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Original Article

TRPM-7 inhibits necroptosis via the PARP-1/RIP-1 signaling pathway and attenuates renal ischemic reperfusion injury in renal tubular epithelial cells

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Abstract: Renal ischemic reperfusion injury (RIRI) plays a significant role in acute kidney injury in native and transplanted kidneys. Some reviews have suggested that the core target of RIRI is mitochondrial injury with Ca^{2+} overload. TRPM-7 is an important non-selective Ca^{2+} ion channel expressed in renal tubular epithelial cells. The expression of TRPM-7 has been demonstrated to play an important role in RIRI. However, the mechanism of TRPM-7 attenuates RIRI has never been investigated. In this study, the expression of TRPM-7 increased in the early stage of RIRI and then gradually decreased at 48 h, 5 days, and 7 days. Expression levels exhibited a positive relationship with acute renal tubular injury grading and the degree of renal function injury. Furthermore, TRPM-7 overexpression promoted PARP-1 expression, inhibited RIP-1 expression, and inhibited necroptosis, which attenuated RIRI in renal tubular epithelial cells. TRPM-7 inhibited necroptosis via the PARP-1/RIP-1 signaling pathway to attenuate RIRI in renal tubular epithelial cells, which indicates that TRPM-7 may play a protective role in RIRI and that the up-regulation of TRPM-7 could be a novel therapeutic strategy for RIRI.

Keywords: Renal ischemic reperfusion injury, TRPM-7, PARP-1, RIP-1, necroptosis

Introduction

Acute kidney injury (AKI) is a common and significant issue in clinical kidney transplantation and renal angioplasty, and is associated with increased kidney morbidity and mortality rates [1]. Renal ischemic reperfusion injury (RIRI) is one of the major factors influencing AKI, owing to the physical structure and function of the kidney. RIRI is typically characterized by increased microvascular permeability, interstitial edema, inflammatory cell infiltration, cell dysfunction and necrosis, renal tubular epithelial cell injury, and a decline in renal function [2, 3]. Necroptosis, known as programmed necrosis, is an important form of cell death that is independent of apoptosis. It is present in AKI [4]. Necroptosis displays as an obvious necrosis feature in pathology, induces an obvious inflammatory reaction [5], and is specifically blocked by the small-molecule compound, Necrostatin-1 (Nec-1) [6]. Compared to apoptosis, necroptosis has been empirically confirmed in RIRI, and is thought to play a more important role in RIRI processes than is currently known [7]. Therefore, exploring the regulatory mechanism of necroptosis in RIRI has important clinical significance with regard to RIRI prevention.

Transient receptor potential melastatin-related 7 (TRPM-7) is an important non-selective Ca^{2+} and Mg^{2+} ion channel that is expressed in renal tubular epithelial cells [8]. When deprived of oxygen and energy, ATP production drops and Na^+/K^+-ATP enzyme function is exhausted, which induces the influx of Na^+ and Ca^{2+} into cells via the TRPM-7 channel [9]. TRPM-7, having kinase activity, likely mediates signal transduction in and out of cells and participates in cells' multiple physiological and pathological functions, such as cellular magnesium homeostasis [10, 11], neurotransmitter release [12], tumor proliferation, and metastasis [13, 14]. Research has shown that TRPM-7 plays an important role in regulating neuron ischemic injury [15, 16]. TRPM-7 also plays an important

role in RIRI [17]; however, the mechanism by which TRPM-7 mediates necroptosis in RIRI is unknown.

In this study, we hypothesized that TRPM-7 may play a role in RIRI through the inhibition of necroptosis in renal tubular epithelial cells. To investigate this hypothesis, we evaluated changes in the expression of TRPM-7, PARP-1 (poly (ADP-ribose) polymerase-1), and RIP-1 (receptor-interacting protein 1) in RIRI Sprague Dawley (SD) rats. Furthermore, we investigated the mechanism by which TRPM-7 inhibits necroptosis and attenuates RIRI.

