Original Research

4-Octyl itaconate alleviates renal ischemia reperfusion injury by ameliorating endoplasmic reticulum stress via Nrf2 pathway

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Impact statement

This study demonstrates that 4-octyl itaconate (4-OI) can protect renal ischemia-reperfusion injury (IRI), one of the most commonly occurring serious complication of inpatients. 4-OI is a cell-permeable derivative of itaconate, which is a by-product of the Krebs cycle and has been regarded as an important link between metabolism and immune defense. For the first time, we demonstrated that 4-OI could mitigate renal histomorphology damage, dysfunction, and endoplasmic reticulum stress (ERS) caused by IRI, both *in vivo* and *in vitro*. Furthermore, this study proved that the protective effect of 4-OI was dependent on the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. We believe that our study makes a significant contribution to the literature because it provides novel insights into the function of 4-OI in the alleviation of ERS. Furthermore, we believe that our study offers a new and reliable therapeutic option for renal IRI.

Abstract

Renal ischemia-reperfusion injury (IRI) is a common clinical complication of multiple severe diseases. Owing to its high mortality and the lack of effective treatment, renal IRI is still an intractable problem for clinicians. Itaconate, which is a metabolite of cis-aconitate, can exert anti-inflammatory and antioxidant roles in many diseases. As a derivative of itaconate with high cell membrane permeability, 4-octyl itaconate (4-OI) could provide a protective effect for various diseases. However, the role of 4-OI in renal IRI is still unclear. Herein, we examined whether 4-OI afforded kidney protection through attenuating endoplasmic reticulum stress (ERS) via nuclear factor erythroid-2-related factor 2 (Nrf2) pathway. To observe the effects of 4-OI on alleviating renal pathologic injury, improving renal dysfunction, decreasing inflammatory cytokines, and reducing oxidative stress, we utilized C57BL/6J mice with bilateral renal pedicle clamped and HK-2 cells with hypoxia/reoxygenation (H/R) exposure in our study. In addition, through western blot assay, we found 4-OI ameliorated renal IRI-induced ERS, and activated Nrf2 pathway. Moreover, Nrf2-knockout (KO) mice and Nrf2 knockdown HK-2 cells were used to validate the role of Nrf2 signaling pathway in 4-OI-mediated alleviation of ERS caused by renal IRI. We demonstrated that 4-OI relieved renal injury and suppressed ERS in wild-type mice, while the therapeutic role was not shown in Nrf2-KO mice. Similarly, 4-OI could exert cytoprotective effect and inhibit ERS in HK-2 cells after H/R, but not in Nrf2 knockdown cells. Our *in vivo* and *in vitro* studies revealed that 4-OI protected renal IRI through attenuating ERS via Nrf2 pathway.

Keywords: 4-Octyl itaconate (4-OI), ischemia-reperfusion injury (IRI), endoplasmic reticulum stress (ERS), nuclear factor erythroid-2-related factor 2 (Nrf2)

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Introduction

Acute kidney injury (AKI) is a common and serious complication of inpatients (10–15%) and its incidence rate among critically ill patients even exceeds 50%.[1] There are multiple factors that are associated with AKI, while ischemia-reperfusion injury (IRI) has been proved to be a crucial etiology.[2] Renal IRI is often secondary to surgical procedures, shock, sepsis, and other conditions that can reduce renal blood flow.[3] However, despite considerable effort, early diagnosis and effective therapy of renal IRI remain a challenge for clinicians. However, without timely and effective therapeutic intervention, renal IRI would progress to chronic kidney disease (CKD), consuming considerable health-care resources.[4] Thus, there is an urgent need to seek a novel efficient treatment modality for renal IRI.

According to previous research, endoplasmic reticulum stress (ERS) of renal tubular cells is crucial in the progression of renal IRI.[5] The endoplasmic reticulum (ER) is the largest organelle of eukaryocytes and plays a significant role in the synthesis of proteins.[6] There are many reasons for unfolded or misfolded proteins to accumulate in ER, and this abnormal

accumulation can induce a stress response, which is also known as ERS.[7] As a basic self-regulation mechanism of cells, ERS is crucial for the viability and function of cells and is associated with the etiology of many diseases.[8] Furthermore, ERS has been demonstrated to be strongly correlated with oxidative stress, inflammation, and tubular epithelial cell apoptosis, all of which are important and influential factors for the pathological processes of renal IRI.[3,9] As a result, ameliorating ERS is a highly effective and feasible treatment modality against renal IRI.

Itaconate (ITA) has been proved to be an important link between metabolism and immunity and has recently gained increasing attention.[10] As a by-product of the Krebs cycle, the synthesis of ITA has been demonstrated to be regulated by aconitate decarboxylase (IRG1).[11] Inflammation can rapidly activate macrophages and subsequently upregulate the expression of IRG1, thus reprogramming the metabolic process to largely produce ITA.[10,12,13] ITA and its cell-permeable derivative 4-octyl itaconate (4-OI) exert obvious antioxidant and anti-inflammatory properties in various diseases through the regulation of different signaling pathways.[10,14–17] However, to date, the effect of 4-OI on renal IRI-induced ERS remains unclear.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a significant anti-inflammatory and antioxidant transcription factor, which is expressed in almost all organs and tissues. More importantly, the activation of the Nrf2 pathway could mitigate ERS.[18] A recent study has discovered that 4-OI can promote the entry of Nrf2 into the nucleus by alkylating cysteine residues of Keap1, which would activate the Nrf2 pathway.[14] Hence, we speculated that 4-OI may activate the Nrf2 pathway during renal IRI.

In this study, we explored whether 4-OI protects renal IRI by alleviating ERS using renal IRI mice and human renal proximal tubular epithelial (HK-2) cells exposure to hypoxia/reoxygenation (H/R). Furthermore, utilizing Nrf2-knockout (Nrf2-KO) mice and transfected Nrf2 small interfering (siRNA) HK-2 cells, we determined whether the Nrf2 pathway was associated with the therapeutic effect of 4-OI.

Materials and methods

Animal model

Jinan Pengyue laboratory Animal Breeding Co. Ltd. supplied C57BL/6J mice (weighing 20–25 g and aged 6–8 weeks). Jiangsu Huachuang Sino Pharma Co. provided well-characterized male Nrf2-KO mice on a C57BL/6J background. All animal experiments strictly followed the laboratory animal welfare ethics principles, and were approved by the Animal Care & Welfare Committee of the Affiliated Hospital of Qingdao University (No. AHQU-MAL20220218). To build a renal IRI model, anesthesia was induced with pentobarbital sodium (50 mg/kg, intraperitoneally) and maintained with 1–3% isoflurane breathing. To expose renal pedicles, the dorsal lumbar incision was made. Bilateral renal pedicles were clamped in all IRI groups. Arterial clamps were removed after 30 min of ischemia. After 24 h reperfusion, mice were euthanized and we collected fresh blood and kidney tissues immediately.[19]

To determine the protective effect of 4-OI, mice were randomly divided into four groups ($n = 5$/group): Sham group, 4-OI group, IRI group, and IRI + 4-OI group. Kidneys and pedicles were exposed but not clamped in the Sham and 4-OI groups. 4-OI (MedChemExpress, USA) dissolved in 20% cyclodextrin in saline was injected intraperitoneally (50 mg/kg) 2 h before operation.[20] The equal amount of saline was injected in the IRI group. Furthermore, to determine the role of the Nrf2 pathway in the 4-OI-mediated ameliorative effect, wild-type (WT) and Nrf2-KO mice were randomly divided into six subgroups ($n = 5$/group): WT + Sham group, WT + IRI group, WT + IRI + 4-OI group, Nrf2KO + Sham group, Nrf2KO + IRI group, and Nrf2KO + IRI + 4-OI group.

