Autophagy activation attenuates renal ischemia-reperfusion injury in rats

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Abstract

Ischemia-reperfusion (I/R) injury is a leading cause of acute kidney injury (AKI), which is a common clinical complication but lacks effective therapies. This study investigated the role of autophagy in renal I/R injury and explored potential mechanisms in an established rat renal I/R injury model. Forty male Wistar rats were randomly divided into four groups: Sham, I/R, I/R pretreated with 3-methyladenine (3-MA, autophagy inhibitor), or I/R pretreated with rapamycin (autophagy activator). All rats were subjected to clamping of the left renal pedicle for 45 min after right nephrectomy, followed by 24 h of reperfusion. The Sham group underwent the surgical procedure without ischemia. 3-MA and rapamycin were injected 15 min before ischemia. Renal function was indicated by blood urea nitrogen and serum creatinine. Tissue samples from the kidneys were scored histopathologically. Autophagy was indicated by light chain 3 (LC3), Beclin-1, and p62 levels and the number of autophagic vacuoles. Apoptosis was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method and expression of caspase-3. Autophagy was activated after renal I/R injury. Inhibition of autophagy by 3-MA before I/R aggravated renal injury, with worsened renal function, higher renal tissue injury scores, and more tubular apoptosis. In contrast, rapamycin pretreatment ameliorated renal injury, with improved renal function, lower renal tissue injury scores, and inhibited apoptosis based on fewer TUNEL-positive cells and lower caspase-3 expression. Our results demonstrate that autophagy could be activated during I/R injury and play a protective role in renal I/R injury. The mechanisms were involved in the regulation of several autophagy and apoptosis-related genes. Furthermore, autophagy activator may be a promising therapy for I/R injury and AKI in the future.

Keywords: Autophagy, ischemia reperfusion, apoptosis, acute kidney injury

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Introduction

Renal ischemia-reperfusion (I/R) injury usually occurs during urological surgery and renal transplant, which require revascularization of the renal artery. I/R injury has been characterized as a common pathological change leading to acute kidney injury (AKI), which remains a potentially life-threatening clinical condition with high morbidity, mortality, and prolonged hospitalization of patients.^{1,2} The mechanisms of I/R injury are multifactorial and may include oxidative stress, mitochondrial Ca^{2+} overload, inflammation, cell apoptosis, necrosis, autophagy, loss of cell polarity, dedifferentiation and proliferation of viable cells, and disruption of the generation of free radicals.^{3–5}

Autophagy was originally defined as “self-eating” based on cell starvation, but it is a highly conserved, physiological, catabolic process involving the bulk lysosomal degradation of cytosolic components, including macromolecules and cytosolic organelles.⁶ Microtubule-associated protein light chain 3 (LC3) and Beclin-1 are two pacemakers in the

autophagic cascade that could lead to autophagy. The ratio of LC3I to LC3II correlates with the extent of autophagosome formation, and LC3II could be a marker of autophagic activity.^{7,8} In addition, the autophagic protein p62 is efficiently degraded by autophagy, and p62 levels could be inversely related to autophagic activity.⁹ Renal tubular cell apoptosis and caspase-3 have been demonstrated to be essential during I/R-induced renal damage.^{10,11} Apoptosis is enhanced with the inactivation of autophagy, indicating that increased autophagy can inhibit apoptosis.¹² Autophagy contributes to the pathogenesis of a number of human diseases, such as cancer, certain neurodegenerative disorders, myopathies, and infectious diseases.^{6,13} The small molecule autophagy enhancers have been reported to be useful for neurodegenerative disorders or other diseases in which autophagy acts as a protective pathway.¹⁴ However, until recently, little was known about the connection between autophagy and kidney diseases and whether the autophagy inducer has a renoprotective effect in I/R injury.

In this experimental study, we investigated the effect of activated autophagy on renal I/R injury in a rat model. Autophagy inhibitor 3-methyladenine (3-MA) could aggravate kidney damage and increase apoptosis, but rapamycin exerted a renoprotective role by increasing autophagy and reducing apoptosis. The mechanisms were associated with the regulation of LC3, Beclin-1, p62, and caspase-3.

