ORIGINAL ARTICLE

Basic Study

Hypoxia inducible factor 1α promotes interleukin-1 receptor antagonist expression during hepatic ischemia-reperfusion injury

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Abstract

BACKGROUND
Ischemia-reperfusion injury (IRI) is a major risk associated with liver surgery and transplantation, and its pathological mechanism is complex. Interleukin-1 receptor antagonist (IL-1ra) can protect the liver from IRI. However, the regulatory mechanism of IL-1ra expression is still unclear.

AIM
To identify the mechanism that could protect the liver in the early stage of IRI.

METHODS
To screen the key genes in hepatic IRI, we performed RNA sequencing and gene enrichment analysis on liver tissue from mice with hepatic IRI. Subsequently, we verified the expression and effect of IL-1ra in hepatic IRI. We also used promoter mutagenesis and chromatin immunoprecipitation assay to search for the trans-

criptional regulatory sites of hypoxia-inducible factor (HIF)-1α. Finally, to explore the protective mechanism of ischemic preconditioning (IP), we examined the expression of HIF-1α and IL-1ra after IP.

RESULTS

We identified IL-1ra as a key regulator in hepatic IRI. The expression of IL-1ra was significantly upregulated after hepatic IRI both *in vivo* and *in vitro*. Furthermore, we found that HIF-1α regulated *Il-1ra* transcription in response to hypoxia. Increased HIF-1α accumulation promoted IL-1ra expression, whereas inhibition of HIF-1α exhibited the opposite effect. We also confirmed a predominant role for hypoxia response element in the regulation of *Il1ra* transcription by HIF-1α activation. Of note, we demonstrated that IP protects against hepatic IRI by inducing IL-1ra expression, which is mediated through HIF-1α.

CONCLUSION

We demonstrated that ischemia or hypoxia leads to increased expression of IL-1ra through HIF-1α. Importantly, IP protects the liver from IRI *via* the HIF-1α–IL-1ra pathway.

Core Tip: Ischemia-reperfusion injury (IRI) is a major risk associated with liver surgery and transplantation, and its pathological mechanism is complex. Interleukin-1 receptor antagonist (IL-1ra) can protect the liver from IRI. The expression of IL-1ra was significantly upregulated after IRI in mice. Hypoxia promoted the expression of IL-1ra. Hypoxia-inducible factor (HIF)-1α accumulation contributed to increased *Il-1ra* transcription. Ischemic preconditioning protected the liver from IRI *via* the HIF-1α-IL-1ra pathway.

INTRODUCTION

Hepatic ischemia (IS)-reperfusion injury (IRI) is a common complication in liver surgery and transplantation[1]. IRI usually results from restoration of the blood supply after brief IS[2]. The sterile inflammatory response induced by hypoxic stress leads to hepatic IRI[3]. The pathogenesis of hepatic IRI is complex and affected by various factors[4,5], which involve the damage-associated molecular patterns, innate immune response, and inflammation. Among them, the inflammation caused by interleukin (IL)-1β plays an important role[6,7]. During hepatic IRI, injured cells activate the inflammasome pathway, allowing the IL-1β precursor to be cleaved into its mature form by caspase-1 and subsequently released. Mature IL-1β is a broad-acting proinflammatory cytokine that increases the recruitment of endothelial adhesion molecules to innate immune cells and promotes the development of inflammatory phenotypes[8]. However, blocking the action of IL-1 can reduce hepatic damage[9,10]. As a natural anti-inflammatory factor, IL-1 receptor antagonist (IL-1ra) is highly expressed in a variety of inflammatory diseases and is closely related to the occurrence and progression of inflammation[11-14]. Several studies have shown a potential protective effect of IL-1ra in inflammation. For example, IL-1ra competes with IL-1β to bind to IL-1 receptor I, thus playing an anti-inflammatory role[15]. However, the transcriptional regulatory mechanism of IL-1ra during IRI remains unclear.

As a transcription factor, hypoxia-inducible factor (HIF)-1α is an important molecule for cell regulation in the hypoxic environment[16,17]. There have been a few reports confirming that HIF-1α plays an irreplaceable role in hepatic IRI[18]. HIF-1α can regulate the expression of multiple genes in cells after hypoxia[19], and consequently reduce organ IRI by regulating metabolism[20]. Furthermore, the mechanism of HIF-1α protection against hepatic IRI deserves further study.

Ischemic preconditioning (IP) was first reported by Murry *et al*[21] in 1986, who demonstrated that multiple IP reduces cell death after coronary artery occlusion. Although the protective effect of IP on hepatic IRI has been subsequently reported[22], its mechanism of action remains unclear. Therefore, it is

necessary to confirm the mechanism underlying this protective effect, which would be of great clinical significance. In the present study, we found that IL-1ra was expressed in hepatic tissue during IRI and was regulated by IS-induced HIF-1α. We also confirmed that the protective effect of IP was exerted precisely *via* the HIF-1α–IL-1ra pathway, leading to inhibition of IL-1β signaling and subsequent reduction in hepatic IRI.

MATERIALS AND METHODS

Reagents and antibodies
2-MeOE2 (S1233) and DMOG (S7483) were purchased from Selleck (China). Percoll (17089101) was purchased from GE Healthcare (United States). Collagenase IV (C8160) was purchased from Solarbio (China). Mouse monoclonal antibody for β-actin (sc-47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). A rabbit monoclonal antibody for IL-1ra (ab124962) was purchased from Abcam (Cambridge, MA, United States). Rabbit antibodies for HIF-1α (36169), BAX (14796), Bcl-2 (3498), and cleaved caspase-3 (9664) were purchased from Cell Signaling Technology (Danvers, MA, United States). ELISA kits for detecting mouse alanine transaminase (ALT) (JL12668), aspartate transaminase (AST) (JL13793), and IL-1ra (JL20255) were purchased from Jonln (China). Recombinant IL-1ra (200-01RA) was purchased from PeproTech (United States).

Animals
Wild-type male C57BL/6 mice, aged 6-8 wk, were purchased from Beijing HFK Bioscience (Beijing, China). The mice were raised in standard conditions[23] and received humanitarian care. The mice were acclimated to the rearing environment for 3-4 d before experimentation.

Animal experiments
Mice were randomly grouped and marked. The mice were weighed and anesthetized with 1% pentobarbital (50 mg/kg). For the hepatic IR experiments, mice were divided into six groups (*n* = 5 each): Sham group: The portal vein was exposed, and no blood vessels were clamped; ischemia reperfusion (IS) 1.5 h group: We only clamped the left and middle lobe vessels for 1.5 h; IR 3-24 h group: Reperfusion was performed for the corresponding time after the vessels had been clamped for 1.5 h; IP group: We briefly clamped the vessel for 10 min and released it for 10 min before IS; this was named an IP cycle; sh*Il1ra* group: Adenovirus carrying *Il1ra* shRNA was injected *via* the tail vein 48 h before IR. At the end of the experiment, the ischemic hepatic lobes were isolated for subsequent processing or stored at -80 °C. The animal study protocol was approved by the Animal Ethical and Welfare Committee (protocol code: IRM-DWLL-2020173 and date of approval: September 15, 2020).

Nonparenchymal cell and hepatocyte isolation
Nonparenchymal cells (NPCs) and hepatocytes were isolated from mice by collagenase digestion and differential centrifugation using Percoll, as previously described[24].

Histological examination of liver tissue
The left lobe samples of mouse livers were fixed in formaldehyde solution, dehydrated, paraffin-embedded, and cut into 4-μm-thick sections. The IL-1ra levels were determined by immunohistochemistry. The histological analysis was performed using the histochemistry score. A section was selected from the left lobe in every liver, and five views (200 ×) were captured for calculation in each section.

