Pannexin 1 mediates ferroptosis that contributes to renal ischemia/reperfusion injury

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**Running title** : *Panx1 mediates ferroptosis in IRI-AKI*

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ABSTRACT
Renal ischemia/reperfusion injury (IRI) is a significant challenge in perioperative medicine and is related to oxidative programmed cell death. However, the role of ferroptosis, a newly discovered form of oxidative cell death, has not been evaluated widely. Pannexin 1 (PANX1), an ATP-releasing pathway family protein, has pro-apoptotic effects during kidney injury. Here, we demonstrate that PANX1 deletion protects against renal IRI by regulating ferroptotic cell death. *Panxl* knockout mice subjected to renal IRI had decreased plasma creatinine, malondialdehyde (MDA) levels in kidney tissues, and tubular cell death (visible as decreased TUNEL-positive renal tubular cells) compared with wildtype (WT) mice. In cultured human kidney 2 (HK-2) cells, silenced *Panxl* expression significantly attenuated ferroptotic lipid peroxidation and iron accumulation induced by the ferroptosis inducer erastin. Moreover, the *Panxl* silencing significantly modulated ferroptosis-related protein expression. Furthermore, *Panxl* deletion induced the expression of a cytoprotective chaperone, heme oxygenase-1 (HO-1), and inhibited ferroptinophagy via the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. In summary, *Panxl* deletion protects against renal IRI by attenuating MAPK/ERK activation in a ferroptotic pathway. Our findings provide critical insights into the role of PANX1 in ferroptotic cell death and highlight a potential therapeutic target for the management of acute kidney injury (AKI) during the perioperative period.

1. Introduction

Acute kidney injury (AKI) is a frequent complication after cardiac surgery, which contributes to increased mortality, and has been related to ischemia/reperfusion injury (IRI)(1). Oxidative damage has been widely studied in IRI-AKI and most of current studies focus on harm mediated by apoptosis, autophagy and necrosis(2-4). Ferroptosis, a newly discovered form of programmed, non-apoptotic cell death...
triggered by oxidative damage, has not been widely investigated in IRI-AKI. Ferroptosis is an iron-dependent type of programmed cell death, triggered by lipid peroxide accumulation in the context of increased reactive oxygen species (ROS) generation and inactivation of glutathione peroxidase 4 (GPX4), a glutathione-dependent enzyme that prevents lipid peroxidation(5). It can be triggered by structurally diverse small molecules (e.g. erastin, sulfasalazine and RSL3) and also prevented by lipophilic antioxidants (CoQ10, Vitam E, ferrostatins and liproxstatins)(6-9). Many regulators such as glutathione peroxidase 4 (GPX4), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), nuclear receptor coactivator 4 (NCOA4) and ferritin light chain 1(FTH1) have been identified to be involved in regulating ferroptosis(10-13). Ferroptosis has been shown to occur IRI-AKI(14). Furthermore, IRI-AKI has been shown to be attenuated after treatment with the ferroptosis specific inhibitor ferrostatin-1 (Fer-1)(14). In addition, the augmenter of liver regeneration (ALR) has been shown to prevent IRI-AKI by limiting ferroptosis through the regulation of the glutathione-glutathione peroxidase (GSH-GPx) system. Pannexin belongs to the ATP-releasing pathway family and is expressed in almost all cell types. The pannexin family consists of three proteins, Panx1, Panx2 and Panx3, all of which form membrane channels. Among them, Panx1 has been extensively investigated(15,16). Panx1 is involved in regulating ATP release as a damage-associated molecular pattern (DAMP) molecule that can activate apoptosis or autophagy signaling in oxidative condition(17,18). ATP binds to the P2Y7 receptor, activating thereby protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling (19), that regulates ferroptosis in Parkinson’s disease(20). Importantly, a reduction in this ATP release-dependent signaling has been shown to protect kidneys from oxidative damage during IRI-AKI(21). Therefore, we hypothesized that panx1 deletion protects against IRI-AKI by limiting ferroptosis-mediated
oxidative damage. Here, we demonstrate for the first time a role of panx1 in ameliorating ferroptosis in the kidney. Our findings provide new insights to understand the mechanism of panx1 in ferroptotic cell death and highlight a new therapeutic target for AKI treatment.

