Ischemic Preconditioning Alleviates Mouse Renal Ischemia/Reperfusion Injury by Enhancing Autophagy Activity of Proximal Tubular Cells

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**Keywords**
Autophagy · Ischemic preconditioning · Renal ischemia/reperfusion injury · Macrophage · Monocyte chemoattractant protein-1

**Abstract**

**Objectives:** Ischemia/reperfusion injury (IRI) is one of the most vital pathogenesis leading to kidney injury but lacks effective prevention and treatment strategies. This study was conducted to investigate the influences of ischemic preconditioning (IPC) on the pathological process of mouse renal IRI (RIRI) and to figure out the role of autophagy of proximal tubular cells (PTCs) in this process. **Methods:** C57BL/6J mice were randomized to three groups, i.e., sham-operated group, ischemia/reperfusion (I/R) group, and IPC + I/R group. Meanwhile, 3-methyladenine, an autophagy inhibitor, was administered when further verification was needed. Histological and functional severity of kidney injury, the autophagy and apoptosis activity of PTCs, as well as the characterization of the immune cell infiltration landscape in kidney tissues were investigated. Furthermore, HK-2 cells and primary cultured PTC were cultured to set up the hypoxic preconditioning and hypoxia/reoxygenation model for in vitro simulation and verification, and a microarray dataset derived from the Gene Expression Omnibus database was analyzed to explore the transcriptome profiles after IPC. **Results:** IPC could significantly attenuate I/R-induced kidney injury functionally and histologically both in the acute and recovery phase of RIRI by enhancing the autophagy activity of PTCs. Cell autophagy could regulate the release of monocyte chemoattractant protein-1, and sequentially decrease macrophages infiltration in kidney tissues in the acute phase of RIRI, thus mediating the reno-protective effect. **Conclusions:** IPC could attenuate mouse RIRI-induced kidney injury. IPC-mediated activation of autophagy of PTCs plays a vital role in affording protection in RIRI-induced kidney injury.

**Introduction**

Ischemia/reperfusion injury (IRI) is one of the most vital pathogenesis leading to kidney injury, especially during the perioperative periods of partial nephrectomy and renal transplantation [1–3]. Multiple strategies, including reno-protective drugs (diuretics, sodium bicarbonate, etc.), renal replacement therapy, and surgical approaches innovation such as hypothermia, limited warm Shun Zhang and Weimin Xia are contributed equally.
ischemia, and zero or segmental ischemia have been brought up to prevent or treat renal IRI (RIRI). However, most of these efforts have yielded limited success [4].

Ischemic preconditioning (IPC) is a kind of endogenous phenomenon that promotes tissue tolerance to IRI by a brief episode of ischemia and subsequent reperfusion before the index ischemic event and may be a highly appealing, nonpharmacological, and practical approach to attenuate RIRI [5]. Since the concept of IPC was first proposed by Murry et al. [6] in 1986, several studies have assessed the organ protective effect of IPC in various tissues and organs as well as across several species [7–11]. Over the past three decades, both experimental and clinical researches have demonstrated that IPC could enhance kidney tolerance to ischemic injury and has therapeutic potential for the prevention and/or reversal of the pathological sequelae associated with acute kidney injury (AKI) [11–13]. Nitric oxide [14], protein kinase C [15], MAP kinase and MAPKAP kinase 2 [16], NF-kB [17], mitochondria [18], microRNA [19], and decreased capacity of immune cells [20], etc., have all been reported implicated in mediating the protective effects of IPC.

The tubular epithelial cells, especially the proximal tubular cells (PTCs) with high metabolic activity, are susceptibility damaged by ischemia injury and play crucial roles in the pathophysiological process of RIRI [21]. Studies have demonstrated that the disarrangement of the balance between cell repair and cell death decides the fate of PTCs and the severity of kidney injury during RIRI. In recent years, autophagy has become a new research hotspot in the field of RIRI because of its distinct regulatory function on cell survival and death [22, 23]. Autophagy is detected to be activated during the earlier stage of RIRI and contributes to a protection role. But excessive autophagy promotes cell apoptosis and cell death [3, 24]. However, whether the autophagy activity of PTCs participates in IPC-induced renoprotection is still ambiguous. Thus, this study was conducted to investigate the influences of IPC on the pathological process of mouse RIRI, as well as to figure out the regulatory role of cell autophagy in this process.