Histological analysis

After routinely embedding the paraformaldehyde-fixed mice kidney in paraffin, tissues were subjected to hematoxylin-eosin (HE) staining on 4 μm sections. Two renal pathologists blinded to treatment groups performed the pathological assessments of renal tissue. The severity of kidney damage was measured using the tubular injury score (scale: 0–4) according to a previous description.[21]

Assessment of renal function

To evaluate renal function, we investigated the concentrations of serum creatinine (SCr) and blood urea nitrogen (BUN). Following the manufacturer's instructions, fresh blood samples were analyzed through an automatic biochemical analyzer (BS-240Vet, Mindray, China).

Measurement of glutathione

The total glutathione (GSH) and oxidized glutathione (GSSG) in cells and renal tissues were measured by a commercial kit (A061-1, Jiancheng, China) according to the manufacturer's instructions. Briefly, homogenates were added to GSH or GSSH assay buffers. For detection of GSSG content, GSH was first eliminated with the thio-scavenging reagent. The total GSH and GSSG concentrations were calculated from a standard curve and normalized to protein content, and the ratio of reductive GSH to oxidative GSH was obtained as ((total GSH – 2GSSG)/GSSG).

Measurement of cytokines

The amounts of IL-1β and IL-6 in serum and supernatant were assessed via the ELISA kits (SEKM-0002 and SEKM-0007; Beijing Solarbio Science & Technology Co., Ltd., China). According to the manufacturer's instructions, 100 μL serum or supernatant have been used to coat the plate. Absorbance of samples was measured by the microplate reader (Bio-Rad, USA) and the IL-1β and IL-6 concentrations were calculated from standard curves.

Immunofluorescence staining

After triple rinsing with phosphate-buffered saline (PBS), the paraformaldehyde-fixed slices were incubated with anti-CHOP antibody (Cell Signaling Technology (CST), #2895) overnight and then with fluorescent-labeled secondary

antibody for 1 h. 4,6-Diamino-2-phenylindole was utilized to identify nuclei. Quantification of the immunofluorescent intensity was evaluated with ImageJ Software (NIH, USA).

Western blotting

Kidney tissues or cells were homogenized with lysis buffer on ice. The lysate was centrifuged at 13,000 rpm for 10 min at 4°C and then the supernatant was collected. The protein levels were assessed by bicinchoninic acid (BCA) assay (Boyetime, China). Furthermore, utilizing the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China), we extracted nuclear protein according to the manufacturer's instructions. Proteins were separated by 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk-TBST (tris-buffered saline with 0.1% Tween 20) for 2 h and incubated with primary antibodies against KIM-1 (1:2000, R&D Systems, AF1817), CHOP (1:1000, CST, #2895), BiP (1:1000, CST, #63411), ATF-6 (1:1000, CST, #65880), PERK (1:1000, CST, #3192), IRE1α (1:1000, CST, #3294), Nrf2 (1:1000, CST, #12721), HO-1 (1:2000, CST, #43966), p-PERK (1:1000, Affinity, DF7576), p-IRE1α (1:1000, Affinity, AF7150), NGAL (1:1000, Affinity, DF6816), Lamin B1 (1:2000, Affinity, BF8009), and β-actin (1:2000, Affinity, AF7018) overnight at 4°C. Next, membranes were incubated with appropriate secondary antibodies for 1 h at room temperature. The blots were visualized with Fusion Fx7 system (Vilber Lourmat, France) and analyzed with Image J (NIH, USA).

Cell treatment

HK-2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. Dulbecco's Modified Eagle's medium (DMEM; Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA) was used to culture cells at 37°C in a 5% CO_2 incubator. HK-2 cells were serum-starved for 12 h in serum-free DMEM and then fresh serum-free DMEM was replaced before exposure to H/R. A three-gas incubator was used to create a hypoxic environment (1% O_2, 94% N_2, and 5% CO_2). Cells were exposed to hypoxia for 24 h. Next, medium was replaced and HK-2 cells were cultured under normoxic conditions (5% CO_2 and 95% air) for 6 h.[22] Different doses of 4-OI were added 2 h prior to H/R exposure. Dimethyl sulfoxide (DMSO) was used for dissolving 4-OI. The cells were split into seven groups: Control group, H/R group, 4-OI group, H/R + 4-OI group, H/R + si-Nrf2 group, H/R + 4-OI + si-Nrf2 group and H/R + 4-OI + si-NC group. Equal amount of DMSO was added in groups without 4-OI. Cells in groups without H/R were incubated under normoxic conditions.

Cell viability

To select the optional concentration of 4-OI, the viability of cells was assessed using the CCK-8 assay (Beyotime, China). Cells were incubated with 10 μL CCK-8 solution per well for 2.5 h at normal temperature. After mixing, the active agent of CCK-8 can be reduced by dehydrogenases in living cells to produce yellow-colored product. The absorbance at 450 nm was recorded by a microplate reader (Bio-Rad, USA).

Transient transfection with siRNA

Nrf2 siRNA (si-Nrf2) and negative control siRNA (si-NC) were designed by Shanghai GenePharma Co, Ltd. (China). Lipofectamine 3000 reagent (Invitrogen, USA) was utilized to transfect cells with siRNAs. Real-time polymerase chain reaction (RT-PCR) was utilized to assess the effectiveness of Nrf2 silencing after 24 h of transfection.

Statistical analysis

All statistical analyses were performed with SPSS, version 24 (SPSS Inc, USA). All values are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with post hoc Bonferroni was used to analyze differences among experimental groups. Statistical significance was indicated by $P < 0.05$.

Results

4-OI protected kidney from IRI *in vivo*

Until being euthanized, all mice survived. To determine whether 4-OI pretreatment relieved renal IRI, we explored the histopathological changes and the tubular injury score based on HE staining. Mice in the IRI group exhibited noticeable pathological damage, such as dilatation of tubules, inflammatory cell infiltration, cast formation, and swelling of tubular cells, compared with the Sham group. 4-OI significantly ameliorated all of these pathological changes (see Figure 1(A)). Two blinded experienced renal pathologists performed the pathological assessments of renal tissue using the tubular scoring system. The tubular injury score of the IRI group was much higher than the Sham group; meanwhile, 4-OI greatly decreased this score ($P < 0.05$; see Figure 1(B)). Next, we detected levels of BUN and SCr to examine renal function (see Figure 1(C) and (D)). In addition, representing novel markers of renal proximal tubular damage, the expression levels of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) in kidney, were determined using western blotting (see Figure 1(H) to (J)). Both the BUN and SCr concentrations, and the NGAL and KIM-1 expressions were dramatically elevated in the IRI group, and 4-OI pretreatment significantly decreased the levels of the four markers ($P < 0.05$). To determine the 4-OI-mediated anti-inflammatory effect, we detected serum IL-1β and IL-6. As shown in Figure 1(E) and (F), exposure to IRI increased IL-1β and IL-6 concentrations, and 4-OI diminished inflammatory cytokine production ($P < 0.05$). In addition, the GSH/GSSG ratio was used to evaluate the level of oxidative stress in the kidney. While IRI could result in an apparent decrease of the GSH/GSSG ratio, this change was mitigated by 4-OI pretreatment ($P < 0.05$). Furthermore, differences between the 4-OI and Sham groups were not significant ($P > 0.05$). Thus, we surmised that 4-OI ameliorates renal pathological damage, improves renal function, and reduces inflammation and oxidative stress.

