Ligustrazine ameliorates acute kidney injury through downregulation of NOD2-mediated inflammation

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Abstract. Ligustrazine has been used to alleviate clinical acute kidney injury (AKI); however, the underlying molecular mechanisms are poorly understood. In order to further elucidate the molecular mechanism underlying its occurrence, the role of nucleotide-binding oligomerization domain-containing 2 (NOD2) in AKI was investigated in the present study, and the results indicated that ligustrazine exerts an important protective effect against AKI *in vivo* by inhibiting the upregulation of NOD2 expression and reducing apoptosis of kidney cells following ischemia/reperfusion injury in rat models. Furthermore, the inhibitory role of ligustrazine on the upregulation of NOD2 and apoptosis of kidney cells induced by $CoCl_2$ and oxygen and glucose deprivation followed by reoxygenation was investigated in *in vitro* experiments. The effect of ligustrazine on NOD2 downregulation was partially blocked by inhibiting autophagy. To the best of our knowledge, the results of the present study are the first to provide evidence that ligustrazine can inhibit NOD2-mediated inflammation to protect against renal injury, which may be in part attributed to the induction of autophagy. These findings may help design and develop new approaches and therapeutic strategies for AKI to prevent the deterioration of renal function.

Introduction

Acute kidney injury (AKI) is a complex pathophysiological process that is associated with the progression of chronic kidney disease. Previous studies have highlighted the role of the innate immune system and inflammatory mechanisms in the progression of AKI, particularly the activation of pattern-recognition receptors (PRRs) (1,2). Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), which are members of the family of PRRs, are known to be involved in kidney ischemia/reperfusion (I/R) injury (3,4), and may represent a potential therapeutic target to abrogate the pathogenesis of AKI. Among the known NLRs, nucleotide-binding oligomerization domain-containing 2 (NOD2) and nucleotide-binding domain and leucine-rich repeat pyrin 3 domain (NLRP3) act as sensors of 'cellular danger' (5), and belong to two different subfamilies based on the nature of their N-terminal domains. These proteins play a key role in the control of inflammatory and immune responses through the modulation of different signaling pathways, including those dependent on nuclear factor (NF)-κB and the caspase-1-mediated cleavage of interleukin (IL)-1β and IL-18, respectively (6,7). Moreover, NOD2 and NLRP3 are extensively involved in the progression of renal diseases (8-12). Suppression of NOD2-mediated immune responses was found to attenuate hypoxia-induced

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Abbreviations: AKI, acute kidney injury; NLRs, nucleotide-binding and oligomerization domain (NOD)-like receptors; I/R, ischemia/reperfusion; PRRs, pattern-recognition receptors; NOD2, nucleotide-binding oligomerization domain-containing 2; NLRP3, nucleotide-binding domain and leucine-rich repeat pyrin 3 domain; BDMCs, bone marrow-derived cells; SCr, serum creatinine; BUN, blood urea nitrogen; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; CQ, chloroquine; CCK-8, Cell Counting Kit-8; H&E, hematoxylin and eosin

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inflammatory effects and apoptosis in proximal tubule epithelial cells (13), and knockout of NLRP3 was shown to inhibit the activation of bone marrow-derived cells and T cells in a mouse IgA nephropathy model (10). Therefore, preventing inflammation mediated by the activation of NLRs may be considered as a potential therapeutic option for AKI.

Ligustrazine is a bioactive alkaloid that is extracted from the Chinese herb *Ligusticum wallichii* Franchat, which has long been used for the treatment of cardiac and cerebral diseases (14). As a calcium antagonist and reactive oxygen species scavenger, ligustrazine can significantly improve cardiac and cerebral blood flow (15,16). It may also be used to alleviate clinical renal injury following AKI. However, the mechanisms underlying its protective effects remain poorly understood. The anti-inflammatory effect of ligustrazine was recently demonstrated in patients with rheumatic heart disease, an allergic asthma mouse model, and a rat model of spinal cord I/R injury (17-19), suggesting that this effect may represent the mechanism through which this compound confers renal protection. The aim of the present study was to investigate whether ligustrazine can inhibit NOD2-mediated inflammation.

Materials and methods

Animal studies. A total of 27 male Sprague-Dawley rats, aged 8 weeks and weighing 280-300 g, were purchased from the Laboratory Animals Center of Shandong University. The animals were housed in standard cages and maintained under standard conditions at a constant room temperature of 20-25˚C, a humidity of 40-70% and a 12/12 h light/dark cycle, with unrestricted access to food and water. The methods for generating a kidney I/R injury model were as follows: The rats were anesthetized via intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Subsequently, the left renal artery and vein were exposed via an abdominal midline incision and separated. Ischemia of the left kidney was induced by occluding the artery with non-traumatic microvascular clamps. The right renal artery was immediately separated from the branch originating from the abdominal aorta and occluded by non-traumatic microvascular clamps. The kidney color then changed from red to black-red on visual inspection, which indicated that the cessation of blood flow was successful. At 50 min after induction of ischemia, the clamps were removed and the color of the kidneys returned to red, indicating reperfusion. The incisions were sutured, followed by the injection of penicillin and saline (30 μl/g body weight) to replenish fluid loss. Reperfusion lasted for 24 h. The rats were allowed to recover from anesthesia between I/R and the endpoint of the experiment 24 h later. Ligustrazine-treated rats were administered ligustrazine hydrochloride (40 mg/kg body weight; Harbin Medisan Pharmaceutical, Co., Ltd.) via intraperitoneal injection once every 6 h during the reperfusion period (20-22). During surgery, all rats were placed on a homeothermic pad to maintain body temperature at 37˚C, and wet warm gauze was used to cover the incision to keep the tissue moist. After 24 h of reperfusion, the rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium, and then rapidly sacrificed using CO_2 asphyxiation, with a fill rate of 20% of the chamber volume/min. Death was confirmed using a combination of criteria, including lack of pulse, breathing, corneal reflex, response to a firm toe pinch and graying of the mucous membranes, which conformed to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition (23). Plasma and tissue samples were collected and stored at -80˚C or fixed in 4% formaldehyde at 4˚C for 24 h for analysis.

Assessment of renal function. Blood from the heart was collected into homemade anticoagulant tubes with 3.8% sodium citrate, and serum was isolated by centrifugation at 3,000 x g for 10 min at 4˚C. Serum creatinine (SCr) and blood urea nitrogen (BUN) were measured using creatinine assay kits and urea assay kits in accordance with the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Histological assessment. Formalin-fixed kidneys were embedded in paraffin, and 4-μm sections were stained with hematoxylin for 5 min and eosin for 1-3 min at room temperature (25˚C). Histological scoring was conducted in a blinded manner. The percent injury in tubules of the outer medulla based on cell blebbing or vacuolization and/or necrosis was scored as follows: 0 (none); 1 (0-10%); 2 (11-25%); 3 (26-45%); 4 (46-75%); or 5 (>75%). A total of 10 high-power fields (magnification, x200) per section were examined by Leica DM 6000 B light microscope (Leica Microsystems GmbH).