2. Results

2.1. Panx1 regulated erastin-induced ferroptosis in HK-2 cells

2.1.1 Increased Panx1 expression levels of HK2 upon erastin

As a special ferroptosis inducer, erastin has been widely studied in ferroptosis for abolishing the import of cysteine, a precursor for glutathione during ferroptosis (22-24). We performed a CCK-8 assay to investigate erastin-mediated cell death in HK-2 cells, and found a significantly decreased cell viability at an erastin concentration of 0.1 µg/ml (Figure 1A). In order to examine whether the induction of ferroptosis by erastin regulates panx1 expression, we next analyzed protein and mRNA expression levels of panx1 in Hk-2 cells. Remarkably, treatment with erastin significantly induced panx1 mRNA (Figure 1B) and protein expression in a time and concentration dependent manner (Figure 1C, D, E and F).

2.1.2. Panx1 silencing inhibits erastin-induced ferroptosis in HK-2 cells, and decreases lipid peroxidation, iron accumulation and mitochondrial membrane potential hyperpolarization

To investigate whether upregulated panx1 confers to ferroptosis induced by erastin, we first measured cell viability after panx1 pharmacologic inhibition or knock-down using a specific shRNA. Similar to results from previous studies (6), several ferroptosis inhibitors (ferrostain-1 and DFO) significantly reversed erastin-induced cell death (Figure 2A). Three sequences of shRNA were designed testing by qPCR (Figure 2B) and Western blot was used to verify the most efficient sequence (Figure 2C). Suppression of panx1 expression both genetically and pharmacologically significantly inhibited
erastin-induced ferroptotic cell death in HK-2 cells (Figure 2 A and D). Examination of the morphology of cells using an inverted microscope and cell death analysis by flow cytometry showed that panx1 knockdown significantly inhibited erastin-induced ferroptotic cell death in HK-2 cells (Figure 2 E, F and G). To characterize cell death by erastin, we measured key characteristics of ferroptosis in HK-2 cells including lipid peroxidation and liable iron levels in HK-2 cells (6). Lipid ROS measured using the dye C11-BODIPY 581/591, and liable iron levels were significantly increased following treatment with erastin. In contrast, panx1 knockdown decreased lipid peroxidation and iron accumulation (Figure 2 H, I and J). Mitochondria play a pivotal role in ferroptosis and it’s membrane potential hyperpolarization is associated with ferroptotic cell death (25). As the results shown that panx1 knockdown decreased mitochondrial membrane potential hyperpolarization (Figure 2 K).

2.1.3 Panx1 regulates the expression of ferroptosis-related proteins in HK2

Previous studies have shown that genes involved in iron and ROS metabolism such as NCOA4, NRF2, HO1 and FTH1, play a role in ferroptosis (10, 12, 13). Therefore, we analyzed the expression of proteins translated by NCOA4, NRF2, HO1, FTH1 and GPX4. In response to erastin, NCOA4 and FTH1 expression increased initially, to then decline (Figure 3 A and B). The expression of NRF2 and HO1 increased significantly throughout the entire 24h period, while GPX4 expression decreased at 12h and 24h (Figure 3 A and B). To further investigate the role of panx1 in regulating ferroptosis, we exposed panx1 knocked down HK-2 cells to erastin. The expression of NCOA4 and FTH1 was inhibited in HK-2 panx1 knockdown cells in response to erastin (Figure 3 C and D). However, panx1 knockdown had no effect on regulating GPX4 and NRF2 expression. In addition, we found that HO1 expression was significantly upregulated in panx1 knockdown cells in response to erastin suggesting that the protective effect of knocking down panx1 may be related to...
up-regulation of antioxidant genes (Figure 3 C and D). Collectively, these findings suggest that panx1 could regulate ferroptosis-related gene involved in iron and ROS metabolism.

