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Anisodamine ameliorates ischemia/reperfusion-induced renal injury in rats through activation of the extracellular signal-regulated kinase (ERK) pathway and anti-apoptotic effect

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Anisodamine exerts significant protective effect on ischemia/reperfusion (I/R) injury in various organs. However, little is known about the mechanisms of anisodamine in renal I/R injury. Activation of extracellular regulated protein kinases (ERK) pathway promotes the repair of renal epithelial cells following oxidant injury. The present study investigated whether the renoprotective role of anisodamine against renal I/R injury in rats was associated with the activation of ERK signaling pathway. Male Sprague-Dawley (SD) rats were separated into the following groups: Sham-operated group, I/R group, anisodamine-treated group, PD98059 (MEK-1/ERK inhibitor)-treated group and anisodamine plus PD98059-treated group. A rat model of renal I/R was established by excising the right kidney and then clamping the left renal pedicle for 45 min followed by reperfusion for 24 h. Serum and renal tissue samples were obtained for assays of the associated morphological, molecular and biochemical parameters. Treatment with anisodamine ameliorated renal I/R injury, as evidenced by improvements of renal histology and kidney function, a decrease in paller's score and apoptosis index. Anisodamine also upregulated the phosphorylation levels of ERK1/2 and its downstream targets, including 90 ribosomal S6 kinase (p90rsk) and Bad, as well as the expression of antiapoptotic Bcl-2 protein, downregulated the expression levels of proapoptotic proteins Bax and cleaved-caspase-3, whereas these effects were greatly abolished by administration of PD98059. In conclusion, the results suggest that anisodamine prevents renal I/R injury in rats as a result of an activation of the ERK signaling pathway and anti-apoptotic properties.

1. Introduction

Acute kidney injury (AKI) is an important life-threatening clinical problem and is associated with increased morbidity and mortality in hospitalized patients, some survivors of AKI might develop chronic kidney disease or end stage renal disease owing to incomplete recovery of tubular cells (Zarbock et al. 2018). Renal ischemia/reperfusion (I/R) injury is the leading causes of AKI as observed after kidney transplantation, cardiovascular surgery, disseminated intravascular coagulation, and septic, as well as hemorrhagic shock (Philipponnet et al. 2018; Parolari et al. 2012; Jorge et al. 2019), Therefore, it is quite necessary to explore the pathogenesis of AKI induced by renal I/R injury and a potential therapeutic approach for this disease.

The underlying mechanisms of the pathophysiology of renal I/R injury is very complex, involving oxidative stress, tissue neutrophil infiltration, excessive production of pro-inflammatory cytokines, and inflammatory responses with eventual death of renal tubular epithelial cells (Diao et al. 2019; Cura-Esquivel et al. 2018). Renal I/R injury-induced loss of tubular epithelial cells by necrosis and apoptosis is a major cause of AKI. Apoptosis, a predominant type of cell death, is the major pathway that leads to the process of cell death during renal I/R damage (Xia et al. 2014; Sari et al. 2020). Thus, inhibiting the apoptosis of renal tubular cells would provide a suitable strategy for preventing renal damage and promoting recovery of renal structure and function from AKI induced by renal I/R.

The tubular epithelial cell-survival or cell death-related molecular pathway or mechanism during the process of renal I/R injury are not completely understood. Mitogen-activated protein kinases (MAPKs), comprising a family of serine-threonine kinases of extracellular regulated protein kinases (ERK), c-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38), play an important role in the transmission of signals from cell membrane to nucleus, which regulates cell survival or death (Su et al. 2019; Cavdar et al. 2017). Several studies have demonstrated that the activation of ERK kinases during the processes of renal I/R injury may be essential for renal epithelial cell survival (Andreucci et al. 2003; di Mari et al. 1999). Furthermore, some therapeutic interventions to prevent or ameliorate renal I/R injury is related to the activation of ERK signal (Chen et al. 2015; Zou et al. 2016). However, there is little controversial reports in terms of the role of ERK signal pathway in renal I/R injury (Alderliesten et al. 2007). Therefore, the exact role of ERK remains to be defined in the recovery process following kidney I/R injury.