Materials and methods

Experimental animals and grouping

In total, 80 male SD rats, aged 5 weeks old and weighing 200 ± 20 g, were used throughout the study. All animals were provided by the Animal Experimental Center of Institute of Field Surgery, Daping Hospital, Third Military Medical University [Production license No. SCXK (Army) 2012-0013; Lot No. R20130706]. All animals were kept in a specific free environment with a temperature of 21-25°C, 65-70% humidity environment with regular light cycles (12 h) during the experiment. Drinking water, feed, and experimental materials were disinfected by sterilization, and the rule of aseptic operation was strictly followed. Our research reported here has been performed with the approval of the Third Military Medical University ethics committee. During the preoperative stage, all animals were allowed free activity, feeding, and drinking. The animals were watched and their weight was recorded daily. Eighty animals were randomly selected from each group using the random number table method according to the basic principle of randomized block design. Animals were anesthetized with 3% pentobarbital sodium (1-1.5 ml/kg body weight, Beijing Propbs Biotechnology co., Ltd., Beijing, China). For the RIRI operation group (n = 40), both kidneys were exposed through flank incisions and mobilized by being dissected free from the perirenal fat, the left kidney was removed, and the right renal pedicle was cross-clamped using a nondestructive artery clamp for 40 minutes. The abdomen was closed, and the rat was placed in a 37°C incubator. The rats were sacrificed at 24 h, 48 h, 5 days, and 7 days (n = 10, respectively). The sham operation (SO) group (n = 10) went through the same surgical procedure as the other animals, including blunt dissection of the renal pedicle; however, renal clamps were not applied. The Nec-1, 2-APB (2-Aminoethoxydiphenyl borate), and Bradykinin groups (n = 10, respectively) were treated by tail intravenous injection with Nec-1 (1.65 mg/kg), 2-APB (1.5 mg/kg), or Bradykinin (1.5 mg/kg), respectively. After 5 min, the SD rats went through the same surgical procedure as the RIRI operation group and were sacrificed at 24 h.

Hematoxylin-Eosin (H&E) staining and immunohistochemical (IHC) analysis

For renal pathological evaluation, fresh kidney tissue samples were fixed in 10% formalin (Sigma, Saint Louis, MO, USA) and embedded in paraffin. For all of the tissue slides, each array spot was 1.5 mm in diameter and 5 μm in thickness. All of the slides were stained with H&E staining, and scored with a semi-quantitative scale designed to evaluate the degree of renal tubular necrosis. The method of scoring criteria was as previously described [18]. Briefly, higher scores represent more severe damage, with the maximum score being 4: 0, normal kidney; 1, minimal damage (0-5% involvement); 2, mild damage (5-25% involvement); 3, moderate damage (25-75% involvement); and 4, severe damage (75-100% involvement). The protein expression levels of TRPM-7, PARP-1, and RIP-1 in the kidney tubules were measured by IHC analysis. Briefly, sections were incubated with a 1:50 dilution of rabbit anti-rat TRPM-7 (Abgent, San Diego, CA, USA), PARP-1, and RIP-1 (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Protein expression was detected according to the manufacturer's protocol using the SP kit and DAB kit (ZSGB-BIO, Beijing, China). Mean optical density (MOD) was quantified using Image ProPlus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Renal function analysis

From all animals, 5 ml blood was collected via the rats heart and centrifuged at 2500× *g* for 10 min to obtain the supernatant serum. Parameters evaluated included serum creatinine (Scr) and blood urea nitrogen (BUN) using an Olympus AU2700 automatic biochemical analyzer (Olympus, Tokyo, Japan).

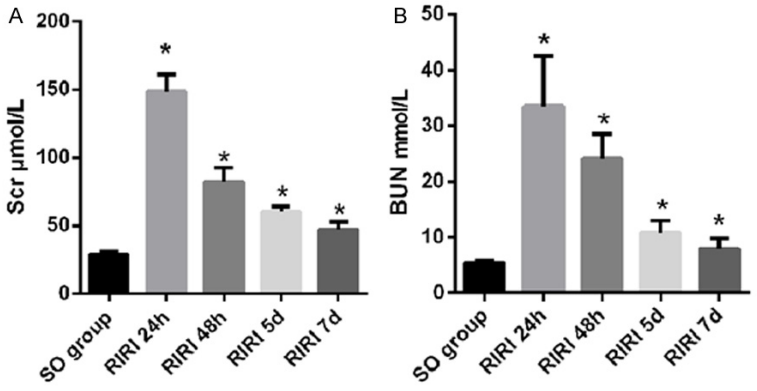

Figure 1. Serum creatinine (A) and blood urea nitrogen (B) values. *P<0.05 compared with SO group. SO group: sham operation group; RIRI 24 h, RIRI 48 h, RIRI 5 days, RIRI 7 days: after the renal ischemic reperfusion injury (RIRI) operation, the rats were sacrificed at 24 h, 48 h, 5 days, 7 days, respectively. The bars indicate the Scr and BUN values, dates are presented as mean ± SD.

