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GuoFeng Shu
Tianzhu County People's Hospital
Shuxiong Xu
xushuxiong123@sina.com
Guizhou Provincial People's Hospital
Yikun Wu
Guizhou Provincial People's Hospital
Yu Xiao
Guizhou Provincial People's Hospital

Research Article

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Deiodinase 1 (DIO1) Attenuates Renal Ischemia-Reperfusion Injury by Suppressing the NLRP3 Inflammasome

Guofeng Shu[1], Yikun Wu[2], Yu Xiao[2], Shuxiong Xu[2,*]
1 Department of Urology, Tianzhu County People's Hospital, Guizhou Province, China
2 Department of Urology, Guizhou Provincial People's Hospital, Guiyang, Guizhou, China
* Corresponding author: Shuxiong Xu, E-mail: xushuxiong123@sina.com

Abstract

Renal ischemia-reperfusion injury (RIRI) is a leading cause of acute kidney injury (AKI), and inflammation driven by excessive NLRP3 inflammasome activation is a key pathological event in its progression. Deiodinase 1 (DIO1), a selenoprotein highly expressed in the kidney, has an undefined role in RIRI. This study aimed to investigate the protective effect of DIO1 against RIRI and the underlying mechanisms. A mouse RIRI model was established by bilateral renal artery clamping for 30 min, and mice were randomized to sham, IR, DIO1 activation (T3 pretreatment), and NLRP3 inhibitor (INF39) groups. Renal function, histopathology, and the expression of DIO1, NLRP3, and IL-1β were assessed 24 h after reperfusion. In vitro, DIO1-overexpressing HK-2 cells subjected to hypoxia-reoxygenation with or without nigericin (NSS) were examined by Western blot. DIO1 expression was markedly downregulated in RIRI kidneys; T3-mediated DIO1 activation significantly improved renal function and histopathology and reduced NLRP3 and IL-1β expression, comparably to INF39. In vitro, DIO1 overexpression significantly downregulated the expression of NLRP3, Caspase-1, GSDMD, IL-1β, IL-18, and p-NF-κB p65. These findings indicate that DIO1 protects against RIRI, likely by suppressing NF-κB/NLRP3 signaling activation and inflammatory cytokine release, and may represent a potential therapeutic target for RIRI.

Keywords: Deiodinase 1; renal ischemia-reperfusion injury; NLRP3 inflammasome; Caspase-1; NF-κB signaling pathway; inflammation

1. Introduction

Acute kidney injury (AKI) is a clinical syndrome of major consequence, and renal ischemia-reperfusion injury (RIRI) is its most important cause. RIRI occurs commonly in kidney transplantation, major cardiac surgery, shock, and severe trauma, and accounts for approximately 60% of hospital-acquired AKI [1,2]. RIRI not only causes acute renal failure but may also progress to chronic kidney disease and even end-stage renal disease, profoundly affecting long-term prognosis [3]. However, apart from supportive care, no specific

interventions are currently available for RIRI; breakthroughs in prevention and treatment depend on a deeper understanding of the core mechanisms of injury.

The inflammatory cascade triggered by ischemia-reperfusion is the central driver of tissue injury in RIRI [4,5]. Within this cascade, the NLRP3 inflammasome occupies a pivotal position. This multi-protein complex is assembled from the sensor protein NLRP3, the adaptor protein ASC, and the effector pro-Caspase-1. Activated Caspase-1 catalyzes the maturation and secretion of IL-1β and IL-18, and cleaves gasdermin D (GSDMD) to form membrane pores and initiate pyroptosis, thereby amplifying inflammatory injury [6-9]. Excessive NLRP3 inflammasome activation is a common pathway in ischemia-reperfusion injury of multiple organs, and agents such as ulinastatin and naringenin have been shown to attenuate RIRI by suppressing inflammasome activation [10-14]. Therefore, identifying endogenous protective molecules capable of effectively restraining NLRP3 inflammasome activation has become a key direction in RIRI research.

As important endogenous selenoproteins, the deiodinases (DIOs) catalyze the deiodination of thyroid hormone T4 to finely tune local triiodothyronine (T3) concentrations, participating in metabolism and immune regulation [15,16]. DIO1 is the only selenoenzyme possessing both outer- and inner-ring deiodination capacity; the selenocysteine in its active center confers unique antioxidant properties, and it is highly expressed in the liver and kidney [17]. Several clues point to a close relationship between DIO1 and renal inflammatory injury. DIO1 participates in antioxidative and metabolic regulation in renal cell carcinoma [18]. In patients with chronic renal failure, DIO1 levels are negatively correlated with the pro-inflammatory cytokine TNF-α [19]. Moreover, IL-1β can downregulate DIO1 expression via the NF-κB and AP-1 pathways [20]. Notably, renal DIO1 expression is markedly reduced during RIRI [21]; however, whether the downregulation of DIO1 is causally linked to NLRP3 inflammasome activation, and whether DIO1 can serve as an endogenous protective molecule against RIRI, remain unexplored.

In summary, the NF-κB/NLRP3 inflammasome axis drives inflammatory injury in RIRI, whereas DIO1, which is highly expressed in the kidney, is inversely associated with inflammatory states, suggesting an as-yet-uncharacterized negative regulatory relationship between the two. We therefore hypothesized that DIO1 is an endogenous protective factor in RIRI, and that upregulation of DIO1 attenuates renal inflammatory injury by suppressing the NF-κB/NLRP3 signaling axis. To test this hypothesis, we constructed a mouse RIRI model with T3-induced DIO1 activation (INF39 as a positive control) in vivo, and employed lentivirus-mediated DIO1 overexpression combined with hypoxia-reoxygenation/NSS

challenge in HK-2 cells in vitro. The hypothesis was verified at multiple levels, including renal function, histopathology, NLRP3 inflammasome components, inflammatory cytokines, and p-NF-κB p65. This study is the first to elucidate the protective role and mechanism of DIO1 in RIRI using dual in vivo and in vitro models. The findings provide experimental evidence and translational prospects for a novel renal anti-inflammatory function of endogenous selenoproteins and for targeting DIO1 in the prevention and treatment of RIRI.