Immunohistochemical examination. Paraffin-embedded renal tissue sections were used for immunohistochemistry and the samples were examined using a Leica DM 6000 B light microscope (Leica Microsystems GmbH). The sections were incubated with primary polyclonal antibodies against NOD2 (cat. no. ab197030, 1:200, Abcam) and caspase 3/cleaved caspase 3 (cat. no. WL02117, 1:100, Wanleibio Co., Ltd.) at 4˚C overnight. Subsequently, the sections were incubated with the secondary HRP-goat anti-rabbit IgG antibodies for 30 min at room temperature (25˚C) according to the rabbit polymer detection system (PV-6001; ZSGB-BIO).

RNA extraction and reverse transcription quantitative PCR (RT-qPCR) analysis. Total RNA was isolated from the kidney using TRNzol reagent (Tiangen Biotech Co., Ltd.) and converted to complementary DNA by RT kit (Tiangen Biotech Co., Ltd.) at 70˚C for 5 min, 37˚C for 5 min, 42˚C for 60 min and 70˚C for 10 min. qPCR reactions (20 μl) were performed with SYBR® Premix Ex Taq™ (Takara Bio Inc.), and the thermocycling conditions were as follows: 94˚C for 5 min, 95˚C for 30 sec, followed by 40 cycles at 59˚C for 30 sec, 72˚C for 30 sec, 72˚C for 10 min and 65-95˚C for 15 min. Bio-Rad iCycler system software, version 3.1 (Bio-Rad Laboratories, Inc.) was used for quantitative data analysis. The specific primers were as follows: Forward, 5'-TACCTGAGAAAG CACCACCG-3' and reverse, 5'-GCACTGACAGCCAAGTAG AACG-3' for NOD2; and forward, 5'-TGCATCCTGCACCAC CAACTGC-3' and reverse, 5'-ACAGCCTTGGCAGCACCAG TGG-3' for the housekeeping gene GAPDH. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative mRNA expression (24).

Cell culture and treatments. Rat proximal tubule epithelial cells (NRK-52E cells, Falcon, BD Biosciences) were cultured in serum-free Dulbecco's modified Eagle's medium (HyClone;

Table I. Physical and biochemical parameters of experimental animals.

Variables	Sham	I/R	I/R + Ligu
Body weight (g)	222±18	220±20	223±15
Blood urea nitrogen (mmol/l)	8.9±0.93	42.1±4. 57[a]	22.7±0.85[b]
Serum creatinine (μmol/l)	69.8±10.26	206.1±25.73[a]	145.6±15.49[b]
N	9	9	9

Values are expressed as mean ± standard error of the mean. [a]P<0.05 vs. scramble rats, was considered statistically significant. [b]P<0.05 vs. I/R rats, was considered statistically significant. Sham, scramble rats; I/R, renal ischemia/reperfusion rats; I/R + Ligu, renal ischemia/reperfusion rats receiving ligustrazine treatment.

GE Healthcare Life Sciences) at pH 7.4 and 37˚C with 5% CO_2. The medium was changed 2 h before all the experiments were performed.

In vitro experiments were performed using two models to mimic hypoxic conditions. The first model included incubating NRK-52E cells with different concentrations of $CoCl_2$ (0, 100, 250 and 500 μM) for 12 h (anoxia) (13). The second model involved oxygen and glucose deprivation (OGD) followed by reoxygenation, in which NRK-52E cells were incubated in a hypoxic environment for 2 h (1%O_2 and glucose-free buffer), which was followed by reoxygenation for 24 h. Subsequently, in vitro reperfusion was achieved by incubating cells in normal medium for 24 h (recovery); ligustrazine (30 and 50 μM) was added immediately after reperfusion in routine culture medium for 24 h. Chloroquine (CQ; Sigma-Aldrich; Merck KGaA), an autophagy inhibitor, was also added at a concentration of 50 μM immediately after reperfusion in routine culture medium for 24 h.

Western blot analysis. Renal cortical tissues and cultured cells were homogenized in ice-cold RIPA lysis buffer with 1 mM phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). Protein quantification was determined by Enhanced BCA Protein Assay kit (P0006, Beyotime Institute of Biotechnology). Equal amounts of protein extract (40 μg) were loaded per lane and separated by 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore). Non-specific binding was blocked by incubation with 5% skimmed milk for 15 min at room temperature. The membranes were incubated with the indicated primary antibodies at 4˚C overnight and subsequently hybridized with horseradish peroxidase-conjugated secondary antibodies (ProteinTech Group, Inc.) for 1 h at room temperature. The bands were visualized using Millipore Immobilon ECL (EMD Millipore). Primary antibodies included those against NOD2 (cat. no. ab197030, 1:1,000, Abcam), LC3A/B (cat. no. 4108, 1:1,000, Cell Signaling Technology, Inc.), CD68 (cat. no. wl01218, 1:100, Wanleibio Co., Ltd.) and β-actin (cat. no. 60008-1-Ig, 1:5,000, ProteinTech Group, Inc.). The second antibodies included HRP-conjugated Affinipure Goat Anti-Mouse IgG (H + L) (cat. no. SA00001-1, 1:3,000, ProteinTech Group, Inc.) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (cat. no. SA00001-2, 1:3,000, ProteinTech Group, Inc.).

Detection of cytokines and chemokines. Chemokines and cytokines in the kidney and cells were measured using rat TNF-α, IL-6 and MCP-1 ELISA kits (RTA00, R6000B and DY3144-05, respectively; R&D Systems, Inc.) according to the procedure recommended by the manufacturer. The samples were read at 450 nm within 30 min by Spectramax Microplate Reader (Molecular Devices, LLC).

TUNEL assays. TUNEL assays were performed according to the manufacturer's instructions (Roche Diagnostics) to detect cell death in the kidney following I/R injury and ligustrazine administration, and in NRK-52E cells in response to different treatments. Samples were visualized using the Leica TCS SPE confocal system (Leica Microsystems GmbH).

Cell viability. Cell Counting Kit-8 (CCK-8) assays (Beyotime Institute of Biotechnology) were performed according to the manufacturer's instructions. NRK-52E cells were plated in 96-well plates and subjected to different treatments. CCK-8 reagents were added and the cells were incubated in a cell incubator at 37˚C for 4 h. The absorbance was measured at 450 nm using the Infinite® 200 PRO multimode microplate reader (M200 Pro/F200 Pro, Tecan Group, Ltd.).

Statistical analyses. Data are expressed as means ± standard error of the mean. The significance of the differences in mean values among groups was examined by two-way ANOVA followed by Bonferroni post hoc tests when >1 variables were compared, and others were performed by one-way ANOVA followed by Duncan's multiple range tests. SPSS software, version 17.0 (SPSS, Inc.) and GraphPad Prism 5 software (GraphPad Software, Inc.) were used for statistical analyses. P<0.05 was considered to indicate statistically significant differences.