Cell culture
Mouse liver parenchymal cells, AML12 (SCSP-550; American Type Culture Collection, Manassas, VA, United States), were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (11965092; Gibco) supplemented with 10% fetal bovine serum (FBS) (10099133; Gibco) and 1% penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO_2. HEK293T cells were cultured in DMEM (11320033; Gibco) supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids, and 2% glutamine.

Cell experiment
AML12 cells were subjected to hypoxia and reoxygenation to simulate the ischemic and reperfusion environment *in vivo*. During hypoxia, cells were cultured in a 37 °C incubator with 95% N_2 and 5% CO_2. After a specific time period, cells were removed and placed back into the 37 °C incubator with 5% CO_2. To interfere with HIF-1α, cells were treated with DMOG or 2-MeOE2 at a concentration of 100 μM for 6 h before subsequent experiments were performed.

Table 1 Primer sequences

Gene	Forward sequence	Reverse sequence
Il1ra	TCTTGGGCATCCACGGG	GAGGCTCACAGGACGGTCAG
β-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
Il1ra pro-2.1K	CGGAGCTCGCCAGCAAGATTTTAAGTGATTCT	CCAGATCTGGGTGAGCTAAACAGGACACAAGGT
Il1ra pro-1.5K	CGGAGCTCCCCAACAACTTCCAGACTTCCCTC	CCAGATCTGGGTGAGCTAAACAGGACACAAGGT
Il1ra pro-1.1K	CGGAGCTCATCAGTCTAAGGCTGGGCAGGGAG	CCAGATCTGGGTGAGCTAAACAGGACACAAGGT
Il1ra pro-0.6K	CGGAGCTCCCCAGCTCAAATGCCACCATTCTC	CCAGATCTGGGTGAGCTAAACAGGACACAAGGT
Il1ra Mut1	TTTATGCACATTCCCTCTTTCAGC	AGAGGGAATGTGCATAAACTTGT
Il1ra Mut2	GATATGGACTTGCCATTTGACTC	AAATGGCAAGTCCATATCTTTCTT

Plasmid construction

The mouse *Il1ra* promoter (-2113/+143) sequences were obtained by polymerase chain reaction (PCR) from mouse genomic DNA and cloned into the pGL3-basic vector (Promega, Madison, WI, United States). Mutagenesis of HRE-I and HRE-II in the mouse *Il1ra* promoter was performed using a Quick-Change® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). The primer sequences are listed in Table 1. The mouse *Il1ra* shRNA plasmid was purchased from Hanbio Biotech-nology (Shanghai, China), and packaged into adenoviral particles.

RNA extraction and quantitative qPCR

Total RNA from liver tissue and AML12 cells was extracted using Trizol (Life Technologies, Carlsbad, CA, United States). cDNA was synthesized using reverse transcriptase (Takara, Japan). The specific product of *il1ra* was amplified by qPCR using the TransStart Green Q-PCR SuperMix Kit (TransGen, China). β-Actin was used as a normalization control. The primer sequences are listed in Table 1.

Immunoblotting assay

Tissues and cell lysates were prepared in RIPA buffer with protease and phosphatase inhibitors. The immunoblotting procedures can be found in the literature[25].

Luciferase assay

Cells were transfected with wild-type or mutant mouse *Il1ra* promoters, followed by treatment with DMOG or 2-MeOE2 under hypoxia in 24-well plates. Lysates were prepared 48 h after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was normalized to the values for Renilla luciferase.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using an EZ-ChIP kit (Millipore, Billerica, MA, United States). The primers and antibodies used in these experiments are shown in Table 1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (San Diego, CA, United States). All the data are presented as the mean ± SEM and represent three or five independent experiments. One-way analysis of variance was used to compare means among treatment groups. Student's *t*-test for unpaired observations was applied. $P < 0.05$ was considered significant.

