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Original Paper

Role of miR-148a in Mitigating Hepatic Ischemia-Reperfusion Injury by Repressing the TLR4 Signaling Pathway via Targeting CaMKIIα *in Vivo* and *in Vitro*

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Key Words
Ischemia-reperfusion injury • Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) • MicroRNA (miRNA) • Toll-like receptor 4 (TLR4) • Hypoxia/reoxygenation

Abstract
Background/Aims: Hepatic ischemia-reperfusion (I/R) injury, which is mainly induced by inflammation and unstable intracellular ions, is a major negative consequence of surgery that compromises hepatic function. However, the exact mechanisms of liver I/R injury have not been determined. Positive crosstalk with the Ca^{2+}/CaMKII pathway is required for complete activation of the TLR4 pathway and inflammation. We previously found that miR-148a, which decreased in abundance with increasing reperfusion time, targeted and repressed the expression of CaMKIIα. In the present study, we examined the role of the miR-148a machinery in I/R-induced Ca^{2+}/CaMKII and TLR4 signaling changes, inflammation, and liver dysfunction *in vivo* and *in vitro*. ***Methods:*** Liver function was evaluated by serum aminotransferase levels and hematoxylin-eosin (HE) staining. Inflammatory factors were detected by enzyme-linked immunosorbent assay. Gene and protein expression were assessed by RT-PCR and western blot. Small interfering RNA was used to silence target gene expression. HE staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling were used to measure hepatic tissue apoptosis. These assays were performed to identify factors upregulated in hepatic I/R injury and downregulated by miR-148a. ***Results:*** We manifested that expression of CaMKIIα and phosphorylation of TAK1 and IRF3 were elevated in hypoxia/reoxygenation (H/R)-treated primary Kupffer cells (KCs) and liver tissue of I/R-treated mice, but these effects were attenuated by treatment with miR-148a mimic and were accompanied by the

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alleviation of liver dysfunction and hepatocellular apoptosis. Luciferase reporter experiments showed that miR148a suppressed luciferase activity by almost 60%. Moreover, knockdown of CaMKIIα in H/R KCs led to significant deficiencies in p-TAK1, P-IRF3, IL-6, and TNF-α, which was consistent with the effects of miR-148a overexpression. Otherwise, the same trend of activation of TAK1 and IRF3 and inflammatory factors *in vitro* was observed in the siTAK1 + siIRF3 group compared with the siCaMKIIα group. ***Conclusion:*** Taken together, we conclude that miR-148a may mitigate hepatic I/R injury by ameliorating TLR4-mediated inflammation *via* targeting CaMKIIα *in vitro* and *in vivo*.

Introduction

Living donor liver transplantation is a valuable and indispensable method for circumventing the shortage of organs, a worldwide problem in the field of organ transplantation. However, various problems remain unsolved, including hepatic ischemia reperfusion (I/R) injury [1, 2], portal hypertension [3], inflammatory responses [4], and graft rejection [5]. The hallmarks of hepatic I/R injury include severe cell death and inflammatory responses that contribute to early graft failure [6]. The activation of inflammatory responses, including macrophage and neutrophil infiltration, cytokine production, and inflammatory molecular events, promotes severe insults that lead to hepatocellular damage. In turn, inflammatory responses can also be initiated and enhanced by cell death [7, 8]. Despite hundreds of investigations addressing this issue, the mechanisms responsible for liver I/R injury still require further exploration.

Toll-like receptors (TLRs), a group of important innate immune system receptors, have been the subject of recent research. Liver I/R injury can activate TLR4 through ATP generation, cellular ion (especially calcium [Ca^{2+}]) homeostasis, and generation of reactive oxygen species [9]. Ca^{2+} plays as a major second messenger role in regulating a broad range of important cellular processes. Many cellular responses to Ca^{2+} signals are induced by a family of multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), among which CaMKII is a ubiquitous serine/threonine protein kinase encoded by four separate genes (α, β, γ, and δ) [10]. TLR4 ligands can significantly trigger elevation of intracellular Ca^{2+} and activation of CaMKII in macrophages. In turn, CaMKII promotes both myeloid differentiation factor 88 (MyD88)-dependent and Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β (TRIF)-dependent proinflammatory factor and type I interferon (IFN) production by regulating transforming growth factor-β-activated kinase 1 (TAK1) and IFN regulatory factor 3 (IRF3) [11]. Therefore, positive crosstalk with the Ca^{2+}/CaMKII pathway is required for complete activation of TLR responses in macrophages, and *vice versa*. However, whether Ca^{2+}/CaMKII signaling participates in inflammation of hepatic I/R by interacting with TLR4 pathway has not been elucidated.

MicroRNAs (miRNAs), a class of highly conserved small endogenous noncoding RNA molecules, as important post-regulators of several biological process, silence targeted mRNAs by binding to their 3′-untranslated regions (3′-UTRs), and are often dysregulated in disease and tumors [12, 13]. In the current study, we focused on the effects of miRNAs on inflammation derived from hepatic I/R injury. Ever since identification of the effects of miRNAs on I/R injury in myocardial ischemia and their association with angiogenesis and inhibition of apoptosis and inflammation [14], there have been more studies of the roles of miRNAs in the regulation of I/R injury and other human hepatic physiological and pathological processes [15]. One miRNA, known as miR-148a, may play an especially important role as a negative regulator of innate responses and antigen presentation in TLR-triggered dendritic cells [16]. In particular, the 3′-UTR of the CaMKIIα mRNA contains the predicted target sites for miR-148a binding, which have been verified in several miRNA databases. Thus, miR-148a might ameliorate I/R by inhibiting TLR4 signaling *via* targeting CaMKIIα.