2.2 Panx1 regulates ferroptosis in ischemia/reperfusion kidney injury

2.2.1 Increased Panx1 expression levels during IRI-AKI

To determine whether Panx1 participate in kidney IRI, we quantified the expression of panx1 in wild type (wt) mice subjected to kidney IRI. Compared with sham mice, both mRNA and protein expression of Panx1 were up-regulated significantly at 6 and 12 h after IRI suggesting that IRI induces Panx1 renal expression (Figure 4 A, B C and D).

2.2.2. Panx1 deletion prevents kidney injury induced by ischemia/reperfusion

The effect of Panx1 gene delection in kidney was confirmed by western blot and immunohistochemistry (Figure 5 A and B). To confirm the role of panx1 in kidney IRI, we compared markers of renal injury in wt vs panx1 knockout mice subjected to I/R. We assessed kidney function by measuring serum creatinine and investigating histological features. Serum creatinine (Scr) and tissue damage were similar between wt and panx1 knockout mice subjected to sham-operation (Figure 5 C). Serum creatinine (Scr) at 24 h after IRI was lower in Panx1 knockout mice than in wt mice at (Figure 5 C). Consistent with the Scr data, Panx1 knockout mice had decreased renal tubular necrosis, congestion and cast formation with significantly lower renal injury scores compared to wt mice subjected to renal IRI. (Figure 5 D and E). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) showed increased renal cell death in WT mice when compared to Panx1 knockouts (Figure 5 F and G). Tissue lipid peroxidation assessed using MDA levels decreased in Panx1 knockout mice compared to WT (Figure 5 H). Taken together, these results suggested that
Panx1 deletion prevent kidney injury after ischemia/reperfusion.

2.2.3. Panx1 regulates the expression of ferroptosis-related protein in renal IRI

Previous studies have proven the occurrence of ferroptosis in kidney injury induced by ischemia/reperfusion(11,14). In our study, we found that ferroptosis-related proteins were up-regulated significantly during IRI (Figure 6 A and B). In addition, Panx1 knockout mice had a significant increase in HO1 expression compared to WT mice after IRI. The expression of ferroptosis-related protein was similar to in vitro results(Figure 6 C and D).

2.3 Panx1 regulates ferroptosis via MAPK/ERK signaling pathway

Previous studies have shown that ATP, as signal transduction molecules, could activate the MAPK pathway in the process of cell proliferation and cell death(26,27). We hypothesized that panx1 downregulates the activity of MAPK signaling in HK-2 cells by regulating ATP release which in turn, can inhibit ferroptosis. The results showed that panx1 deletion significantly attenuated phosphorylation of ERK expression exposed to Erastin but had almost no effect on phosphorylation of p38 and JNK levels (Figure 6 E and F). These results indicate that panx1 regulates the activity of MAPK/ERK signaling in HK-2 cells and lead to ferroptosis. We hypothesized that the panx1 channel releases ATP under oxidative stress conditions and then ATP combined to P2X7R to activate MAPK pathway resulting ferroptotic cell death. In order to test our hypothesis, we conducted ERK inhibitor (LY3214996, 10µM) and P2Y7 inhibitor (A-74003, 10µM) to HK2 cells exposed to Erastin. The result shown that inhibiting P2Y7 receptor significantly decreased phosphorylation of ERK. Inhibiting ERK significantly decreased NCOA4 while increased HO1 expression in the HK2 cells exposed to Erastin(Figure 6 G and H). Both of the two inhibitor decrease GPX4 expression suggesting that the phosphorylation of ERK has two sides of effect and the antioxidant effect of
GPX4 may relatively weak (Figure 6 G and H). Treatment of ZnPP (zinc protoporphyrin-9), a specific inhibitor of HO-1 had no marked effects on ERK levels suggesting a MAPK/ERK–HO-1 hierarchy as a response to Erastin induction (Figure 6 I and J). Taking together the protective effect of panx1 deletion in ferroptosis is via activating MAPK/ERK pathway to inhibit NCOA4-mediated ferroptinophagy and increasing antioxidant gene HO1 expression.