RESULTS

Screening of protective genes during hepatic IRI

To screen key genes that potentially play protective roles in the liver in IRI, we constructed a mouse model of hepatic IRI and performed transcriptome sequencing using mRNAs of the liver from the Sham, IS 1.5 h, and IR 3 h groups (Figure 1A). Gene enrichment analysis showed that, after IR 3h, a group of genes enriched in the IL-1 pathway were upregulated (Figure 1B). Expression of 25 genes was significantly increased (> 2-fold) in both IS and IR periods (Figure 1C). These genes were then ranked in descending order of ischemic expression abundance, and *il1ra*, as a key antagonist of the IL-1 signaling, was shown to be one of the highly differentially expressed genes (Figure 1D). Thus, we chose *il1ra* for further investigation on its regulatory mechanism in the hepatic IRI.
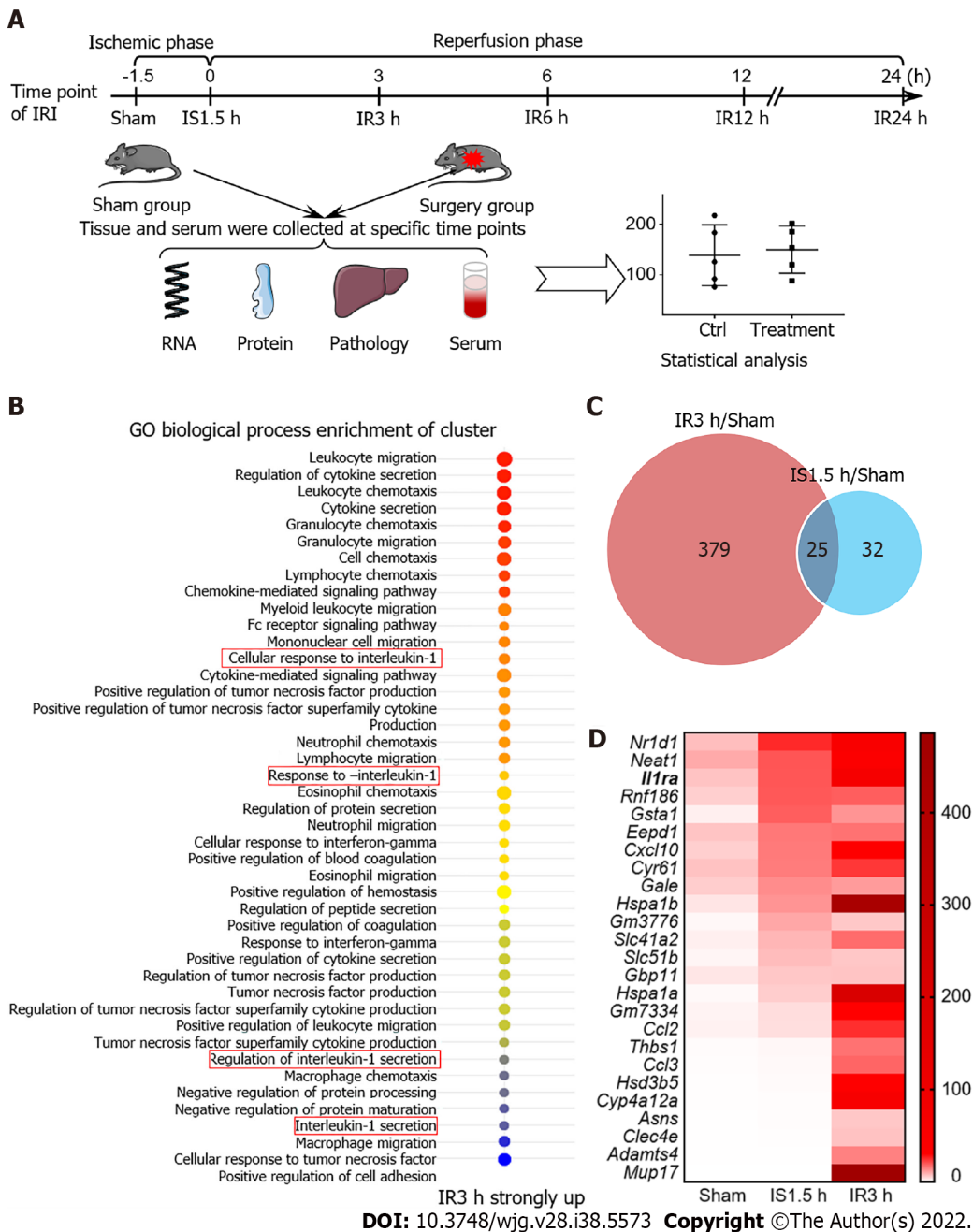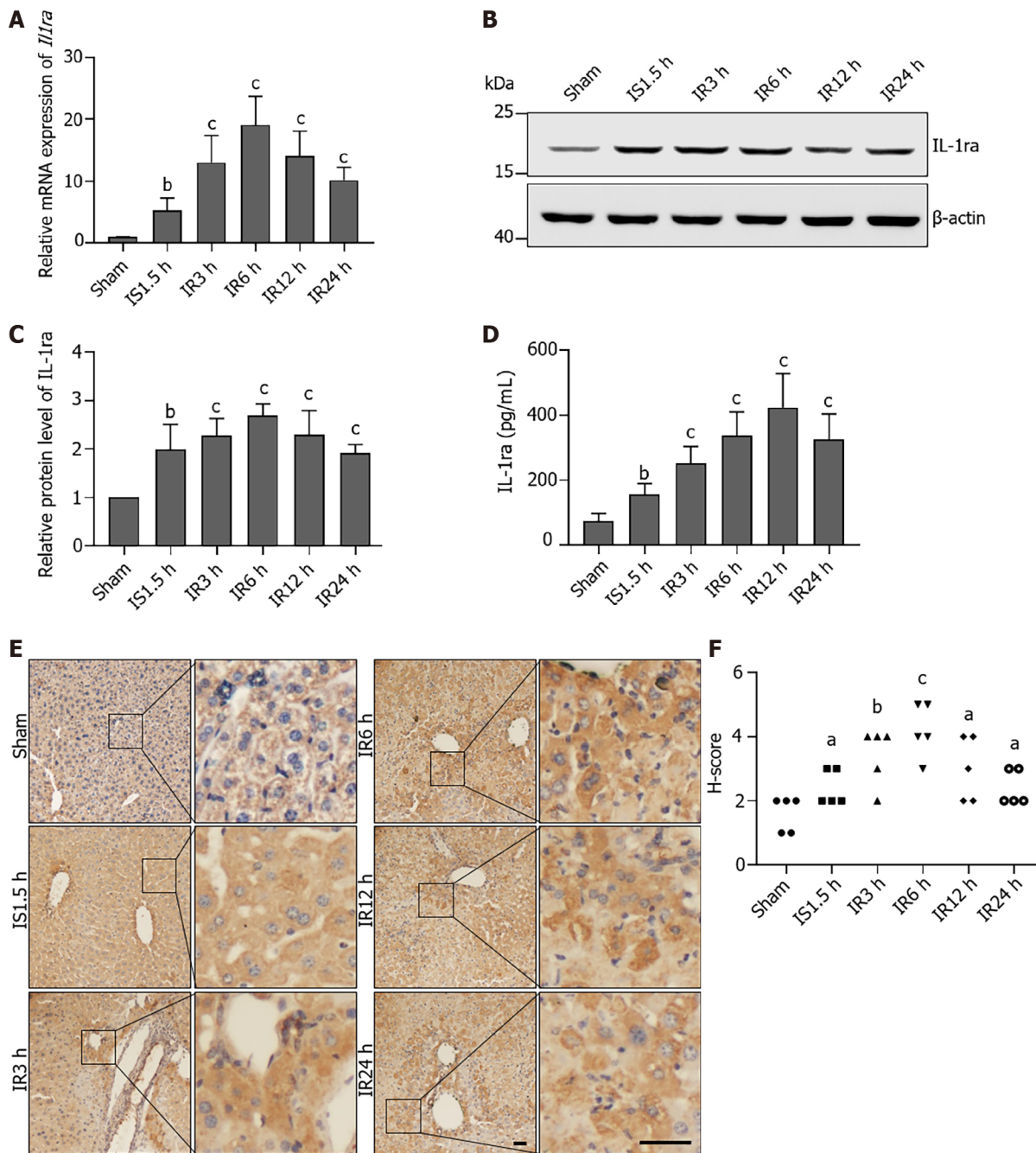
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Figure 1 Construction of a mouse model of hepatic ischemia-reperfusion injury and gene enrichment analysis. A: Model of ischemia-reperfusion injury (IRI) in mice; B: GO enrichment analysis upon IR for 3 h; C: Venn diagram shows that the expression of genes for IR 3 h and ischemia (IS) 1.5 h was more than 2-fold than that in the Sham group; D: Twenty-five genes were elevated in both IR 3 h and IS 1.5 h groups according to IS 1.5 h expression level. IS: Ischemia; IR: Ischemia-reperfusion; IRI: Ischemia-reperfusion injury.

IL-1ra expression is elevated during hepatic IRI

We detected IL-1ra expression in mice with IRI. This confirmed that mRNA expression of *Il1ra* was increased in mice that experienced hepatic IRI as compared with the Sham group (Figure 2A). Upregulation of IL-1ra in liver tissue was confirmed at the protein level by immunoblotting (Figures 2B and C), ELISA (Figure 2D), and immunohistochemistry (Figures 2E and F), demonstrating that expression of IL-1ra is significantly elevated in response to hepatic IRI in mice.