Anisodamine is a competing blocking drug for the muscarinic receptor with low toxic and side effects. Previous studies have

shown that besides the competitive action of M receptor, anisodamine has a variety of biological effects such as anti-shock, anti-oxidation, anti-inflammatory and immune regulation (Yu et al. 2019; Li et al. 2019). Several reports demonstrated that anisodamine reduced tissue injury in models of ischemia/reperfusion in several organs, including the gut, brain and heart (Hu and Sheng 2002; Chen and Zeng 2000; Yao et al. 2018). Yu et al.'s study showed that anisodamine also exhibited protective effect on renal I/R injury (Yu et al. 2002). However, it has not been confirmed whether activation of the ERK signaling pathway is involved in the renoprotective effects of anisodamine against renal I/R injury in rats.

Based on these findings, the aim of the current study was to investigate the renoprotective effects of anisodamine using a rat model of renal I/R injury and to further identify the mechanisms underlying the protection of anisodamine against kidney injury induced by I/R.

2. Investigations and results

2.1. *Effects of anisodamine on renal function*

The rats with renal I/R injury were observed to have a significant increase in the levels of SCr (Fig. 1A), BUN (Fig. 1B), NGAL (Fig. 1C) and Cys C (Fig. 1D) as compared to the sham-operated rats. In the anisodamine-treated group, there was a significant decrease in these indices as compared to that in the I/R group, whereas these reductions in these parameters induced by anisodamine became blunted in the presence of PD98059.

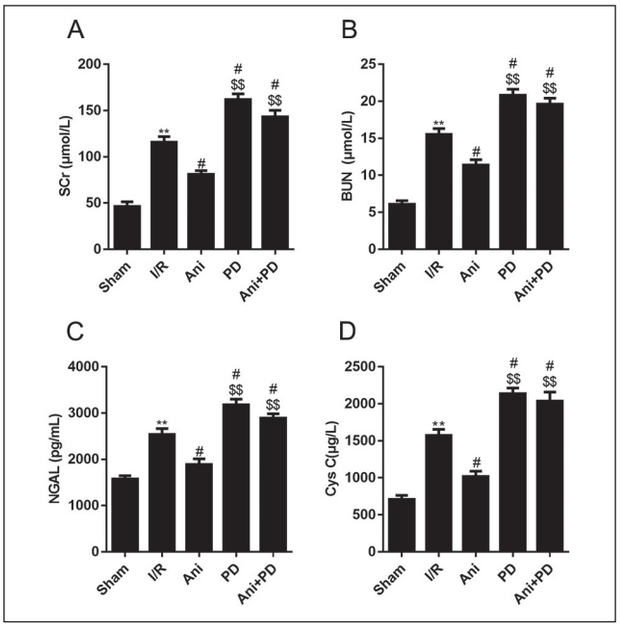

Fig. 1: Effect of anisodamine on renal function of rats with renal ischemia/reperfusion injury. The levels of SCr (A), BUN (B), NGAL (C), Cys C (D) in each group. Data were presented as mean±SEM (n = 6). $^{**}P < 0.01$ *vs.* Sham group. $^{\#}P < 0.05$ *vs.* I/R group. $^{\$\$}P < 0.01$ *vs.* Ani group.

2.2. *Effects of anisodamine on renal histomorphology*

As shown in Fig. 2, kidneys of sham-operated rats displayed a normal morphologic appearance. The kidneys from the I/R group showed significant changes in pathology and morphology, as evidenced in cell swelling, detachment, vacuolization, partly necrosis of tubular epithelial cells, tubular lumen dilatation, cast formation (Fig. 2A), and high level of paller's score (Fig. 2B). In contrast, the kidneys of rats that received anisodamine therapy revealed mild renal histologic damage and a significant decrease in paller's score as compared to the kidneys of rats in the I/R group, and these attenuation of histomorphological injury induced by anisodamine was partially reversed by the treatment of PD98059 (Fig. 2A and B).