Materials and Methods

Mice and Animal Models

Eight- to 12-week-old male C57BL/6J mice weighing 20–25 g were purchased from the animal experiment center (Super-B&K Laboratory Animal Corp. Ltd.). Mice were randomized into three groups, i.e., sham-operated group (hereinafter referred to as sham group for short), ischemia/reperfusion (I/R) group, and IPC + I/R group (IPC group for short). The ideograph of IPC and RIRI strategy was shown in Figure 1a. I/R procedure was reported before [25](detailed in online supplementary methods (for all online supplementary material, see www.karger.com/doi/10.1159/000521850)).

In addition, the group of 3-methyladenine (3-MA, an autophagy inhibitor) + IPC + I/R (3-MA group for short) was complemented to verify the effect of autophagy in the pathological process of RIRI and IPC when needed. The ideograph of 3-MA management was shown in Figure 1a. Briefly, 3-MA (30 mg/kg, Sigma, M9281) was given intraperitoneally (i.p.) 12 h and 1 h before IPC and continued for 3 days from day 1 to day 3 after IPC.

Histology and Kidney Injury Scoring

Histologic damage in the cortex and H&E-stained kidney sections was scored by counting the percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation as follows: 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–50%; 4 = 51–75%; 5 ≥ 75% [26]. Ten randomly selected fields (original magnification, ×200) per kidney were used for counting.

Transcriptome Analysis

The detailed analysis methods can be found in online supplementary methods.

Cell Culture and in vitro Model

HK-2 human renal cortical PTCs were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China) and were cultured in DMEM/F-12 (ThermoFisher, 12400024). Four subgroups of in vitro study were established, i.e., control group, hypoxia/reoxygenation (H/R) group, hypoxic preconditioning (HPC) + H/R group (HPC group for short), and 3-MA + HPC + H/R group (3-MA group for short). The establishment of in vitro HPC and H/R model was as reported by Xie et al. [27]. In brief, oxygen and glucose deprivation (OGD) of HK-2 cells was induced by changing the medium to serum/glucose-free DMEM/F-12 medium and then incubated in a chamber flushed with 94% N2 and 5% CO2. After 15 h of OGD, the medium was replaced with fresh oxygenated medium containing serum and glucose, and the cells were returned to normoxic conditions for 2 h. For the delayed HPC group, cells were subjected to OGD for 6 h, followed by 24 h of reoxygenation before prolonged H/R injury. For the 3-MA subgroup, 3-MA (10 μM) was added to the medium 1 h before experiment and was continuously used throughout the HPC treatment to suppress cell autophagy.

Statistical Analysis

Statistical comparisons were performed using the statistical software GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The intergroup differences were tested using one-way or two-way analysis of variance. Statistical significance was defined as a *p* < 0.05. Other methods can be found in online supplementary materials.

Results

IPC Attenuated I/R-Induced AKI

The delayed IPC strategy was performed as shown in Figure 1a. A brief episode of 15 min of ischemia was formulated to establish the delayed IPC model and that bi-
Fig. 1. Functional and histological change of kidney in the acute phase of RIRI. 

**a** Ideograph of DIPC and RIRI strategy. 

**b, c** Dynamic change patterns of BUN and sCr after RIRI (n = 6/group). 

**d** H&E-stained histological images of kidneys and quantification of tubular damage by counting the percentage of tubular that displayed tubular necrosis, cast formation, and tubular dilation, etc., as follows: 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–50%; 4 = 51–75%; 5 ≥ 75%; ten randomly selected fields (original magnification, ×200) per kidney were used for counting (n = 6/group). 

