Original Article

TRPC6 ameliorates renal ischemic reperfusion injury by inducing Zn^{2+} influx and activating autophagy to resist necrosis

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Background: Renal ischemic reperfusion injury (RIRI) is the most hackneyed cause of acute renal injury with high incidence. As a slit diaphragm (SD), TRPC6 (transient receptor potential channel 6) can maintain the structure and function of glomerular podocytes, and its activation has been reported to prominently alleviate ischemia reperfusion (I/R). However, the specific mechanism of TRPC6 in RIRI is uncertain.

Methods: The TRPC6 specific shRNA or overexpressing plasmid was used to decrease or increase TRPC6 level in HK-2 cells, respectively. Subsequently, the OGD/R (oxygen-glucose deprivation and re-oxygenation) HK-2 cells and RIRI model rats was established to examine the effect of TRPC6 in RIRI *in vitro*. After processing, viability was confirmed with MTT; cell necrosis was analyzed with flow cytometry; necrosis and autophagy-related proteins were verified with Western blot; free Zn^{2+} was tested with an Zn^{2+} fluorescent probe; and cell autophagy was monitored with MDC (monodansylcadaverine) method. Furthermore, TRPC6 agonist (OGA) or TRPC6 inhibitor (SKF96365) were introduced to increase or inhibit the activity of TRPC6 in RIRI model rats, and the kidney injury was assessed with H&E staining and RIP1 and PARP-1 expressions were examined with IHC (immunohistochemistry) staining.

Results: Our results verified TRPC6 could markedly enhance viability, Zn^{2+} influx, and autophagy, and suppressed necrosis in OGD/R HK-2 cells. In addition, increase of Zn^{2+} or autophagy activation produced similar results to TRPC6 overexpression in viability, autophagy, and necrosis of OGD/R HK-2 cells. Rescue experiment results also showed TRPC6 could prevent necrosis and facilitate Zn^{2+} influx and autophagy of OGD/R HK-2 cells by inducing Zn^{2+} influx and autophagy. Moreover, TRPC6 could ameliorate kidney injury, block necrosis, and enhance autophagy in RIRI model rats by promoting Zn^{2+} influx and autophagy.

Conclusions: TRPC6 could prevent necrosis and induce autophagy to alleviate RIRI by accelerating Zn^{2+} influx and autophagy. This shows TRPC6/Zn^{2+} influx/autophagy might be a novel therapeutic strategy for RIRI.

Keywords: TRPC6; renal ischemic reperfusion injury (RIRI); Zn^{2+} influx; autophagy; necrosis

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Introduction

Ischemia reperfusion injury (IRI) may occur following acute hemorrhage, severe trauma, toxic shock, or kidney transplantation (1,2). Due to the characteristics of high perfusion and high metabolism, the kidney is extremely sensitive to ischemia and hypoxia stimulation and prone to IRI (3), and research has shown renal IRI (RIRI) is one of the major factors affecting renal function recovery and long-term survival after renal ischemia or transplantation (4).

Therefore, further exploration of the pathogenesis of RIRI and effective treatment strategies is of clinical significance.

As a member of the transient receptor potential family of calcium channels, transient receptor potential channel 6 (TRPC6) is a nonselective cation channel and a key molecule of slit diaphragm (SD) (5,6). It was reported that TRPC6 could be involved in the intracellular flow of Ca^{2+}, Fe^{2+}, and Zn^{2+} (7,8), and Zn^{2+} had an antioxidant role and could prevent renal cell apoptosis in IRI (9). Thus, we speculated that TRPC6 could restrain programmed cell necrosis by mediating Zn^{2+} influx and play a protective role in RIRI. However, the regulatory role of Zn^{2+} on programmed cell necrosis in RIRI is not entirely clear. Role of Zn^{2+} in the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway to protect against renal I/R injury has been investigated in published research (10). It has been observed that I/R injury results in significant increase in the serum levels of blood urea nitrogen (BUN) and creatinine and increase in the fractional excretion of sodium (FENa) which is decreased by zinc chloride ($ZnCl_2$) supplementation (11,12). Besides, Zn^{2+} also improved glomerular filtration rate value, Cr-clearance, and urine flow rate beyond renal I/R induced renal injury (12,13). Furthermore, Major antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione (GSH) decreased after renal I/R-induced injury. It has been suggested that Zn^{2+} supplementation stimulates the activity of these enzyme (14). However, it is still unclear whether Zn^{2+} is the key regulator of TRPC6 in renal ischemic reperfusion injury.

Autophagy is a highly conserved metabolic mechanism in the organic evolutionary process (15), and plays a fatal regulatory role in programmed cell necrosis (16). In addition, it has been recently disclosed that Zn^{2+} can induce autophagy (17) and can protect RIRI by accelerating autophagy (18). However, it is still unclear whether Zn^{2+} can participate in the suppression of TRPC6 on cell necrosis by regulating cell autophagy.

TRPC6 is a receptor-activated nonselective cation channel that is homogeneously expressed throughout the central nervous system and peripheral tissues, including the kidneys (19). It has been suggested to play a significant role in I/R injury of the lungs, retinas, and brain (20,21). Our previous study identified TRPC6 as an up-regulated differentially expressed gene in the pathogenesis of I/R injury, and protected against I/R injury, suggesting that it may be a potential novel target for therapy (22). However, the underlying mechanism of TRPC6 in renal I/R injury has not been investigated. In current study, we further verified the influences of TRPC6 on necrosis, Zn^{2+} influx, and autophagy in oxygen-glucose deprivation and re-oxygenation (OGD/R) HK-2 cells and RIRI model rats.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-5837/rc).

Methods

Establishment of OGD/R HK-2 cells

HK-2 cells were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. For the OGD/R model, HK-2 cells were grown in an airtight container with ischemia simulation medium (0.338 mmol/L Na_2HPO_4, 136.89 mmol/L NaCl, 5.37 mmol/L KCl, 0.44 mmol/L KH_2PO_4, 4.166 mmol/L $NaHCO_3$, and 5 mmol/L D-glucose; pH =6.8), 5% CO_2, and 95% N_2. The airtight container was then plated into an anoxic incubator (5% CO_2, 1% O_2, and 94% N_2) at 37 ℃ for 2 h, and the ischemia simulation medium was then replaced with conventional medium. Cells were then incubated under normal culture conditions (37 ℃ and 5% CO_2) for 3 h (22).

Cell treatment

Negative control (NC), TRPC6 shRNAs (sh-TRPC6), and TRPC6-overexpressed plasmid (OE-TRPC6) were gained from HanBio Biotechnology (Shanghai, China). The sequence for TRPC6 shRNA was 5'-AAGAAGUUCUGUAAUUUCCAG-3' (sense) and 5'-GGAAAUUACAGAACUUCUUCU-3' (anti-sense). OGD/R HK-2 cells were then transfected with these plasmids with Lipofectamine 3000 (Invitrogen) for 48 h, and the cells were also treated with 0.3 mM $ZnSO_4$, TPEN, rapamycin, or 3-MA, respectively.

Establishment of RIRI model rats

A total of 36 male SD rats (age: 6–8 weeks; bodyweight: 201±38.3 g) were provided by the Experimental Animal Center of Army Medical University (Chongqing, China), and kept in a standard comfortable environment. Animal experiments were approved by the Animal Care and Use Committee of the Army Medical University [with two institutional licenses SCXK(YU)20170002 and SYXK(YU)20170002], in compliance with the guidelines

of the Army Medical University for the care and use of animals. A protocol was prepared before the study without registration. These rats were divided into a sham group (n=6) and I/R group (n=30). After the abdominal cavity of rats in the IR group was opened, the bilateral renal pedicles were closed with vascular clips to block the renal blood supply for 30 min, after which the vascular clips were then removed to restore renal blood perfusion and the wound sutured. In the sham group, after opening the abdominal cavity, the kidney pedicle was not clamped, and the kidneys were exposed for 30 min before suturing. RIRI model rats were then treated with the TRPC6 agonist (OGA), TRPC6 inhibitor (SKF96365), SKF96365 and 15 mg/kg $ZnSO_4$, SKF96365, and rapamycin.