2. Materials and Methods

2.1. Experimental animals and cell line

Twenty-four healthy male C57BL/6 mice (6-8 weeks old, 20-22 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (license number: SCXK (Liao) 2020-0001). All mice were housed in a standardized animal facility (temperature 20-24°C, humidity 40%-60%, 12 h light/dark cycle) with free access to food and water, and were allowed to acclimatize for 1 week before the experiments. All animal experiments were approved by the Animal Ethics Committee of Guizhou Medical University (approval number: 2305126) and performed in strict accordance with the guidelines for the use and ethics of laboratory animals. The human renal tubular epithelial cell line HK-2 was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China; CL-0222).

2.2. Main reagents and instruments

Triiodothyronine (T3, HY-A0070A), the NLRP3 inflammasome inhibitor INF39 (HY-137574), and the NLRP3 inflammasome agonist nigericin sodium salt (NSS, HY-100381) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Rabbit anti-mouse polyclonal antibodies against NLRP3 (#15101), Caspase-1 (#24232), GSDMD (#39754), IL-1β (#12703), IL-18 (#57058), p-NF-κB p65 (#3034), NGAL (#44058), and DIO1 (#ab224426) were purchased from Cell Signaling Technology (Danvers, MA, USA) and Abcam (Cambridge, UK). HRP-conjugated goat anti-rabbit IgG secondary antibody (ZB-2301) was purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). The ECL chemiluminescence kit (P0018S) was purchased from Beyotime Biotechnology (Shanghai, China). TRIzol (9108) and qRT-PCR kits (RR047A) were purchased from TaKaRa (Shiga, Japan). Assay kits for serum creatinine (Scr) and blood urea nitrogen (BUN) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The main instruments included a refrigerated centrifuge (Eppendorf 5810R, Germany), a microplate reader (Thermo Multiskan FC, USA), electrophoresis and transfer systems (Bio-Rad, USA), a chemiluminescence imaging system

(Tanon 5200, China), and a real-time PCR system (Applied Biosystems 7500, USA).

2.3. Animal grouping and establishment of the RIRI model

The 24 mice were randomly divided into four groups (n = 6 per group) using a random number table: the sham group (S group), the IR group, the DIO1 activation group (DIO1 group), and the NLRP3 inflammasome inhibitor group (INF39 group). Two hours before surgery, the DIO1 group received an intraperitoneal injection of T3 (0.1 mg/kg) to activate DIO1 expression [22], and the INF39 group received INF39 (12.5 mg/kg) to inhibit the NLRP3 inflammasome [23]. The S and IR groups received an equal volume of normal saline. After anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), a dorsal midline incision was made to expose both renal arteries. In the IR, DIO1, and INF39 groups, both renal arteries were clamped with non-invasive micro-artery clamps for 30 min to induce renal ischemia, whereas the renal arteries were only isolated without clamping in the S group. After removal of the clamps and restoration of perfusion, the incisions were sutured. The mice were euthanized by an overdose of sodium pentobarbital 24 h after surgery, and orbital venous blood and kidney specimens were collected for subsequent analyses.

2.4. Cell culture and grouping

HK-2 cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO2. Cells in the logarithmic growth phase were randomly divided into two groups: the empty vector control group (C group) and the DIO1 overexpression group (OE group). The OE group was transfected with lentivirus carrying the DIO1 gene (pLVX-Puro-DIO1), and the C group was transfected with the empty vector (pLVX-Puro). Forty-eight hours after transfection, stably transfected cell lines were selected with 2 μg/mL puromycin for 7 days, and transfection efficiency was verified by qRT-PCR and Western blot. Each group was further divided into two subgroups: the hypoxia-reoxygenation subgroup (HR subgroup, treated with an equal volume of PBS) and the NSS subgroup (pretreated with 10 μM NSS for 1 h). All cells were cultured in a tri-gas incubator (1% O2, 5% CO2, 94% N2) for 5 h of hypoxia, followed by 24 h of reoxygenation under normal conditions, and then collected for subsequent analyses [24].

2.5. Serum biochemical analysis

Orbital venous blood was collected from mice in each group and allowed to stand at room temperature for 30 min, and serum was separated by centrifugation at 3,000 rpm for 15 min. Serum Scr and BUN levels were measured using an automatic biochemical analyzer (Hitachi

7100, Japan) strictly according to the manufacturer's instructions.

2.6. Histopathological examination

Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned at 4 μm thickness, followed by routine hematoxylin-eosin (HE) staining. Renal tubular pathological changes were observed under a light microscope. Tubular injury was scored independently by two pathologists in a blinded manner according to the following semi-quantitative criteria [25]: 0 = no injury; 1 = mild injury (<25% of tubules involved); 2 = moderate injury (25%-50%); 3 = severe injury (51%-75%); and 4 = extremely severe injury (>75%). Ten high-power fields (×400) were randomly selected from each section for scoring, and the mean score was calculated as the injury score of that sample.