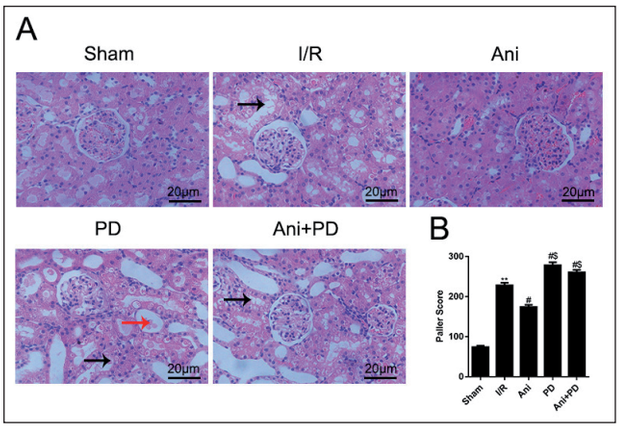

Fig. 2: Effect of anisodamine on renal histology of rat with renal ischemia/reperfusion injury. Black arrows indicate the formation of cast. Red arrows indicate that the renal tubular epithelial cells showed severely swollen, vacuolar degeneration or necrosis. (A) Representative microphotographs came from the kidneys of the each group; (B) Paller's score of renal injury in each group. Data were presented as mean±SEM (n = 6). $^{**}P < 0.01$ *vs.* Sham group. $^{\#}P < 0.05$ *vs.* I/R group. $^{\$}P < 0.05$ *vs.* Ani group.

2.3. *Effect of anisodamine on apoptosis of renal tubular epithelial cell induced by I/R*

TUNEL assay was used to investigate renal tubular epithelial cell apoptosis. The results showed that few apoptotic renal tubular epithelial cells were observed in the Sham group, while a large number of apoptotic cells were seen in I/R group compared with sham group (Fig. 3A and B). Administration of anisodamine reduced the degree of apoptosis of renal tubular epithelial cells (Fig. 3A and B), indicating that anisodamine play a renoprotective role through anti-apoptotic activity. Similarly, the anti-apoptotic actions of anisodamine was partially abolished by application of PD98059 (Fig. 3A and B).

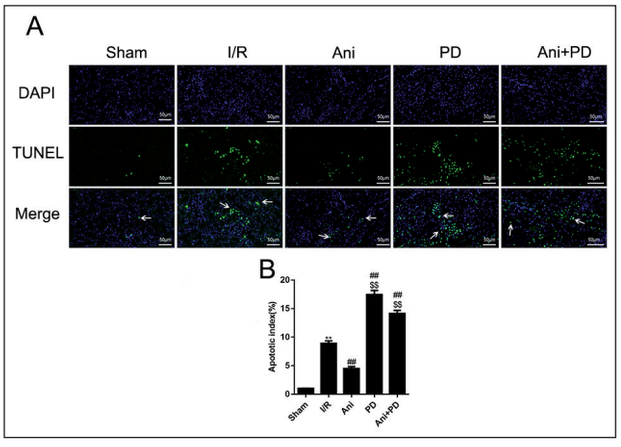

Fig. 3: Effect of anisodamine on cell apoptosis of renal tubular epithelial cells in rats with ischemia/reperfusion injury. The arrows represent the apoptotic cells. (A) The representative TUNEL section. (B) The apoptosis index. Data were presented as mean±SEM (n = 6). $^{**}P < 0.01$ *vs.* Sham group. $^{\#\#}P < 0.01$ *vs.* I/R group. $^{\$\$}P < 0.01$ *vs.* Ani group.

2.4. *Effect of anisodamine on expression of Bcl-2, Bax and cleaved caspase-3 proteins during renal I/R injury*

To further investigate the anti-apoptotic effect of anisodamine in an I/R kidney, the renal expressions of Bcl-2, Bax and cleaved caspase-3 were evaluated. Western blotting analysis showed that the renal Bcl-2 protein expression level (Fig. 4A) decreased and that of Bax (Fig. 4B) and cleaved caspase-3 (Fig. 4C) increased in

I/R group compared to sham-operated kidneys. However, anisodamine treatment prevented the decreasing effect on Bcl-2 level (Fig. 4A), the increasing effects on Bax (Fig. 4B) and cleaved caspase-3 levels (Fig. 4C). However, these alterations in protein expressions were almost reversed by PD98059 (Fig. 4A-C).

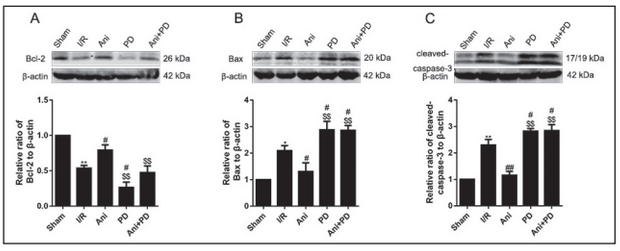

Fig. 4: Effect of anisodamine on the expression of Bcl-2, Bax and cleaved caspase-3 in renal tissues from rats with ischemia/reperfusion injury. Western blotting bands and relative protein levels of Bcl-2 (A), Bax (B), and cleaved caspase-3 (C). β-actin was used as an internal control. Data were presented as mean±SEM (n = 3). $^{*}P < 0.05$ *vs.* Sham group. $^{\#}P < 0.05$ *vs.* I/R group. $^{\$\$}P < 0.01$ *vs.* Ani group.