3. Discussion

Ferroptosis is a novel type of programmed cell death characterized by iron dependent increase in ROS, which is known to play crucial roles in cellular proliferation, senescence and differentiation (22). The most important finding of this study is the identification of an important role of Panx1 in regulating ferroptosis in the kidney, whereby, the absence of panx1 channel protects renal tubular epithelial cells from erastin-induced ferroptosis and kidney injury for IRI. Pannexin channels are known to play pathological roles in oxidative stress, inflammation and cell death (28). Previous studies have reported that P2Y7, receptors of panx1, triggered cell death which can be reversed by inhibition ferroptosis production of NADPH oxidases-generated ROS which suggesting a role of panx1 in ferroptosis (29,30). Decreasing lever of GSH leads to lipid peroxidation which can be tested by a lipophilic fluorescent dye (C11-BODIPY 581/591) in vitro (31). Indeed, the present study showed that panx1 knockdown decreases lipid peroxidation in HK-2 exposed to erastin. Iron participates in several biologic functions including ferroptosis. For instance, increased ROS causes lipid peroxidation and triggers ferroptosis, which can be suppressed using deferoxamine, an iron chelator (9). In addition, higher levels of iron transport proteins increase iron mediated ROS production leading to increased ferroptosis (32,33). In this study observed that iron metabolism related genes, such as NCOA4 and FTH1 which maintain
cellular labile iron contents, promote the accumulation of cellular ROS and ferroptotic cell death via the “ferroptinophagy” pathway (13,34). Importantly, panx1 knockdown reduced iron accumulation, and reduced iron regulatory gene expression after exposure to erastin, suggesting that panx1 regulates ferroptosis by decreasing cellular iron level. Previous studied reported that renal proximal tubular cells have limited biosynthetic capacity for GSH in vitro and GSH metabolism may not a requirement for iron-dependent lipid peroxidation(35,36). Blocking system xct inhibits cysteine-dependent glutathione(GSH) synthesis and also inhibits the transplasma membrane cysteine redox shuttle(37,38).Both effects impair cellular antioxidant defenses, thereby facilitating mitochondrial metabolism disfunction and toxic ROS accumulation(6). In regard to erasin-induced HK-2 cell death, it might due to abolishing transplasma membrane cysteine redox shuttle which leading mitochondrial disfunction. The role of mitochondria have been proved to be a motivator in ferroptosis(25,39).Our results shown that panx1 knockdown decreased mitochondrial membrane potential hyperpolarization suggesting that the protect effect of panx1 deletion in erasin-induced ferroptotic cell death might due to defending mitochondria disfunction caused by system xct blocking.

Previous study has investigated the role of ferroptosis in the pathogenesis of IRI mediated kidney injury(14). In present study, we shown that the Panx1 channels in the pathophysiologic pathway of ferroptosis-mediated IRI-AKI, because IRI increases the expression of the Panx1 channel and knocking out the channel decreases renal injury. Our data suggests that the absence of the Panx1 channel may protect by limiting lipid peroxidation. We further demonstrate that panx1 deletion further protects the kidney by ameliorating ferroptosis, not through upregulation of antioxidant genes like GPX4(40) or Nrf2(10), but by upregulating HO-1 expression, which is known to protect against ferroptosis (12,41). Furthermore,
panx1 deletion decreased ferroptosis related proteins like NCOA4 and FTH1, otherwise upregulated with IRI. These results illustrated that panx1 could reduce lipid peroxidation to alleviate kidney injury under oxidative stress in vivo. Whether this protected effect of panx1 deleting after I/R in vivo was related to ferroptotic cell death? We further investigated some ferroptosis related protein during ischemia reperfusion in panx1-/— mice. GPX4 can neutralize lipid peroxides and protect membrane fluidity by using glutathione, as a cofactor of GPX4, to protect cells and membranes against peroxidation(40). Nuclear factor-E2-related factor 2 (Nrf2) is responsible for regulating hundreds of antioxidant genes and have been identified as a ferroptosis regulator(10). The results shown that panx1 deleting have no effect on their expression suggesting panx1 could be downstream gene of them. Heme oxygenase-1 (HO-1)regulating cellular defensive response against oxidative stress have proved to play an important role in ferroptosis(12,41). In our study, we found the protective effect of panx1 deleting on ferroptosis was through up-regulating HO1 expression. NCOA4 and Ferritin(FTH1), an iron regulatory protein, regulate ferroptosis in a selective autophagy pathway, so called “ferritinophagy” which degrades FTH1 to release free iron(42). The results were consistent with experiment in vitro and indicated that panx1 deletion protects the kidney from oxidative damage during IRI by decreasing ferroptosis through modulation of cellular iron levels. Panx1 was also involved in regulating ATP release which can activate apoptosis or autophagy signaling (17,18). Extracellular ATP can act as a paracrine molecule to activate P2X7R which can regulate various signaling pathway such as AMPK and MAPK signaling pathway(43). Previous studies have shown that ferroptosis could activate the MAPK pathway through MEK (20). Our data suggests that the panx1 channel releases ATP under oxidative stress conditions and then ATP combined to P2X7R to activate MAPK pathway.
resulting ferroptotic cell death (Figure 7). We investigated the signal pathway of MAPK regulating ferroptosis. The results demonstrated that panx1 deletion markedly inactivated the MAPK/ERK pathway. The presence of panx1 can inhibit HO1 expression by activating the MAPK/ERK pathway. Taken together, our data suggests that panx1 deletion contributed to modulate the activation of MAPK/ERK and HO1 signaling pathways, resulting in the inhibition of ferroptotic cell death during IRI.