Renal damage rating scale

The Histological Score of the Kidney (HSK) was microscopically evaluated by the same pathologist. The section of the damaged kidney was evaluated based on tubule cell swelling, nuclear pyknosis (apoptosis), and nucleolysis (necrosis). Renal injury was divided into 4 grades: 0= normal; 1 point = minor damage (less than 1/3 nuclear pyknosis or nucleolysis); 2 points = moderate damage (1/3 to 2/3); and 3 points = severe damage: greater than 2/3.

MTT assay

OGD/R HK-2 cells (1×10^5 cells/mL) in each group were evenly inoculated into 96-well plates and incubated for 48 h, then 20 μL MTT (5 mg/mL; Sigma-Aldrich, Cat. no. 298-93-1) was added to each cell before incubation for 3 h. After the supernatant was discarded, 150 μL DMSO was added to the cells and shaken for 10 min. A microplate analyzer was used to confirm the absorbance at 490 nm.

Flow cytometry

The cell necrosis rate was tested using an Annexin V-FITC/PE kit (Sigma). By referencing the instructions, the treated OGD/R HK-2 cells were collected and suspended with 100 μL 1× binding buffer, then disposed of with 5 μL FITC-Annexin V and 5 μL PE solution for 15 min in the dark. Finally, the number of necrotic cells was monitored using FACS Calibur flow cytometry (BD Biosciences).

Western blot

We first obtained total proteins from the treated OGD/R HK-2 cells or kidney tissues of RIRI model rats, which had also been ground, using RIPA buffer (Beyotime, Shanghai, China). After quantification, the protein (40 μg) was detached via electrophoresis on 10% SDS-PAGE, then transferred to PVDF membrane (Millipore). After sealing, the treated membranes were then soaked with primary antibodies at 4 ℃ overnight and secondary antibody for 2 h. Finally, the protein blots were visualized with the ECL system (Thermo Fisher Scientific). All antibodies were obtained from Abcam (USA).

Detection of Zn^{2+} and autophagy

Based on the specifications provided by the supplier, free Zn^{2+} was examined with a Zn^{2+} detection kit (sigma-Aldrich, Cat. No. MAK032) and cell autophagy was tested by the MDC method with an autophagy staining kit (Solarbio, cat. no. G1170).

H&E staining

Kidney tissues from rats were collected after sacrifice and subjected to a series of treatments including fixation (4% paraformaldehyde), dehydration (gradient alcohol), and paraffin embedding, before being cut into 4-μm slices. After dewaxing, the slices were further addressed with xylene I, xylene II, gradient ethanol, and distilled water, then dyed with Harris hematoxylin, 1% hydrochloric acid alcohol, 0.6% ammonia, and eosin. Through dehydration and transparency, the degree of kidney tissue injury was observed under a light microscope.

Immunohistochemistry (IHC) assay

Slices were processed with 3% hydrogen peroxide and EDTA after dewaxing, then disposed of primary antibodies (anti-RIP1 and anti-PARP-1) at 37 ℃ for 1 h and secondary antibody for 30 min. DAB, hematoxylin, and 0.1% hydrochloric acid were then added to slices, and after dehydration, the results were confirmed with a light microscope.

Statistical analysis

All data repeated three times are represented as mean ± SD. Data were counted with one-way analysis of variance and $P<0.05$ denoted that difference was statistically significant.

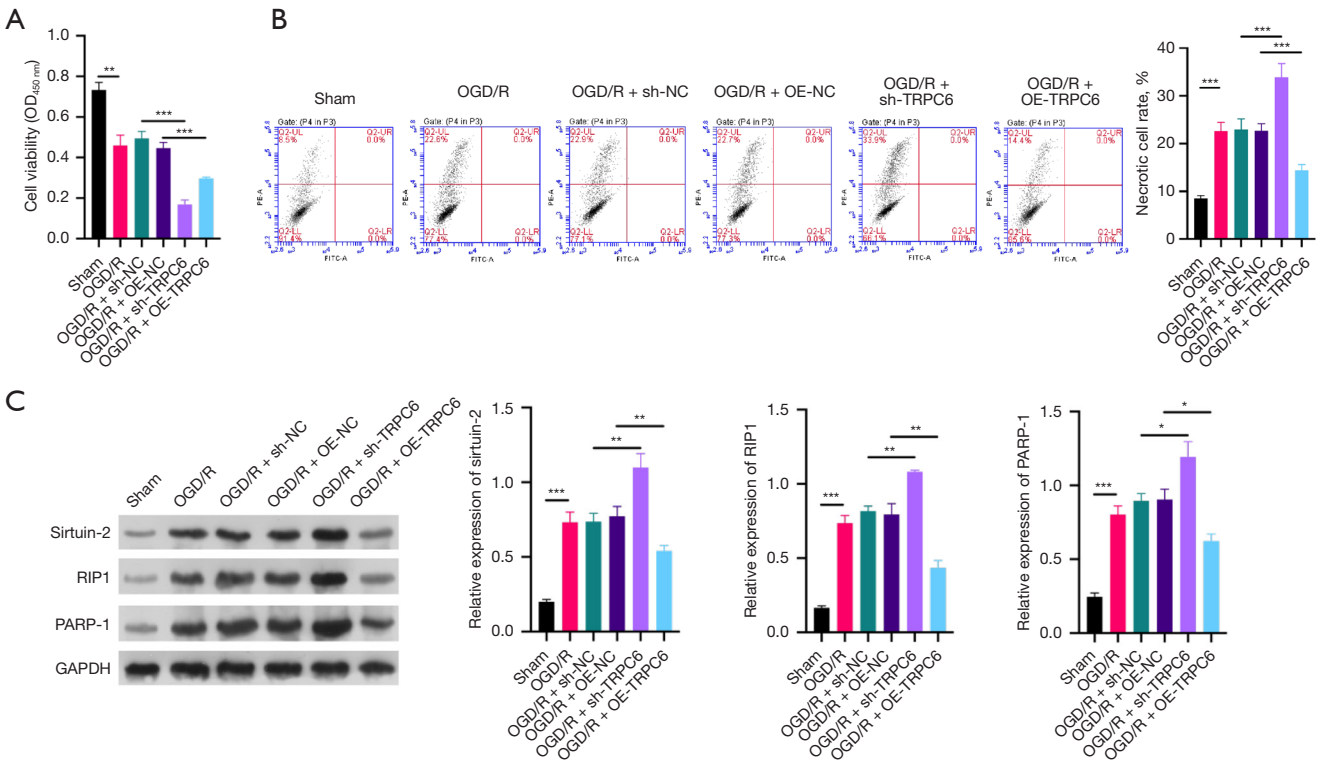

Figure 1 TRPC6 markedly enhanced viability and resisted necrosis in OGD/R HK-2 cells. HK-2 cells were first induced with OGD/R, and then transfected with sh-TRPC6 and OE-TRPC6, respectively. (A) MTT exhibited the change in viability; (B) flow cytometry displayed the change in the degree of cell necrosis; (C) Western blot presented the changes in Sirtuin-2, RIP1, and PARP-1 expressions, and the relative quantification was confirmed. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Results

TRPC6 markedly enhanced viability and resisted necrosis in OGD/R HK-2 cells

TRPC6 has been reported to participate in I/R injury of target organs, and we further explored its impact on RIRI. Firstly, we constructed an OGD/R cell model with HK-2 cells as previously reported (23), and, as displayed in *Figure 1A*, cell viability was observably reduced in the OGD/R group compared to the sham group. In addition, silence of TRPC6 caused a significant decrease in the viability of OGD/R HK-2 cells, while TRPC6 overexpression exerted an opposite effect to TRPC6 silencing on cell viability. Flow cytometry data revealed cell necrosis was markedly aggrandized in the OGD/R group compared to the sham group and could be signally motivated by TRPC6 silencing and decreased by TRPC6 overexpression in OGD/R HK-2 cells (*Figure 1B*). Necrosis-related proteins (Sirtuin-2, RIP1, and PARP-1) were also dramatically upregulated in the OGD/R group compared to the sham group, and the expression of these proteins could be notably up-regulated by TRPC6 silencing and prominently down-regulated by TRPC6 overexpression in OGD/R HK-2 cells (*Figure 1C*). On balance, our data disclosed that upregulation of TRPC6 could result in a remarkable increase in viability and a noteworthy decrease in necrosis of OGD/R HK-2 cells.