Although miR-148a has been widely studied with regard to its role in carcinoma and inflammation, no *in vivo* or *in vitro* studies have heretofore been published regarding the role

of miR-148a in liver I/R injury. Previously, we had clarified that the expression of CaMKIIα and miR-148a were negatively correlated to reperfusion time [17]. In the present study, we attempted to investigate whether miR-148a plays a vital role in alleviating liver I/R injury by suppressing TLR4-mediated inflammation. Moreover, we aimed to examine the effects of miR-148a mimics or miR-148a inhibitors on CaMKIIα expression and TLR4-mediated inflammation in hepatic I/R injury and in an H/R treated Kupffer cells (KCs). Taken together, our results suggest that miR-148a may alleviate hepatic I/R injury by repressing the TLR4 signaling pathway *via* targeting CaMKIIα both *in vivo* and *in vitro*.

Materials and Methods

Animals

Male C57BL/6J mice (6–8 weeks old) were purchased from the Animal Center of Chongqing Medical University. All experimental protocols were approved by the Committee on Animal Research at the Chongqing Medical University. Mice were maintained under a 12-h light-dark cycle with food and water *ad libitum* in a temperature-controlled environment (22 ± 2°C and 55 ± 10% relative humidity). The animals were maintained under laboratory conditions for an acclimatization period of 7 d before conducting the experiments.

Isolation and culture of KCs

KCs were isolated from C57BL/6J mice livers as previously described [18]. Shortly after puncturation of the portal vein, the liver was perfused with phosphate-buffer solution (Boshide, Wuhan, China) at 37°C. The liver was then perfused with 20 mL 0.1% collagenase IV solution (Sigma-Aldrich, St. Louis, MO) for 10 min. After removal of hepatic envelope, the cells were filtered, centrifuged at various speeds, and washed several times. Cells were then plated in 6-well culture plates for 2 h at 37°C, after which the nonadherent cells were removed. The adherent KCs were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL). All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Animal surgery

A warm segmental (70%) hepatic I/R model was generated as previously described [19]. Briefly, after the mouse was anesthetized using 0.1% pentobarbital sodium and laparotomized, the arterial and portal venous blood were clamped for 60 min. Reperfusion was then performed by removing the clamp. A warming pad was provided to maintain the body temperature at 37°C. Surgery in the sham group only exposed the portal vein for 1 h without hepatic ischemia. By the end of the predetermined period following reperfusion, mice were anesthetized and euthanized for tissue and plasma collection by exposure to a high concentration of CO_2. All tissue and sera were collected 8 h after reperfusion, except for detection of miR-148a mRNA abundance at various reperfusion times.

Hypoxia/reoxygenation model

The hypoxia/reoxygenation (H/R) model was created as previous described [20]. Hypoxia preconditioning was performed by culturing cells in a tri-gas incubator with a 1% oxygen concentration according to the manufacturer's instructions. The cells were then placed in a normoxic humidified incubator for various periods. All experiments were performed at 37°C.

Quantitative real-time PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed in a reaction mixture. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed following the manufacturer's protocol. The expression data were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To determine miRNA expression, total RNA was reverse-transcribed, and the resulting cDNA mixed with miRNA-specific TaqMan primers (GenePharma, Shanghai, China) and TaqMan Universal PCR Master Mix (GenePharma). U6 was used as an internal

control for normalization of miRNA levels. The primers used for these experiments are shown as follows: TAK1 forward, 5′-GCTCGCTGGTAGTGGTGTT-3′, and reverse, 5′-GCCCCACTGTGAATCTGAAA-3′; IRF3 forward, 5′-TACACTGAGGACTTGCTGGAGGT-3′, and reverse, 5′-AAGATGGTGGTCTCCTGATCC-3′; CaMKIIα forward, 5′-ACCTGCAC CCGATTCACAG-3′, and reverse, 5′-TGGCAGCATACTCCTGACCA-3′; miR-148a forward, 5′-ATGCTCAGTGCACTACAGAA-3′, and reverse, 5′-GTGCAGGGTCCGAGGT-3′; GAPDH forward, 5′-AGCGAGACCCCACTAACA-3′, and reverse, 5′-GGGGCTAAGCAGTTGGTG -3′; U6 forward, 5′-CTCGCTTCGGCAGCACA-3′, and reverse, 5′-AACGCTTCACGAATTTGCGT-3′. The comparative threshold cycle (Ct) method was used to measure the relative expression, where $2^{-\Delta\Delta Ct}$ represents the fold-change in expression, as previously described [21].

