcineurin.[15]

As a natural selective inhibitor of calcineurin, Cabin1 is an ideal candidate to alleviate renal cell damage. Cabin1 plays a key role in T cell activation, myocardial hypertrophy, and neuronal development via inhibiting the activation of calcineurin.[16] It was reported that calpain-dependent cleavage of Cabin1 induced the activation of calcineurin, which resulted in human T lymphoma cells death.[17] In patients who suffered from congestive heart failure, calpain-dependent cleavage of Cabin1 induced myocardial cell damage.[18] While upregulated Cabin1 with human Cabin1 in rheumatoid arthritis mice attenuated inflammation.[19] Our previous study indicated calcineurin-specific inhibitor, tacrolimus, alleviated podocyte damage and stabilized the expression of Cabin1 in 5/6 nephrectomized rats.[7] This study was the first to report RTECs injury and Cabin1 protein overexpression occurred in a time-dependent manner. Whether Cabin1 alleviates RTEC injury via inhibiting the activation of calcineurin needs to be further investigated.

Moreover, Cabin1 negatively regulates p53 on its target promoter via its N-terminus in tumor cells.[4] Ying and coworkers indicated Cabin1 inhibited lymphoma cells apoptosis by restraining the activation of p53.[20] Genotoxic stress induced Cabin1 undergone phosphorylation and ubiquitination dependent degradation, released p53 for transactivation.[21] Our previous study found both