As the unique properties of GSH equilibrium in renal proximal tubular cells in vitro (35), it might due to the inactivation of system Xc- when cells got immortalized in regard to erasin-induced HK-2 cell death. There may be involved GSH metabolism during ferroptosis in vivo. Although we have established that whole organism Panx1KO is protective against IRI, it is unclear if this mechanism is specific to the kidney. A conditional, renal-specific knock out panx1 would help further dissect these mechanisms specifically in IRI-AKI. Also, whether Panx1 inhibition could protect from other models of AKI, for example, nephrotoxins, obstruction, and sepsis, needs further investigation. Nevertheless, our data suggests that Panx1 inhibition could be a novel therapeutic strategy to attenuate AKI. Further studies are needed to explore safety and effective Panx1-selective inhibitors for therapy in patients with AKI.

In summary, we have shown the role of the Panx1 channel in the regulation ferroptosis in the kidney in response to IRI. Released ATP from panx1 channel may act as a DAMP that activates ferroptosis via MAPK/ERK signaling and regulates NCOA4-mediated ferroptinophagy and antioxidant gene HO1 expression (Figure 7). Therefore, Panx1 inhibition may be a promising therapeutic target to ameliorate AKI in IRI.

4. Materials and Methods

4.1. Antibodies and Reagents

The antibodies to NRF2 (#ab62352), Panx1 (#ab139715), GPX4(ab125066) and NCOA4 (#ab) were obtained from Abcam...
The antibodies to Panx1 (#91137), Actin (#3700), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #5174), and β-tubulin (#2146) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies to NRF2 (#19693), ERK1/2 (#16443), JNK (#66210), JNK (#614064), and HO1 (#27282) were taken from Proteintech (Rosemont, IL, USA). The antibodies to p-p38 (#AF4001), p-JNK (#AF3318), p-ERK (#AF1015) were obtained from Affinity Biosciences (Cincinnati, OH, USA). The antibodies to FTH1 (#sc-28359) and NCOA4 (#sc-373739) were purchased from Santa Cruz. Deferoxamine (#D9533) and Panx peptide (#SML2082) were taken from Sigma (St. Louis, MO, USA). Erastin (#E7781), ferrostatin-1 (#S7243) were obtained from Selleck Chemicals (Houston, TX, USA). ZnPP (#B6431) were obtained from APExBIO (Houston, USA).

4.2. Cell culture

Human kidney-2 (HK-2) cell lines was purchased from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in Minimum Essential Medium (MEM, HyClone) with 5 ng/mL of human recombinant epidermal growth factor (EGF, Novus), 10% fetal bovine serum (FBS, Gibco), and a penicillin–streptomycin supplement. The cells were maintained in a humidified incubator at 37°C and 5% CO₂.