2.7. Immunohistochemistry and immunofluorescence staining

Paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase was inactivated with 3% H2O2 for 15 min, and sections were blocked with normal goat serum for 30 min at room temperature. Primary antibodies against NLRP3 (1:200), IL-1β (1:200), and DIO1 (1:200) were applied and incubated overnight at 4°C. The next day, HRP-conjugated or fluorochrome-conjugated secondary antibodies (1:500) were applied, followed by DAB color development or DAPI counterstaining, and the sections were observed and photographed under a microscope. The percentage of positive cells and the mean optical density were analyzed using Image-Pro Plus 6.0 software. Five high-power fields were randomly selected from each section for semi-quantitative analysis.

2.8. Western blot analysis

Kidney tissues or HK-2 cells were lysed in RIPA lysis buffer containing protease inhibitors on ice for 30 min, and the lysates were centrifuged at 12,000 × g and 4°C for 15 min to collect the supernatant. Protein concentrations were determined by the BCA method. Thirty micrograms of protein per lane were separated by 10% or 12% SDS-PAGE and transferred to PVDF membranes, which were blocked with 5% non-fat milk at room temperature for 1 h. The membranes were incubated overnight at 4°C with primary antibodies against NLRP3, Caspase-1, GSDMD, IL-1β, IL-18, p-NF-κB p65, NGAL, and DIO1 (all 1:1,000). After washing with TBST (3 × 10 min), the membranes were incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature, developed with ECL, and imaged using a chemiluminescence imaging system. β-actin (1:5,000) was used as the internal reference, and

band gray values were analyzed using ImageJ software. The relative expression of target proteins was calculated as the gray value of the target protein divided by that of β-actin. All experiments were independently repeated three times.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HK-2 cells using TRIzol, and cDNA was synthesized according to the instructions of the PrimeScript RT kit. GAPDH was used as the internal reference, and real-time quantitative PCR was performed using TB Green Premix Ex Taq. The primer sequences were as follows: DIO1 forward, 5'-TCTGCTCAAGCACGTGAAAAA-3' and reverse, 5'-GTAGCGCATAATTGGCTTGC-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'. The amplification conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Melt curve analysis was performed to confirm amplification specificity. The relative expression of DIO1 mRNA was calculated using the 2-ΔΔCt method. All experiments were independently repeated three times.

2.10. Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 10.1.2 software (GraphPad Software, San Diego, CA, USA). All measurement data are expressed as mean ± standard deviation. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. $P < 0.05$ was considered statistically significant. All experiments were independently repeated at least three times.

3. Results

3.1. DIO1 activation affects the expression of DIO1 and inflammation-related factors in kidney tissues of RIRI mice

To determine whether DIO1 is involved in the pathogenesis of RIRI and its relationship with NLRP3 inflammasome activation, we first performed immunofluorescence staining of DIO1 in kidney tissues from each group (Figure 1a). DIO1-positive expression was strong in the S group but was significantly reduced in the IR group ($P < 0.05$). Compared with the IR group, DIO1-positive expression was significantly restored in the DIO1 group (T3 pretreatment) ($P < 0.05$) and was partially restored in the INF39 group ($P < 0.05$). These findings indicate that RIRI downregulates renal DIO1 expression, whereas T3 pretreatment effectively activates

endogenous DIO1. To further define the activation status of the NLRP3 inflammasome in RIRI and the effect of DIO1 activation thereon, immunohistochemical staining of NLRP3 and IL-1β was performed (Figure 1b). The percentages of NLRP3- and IL-1β-positive cells in renal tubules were significantly increased in the IR group compared with the S group ($P < 0.05$). Compared with the IR group, these percentages were significantly decreased in the DIO1 and INF39 groups ($P < 0.05$), with no significant difference between the latter two groups. These results indicate that DIO1 downregulation during RIRI is accompanied by NLRP3 inflammasome activation; DIO1 activation effectively suppresses the expression of NLRP3, IL-1β, and other inflammation-related factors, with an effect comparable to that of the NLRP3 inhibitor INF39, suggesting a negative regulatory relationship between DIO1 and NLRP3 inflammasome activation.