**e, f** KIM-1 expressions in the renal cortex (2 days after the index ischemia), detecting by immunofluorescence staining and Western blot (n = 6/group). The grouping of blots cropped from different parts of the same gel. Representative data are presented. DIPC, delayed ischemic preconditioning; SAC, sacrifice. * meant p < 0.05, ** meant p < 0.01, *** meant p < 0.001, **** meant p < 0.0001. Scale bar: 50 μm (**d), 20 μm (**e).
lateral 30 min of ischemia was chosen to construct the severe RIRI model. Following the reperfusion phase, serum creatinine (sCr) and blood urea nitrogen (BUN) of both IPC group and I/R group mice presented an obvious increase, with the peak concentrations appearing at 48 h post the kidney ischemia, and then decreased gradually. But the mice in IPC group showed dramatically lower values of both sCr (0.89 ± 0.05 mg/dL vs. 1.08 ± 0.04 mg/dL at 48 h, p < 0.01) and BUN (63.14 ± 3.37 mg/dL vs. 97.00 ± 2.16 mg/dL at 48 h, p < 0.001) when compared with I/R group in acute phase of RIRI. From 1 week after the index ischemic injury, both sCr and BUN could return to a platform value by degrees and be sustained stably thereafter. Though it was not statistically significant, both platform sCr and BUN of mice in the I/R group were higher than in the IPC group (Fig. 1b, c).

The time point of day 2 post ischemia was chosen to evaluate the acute phase of RIRI. Histologic analysis with H&E staining sections revealed more PTCs damage in I/R kidneys when compared with IPC kidneys by counting the percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation, etc. (Fig. 1d). Results of immunofluorescence staining and Western blot also exhibited a higher expression of kidney injury molecule-1 (KIM-1) in the I/R group (Fig. 1e, f). KIM-1 is a type 1 transmembrane protein, whose expression is markedly and promptly up-regulated in the apical membrane of PTCs when kidney injury occurs [25]. As expected, IPC significantly attenuated I/R-induced PTCs KIM-1 expression in the early phase when compared with those without IPC. But intriguingly, the mice in the sham group also showed KIM-1 expression in PTCs, implying that unavoidable blood loss during the operation could also lead to kidney injury. Taken together, these data indicated that IPC could attenuate I/R-induced kidney injury in the acute phase.

**IPC Alleviated Tubulointerstitial Fibrosis and Collagen Deposition**

The long-term effect of IPC on the recovery phase of RIRI was also investigated. Mice were allowed for reperfusion for 6 weeks, and the overall survival rate was recorded. As shown in Figure 2a, mice were prone to death within 2 weeks after RIRI, and I/R mice presented higher mortality when compared with IPC mice (50% vs. 20%). In the late 4 weeks after RIRI, no mice deaths were recorded.

Six weeks after the bilateral ischemia, the mice were sacrificed. Mice in I/R group suffered from an extremely serious atrophic kidney manifesting a lower left kidney weight/body weight than IPC group (0.45 ± 0.09% vs. 0.74 ± 0.13%, p < 0.01) (Fig. 2b). Meanwhile, IPC led to decreased renal interstitial fibrosis which was confirmed by picrosirius red staining and immunofluorescence staining of collagens I and IV (Fig. 2c-e). These data further indicated a protective role of IPC in the recovery phase of RIRI.

**Transcriptome Analysis Indicated an Enhanced Autophagy Activity after IPC**

To investigate the transcriptional changes induced by IPC, we analyzed the I/R group and IPC group data from GSE39548 after a careful review. The two groups showed great differences in gene expressions (Fig. 3a). After removing the transcripts without gene symbol or gene ID, and setting the fold change (logFC >1) and adjusted p value cutoff (adjusted p < 0.05), a set of 2,385 differentially down-regulated and 424 up-regulated genes were identified. These genes were submitted to functional enrichment analysis through Gene Ontology analysis. Apart from the reported metabolism-related, oxidation reduction-related genes, etc. [28, 29], many gene biological processes concerning autophagy were enriched (Fig. 3b; online suppl. Table 2), which indicated a role of autophagy in kidney injury after IPC. I/R group and IPC group showed obviously different expression patterns of autophagy-related genes (Fig. 3c-d). The key genes regulating the formation of phagosomes and autophagosomes including autophagy related 10 (Atg10), lysosomal-associated membrane protein 2 (Lamp2), RB1 inducible coiled-coil 1 (Rb1cc1), etc., were all upregulated in the IPC group. We further explored the interactions of the proteins encoded by the genes above (Fig. 3e). The top ten genes evaluated by connectivity degree in the protein-protein interaction network were identified (Fig. 3f; online suppl. Table 3). Of note, chemokine (C-C motif) ligand 2 (Ccl2), also named monocyte chemoattractant protein-1 (MCP-1), a chemokine regulating monocyte and macrophage chemotaxis was significantly down-regulated and showed a high connectivity degree.