Hepatocytes and hepatic NPCs both express high levels of IL-1ra after IRI

The liver is composed of several different embryonic-derived cell types, including hepatocytes, bile duct epithelial cells, stellate cells, Kupffer cells, and sinusoidal endothelial cells[26]. The liver consists of 80% hepatocytes, and 20% of the other cells are collectively called NPCs. Hepatocytes are the major epithelial cell population of the liver and perform various physiological functions. Therefore, we aimed to identify whether there is a difference in the expression of IL-1ra in hepatocytes and NPCs after IRI. To do so, gradient centrifugation was used to separate hepatocytes and NPCs from mouse liver tissue, and
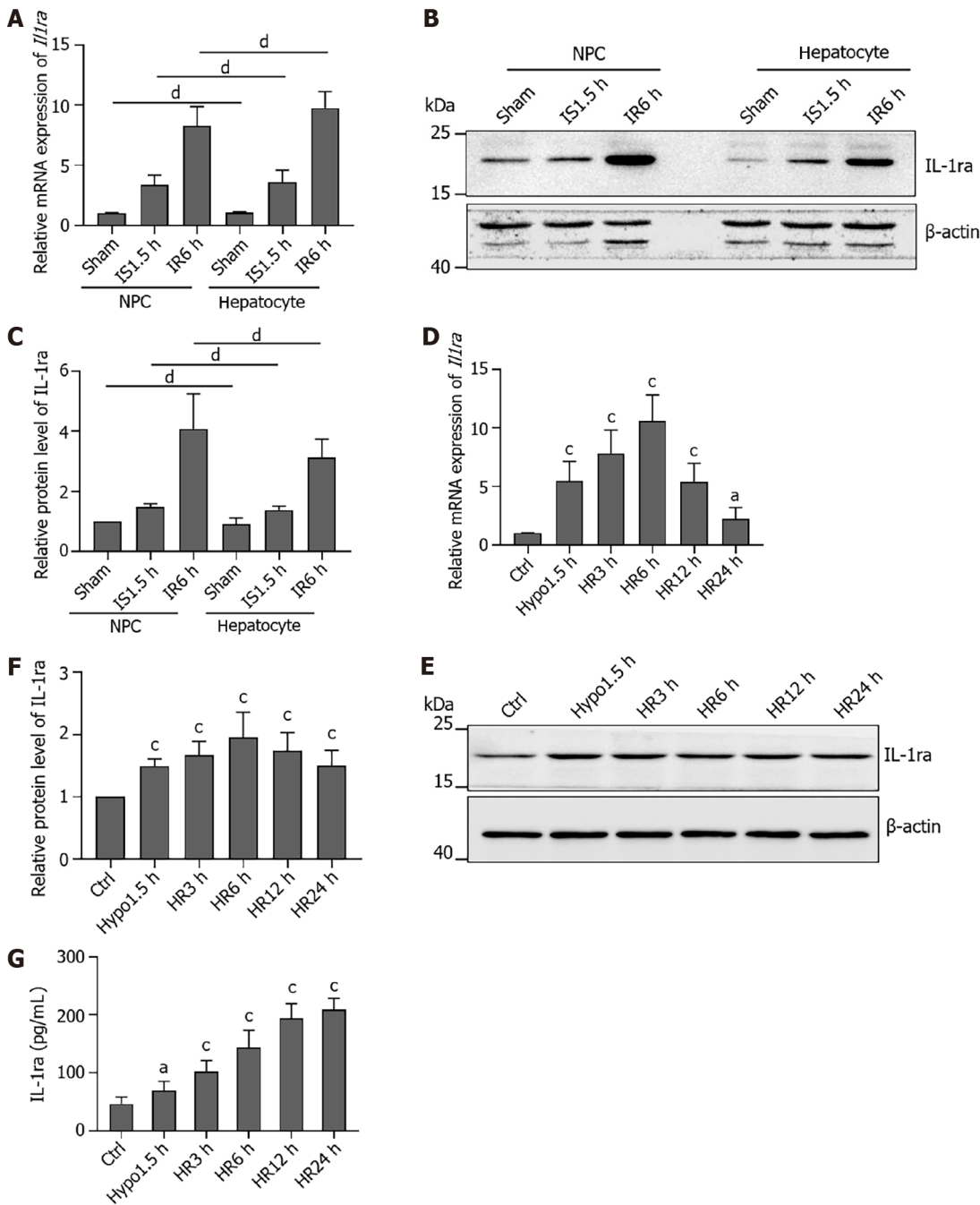
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Figure 2 Interleukin-1 receptor antagonist expression is elevated after hepatic ischemia-reperfusion injury in mice. A: Relative mRNA levels of interleukin-1 receptor antagonist (*Il1ra*) in the liver upon ischemia-reperfusion injury (IRI); B: Protein levels of IL-1ra in the liver upon IRI; C: Statistical analysis of protein levels of IL-1Ra upon IRI; D: Protein content of IL-1ra in serum upon IRI; E. Representative images of immunohistochemical staining for IL-1ra in the liver upon IRI. Scale bars, 100 μm; F: Statistical analysis of immunohistochemical scores. Indicated *P* values were calculated using a two-tailed unpaired Student's *t*-test. Data are presented as the mean ± SEM. Data are representative of five independent experiments. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. IS: Ischemia; IR: Ischemia-reperfusion; IL-1ra: Interleukin-1 receptor antagonist.

expression of IL-1ra was detected. There was no significant difference in the expression of IL-1ra at both the mRNA (Figure 3A) and protein (Figures 3B and C) levels between hepatocytes and NPCs after IR treatment.

To confirm these results *in vitro*, we performed the hypoxia–reoxygenation experiments in the mouse hepatic cell line AML12 to simulate IRI. We found that the mRNA (Figure 3D) and protein (Figures 3E and F) expression of IL-1ra was increased in AML12 cells upon hypoxia and reoxygenation. Similarly, the content of IL-1ra in the culture medium of the hypoxia-reoxygenation group was significantly higher than that in the control group (Figure 3G). Together, these data suggested that IL-1ra is highly expressed in both hepatocytes and NPCs after IRI.

Figure 3 Interleukin-1 receptor antagonist is expressed in both mouse hepatocytes and nonparenchymal cells. A: Relative mRNA levels of interleukin-1 receptor antagonist (*Il1ra*) in hepatocytes and nonparenchymal cells (NPCs) after ischemia-reperfusion injury (IRI); B: Protein levels of IL-1ra in hepatocytes and NPCs after IRI; C: Statistical analysis of protein levels of IL-1Ra in hepatocytes and NPCs after IRI; D: Relative mRNA levels of *Il1ra* in AML12 cells upon hypoxia-reoxygenation (HR); E: Protein levels of IL-1ra in AML12 cells upon HR; F: Statistical analysis of protein levels of IL-1Ra in AML12 cells upon HR; G: Protein content of IL-1ra in culture medium upon HR. Indicated *P* values were calculated using two-tailed unpaired Student's *t*-tests. Data are presented as the mean ± SEM. Data are representative of five independent experiments. $^{ns}P > 0.05$, $^{a}P < 0.05$, $^{b}P < 0.001$. HR: Hypoxia-reoxygenation; NPCs: Nonparenchymal cells; IL-1ra: Interleukin-1 receptor antagonist.

IL-1ra expression is upregulated upon IS and hypoxia

To verify the regulation of IL-1ra expression in the early stage of hepatic IRI, we constructed hypoxic models at different time points *in vivo* and *in vitro*. Compared with the Sham group, ischemic treatment in mice increased IL-1ra expression in a time-dependent manner at both the mRNA (Figure 4A) and protein (Figures 4B-D) levels. Similar results were also revealed in AML12 cells (Figures 4E-H), demonstrating that IS and hypoxia could promote the expression of IL-1ra.

HIF-1α promotes IL-1ra expression under hypoxic conditions

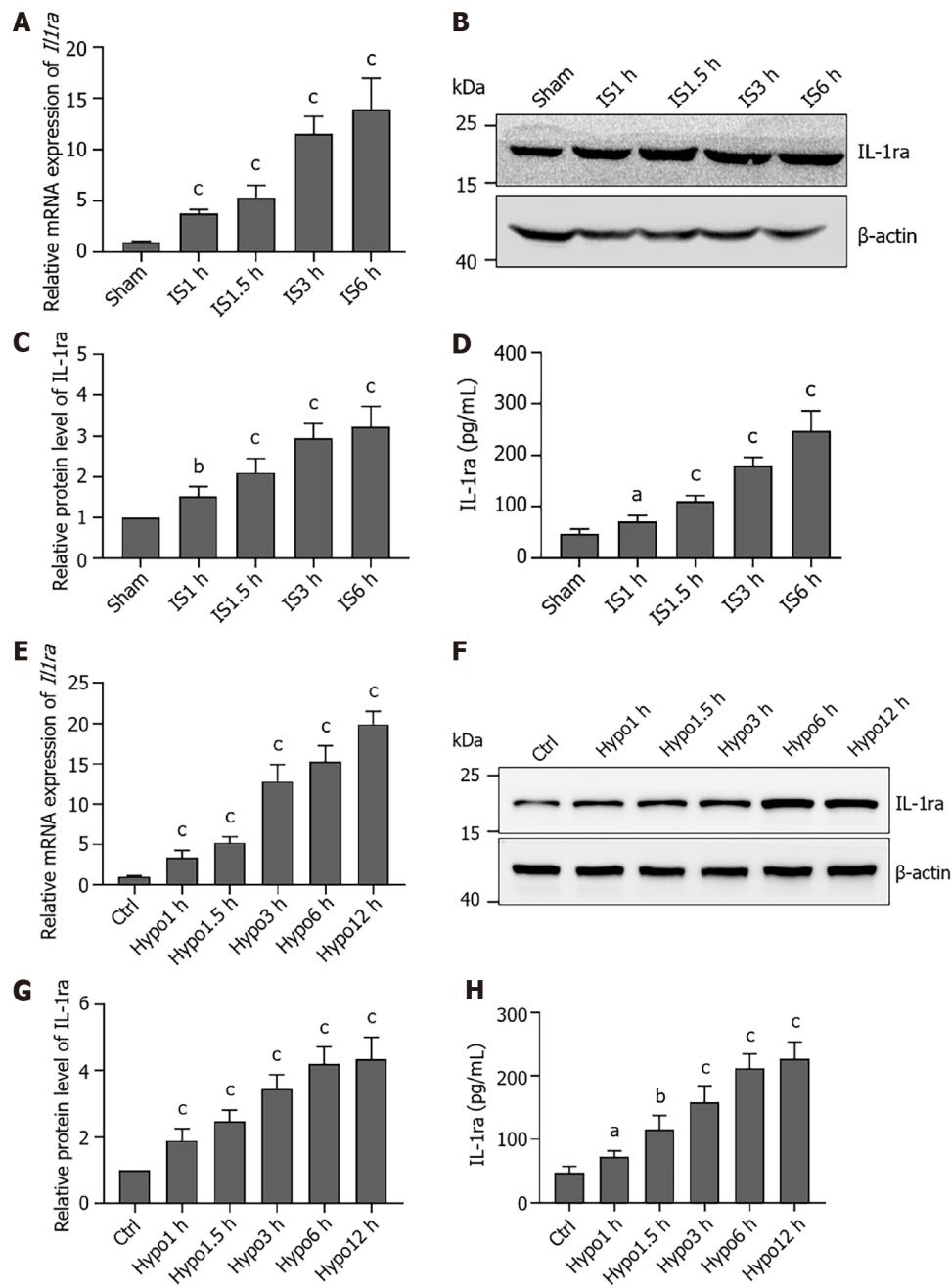HIF-1α, as a transcriptional regulator, plays an important role under hypoxic conditions. Therefore, we