4.3. Cell viability assay and cell death analysis

Cell viability was evaluated using a Cell Counting Kit 8 (Dojindo Molecular Technologies, Kumamoto, Japan) assay. Cells were cultured in 96-well plates and treated with the specified compounds for the indicated times. The plates were incubated for an additional 2 h at 37°C. The absorbance at 450 nm was measured with a microplate reader (ELX-800; BioTek, Winooski, VT). Cell death analysis of ferroptosis was determined followed the method described by Chen et al. [25]. After treatments, cells were collected and stained with Annexin V-FITC reagent (BioVision) and 7-AAD.
(Cayman) and subjected to flow cytometry. The percentages of dead cells were quantified with CellQuest software.

4.4 Lipid peroxidation, labile iron pool and MMP detecting

A special fluorescent dyes, BODIPY581/591 (ThermoFisher Scientific) is for testing lipid peroxidation in vitro. BODIPY581/591 (1 mM) incubated for 10 min at 37 °C. The cells were washed with PBS three times, and the intracellular fluorescence was measured by flow cytometry. Evaluating lipid peroxidation in vivo used TBARS method to test MDA level. The total cellular labile iron pool was detected based on the calcein-acetoxyethyl ester method(44). The cells were washed twice with PBS followed by incubation of 0.05 μM calcein-acetoxyethyl ester (Enzo) for 15 min at 37°C. Then, the cells were washed with PBS and incubated with or without Deferiprone (100 μM) for 1 h at 37°C. After trypsinization, the cells were collected and analyzed by flow cytometry. The levels of the liable iron pool were calculated by the difference in cellular mean fluorescence with and without deferiprone incubation. Mitochondrial membrane potential (MMP) measurement using a JC-1 kit according to manufacturer’s instructions (J8030; Solarbio). Cells were treated as indicated, and then 10μg/mlJC-1 was added and incubated for 30 min. Labeled cells were trypsinized and resuspended in PBS plus 2% FBS. Fluorescence was analyzed using a flow cytometer.

4.5 ShRNA and transfection protocol

Human Panx1-shRNA (Sequence:sense(5'-3')-GCAGCUGCUCCUCAUUUUTT; sense (3'-5')-AAUGAUGAGUGUCAGCGGTT) plasmids were purchased from tGenePharma biotechnology company (China). Cancer cells were infected with specific shRNA viral-contained supernatant in the presence of polybrene (8 μg/ml). After 24 h of incubation at 37°C, the medium was replaced with complete medium containing puromycin.
(2 µg/ml). The cells were prepared for tests and harvested based on the experiments that were required.

4.6 Animals and kidney IRI model
Panx1-/- mice were purchased from the GemPharmatech Co., Ltd company (China). Panx1-/- were then mated to C57BL/6 mice yielding Panx1-/- mice. IRI surgery was performed to induce AKI as previously described [20]. Briefly, C57BL/6 mice (male, 10–15 weeks old) were food-deprived for 12 h before the procedures and were anaesthetized with intraperitoneal injection of 1% sodium pentobarbital solution (40 mg/kg). Using amidline abdominal incision, bilateral renal IRI was induced by clamping renal pedicles for 30 min. After removal of the clamp, the kidneys were inspected for confirming reperfusion. Body temperature was maintained at 37 °C throughout the procedure. As a control, sham-operated mice underwent the same procedure, except clamping of the renal pedicles. Heparinized blood was centrifuged at 2,000rpm for 10 min to separate the plasma to detect serum creatinine. The concentration of creatinine in serum was measured using commercial kit reagents (Institute of Jiancheng Bioengineering, Nanjing, China). The absorbance at 546 nm was detected by a multimode plate reader (PerkinElmer). For tissue analyses, kidneys were harvested, and one half of the harvested kidneys was fixed in 4% paraformaldehyde and processed for H&E staining analysis. The Jablonski renal injury score (scale: 0-4) for histology grading was used to grade renal tubular necrosis 24 hours after renal IR. All experiments were performed in accordance with Chinese legislation on the use and care of laboratory animals and were approved by the Animal Care and Use Committee of Wuhan University.