Results

Ligustrazine protects against AKI by suppressing tubular damage and inflammatory response following I/R in a rat model. Compared with the sham-operated group, I/R resulted in higher levels of SCr and BUN in rats, which was reduced by ligustrazine treatment (Table I). Histological examination following H&E staining revealed severe morphological kidney injury, with scattered single cell necrosis or desquamation of proximal tubular cells with intact basement membranes, loss
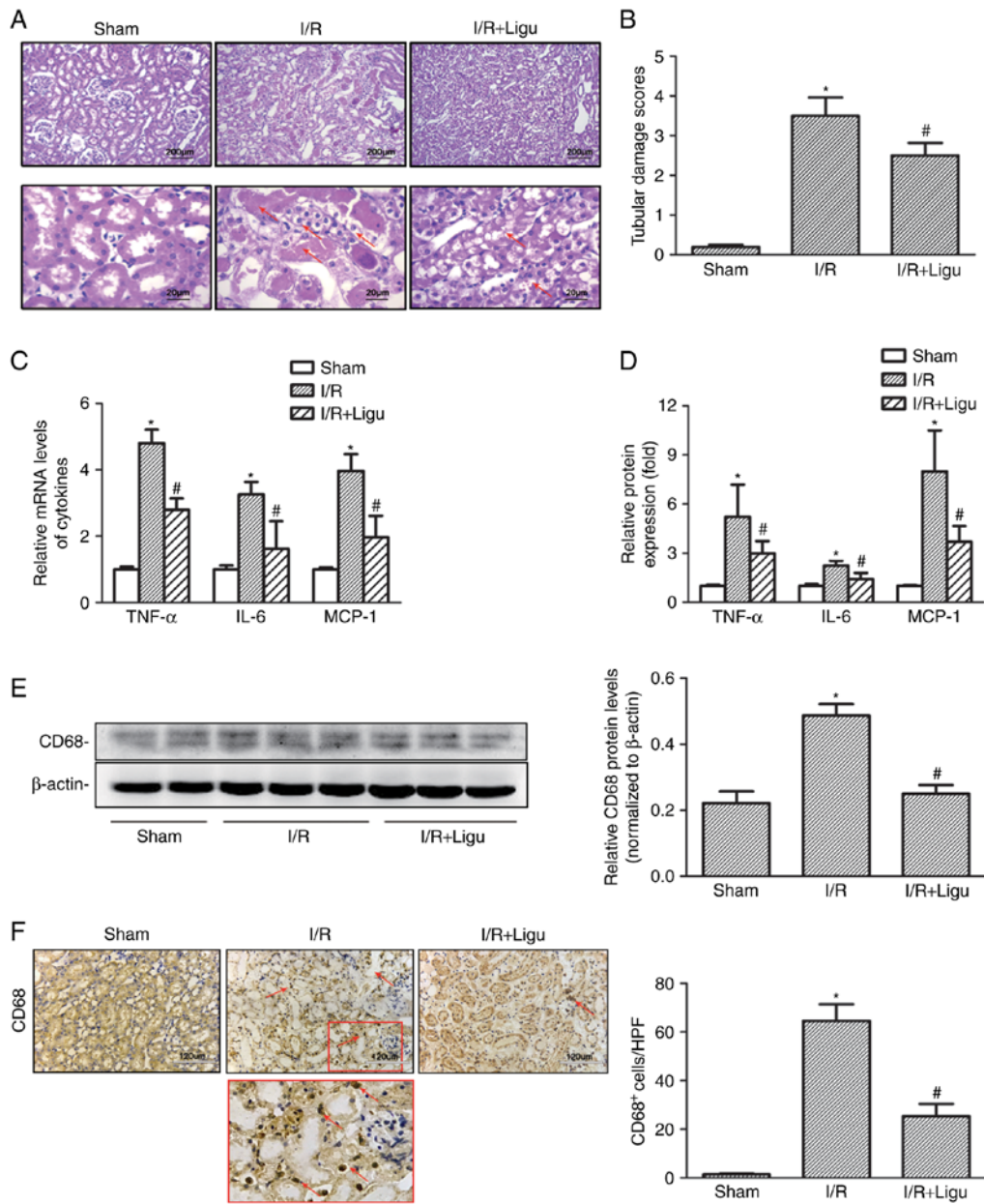
Figure 1. Ligustrazine protects against AKI by suppressing tubular damage and inflammatory responses following I/R in a rat model. (A) Representative photomicrographs showing the morphological changes of kidneys from different groups of rats. Red arrows indicated morphological kidney injury with scattered single cell necrosis or desquamation of proximal tubular cells with intact basement membranes, loss of the brush border and tubule dilation. Bars, 200 μm at lower magnification (x40). Bars, 20 μm at higher magnification (x400). (B) Quantitative assessment of tubular damage from different groups of rats. (C) RT-qPCR analysis showing the levels of proinflammatory mediators (TNF-α, IL-6 and MCP-1) in different groups of rats. (D) ELISA was used to evaluate the levels of proinflammatory mediators in different groups of rats. (E) Representative western blots and summarized data showing the protein levels of CD68 in kidneys from different groups. (F) Representative photomicrographs of CD68 IHC staining in kidneys from different groups. Bars, 120 μm (magnification, x200). *P<0.05 vs. sham-operated rats (n=9), #P<0.05 vs. I/R rats (n=9). Ligu, ligustrazine; AKI, acute kidney injury; I/R, ischemia/reperfusion; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein.

of the brush border and tubule dilatation subsequent to I/R injury, which were alleviated in the ligustrazine treatment group (Fig. 1A and B). Ligustrazine was also found to reduce the levels of pro-inflammatory mediators, including tumor necrosis factor (TNF)-α, IL-6 and monocyte chemoattractant protein (MCP)-1, in renal tissue after I/R injury, based on RT-qPCR analysis (Fig. 1C and D). Furthermore, western blot analysis demonstrated that the protein levels of CD68 were also suppressed by ligustrazine (Fig. 1E), and the number of infiltrating CD68+ macrophages was also decreased in the ligustrazine treatment groups (Fig. 1F). Therefore, the

downregulation of the aforementioned cytokines and CD68+ macrophages indicated that ligustrazine may protect against AKI by suppressing tubular damage and inflammatory response after I/R in a rat model.