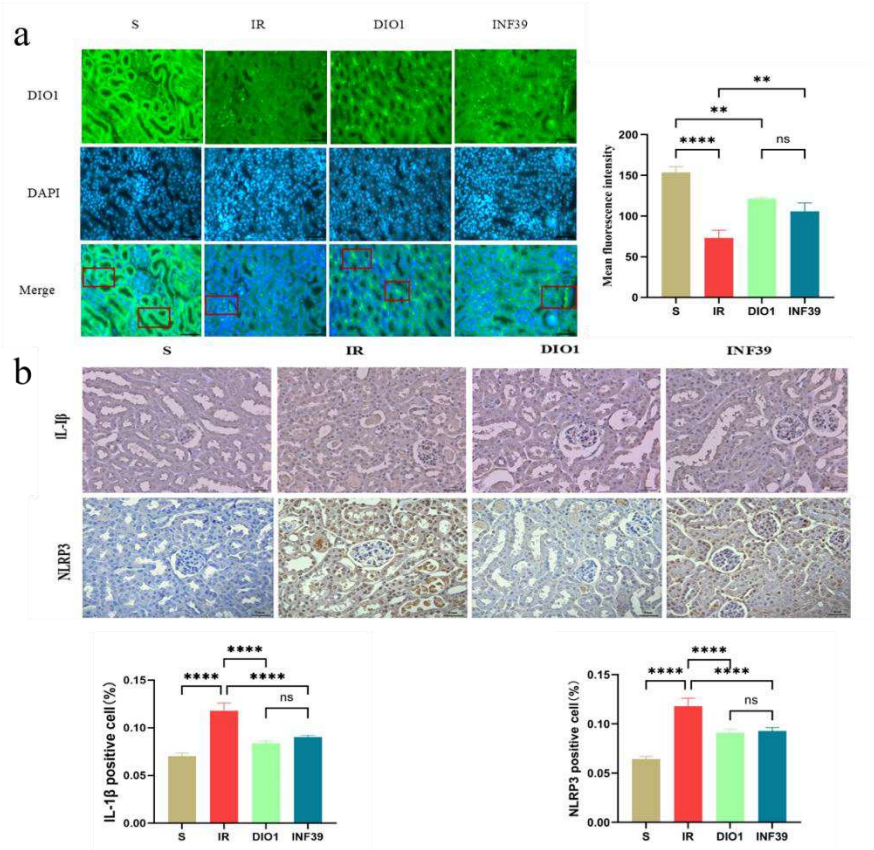


Figure 1. Effects of DIO1 activation on the expression of DIO1 and inflammation-related factors in kidney tissues of RIRI mice

Note: (a) Immunofluorescence staining of DIO1 (green fluorescence) in the renal cortex (scale bar, 50 μm) and quantification of positive expression; (b) immunohistochemical staining of NLRP3 and IL-1β (×400; scale bar, 50 μm) and percentages of positive cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. S, sham group; IR, renal IR injury group; DIO1, renal IR injury with DIO1 activation group; INF39, renal IR injury with NLRP3 inflammasome inhibitor group.

3.2. DIO1 activation improves renal function and attenuates tissue injury in RIRI mice

To evaluate the protective effect of DIO1 activation against RIRI-induced renal dysfunction, serum BUN and Scr levels were measured 24 h after reperfusion (Figure 2a, b). Serum BUN and Scr levels in the IR group were significantly higher than those in the S group (both $P < 0.05$), and were significantly decreased in the DIO1 and INF39 groups compared with the IR group ($P < 0.05$), with no significant difference between the latter two groups ($P > 0.05$). To further assess the effect of DIO1 activation on histopathological injury, HE staining was performed (Figure 2c). The S group showed normal tubular architecture. The IR group exhibited typical lesions, including tubular dilation, atrophy and degeneration, cast formation, and brush border loss, with an injury score significantly higher than that of the S group ($P < 0.05$). These lesions were markedly attenuated in the DIO1 and INF39 groups, with significantly lower injury scores (both $P < 0.05$). In addition, Western blot was used to detect NGAL, an early sensitive marker of AKI (Figure 2d). NGAL protein expression in kidney tissues was significantly higher in the IR group than in the S group ($P < 0.05$) and was significantly decreased in the DIO1 and INF39 groups ($P < 0.05$). These results indicate that DIO1 activation significantly ameliorates RIRI-induced renal dysfunction and tubular injury, confirming a clear protective effect against RIRI.

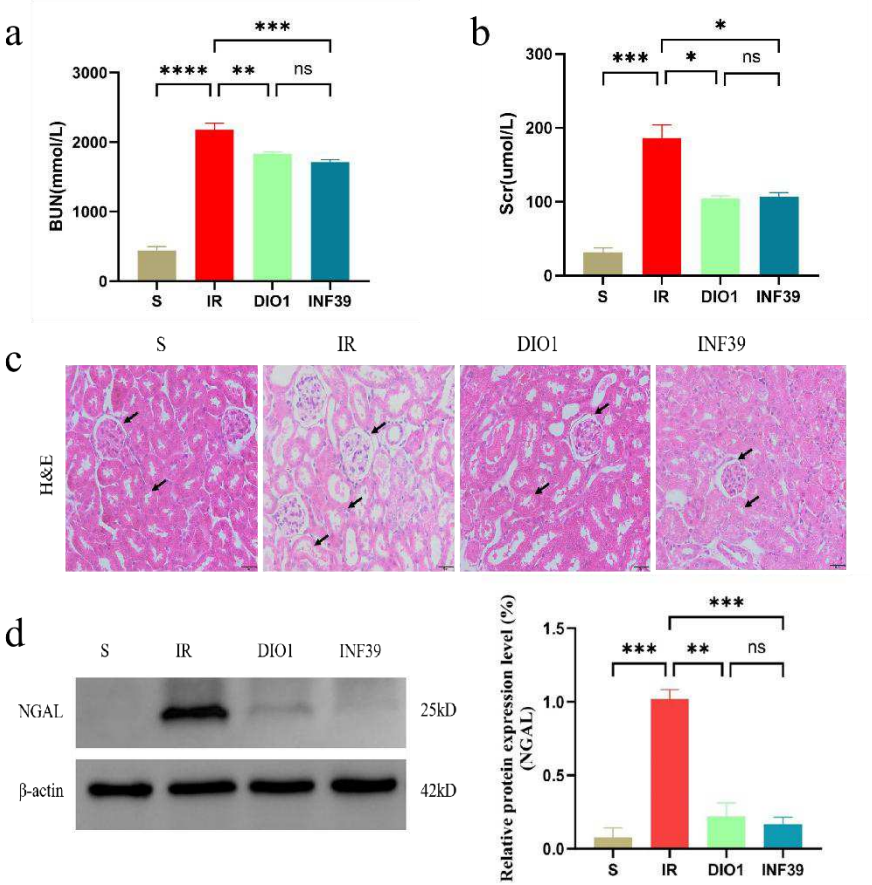


Figure 2. DIO1 activation improves renal function and attenuates kidney injury in RIRI mice

Note: (a) Serum BUN levels; (b) serum Scr levels; (c) HE staining of kidney tissues (×400; scale bar, 50 μm); (d) NGAL protein expression by Western blot and quantification (normalized to β-actin). *P < 0.05, **P < 0.01, ***P < 0.001. S, sham group; IR, renal IR injury group; DIO1, renal IR injury with DIO1 activation group; INF39, renal IR injury with NLRP3 inflammasome inhibitor group.