Materials and methods

Experimental animal preparation and renal I/R model

Adult male Wistar rats (250–300 g) were originally purchased from the Laboratory Animal Center of Peking University Health Science Center and maintained in the animal facility of the Peking University Health Science Center. The rats were kept in a temperature-controlled environment (20–22°C) under a 12-h light/dark cycle with rodent chow and tap water *ad libitum*. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Forty male Wistar rats were randomly divided into four groups: Sham ($n=10$), rats subjected to identical surgical procedures without renal arterial clamp; I/R ($n=10$), rats underwent renal ischemia for 45 min followed by 24 h reperfusion and received the same volume of isotonic saline 15 min before the renal artery clamp was applied; I/R+3-MA ($n=10$), rats received an intravenous (intravenous) injection of 3-MA (Sigma Life Science, USA) (30 mg/kg) 15 min before the renal artery clamp was applied; I/R + Rap ($n=10$), rats received an intravenous injection of rapamycin (LC Laboratories, USA) (1 mg/kg) 15 min before the renal artery clamp was applied.

Rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (50 mg/kg). The two dorsal regions were shaved and cleaned with povidone-iodine. We performed dorsal incisions to expose both the right and left kidneys; the right kidney was removed. The left renal artery was separated and occluded with a non-traumatic microvascular clamp for 45 min. The clamp was then removed and the kidney observed for 4–5 min to ensure that reperfusion was established successfully. The wounds were sutured and reperfusion continued for 24 h.

Organ and tissue sample collection

Blood samples were drawn from the orbital sinus under light anesthesia at the end of the renal reperfusion (24 h). The remaining left kidneys were removed and sliced in half using a coronal cut and the rats decapitated. Blood samples were centrifuged at 3000g for 15 min and the serum samples stored at -80°C for further analysis of blood urea nitrogen (BUN) and serum creatinine (SCr). Half of each kidney sample was fixed in formalin solution (10%) for hematoxylin-eosin (H-E) staining, transmission electron microscopy and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, and immunohistochemical analysis. The other half of each sample was frozen in liquid nitrogen and stored at -80°C for further studies.

Measurement of renal function

Renal function was assessed by BUN and SCr levels using commercial kits from an Olympus AU2700 Analyzer (Olympus, Japan).

Histological examination

The renal tissue specimens were fixed by immersion in formalin solution (10%), dehydrated in alcohol, paraffin embedded, cut in fine (4 mm) sections using a microtome, stained with H-E according to standard procedures, and mounted on a glass slide. Two sections per kidney were evaluated under a standard light microscope. All samples were coded, and an experienced renal pathologist examined the specimens in a blinded fashion. Pathological scoring (0–5) was used to assess the degree of I/R-induced tubulointerstitial injury, which was defined as tubular epithelial cell swelling, tubular atrophy, tubular dilatation, vacuolization, loss of brush border, cellular infiltration, hyperemia in intertubular and glomerular blood vessels, cast formation, and desquamation. Ten areas in randomly selected renal tubules from the outer medulla of the kidney were examined at $\times 200$ magnification. Pathological scoring ranged from 0 to 5 points based on the percentage of injury area as follows: 0, normal; 1, injury area $<10\%$; 2, injury area $>10\%$ but $<25\%$; 3, injury area $>25\%$ but $<50\%$; 4, injury area $>50\%$ but $<75\%$; 5, injury area $>75\%$.^{15,16}

TUNEL staining

Apoptosis was assessed by the TUNEL assay using the In Situ Cell Death Detection Fluorescein Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Paraffin sections (4 mm) were deparaffinized, permeabilized with 20 mg/mL proteinase K at room temperature for 15 min and 0.2% triton X-100/PBS for 15 min at 4°C , and then incubated with 3% hydrogen peroxide. Next, the sections were incubated with a mixture of nucleotides and TdT enzyme for 60 min at 37°C . We pre-treated positive controls displaying the characteristic morphology of apoptosis with 1 U/mL deoxyribonuclease and counted as apoptotic. Negative controls were incubated without terminal transferase. The final count was expressed as the percentage of total cells detected by visualization under a fluorescence microscope. Finally, apoptotic cells were quantified as the average number of epithelial cells with bright green signals counted in 10 consecutive fields under $\times 200$ magnification.

Immunohistochemistry

Immunohistochemical staining for caspase-3 and Beclin-1 in the renal cortex was detected using commercial assay kits. We deparaffinized 4-mm-thick paraffin sections heated in citrate buffer, and then incubated them with primary antibodies against caspase-3 (rabbit anti-rat, 1:800; Cell Signaling Technology, USA) and Beclin-1 (rabbit anti-rat, 1:800; Thermo Fisher Scientific, USA). Subsequently, the sections were incubated with secondary antibodies for 30 min at 37°C . Color was developed using 3,3'-diaminobenzidine tetrahydrochloride, followed by nuclear

counterstaining with hematoxylin. Positive and negative controls were included in each step. Expression of the caspase-3 and Beclin-1 proteins was evaluated by calculating the immunostaining score for both the intensity (0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining) and proportion (0, none; 1, <10%; 2, 10–50%; 3, 51–80%; 4, >80%).¹⁷ Results were evaluated by two independent investigators in a blinded fashion. Five visible areas from each sample were scored randomly in each section.