Cell transfection

KCs were plated at a density of 2–4 × 10^5 cells/mL in 6-well culture plates and cultured for 24 h, then transfected with the 50 nM miR-148a mimic, an miR-148a inhibitor, or a negative control (GenePharma) using Lipofectamine 2000 (Invitrogen) for 24 h, referencing the manufacturer's guide. CaMKIIα, TAK1, and IRF3 small interfering RNA (siRNA; GenaPharma) were transfected into KCs for 24 h. The RNAi sequences are shown as follows: siCaMKIIa: 5′-CACCACCATTGAGGACGAA-3′; siTAK1: 5′-UGGCUUAUCUUACACUGGA-3′; siIRF3: 5′-GGTTGTTCCTACATGTCTTAA-3′. H/R treatment was subsequently performed.

3′-UTR luciferase reporter assays

The pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) was used to perform 3′-UTR luciferase reporter assays. Wild-type and mutant CaMKIIα 3′-UTR luciferase reporter vectors were constructed by amplifying the mouse CaMKIIα mRNA 3′-UTR, then cloning it into the *Xba*I site of the pmirGLO vector. KCs were co-transfected for 24 h with 80 ng luciferase reporter plasmid, 40 ng thymidine kinase promoter-*Renilla* luciferase reporter plasmid, and miR-148a mimic or inhibitor (final concentration, 20 nM) using Lipofectamine 2000 according to the manufacturer's instructions. Firefly luciferase and *Renilla* luciferase luminescence were measured using the Dual-Glo Luciferase Reporter Assay System (Promega) and a GloMax 20/20 Luminometer (Promega). Data were normalized for transfection efficiency by dividing firefly luciferase activity by *Renilla* luciferase activity as described previously [22].

Enzyme-linked immunosorbent assay

Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) are indices of hepatocellular injury. To determine the release of the inflammatory cytokines ALT and AST, cell supernatants and serum were collected. Release of interleukin (IL)-6 and TNF-α were detected using the IL-6 Mouse ELISA Kit and the Mouse TNF-alpha ELISA Kit (Thermo Fisher Scientific, Waltham, MA), and release of ALT and AST were determined using the SGPT ELISA Kit (Enzyme-linked biological technology, Shanghai, China) according to the manufacturers' manuals.

Western blot

Protein blotting was performed as the manufacturer's instructions described. Protein samples were harvested from mice livers and KC cells by lysis buffer (Beyotime, Nantong, China). The Bradford method was performed to measure protein concentrations. The protein samples were separated by 5–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and gels were then transferred to a polyvinylidene fluoride membranes (Millipore, Burlington, MA). After blocking the membranes for 60 min with 5% skim milk, they were incubated overnight at 4°C with anti-CaMKIIα antibody (Cell Signaling Technology, Danvers, MA), anti-TAK1 antibody (Cell Signaling Technology), anti-p-TAKI antibody (Cell Signaling Technology), anti-IRF3 antibody (Cell Signaling Technology), or anti-p-IRF3 antibody (Cell Signaling Technology), and anti-β-ACTIN antibody (XinBoSheng, China). After the membranes were washed, they were probed with the corresponding secondary antibodies before developing them by enhanced chemiluminescence using a Chemiluminescent Detection Kit (Advansta, Menlo Park, CA).

Histology and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Formalin-fixed and paraffin-embedded mice liver specimens were cut into 4 micrometers sections, stained with hematoxylin and eosin (HE), and observed with light microscopy. Suzuki's criteria were used to evaluate the grade of hepatic injury [23]. Apoptosis of hepatocytes was evaluated by an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) based on terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The number of TUNEL-positive cells was counted under 100× magnification to quantify the degree of apoptosis.

Ago-miR-148a therapy in mice

The C57BL/6J mice were treated by tail vein injection with ago-miR-148a (5 mg/kg; GenePharma), antago-miR-148a, and control ago-miRNA in saline, as previously described [24]. After 24 h, 60 min of ischemia and 6 h of reperfusion were performed.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons tests were conducted for multiple-group comparisons, and Student's *t*-test was used to evaluate the significance of differences between treatments and controls. In all analyses, differences at $P < 0.05$ were considered statistically significant.

Results

Upregulation of CaMKIIα, p-TAK1, and p-IRF3 in KCs following H/R

To assay the role of CaMKIIα, p-TAK1, and p-IRF3 expression in primary KCs under H/R conditions, which is a well-established *in vitro* model of I/R. According to the results of our previous study [25], we chose 12 h of hypoxia and 4 h of reoxygenation as the parameters for further experiments. Western blot analysis revealed that following H/R, amounts of CaMKIIα, p-TAK1, and p-IRF3 protein in KCs significantly increased, but amounts of TAK1 and IRF3 protein did not increase (Fig. 1A and B). Our data suggested that KCs subjected to H/R exhibited elevated levels of CaMKIIα and activation of TAK1 and IRF3, which are consistent with the effects of hepatic I/R *in vivo*.