Western blot analysis

The protein levels of TRPM-7, PARP-1, and RIP-1 were determined by Western blotting. Protein extracted from tissues was separated on 10% SDS-PAGE and transferred to PVDF membranes. After being blocked with 5% non-fat milk in TBS for 3 hours, the membranes were incubated with a 1:1000 dilution of rabbit anti-rat TRPM-7 (Abgent, San Diego, CA, USA), or a 1:1000 dilution of PARP-1, and RIP-1 (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by incubation for 40 min with a 1:5000 dilution of goat anti-rabbit HRP, which was used as a secondary antibody (BOSTER, Wuhan, Hubei, China). Proteins were visualized using ECL (Thermo Scientific Pierce ECL Plus, Thermo Scientific, Rockford, IL, USA). β-actin was used as a loading control for comparison between samples. The densitometric analysis was performed using Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). The results of densitometric analysis were expressed as a relative ratio of the target protein to reference protein.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (IBM Inc., USA). Continuous variables are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the differences between groups, complemented by *LSD* test to discover significant intergroup differences. P values <0.05 were considered statistically significant.

Results

Renal function changes

To investigate the effects of RIRI on renal function, we analyzed Scr and BUN values, which are routinely determined for the assessment of kidney function (**Figure 1**). Compared to those in the SO group, Scr and BUN values were significantly higher following RIRI at 24 h, 48 h, 5 days, and 7 days (P<0.01). Scr and BUN values were highest at 24 h and gradually decreased at 48 h, 5 days, and 7 days.

Renal pathological changes

Pathological changes were analyzed by H&E staining as shown in **Figure 2**. Kidney tissue structures were normal in the SO group: renal tubular epithelial cells were neatly arranged and not shedding. At 24 h post-RIRI, the renal tubular epithelial cells demonstrated marked swelling, shedding, and dissolution, and the basement membrane appeared bare and fractured. There were many inflammatory cell infiltrations in the renal interstitium. At 48 h post-RIRI, new renal tubule cells were present. At 5 days post-RIRI, the occurrence of new renal tubule cells had markedly increased, and only part of the basement membrane was bare. At 7 days post-RIRI, most of the kidney tissue structure had reverted to normal. The score for renal tubular damage was semi-quantitative. Compared to the SO group, the degree of renal tubular damage was significantly increased at 24 h, 48 h, 5 days, and 7 days post-RIRI (P< 0.05). Scores for renal tubular damage were highest at 24 h and gradually decreased at 48 h, 5 days, and 7 days.

Expression of TRPM-7, PARP-1, and RIP-1 in RIRI

The protein expression levels of TRPM-7, PARP-1, and RIP-1 in the RIRI kidney tubules were