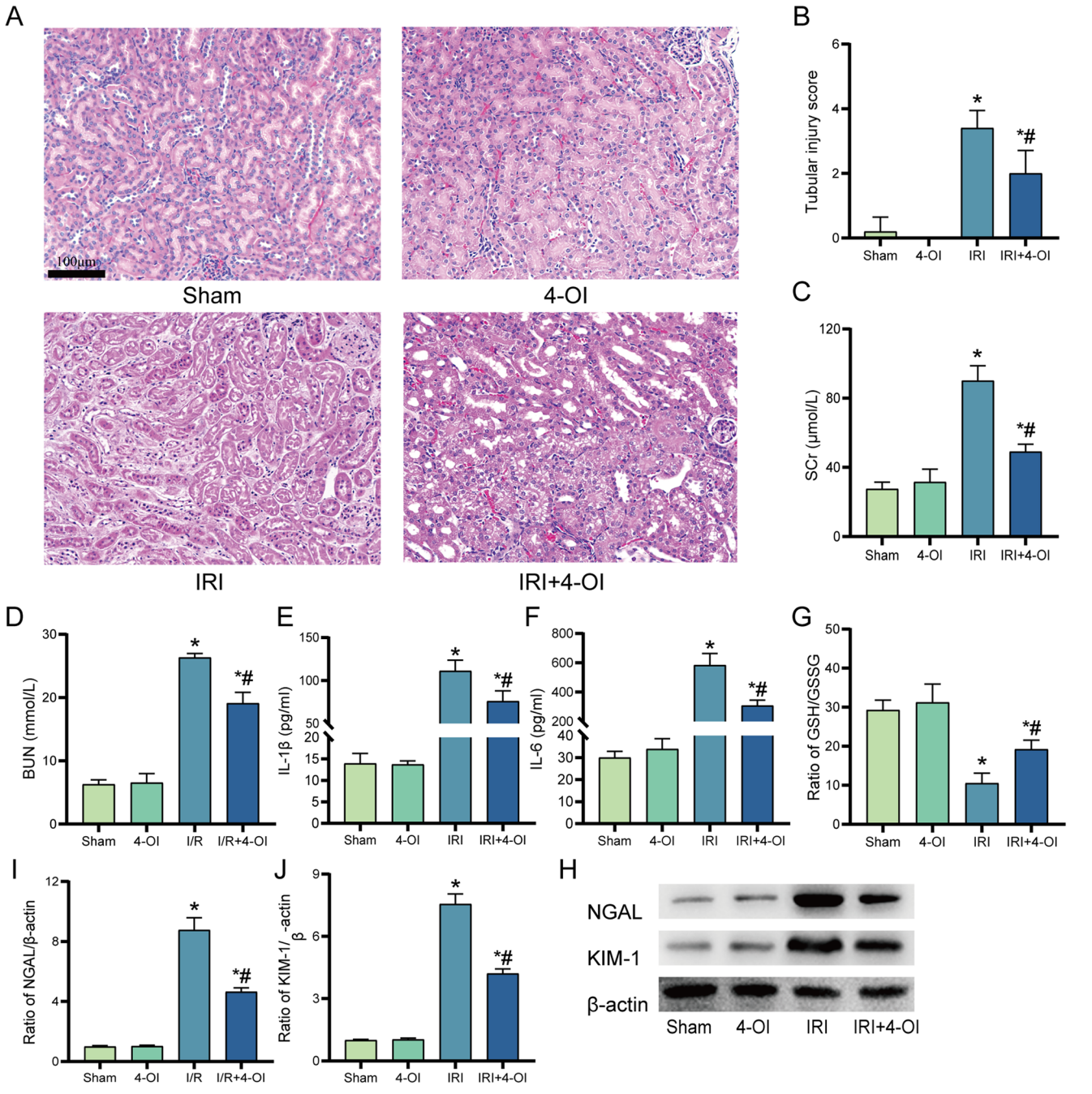

Figure 1. 4-OI protected kidney from IRI *in vivo*. (A) HE staining was applied to assess the renal histopathological changes after IRI pretreated with or without 4-OI (×200). Scale bar: 100 μm. (B) Tubular injury scores were evaluated by two blinded pathologists based on HE staining. (C) and (D) Scr and BUN concentrations of mice in different treatment groups. (E) and (F) Serum IL-1β and IL-6 levels were measured using ELISA. (G) The ratio of GSH/GSSG in renal tissues was detected using a Total-GSH/GSSG assay kit. (H) to (J) The NGAL and KIM-1 expression levels of kidney tissues in each group were determined using western blotting. Each result was replicated in three independent experiments. Data in bar graphs are presented as mean ± SEM ($n = 3$–5).
*$P < 0.05$, relative to Sham group; #$P < 0.05$, relative to IRI group.

4-OI mitigated ERS and activated the Nrf2 pathway in mice exposed to renal IRI

Given that ERS plays an important role in the progression of renal IRI, we determined whether 4-OI attenuated ERS caused by renal IRI (see Figure 2(a) and (b)). We used immunofluorescence (IF) analysis to explore relative CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) protein expression level. IRI could significantly increase the CHOP expression in the renal tissues, whereas 4-OI pretreatment mitigated this change ($P < 0.05$). To investigate further, we determined the expression levels of ERS-related proteins, including activating transcription factor 6 (ATF6), binding immunoglobulin protein (BiP), CHOP, and phosphorylation levels of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1α (IRE1α) using western blotting (see Figure 2(b) to (e) and (g)). The results revealed