TRPC6 induced Zn^{2+} influx and autophagy in OGD/R-mediated HK-2 cells

TRPC6 has been reported to participate in Zn^{2+} influx, and Zn^{2+} can induce autophagy. In this study we further confirmed whether TRPC6 could affect Zn^{2+} influx and autophagy in OGD/R HK-2 cells. Firstly, the data signified that free Zn^{2+} was signally increased in the OGD/R group compared to the sham group, and silence of TRPC6 could result in a conspicuous decrease in free Zn^{2+}, while TRPC6 overexpression could result in a distinct increase in free Zn^{2+} of OGD/R HK-2 cells (*Figure 2A*). Secondly, the results from MDC denoted that cell autophagy was

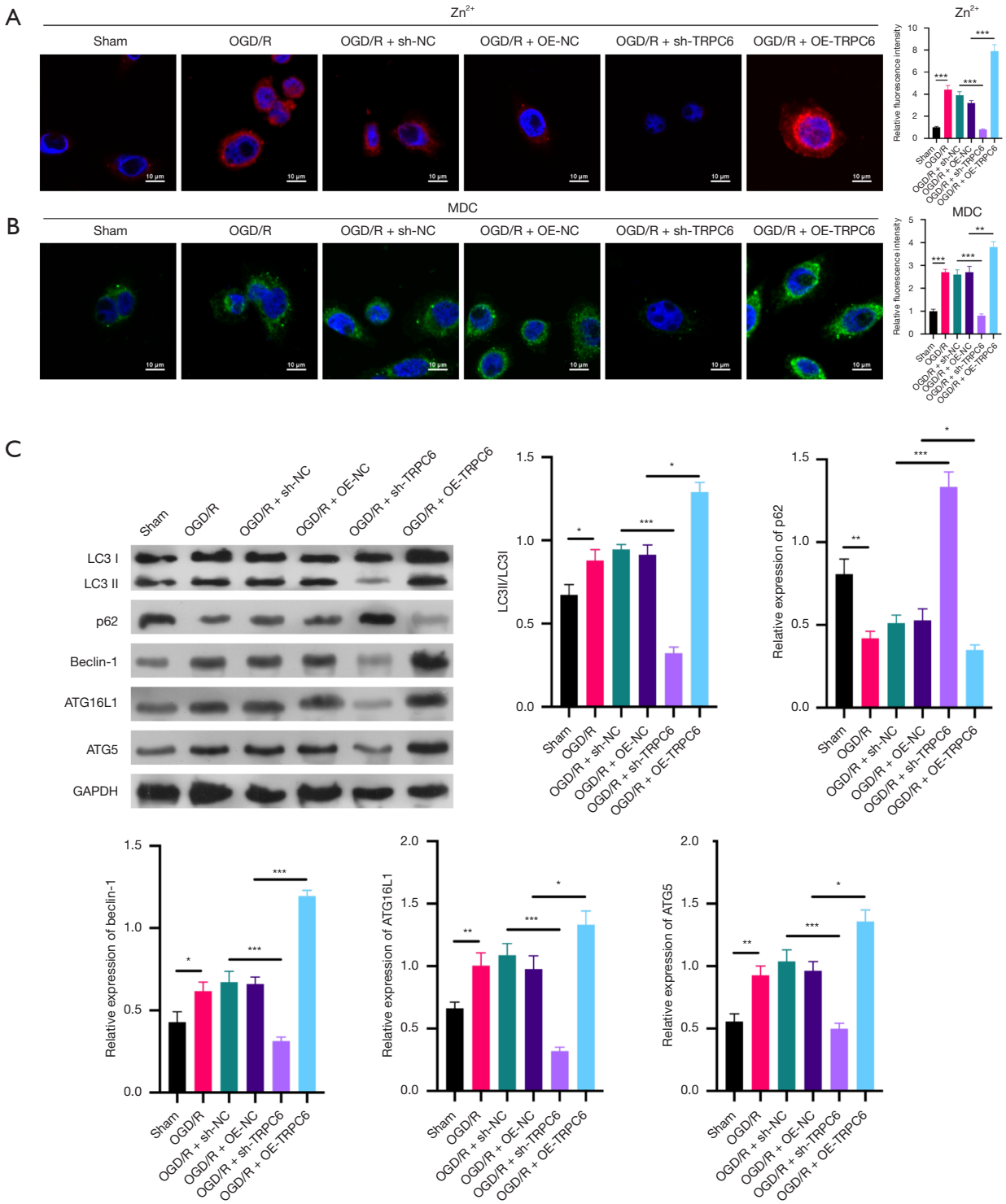

Figure 2 TRPC6 induced Zn^{2+} influx and autophagy in OGD/R-mediated HK-2 cells. sh-TRPC6 and OE-TRPC6 were transfected into OGD/R-induced HK-2 cells, respectively. (A) Free Zn^{2+} was monitored through a Zn^{2+} fluorescent probe in processed HK-2 cells; (B) cell autophagy was identified via MDC method; (C) LC3 I/II, p62, beclin-1, ATG16L1, and ATG5 expressions were tested by the application of Western blot. *, P<0.05; **, P<0.01; ***, P<0.001.