Ligustrazine inhibits the upregulation of NOD2 following I/R. RT-qPCR (Fig. 2A) and western blot (Fig. 2B) analyses demonstrated that the upregulation of NOD2 expression following I/R injury was inhibited by ligustrazine treatment at both the mRNA and protein levels, respectively. Immunohistochemical staining also identified high expression of NOD2 in the
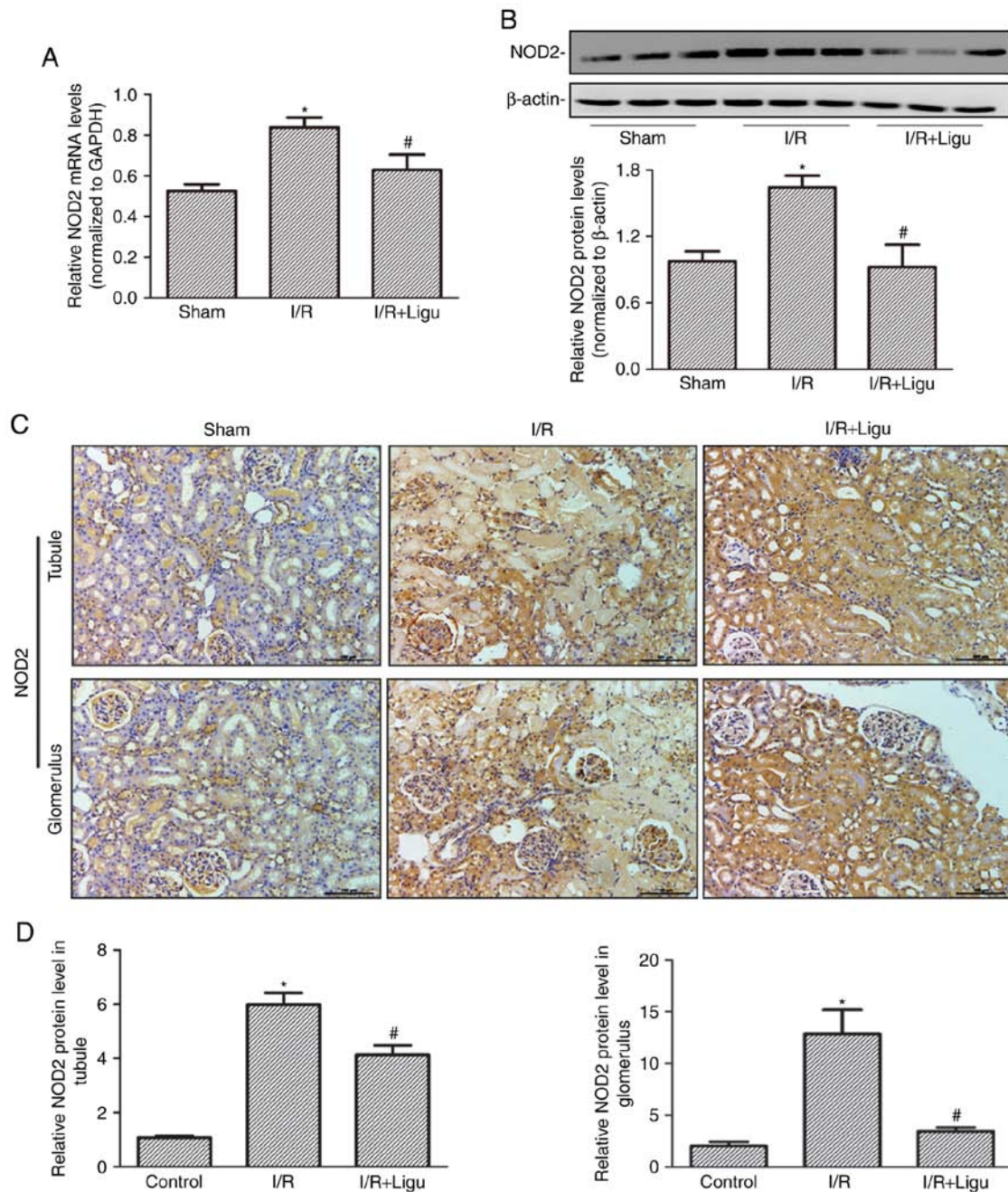
Figure 2. Ligustrazine inhibits the upregulation of NOD2 following I/R in a rat model. (A) Relative quantitation of mRNA levels of NOD2 by RT-qPCR analysis in kidneys from different groups. (B) Representative western blots and summarized data showing the protein levels of NOD2 in kidneys from different groups. (C) Representative photomicrographs of NOD2 IHC staining in the tubules and glomeruli of kidneys from different groups. Bars, 100 μm (magnification, x200). (D) Quantitative analysis of IHC staining showing increased expression of NOD2 in the tubules and glomeruli of rats after I/R, which was significantly decreased with ligustrazine administration. $^*P<0.05$ vs. sham-operated rats (n=9), $^{\#}P<0.05$ vs. I/R rats (n=9). Ligu, ligustrazine; I/R, ischemia/reperfusion; NOD2, nucleotide-binding oligomerization domain-containing 2; IHC, immunohistochemistry.

tubules and glomerulus following I/R, which was inhibited by ligustrazine treatment (Fig. 2C and D).

Ligustrazine inhibits apoptosis of kidney cells following I/R. Ligustrazine reduced cell death, as demonstrated by TUNEL staining and analysis (Fig. 3A). The inhibition of apoptosis by ligustrazine was further confirmed by caspase 3/cleaved caspase 3 immunohistochemistry (Fig. 3B) and western blotting (Fig. 3C). These results collectively indicated that ligustrazine exerted a renoprotective effect by reducing apoptosis of kidney cells and promoting renal function following I/R injury.

Ligustrazine suppresses NOD2 expression induced by CoCl₂ or hypoxia in rat NRK-52E cells. Proximal tubule injury is prominent during AKI; therefore, rat proximal tubule epithelial cells (NRK-52E cells) were treated with CoCl₂ and OGD to model the effect of AKI induced by hypoxia *in vitro*. The upregulation of NOD2 expression with different concentrations of CoCl₂ was demonstrated by western blot analysis (Fig. 4A). The expression of NOD2 was shown to increase after OGD treatment followed by reoxygenation for 24 h (Fig. 4B). The effect of ligustrazine on the upregulation of NOD2 expression was then detected in both *in vitro* models in NRK-52E cells.
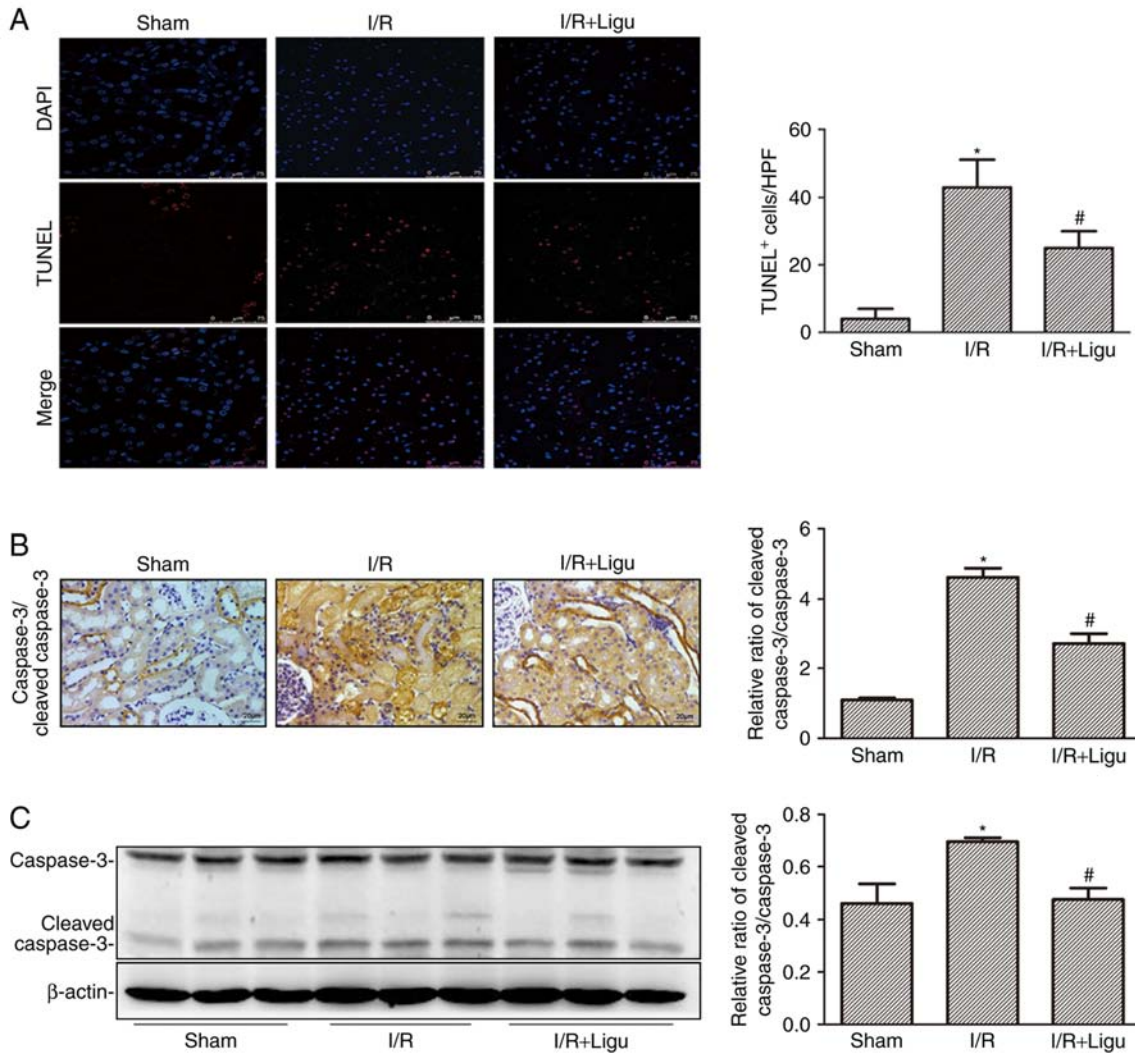
Figure 3. Ligustrazine inhibits kidney apoptosis after I/R. (A) TUNEL assays were performed to assess renal cell death. Nuclei were visualized using DAPI staining. Quantitative analysis of TUNEL+ cells (numbers per high-power field). Bars, 75 μm. (B) Representative photomicrographs of caspase 3/cleaved caspase 3 immunohistochemical staining in kidneys from different groups. Bars, 20 μm (magnification, x400). (C) Representative western blots and summarized data showing the levels of the ratio of caspase 3/cleaved caspase 3 in kidneys from different groups. *P<0.05 vs. sham-operated rats (n=9), #P<0.05 vs. I/R rats (n=9). Ligu, ligustrazine; I/R, ischemia/reperfusion.