**IPC Enhanced Autophagy Activity of PTCs and Inhibited Cell Apoptosis Both in vivo and in vitro Study**

Based on the results of transcriptome analysis that IPC might alleviate mouse RIRI by enhancing the autophagy activity of PTCs, the autophagy and apoptosis activity of PTCs were evaluated in vivo. Results of Western blot presented the autophagy activity of PTCs in IPC kidneys was significantly enhanced, indicated by increased LC3II/
GAPDH ratio and decreased p62 expression when compared with the mice in the I/R group. Meanwhile, the detection of apoptosis activity exhibited a reverse result, presenting a decreased Bax and increased Bcl-2 expression in the IPC group (Fig. 4a-e), which furthermore verified the above findings.

Fig. 2. Tubulointerstitial fibrosis and collagen deposition in the chronic phase of RIRI. a Overall survival rate after operation (n = 10/group). b The severity of kidney atrophy, represented by left kidney weight/body weight (%) on the sacrificed day (n = 5/group). c–e The severity of tubulointerstitial fibrosis, detecting by picrosirius red staining and immunofluorescence staining of collagen IV and I. The time point for assessment was 42 days after the index ischemic injury (n = 5/group). Five randomly selected fields per kidney were taken. The data were presented as mean ± SD. ** meant p < 0.01, *** meant p < 0.001, **** meant p < 0.0001. Scale bar: 100 μm (c), 10 μm (d, e).
Gene expression analysis was performed using microarray data from GSE39548, comparing IPC versus IR conditions. The volcano plot (a) shows a significant number of genes with fold changes greater than 2 and p-values adjusted for multiple testing less than 0.05. The heatmap (c) provides a visual representation of gene expression changes across different conditions, with genes upregulated (Up) in blue and downregulated (Down) in red. The bar graph (d) illustrates the gene count for each log2 fold change category, with p-adjusted values indicating statistical significance. Network analysis (e) and (f) reveals interconnected pathways related to macroautophagy and autophagy regulation, with key genes such as Casp3, Tnsf10, Fas, Bcl2l1, Myc, Rb1cc1, and Foxo3 highlighted in the network diagrams.
In addition, HK-2, a human PTC cell line was treated with HPC and H/R in this study as in vitro validation [27]. HPC group showed higher cell viability when compared with the H/R group (77.19% ± 5.53% for HPC group vs. 45.33% ± 5.28% for H/R group, p < 0.01), and this difference could be counteracted, while 3-MA was administrated (58.4% ± 3.72% for 3-MA group) (Fig. 5a). Intriguingly, apoptosis (including both early and late apoptosis) of PTCs in the HPC group was statistically suppressed when compared with the H/R group (9.12 ± 0.70% for HPC group vs. 21.22 ± 2.52% for H/R group, p < 0.001), and 3-MA could counteract the difference as well (15.50 ± 2.29 for 3-MA group) (Fig. 5b), which was mutually complementary with the results of autophagy detection. Moreover, the results of Western blot also showed similar results to that of in vivo (Fig. 5c, d).

In addition to pharmacological inhibition of autophagy, Atg5 (a critical autophagy-related gene) knockdown down HK-2 cells was also treated with HPC and H/R in vitro (online suppl. Fig. S1). Atg5 knock-down HK-2 cells showed similar cell viability whether treated or not treated with HPC (Fig. 5e). The cell viability imaging showed fewer dead cells in the HPC group of control HK-2 cells. However, in Atg5 knock-down HK-2 cells, the protective effect was canceled (Fig. 5f, g). These data strongly suggested a predominant role of autophagy in IPC-induced renoprotection against I/R injury.