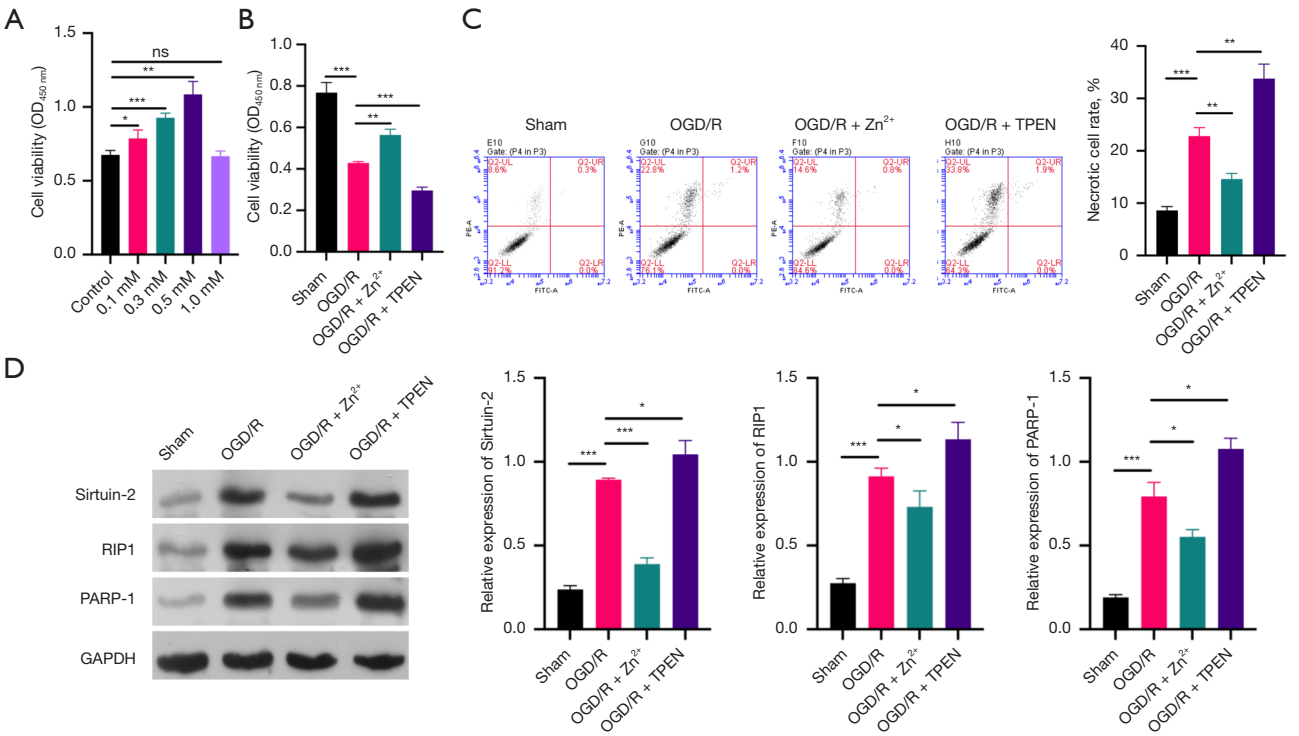

Figure 3 Increase of Zn^{2+} induced viability and reduced necrosis in HK-2 cells with OGD/R. (A) MTT was conducted to assess cell viability in OGD/R-mediated HK-2 cells after processing with 0, 0.1, 0.3, 0.5, and 1.0 mM $ZnSO_4$. OGD/R HK-2 cells were then administrated with 0.3 mM $ZnSO_4$ and Zn^{2+} chelating agent (TPEN), respectively; (B) MTT was applied to test cell viability; (C) flow cytometry was conducted to evaluate cell necrosis; (D) Western blot was adopted to monitor the levels of necrosis-related proteins. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

memorably elevated in the OGD/R group compared to the sham group, and cell autophagy could be observably repressed by TRPC6 silencing and markedly accelerated by TRPC6 overexpression in OGD/R HK-2 cells (*Figure 2B*). Thirdly, Western blotting data showed that relative to the sham group, LC3II/I, beclin-1, ATG16L1, and ATG5 were dramatically upregulated, and p62 was prominently downregulated in the OGD/R group, and the changes in these proteins mediated by OGD/R could be notably attenuated by TRPC6 silencing and enhanced by TRPC6 overexpression in HK-2 cells (*Figure 2C*). Consequently, we proved that an increase of TRPC6 could also exhibit inductive effects in Zn^{2+} influx and autophagy of OGD/R-mediated HK-2 cells.

Increase of Zn^{2+} induced viability and reduced necrosis in HK-2 cells with OGD/R

Subsequently, we also identified the influence of Zn^{2+} on the viability and necrosis of OGD/R HK-2 cells. We first screened the optimal concentration of Zn^{2+} on OGD/R HK-2 cells through MTT, and the data manifested that cell viability was signally enhanced in 0.1, 0.3, and 0.5 mM $ZnSO_4$ treatment groups versus that in the control group and was highest in the 0.3 mM $ZnSO_4$ group (*Figure 3A*). Thus, 0.3 mM $ZnSO_4$ was adopted to construct the Zn^{2+} treatment group in OGD/R HK-2 cells. We also applied Zn^{2+} chelating agent (TPEN) to treat OGD/R HK-2 cells, and as displayed in *Figure 3B*, cell viability in OGD/R HK-2 cells was markedly raised in the Zn^{2+} group and observably decreased in the TPEN group. Data also indicated that Zn^{2+} treatment memorably reduced the necrosis rate of OGD/R HK-2 cells while TPEN treatment notably heightened their necrosis rate (*Figure 3C*). Similarly, we proved that the levels of Sirtuin-2, RIP1, and PARP-1 could be prominently downregulated by Zn^{2+} treatment and dramatically upregulated by TPEN treatment in OGD/R HK-2 cells (*Figure 3D*). Therefore, these data showed that like TRPC6

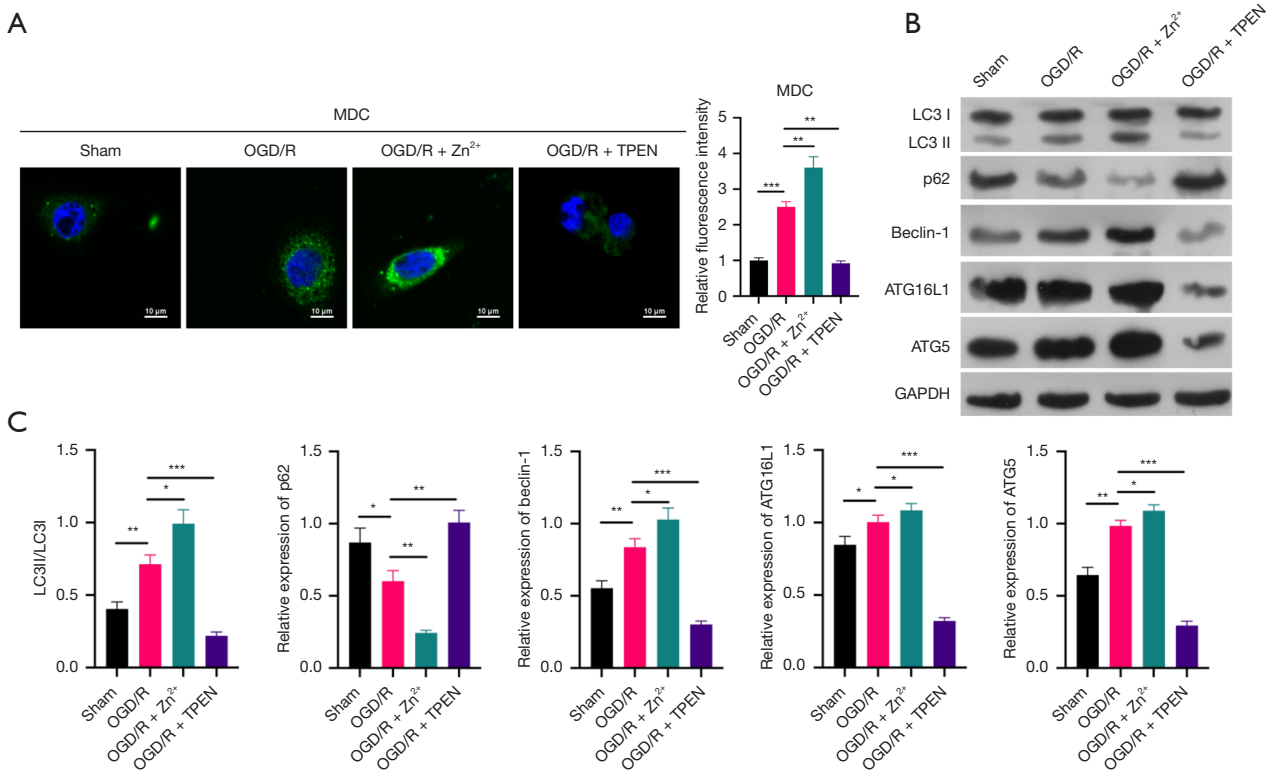

Figure 4 Increase of Zn^{2+} heightened autophagy in OGD/R HK-2 cells. 0.3 mM $ZnSO_4$ and TPEN were applied to treat OGD/R HK-2 cells. (A) A Zn^{2+} fluorescent probe was utilized to confirm the change of free Zn^{2+}; (B) Western blot presented the expression changes of autophagy-associated proteins; (C) these proteins were quantified in view of gray values in Western blot. *, P<0.05; **, P<0.01; ***, P<0.001.

overexpression, the introduction of Zn^{2+} could accelerate the viability of OGD/R HK-2 cells and prevent necrosis.