Western blot analysis revealed that ligustrazine at 50 μM was able to significantly inhibit the upregulation of NOD2 expression induced by CoCl$_2$ (500 μM) and OGD followed by reoxygenation for 24 h (Fig. 4C and D).

Ligustrazine-mediated NOD2 downregulation is blocked by inhibiting autophagy in NRK-52E cells. Inflammation and autophagy are two inextricably linked and important pathophysiological processes. Since autophagy was previously shown to protect the proximal tubule from degeneration and acute ischemic injury (25), it was inferred that the anti-inflammatory effect of ligustrazine was mediated by autophagy. Consequently, this was tested in *in vitro* studies.

The differential autophagy response to CoCl$_2$-induced hypoxia in rat NRK-52E cells was demonstrated by western blotting of LC3A/B-II/I. The ratio of LC3A/B-II/I increased after treatment with a lower concentration of CoCl$_2$, indicating that this treatment could induce autophagy, whereas at a concentration of 500 μM CoCl$_2$, the LC3A/B-II/I ratio was significantly decreased compared with that with 100

and 250 μM, indicating that the induction of autophagy was impaired with CoCl$_2$ treatment at higher concentration (Fig. 5A). Moreover, the ratio of LC3A/B-II/I following CoCl$_2$ treatment at a concentration of 500 μM increased significantly with 50 μM ligustrazine (Fig. 5B), suggesting that the impaired autophagy recovered following ligustrazine treatment.

Next, the association between the inhibition of inflammation and the induction of autophagy was addressed. Autophagy was inhibited with CQ. As an inhibitor of autophagy, CQ raises the lysosomal pH and ultimately inhibits the fusion between autophagosomes and lysosomes, thus preventing the maturation of autophagosomes into autolysosomes, and blocking a late step of macroautophagy. Thus, an increase in LC3-I and LC3-II is expected in the presence of CQ (26,27). Following CoCl$_2$ treatment (500 μM), the inhibitory effect of ligustrazine on the protein levels of NOD2 was diminished when autophagy was inhibited, as determined by western blot analysis (Fig. 5C); moreover, western blot analysis demonstrated that, after OGD treatment followed by reoxygenation (24 h), the inhibitory
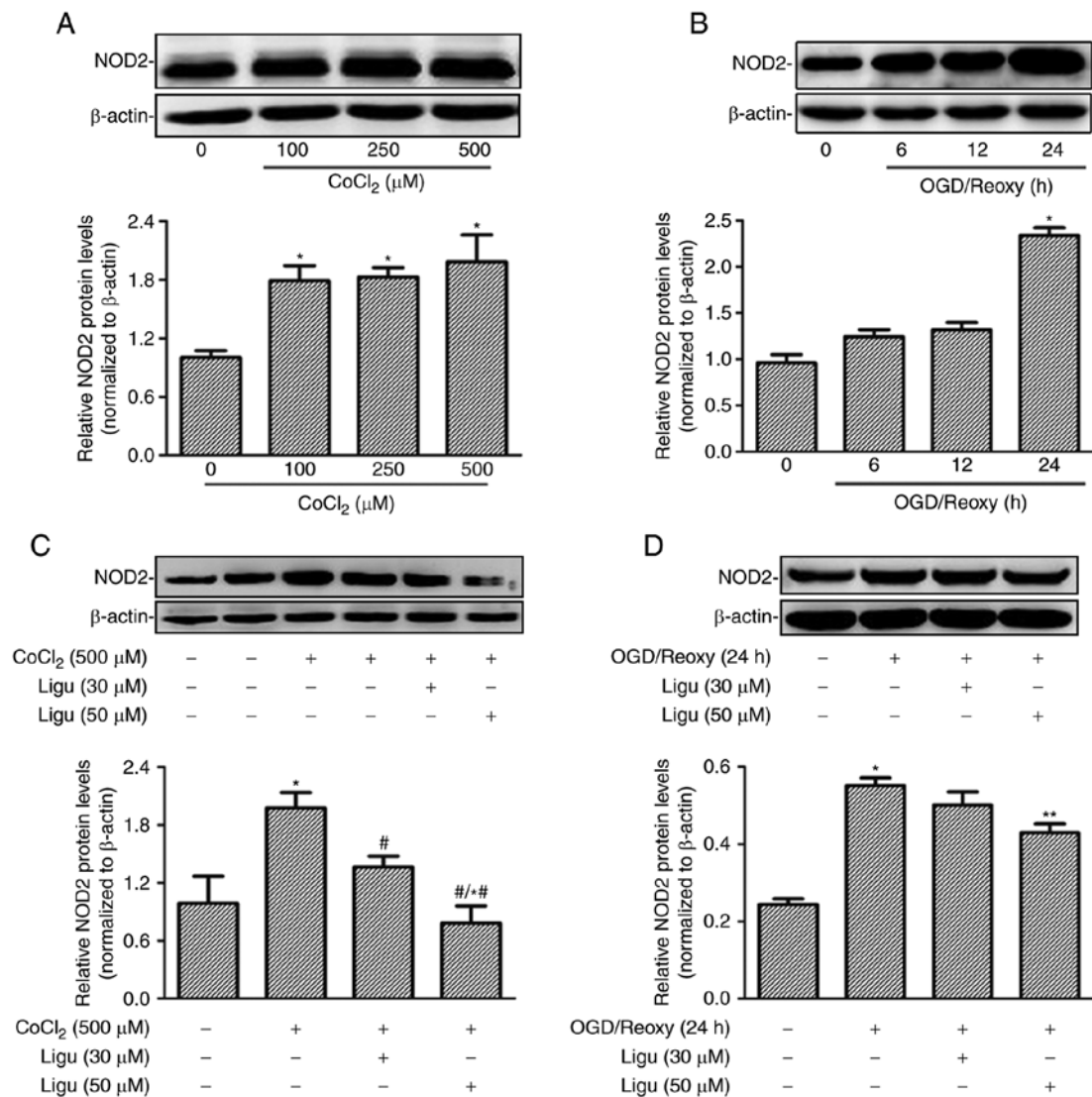
Figure 4. Ligustrazine inhibits NOD2 expression response to different hypoxia models in rat proximal tubule epithelial cells (NRK-52E cells). (A) Representative western blots and summarized data showing the protein levels of NOD2 in response to CoCl₂ treatment at different concentrations. The treatment duration of CoCl₂ was 12 h. (B) Representative western blots and summarized data showing the protein levels of NOD2 in response to different recovery times following OGD. (C) Representative western blots and summarized data showing the protein levels of NOD2 in response to ligustrazine (30 and 50 μM) after CoCl₂ treatment. (D) Representative western blots and summarized data showing the protein levels of NOD2 in response to ligustrazine (30 and 50 μM) after OGD treatment followed by reoxygenation (24 h). *P<0.05 vs. control, #P<0.05 vs. CoCl₂ treatment group (100 and 250 μM), *,#P<0.05 vs. ligustrazine treatment group (30 μM), **P<0.05 vs. OGD treatment group. All the experiments were performed in triplicate. Ligu, ligustrazine; I/R, ischemia/reperfusion; NOD2, nucleotide-binding oligomerization domain containing 2; OGD, oxygen and glucose deprivation; reoxy, reoxygenation.