Western blot analysis

Western blotting was used to quantify the levels of LC3, Beclin-1, and p62 proteins in the kidney tissue. The samples were weighed and protein extracted as follows. Profound hypothermia-preserved (−80°C) kidney tissues were homogenized in radio immunoprecipitation assay (RIPA) cleavage solution. The homogenates were centrifuged at 12,000g for 10 min at 4°C. Supernatants were collected and protein concentrations measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, USA). The protein samples were resuspended in 5×loading buffer, denatured for 5 min at 100°C, separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes by electroelution (100 mA, 90 min, moist electrotransfer). The blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 37°C for 2 h, and then incubated with primary antibodies against LC3 (1:1000; Sigma Life Science, USA), Beclin-1 (1:1000; Cell Signaling Technology, USA), and β-actin (1:1000; ABGENT, USA) at 4°C overnight. After incubation with the primary antibody, the membranes were washed with TBST and exposed to horseradish peroxidase-conjugated secondary antibody (1:10000; LI-COR, USA) for 1 h at room temperature. After several washes with TBST, immunoreactive proteins were visualized using the Odyssey Imaging System (LI-COR, USA). All experiments were performed in triplicate. To facilitate comparisons, the densitometry values were expressed as the target protein/β-actin ratio.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the renal tissue using TRIzol reagent (Invitrogen Life Technologies, 15596-026) according to the manufacturer's instructions, and 2 μg was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Life Technologies, USA). The thermal profile for SYBR Green polymerase chain reaction (PCR) was as follows: denaturation for 10 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 58°C, and 40 s at 72°C. The levels of LC3, Beclin-1, p62, and β-actin mRNA were detected using ABI 7500 (Applied Biosystems, USA) and SYBR Green chemistry using the following sequences: LC3 forward primer, 5'-CATGCCGTCCGAGAAGACCT-3'; LC3 reverse primer, 5'-GATGAGCCGGACATCTTCCACT-3' (GenBankTM accession number, NM022867.2); Beclin-1 forward primer, 5'-TTGGCCAATAAGATGGGTCTGAA-3'; Beclin-1 reverse primer, 5'-TGTCAGGGACTCCAGATACGAGTG-3' (GenBankTM accession number, NM001034117.1); p62

forward primer, 5'-GCCCTGTACCCACATCTCC-3'; p62 reverse primer, 5'-CCATGGACAGCATCTGAGAG-3'; β-actin forward primer, 5'-GGAGATTACTGCCCTGGC TCCTA-3'; β-actin reverse primer, 5'-GACTCATCGTACT CCTGCTTGCTG-3'. The PCR products were normalized to β-actin levels for each sample in duplicate wells. Amplification products were monitored by its dissociation curve. The mean cycle threshold value (Ct) was used to analyze gene expression. The relative quantitation of target genes relative to the number of β-actin gene copies was calculated using $2^{-\Delta\Delta C_t}$. The quantitative real-time PCR (qRT-PCR) assays were performed in triplicate.

Transmission electron microscopy

Kidney samples of approximately 1 mm³ were clipped by ophthalmic scissors and immersed in 2.5% glutaraldehyde. The tissues were rinsed, fixed, dehydrated, soaked, embedded, and sliced. The autophagosome ultrastructure was observed under a transmission electron microscope.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Between-group comparisons were performed using one-way analysis of variance (ANOVA). All analyses were performed using GraphPad Prism Version 5.0 (San Diego, USA); $P < 0.05$ was considered significant.

Results

Increases in autophagosomes after renal I/R injury

The BUN and SCr levels were higher (Figure 1), and the histological score significantly increased after I/R injury (Figure 2(B)). To evaluate the level of autophagy activation, we examined autophagy-related proteins LC3, Beclin-1, and p62 using qRT-PCR and Western blot. Beclin-1-positive cells were increased after I/R injury (Figure 3). I/R injury also led to a significant increase in LC3II and Beclin-1, and the expression of p62 was decreased, but not significantly (Figures 4 and 5). The structures of autophagosomal vacuoles were revealed by high magnification (×10,000) electron microscopy, which is the gold standard for detecting autophagy. Autophagic vacuoles were observed in the I/R group (Figure 6(A) and (B), white chevrons). Taken together, these results confirmed the occurrence of autophagy in this renal I/R model.