Increase of Zn^{2+} heightened autophagy in OGD/R HK-2 cells

Just as TRPC6 has a prominent induction role on autophagy of OGD/R HK-2 cells, we also explored the influence of Zn^{2+} on cell autophagy. The MDC data first exhibited that cell autophagy could be notably strengthened by Zn^{2+} addition and observably weakened by TPEN addition in OGD/R HK-2 cells (*Figure 4A*). Data also denoted that introduction of Zn^{2+} could cause a striking increase in LC3II/I, beclin-1, ATG16L1, and ATG5 levels, and an outstanding reduction in p62 level in OGD/R HK-2 cells, and the increase of TPEN could also cause the opposite effect of Zn^{2+} on the expression of these proteins (*Figure 4B,4C*). Overall, these data verified that the increase of Zn^{2+} alone also had a remarkable accelerative role on autophagy in OGD/R HK-2 cells.

Autophagy activation accelerated viability and restrained necrosis in OGD/R HK-2 cells

As both TRPC6 and Zn^{2+} were seen to induce autophagy, we further investigated whether autophagy change alone could affect the viability and necrosis of OGD/R HK-2 cells. The autophagy activator (rapamycin) and autophagy inhibitor (3-MA) were utilized to treat OGD/R HK-2 cells, respectively, and MTT data showed that compared to the OGD/R + DMSO group, cell viability was memorably increased in the OGD/R + rapamycin group and signally degraded in the OGD/R + 3-MA group (*Figure 5A*). In addition, flow cytometry results showed that relative to the OGD/R + DMSO group, the necrosis rate was prominently lowered in the OGD/R + rapamycin group and markedly elevated in the OGD/R + 3-MA group (*Figure 5B*). The results also showed that Sirtuin-2, RIP1, and PARP-1 expressions were lower in the OGD/R + rapamycin group and higher in the OGD/R + 3-MA group than in the OGD/R + DMSO group (*Figure 5C*). MDC results also signified

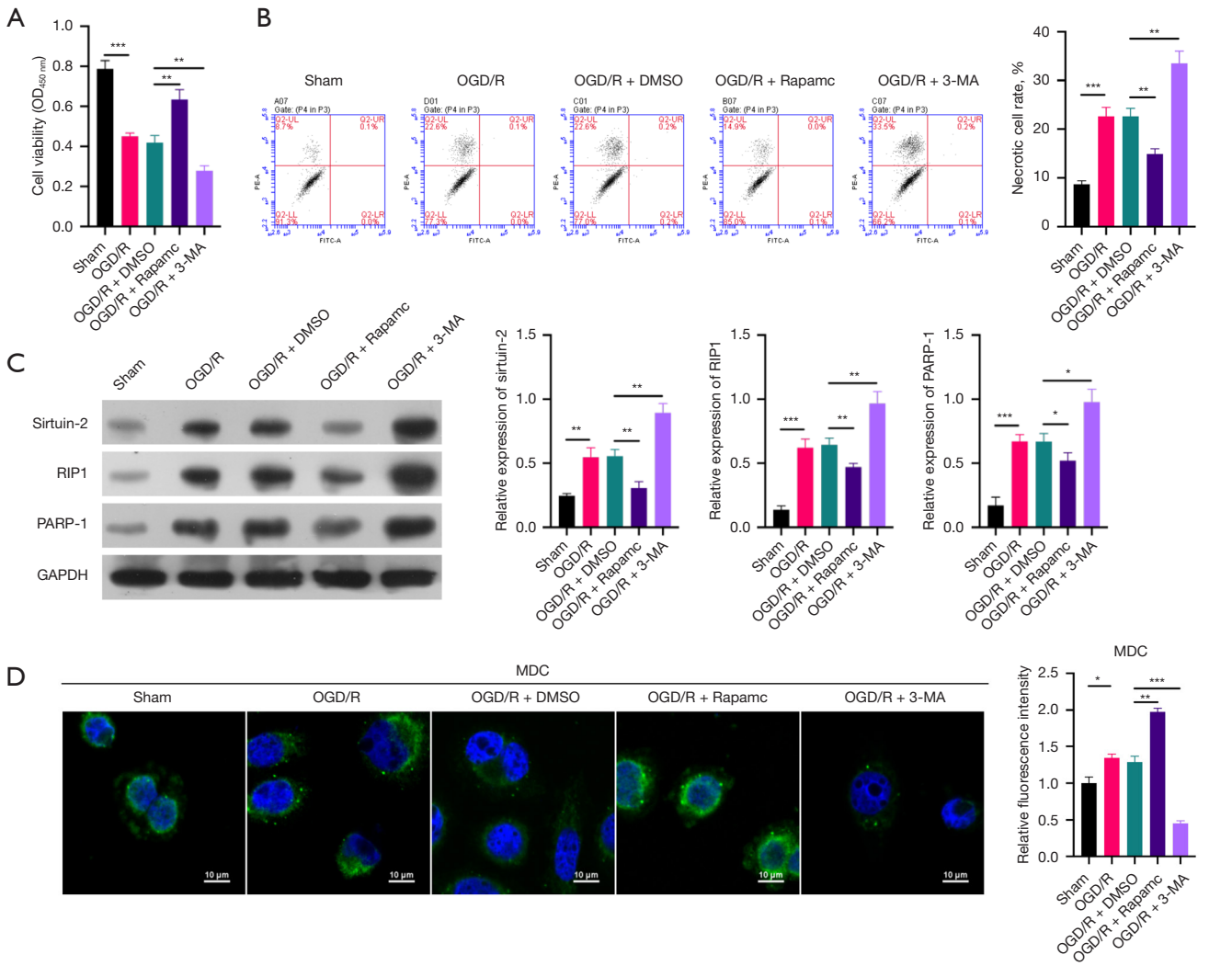

Figure 5 Autophagy activation accelerated viability and restrained necrosis in OGD/R HK-2 cells. OGD/R HK-2 cells were disposed of autophagy activator (rapamycin and autophagy inhibitor (3-MA), respectively. (A) MTT showed the change in cell viability; (B) flow cytometry presented the change in cell necrosis; (C) Western blot demonstrated the change in necrosis-related proteins; (D) MDC method displayed the change in cell autophagy. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

that cell autophagy could be notably induced by rapamycin and dramatically restrained by 3-MA in OGD/R HK-2 cells (*Figure 5D*). These results proved that activation of autophagy alone, like TRPC6 and Zn^{2+}, could also alleviate RIRI.