CaMKIIα modulates H/R-induced inflammatory responses in KCs via inhibiting phosphorylation of TAK1 and IRF3

To determine the effect of CaMKIIα on the activation of TAK1 and IRF3 and H/R-related inflammation in KCs, KCs were transfected with siCaMKIIα, siTAK1, and siIRF3, respectively. Western blotting and qRT-PCR were performed to evaluate the efficacy of transfection. The expression of CaMKIIα, TAK1, and IRF3 all decreased when siRNAs were transfected into KCs (Fig. 1C and D). Immunoassays were used to evaluate protein expression in KCs transfected to express various combinations of these siRNAs. Compared with the control group, expression of CaMKIIα and activation of TAK1 and IRF3 were elevated in the H/R group. When we transfected siCaMKIIα into KCs in the H/R group, the expression of CaMKIIα, p-TAK1, and p-IRF3 revised. Interestingly, the expression of TAK1 and IRF3 remained not change. Moreover, compared with the H/R group, the expression of TAK1, IRF3, p-TAK1, and p-IRF3 decreased in the siTAK1 + siIRF3 + H/R group, while the expression of CaMKIIα was almost the same as in the H/R group. Otherwise, the expression of p-TAK1 and p-IRF3 remained close to the same as in the siCaMKIIα + H/R group and siTAK1 + siIRF3 + H/R groups. The observed trends in expression of the main inflammatory factors in TLR4 signaling, IL-6 and TNF-α, were consistent with the observed alteration in expression of p-TAK1 and p-IRF3. These results imply that CaMKIIα may inhibit H/R-induced inflammation in KCs by targeting p-TAK1 and p-IRF3 (Fig. 1E).

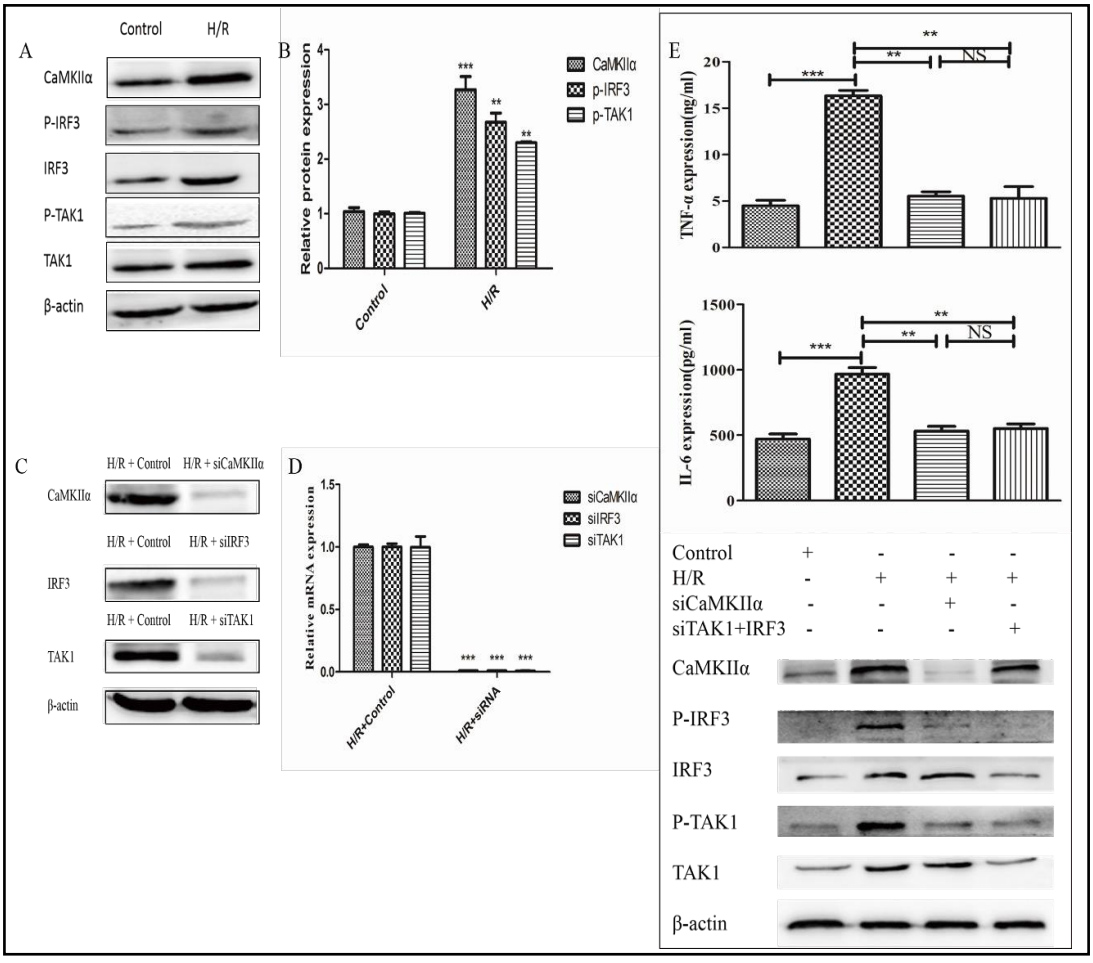

Fig. 1. CaMKIIα regulates H/R-induced inflammatory responses in KCs via inhibiting activation of TAK1 and IRF3. A and B) qRT-PCR analysis and western blotting of CaMKIIα, TAK1, p-TAK1, IRF3, and p-IRF3 in primary KCs after H/R. C and D) qRT-PCR and western blotting were performed to evaluate the efficacy of RNA interference in H/R-treated primary KCs after transfection with siCaMKIIα, siTAK1, or siIRF3. ** P<0.01, *** P<0.001 compared with its relative protein. E) Expression of CaMKIIα, TAK1, p-TAK1, IRF3, and p-IRF3 was detected by immunological assays, while the release of TNF-α and IL-6 in culture supernatants was measured by ELISA. Results are the mean ± SD. Error bars represent SD. One-way ANOVA, t-tests, and Tukey-Kramer multiple comparisons tests were performed. NS, not significant; ** P<0.01; *** P<0.001.