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Figure 4 Interleukin-1 receptor antagonist expression is elevated during ischemia or hypoxia. A: Relative mRNA levels of interleukin-1 receptor antagonist (*Il1ra*) in the liver upon ischemia; B: Protein levels of IL-1ra in the liver upon ischemia; C: Statistical analysis of protein levels of IL-1ra upon ischemia; D: Protein content of IL-1ra in serum upon ischemia; E: Relative mRNA levels of *Il1ra* in AML12 cells upon hypoxia; F: Protein levels of IL-1ra in AML12 cells upon hypoxia; G: Statistical analysis of protein levels of IL-1Ra in AML12 cells upon hypoxia; H: Protein content of IL-1ra in culture medium upon ischemia. Indicated *P* values were calculated using two-tailed unpaired Student's *t*-tests. Data are presented as the mean ± SEM. Data are representative of five independent experiments. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. Hypo: Hypoxia; IS: Ischemia; IL-1ra: Interleukin-1 receptor antagonist.

speculated that HIF-1α would regulate the expression of IL-1ra. Thus, we moved to evaluate the relationship between HIF-1α and IL-1ra expression in hepatic IRI. Increased expression of HIF-1α was observed upon ischemic treatment in mice in a time-dependent manner (Figures 5A and B). Similar results were confirmed in AML12 cells (Figures 5C and D). Immunofluorescence analysis indicated that HIF-1α was localized in the nuclei of AML12 cells after 1.5 h of hypoxia (Figure 5E).

We treated AML12 cells with 2-MeOE2 (a specific HIF-1α inhibitor) or DMOG (a specific HIF-1α agonist). 2-MeOE2 reduced the expression of *Il-1ra* mRNA during hypoxia, while DMOG increased *Il-1ra* expression under normoxia (Figure 5F). Immunoblotting (Figures 5G-I) and ELISA (Figure 5J) confirmed that 2-MeOE2 reduced the protein levels of HIF-1α and IL-1ra in AML12 cells; however, DMOG had the opposite effect. These results indicated that HIF-1α independently promotes the expression of IL-1ra.
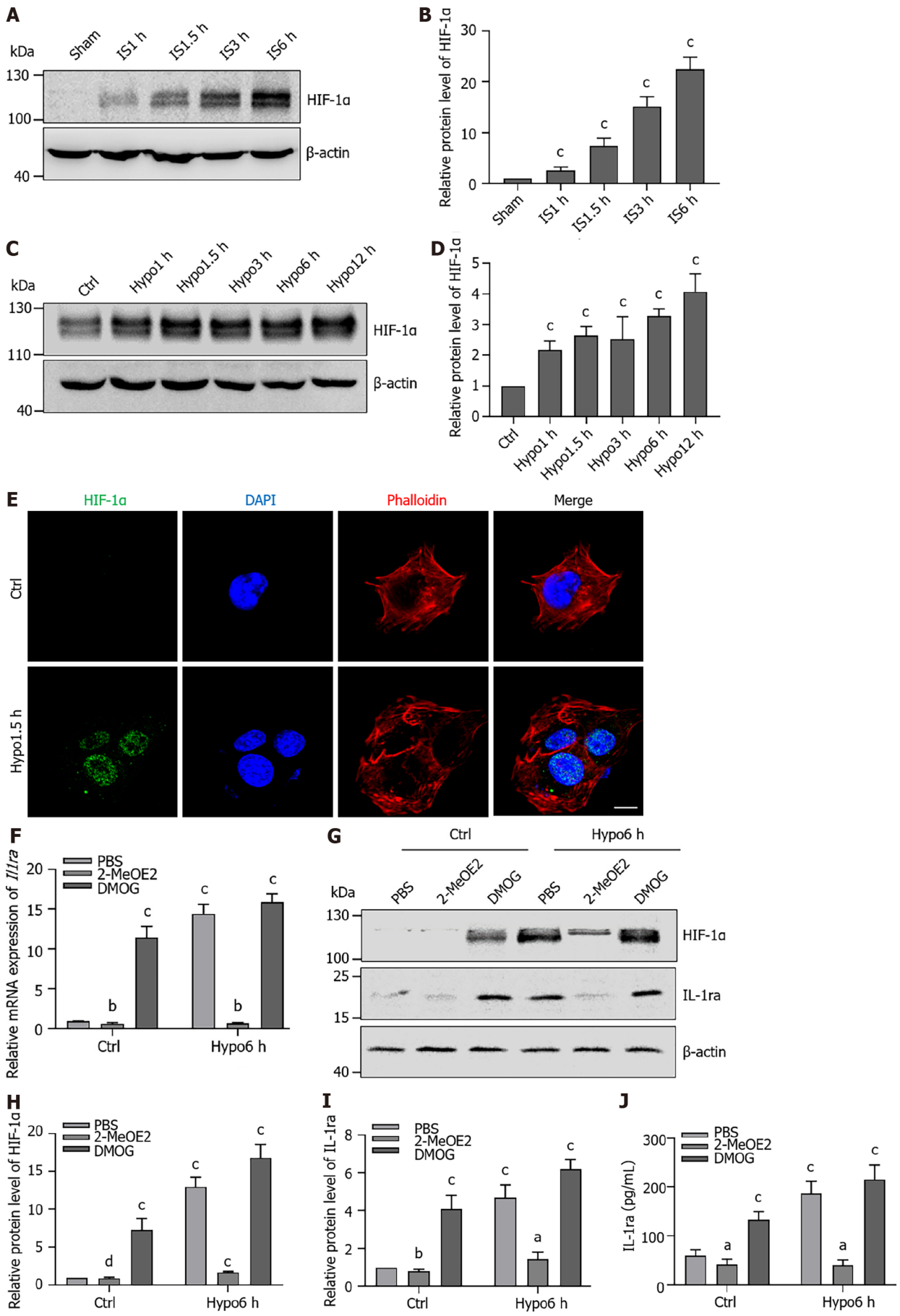
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Figure 5 Hypoxia-inducible factor-1α promotes the expression of interleukin-1 receptor antagonist during ischemia or hypoxia. A: Protein