TRPC6 prevented necrosis of OGD/R HK-2 cells by promoting Zn^{2+} influx and activating autophagy

Based on the effect of TRPC6 overexpression, Zn^{2+} addition and autophagy activation on preventing necrosis and inducing autophagy in OGD/R HK-2 cells, the rescued experiment was then adopted to further explore the possible relationship between the three in cell viability and necrosis. Firstly, TRPC6 overexpression-mediated enhancement of cell viability could be markedly reversed by TPEN or 3-MA, and TRPC6 silencing-mediated suppression of cell viability could be memorably weakened by Zn^{2+} or rapamycin in OGD/R HK-2 cells (*Figure 6A*). Secondly, either TPEN or 3-MA could prominently attenuate the inhibition of cell necrosis mediated by

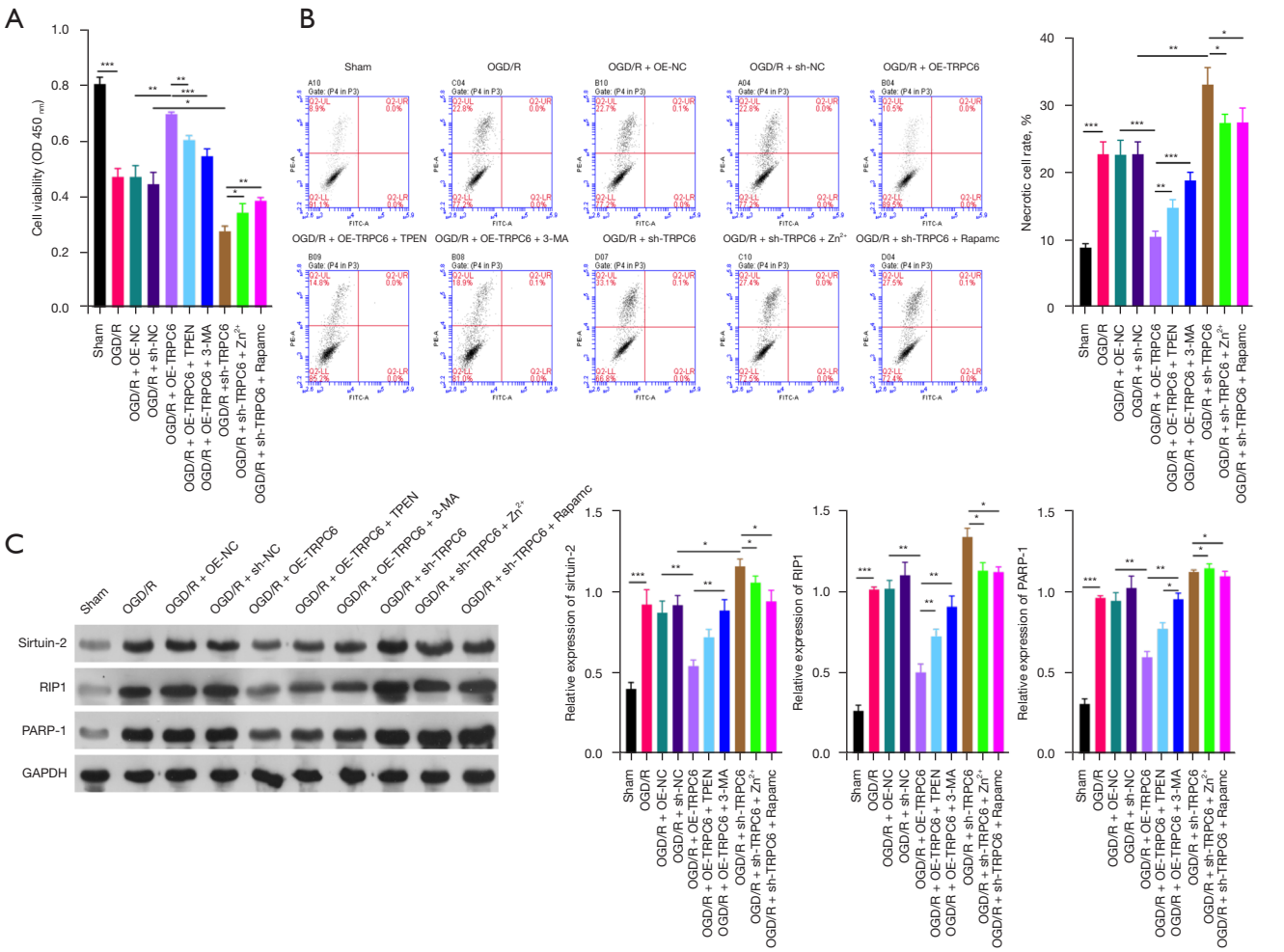

Figure 6 TRPC6 prevented necrosis of OGD/R HK-2 cells by promoting Zn^{2+} influx and activating autophagy. OGD/R HK-2 cells were co-treated with OE-TRPC6 and TPEN, OE-TRPC6 and 3MA, sh-TRPC6 and Zn^{2+}, or sh-TRPC6 and rapamycin, respectively. (A) MTT was carried out to assess cell viability; (B) flow cytometry was applied to determine cell necrosis; (C) Western blot was conducted to identify the expressions of necrosis-related proteins. *, P<0.05; **, P<0.01; ***, P<0.001. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

TRPC6 overexpression, and either Zn^{2+} or rapamycin could dramatically reverse the induction of cell necrosis mediated by TRPC6 silencing in OGD/R HK-2 cells (*Figure 6B*). Thirdly, TRPC6 overexpression-mediated downregulation of Sirtuin-2, RIP1, and PARP-1 could also be signally blunted by TPEN or 3-MA, and TRPC6 silencing-mediated upregulation of Sirtuin-2, RIP1, and PARP-1 also could be significantly weakened by Zn^{2+} or rapamycin in OGD/R HK-2 cells (*Figure 6C*). Generally, these findings certified that TRPC6 could facilitate viability and suppress necrosis of OGD/R HK-2 cells by inducing Zn^{2+} influx and autophagy.

TRPC6 promoted Zn^{2+} influx and autophagy in OGD/R HK-2 cells by accelerating Zn^{2+} influx and autophagy

We also analyzed the regulatory relationships among TRPC6 overexpression, Zn^{2+} addition, and autophagy activation through rescued experiment. As displayed in *Figure 7A*, either TPEN or 3-MA could signally prevent the acceleration of Zn^{2+} influx mediated by TRPC6 overexpression, and either Zn^{2+} or rapamycin could notably reduce the suppression of Zn^{2+} influx mediated by TRPC6 silencing in OGD/R HK-2 cells (*Figure 7A*). Subsequently, Western blot data indicated that upregulation of LC3II/I,