4.7 Western blot analysis and qRT-PCR
Total protein was extracted from HK-2 cells and mice kidney tissues. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore). After blocking with 5% non-fat milk, the membrane was incubated with specific antibody. All western blot data were repeated 3 times. Total RNA was extracted from HK-2 cells, macrophage and kidney tissues by YPH EASY spin tissue/cell RNA quick extraction kit (YPH, Beijing China). In addition, mRNA reverse transcription (RT) was performed by the ReverTra Ace Kit (Toyobo, Osaka, Japan). The cDNA then served as the template for SYBR real-time polymerase chain reaction (PCR). Primer sequences are listed as below(5′-3′):human
panx1 primer sense, 5′-CCACCGAGCCCAAGTTCAA-3′, and anti-sense, 5′-GGAGAAGCAGCTTATCTGGGT-3′; Mouse Panx1 primer sense, 5′-GCAATACTACACGGAGGAGCCTTC-3′, and anti-sense, 5′-CACCACCCACTGTTCGCTG-3′; All reactions were run in triplicate on the Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

4.8 Immunohistochemistry and TUNEL assay
Immunofluorescence and immunohistochemistry staining was carried out as previously described (45). The antibodies were Panx1 antibody (1:100, Abcam). The sections were visualized using a laser-scanning confocal microscope (Olympus FluoView™ FV1000, Tokyo, Japan). For the detection of TUNEL-positive cells, the ApopTag Peroxidase In Situ Apoptosis Detection Kit was used according to the manufacturer’s instructions (S7100; Serologicals, Millipore).

4.9 Statistical analysis
All data were presented as mean ± s.d. unless stated otherwise. Data were analyzed using student’s t-test were used for comparison between two groups. A p-value < 0.05 was considered statistically significant for all experiments. Statistical analysis was performed using GraphPad Prism 5.0 software.

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Author Contributions:
Conceived the project, designed the project, extract and analyzed data, drafted the manuscript and approved the final manuscript: J.S.

Drafted the part of discussion and background of manuscript: Y.Z.

Conducted the experiments: J.S., X.J., M.L., C.Y., H.Z., Q.X., and J.Y.

Designed the project, edited the manuscript and approved the final version: H.G, R.M, and Z.P.

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Figure 1. Increased Panx1 expression during erastin-induced ferroptosis in HK2

(A) HK-2 treated with erastin in different concentration for 24h were subjected to cell viability test.

(B) HK-2 treated with erastin at the indicated concentrations and time intervals for testing mRNA expression by quantitative PCR vs control.

(C) Western blot assay was utilized to detect Panx1 expression in different time point.

(D) Relative expression of gray values for Figure 1C (n=3, mean ± SD, *p < 0.05)

(E) HK-2 treated with erastin at the indicated concentrations and time intervals for testing protein expression by western blot assay.

(F) Relative expression of gray values for Figure 1E (n=3, mean ± SD, *p < 0.05)
Figure 2. Panx1 knockdown inhibits erastin-induced ferroptosis in HK-2

(A) HK-2 treated with erastin (0.25 µg/ml), ferroptosis inhibitor of ferrostatin (5 µM) and DFO (100 µM), and Panx1 specific inhibitor of Panx Peptide (10 µM) for 24 h were subjected to cell viability test.

(B) Different shRNA sequence of Panx1 were tested by quantitative PCR vs control
(C) The third of Panx1 was chosen according to qPCR with most effective knockdown function and confirmed by Western blot with 2 separate transfection experiments.

(D) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) and for 24 hours and cell viabilities were assayed (n=3, *p < 0.05)

(E) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) and for 24 hours and cell morphology was observed by inverted microscope.”.” represents 20µm.

(F) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) and for 18 hours. For Annexin V/7-AAD staining, cells were collected and the proportion of 7-AAD-positive cells (dead) was determined by flow cytometry.

(G) Data shown from three independent experiments of Figure 2F compared to the control. (mean ± SEM,*P < 0.05)

(H) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) for 18 hours and loaded BODIPY581/591 for lipid ROS generation analysis. The fluorescent signal was detected flow cytometry.

(I) Data shown from three independent experiments of Figure 2H compared to the control. (mean ± SEM,*P < 0.05)

(J) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) for 24 hours and liable iron levels were assayed (n=3, *p < 0.05).