3.3. DIO1 overexpression suppresses NLRP3 inflammasome-related protein expression in hypoxia-reoxygenation-treated HK-2 cells

To directly verify the inhibitory effect of DIO1 on NLRP3 inflammasome activation in vitro, we first established an HK-2 cell line stably overexpressing DIO1 (OE group) by lentiviral transfection. The transfection efficiency was verified by Western blot and qRT-PCR (Figure 3a, b). DIO1 protein and mRNA expression were significantly upregulated in the OE group compared with the C group ($P < 0.05$), confirming successful overexpression. The two groups were then subjected to hypoxia-reoxygenation (HR subgroups) or combined NLRP3 agonist NSS treatment (NSS subgroups), and the expression of NLRP3 inflammasome-related proteins was detected by Western blot (Figure 3c). Compared with the C-HR subgroup, the relative protein expression levels of NLRP3, Caspase-1, and GSDMD in the OE-HR subgroup were significantly decreased (all $P < 0.05$). Compared with the C-NSS subgroup, the expression of these proteins in the OE-NSS subgroup was also significantly decreased ($P < 0.05$). Within both the C and OE groups, protein expression in the NSS subgroups was significantly higher than that in the corresponding HR subgroups ($P < 0.05$). These results indicate that hypoxia-reoxygenation and NLRP3 agonist treatment induce the upregulation of NLRP3 inflammasome-related proteins in HK-2 cells, and that DIO1 overexpression effectively suppresses this process, further corroborating the negative regulatory role of DIO1 at the cellular level.

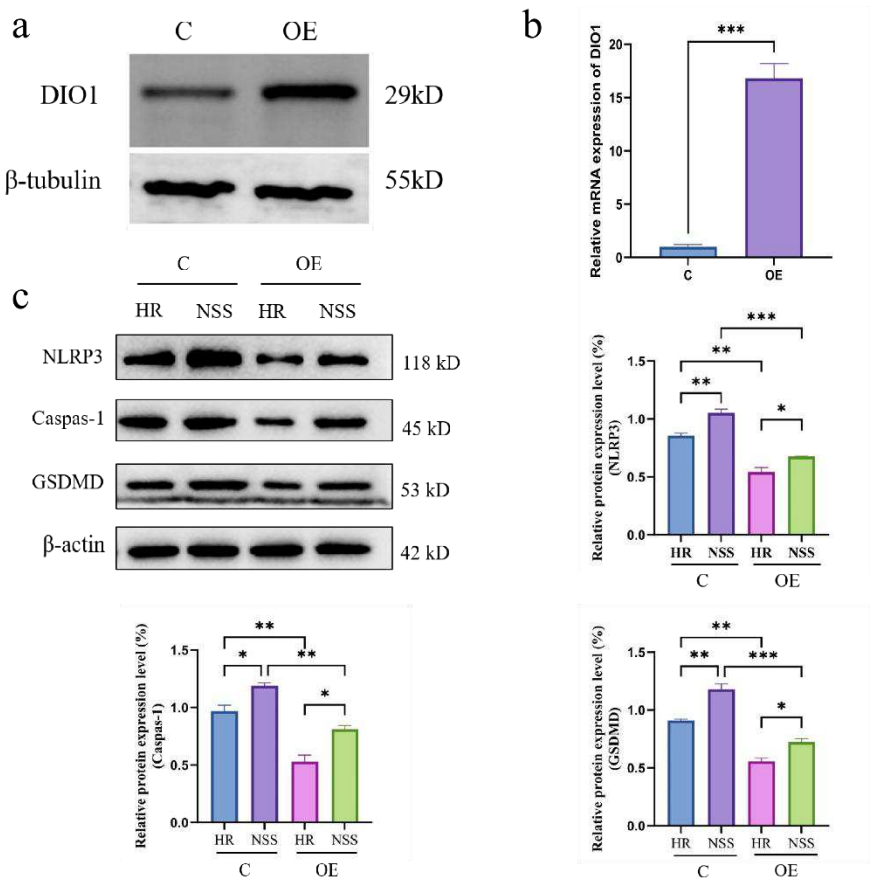


Figure 3. DIO1 overexpression suppresses NLRP3 inflammasome-related protein expression in hypoxia-reoxygenation-treated HK-2 cells

Note: (a) Verification of DIO1 overexpression by Western blot; (b) verification of DIO1 overexpression by qRT-PCR; (c) expression and quantification of NLRP3 inflammasome-related proteins. **P < 0.01, ***P < 0.001, ****P < 0.0001. C, empty vector control group; OE, DIO1 overexpression group; HR, hypoxia-reoxygenation + PBS subgroup; NSS, hypoxia-reoxygenation + NSS subgroup.