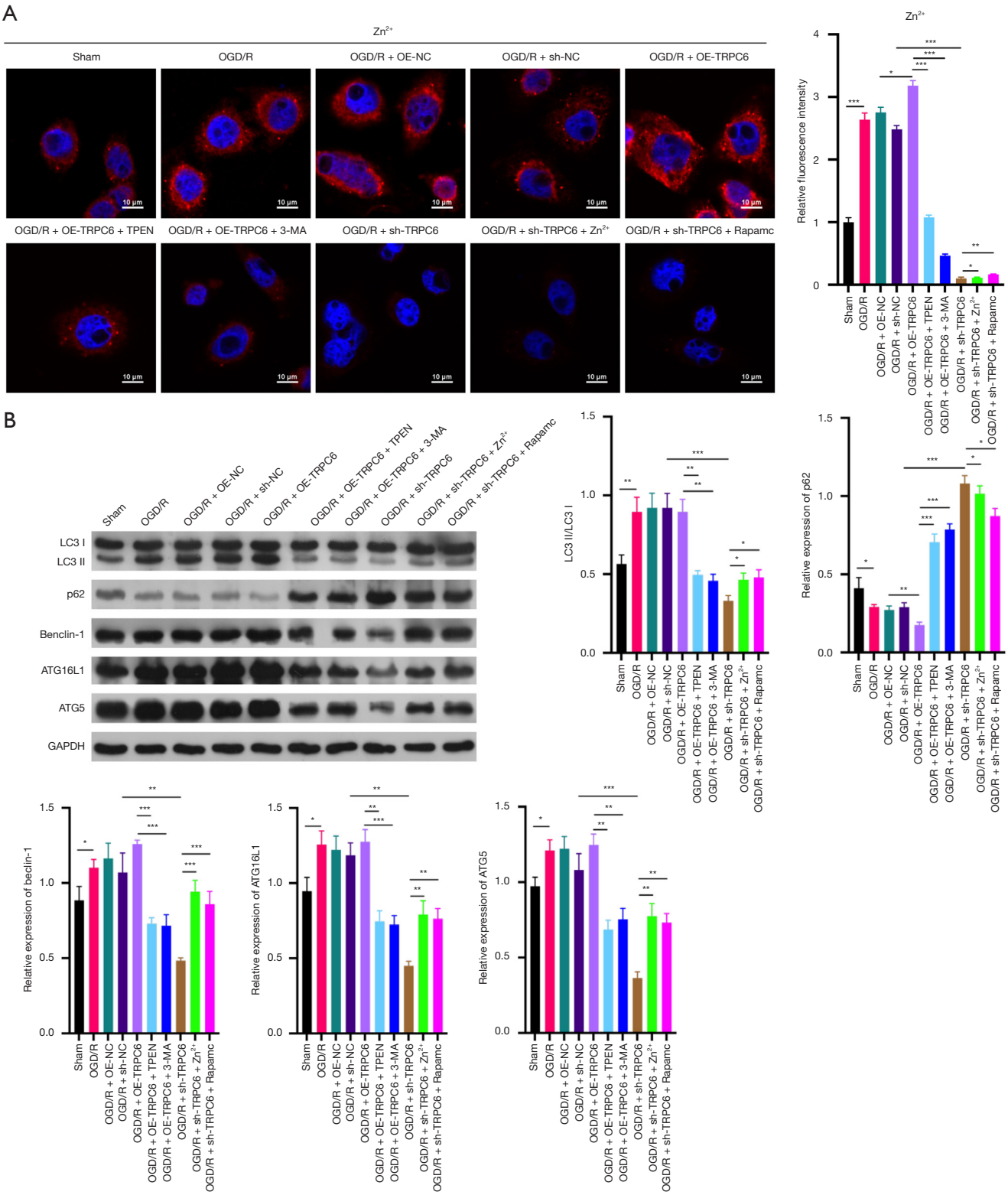

Figure 7 TRPC6 promoted Zn^{2+} influx and autophagy in OGD/R HK-2 cells by accelerating Zn^{2+} influx and autophagy. OGD/R HK-2 cells were co-processed as shown in *Figure 6*. (A) The change of free Zn^{2+} was confirmed with a Zn^{2+} fluorescent probe; (B) Western blotting analysis of autophagy-associated proteins. *, P<0.05; **, P<0.01; ***, P<0.001.

beclin-1, ATG16L1, and ATG5, and downregulation of p62 which were mediated by TRPC6 overexpression could be dramatically decreased by TPEN or 3-MA, and downregulation of LC3II/I, beclin-1, ATG16L1, ATG5, and upregulation of p62, which were mediated by TRPC6 silencing could be memorably reversed by Zn^{2+} or rapamycin in OGD/R HK-2 cells (*Figure 7B*). Consequently, the data disclosed that TRPC6 could also enhance Zn^{2+} influx and autophagy of OGD/R HK-2 cells by encouraging Zn^{2+} influx and autophagy.

TRPC6 ameliorated kidney injury in RIRI model rats by regulating Zn^{2+} influx and autophagy

We further studied the impacts of TRPC6, Zn^{2+}, and autophagy on kidney injury *in vivo* using an RIRI rat model (I/R group). After processing with the TRPC6 agonist (OGA), TRPC6 inhibitor (SKF96365), SKF96365, and $ZnSO_4$, SKF96365, and rapamycin, the data exhibited that the damage rating scale of rats was observably elevated in the I/R group versus that in the sham group; relative to the I/R group, the damage rating scale was markedly lowered in the I/R + OGA group and prominently increased in the I/R + SKF96365 group; and compared to the I/R + SKF96365 group, either ZnSO4 or rapamycin could signally reduce the damage rating scale in I/R rats after administration with SKF96365 (*Figure 8A*). H&E staining results also signified that in the sham group, kidney cells were arranged neatly and the structure was complete, while in the I/R group, vast inflammatory cells were infiltrated in the interstitium of the kidney, the lumen of the renal tubules was dilated, the renal cells were exfoliated or necrotic, and the nuclei were displaced in different degrees. Further, in the I/R + OGA group, the degree of renal injury was observably reduced in I/R rats, and in the I/R + SKF96365 group, the degree of renal injury was markedly increased in I/R rats. After treatment with $ZnSO_4$ or rapamycin, the degree of renal injury mediated by SKF96365 could also be memorably attenuated in I/R rats (*Figure 8B*). Thus, we concluded that TRPC6 could alleviate kidney injury of RIRI rats by inducing Zn^{2+} influx and autophagy.

TRPC6 weakened necrosis and enhanced autophagy in RIRI model rats by promoting Zn^{2+} influx and autophagy

More importantly, we further verified the influences of TRPC6, Zn^{2+}, and autophagy on necrosis and autophagy in the renal tissues of RIRI rats. As manifested in *Figure 8B-8E*, Sirtuin-2, RIP1, and PARP-1 expressions were memorably raised in I/R group relative to that in sham the group; compared with the I/R group, Sirtuin-2, RIP1, and PARP-1 expressions were also dramatically decreased in the I/R+OGA group and observably increased in the I/R + SKF96365 group; and compared to the I/R + SKF96365 group, either $ZnSO_4$ or rapamycin could also prominently downregulate Sirtuin-2, RIP1, and PARP-1 expressions in I/R rats after administration with SKF96365. The data also displayed that LC3II/I, beclin-1, ATG16L1, and ATG5 expressions were higher and p62 was lower in the I/R group than in the sham group; OGA treatment could increase LC3II/I, beclin-1, ATG16L1, and ATG5 expressions and decrease p62 expression in I/R rats; and SKF96365 had an opposite effect to OGA on these autophagy-related proteins. At the same time, SKF96365-medaited downregulation of LC3II/I, beclin-1, ATG16L1, and ATG5, and upregulation of p62 could be notably reversed by ZnSO4 or rapamycin treatment in I/R rats (*Figure 8F*). In summary, these results revealed that TRPC6 also could improve RIRI *in vivo* by accelerating Zn^{2+} influx and autophagy.

Discussion

RIRI is one of the frequent risk factors causing acute kidney injury (AKI) (24). Clinically, RIRI is often associated with procedures such as cardiopulmonary surgery, kidney transplantation, and artery bypass surgery (25). As RIRI has a serious impact on the prognosis of patients, and there is no effective therapy strategy (26), it is of great clinical value to actively explore its pathological mechanism and effective targeted therapy strategies. Studies show that RIRI can cause multiple pathophysiological changes including leukocyte activation and endothelial cell dysfunction, causing inflammation and tissue injury (25,27), and renal tubular epithelial cells (such as HK-2 cells) are the main sites of IRI due to their active metabolism (28). In this study, we adopted an internationally recognized RIRI *in vitro* model (OGD/R) and I/R *in vivo* model to further investigate the possible pathological processes in RIRI. The results signified that OGD/R could result in a prominent inhibition in viability, Zn^{2+} influx, and autophagy and a noteworthy promotion in necrosis of OGD/R HK-2 cells, indicating the successful build of OGD/R HK-2 cells. In addition, we also discovered that the degree of kidney injury was also aggravated in RIRI model rats, further suggesting a successful RIRI model in rats had been established.