2.5. Effects of anisodamine on ERK signaling pathway activity after renal I/R injury

To investigate whether the anti-apoptotic effect of anisodamine was associated with the ERK signaling pathway, the protein levels of ERK signaling pathway were detected using Western blotting. The phosphorylation levels of ERK (Fig. 5A), p90rsk (Fig. 5B) and Bad (Fig. 5C) in kidney tissues decreased significantly in I/R Sham group compared to the group. The kidneys of rat treated with anisodamine exhibited significantly increased phosphorylation levels of ERK (Fig. 5A), p90rsk (Fig. 5B) and Bad (Fig. 5C) compared to the kidneys of rats in the I/R group, whereas, these enhanced levels of phosphorylation of ERK, p90rsk and Bad were reduced by administration of PD98059, suggesting that the anti-apoptotic effect of anisodamine may involve the ERK/p90rsk/Bad signaling pathway.

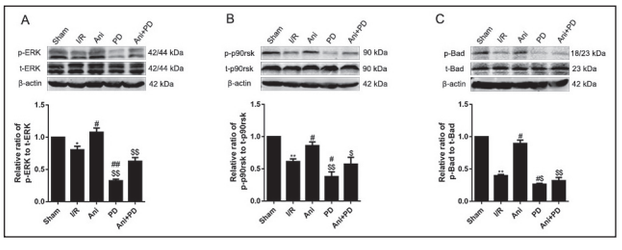

Fig. 5: Effect of anisodamine on ERK signaling pathway activity in renal tissue of rats with ischemia/reperfusion injury. Western blotting bands and relative protein levels of p-ERK1/2 (A), p-p90rsk(B), and p-Bad(C). β-actin was used as an internal control. Data were presented as mean±SEM (n = 3). $^{**}P < 0.01$ *vs.* Sham group. $^{\#}P < 0.05$ *vs.* I/R group. $^{\$}P < 0.05$ or $^{\$\$}P < 0.01$ *vs.* Ani group.

3. Discussion

The present study showed that treatment with anisodamine effectively ameliorated histopathological kidney damages, improved renal function and inhibited tubular cell apoptosis in a rat model of renal I/R injury. This is the first study to demonstrate that the mechanism underling the renoprotective effect of anisodamine is mediated through activation of the ERK signaling pathways.

Anisodamine, a kind of *Belladonna* alkaloid, was firstly separated from nightshade plant scopolamine, and subsequently synthesized by Chinese scientists in 1975 (Xie et al. 1975). As a non-specific muscarinic cholinoceptor antagonist, it has been employed in traditional Chinese medicine for the treatment of septic shock (Zhang et al. 2015), circulatory disorders, organophosphorus poisoning, migraine, acute gastric or intestinal spasm-like pain, and respiratory dysfunction (Poupko et al. 2007; Niu et al. 2018; Huang et al. 2002). It was also previously reported that anisodamine treatment significantly reduces kidney damages in a variety of experimental rat models of AKI induced by glycerol, lipopolysaccharide rhabdomyolysis, and I/R injury (Li et al. 2019; Wan et al. 2020; Yuan et al. 2017). In agreement with these results, we found that treatment with anisodamine effectively ameliorated histopathological kidney damages, improved renal function, as demonstrated by decreases in the levels of SCr, BUN, NGAL and Cys C. Meanwhile, we had explored the molecular mechanisms underlying the renoprotective effects of anisodamine against renal I/R injury in rats.

Apoptosis, known as autonomously programmed cell death, is characterized by sequential morphological events, including condensation of chromatin, cell shrinkage, nuclear DNA fragmentation and ultimately the formation of apoptotic bodies. Many studies have provided evidence that the apoptosis of renal tubular epithelial cells contributes to a major pathogenic mechanism leading to AKI and determines the progression of renal I/R injury (Xia et al. 2014; Sari et al. 2020). Thus, the inhibition of tubular cell apoptosis following renal I/R can be one of the important strategies for renoprotection.