**Autophagy Inhibitor Attenuated the Renoprotection Effect of IPC in vivo**

We further investigated whether the renoprotection effect of IPC in vivo could be reversed by 3-MA. As shown in Figure 6a, 3-MA was administrated 12 h and 1 h before IPC and continued for 3 days from day 1 to day 3 after IPC. Results showed that IPC could alleviate I/R-induced AKI functionally and histologically, but this reno-protection effect could be counteracted by 3-MA (Fig. 6b-d). Consistent with the results of in vitro study, the autophagy activity was inhibited when 3-MA was applied in vivo (Fig. 6e). Results of the TUNEL assay also presented that the number of apoptotic cells was significantly lower in the IPC group (11 ± 4 cells/high power field [HPF, 400×]) than in the I/R group (32 ± 4 cells/HPF) and 3-MA group (24 ± 6 cells/HPF) (Fig. 6f). Taken together, these findings confirmed that IPC was involved in the renoprotection effect against RIRI in an autophagy-dependent manner.

**IPC Inhibited MCP-1 Release and Renal Macrophage Infiltration in the Acute Phase of RIRI**

Since the pro-inflammatory chemokine MCP-1 was shown downregulated after IPC according to the transcriptome analysis, we tested the mRNA levels of MCP-1 and other pro-inflammatory cytokines, including *Tnfα*, *Il1b*, *Il6*, etc., in kidney tissues. Meanwhile, toll-like receptors (TLRs) were well known to play a fundamental role in innate immune responses [30], so mRNA levels of *Tlr2* and *Tlr4* in kidney tissues were also detected. As shown in Fig. 7a–g, mRNA levels of MCP-1 and *Tnf* were significantly downregulated in the IPC group, but other cytokines including *Il1b*, *Il6*, *Tgfb1*, and TLRs showed limited differences between groups. To test whether IPC reduced MCP-1 expressed by PTC per se, primary cultured mice PTCs and HK-2 cells were treated with HPC and/or H/R in vitro and RNA expressions levels of MCP-1 were detected. Both primary cultured mice PTCs and HK-2 cells represented an obviously lower MCP-1 mRNA level in the HPC group, which were autophagy-dependent (Fig. 7h, i). MCP-1 is a potent chemotactic factor for monocytes, macrophages, and other immune cells, which plays an important role in kidney injury [31]. As a result, we further investigated whether the lower level of MCP-1 in the IPC group resulted in decreased macrophages infiltration. As shown in Figure 7j, the IPC kidneys showed significantly lower amounts of macrophages infiltration at 2 days after RIRI (12 ± 3 cells/HPF for IPC group and 15 ± 2 cells/HPF for I/R group, p < 0.05). Besides, flow cytometry analysis also confirmed a decreased number of macrophages infiltration in the IPC group (online suppl.}

**Fig. 3.** Transcriptome analysis of I/R group and IPC group data from GSE39548. a Volcano plots of DEGs between the I/R group and IPC group. Data points in red represented upregulated and blue represented downregulated genes. Genes without any significant difference were in black. The differences were set as logFC >1 and adjusted p value <0.05. b GO enrichment analysis for DEGs. The biological process concerning autophagy was presented. c Heatmap of the autophagy-related genes in the DEGs. Upregulated genes were in red and downregulated genes were in blue. Genes without any significant difference were in white. d Bar plot of the expressions of autophagy-related genes in the DEGs between the I/R group and IPC group. Upregulated genes were in yellow and downregulated genes were in blue. e PPI network of autophagy-related genes in the DEGs. The balls represented the gene nodes; the connecting lines represented the interactions. f Top ten genes that show the highest degree of connectivity in the PPI network. DEG, differential gene; GO, Gene Ontology; PPI, protein-protein interaction.
To enhance the persuasiveness of the results, the immune cell infiltration landscape in kidney tissues was also characterized, including CD4+ T cell, CD8+ T cell, natural killer cell, and dendritic cell (online suppl. Fig. S2b).