TRPC is a non-selective cationic infiltration channel

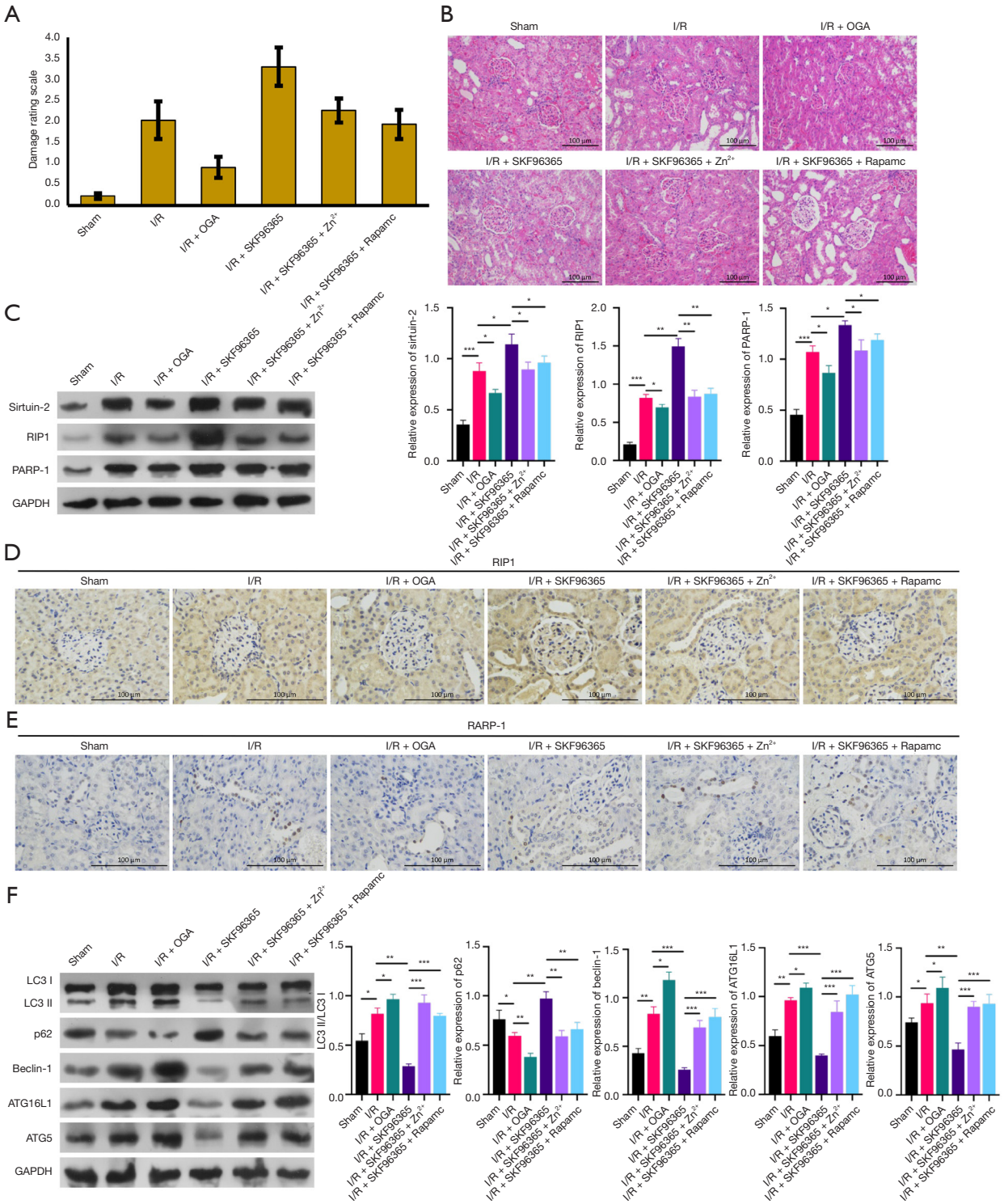

Figure 8 TRPC6 ameliorated kidney injury, weakened necrosis, and enhanced autophagy in RIRI model rats by regulating Zn^{2+} influx and autophagy. RIRI model rats were established and addressed with TRPC6 agonist (OGA), TRPC6 inhibitor (SKF96365), SKF96365 and 15 mg/kg $ZnSO_4$, and SKF96365 and rapamycin. (A) The damage rating scale of rats was assessed in each group; (B) kidney injury in rats was evaluated using H&E staining with magnification 200×; (C) the expression changes in Sirtuin-2, RIP1, and PARP-1 were tested with Western blot; (D,E) IHC staining was performed to monitor the expression changes of RIP1 and PARP-1, magnification 400×; (F) the levels of autophagy-associated proteins were identified via Western blot. *, P<0.05; **, P<0.01; ***, P<0.001.

widely expressed in the heart, kidney, brain, and other vital organs (29). In the kidney, TRPC6 is mainly expressed in mature podocytes, glomerular mesangial cells, and renal vascular smooth muscle cells, especially near SD (30). Studies have certified that overactivation of TRPC6 can increase the concentration of Ca^{2+} in podocytes, leading to their injury and dysfunction, and even glomerular disease (31,32). TRPC6 is also associated with glomerulosclerosis and diabetic nephropathy (33) and has been certified to be relevant to the podocyte response to ischemic injury (6). In the current study, we further confirmed that TRPC6 overexpression could be weakened and TRPC6 silencing could enhance the inhibition of viability, Zn^{2+} influx, and autophagy, and the induction of necrosis in OGD/R HK-2 cells or RIRI model rats. Thus, we proved that TRPC6 might serve an ameliorating action on RIRI.

A recent study also indicated that TRPC6 overexpression contributed to the accumulation of intracellular Zn^{2+} (34). Activation of TRPC6 could induce the mitochondria to release Zn^{2+} (35). Zinc element is one of the essential trace elements with abundant content, and it was reported that Zn^{2+} can participate in antioxidant reactions, nerve conduction, and regulation of the activities of various enzymes and growth factors (36). Studies have also demonstrated that Zn^{2+} is a key ion mediating cerebral ischemia injury (37). Reduction of zinc transporters could cause myocardial IRI (37); zinc preconditioning had a protective effect on RIRI in a preclinical sheep model (38); and Zn^{2+} could protect myocardial IRR by regulating endoplasmic reticulum stress (ERS) (39). In our study, we further testified that an increase of Zn^{2+} could enhance viability and autophagy and restrain necrosis in OGD/R HK-2 cells or RIRI model rats, which were mediated by TRPC6.

Autophagy is both a mechanism of cell death and a self-protection mechanism of cells (40), and has been proved to play a key role in tumor, aging, and neurodegeneration (41). In the process of autophagosome formation, multiple ATG proteins are needed, and studies have confirmed that up-regulation of Beclin1 expression can induce autophagy (42). Atg12 and LC3 also play an important role in the formation of autophagosomes, with the former activated by Atg7, initiating the binding process and allowing Atg12 to interact with other factors leading to the formation of the Atg12-Atg5-Atg16L1 complex. At the same time, ATG7 can also promote the modification of LC3 I to LC3 II (43). Studies have shown that the mechanism of necrosis is related to PAPR (44), and that activating PARP requires a large amount of ATP, and that the inhibition of autophagy to necrosis is mainly manifested in the maintenance of energy (45). Autophagy has also been reported to play a dual role in RIRI (46). While activation of autophagy during RIRI has been observed in many experiments *in vivo* and *in vitro* (47) it is still controversial whether up-regulated autophagy has a protective effect on the kidney or aggravates kidney injury. On the one hand, autophagy can degrade abnormal proteins and organelles in abnormal cells, preventing the accumulation of harmful substances. On the other hand, a high level of autophagy can damage organelles and cause them to transform into autophagy death (48). In our study, we also proved that cell autophagy was notably enhanced in both OGD/R HK-2 cells and RIRI model rats, and autophagy activation could induce autophagy and lower necrosis which were mediated by TRPC6 and Zn^{2+}. Therefore, we speculated that cell autophagy was activated in OGD/R HK-2 cells or RIRI model rats to exert a role in self-protection, and overexpression of TRPC6 could further enhance the protective effect of autophagy on cells.

Conclusions

Our findings suggest TRPC6 can promote RIRI progression by activating Zn^{2+} influx and autophagy, which might provide new ideas and therapeutic targets for RIRI therapy.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-5837/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-5837/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Animal Care and Use Committee of the Army Medical University [with two institutional licenses SCXK(YU)20170002 and SYXK(YU)20170002], in compliance with the guidelines of the Army Medical University for the care and use of animals.

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