effect of ligustrazine on NOD2 levels was suppressed when autophagy was inhibited (Fig. 5D).

Ligustrazine-mediated downregulation of inflammation and apoptosis is blocked by inhibiting autophagy in NRK-52E cells in vitro. Furthermore, RT-qPCR analysis demonstrated that ligustrazine treatment inhibited the production of pro-inflammatory mediators, including TNF-α, IL-6, and MCP-1, and this effect was blocked when autophagy was inhibited, after OGD treatment followed by reoxygenation for 12 h (Fig. 6A). These results suggest that the anti-inflammatory effect of ligustrazine depends to a certain extent on the induction of autophagy. CCK-8 assays demonstrated that cell viability in response to ligustrazine decreased after autophagy was inhibited by CQ (Fig. 6B). TUNEL assays were also performed to assess the effect of ligustrazine on

cell death induced by CoCl₂ treatment. Following inhibition of autophagy, the effect of ligustrazine on reducing cell death was attenuated (Fig. 6C).

Discussion

The present study demonstrated that ligustrazine downregulates autophagy-induced NOD2 expression, attenuates cell death and improves renal function following I/R injury in a rat model. Furthermore, to the best of our knowledge, the present study was the first to identify that the inhibitory effect of ligustrazine on NOD2 depends on the induction of autophagy in NRK-52E cells treated with CoCl₂ in vitro.

I/R injury is a common clinical problem that has been attracting increasing attention in an attempt to delineate putative triggers of renal injury and design novel therapeutic
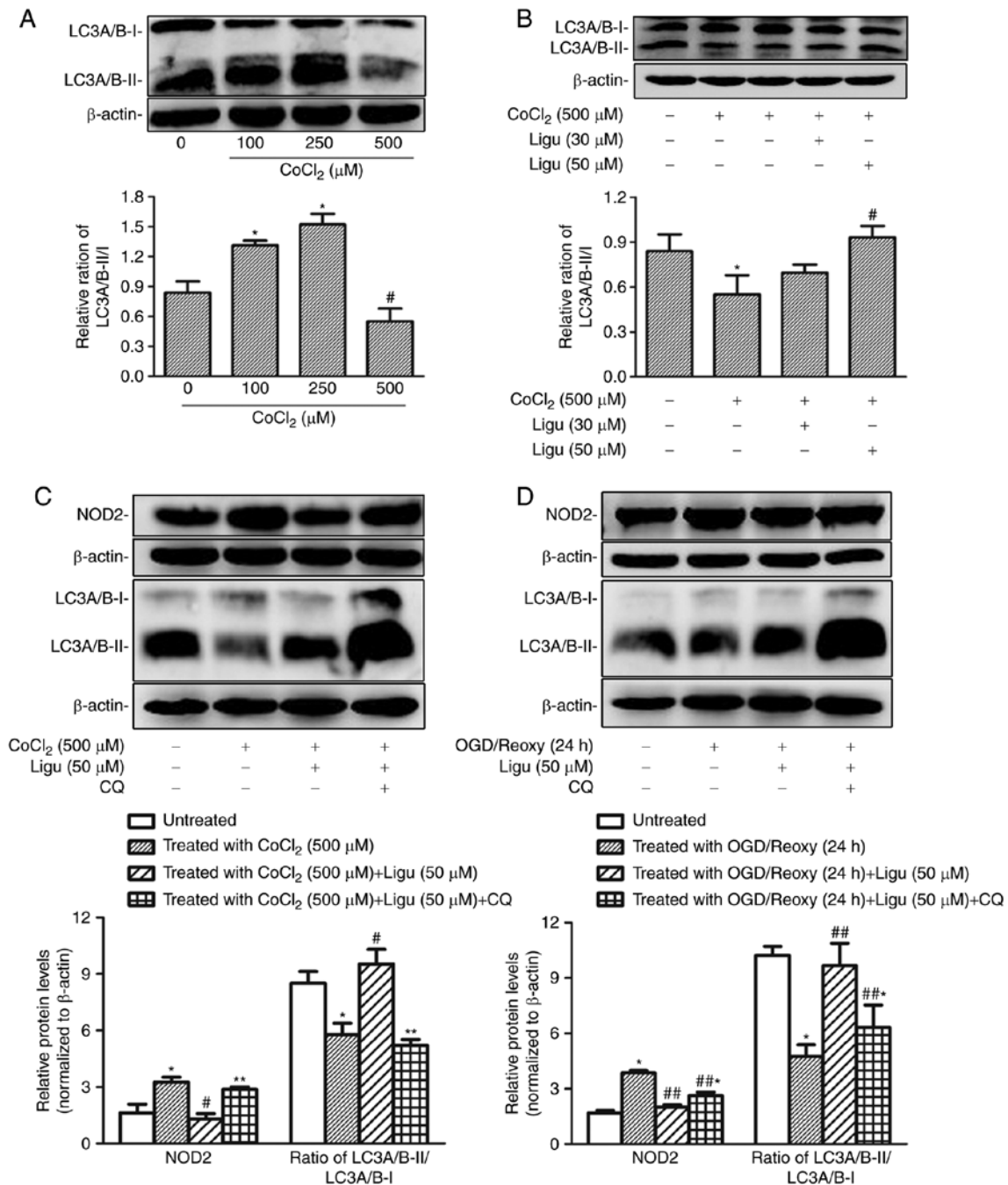
Figure 5. Ligustrazine-mediated NOD2 downregulation is blocked by inhibiting autophagy in NRK-52E cells *in vitro*. (A) Representative western blots and summarized data showing the relative ratio of LC3A/B-II/I in response to $CoCl_2$ treatment at different concentrations. The treatment duration of $CoCl_2$ was 12 h. (B) Representative western blots and summarized data showing the relative ratio of LC3A/B-II/I in response to ligustrazine (30 and 50 μM) after $CoCl_2$ treatment. The duration of $CoCl_2$ treatment was 12 h and the duration of ligustrazine treatment was 24 h. (C) Representative western blots and summarized data showing the protein levels of NOD2 in response to ligustrazine (50 μM) after $CoCl_2$ treatment when the autophagy was inhibited by CQ. (D) Representative western blots and summarized data showing the protein levels of NOD2 in response to ligustrazine after OGD treatment followed by reoxygenation (24 h) when the autophagy was inhibited by CQ. [*]P<0.05 vs. control, [#]P<0.05 vs. $CoCl_2$ treatment group, [**]P<0.05 vs. ligustrazine (50 μM) after $CoCl_2$ treatment group, [##]P<0.05 vs. OGD treatment followed by reoxygenation (24 h) group. [##,*]P<0.05 vs. ligustrazine (50 μM) after OGD treatment followed by reoxygenation (24 h) group. All the experiments were performed in triplicate. Ligu, ligustrazine; I/R, ischemia/reperfusion; reoxy, reoxygenation; NOD2, nucleotide-binding oligomerization domain-containing 2; OGD, oxygen and glucose deprivation; CQ, chloroquine.