miR-148a reduces H/R-mediated inflammatory responses in KCs

To investigate the effect of miR-148a on inflammation in KCs, we first detected the expression of miR-148a in hepatic I/R treated mice. As shown in Fig. 2A, the expression of miR-148a decreased as a function of time following reperfusion, achieving minimum levels at 9 h. To elucidate the role of miR-148a on the production of proinflammatory mediators, KCs were then transfected with miR-148a mimic, inhibitor, or control, and transfection efficiency was measured by qRT-PCR. The expression of miR-148a significantly increased in the miR-148a mimic-transfected group but decreased in the miR-148a inhibitor transfected group as compared with the miR-148a control group (Fig. 2B). Next, we performed ELISA assays to detect the release of inflammatory factors in supernatant. Data showed that miR-148a overexpression attenuated the production of the proinflammatory cytokines TNF-α and IL-6 in H/R-induced KCs, and that knocking down miR-148a aggravated this inflammation (Fig. 2C and D). Western blotting was then conducted to evaluate the protein expression. We found that the expression of CaMKIIα, p-TAK1, and p-IRF3 was elevated in the miR-148a mimic + H/R group, but decreased in the miR-148a inhibitor + H/R group (Fig. 2E-H).

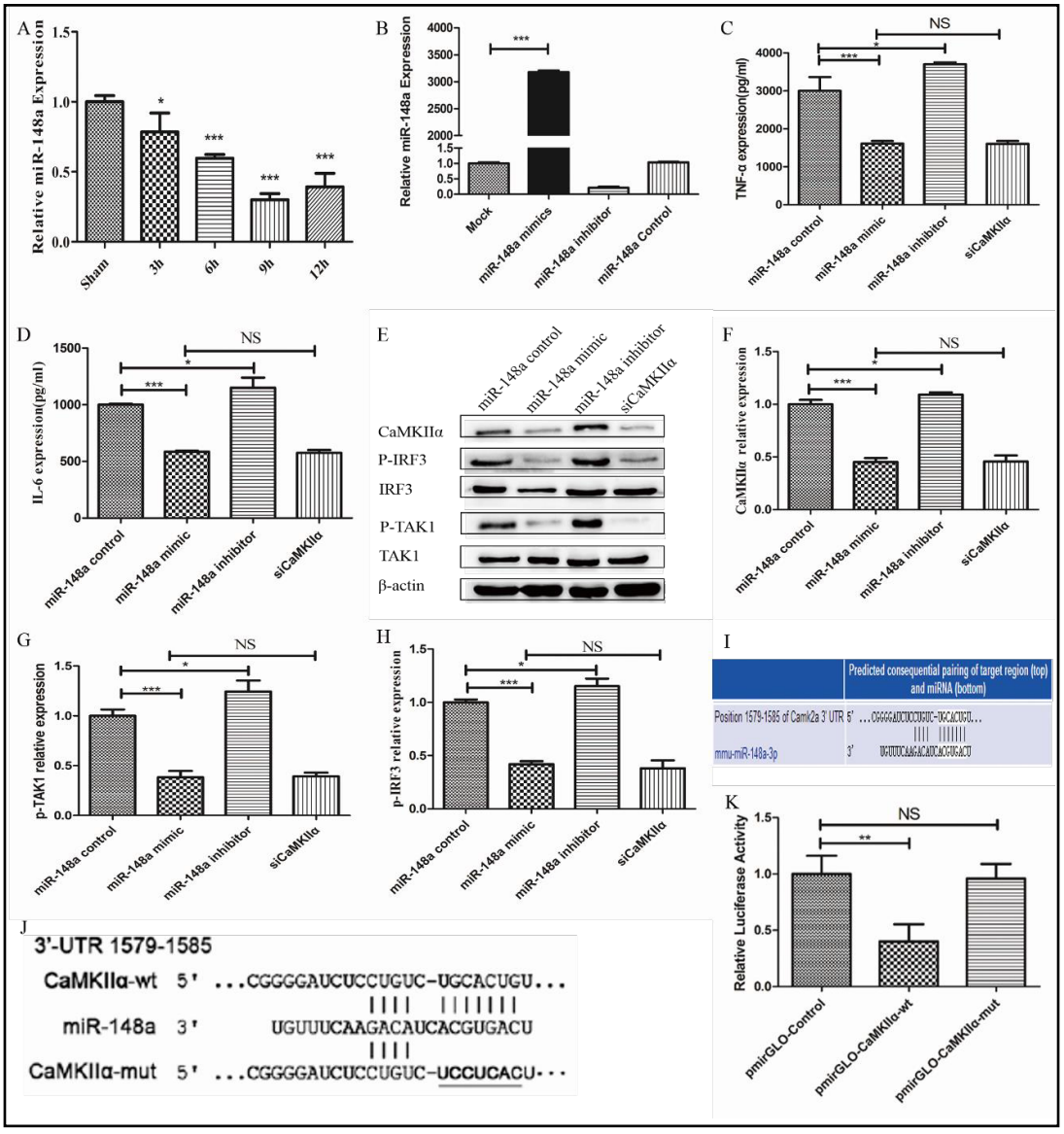

Fig. 2. miR-148a reduces inflammation induced by H/R directly via targeting CaMKIIα and indirectly via inhibiting the phosphorylation of TAK1 and IRF3 in primary KCs. A) Expression of miR-148a in liver tissue of I/R treated mice at various reperfusion times (n = 3–4 mice per group). B) qRT-PCR was performed to evaluate the efficacy of miR-148a transfection in KCs (n = 3–4 mice per group). C and D) ELISA of TNF-α and IL-6 in supernatants of KC cultures. E-H) Immunoblotting of CaMKIIα, TAK1, p-TAK1, IRF3, and p-IRF3 and results of statistical analysis. Primary KCs were transfected to express various combinations of these siRNAs and the miR-148a mimic, miR-148a inhibitor, or its negative control for 24 h before H/R. I) TargetScan 7.1 was performed to show the relation between miR-148a and CaMKIIα. J and K) Sequence alignment of miR-148a and its putative and mutated target sites in the 3′-UTR of the mouse CaMKIIα mRNA are shown. Results are the mean ± SD. One-way ANOVA and Tukey-Kramer multiple comparisons tests were performed. NS, not significant; * P<0.05; ** P<0.01; *** P<0.001.

miR-148a regulates inflammation in KCs by directly targeting CaMKIIα

To evaluate whether CaMKIIα is the main target of miR-148a influencing inflammation induced by H/R in KCs, we first used the miRNA target prediction software TargetScan 7.1 to identify putative miR-148a binding sequences in the 3′-UTR of CaMKIIα (Fig. 2I). A luciferase reporter experiment was then conducted to confirm this hypothesis. Our data showed that miR148a suppressed luciferase activity by almost 60% (Fig. 2J and K). Moreover, we also conducted siCaMKIIα + H/R and miR-148a + H/R treated KCs to elucidate whether CaMKIIα is the main target of miR-148a affecting the inflammatory cascade in H/R treated KCs. There