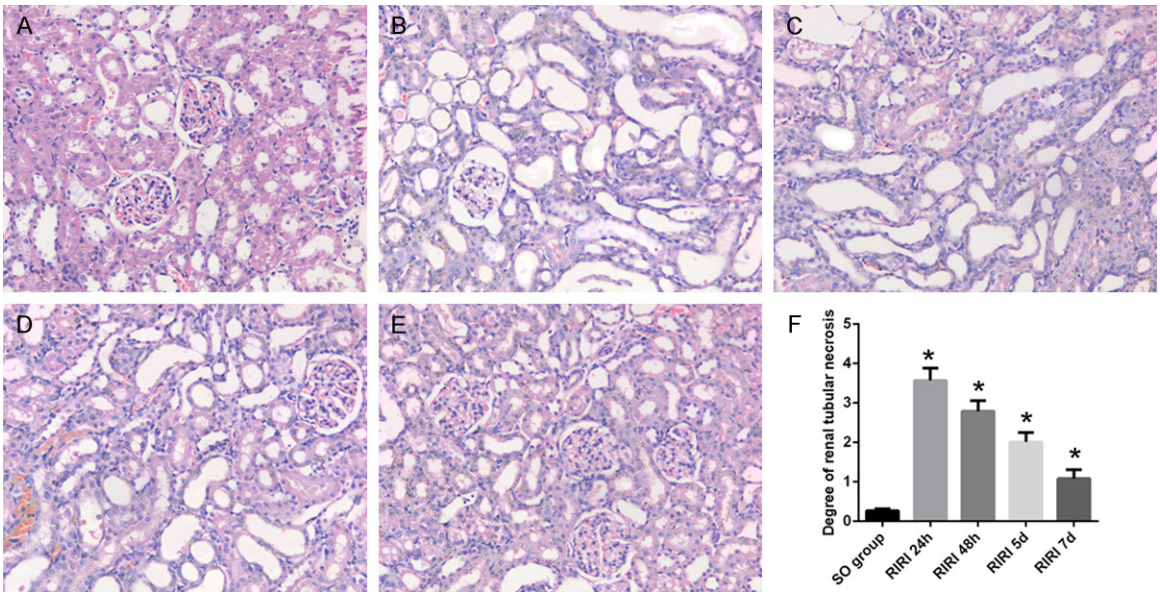

Figure 2. Pathological changes analyzed by Hematoxylin & Eosin (H&E) stain. A-E: Representative images of H&E stain in SO group, RIRI 24 h, RIRI 48 h, RIRI 5 days, and RIRI 7 days, respectively (400×). F: The degree of renal tubular damage is presented as mean ± SD, the bars indicate the degree of renal tubular damage. *P<0.05 compared with SO group. SO group: sham operation group; RIRI 24 h, RIRI 48 h, RIRI 5 days, RIRI 7 days: after the renal ischemic reperfusion injury (RIRI) operation, the SD rats were sacrificed at 24 h, 48 h, 5 days, and 7 days, respectively.

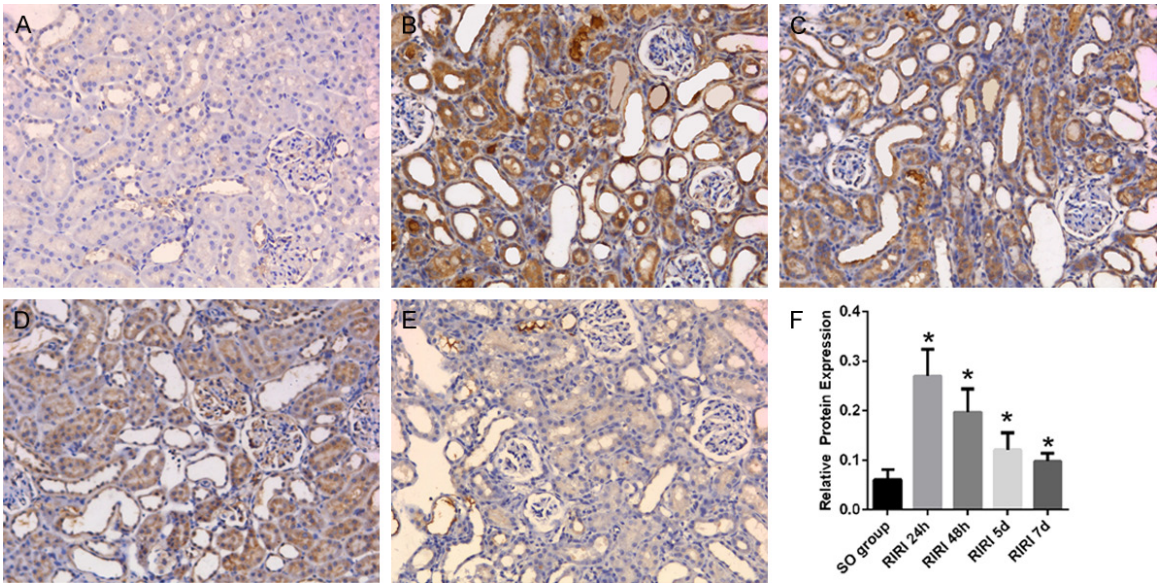

Figure 3. Expression of TRPM-7 in renal ischemic reperfusion injury (RIRI). A-E: Representative images of IHC stain of TRPM-7 in SO group, RIRI 24 h, RIRI 48 h, RIRI 5 days, RIRI 7 days, respectively (400×). F: The results of TRPM-7 expression are presented as mean ± SD, the bars indicate the TRPM-7 protein expression levels, *P<0.05 compared with SO group. SO group: sham operation group; RIRI 24 h, RIRI 48 h, RIRI 5 days, RIRI 7 days: after the RIRI operation, the SD rats were sacrificed at 24 h, 48 h, 5 days, and 7 days, respectively.