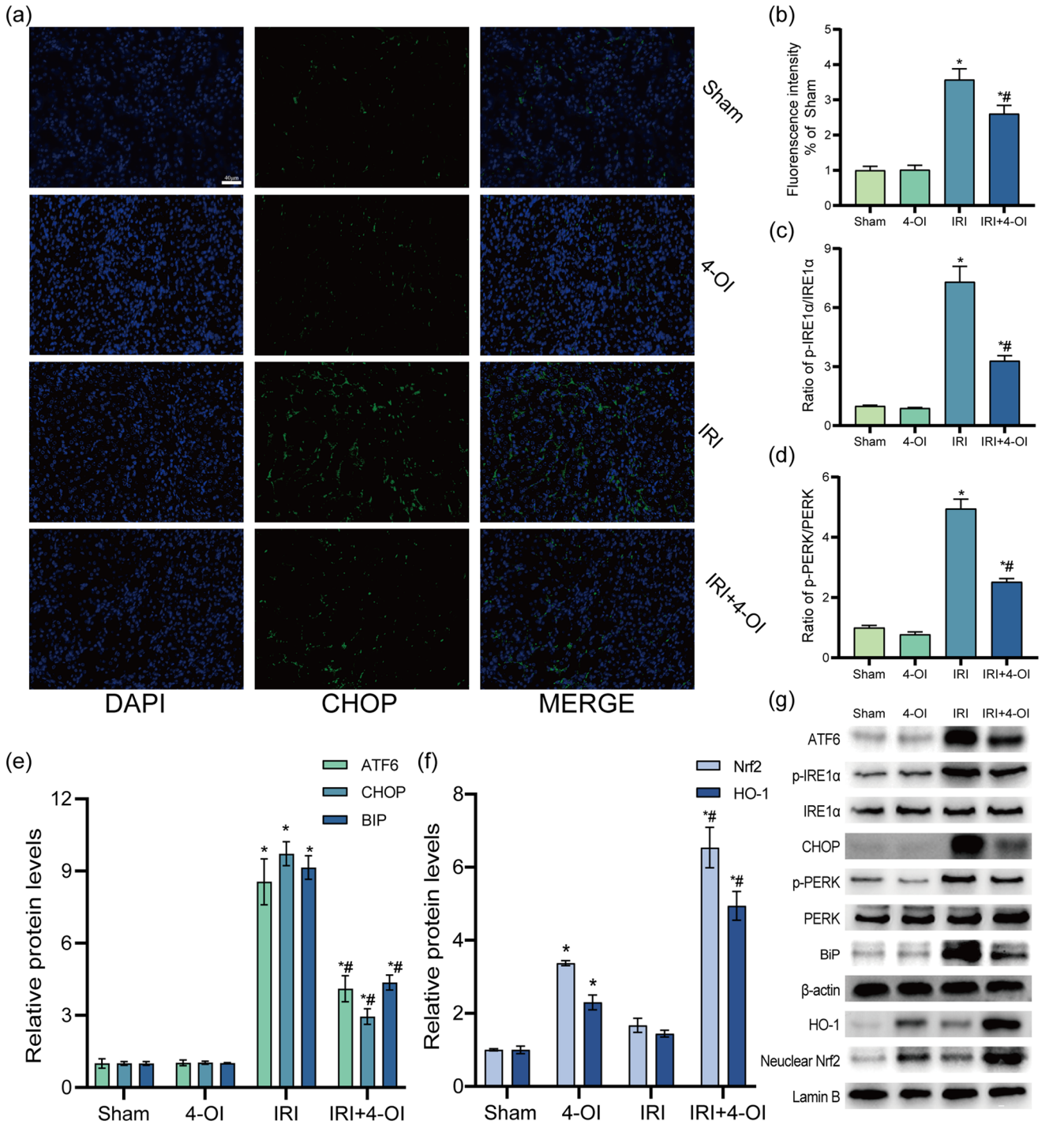

Figure 2. 4-OI mitigated ERS and activated the Nrf2 pathway in mice exposed to renal IRI. (a) Representative immunofluorescence staining of CHOP in renal tissues (×400). Scale bar: 40 μm. (b) Immunofluorescence intensity of CHOP (green) in each group was quantified using ImageJ ($n=5$). (c) and (d) Relative PERK and IRE1α phosphorylation levels in each group were quantified using phosphorylated proteins/total proteins and phosphorylated proteins were normalized to total proteins. (e) Relative ATF6, BiP, and CHOP expression levels in kidney tissues. (f) Nuclear Nrf2 and total HO-1 expression levels in kidney tissues. (g) Representative western blotting images were exhibited. Each result is presented as mean ± SEM ($n=3$).
*$P<0.05$, relative to Sham group; #$P<0.05$, relative to IRI group.

that IRI could obviously upregulate the expression levels of CHOP, BiP, and ATF6 ($P<0.05$). Meanwhile, the ratios of p-PERK/PERK and p-IRE1α/IRE1α in renal tissues were also increased ($P<0.05$). However, 4-OI pretreatment partially alleviated this trend ($P<0.05$). To determine whether 4-OI could activate the Nrf2 pathway during renal IRI, the nuclear accumulation level of Nrf2 and its downstream target heme oxygenase (HO-1) expression level were examined. As shown in Figure 2(f), 4-OI pretreatment upregulated the levels of total HO-1 and nuclear Nrf2 ($P<0.05$). In summary, these results show that 4-OI alleviates ERS and activated the Nrf2 pathway *in vivo*.

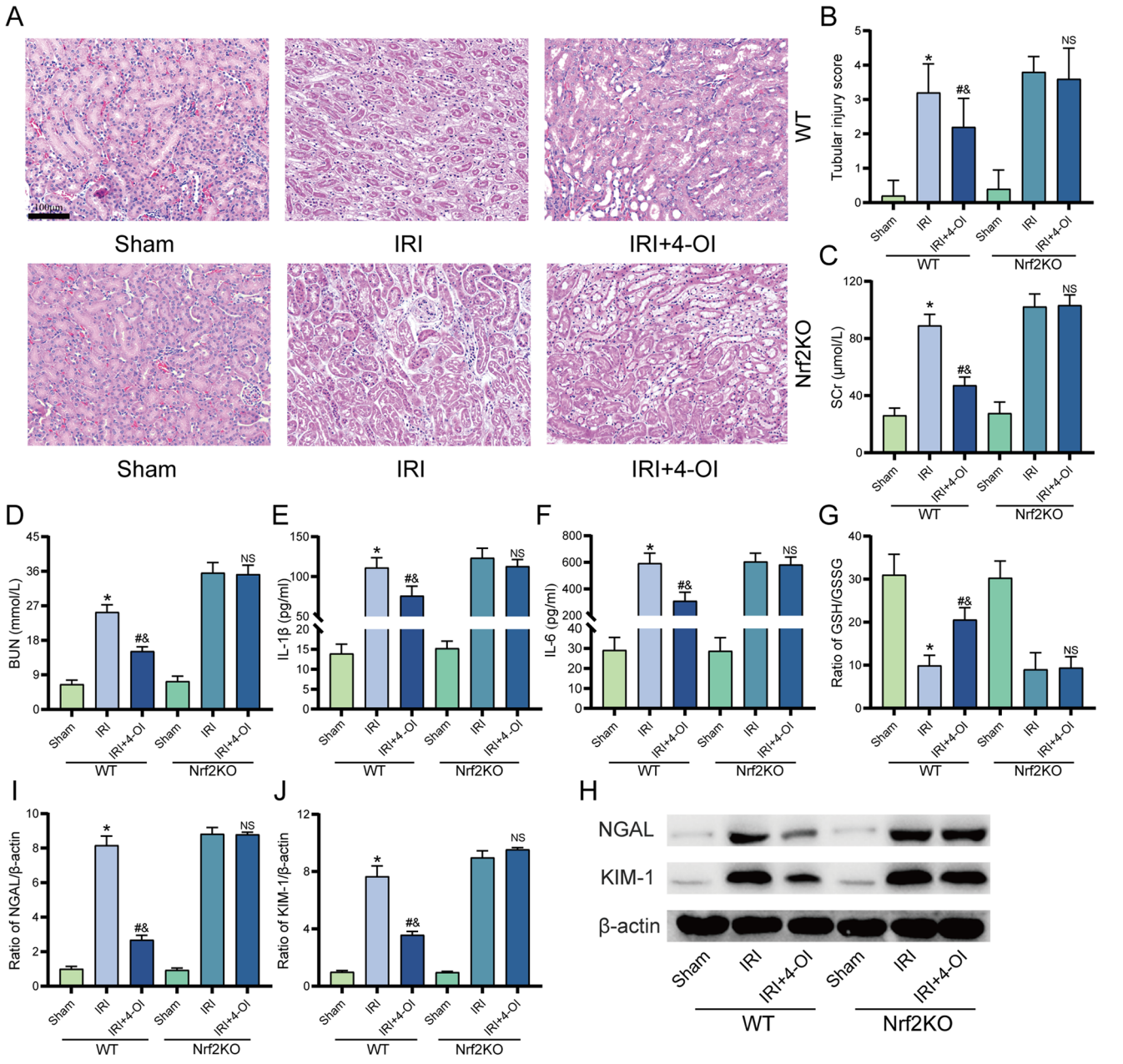

Figure 3. The protective effect of 4-OI was partially counteracted in Nrf2-KO mice. (A) The representative histopathological changes of kidney tissues stained with HE (×200). Scale bar: 100 μm. (B) Tubular injury scores were analyzed according to HE staining. (C) and (D) Scr and BUN concentrations. (E) and (F) Serum IL-1β and IL-6 concentrations. (G) The GSH/GSSG ratio in renal tissues. (H) to (J) NGAL and KIM-1 expression levels in renal tissues were determined using western blotting ($n=3$). Data in bar graphs are presented as mean ± SEM ($n=3$–5).
*$P<0.05$, relative to WT + Sham group; #$P<0.05$, relative to WT + IRI group; &$P<0.05$, relative to Nrf2KO + IRI + 4-OI group; NS$P>0.05$, relative to Nrf2-KO + IRI.