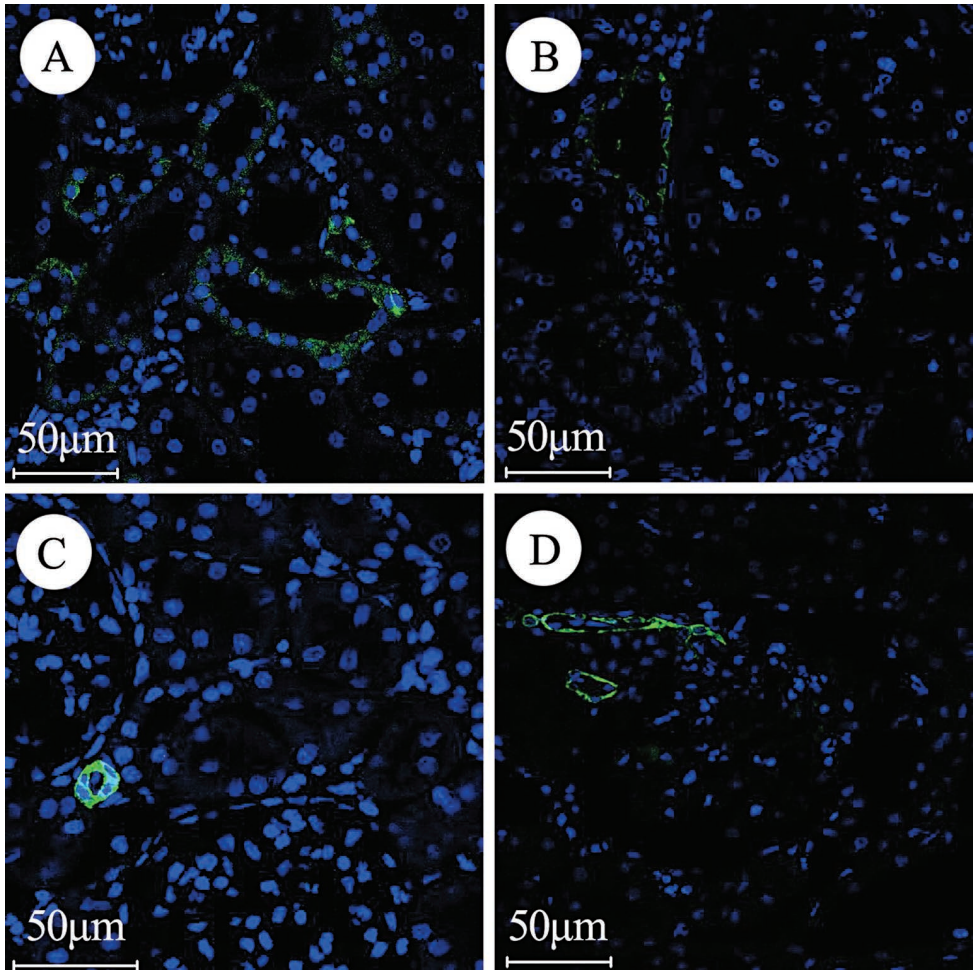

Figure 4. The distribution of E-cadherin and α-smad in 5/6 nephrectomized rats at 8 weeks after the operation. A, In the sham-operated rats, E-cadherin evenly linear distributed along the renal tubular lumen. B, In the 5/6 nephrectomized rats, the distribution of E-cadherin became dot like. C, In the sham-operated rats, α-smad merely located at afferent arteriole. D, In the 5/6 nephrectomized rats, the expression of α-smad was much more significant (× 400).

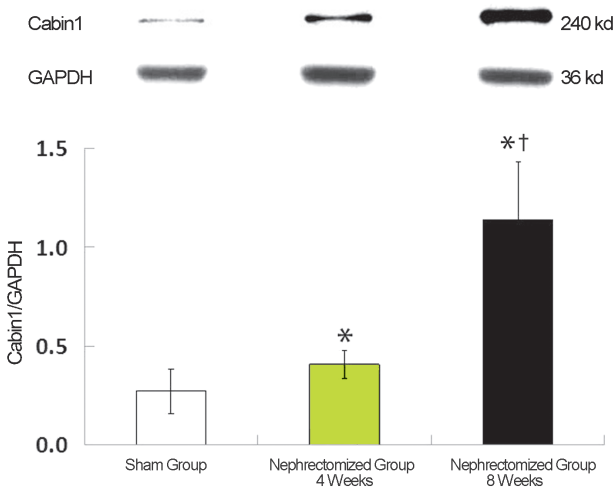

Figure 5. Cabin1 protein was significantly increased in the remnant kidney of 5/6 nephrectomized rats (Western blot analysis).
*P < .05 compared to the sham group
†P < .05 compared to 4 weeks after the operation

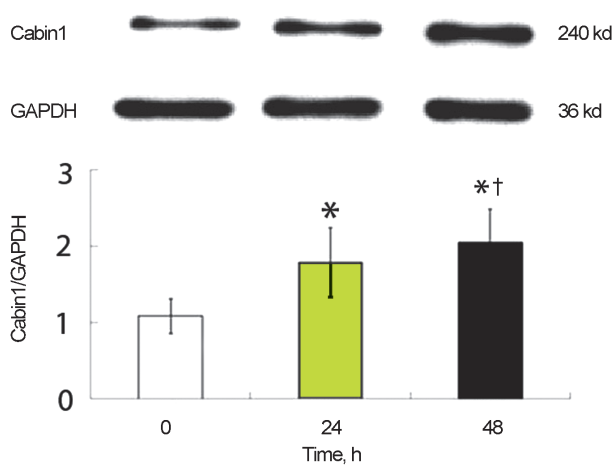

Figure 6. Cabin1 protein was significantly increased in angiotensin II-injured renal tubular epithelial cells. Renal tubular epithelial cells were collected for Western blot at 24 and 48 hours after stimulating by angiotensin II.
*P < .05 compared to the start time
†P < .05 compared to 24 hours

Cabin1 and p53 upregulated in 5/6 nephrectomized rats and angiotensin II-injured podocyte, while knocked-down Cabin1 with small interfering RNA induced the downregulation of p53.[6] Cabin1 may have different function and mechanism in tumor cells and normal cells. Numerous studies suggest that p53 is a key factor which promotes mitochondrial dysfunction and renal cell apoptosis. Vashistha and colleagues found that knocked-out p66 gene in diabetic nephropathy mice significantly alleviated mitochondrial dysfunction by inhibiting the transcriptional activity of p53.[22,23] Furthermore, researchers found that the acetylation of p53 at lysine 120 upregulated apoptotic protease activating facter-1 and sensitized mitochondrial apoptotic pathway via Caspase-9 activation.[24,25] Whether Cabin1 is a key factor in RTEC injury by regulating p53 needs to be proved by our further study.

CONCLUSIONS

Our results showed that RTEC injury and Cabin1 protein overexpression occurred in a time-dependent manner both in vitro and in vivo. Cabin1 may become a potential molecular target in RTEC injury.

CONFLICT OF INTEREST

None declared.

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