3.4. DIO1 overexpression suppresses inflammatory cytokine expression and NF-κB activation in hypoxia-reoxygenation-treated HK-2 cells

To clarify the effect of DIO1 overexpression on downstream inflammatory cytokine release and upstream NF-κB signaling activation, Western blot was performed to detect the protein expression of IL-18, IL-1β, and p-NF-κB p65 in each group (Figure 4). Compared with the C-HR subgroup, the relative protein expression levels of IL-18 and IL-1β in the OE-HR subgroup were significantly decreased (both $P < 0.05$). Compared with the C-NSS subgroup, IL-18 and IL-1β expression in the OE-NSS subgroup was also significantly decreased ($P < 0.05$). Within both the C and OE groups, cytokine expression in the NSS subgroups was significantly higher than that in the corresponding HR subgroups ($P < 0.05$). Meanwhile, compared with the corresponding C subgroups, p-NF-κB p65 protein expression was

significantly decreased in both the OE-HR and OE-NSS subgroups ($P < 0.05$), and p-NF-κB p65 expression in the NSS subgroups was significantly higher than that in the HR subgroups within both groups ($P < 0.05$). The expression trend of p-NF-κB p65 was highly consistent with those of downstream cytokines and NLRP3 inflammasome-related proteins. These results indicate that DIO1 overexpression concomitantly suppresses NF-κB signaling activation and inflammatory cytokine release, suggesting that DIO1 attenuates hypoxia-reoxygenation-induced inflammatory injury in renal tubular epithelial cells by inhibiting the NF-κB/NLRP3 signaling axis.

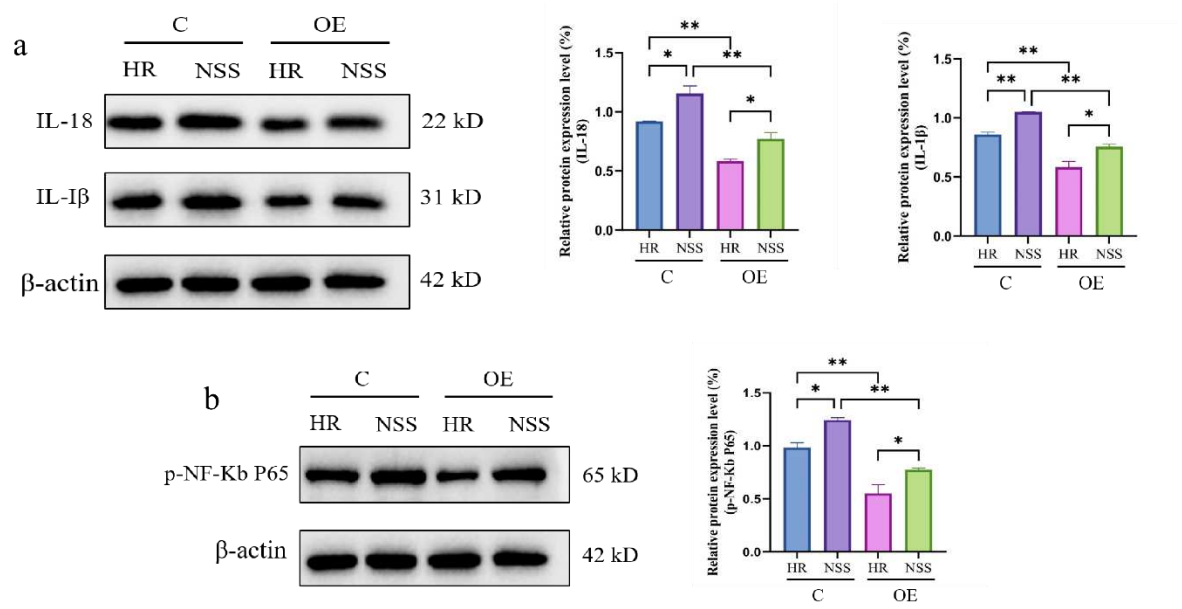


Figure 4. DIO1 overexpression suppresses inflammatory cytokine expression and NF-κB activation in hypoxia-reoxygenation-treated HK-2 cells

Note: (a) Expression and quantification of IL-18 and IL-1β proteins; (b) expression and quantification of p-NF-κB p65 protein. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. C, empty vector control group; OE, DIO1 overexpression group; HR, hypoxia-reoxygenation + PBS subgroup; NSS, hypoxia-reoxygenation + NSS subgroup.

4. Discussion

RIRI is the main pathological process of AKI, and inflammatory responses, particularly the central event of NLRP3 inflammasome activation, play a key role in its pathogenesis [1,26]. In this study, using a mouse RIRI model and a hypoxia-reoxygenation HK-2 cell model, we systematically investigated the protective effect of DIO1 against RIRI and the underlying molecular mechanisms in vitro and in vivo. The main findings are as follows. First, DIO1 expression was significantly downregulated in kidney tissues of RIRI mice, and DIO1 activation significantly ameliorated RIRI-induced renal dysfunction and histopathological

injury. Second, DIO1 activation or overexpression significantly downregulated the expression of NLRP3 inflammasome components (NLRP3, Caspase-1, GSDMD) and downstream inflammatory cytokines (IL-1β, IL-18). Third, DIO1 may suppress NLRP3 inflammasome activation and inflammatory cytokine release by inhibiting NF-κB signaling, thereby attenuating the inflammatory response in RIRI.

As an important selenoprotein, DIO1 plays a central role in thyroid hormone metabolism and participates in various physiological and pathological processes by regulating local T3 levels [15-17]. In this study, immunofluorescence staining revealed that DIO1 expression in the kidneys of IR mice was significantly lower than that in the S group (Figure 1), consistent with the report by Wu et al. [21] that DIO1 mRNA expression is downregulated during RIRI, suggesting that DIO1 is involved in the pathogenesis of RIRI. Thyroid hormone T3 is a positive regulator of DIO1 that induces DIO1 transcription through thyroid hormone receptors [27]. In this study, T3 pretreatment significantly upregulated DIO1 expression in the DIO1 group compared with the IR group, confirming that T3 effectively activates DIO1 in vivo. Regarding renal function, serum Scr and BUN levels and renal NGAL protein expression in the DIO1 group were significantly lower than those in the IR group (Figure 2), with markedly alleviated tubular injury, indicating a significant protective effect of DIO1 activation against RIRI. The NLRP3 inhibitor INF39 was included as a positive control. DIO1 and INF39 showed no significant differences in renal function, injury scores, or NLRP3/IL-1β expression (Figure 1), strongly suggesting that the protective effect of DIO1 against RIRI is associated with the suppression of NLRP3 inflammasome activation.