The protective effect of 4-OI was partially counteracted in Nrf2-KO mice

To elucidate whether 4-OI exerts its therapeutic effects via the Nrf2 pathway, we utilized Nrf2-KO mice in our study. The Nrf2KO + IRI + 4-OI group exhibited more severe tubular damage and higher tubular injury scores than the WT + IRI + 4-OI group ($P<0.05$; see Figure 3(A) and (B)). The improvement of 4-OI on renal histomorphology damage was partially reversed in Nrf2-KO mice. Similarly, the positive impact of 4-OI on renal dysfunction was offset in Nrf2-KO mice. In addition, we tested whether Nrf2-KO could block the anti-inflammatory and antioxidant properties of 4-OI (see Figure 3(E) to (G)). Compared with the WT + IRI + 4-OI group, the IL-1β and IL-6 concentrations were higher, and the GSH/GSSG ratio was lower in the Nrf2KO + IRI + 4-OI group ($P<0.05$). Meanwhile, differences between the Nrf2-KO + IRI and Nrf2KO + IRI + 4-OI groups were not significant ($P>0.05$). Taken together, our results suggest the therapeutic effect of 4-OI on renal IRI is associated with the Nrf2 pathway.

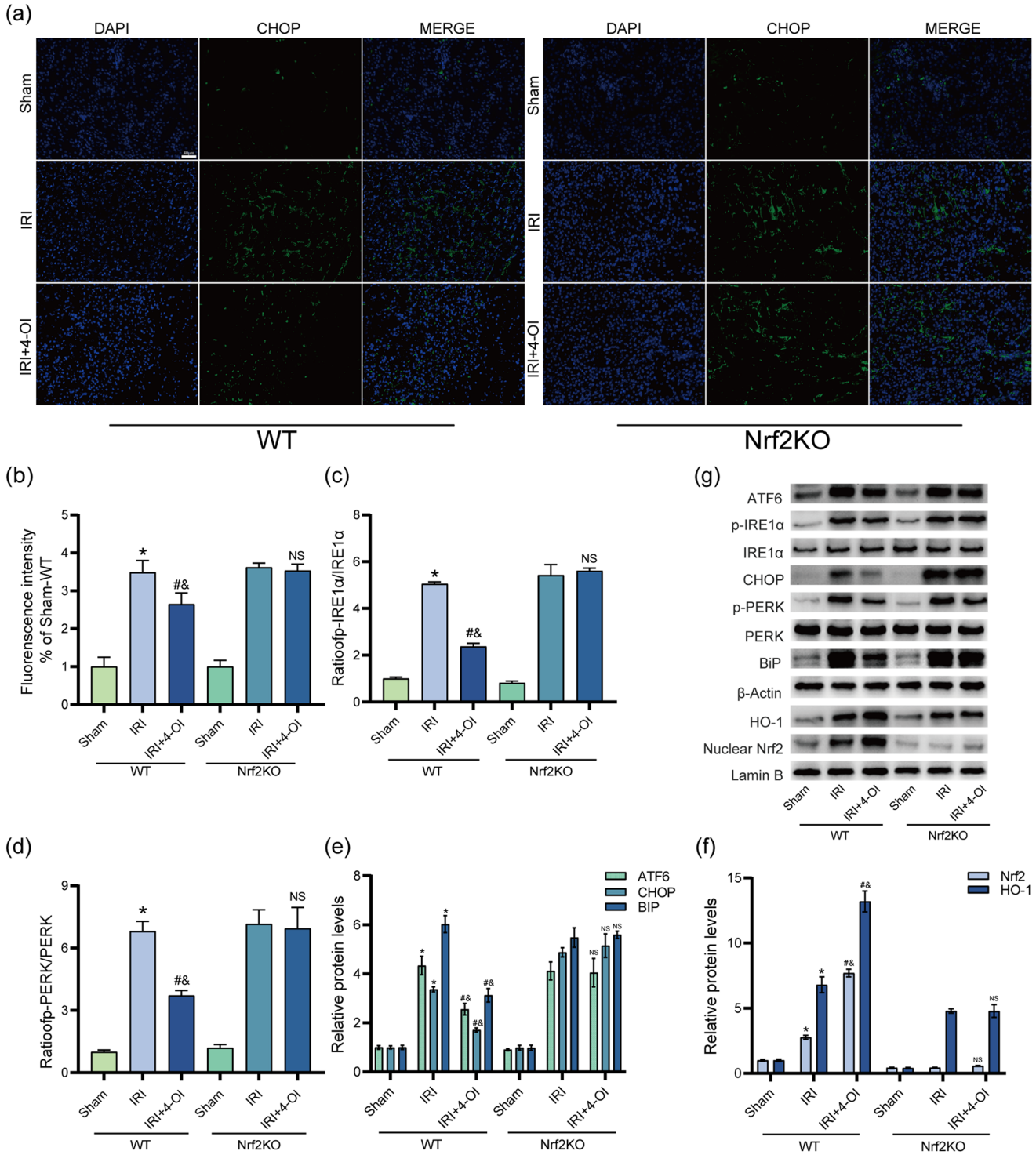

Figure 4. The effects of 4-OI-afforded preservation of IRI-induced ERS was partially counteracted in Nrf2-KO mice. (a) Representative immunofluorescence staining of CHOP in renal tissues (×400). Scale bar: 40 μm. (b) Relative CHOP levels were assessed with immunofluorescent intensity in each group ($n=5$). (c) and (d) Relative PERK and IRE1α phosphorylation levels in different groups. (e) Relative ATF6, BiP, and CHOP expression levels in kidney tissues. (f) Relative Nuclear Nrf2 and total HO-1 expression levels in kidney tissues. (g) Representative western blotting images. Data in (c-f) are presented as mean ± SEM from three independent experiments.
*$P<0.05$, relative to WT + Sham group; #$P<0.05$, relative to WT + IRI group; &$P<0.05$, relative to Nrf2KO + IRI + 4-OI group; NS$P>0.05$, relative to Nrf2-KO + IRI.

The effects of 4-OI-afforded preservation of IRI-induced ERS was partially counteracted in Nrf2-KO mice

Next, we wanted to explore whether 4-OI-mediated alleviation of ERS was dependent on the Nrf2 pathway. The results of IF analysis revealed that Nrf2-KO mice, both those treated with IRI and those with 4-OI, showed much higher expression levels of CHOP than WT mice ($P<0.05$; see Figure 4(a) and (b)). For further validation, we utilized western blotting to assess proteins levels (see Figure 4(g)). As we expected, the results of western blots were broadly consistent with