levels of hypoxia-inducible factor (HIF)-1α in the liver upon ischemia; B: Statistical analysis of protein levels of HIF-1α in the liver upon ischemia; C: Protein levels of HIF-1α in AML12 cells upon hypoxia; D: Statistical analysis of protein levels of HIF-1α in AML12 cells upon hypoxia; E: Immunofluorescence staining for HIF-1α in AML12 cells upon hypoxia for 1.5 h. Scale bars, 10 μm; F: Relative mRNA levels of interleukin-1 receptor antagonist (*Il1ra*) in AML12 cells treated with 2-MeOE2 (100 μM) and DMOG (100 μM) upon hypoxia; G: Protein levels of HIF-1α and IL-1ra in AML12 cells treated with 2-MeOE2 and DMOG upon hypoxia; H: Statistical analysis of protein levels of HIF-1α in AML12 cells treated with 2-MeOE2 and DMOG upon hypoxia; I: Statistical analysis of protein levels of IL-1ra in AML12 cells treated with 2-MeOE2 and DMOG upon hypoxia; J: Protein content of IL-1ra in culture medium of in AML12 cells treated with 2-MeOE2 and DMOG upon hypoxia. Indicated *P*-values were calculated using two-tailed unpaired Student's *t*-tests. Data are presented as the mean ± SEM. Dots depict individual samples. $^{ns}P > 0.05$, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. Hypo: Hypoxia; IS: Ischemia; HIF: Hypoxia-inducible factor; IL-1ra: Interleukin-1 receptor antagonist; IgG: Immunoglobulin G.

HIF-1α transcriptionally upregulates IL-1ra expression

To investigate the regulatory mechanism of IL-1ra by HIF-1α, we transfected a mouse *Il1ra* promoter-reporter construct into AML12 cells (Figure 6A). Upon treatment with hypoxia, we found that *Il1ra* promoter activity was significantly increased (Figure 6B). To explore the transcriptional regulatory elements of the *Il1ra* promoter in response to hypoxia, we generated a series of truncated *Il1ra* promoter–reporter constructs. *Il1ra*-p-2.1k promoter activity was significantly increased upon hypoxic treatment, while the truncated promoter activity of *Il1ra*-p-1.5k, *Il1ra*-p-1.1k, and *Il1ra*-p-0.6k was not altered by hypoxia (Figure 6C).

We used the JASPAR database to predict the binding site of HIF-1α on the *Il1ra*-p-2.1k promoter and found two possible binding elements, HRE I (-2018/-2011) and HRE II (-1895/-1888) (Figure 6D). Site-directed mutagenesis showed that mutation of HRE II did not affect hypoxia-induced activation of the *Il1ra* promoter, while mutation of either HRE I or HRE I + II eliminated this effect (Figure 6D). Importantly, we used the ChIP assay to verify that HIF-1α was able to be recruited to the HRE I element in the *Il1ra* promoter (Figures 6E and F).

IP protects the liver from IRI via the HIF-1α-IL-1ra pathway

It has been reported that IP can alleviate hepatic IRI[27]. However, the protective mechanism of IP is not yet clear. Here, we speculated that IL-1ra might play a potential role in the regulation of IP. Thus, the mice were subjected to IP with the indicated cycles. Compared with the control group, the IP group had significantly lower ALT and AST levels in their serum, which were further reduced by increased IP cycles (Figures 7A and B). Similar results of IL-1ra upregulation were observed by immunoblotting (Figures 7C and D) and ELISA (Figure 7E).

To verify whether IL-1ra plays a protective role in IP, we injected adenovirus carrying *Il1ra* shRNA *via* the tail vein to knock down the expression of IL-1ra in mice. Expression of IL-1ra in the liver and serum of mice injected with adenovirus was significantly decreased (Figures 7F-H). The ALT and AST levels were increased by knockdown of IL-1ra in the liver (Figures 7I and J).

It has been reported that HIF-1α plays an important role in IP[28], and we thus speculated that the expression of IL-1ra in IP is regulated by HIF-1α. We found that IP induced accumulation of HIF-1α in the liver (Figures 7K-M); however, 2-MeOE2 significantly attenuated this effect (Figures 7M-P). These results collectively suggested that IP might exert a protective effect on the liver from IRI through regulating the HIF-1α–IL-1ra signaling.

DISCUSSION

Hepatic IRI is a common complication of liver surgery. Previous studies have demonstrated that the IL-1 signaling pathway plays a pivotal role in the regulation of hepatic IRI. Here, we extended the study, showing that the expression of IL-1ra, an inhibitor of the IL-1 signaling pathway, is increased in response to hepatic IRI, especially in the ischemic stage. Mechanistically, hypoxia induces the transcriptional expression of IL-1ra through HIF-1α accumulation. Of note, we proved that IP could protect the liver from IRI by promoting IL-1ra expression in a HIF-1α-dependent manner. Thus, our study has provided additional evidence for the regulatory mechanism of IL-1ra during IS, indicating a practical strategy for alleviating hepatic IRI.