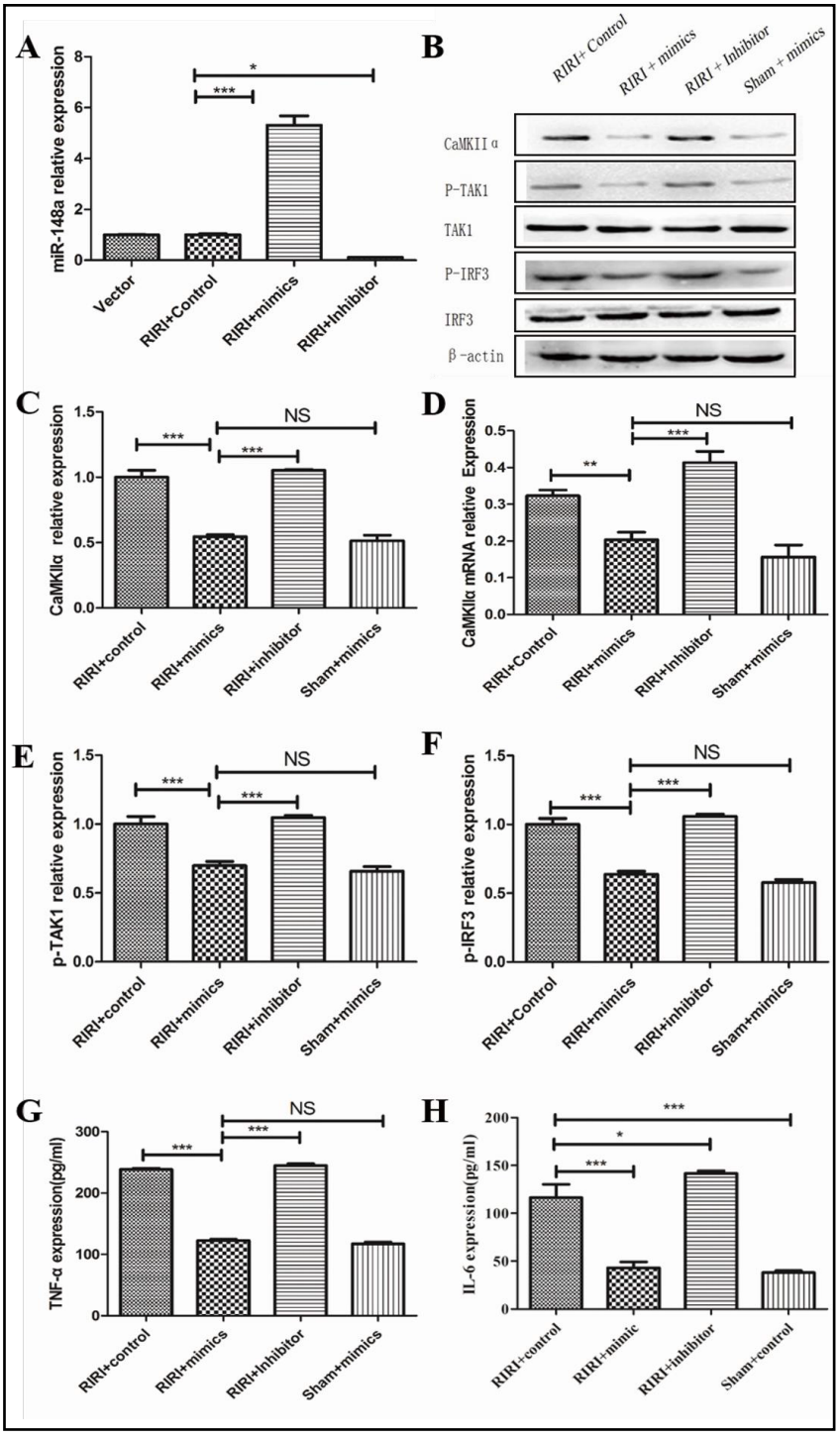

Fig. 3. miR-148a represses hepatic inflammatory responses resulting from liver I/R in C57BL/6J mice. Male mice (n = 3–4 mice per group) were treated with ago-miR-148a, antago-miR-148a, ago-miRNA control, or vector for 24 h, then subjected to I/R. A) qRT-PCR for miR-148a. B-F) qRT-PCR for CaMKIIα and western blot analysis of CaMKIIα, TAK1, p-TAK1, IRF3, and p-IRF3. G and H) ELISA of TNF-α and IL-6 in serum of mice. Results are the mean ± SD. One-way ANOVA and Tukey-Kramer multiple comparisons tests were performed. NS, not significant; * P<0.05; ** P<0.01; *** P<0.001.

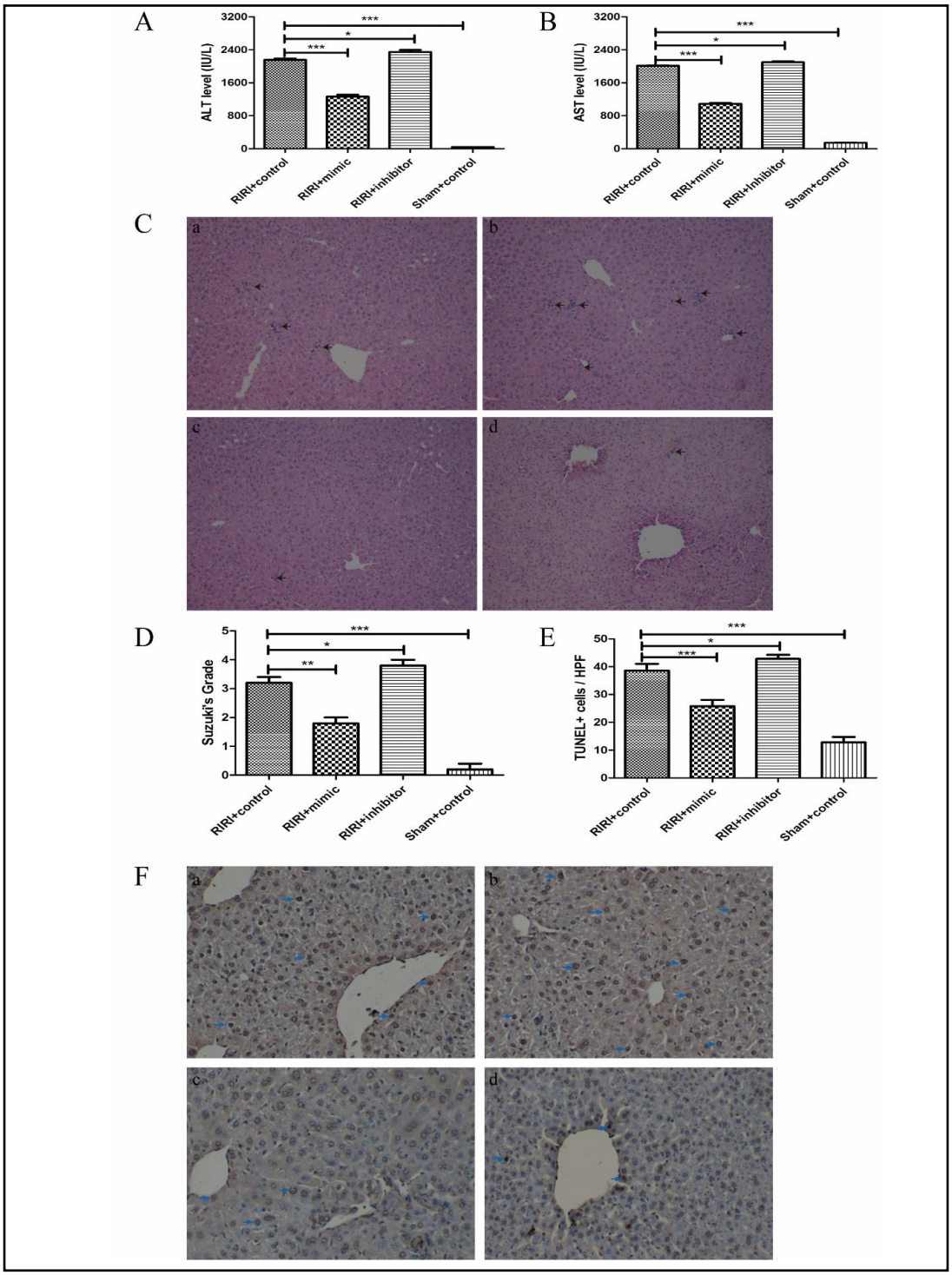

Fig. 4. Transfection of ago-miR-148a improves hepatocellular function after liver I/R injury in vivo. Male mice (n = 3–4 mice per group) were treated with ago-miR-148a, antago-miR-148a, ago-miRNA control for 24 h, then subjected to I/R. A and B) ELISA of ALT and AST in serum of experimental mice. C and D) Representative hematoxylin-eosin staining (100× magnification, black arrow points to the inflammatory infiltration) and Suzuki's grades. E and F) TUNEL of liver tissue (200× magnification, blue arrow points to TUNEL-positive cell), with results of statistical analysis. a, RIRI + control; b, RIRI+ inhibitor; c, RIRI + mimic; d, Sham + control. Results are the mean ± SD. One-way ANOVA and Tukey-Kramer multiple comparisons tests were performed. * P<0.05; ** P<0.01; *** P<0.001.