measured by IHC and western blot analysis. The expression level of TRPM-7 was significantly increased in RIRI at 24 h, 48 h, 5 days, and 7 days compared to the SO group (**Figure 3**) (P<0.05). The expression of TRPM-7 was rapidly up-regulated at 24 h following RIRI, followed by a decline at 48 h, 5 days, and 7 days. The expression of PARP-1 and RIP-1 showed the same varying trend as TRPM-7 expression (**Figures 4** and **5**).

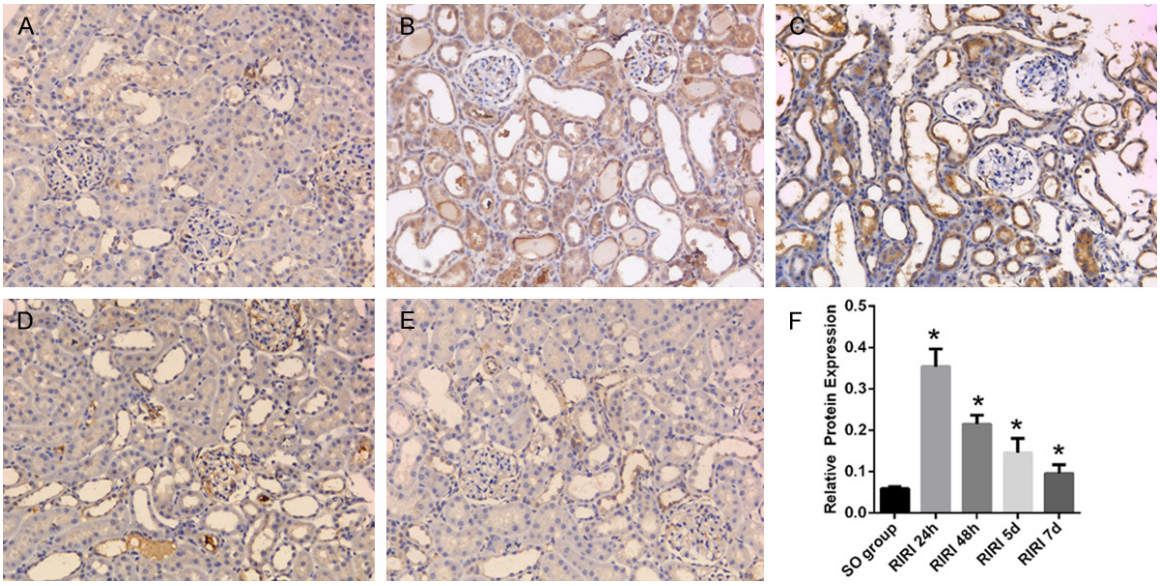

Figure 4. Expression of PARP-1 in renal ischemic reperfusion injury (RIRI). A-E: Representative images of immunohistochemical stain of PARP-1 in SO group, RIRI 24 h, RIRI 48 h, RIRI 5 days, and RIRI 7 days, respectively (400×). F: The results of PARP-1 expression are presented as mean ± SD, the bars indicate the PARP-1 protein expression levels, **P*<0.05 compared with SO group. SO group: sham operation group; RIRI 24 h, RIRI 48 h, RIRI 5 d, RIRI 7 d: after the RIRI operation, the SD rats were sacrificed at 24 h, 48 h, 5 days, and 7 days, respectively.