It should be noted that T3 pretreatment, rather than DIO1 gene overexpression, was used to upregulate DIO1 expression in the animal experiments, based on the following considerations. First, T3 is a physiological positive regulator of DIO1 that directly induces DIO1 transcription through thyroid hormone receptors [27]; T3 pretreatment thus represents a physiological strategy to activate endogenous DIO1, closely resembling the body's own regulatory mode rather than exogenous gene overexpression. Second, achieving kidney-specific DIO1 overexpression in mice requires kidney-targeted adeno-associated virus transduction or DIO1 conditional transgenic mice, which involve long preparation periods and unstable transduction efficiency in tubular epithelial cells, and were not feasible under the conditions of this study. Third, from a translational perspective, T3 is an approved thyroid hormone drug, and pharmacological activation of endogenous DIO1 offers greater translational feasibility. Of note, thyroid hormone can also modulate the renal immune microenvironment and innate immune cell function [34,35], providing additional support for the anti-

inflammatory effect of T3 pretreatment in RIRI. Meanwhile, the in vitro experiments directly confirmed the causal protective effect of DIO1 through lentivirus-mediated overexpression, complementing the in vivo T3 activation strategy; the consistent results obtained through these two approaches strengthen the reliability of our conclusions. Admittedly, as a systemic hormone, T3 exerts broad metabolic and hemodynamic effects, and renoprotection partially independent of DIO1 cannot be entirely excluded. Future studies will employ renal tubule-specific DIO1 conditional overexpression mice or DIO1 knockdown combined with T3 rescue experiments to further clarify the causal contribution of DIO1.

Excessive NLRP3 inflammasome activation is a central event in the inflammatory cascade during ischemia-reperfusion injury: activated Caspase-1 promotes the maturation and release of IL-1β and IL-18 and cleaves GSDMD to initiate pyroptosis, thereby aggravating tissue inflammatory damage [6,8,9,26]. In the in vivo experiments, immunohistochemistry showed that positive NLRP3 and IL-1β expression in the DIO1 group was significantly lower than that in the IR group (Figure 1). In vitro, DIO1 overexpression significantly downregulated the expression of NLRP3, Caspase-1, GSDMD, IL-1β, and IL-18 in hypoxia-reoxygenation-treated HK-2 cells (Figures 3, 4). These results indicate that DIO1 effectively inhibits NLRP3 inflammasome activation and reduces downstream cytokine release, thereby attenuating RIRI. It should be pointed out that this study detected only the expression of full-length GSDMD and did not directly assess pyroptosis-executing events such as GSDMD cleavage, membrane integrity, and LDH release. Therefore, whether DIO1 directly inhibits pyroptosis remains inconclusive and requires further investigation. Notably, recent studies have confirmed that suppression of renal pyroptosis significantly attenuates RIRI [37,38]. The NF-κB signaling pathway is a key upstream regulator of NLRP3 inflammasome activation and promotes inflammasome assembly by upregulating NLRP3 and pro-Caspase-1 expression [28,29]. In multiple ischemia-reperfusion injury models, NF-κB activation has been shown to be closely associated with excessive NLRP3 inflammasome activation [30-32,37]. In this study, DIO1 overexpression significantly reduced p-NF-κB p65 protein expression in hypoxia-reoxygenation-treated HK-2 cells (Figure 4), and its expression trend was consistent with those of NLRP3 inflammasome-related proteins and inflammatory cytokines. These findings suggest that DIO1 inhibits NLRP3 inflammasome activation by suppressing the NF-κB pathway, thereby attenuating the inflammatory response. Considering the previous findings that DIO1 is negatively correlated with TNF-α [19] and that IL-1β can downregulate DIO1 mRNA via the NF-κB pathway [20], the present study further supports the existence of a negative feedback regulatory relationship between DIO1 and the NF-κB/NLRP3 signaling pathway.

The findings of this study have important implications for the clinical prevention and treatment of RIRI. Current strategies mainly include ischemic preconditioning, pharmacological interventions (e.g., antioxidants and calcium channel blockers), and optimization of surgical protocols, but their efficacy is limited and lacks specificity [33]. Recently, renal-targeted delivery strategies against the NF-κB/NLRP3 pathway, such as ultrasound-responsive nanoparticles, have shown promising prospects [36]. As an endogenous protective factor, DIO1 has the following translational advantages. First, DIO1 is a selenoprotein endogenously expressed in humans, with a favorable safety profile. Second, DIO1 can be activated by marketed thyroid hormone drugs such as T3, implying a short drug development cycle. Third, DIO1 simultaneously inhibits NF-κB signaling activation and NLRP3 inflammasome-mediated inflammatory responses, offering multi-target advantages. This study provides an experimental basis for developing novel DIO1-targeted strategies for the prevention and treatment of RIRI.