Previous studies have shown that hepatic IRI not only causes damage to the liver itself, but also leads to the abnormal functioning of various organs, and induces systemic inflammatory response syndrome [29]. Therefore, the question of how to reduce or even eliminate hepatic IRI has received constant attention among clinicians and researchers. Hepatic IRI is a complex pathophysiological process with numerous and relational influencing factors. Among them, the early inflammatory response plays a crucial role in the occurrence and development of hepatic IRI[30], because it is located in the upstream region of the injury response chain. Upon activation, downstream factors often promote each other and thus aggravate the injury[31]. If the activation of early inflammation can be reduced or even blocked, it may be possible to prevent the occurrence and development of hepatic IRI at source.
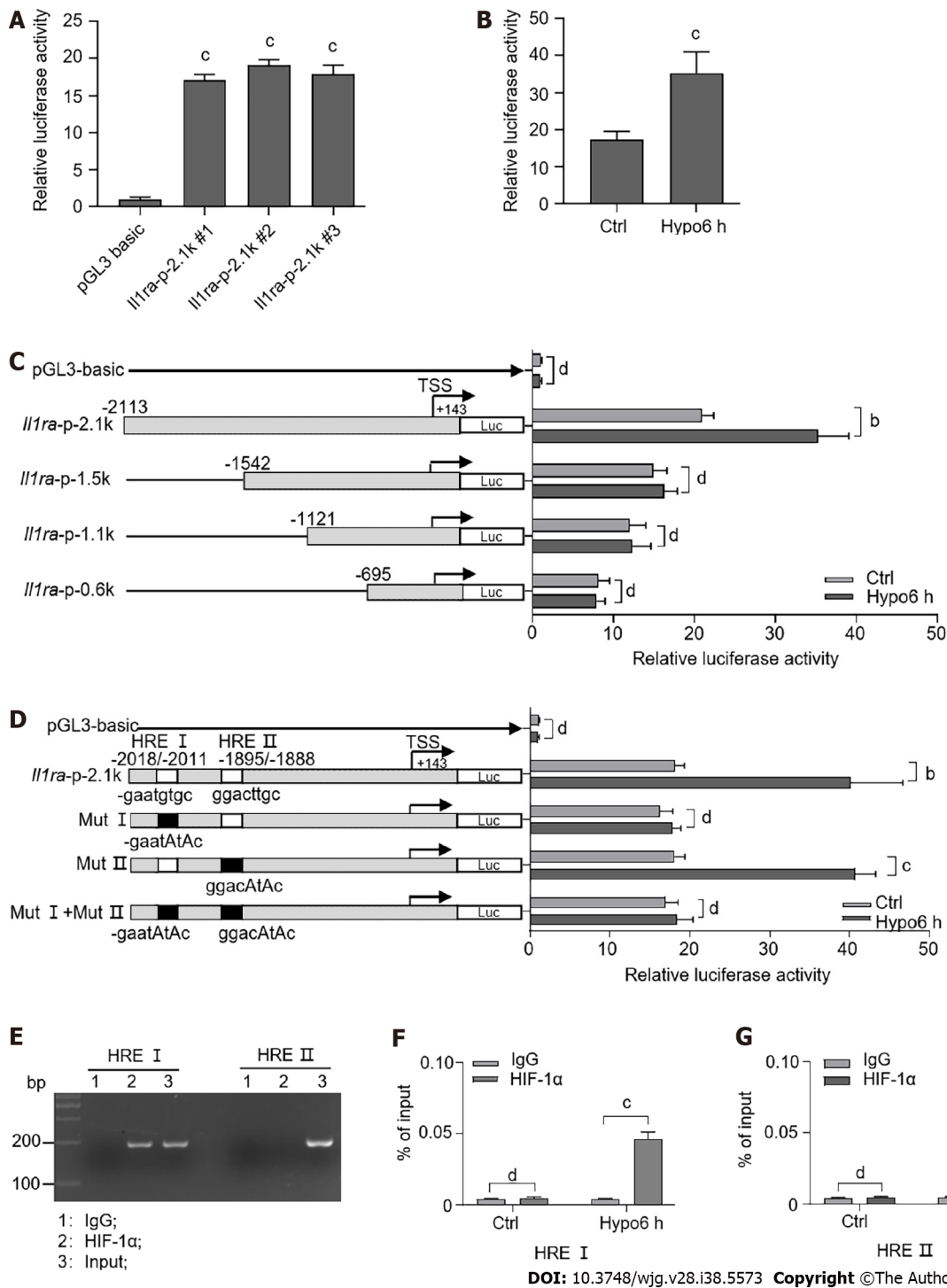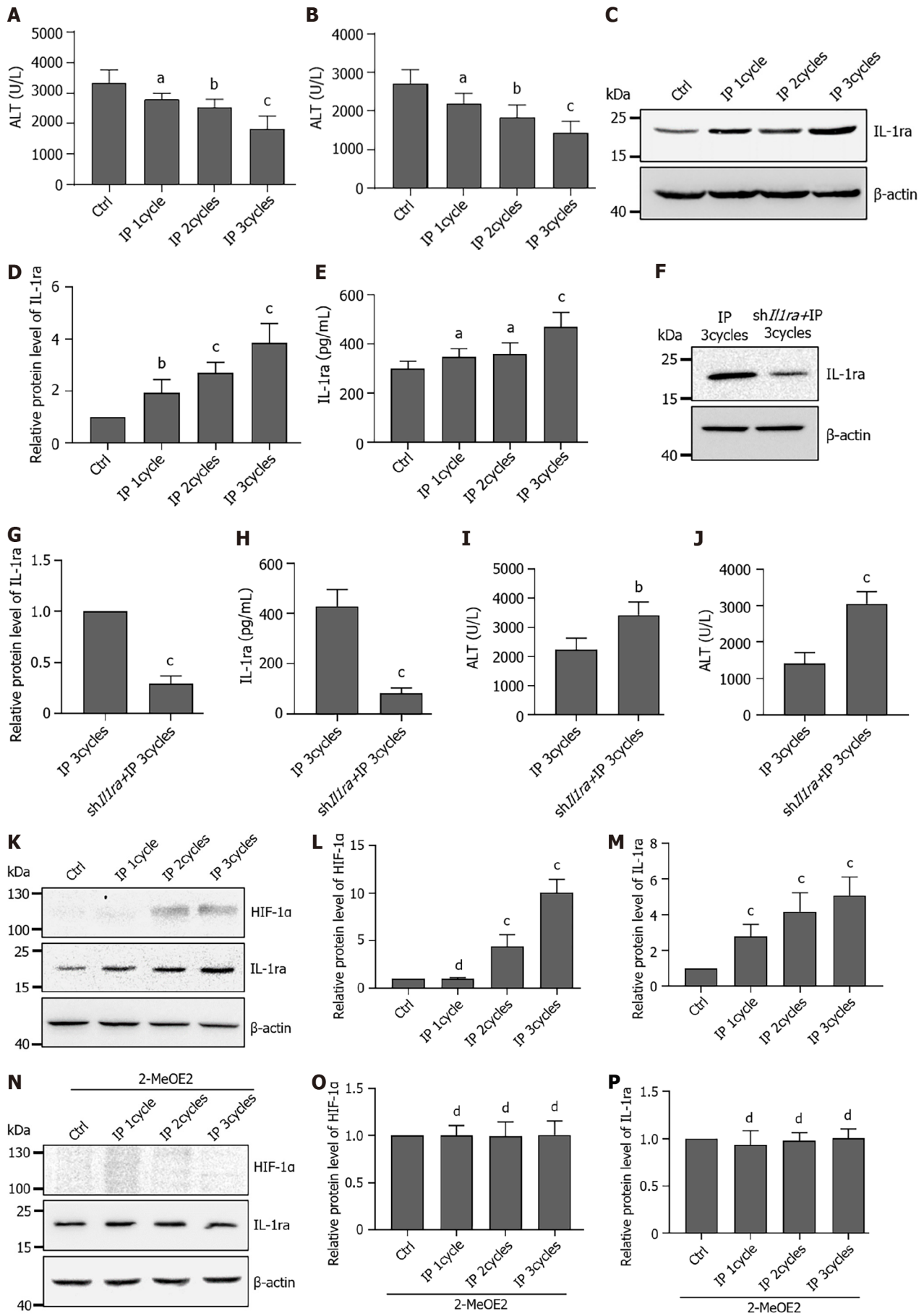
Figure 6 Hypoxia-inducible factor transcriptionally upregulates interleukin-1 receptor antagonist expression. A: Luciferase assay for the wild-type promoter (-2113/+143) of interleukin-1 receptor antagonist (*Il1ra*) in AML12 cells; B: Luciferase assay for the wild-type promoter of *Il1ra* in AML12 cells upon hypoxia; C: Luciferase assay for wild-type and truncated promoters of *Il1ra* in AML12 cells upon hypoxia; D: Luciferase assay for wild-type (-2113/+143) and HRE-mutated promoters of *Il1ra* upon hypoxia; E: Chromatin immunoprecipitation assay for hypoxia-inducible factor (HIF)-1α binding to HRE sites upon hypoxia; F and G: Quantitative polymerase chain reaction analysis for the recruitment of HIF-1α to the endogenous *Il1ra* promoter in AML12 cells upon hypoxia. Indicated *P*-values were calculated using two-tailed unpaired Student's *t*-tests. Data are presented as the mean ± SEM. Data are representative of independent experiments. $^{ns}P > 0.05$, $^{a}P < 0.01$, $^{b}P < 0.001$. Hypo: Hypoxia; IS: Ischemia; HIF: Hypoxia-inducible factor; IL-1ra: Interleukin-1 receptor antagonist; IgG: Immunoglobulin G.

Current studies have shown that many proinflammatory cytokines are released in the early stage of hepatic IRI[4]. For example, blocking the activation of proinflammatory IL-1 signaling significantly prevents IRI[32]. IL-1 is a major inflammatory response mediator[33]. Experimental results have shown that large amounts of IL-1β and tumor necrosis factor-α are produced in the tissue after hepatic IS and reperfusion[34]. At the same time, this can also stimulate the secretion of inflammatory cells, leading to the inflammatory destruction of local tissues. IL-1β and the nucleotide-binding and leucine-rich repeat protein 3 (NLRP3) inflammasome play a role through high mobility group box 1 protein[35], nuclear