During I/R injury, apoptosis was activated in the renal tissues. TUNEL-positive cells were rarely observed in kidneys from the Sham group (Figure 2(A–e)). An increased number of TUNEL-positive cells were observed in renal sections from the I/R group compared to the Sham group (Figure 2(B)). Similarly, the expression of caspase-3 was increased in the I/R group compared to the Sham group (Figure 3).

Effect of 3-MA on renal I/R injury

qRT-PCR and Western blot analyses showed that, compared to the I/R group, LC3 and Beclin-1 were down-regulated with 3-MA pretreatment, but p62 was up-regulated

Figure 1 Rat renal function after I/R injury. BUN levels (A) and SCr levels (B). Data were shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Sham group; ### $P < 0.001$ versus I/R group.

3-MA: 3-methyladenine; I/R: ischemia reperfusion; BUN: blood urea nitrogen; SCr: serum creatinine

Figure 2 Rat kidney H-E and TUNEL staining. Representative images of renal histology with H-E staining (original magnification $\times 200$) (A(a) to (d)). The Sham group showed normal renal cortical tissue structure (A(a)), marked changes of early-stage acute tubular necrosis, including loss of brush border cells, nuclear pyknosis and flattening of the tubular epithelium in the I/R group and I/R + 3-MA group (A(b) and (c)). Representative immunofluorescence pictures of TUNEL staining for apoptotic cells of kidney samples, nuclei of TUNEL-positive cells are stained green (original magnification $\times 200$) (A(e) to (h)). Mean number of TUNEL-positive cells per high-power field (HPF $\times 200$) (B). Data were shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Sham group; # $P < 0.05$, ## $P < 0.01$ versus I/R group. H-E: hematoxylin-eosin; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; I/R: ischemia reperfusion; 3-MA: 3-methyladenine

(Figures 4 and 5). Immunohistochemical analysis also revealed a higher Beclin-1 score (Figure 3), and high magnification ($\times 10,000$) electron microscopy showed fewer autophagic vacuoles but more apoptotic features with 3-MA pretreatment (Figure 6(A-c), arrows). The number of TUNEL-positive cells was significantly

increased, and the caspase-3 score was significantly higher in the I/R + 3-MA group compared to the I/R group (Figures 2 and 3).

Consistent with the functional data, I/R-induced renal damage was aggravated by 3-MA with high BUN and SCr levels (Figure 1). The histopathological features worsened

Figure 3 Representative of immunoblotting images of caspase-3 (A(a) to (d)) and Beclin-1 (A(e) to (h)) (original magnification $\times 200$). Quantification of the percentage of caspase-3 expression and Beclin-1 expression of all groups (B). Compared with the I/R group, the caspase-3 score was higher, but the Beclin-1 score was lower when pretreated with 3-MA. The caspase-3 score was lower, but Beclin-1 score was higher when pretreated with rapamycin. Data were shown as the mean $\pm$ SD. * $P < 0.05$, *** $P < 0.001$ versus Sham group; # $P < 0.05$, ## $P < 0.01$ versus I/R group. I/R: ischemia reperfusion; 3-MA: 3-methyladenine

Figure 4 Expression of mRNA levels of LC3, Beclin-1, and p62 detected by qRT-PCR. The mRNA expressions of LC3 and Beclin-1 were increased but p62 was decreased after I/R injury. Compared with the I/R group, 3-MA pretreatment with higher level of p62 but lower levels of LC3 and Beclin-1, rapamycin pretreatment with higher levels of LC3 and Beclin-1 and lower level of p62. Data were shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ versus Sham group; # $P < 0.05$, ## $P < 0.01$ versus I/R group. I/R: ischemia reperfusion; 3-MA: 3-methyladenine

with karyorrhexis, karyolysis, severe vacuolar degeneration, atrophy, tubular structure degeneration, and acute tubular necrosis. The histomorphological score was higher in the I/R + 3-MA group than in the I/R group (Figure 2).