strategies. Ligustrazine is a natural small-molecule compound that was previously characterized and found to be associated with few side effects and favorable bioavailability *in vivo* (28), and determining its targets and mechanism of action may be beneficial for its clinical use. Recently, the anti-inflammatory role of ligustrazine was demonstrated in patients with rheumatic heart disease, a mouse model of allergic asthma and

after spinal cord I/R injury in rats (17,18,29), suggesting that these anti-inflammatory effects may underlie its renoprotective properties. The reduced level of pro-inflammatory mediators and infiltration of CD68^{+} macrophages in renal tissue by ligustrazine following I/R indicated that ligustrazine protects against AKI by suppressing inflammatory response. Furthermore, ligustrazine was found to suppress the expression
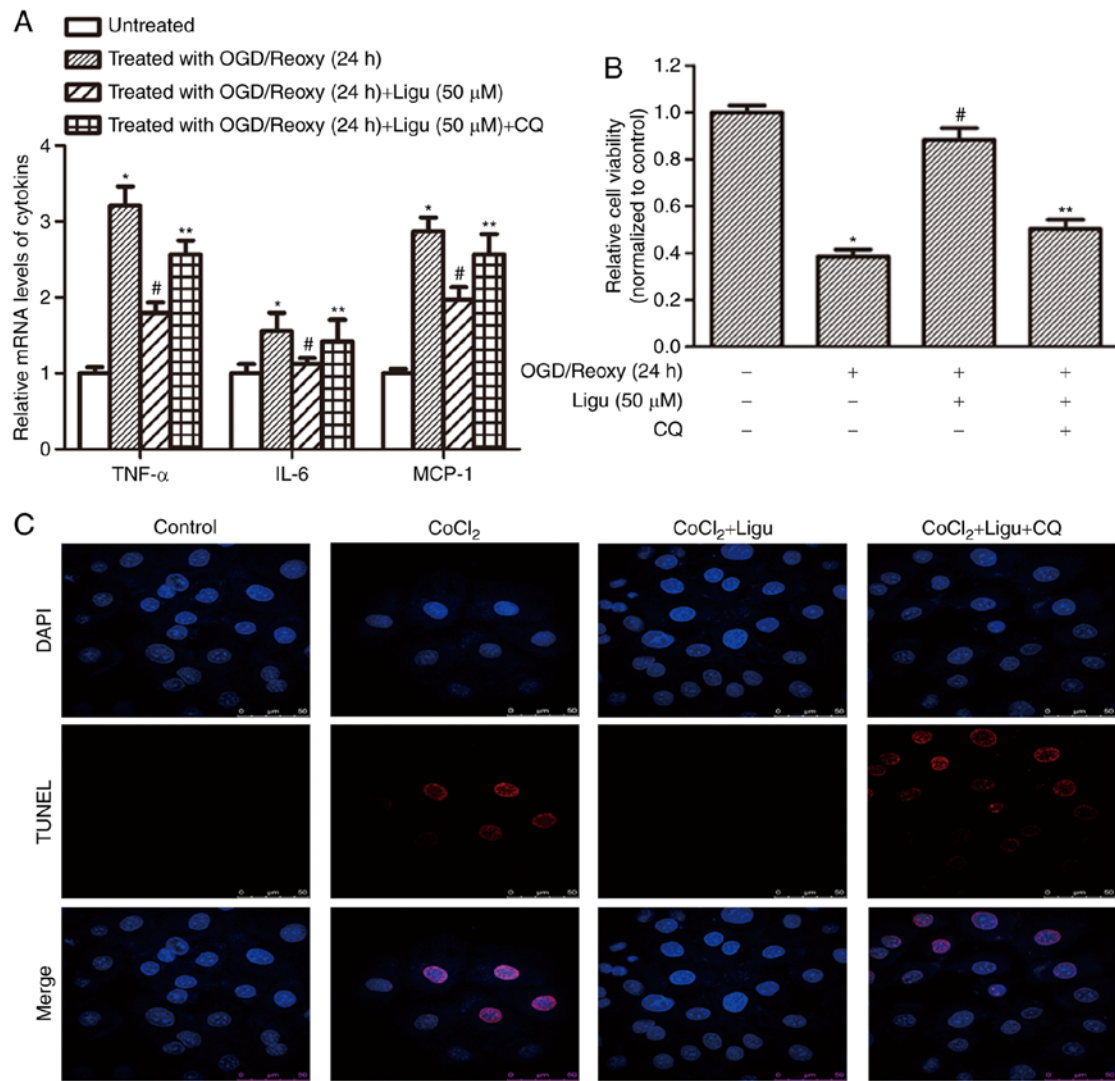
Figure 6. Ligustrazine-mediated downregulation of inflammation and apoptosis is blocked by inhibiting autophagy in NRK-52E cells *in vitro*. (A) RT-qPCR analysis demonstrated that ligustrazine treatment suppressed the levels of proinflammatory mediators, including TNF-α, IL-6 and MCP-1, and this effect was attenuated when autophagy was inhibited by CQ. The duration of reoxygenation was 24 h. (B) CCK-8 assays demonstrated that cell viability in response to ligustrazine after OGD followed by reoxygenation for 24 h was blocked when the autophagy was inhibited by CQ. (C) TUNEL assays showing the changes of cell death in response to ligustrazine after $CoCl_2$ treatment when autophagy was inhibited by CQ. The treatment duration of $CoCl_2$ was 12 h, and that of ligustrazine and CQ was 24 h. *P<0.05 vs. control, #P<0.05 vs. OGD treatment, **P<0.05 vs. ligustrazine treatment group. All the experiments were performed in triplicate. Ligu, ligustrazine; I/R, ischemia/reperfusion; reoxy, reoxygenation; OGD, oxygen and glucose deprivation; CQ, chloroquine; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein.

of NOD2 and enhance autophagy in the injured kidney cortex following I/R injury in rats.