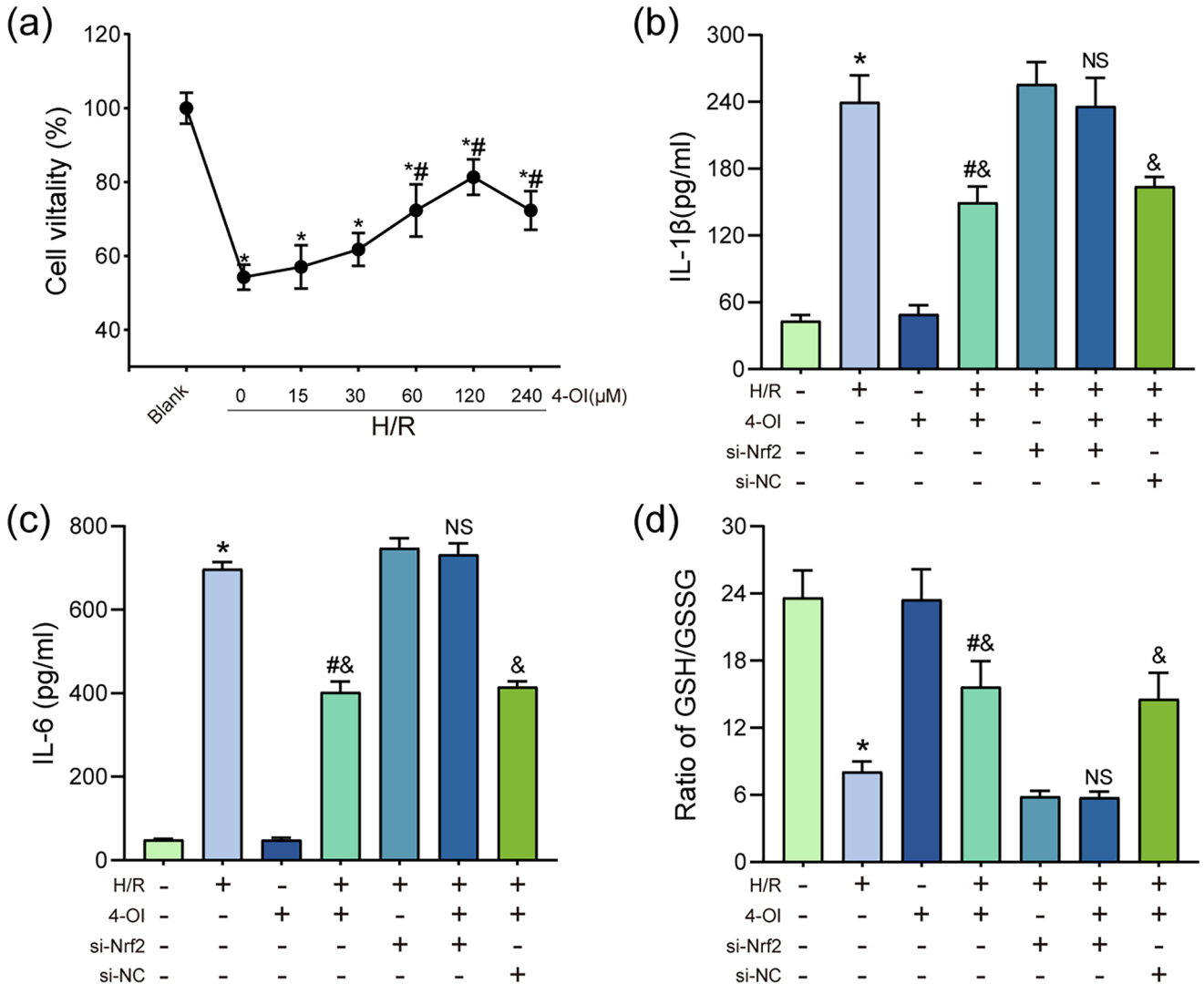

Figure 5. Nrf2 knockdown counteracted 4-OI-mediated protection on H/R-stimulated inflammation and oxidative damage *in vitro*. (a) The viability of HK-2 cells cultivated with different doses of 4-OI prior to H/R was assessed using a CCK-8 assay. $^{*}P < 0.05$, relative to Blank group; $^{\#}P < 0.05$, relative to 4-OI = 0 and H/R group. (b) and (c) IL-1β and IL-6 concentrations in the cell supernatant. (d) Intracellular GSH/GSSG ratio. Data in (b) and (d) are presented as mean ± SEM from three individual experiments. $^{*}P < 0.05$, relative to Blank group; $^{\#}P < 0.05$, relative to H/R group; $^{\&}P < 0.05$, relative to H/R + 4-OI + si-Nrf2 group; $^{NS}P > 0.05$, relative to H/R + si-Nrf2 group.

those of IF staining. Compared with the WT + IRI + 4-OI group, the phosphorylation levels of PERK and IRE1α in the Nrf2KO + IRI + 4-OI group were higher ($P < 0.05$; see Figure 4(b) and (c)). Not surprisingly, the CHOP, ATF6, and BiP expression levels exhibited the same results ($P < 0.05$; see Figure 4(e)). Meanwhile, the expression of HO-1 and ERS-related proteins between the Nrf2KO + IRI and Nrf2KO + IRI + 4-OI groups showed no significant differences ($P > 0.05$). Our data illustrate that the effect of 4-OI on alleviating ERS is associated with the Nrf2 pathway.

Nrf2 knockdown counteracted 4-OI-mediated protection on H/R-stimulated inflammation and oxidative damage *in vitro*

For further validation, we performed *in vitro* experiments using H/R-exposed HK-2 cells. We imitated the IRI procedure by subjecting cells to 24 h hypoxia and 6 h reoxygenation. The effects of 4-OI treatment in different concentrations on H/R-exposed HK-2 cells were evaluated based on the cellular viability, using CCK-8 assay. Before H/R, 0, 15, 30, 60, 120, or 240 μM of 4-OI were added and incubated for 2 h. As shown in Figure 5(a), a progressive increase of cell viability was detected with different doses of 4-OI pretreatment and reached the highest effect at a dose of 120 μM in H/R-treated cells ($P < 0.05$). A total of 120 μM 4-OI was used in the subsequent experiments. Next, the anti-inflammatory properties of 4-OI were examined *in vitro* (see Figure 5(b) to (d)). H/R exposure could greatly increase the levels of IL-1β and IL-6, while 4-OI reversed this trend ($P < 0.05$). Similarly, the reduced GSH/GSSG ratio caused by H/R was improved by 4-OI ($P < 0.05$). Nrf2 knockdown could reverse the anti-inflammatory and antioxidant effects of 4-OI ($P > 0.05$), while NC siRNA could not ($P < 0.05$). All in all, our results demonstrate that 4-OI exerts the anti-inflammatory and antioxidant effect on H/R-stimulated cells by activating the Nrf2 pathway.