Effect of rapamycin on renal I/R injury

We also tested the effects of autophagy activator rapamycin, a pharmacological inhibitor of mammalian target of rapamycin (mTOR), in the kidneys after I/R injury. As shown in Figures 3 to 5, rapamycin significantly up-regulated LC3 and Beclin-1 but down-regulated p62. The number of autophagic vacuoles was higher in the I/R + Rap group compared to the I/R group (Figure 6). Figures 2 and 3 show that rapamycin pretreatment markedly reduced both the level of caspase-3 and the number of TUNEL-positive cells, which suggests that rapamycin could attenuate I/R-induced apoptotic cell death in the kidneys.

As shown in Figure 1, pretreatment with rapamycin significantly reduced the BUN and SCr levels compared to the I/R group. Rapamycin also attenuated tubular damage as indicated by histological examination with a lower

Figure 5 Expression of protein levels of LC3, Beclin-1, and p62 detected by Western blot. Representative blots of LC3 I/II, Beclin-1, and p62, β -actin was used as a loading control (A). The relative densities of the bands in each lane were analyzed and normalized to β -actin (B to D). The data were expressed as percentage of the Sham group, higher protein levels of p62 but lower of LC3 and Beclin-1 in the I/R + 3-MA group, higher protein levels of LC3 and Beclin-1 and lower of p62 in the I/R + Rap group. Data were shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Sham group, ** $P < 0.01$, *** $P < 0.001$ versus I/R group. I/R: ischemia reperfusion; 3-MA: 3-methyladenine

Figure 6 Ultrastructural changes in renal tubular cells after I/R injury. Representation of high magnification of electron micrographs showing ultrastructural changes structures (A). The Sham group showing healthy nuclei, endoplasmic reticulum, mitochondria, lysosomes, and cytoplasmic Golgi complexes (A(a)). Autophagic vacuoles containing whorls of membranous material and some cytoplasm (white chevrons) were found in the I/R group (A(b)). Apoptosis with disrupted cell membranes displaying cell shrinkage, nuclear and chromatin condensation or aggregation at the edge of the nucleus in the I/R + 3-MA group (white arrows) (A(c)). More multiple double- or multiple-membrane autophagic vacuoles containing cytoplasm or undigested organelles were identified in the I/R + Rap group (white arrows) (A(d)). Quantification of the number of autophagic vacuoles per 100 μm^2 cytoplasm (B). Data were shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Sham group; # $P < 0.05$, ## $P < 0.01$ versus I/R group. I/R: ischemia reperfusion; 3-MA: 3-methyladenine

histomorphological score for renal injury (Figure 2), suggesting a renoprotective role of autophagy in this I/R model.

Discussion

In this study, we established a model of rat renal I/R injury and found that autophagy is activated in the renal tubule after I/R injury. Twenty-four hours after I/R injury, moderate renal failure was induced with increased BUN and SCr. The inhibition of autophagy by 3-MA induced a more severe loss of renal function, whereas activation of autophagy by rapamycin protected against the injury. The role of autophagy in AKI remains to be determined.¹⁸ Several reports support autophagy ameliorating AKI caused by renal insults such as I/R injury.^{19–21} These results suggest that autophagy is a protective mechanism in renal I/R models and that the activation of autophagy could be effective in I/R injury, even AKI.

AKI is a complex disorder comprising multiple causative factors that occurs in a variety of settings with varied clinical manifestations ranging from a minimal elevation in SCr to anuric renal failure.²² Studies have shown that patients with AKI have a high fatality rate of approximately 40–80% in the intensive care setting.^{23,24} Approximately 13% of AKI patients develop chronic kidney disease (CKD) over a three-year period.²² Renal I/R injury is a major cause of AKI in the clinical setting, which contributes to the high morbidity of patients, especially those subjected to partial nephrectomy, major vascular surgical interventions, or renal transplantation; the extent of I/R injury significantly affects the function of grafts. The tubular damage is associated with the severity of histopathological changes and increased serum BUN and SCr levels.²⁵ In our study, higher BUN, SCr, and histopathological scores were detected in the kidney after I/R injury and could be used to evaluate renal function and the level of injury. In addition, autophagy and apoptosis are concomitantly expressed in the rat kidney after I/R injury.