PRRs have been suggested to be important triggers of ischemic injury (30,31). NOD2 is a well-characterized member of the NLR family, which mediates the activation of NF-κB and mitogen-activated protein kinases in response to muramyl dipeptide, a peptidoglycan motif that is present in all gram-positive and gram-negative bacteria (32). The activation of NOD2 mainly leads to the production of pro-inflammatory cytokines and the expression of co-stimulatory and adhesion molecules, which are dependent on NF-κB activation (33). NOD2 was suggested to not only promote renal injury by exacerbating inflammation and podocyte insulin resistance during diabetic nephropathy, but also to participate in renal I/R, which is negatively regulated by progranulin, a protective autocrine growth factor involved in AKI (11,13). In the present study, it was confirmed that the expression of NOD2 increases

in the injured kidney cortex following renal I/R injury and in NRK-52E cells treated with $CoCl_2$, a chemical reagent that promotes a cellular anaerobic state *in vitro*. Therefore, it was hypothesized that NOD2 may serve as a therapeutic target for AKI. The present study was the first to demonstrate that ligustrazine is associated with the innate immune response via NOD2.

To address the mechanism through which ligustrazine suppresses NOD2 expression, autophagy, an important physiological process that occurs during AKI, was investigated. In view of the current data, autophagy induction in response to multiple stresses induced by AKI is cytoprotective (34). *In vitro*, different levels of autophagy were demonstrated in NRK-52E cells treated with different concentrations of $CoCl_2$. With a high concentration of $CoCl_2$, the ratio of LC3A/B-II/I was significantly lower compared with that with low concentrations of $CoCl_2$, suggesting that a high concentration of

$CoCl_2$ (500 μM) can cause serious cell damage through the induction of hypoxia and subsequent reoxygenation. Thus, autophagy was impaired to the extent that its protective effect against cell injury was lost. Therefore, a high concentration of $CoCl_2$ was employed to test the effect of ligustrazine on the reactivation of autophagy. The results demonstrated that autophagy inhibition was significantly reversed by ligustrazine; in addition, the expression of NOD2 was also inhibited. We hypothesized that there is a functional association between NOD2 and autophagy. To confirm this hypothesis, autophagy was inhibited using an autophagy inhibitor, and the suppression of NOD2 by ligustrazine was partially abrogated. This indicated that the anti-inflammatory effect of ligustrazine depends on the induction of autophagy, at least to some extent. These results also provided more evidence supporting that NOD2 is a key mediator through which autophagy regulates the innate immune response and inflammation (35). Mammalian target of rapamycin (mTOR) is a key negative regulator of autophagy. Ligustrazine was previously shown to disrupt phosphoinositide 3-kinase/AKT/mTOR signaling in angiotensin II-activated hepatic stellate cells (36). Ligustrazine was also reported to prevent apoptosis by promoting autophagy in an AMP-activated protein kinase- and mTOR pathway-dependent manner in bone marrow mesenchymal stem cells (37).

Autophagy consists of an intracellular degradation system that is required for cellular homeostasis, and basal autophagy is important for proximal tubule homeostasis. Cellular stress during AKI, including hypoxia, oxidative injury and nutrient deprivation, contributes to the induction of autophagy. Conditional kidney proximal tubule-specific Atg5- or Atg7-knockout mice were used to demonstrate the renoprotective effect of autophagy following renal I/R injury (25,38). However, the precise mechanisms underlying autophagy during AKI are unclear. In addition to the fact that the effect of ligustrazine on NOD2 downregulation was partially blocked by inhibiting autophagy, other studies also demonstrated the association of autophagy with NLRs in immune cells, which included the identification of cross-talk-related processes. The autophagy-related protein ATG16L1 was found to suppress inflammation selectively induced by the activation of NOD2 (35,39,40), and autophagy was also found to play an important role as a macrophage-intrinsic negative regulator of the NLRP3 inflammasome (32,33). Thus, the autophagic machinery comprises a key cellular monitoring system that prevents excessive NLRP3 inflammasome activation. Moreover, NOD2 and NLRP4 can regulate autophagic processes by associating with the autophagy-related protein Beclin 1 (41). However, the detailed underlying mechanisms remain elusive.

Hypoxia-induced tubular epithelial damage is prominent during renal I/R injury, whereas post-reperfusion inflammation is a pathognomonic characteristic, involving multiple cell types and cell signals (8). Furthermore, glomerular injury and the excessive expression of adhesion molecules followed by leukocyte infiltration must be taken into consideration. A limitation of the present study was that the effect of ligustrazine on glomerular injury was not extensively addressed; in addition, whether ligustrazine regulates the association between NOD2 and autophagy in renal immune cells was not discerned. In the future, the effect of ligustrazine on the activation of NOD2 in glomerular cells such as podocytes, endothelial cells, or mesangial cells should be addressed. Another limitation was the intervention time for ligustrazine. In the pre-experiment, we constructed models of three time points, namely 24, 48 and 72 h, according to different reperfusion time. H&E staining revealed that kidney injury in rats was the most severe at 24 h after I/R; 48 h later, the kidney injury in rats did not become worse, and gradually recovered with the prolongation of reperfusion time. This was in accordance with previous findings (42). Therefore, in terms of timing, we focused on 24 h after reperfusion. Pre-treatment with drugs prior to ischemia was previously found to promote renal function and attenuate inflammation after renal I/R injury (13). In the present study, we only investigated the effect of ligustrazine treatment after ischemia, whereas the effect of ligustrazine pre-treatment was not assessed. Additional studies must be conducted to explore the regulation of inflammation by autophagy following pre-treatment with ligustrazine.

Apoptosis is a major pathological process in AKI. There is cross-talk between autophagy and apoptosis in addition to inflammation (34,43). In the present study, ligustrazine helped to preserve renal function in a model of I/R injury in part by reducing apoptosis. *In vitro*, the effect of ligustrazine on inhibiting cell death was diminished after autophagy was suppressed. Thus, ligustrazine may be implicated in homeostasis and renal pathophysiology via an interaction between autophagy and apoptosis. Of note, although it was demonstrated that the inhibition of inflammation and apoptosis by ligustrazine partly depended on the induction of autophagy, other researchers have demonstrated that ligustrazine pre-treatment may reverse the increase in autophagy observed in an arsenic-induced nephrotoxicity cell model, which is considered to induce necrosis (44). These opposite results suggest that autophagy is a complex process, and its role in the protection against diseases requires comprehensive consideration. Thus, interfering with the regulation of inflammation by autophagy may be of great value in the treatment for kidney injury (45).

In conclusion, the present study was, to the best of our knowledge, the first to provide evidence that ligustrazine can protect against renal injury after AKI by suppressing NOD2-mediated inflammation, which is partly dependent on the induction of autophagy. Ligustrazine was investigated for its potential anti-inflammatory effects and its ability to induce autophagy, which may broaden its clinical application spectrum for immune- and autophagy-related diseases.

Authors' contributions

GJ, RX, WY and PD were responsible for study design, data collection, statistical analysis and manuscript preparation; LZ, WS, XM, HH, YH and LW were responsible for partial data collection, statistical analysis and literature search; PD supervised the entire project, study design, data analysis and writing of the manuscript. All authors have reviewed and approved the final manuscript.

Availability of materials and data

The data that support the findings of the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Binzhou Medical University (protocol no. 2015-008).

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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