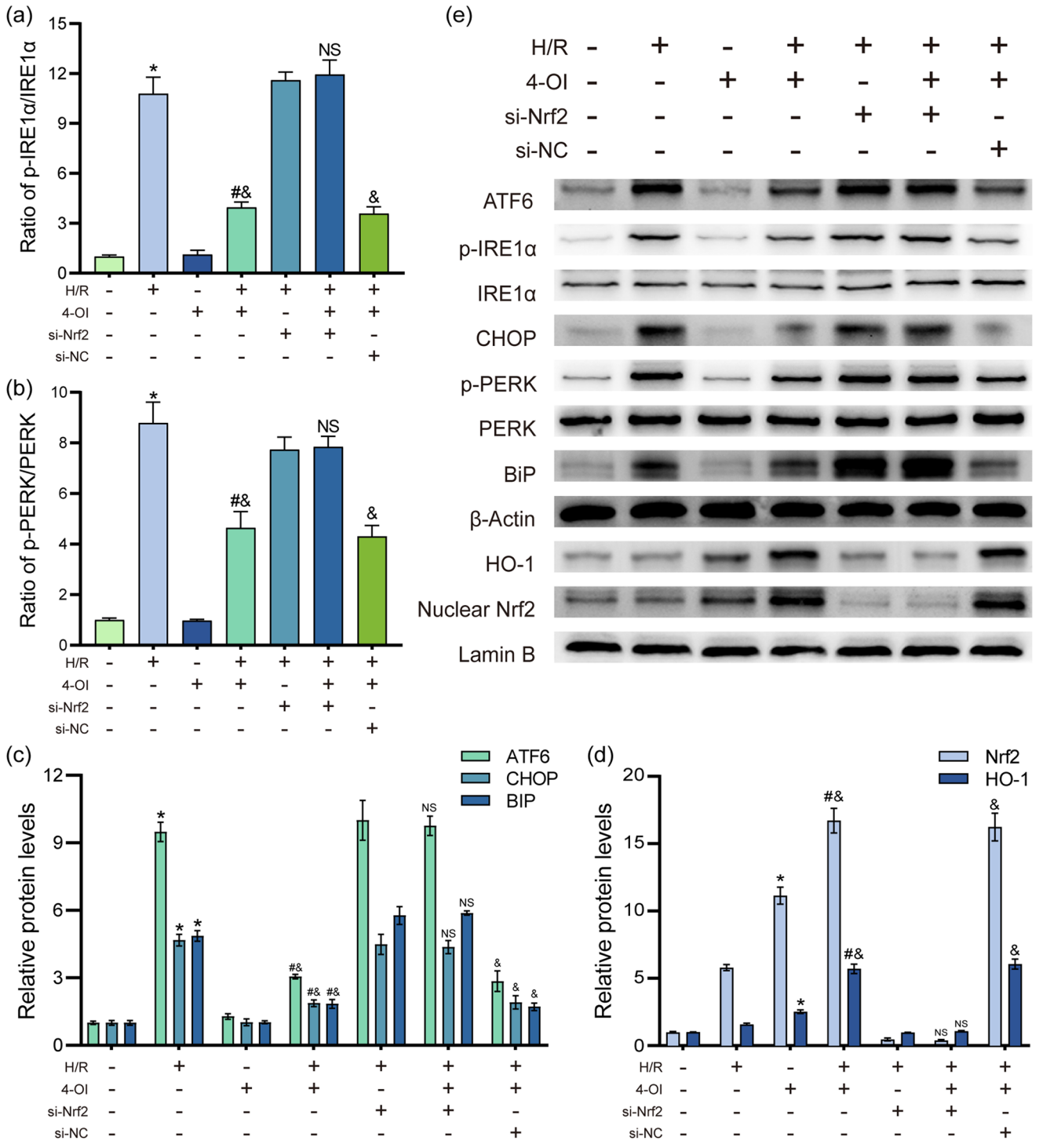

Figure 6. Nrf2 knockdown partially counteracted the ERS-alleviating effect of 4-OI on H/R-stimulated HK-2 cells. (a) and (b) Relative PERK and IRE1α phosphorylation levels in different groups and phosphorylated proteins were normalized to total proteins. (c) Relative ATF6, BiP, and CHOP expression levels. (d) Relative nuclear Nrf2 and total HO-1 expression levels. (e) Representative western blotting images. Data are presented as mean ± SEM from three independent experiments.
*$P < 0.05$, relative to Blank group; #$P < 0.05$, relative to H/R group; &$P < 0.05$, relative to H/R + 4-OI + si-Nrf2 group; NS$P > 0.05$, relative to H/R + si-Nrf2 group.

Nrf2 knockdown partially counteracted the ERS-alleviating effect of 4-OI on H/R-exposed HK-2 cells

For further validation, we tested whether the Nrf2 pathway was associated with the ERS-alleviating effect of 4-OI *in vitro*. We detected ERS-related protein expression levels (see Figure 6(a) to(e)). H/R stimulation could dramatically upregulate the expression levels of CHOP, ATF6, and BiP, and increase the levels of PERK and IRE1α phosphorylation, whereas 4-OI could reduce these increases ($P < 0.05$). In addition, as we expected, this role of 4-OI could be shown in the H/R + 4-OI + si-NC group ($P < 0.05$), but not in the H/R + 4-OI + si-Nrf2 group ($P > 0.05$). Overall, 4-OI alleviates ERS caused by H/R via the Nrf2 pathway *in vitro*.

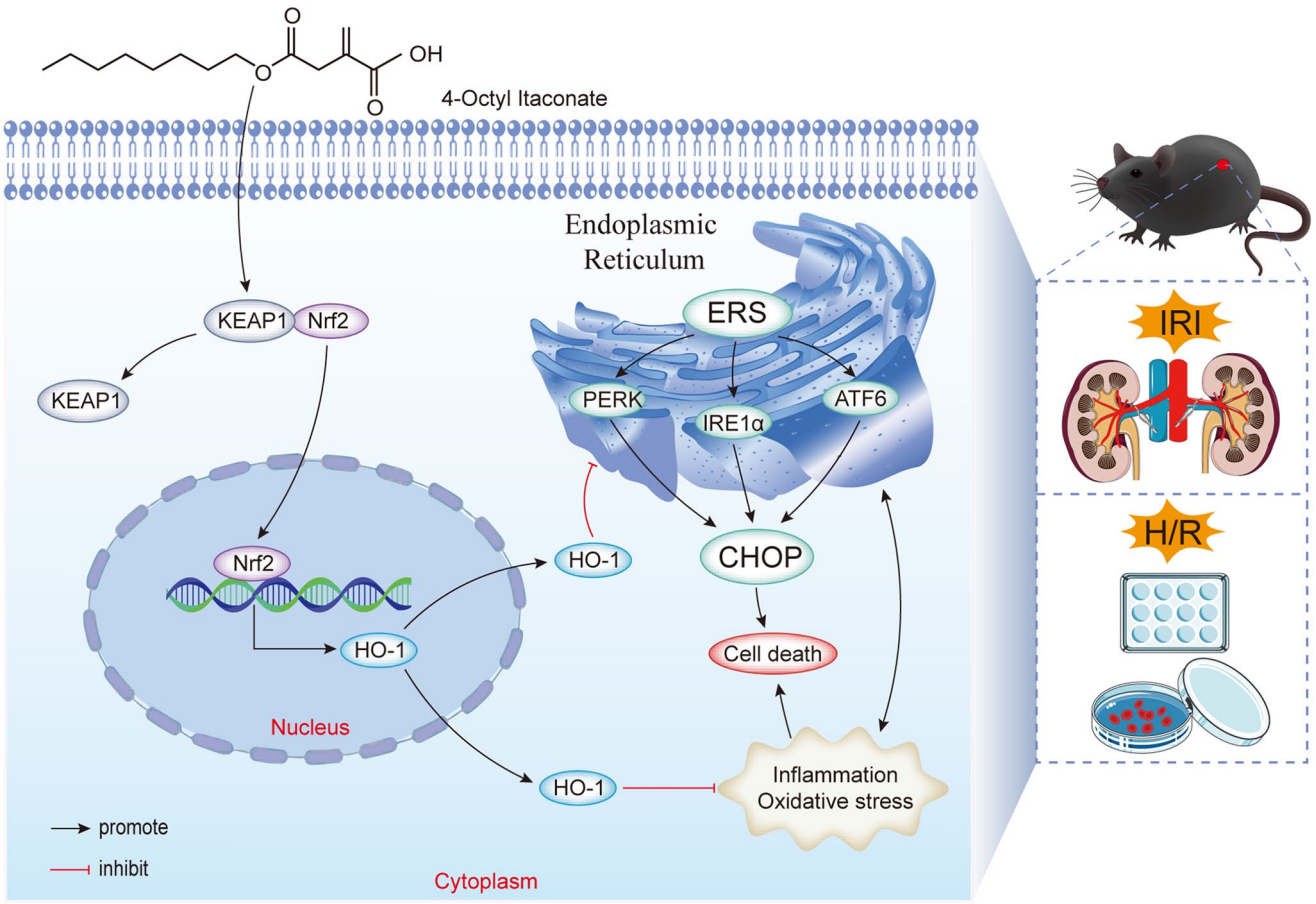

Figure 7. Graphical illustration of 4-OI-mediated protection on renal IRI.

Discussion

Our study demonstrates that 4-OI can alleviate renal IRI by alleviating ERS through activating the Nrf2 pathway, both *in vivo* and *in vitro* (see Figure 7). First, we assessed the therapeutic role of 4-OI in renal histomorphology damage, dysfunction, and ERS *in vivo*. Our results revealed that 4-OI mitigated IRI and attenuated IRI-induced ERS, while it activated the Nrf2 pathway *in vivo*. Furthermore, we found that the 4-OI-mediated protection could not be shown in Nrf2-KO mice. Similarly, 4-OI could alleviate inflammation, oxidative stress, and ERS caused by H/R *in vitro* and the therapeutic effect was blocked by siRNA-mediated silencing of Nrf2 *in vitro*. Therefore, our study shows that 4-OI protects renal IRI by mitigating ERS via the Nrf2 pathway.