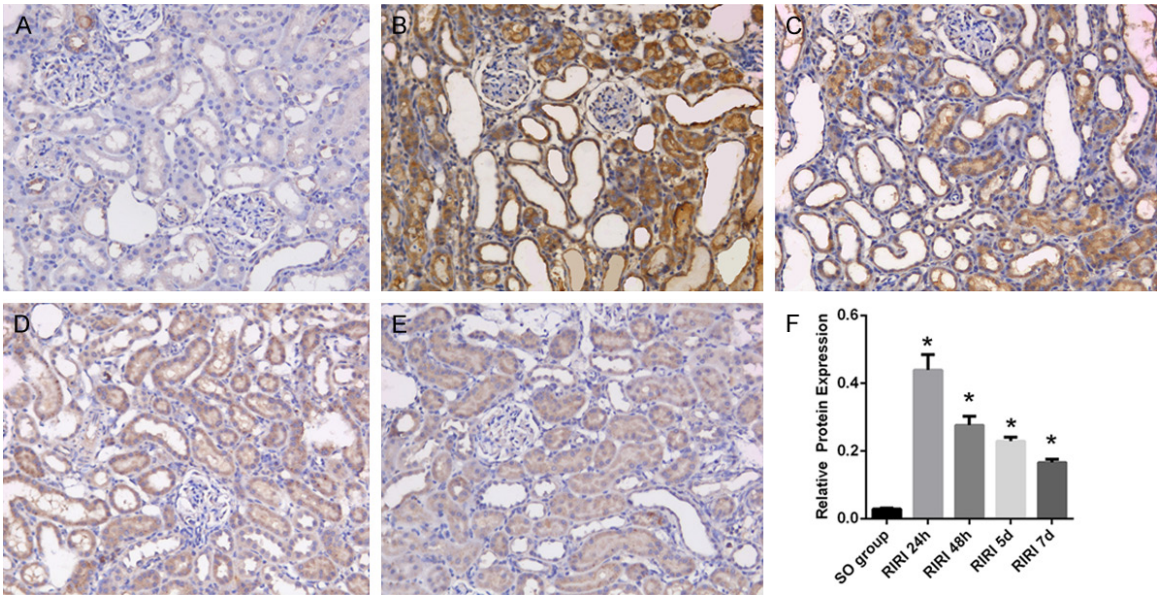

Figure 5. Expression of RIP-1 in renal ischemic reperfusion injury (RIRI). A-E: Representative images of immunohistochemical stain of RIP-1 in SO group, RIRI 24 h, RIRI 48 h, RIRI 5 days, and RIRI 7 days, respectively (400×). F: RIP-1 expression values are presented as mean ± SD, the bars indicate the RIP-1 protein expression levels, **P*<0.05 compared with SO group. SO group: sham operation group; RIRI 24 h, RIRI 48 h, RIRI 5 d, and RIRI 7 d: after the RIRI operation, the SD rats were sacrificed at 24 h, 48 h, 5 days, and 7 days, respectively.

TRPM-7 ameliorates RIRI via up-regulating PARP-1 and down-regulating RIP-1

To reveal the underlying molecular mechanism for the effects of TRPM-7 on RIRI, we examined the effects of TRPM-7 on PARP-1, RIP-1, and renal function. In this study, we used Bradykinin (a non-specific agonist that activates TRPM-7 expression), 2-APB (a non-specific blocker that inhibits TRPM-7 expression), and Nec-1 (a spe-

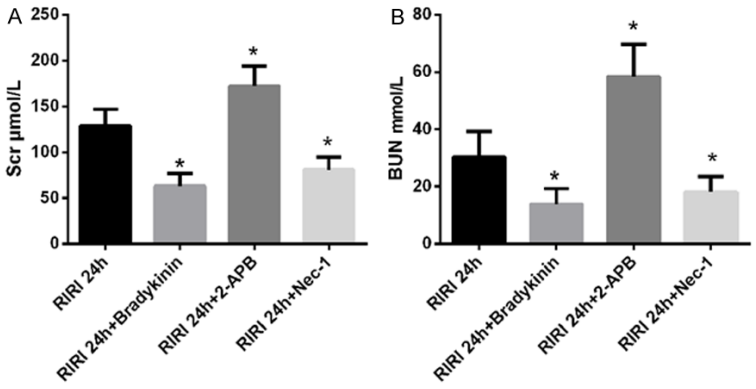

Figure 6. Serum creatinine (A) and blood urea nitrogen (B) values in the RIRI 24 h, Bradykinin, 2-APB (2-Aminoethoxydiphenyl borate), and Nec-1 (Necrostatin-1) groups. The bars indicate the Scr and BUN values, dates are presented as mean ± SD. **P*<0.05 compared with RIRI 24 h group.