**Discussion**

AKI is a common critical illness with high risks of morbidity and mortality. Around 50% will eventually develop chronic kidney disease, and 8.1% of these patients will progress to end-stage renal disease and require dialysis or renal transplantation [32, 33]. Plenty of pathogenesis etiologies have been reported causing AKI, and IRI is one of the most important.

Autophagy is the process through which parts of the cell are degraded in the lysosome [34]. Autophagy is crucial for cell survival during RIRI. IPC has a strong renoprotective effect during RIRI partially due to the promotion of autophagy activity [27, 35], but it still remains controversial [36]. Thus, a bilateral IPC + I/R mouse model was established to investigate the influences of IPC on the pathological process of mouse bilateral RIRI and autophagy activity of PTCs, as well as to figure out the regulatory role of cell autophagy in this process. We found that

![Fig. 5. HPC enhanced autophagy activity of PTCs and inhibited cell apoptosis in vitro.](image-url)

**Fig. 5.** HPC enhanced autophagy activity of PTCs and inhibited cell apoptosis in vitro. a HK-2 cells were treated with HPC or H/R (2 days after HPC) as described in methods. 3-MA (10 mM) was used to inhibit autophagy. Cell viability assay of HK-2 cells was evaluated by CCK-8. b Determination and quantitative analysis of apoptotic HK-2 cells by Annexin V-PI FACS analysis. c, d Detection of autophagy and apoptosis activity of HK-2 cells by Western blot. The grouping of blots cropped from different parts of the same gel. Data presented were from at least 3 independent experiments and were shown as mean ± SD. e–h Quantification of the Western blot results.

![Fig. 4. IPC enhanced autophagy activity of PTCs and inhibited cell apoptosis in the acute phase of RIRI.](image-url)

**Fig. 4.** IPC enhanced autophagy activity of PTCs and inhibited cell apoptosis in the acute phase of RIRI. a Detection of autophagy and apoptosis activity in the renal cortex (2 days after the index ischemia, n = 6/group) by Western blot. Data presented were from at least 3 independent experiments and were shown as mean ± SD. b–e Quantification of the Western blot results.
IPC-mediated activation of autophagy of PTCs plays a vital role in affording protection in I/R-induced kidney injury both at the acute phase and chronic phase, providing a hypothesis to promote cell autophagy at the appropriate timing and intensity so as to alleviate renal injury and sustain cell survival of the kidney.

An excessive and inflammation response has been elucidated as the key mechanisms of renal ischemia/reperfusion injury [37]. The cellular damage and its associated molecular products are thought to be key triggers for inflammation after acute tissue injury. Renal parenchymal cells, such as renal tubular epithelial cells and endothelial cells, play an essential role in the earliest phases of AKI by releasing damage-associated molecular patterns (DAMPs) and inflammatory cytokines like MCP-1, TNF-α, and IL-6, which can recruit inflammatory cells such as neutrophils, monocytes/macrophages, and lymphocytes into kidney [38].

MCP-1, a member of the C-C chemokine family, regulates the migration and infiltration of monocytes/macrophages, memory T lymphocytes, and natural killer cells [39]. MCP-1 is produced by a variety of cell types in the kidney, including epithelial, endothelial, and immune cells [40, 41]. In our study, we found the elevated autophagy of PTC significantly reduced the expression of MCP-1 in the HPC group. The expression of MCP-1 may be regulated by autophagy in several ways. Reactive oxygen species (ROS) is considered the critical cause of I/R injury. ROS can induce MCP-1 expression in PTCs [42]. Autophagy protects kidney tubular from death or necroptosis after I/R through the removal of ROS-producing mitochondria [18], thus reducing the MCP-1 expression. Lysosomal rupture strongly activates inflammation and aggravates kidney injury. Autophagy reduced the release of pro-inflammatory cytokines and protects against lysosomal rupture-induced inflammatory injuries by engulfing damaged lysosomes [43]. DAMPs activate the inflammasome and NF-kappa B pathway, which mediates the expression of MCP-1 in the kidney during IRI [44]. Autophagy also suppresses the release of DAMPs by cellular protection and DAMPs degradation [45].