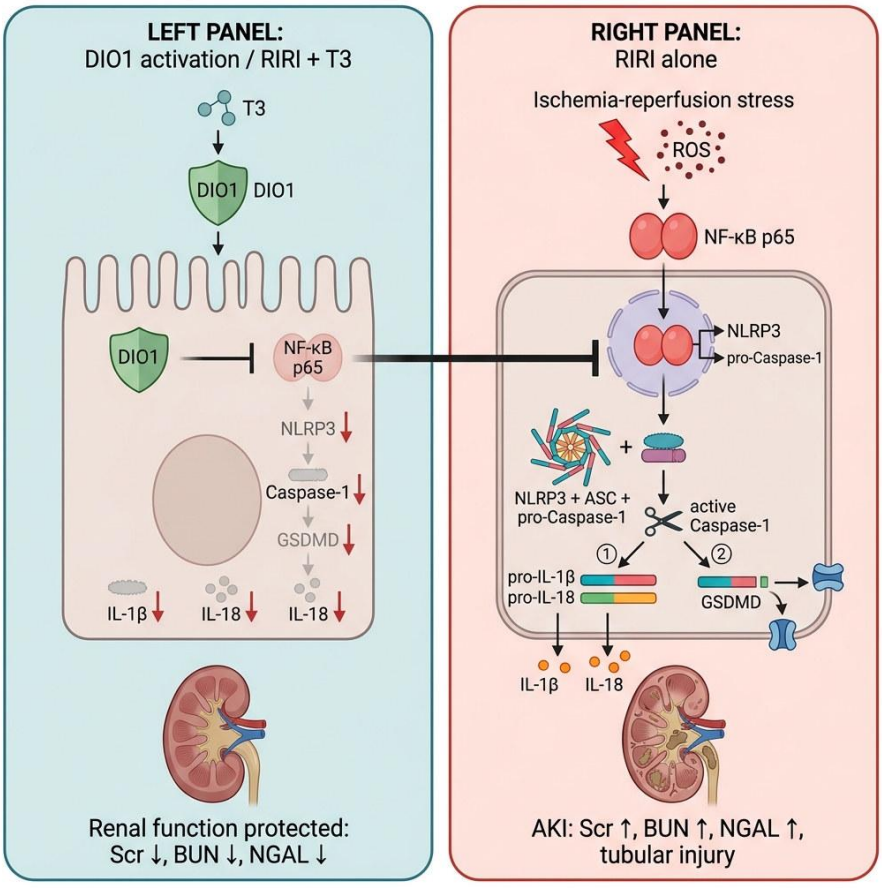


Figure 5. Schematic diagram of the mechanism by which DIO1 attenuates renal ischemia-reperfusion injury by suppressing the NF-κB/NLRP3 signaling pathway

Note: Ischemia-reperfusion activates NF-κB p65 and upregulates the transcription of NLRP3 and pro-Caspase-1, promoting NLRP3 inflammasome assembly and Caspase-1 activation; activated Caspase-1 cleaves GSDMD to form membrane pores and drives the maturation and release of IL-1β and IL-18,

aggravating tubular epithelial injury (right). T3-induced activation or overexpression of DIO1 inhibits NF-κB p65 activation, blocking NLRP3 inflammasome activation and downstream cytokine release, thereby attenuating RIRI (left). ⊣ denotes inhibition. T3, triiodothyronine; RIRI, renal ischemia-reperfusion injury.

This study has several limitations: (1) the animal experiments were validated only in male C57BL/6 mice, which may affect the generalizability of the conclusions; (2) the in vitro experiments used the HK-2 cell line, which cannot fully recapitulate the in vivo immune microenvironment and hemodynamic conditions; (3) only the expression levels of NLRP3 inflammasome-related proteins (NLRP3, Caspase-1, GSDMD) were detected, without direct assessment of pyroptosis-executing events such as GSDMD cleavage, membrane integrity, or LDH release, so the direct effect of DIO1 on pyroptosis remains to be elucidated; (4) T3 pharmacological activation was used to upregulate DIO1 in vivo, and the systemic pleiotropic effects of T3 may confer DIO1-independent renoprotection; moreover, no DIO1 knockout/knockdown controls were included, so the causal protective role of DIO1 in vivo awaits direct verification by genetic intervention; (5) the non-canonical inflammasome pathway mediated by Caspase-4/5/11 was not investigated; (6) the long-term effects of DIO1 on chronic kidney injury (e.g., renal fibrosis) were not assessed; and (7) individual differences in baseline thyroid hormone levels were not controlled. Future studies will enlarge sample sizes to verify DIO1 expression changes in patients with clinical RIRI. Renal tubule-specific DIO1 conditional overexpression mice and pyroptosis-specific assays to further clarify the precise molecular mechanisms by which DIO1 regulates the NF-κB/NLRP3 signaling pathway.

5. Conclusion

(1) DIO1 expression is significantly downregulated in kidney tissues of RIRI mice, and DIO1 activation significantly ameliorates RIRI-induced renal dysfunction and histopathological injury.

(2) The protective mechanism of DIO1 against RIRI is associated with the suppression of NLRP3 inflammasome activation and the reduction of IL-1β and IL-18 release.

(3) The protective effect of DIO1 against RIRI may be attributed to the inhibition of NF-κB signaling pathway activation.

Ethics statement

All animal experiments were approved by the Animal Ethics Committee of Guizhou Medical University (approval number: 2305126) and conducted in strict accordance with the guidelines for the use and ethics of laboratory animals. This study did not involve human

participants.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Author contributions

Guofeng Shu: experimental operations, data curation, statistical analysis, and manuscript drafting; Yikun Wu: experimental guidance, data analysis, and manuscript revision; Shuxiong Xu: study design, manuscript review, and funding acquisition; Yu Xiao: assistance with experiments, literature search and collection, and manuscript formatting check. All authors have read and approved the final manuscript.

Data availability

The original data of this study are available from the corresponding author upon reasonable request.

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