To investigate the anti-apoptotic effect of anisodamine on renal I/R injury, Bcl-2, Bax and cleaved-caspase-3 in kidney were determined by Western blotting, and TUNEL staining was used to assess tubular cell apoptosis. Our results showed that anisodamine significantly inhibited apoptosis of tubular epithelial cell caused by I/R injury as demonstrated by decreases in apoptosis index, the expression levels of pro-apoptotic members of Bax and cleaved-caspase-3 protein, and increase in the expression level of anti-apoptotic Bcl-2 protein in rat kidney tissues. Moreover, we also found that inhibition of ERK activation using PD98059, a specific inhibitor of MEK-1/ERK, abolished the anti-apoptotic effect of anisodamine. Previous studies have demonstrated that anisodamine prevented myocardial cell apoptosis both in a myocardial I/R rat model and I/R injury of patients with acute myocardial infarction (Yao et al. 2018). Additionally, recent studies have indicated that anisodamine exerts an anti-apoptotic effect on the kidney in the rat model of chemical-induced AKI (Li et al. 2019; Wan et al. 2020; Yuan et al. 2017). These results support the hypothesis that anisodamine exhibits antiapoptotic effects through activation of ERK pathway, and then reducing renal tissue damage.

The Bcl-2 family of proteins are key regulators of the mitochondrial pathway of apoptosis in many cellular systems, including antiapoptotic proteins, and proapoptotic proteins. Overexpression of Bcl-2 protein inhibits opening of mitochondrial permeability transition pore and apoptosis-associated cytochrome c release from mitochondria and subsequent caspases activation. In contrast, the combination of pro-apoptotic Bax and mitochondria induces release of cytochrome c from mitochondria, which triggers activation of caspases and results in apoptosis (Narita et al. 1998). Caspase-3, a terminal protein in the apoptotic cascade, is a key effector protease responsible for apoptosis execution (Stevenson et al. 2018). Results from the current study suggest that anisodamine treatment attenuates renal I/R injury via inhibition of Bcl-2/Bax/cleaved-caspase-3 apoptotic signaling pathways.

The signaling pathways involving in cell death or survival after renal I/R are not well studied. ERK, one of the primary components of the MAPKs pathway has been shown to be implicated in promoting cell survival and inhibiting apoptosis for a damaged kidney after I/R, which determines the progression of renal I/R injury (Chen et al. 2015). It has been reported that the activation of ERK directly phosphorylates and activates p90rsk through selection of different phosphorylation substrates, which, in turn, activates various signaling events in the recovery of acute kidney injury (Lin et al. 2019). The activation of p90rsk increases its pro-apoptotic protein substrate Bad activation (Edwin and Patel 2008). Phosphorylated form of Bad is associated with the dissociation of Bad from pro-survival Bcl-2 proteins and the association of Bad with members of the 14-3-3 family of proteins, an event that improves cell survival and inhibits cell apoptosis in kidney tissue (Zha et al. 1996). Additionally, previous studies have shown that

nonphosphorylation of Bad within the membrane is directly linked to activation of the caspase-3 apoptotic cascade (Springer et al. 2000). In agreement with these results, we found that kidneys from renal I/R injury in rats exhibited decreases in the levels of specific phospho-proteins, including phospho-ERK, phospho-p90rsk and phospho-Bad, the latter two phosphoproteins are the key molecule downstream of the ERK signaling pathway, whereas treatment with anisodamine markedly increased the phosphorylation expressions of ERK, p90rsk and Bad, and PD98059 reduced the enhancements of phosphorylation levels of ERK, p90rsk and Bad triggered by anisodamine treatment, suggesting that ERK activation may be essential for cellular survival responses and inhibition of cell apoptosis.

In conclusion, the findings in this study revealed that anisodamine exerted a protective role in renal I/R injury in rats, and the mechanism of this is at least in part, related to the activation of ERK/p90rsk/Bad signaling, thereby reducing apoptotic cell death, and eventually ameliorating renal I/R injury. These findings potentially provide the basis of using anisodamine for the treatment of renal I/R injury in the future.

4. Experimental

4.1. Animals and experimental protocols

Male Sprague-Dawley (SD) rats weighing 180–220 g, were purchased from Jinan pengyue experimental animal breeding co. LTD (Jinan, China). The animals housed on a 12/12 light-dark cycle were provided food and water *ad libitum* during the experiments. All animal experiments were conducted in accordance with the principles provided by the National Institute of Health Guideline for the Care and Use of Laboratory Animals. The approval to proceed with this experiment was issued by the Animal Ethics Committee of Xuzhou Medical University.