Brooks and colleagues [46] reported that monocytes and macrophages were important effectors of the innate immune response after RIRI. Therefore, we hypothesized that the reno-protective effect of IPC-mediated activation of autophagy of PTCs was by inhibiting MCP-1 release and further decreased the macrophages infiltration in kidney tissues. This assumption was verified by staining and flow cytometry. The decreased macrophages and immune cells in turn produced decreased MCP-1, which caused a dramatically lower level of MCP-1 in the IPC group. However, since MCP-1 plays an essential role in routine immune surveillance and immune modulation, and in clearing acute viral infections, any attempts to reduce MCP-1 production to achieve beneficial effects in I/R-induced kidney injury should be carefully weighed because of its role in the maintenance of health.

**Conclusions**

The present study determines that IPC plays a protective role in the pathological process of RIRI. IPC-mediated activation of autophagy of PTCs plays a vital role in affording protection in I/R-induced kidney injury by inhibiting MCP-1 release and renal macrophages infiltration. Thus, promoting autophagy activity might be novel therapy to alleviate I/R-induced kidney injury. Further studies are still needed to gain insights into the specific molecular mechanisms whereby IPC mediates autophagy and protects cells from RIRI.

**Statement of Ethics**

All animal procedures were approved by the Animal Ethics Committee of Xinhua Hospital (XHCE-F-2020-181). Animal experiments were performed in accordance with the Health Guidelines of the National Institutes for the Care and Use of Laboratory Animals.

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**Fig. 6.** Autophagy inhibitor attenuated the reno-protective effect of IPC in vivo study. a Ideograph of 3-MA administration strategy. b H&E-stained histological images of kidneys and quantification of tubular damage (n = 6/group). Ten randomly selected fields (original magnification, ×200) per kidney were used for counting. c, d sCr and BUN levels 2 days after RIRI (n = 6/group). e Detection of autophagy activity in the renal cortex (2 days after the index ischemia) by Western blot. The grouping of blots cropped from different parts of the same gel. Data presented were from at least 3 independent experiments and were shown as mean ± S.D. f Detecting and quantification of TUNEL* cells in kidneys (n = 6/group). Five randomly selected fields per kidney (original magnification, ×400) were taken to calculate the average TUNEL* cells per HPF. The data were presented as mean ± SD, SD, standard deviation. * meant p < 0.05, ** meant p < 0.01, *** meant p < 0.001, **** meant p < 0.0001. Scale bar: 50 μm (b), 20 μm (f)
Fig. 7. IPC inhibited MCP-1 release and renal infiltrating macrophages in the acute phase of RIRI. **mRNA levels of MCP-1, Tnf, Il1b, Il6, Tgfb1, Tlr2, and Tlr4 in the renal cortex, detected by qRT-PCR (n = 6/group). One of the mice in the sham group was used as control, and the relative expressions were calculated by dividing the experimental value by the control value. h, i HK-2 cells and primary cultured PTC were treated with HPC or H/R. 3-MA (10 mM) was used to inhibit autophagy. MCP-1 mRNA expressions were detected by qRT-PCR. j Immunohistochemical staining of F4/80⁺ cells in kidneys (n = 6/group). Five images per kidney (original magnification, ×400) were taken randomly to calculate the average F4/80⁺ cells per HPF. The data were presented as mean ± S.D. The white arrows indicated the F4/80⁺ cells. qRT-PCR, quantitative real-time RT-PCR; Tnf, tumor necrosis factor; Il1b, interleukin 1 beta; Il6, interleukin 6; Tgfb1, transforming growth factor beta 1; Tlr2, toll-like receptor 2; Tlr4, toll-like receptor 4; ns, no significance; S.D., standard deviation. *mean p < 0.05, ** mean p < 0.01, **** mean p < 0.0001. Scale bars: 20 μm (h).
Conflicts of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

S. Zhang and W.M. Xia: study design, perform the experiments, data analysis, manuscript writing, and editing. H.Q. Duan and X.Y. Li: help to perform the experiments, data analysis. S.B. Qian and H.B. Shen: study design, data analysis, manuscript writing and editing, and supervision.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding authors, Subo Qian and Ha-ibo Shen, upon reasonable request.

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