cific blocker that inhibits necroptosis) to treat the RIRI mice. To investigate the effects of Bradykinin, 2-APB, and Nec-1 treatment on renal function, we analyzed Scr and BUN levels (**Figure 6**). Compared to the RIRI 24 h group, Scr and BUN values were significantly higher in the 2-APB group (*P*<0.01), and decreased in the Bradykinin and Nec-1 groups (*P*<0.01). The Bradykinin and Nec-1 treatments may ameliorate RIRI, while the 2-APB treatment may exacerbate RIRI. Results for the expression of TRPM-7, PARP-1 and RIP-1 in the Bradykinin, 2-APB, and Nec-1 groups were shown in **Figure 7**. Compared with the RIRI 24 h group, the expression of TRPM-7 and PARP-1 did not change in Bradykinin, 2-APB, and Nec-1 group (*P*>0.05). Compared with the RIRI 24 h group, the expression of PARP-1 cleavage was significantly up-regulated in the Bradykinin group (*P*<0.05), significantly down-regulated in the 2-APB group (*P*<0.05), and did not change in the Nec-1 group (*P*>0.05). Compared with the RIRI 24 h group, the expression of RIP-1 was significantly down-regulated in the Bradykinin and Nec-1 groups (*P*<0.05), and significantly up-regulated in the 2-APB group (*P*<0.05).

Discussion

RIRI plays a significant role in AKI in native and transplanted kidneys [1]. Some reviews have suggested that mitochondrial injury with Ca^{2+} overload is one of the most important factors in RIRI [1]. TRPM-7 is an important non-selective Ca^{2+} ion channel expressed in renal tubular epithelial cells [19]. In this study, we found that the expression of TRPM-7 increased in the early stage of RIRI and then gradually decreased at 48 h, 5 days, and 7 days post-operation. The change in Scr and BUN values showed the same trend as TRPM-7 expression. These results suggest that there was a positive relationship between changes in TRPM-7 expression and acute renal tubular injury grading. Our findings support the use of TRPM-7 as a diagnostic gene. Several studies have suggested that TRPM-7 is up-regulated in the early stage of RIRI and other ischemia reperfusion injuries (IRI) [17], which concurs with our results.

To investigate the effect of TRPM-7 on RIRI, we explored the downstream signaling pathway of TRPM-7, and contributed to the understanding of its role in RIRI progression and attenuation. Compared to apoptosis, necroptosis has been empirically confirmed in RIRI, and is thought to play a more important role in RIRI processes than is currently known [7]. RIP-1 is the key regulating factor of necroptosis, and RIP-1 silencing can inhibit necroptosis [20, 21]. Sirtuin-2 could de-acetylate RIP-1, which is necessary for the formation of the RIP1-RIP3 complex that leads to necroptosis [22, 23]. Sirtuin-2 enzyme activity depends on the level of metabolic intermediates (e.g. NAD+) [24]. A decrease in NAD+ activity has been related to a lack of cellular energy and oxidative stress. Meanwhile, research has found that PARP-1 and sirtuin-2 are both belong to the NAD+ dependent ADP-ribosyltransferase, which depend on the cofactor NAD+ level and are regulated by NAD+ stability [25]. In the presence of NAD+, both PARP-1 and sirtuin-2 competitively inhabited each other. In this study, we determined the expression of the necroptosis-related proteins, RIP-1 and PARP-1, and found that PARP-1 and RIP-1 expression increased significantly after the early stage of RIRI and then gradually decreased at 48 h, 5 days, and 7 days.

2-APB, a nonspecific TRPM-7 inhibitor, inhibited TRPM-7 expression, while Bradykinin, a nonspecific TRPM-7 agonist, increased TRPM-7 ex-