Thirty SD rats were randomly separated into five groups with six animals in each group, namely sham group, I/R group, anisodamine (10 mg/kg) treatment group (Ani group), PD98059 (MEK-1/ERK inhibitor) treatment group (PD group) and Ani+PD98059 group. Renal I/R injury was induced as follows: Rats were anesthetized by intraperitoneal injection of pentobarbital sodium 50 mg/kg body weight). A longitudinal incision was made along the abdominal midline. An atraumatic clip was used to interrupt blood supply of left kidney. After 45 min of left renal ischemia, the clip was removed to initiate renal reperfusion. Sham group rats underwent the same protocol without renal vascular occlusion. Anisodamine was injected into rat tail vein at 10 min before modeling; and PD98059 (1 mg/kg) was given by the same route at 15 min before left renal reperfusion. Rats in Sham group were injected with an equal volume of sterile saline via tail-vein.

4.2. Sample collection

All rats were anesthetized using 3% pentobarbital sodium (50 mg/kg body weight) to collect blood from the abdominal aorta after reperfusion for 24 h. Then, the left kidney was taken right away, one part of the kidney was fixed in 4% paraformaldehyde for subsequent histological analysis. The remaining parts of the kidneys were immediately stored at -80 °C for Western blot analysis. Blood samples were centrifuged at 3000 RPM /min for 15 min, and the supernatant was collected and packed separately, stored at -80 °C for biochemical kit detection and metabolomics analysis.

4.3. Biochemical analysis

Serum creatinine (SCr) and blood urea nitrogen (BUN) levels were determined using a picric acid method and an urease assay, respectively (Jiancheng Bioengineer-ing Institute, Nanjing, China). serum neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C (Cys C) levels were measured by specific enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Mbbiology Co., Ltd, Jiangsu, China).

4.4. Hematoxylin - eosin(H&E) staining

The paraformaldehyde-fixed kidney tissues were placed in the tissue embedding basket, and embedded in paraffin. Subsequently, sections of 4-μm thick paraffin of renal tissues were made and were routinely stained with dyes of hematoxylin and eosin (H&E staining). Then the morphological changes of kindney tissue were examined using an Olympus BX43F microscope (Tokyo, Japan), and the degree of renal tubular damage was assessed by paller scoring method (Paller et al. 1984).

4.5. TUNEL assay

Apoptosis of renal tubular epithelial cell was detected by the terminal deoxyribonucleotide transferase- (TdT-) mediated dUTP nick end labeling (TUNEL) detection kit (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's insturctions. In summary, paraffin sections were deparaffinized, permeabilized with proteinase K and quenched with 3% H_2O_2 in methanol for 30 min at 37 °C. Next, 50 μl of TdT reaction mixure (containing TdT and fluorescein-labelled nucleotides) was added to each section and incubated for 30 min at 37 °C. The slices were sealed by anti-fluorescence quenching liquid and then were analyzed under an Olympus BX43F fluorescence microscope (Olympus Corporation, Tokyo, Japan). The renal tubular epithelial cells were counted in five randomized and non-overlapping high magnification fields, and the apoptosis of renal tubular epithelial cell was represented as apoptosis index (AI) calculated as follows: AI = the number of TUNEL-positive cells/the total number of renal tubular epithelial cells.

4.6. Western blotting analysis

All primary antibodies were from Cell Signaling Technologys (MA, USA), except for anti-p-p90rsk (ab32413; Abcam Inc., UK) and anti-p-Bad A0208; ABclonal Inc., UK). Tissue samples of renal tissues were lysed in RIPA buffer (Beyotime, Shanghai, China), and the mixture was centrifuged at 4 °C and 10,000 x g for 15 min. The supernatant was collected, and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (50 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 3% BSA in PBS, the membranes were incubated with primary antibodies β-actin, p-p90rsk/p90rsk, p-ERK/ERK, Bcl-2, Bax, p-Bad/Bad, and anti-cleaved-caspase-3 overnight at 4 °C, respectively. Afterward, the primary antibodies were washed with PBS three times (5 min each). The membranes were incubated for 1 h at room temperature with Near-infrared fluorescence-conjugated secondary antibodies (V926-32210; Vicmed Biotech Co. Ltd.) Finally, the protein bands were scanned and quantified using Image J software (version 1.48; National Institutes of Health, Bethesda, Md, USA).

4.7. Statistical analysis

Data are expressed as the mean±standard error of the mean. Statistical analysis was performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Comparison between groups was conducted using one-way analysis of variance, followed by Tukey's test. Differences with $P<0.05$ were considered to be statistically significant.

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Conflicts of interest: None declared.

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