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Figure 7 Ischemic preconditioning protects the liver by promoting interleukin-1 receptor antagonist expression *via* hypoxia-inducible

factor-1α. A: Serum alanine transaminase (ALT) levels in mice undergoing ischemia-reperfusion injury (IRI) after different ischemic preconditioning (IP) cycles; B: Serum aspartate transaminase (AST) levels in mice undergoing IRI after different IP cycles; C: Protein levels of interleukin-1 receptor antagonist (IL-1ra) in the liver after different IP cycles; D: Statistical analysis of protein levels of IL-1ra after different IP cycles; E: Protein content of IL-1ra in serum after different IP cycles; F: Protein levels of IL-1ra in the liver with sh*Il1ra* injection after three IP cycles; G: Statistical analysis of protein levels of IL-1ra with sh*Il1ra* injection after three IP cycles; H: Protein content of IL-1ra in serum with sh*Il1ra* injection after three IP cycles; I: Serum ALT levels in mice with sh*Il1ra* injection after three IP cycles; J: Serum AST levels in mice with sh*Il1ra* injection after three IP cycles; K: Protein levels of hypoxia-inducible factor (HIF)-1α and IL-1ra in the liver after different IP cycles; L: Statistical analysis of protein levels of HIF-1α after different IP cycles; M: Statistical analysis of protein levels of IL-1ra after different IP cycles; N: Protein levels of HIF-1α and IL-1ra in the liver with 2-MeOE2 treatment after different IP cycles; O: Statistical analysis of protein levels of HIF-1α with 2-MeOE2 treatment after different IP cycles; P: Statistical analysis of protein levels of IL-1ra with 2-MeOE2 treatment after different IP cycles; Indicated *P* values were calculated using two-tailed unpaired Student's *t*-tests. Data are presented as the mean ± SEM. Data are representative of five independent experiments. $^{ns}P > 0.05$, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. IP: Ischemic preconditioning; AST: Aspartate transaminase; ALT: Alanine transaminase; IL-1ra: Interleukin-1 receptor antagonist; HIF: Hypoxia-inducible factor.

factor-κB and Toll-like receptor 4 in lesions caused by warm IRI[7]. Of note, NLRP3 may be involved in IRI independently of the inflammasome pathway by recruiting neutrophils. Macrophages secrete pro-IL-1β, which is subsequently cleaved and activated by neutrophil-derived proteases in a mouse model. Mature IL-1β is required to induce inflammation during hepatic IR, and the interaction between macrophages and neutrophils is essential in this process[6]. Therefore, the antagonism of proinflammatory IL-1 cytokines has important therapeutic potential for reducing IRI.

IL-1ra is a naturally occurring polypeptide. Recent research has focused on the role of IL-1ra in various pathophysiological states or the effects of recombinant IL-1ra administration in animals and humans[36]. It has been reported that the delivery of the *Il-1ra* gene to the rat liver *via* the adenovirus vector or lipofection can significantly reduce IR-induced proinflammatory cytokine production and hepatocyte injury[15]. This is consistent with our results showing that the knockdown of IL-1ra significantly increased the damage during hepatic IRI, suggesting a potential inhibitory effect of IL-1ra on inflammation in response to hepatic IR.

In the process of IRI, IS is a prerequisite. It is precisely regulated because the depletion of oxygen and energy in the ischemic period cause a series of injuries in the subsequent reperfusion period. In the state of hepatic IS, the metabolic mode switches from aerobic to anaerobic, the redox process in hepatocytes is blocked, the ATP-dependent cellular metabolic activity gradually stops, and the intracellular ATP is rapidly depleted. At the same time, the absence of oxygen causes HIF-1α to gradually accumulate. The increased transport of HIF-1α into the nucleus triggers the expression of genes involved in oxygen transport, oxygen utilization, glycolytic metabolism, cell death, cell survival, and other processes that affect cell survival during IS. Recent studies have demonstrated that HIF-1α can alleviate IRI by regulating the expression of inducible NO synthase[37]. HIF-1α may also enhance tissue anti-inflammatory effects by increasing heme oxygenase-1 expression, thereby reducing damage[38]. In this study, we further showed that HIF-1α, as an important protective factor in IRI, could protect the liver from injury by promoting the expression of IL-1ra and subsequently inhibiting activation of the IL-1 signaling pathway. In terms of the mechanism, we found that HIF-1α recognizes and binds to the basic helix-loop-helix protein sequence in the *Il1ra* promoter region, resulting in the increased transcription of *Il1ra*.

Recent studies have shown that IP has broad protective effects against IRI[39]. However, due to the complexity of IRI, the underlying cellular and molecular mechanisms of IP remain largely unknown. In the present study, we provided evidence that IP promotes the expression and release of IL-1ra into the hepatic microenvironment, thus blocking the downstream activation of IL-1β and protecting the liver from the cascading effects of inflammatory factors. We demonstrated that IP can induce the accumulation of HIF-1α, which increases with the number of IP cycles. These results would explain the protective effect of IP at a mechanistic level and lay a theoretical foundation for the better application of IP.

CONCLUSION

Our study demonstrated a protective effect of IL-1ra on hepatic IRI through the transcriptional regulation of IL-1ra by HIF-1α. Importantly, we found that IP can regulate the expression of IL-1ra through HIF-1α at early stage of hepatic IRI, thereby blocking the inflammatory IL-1 signaling pathway to protect the liver from IRI (Figure 8).
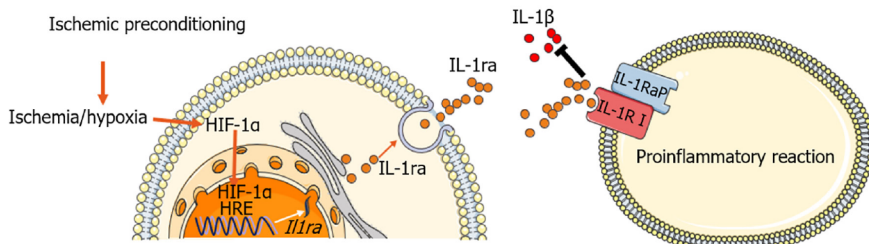
Figure 8 Model of ischemic preconditioning-induced hypoxia-inducible factor-1α-interleukin-1 receptor antagonist signaling to alleviate hepatic ischemia-reperfusion injury. Ischemic preconditioning promotes the accumulation of hypoxia-inducible factor (HIF)-1α, which subsequently increases HIF-1α entry into the nucleus and promotes the transcriptional expression of interleukin-1 receptor antagonist (IL-1ra). In turn, this inhibits the pro-inflammatory effects of IL-1β and eventually protects against hepatic ischemia-reperfusion injury. HIF: Hypoxia-inducible factor; IL-1ra: Interleukin-1 receptor antagonist.

ARTICLE HIGHLIGHTS

Research background

Ischemia-reperfusion injury (IRI) is associated with transplant failures, graft dysfunction, and a relatively poor prognosis. Interleukin-1 receptor antagonists (IL-1ra) play an important role in protecting the liver from IRI. However, the mechanism of its regulatory expression remains unclear.

Research motivation

Inhibition of hepatic inflammation by promoting the expression of IL-1ra is one of the key targets for the treatment of hepatic IRI.

Research objectives

To investigate the regulatory mechanism of hepatocyte-derived IL-1ra expression in the process of hepatic IRI to provide a therapeutic target for hepatic IRI.

Research methods

A 70% hepatic IRI model was established in mice, and AML12 cells were subjected to hypoxia/reoxygenation for the simulation of IRI *in vitro*. The *ll-1ra* promoter-reporter system was constructed to detect the regulatory effect of hypoxia. The ischemic preconditioning (IP) model was established to investigate its regulatory effect on IL-1ra.

Research results

IL-1ra is a key regulator of hepatic IRI. Il-1ra expression was significantly up-regulated after hepatic IRI *in vivo* and *in vitro*. Hypoxia inducible factor (HIF)-1α regulates *ll-1ra* transcription during hypoxia. IP prevents hepatic IRI by inducing the expression of IL-1ra, which is mediated by HIF-1α.

Research conclusions

Ischemia or hypoxia leads to increased IL-1ra expression through HIF-1α. In addition, IP protects the hepatic tissue from IRI through the HIF-1α-IL-1ra pathway.

Research perspectives

HIF-1α-IL-1ra pathway is a potential mechanism of hepatic protection during hepatic IRI, and also a key target for the treatment of hepatic IRI.

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FOOTNOTES

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