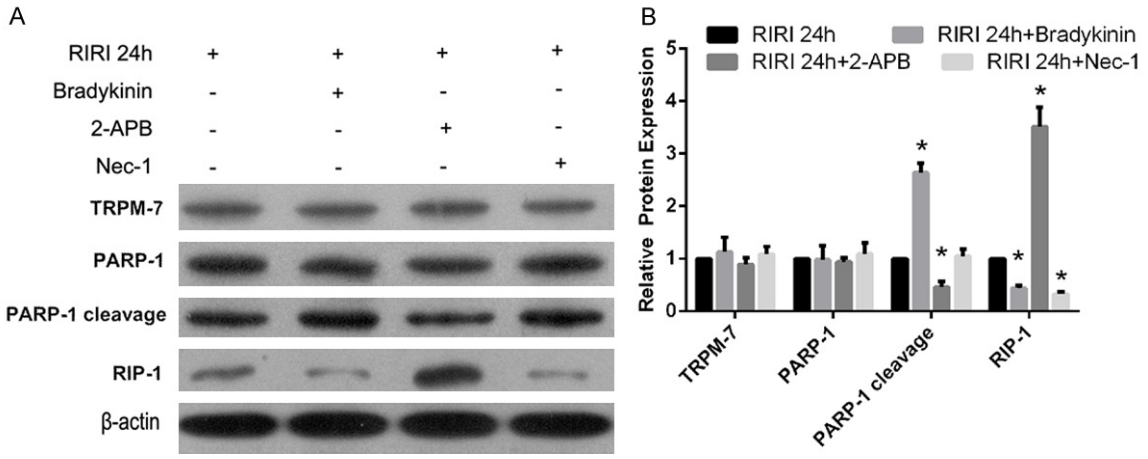

Figure 7. Expression of TRPM-7, PARP-1, and RIP-1 in the RIRI 24 h, Bradykinin, 2-APB (2-Aminoethoxydiphenyl borate), and Nec-1 (Necrostatin-1) groups. A: Representative images of TRPM-7, PARP-1, and RIP-1 expression detected by western blot. B: TRPM-7, PARP-1, and RIP-1 expression values are presented as mean ± SD. **P*<0.05 compared with RIRI 24 h group. RIRI 24 h: after the RIRI operation, the SD rats were sacrificed at 24 h. Bradykinin, 2-APB, and Nec-1 groups: before the RIRI operation, the SD rats were treated by tail intravenous injection with Nec-1 (1.65 mg/kg), 2-APB (1.5 mg/kg), or Bradykinin (1.5 mg/kg). After 5 min, the SD rats went through the same surgical procedure as the RIRI operation group and were sacrificed at 24 h.

pression. In this study, we injected RIRI rats with a pre-treatment of 2-APB or Bradykinin. In the 2-APB group, the expression of TRPM-7 and PARP-1 were significantly down-regulated, the expression of RIP-1 was significantly up-regulated, and Scr and BUN values were significantly higher. In the Bradykinin group, the expression of TRPM-7 and PARP-1 were significantly up-regulated, the expression of RIP-1 was significantly down-regulated, and Scr and BUN values were significantly lower. These results suggest that inhibition of TRPM-7 expression can promote RIRI; in contrast, enhanced TRPM-7 expression can inhibit RIRI. This indicates that TRPM-7 may activate PARP-1 and inhibit RIP-1 to attenuate RIRI in renal tubular epithelial cells. These results were dramatically different from the other studies, which showed that suppression of TRPM-7 may alleviate kidney injury in RIRI [26]. The difference in results may be associated with the function of PARP-1. PARP-1 has a dual function to promote cell survival or death. Under moderate activation, PARP-1 may contribute to cellular recovery following sub-lethal, transient global ischemia [27]. When PARP-1 is potently activated by DNA strand nicks and breaks, apoptosis-inducing factor (AIF) is released by the mitochondria to induce caspase-independent cell death [28]. In this study, TRPM-7 moderately activated PARP-1 to protect the renal tubular epithelial cells from damage.

In addition, to investigate the effect of TRPM-7 on necroptosis in RIRI, we treated RIRI rats with Nec-1, an inhibitor of necroptosis. In the Nec-1 group, expression of TRPM-7 and PARP-1 was not significantly different compared to the RIRI 24 h group, while the expression of RIP-1 was significantly reduced and Scr and BUN values were significantly lower. These results further support that TRPM-7 can activate PARP-1 and inhibit RIP-1 to attenuate RIRI in renal tubular epithelial cells.

In conclusion, these results suggest that TRPM-7 inhibits necroptosis by activating the PARP-1/RIP-1 signaling pathway to attenuate RIRI in renal tubular epithelial cells, which indicates that TRPM-7 may play a protective role in RIRI and that up-regulation of TRPM-7 could be a novel therapeutic strategy for RIRI.

Disclosure of conflict of interest

None.

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