Renal IRI is a principal reason accounting for AKI and following CKD[23] and is characterized by the damage of renal tissue and the renal dysfunction.[24] Previous studies have shown that the severity of renal IRI is strongly associated with inflammation and oxidative stress.[25] Our *in vivo* model with clamping of bilateral renal pedicles reproduces the main characteristics of human renal IRI, with severe renal histopathological damage, elevated tubular injury scores, and decline of renal function, accompanied by reducing oxidative stress and increased inflammation.[26] We utilized HK-2 cells subjected to H/R to perform experiments *in vitro*.

As a by-product of the Krebs cycle, ITA is decarboxylated from cis-aconitate by IRG1.[27] Growing research findings show that the changes of cellular metabolism are intricately associated with immune activation.[28] Recently, ITA has gained significant attention as a key immunoregulatory molecule, and has been considered as a novel and crucial bridge between metabolism and immune response.[20,29,30] ITA has been indicated to regulate inflammatory responses and macrophage activation through modulating mitochondrial function and synthesis of succinate, a pro-inflammatory cytokine.[16] In addition, ITA can show its immunoregulatory effects in the treatment of autoimmune diseases via regulating the IκBζ-ATF3 inflammatory axis.[15] Besides, ITA has also been reported to exert an antioxidant effect in many studies.[31,32] However, ITA cannot pass cell membranes easily because of its high polarity, so 4-OI was synthesized to conquer the limitations of ITA. 4-OI and ITA had similar thiol reactivity, and it could be hydrolysed to ITA by esterases inside cells, making it an appropriate cell-permeable ITA substitute.[14] More importantly, 4-OI has been proved to protect from various diseases. Herein, for the first time, we examined the therapeutic role of 4-OI in renal IRI.

The analyses of HE staining showed that 4-OI could dramatically reduce tubular epithelial swelling, decrease the level of inflammatory cell infiltration, and ameliorate the loss of brush border. The levels of SCr and BUN are widely used as markers of renal function, and largely

reflect glomerular filtration.[33] The decreased levels of SCr and BUN revealed that 4-OI could improve IRI-induced impaired renal function. Recently, KIM-1 and NGAL have been regarded as the early diagnostic biomarkers of renal IRI, on account of the rapidly elevated expression in kidney tissues and blood.[34] The KIM-1 expression level in the kidney is a specific and sensitive diagnostic marker and a reliable prognostic marker for AKI.[35] NGAL in serum and kidney is a marker of active renal injury and its level can reflect the severity of renal injury.[36,37] Herein, the reduced KIM-1 and NGAL expression levels in renal tissues indicated that 4-OI pretreatment could relieve renal damage. Oxidative stress and interstitial inflammation are crucial in the pathological processes of renal IRI, so ameliorating these pathophysiologic features is the major goal of treatment.[38,39] The decreasing IL-1β and IL-6 levels illustrated that 4-OI could alleviate IRI-induced inflammation. Besides, 4-OI could also diminish IRI-induced oxidative stress based on the GSH/GSSG ratio. In addition, our data revealed that mice pretreated with 4-OI showed no evidence of toxic side effects, compared with the Sham group. According to our results, 4-OI may be a new option for renal IRI therapy.

The ER is a specific organelle of eukaryocytes, which modulates the synthesis, folding, and assembly of numerous proteins.[8] Unfolded polypeptide chains enter ER to form proteins, and the level of this flux into the ER lumen, which is determined by the physiological state and environmental conditions of cells, changes rapidly. Cells can regulate ER protein-folding ability to handle this dynamic changes[7]. If the protein-processing capacity of ER does not match the increasing demand for protein synthesis or is disturbed by alterations in the intracellular and extracellular environment, this will cause the abnormal accumulation of unfolded or misfolded proteins in ER, known as ERS. Subsequently, unfolded protein responses (UPRs) are activated by ERS.[7,8] At first, the activation of UPR can improve the function of ER to adapt ERS and help cells survive. However, if UPR fails to adapt ERS, irremediable ERS would induce cell death.[40,41] ATF6, PERK, and IRE1α, which are all transmembrane protein sensors, comprise the three main branches of the UPR pathway.[41] BiP binds to three sensors and makes them inactive in resting cells. The increasing level of unfolded and misfolded proteins causes the dissociation between BiP and the sensors, which activates the sensors. An elevated protein expression level of BiP is considered as a significant marker of ERS.[7] CHOP is a crucial regulator of ERS-mediated apoptosis.[42] In this study, we determined the expressions of ERS-related proteins through immunofluorescence and western blotting. Recent studies have reported that the prolonged or excessive ERS aggravated renal IRI, while therapies targeting ERS could reduce renal tissue damage caused by IRI.[43] Our results are consistent with the previous study. Renal IRI greatly elevated the BiP, CHOP, and ATF6 expression and the phosphorylation levels of PERK and IRE1α, indicating that IRI could induce ERS. More importantly, 4-OI pretreatment remarkably downregulated the BiP, CHOP, and ATF6 expressions, accompanied by reducing the phosphorylation levels of PERK and IRE1α. Our results, therefore, show that 4-OI can relieve ERS so as to ameliorate renal IRI.

As a significant transcription factor, Nrf2 is present in nearly all human organs. In addition, Nrf2 is crucial to the protection against inflammation and oxidative stress, which are pathologic characteristics underlying diseases of the kidney, lung, liver, and cardiovascular system.[44] Previous studies have reported that Nrf2 could alleviate IRI-induced tissue damage, through its anti-inflammatory and antioxidant abilities.[45–47] Nrf2 can upregulate the expression of many notable cytoprotective genes by binding antioxidant response elements.[47] 4-OI can alkylate the cysteine residues of KEAP1, which releases Nrf2 from KEAP1 and increases the entry of Nrf2 into the nucleus, accompanied by upregulating the expression of downstream target genes.[14] Our findings were consistent with previous studies. We detected that the nuclear Nrf2 accumulation was evaluated by 4-OI during renal IRI. In addition, total HO-1 expression was upregulated significantly, indicating that 4-OI activated the Nrf2 signaling pathway. HO-1, which is an important downstream gene of Nrf2, has significant anti-inflammatory and antioxidant properties, and plays a significant role in inhibiting ERS and maintaining mitochondrial dynamic equilibrium.[48,49] Furthermore, to validate the role of the Nrf2 pathway in the 4-OI-mediated alleviation of ERS during renal IRI, Nrf2-KO mice and Nrf2-siRNA-transfected HK-2 cells were utilized in our study. As expected, the effects of 4-OI on alleviating kidney tissues damage, and improving renal dysfunction were partially blocked in Nrf2-KO mice, based on the elevated renal pathological injury, higher tubular injury scores, increased SCr and BUN levels, and upregulated KIM-1 and NGAL expressions. Meanwhile, Nrf2-KO increased the serum concentrations of IL-1β and IL-6, reduced the GSH/GSSG ratio, accompanied by upregulating the expression of ATF6, BiP, CHOP, and elevated the phosphorylation levels of PERK and IRE1α. Similarly, the beneficial roles of 4-OI in inflammation, oxidative stress and ERS were partially reversed by Nrf2 knockdown *in vitro*. These findings illustrate that 4-OI ameliorates renal IRI by activating the Nrf2 pathway. Of note, our experiments examined the therapeutic effects of 4-OI only 24 h after IRI, and more time spots need to be explored further. In addition, to confirm the clinical efficacy of 4-OI in treatment of renal IRI, a clinical trial with larger sample size is essential in the future.

Overall, our study elucidates that 4-OI attenuates renal IRI by alleviating ERS via activating the Nrf2 signaling pathway. Due to its good efficacy and relative non-toxicity, 4-OI may be a novel and reliable treatment candidate for renal IRI.

AUTHORS CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data, and review of the article; X-KL and H-JY conducted the experiments and wrote the article.

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