Autophagy is an evolutionarily conserved catabolic process characterized by the degradation of long-lived proteins, misfolded or aggregated proteins, and intracellular organelles. In this process, the formation of a double-membrane structure (autophagosomes) engulfs and delivers cellular contents to the lysosome for degradation. The expression levels of autophagosome-labeled LC3 and Beclin-1 proteins were thought to be markers of autophagic flux. LC3 is the most important and reliable marker of autophagy,²⁶ as the conversion of LC3I to LC3II is considered to be closely correlated with the extent of autophagosome formation.⁷ Beclin-1 is a unique autophagy-related protein thought to be important in the recruitment of other autophagic proteins during expansion of the pre-autophagosomal membrane and structure.^{13,27,28} Similarly, as one of the key autophagy substrates, p62 (SQSTM1 or A170) could be used to assess autophagic flux in some contexts. This protein is selectively incorporated into autophagosomes through LC3 binding via its LC3-interacting domain and efficiently degraded by autophagy. In both cell cultures and animal models of I/R injury, the activation

of autophagy in tubular cells has been demonstrated,^{19,20,29–33} indicating that I/R could increase the autophagic flux, and we thought that autophagy may play a key role in renal I/R injury. Recently, more attention has focused on the role of autophagy in I/R injury, but whether the induction of autophagy provides protection or aggravates tubular damage is also debatable.

In this study, we used 3-MA and rapamycin to explore the role of autophagy. 3-MA is a class III phosphatidylinositol 3-kinase (PI3K) inhibitor that can block initial autophagic sequestration and autophagosome formation.^{34,35} Thus, 3-MA potentially blocks the initial autophagic sequestration and down-regulates autophagic activity at the early stage; it has been used to understand the role of autophagy.^{20,30,36} Pretreatment with 3-MA resulted in the lowest expression of LC3 and Beclin-1, highest levels of p62, and a reduced number of autophagic vacuoles, indicating that autophagy was inhibited by 3-MA. 3-MA also exacerbated kidney injury with the highest BUN and SCr levels and histological scores. The suppression of autophagy sensitizes tubular cells to apoptosis induced by I/R injury, resulting in the highest expression of caspase-3 and more TUNEL-positive cells. Therefore, inhibition of autophagy by 3-MA worsened I/R injury. In contrast, the data suggest that rapamycin-induced autophagy after I/R injury exerts a strong protective effect on the rat kidney. Rapamycin is a macrolide antibiotic originally used as an antifungal agent, but later as an inducer of autophagy by inhibition of mTOR.^{37,38} The highest expression of LC3 and Beclin-1, lowest p62 expression, and increased number of double- or multiple-membrane autophagic vacuoles indicated that autophagy was activated by rapamycin. In kidney tissues, this induction of autophagy protects against tubular I/R injury,³⁰ regulates podocyte homeostasis,³⁹ and mediates caloric restriction-induced protection of renal aging.⁴⁰ Studies have shown that renal tubular damage and cell apoptosis often develop together with renal I/R injury,⁴¹ and apoptosis has become an important factor for cell death. Apoptosis is often described as programmed cell death, which can be evaluated by caspase-3 activity and the number of TUNEL-positive cells. Caspase-3 is an important effector caspase and plays an important role during apoptosis in renal I/R injury.⁴² Researchers have shown that caspase-3 positively correlates with renal structure and function.⁴³ In the I/R + Rap group, the BUN and SCr levels were reduced, the histopathological scores decreased, and TUNEL staining and caspase-3 expression decreased. The induction of autophagy by rapamycin could effectively suppress apoptosis. Thus, autophagy activation could ameliorate renal I/R injury. Taken together, our results provide evidence suggesting that autophagy is a renoprotective mechanism for cell survival in I/R injury.

Among mTOR inhibitors, rapamycin ensures cell growth inhibition and cell death induction in lymphoma cell lines and malignant glioma, breast cancer, renal cell carcinoma, and mesothelioma.⁴⁴ Novel pathways and L-type Ca²⁺ channel antagonists could induce autophagy via a cyclical mTOR-independent pathway. This pathway has many potential points where autophagy can be induced and is useful for therapeutic correlations with Huntington's

disease using cell, fly, and zebrafish models. Because of the involvement of autophagy in many different disease conditions, drugs that could be used to manipulate autophagy for therapeutic purposes are necessary.

In conclusion, our data showed that autophagy was moderately induced by I/R injury. Further, enhanced autophagy by rapamycin exerts a renoprotective role via inhibiting the tubular cell apoptosis during the renal I/R injury. Although the precise protective mechanisms of activated autophagy need further study, the inducer of autophagy might serve as a promising therapeutic agent in renal I/R injury.

Author contributions: Y-LZ conducted and designed the experiments, performed research, analysis, and interpretation of the results, drafted the manuscript, and provided final approval of the version to be published. JZ, L-YC, and SY participated in the design of the study and provided general support.

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