(K) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) for 18 hours and loaded JC-1 for mitochondrial membrane potential.
Figure 3 Panx1 regulates the expression of ferroptosis-related protein in HK2

(A) Ferroptosis-related protein expression in different time point were assayed using western blot.

(B) Relative expression of gray values for Figure 3A(n=3, mean ± SD, *p < 0.05)

(C) Panx1 knockdown regulated ferroptosis-related protein expression testing by western blot.

(D) Relative expression of gray values for Figure 3C(n=3, mean ± SD, *p < 0.05)
Figure 4 Increased Panx1 expression levels during IRI AKI

(A) The mRNA expression of Panx1 were during IRI in different time point assayed by Q-PCR (n=3, *p < 0.05).

(B) The protein levels of Panx1 were assayed using western blot (n=3, *p < 0.05).

(C) Relative expression of gray values for Figure 4B(n=3, mean ± SD, *p < 0.05).

(D) The protein levels of Panx1 were assayed using immunohistochemistry. bar represents 50µm.
**Figure 5 Panx1 deletion prevent kidney injury induced by I/R**

(A) The Panx1 knockout mice was confirmed by the protein expression in kidney using western blot with 3 mice vs wt.

(B) The Panx1 knockout mice was confirmed by the protein expression in kidney using immunohistochemistry.
(C) Mice were subjected to sham-operation or to 30min renal ischemia and 24-hour reperfusion. Serum creatinine was tested by kit. (n=6, *p < 0.05)

(D) H&E images of mice subjected to 30min renal ischemia and 24-hour reperfusion. Scale bars:20 μm.

(E) The Jablonski scale renal injury score (scale: 0-4) for histology grading was used to grade renal tubular necrosis. (n=3, *p < 0.05)

(F) Cell death was analysis by TUNEL in the kidney of mice. bars:20 μm.

(G) TUNEL positive renal tubular cells was collected from three independent experiments of Figure F (n=3, *p < 0.05).

(H) Lipid peroxidation in kidney was assessed by MDA level (n=6, *p < 0.05).
Figure 6 Panx1 regulates ferroptosis in IRI and mediated alternative activation via MAPK/ERK and HO1 pathway in HK2

(A) Western blot assay was utilized to detect ferroptosis related protein expression in IRI mice kidney.

(B) Relative expression of gray values for Figure 6A (n=3, mean ± SD, *p < 0.05)

(C) Western blot analysis of ferroptosis-related protein expression in Panx1 knockout and WT mice subjected to IRI or not.
(D) Relative expression of gray values for Figure 6C (n=3, mean ± SD, *p < 0.05)

(E) Indicated knockdown HK2 cells were treated with erastin (0.25µg/ml) for 24h. Western blot analysis of AMPK, P-AMPK, p38, p-p38, ERK, p-ERK, JNK, and p-JNK protein expression.

(F) Relative expression of gray values for Figure 6E (n=3, mean ± SD, *p < 0.05)

(G) Western blot analysis of ferroptosis-related protein expression in HK2 cells exposed to erastin (0.25µg/ml) with ERK inhibitor (LY3214996, 10µM) or P2Y7 inhibitor (A-740003, 10µM).

(H) Relative expression of gray values for Figure 6G (n=3, mean ± SD, *p < 0.05)

(I) Western blot analysis of ERK pathway in HK2 cells exposed to erastin (0.25µg/ml) with ERK inhibitor (LY3214996, 10µM) or HO1 inhibitor (ZnPP, 5µM).

(J) Relative expression of gray values for Figure 6I (n=3, mean ± SD, *p < 0.05)

Figure 7 Schematic of proposed mechanisms for panx1 regulating ferroptosis in renal IR injury
When HK2 cells are exposed to ischemia or hypoxia, the Panx1 channel on the membrane open up and release ATP. Extracellular ATP as a paracrine molecule combines to P2Y7 receptors to activate MAPK/ERK signaling pathway which regulate NCOA4-mediated ferroptinophagy and antioxidant gene HO1 expression.
Pannexin 1 mediates ferroptosis that contributes to renal ischemia/reperfusion injury
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