was no statistically significant differences between these two groups (Fig. 2C-H). These results imply that miR-148a mainly binds directly to the 3′-UTR of CaMKIIα to decrease phlegmonosis in H/R-induced KCs.

Effect of miR-148a on liver function after hepatic I/R injury.

To verify the effect of miR-148a on hepatic I/R injury-related liver function, male mice were treated with ago-miR-148a (a chemically-modified miR-148a), antago-miR-148a, or control ago-miRNA by tail vein injection before subjecting mice to I/R. Using qRT-PCR analysis, we measured miR-148a expression in mouse livers and found that pretreatment of mice with ago-miR-148a resulted in a significant increase in miR-148a expression, while preconditioning with antago-miR-148a resulted in a decrease in miR-148a expression compared to control ago-miRNA-treated mice (Fig. 3A). Overexpression of miR-148a led to reduced CaMKIIα expression and phosphorylation of TAK1 and IRF3 in hepatic tissue after I/R (Fig. 3B-F). Likewise, serum levels of TNF-α and IL-6 decreased in ago-miR-148a-treated mice following I/R, compared with the antago-miR-148a and control ago-miRNA groups (Fig. 3G and H). To elucidate the role of miR-148a on hepatocellular function after I/R, serum ALT and AST levels were determined in treated mice following liver I/R. As shown in Fig. 4A and B, compared with the control ago-miR-148a group, treatment with ago-miR-148a significantly inhibited the release of ALT and AST induced by I/R treatment, but treatment with the miR-148a antagomir promoted release of ALT and AST upon I/R treatment. Further, HE and TUNEL staining were further utilized to evaluate the severity of liver I/R injury. Compared with the ago-miR-148a control group, amelioration of inflammatory cell infiltration and reduction of TUNEL-positive hepatic cells were found in mice overexpressed miR-148a, while these processes were more severe in the antago-miR-148a group after hepatic I/R (Fig. 4C and F). Thus, our results indicate that miR-148a administration can protect the murine liver from I/R injury partially *via* inactivation of the TLR4 and Ca^{2+}/CaMKII signaling pathways.

Discussion

In this study, we verified that miR-148a ameliorated hepatic I/R injury and reduced liver cell apoptosis and aminotransferase release by suppressing the release of proinflammatory factors through the repression of CaMKIIα. To our knowledge, it is first time that the vital role of miR-148a in counteracting hepatic I/R injury by targeting CaMKIIα and its crosstalk with TLR4 signaling has been demonstrated.

To investigate the effect of miR-148a on liver I/R injury *in vivo* and *in vitro*, we transfected mice and KCs with ago-miR-148a and miR-148a mimic to overexpress miR-148a in liver and KCs. miR-148a overexpression significantly decreased the levels of inflammatory factors TNF-α and IL-6 and apoptosis during H/R, indicating that miR-148a is an anti-inflammatory and anti-apoptotic agent. Antago-miR-148a consistently reduced the release of TNF-α and IL-6 and hepatocellular apoptosis, then attenuated hepatic function. To analyze the mechanism of these effects, we transfected KCs with miR-148a mimic, miR-148a inhibitor, or negative control miRNA into KCs. We found that transfection of miR-148a significantly suppressed CaMKIIα, p-TAK1, and p-IRF3 expression, but did not influence TAK1 and IRF3 expression in the KC H/R model. Furthermore, miR-148a likely interfered with the expression of CaMKIIα through interaction with the 3′-UTR of CaMKIIα, which was confirmed using TargetScan 7.1 software and was consistent with the luciferase results. A previous study reported by Cao et al. also supports our findings [16]. Because miR-148a-3p can regulate more than 600 different genes, we compared the effects of siCaMKIIα transfection with those of miR-148a transfection on H/R-treated KCs. There were no statistical differences in expression of CaMKIIα, p-TAK1, and p-IRF3 proteins and inflammatory cytokines between KCs transfected with siCaMKIIα or miR-148a, indicating that CaMKIIα might be the main target of miR-148a for regulating inflammation in H/R treated KCs. Consistently, the overexpression of miR-148a *in vivo* caused by ago-miR-148a transfection also decreased the levels of CaMKIIα,

p-TAK1, and p-IRF3. Moreover, our results showed that the downregulation of CaMKIIα, p-TAK1, and p-IRF3 by miR-148a reduced the release of the proinflammatory factors TNF-α and IL-6. In consideration of the above-mentioned findings, we conclude that administration of miR-148a inhibits liver I/R-triggered inflammation *via* dephosphorylation of TAK1 and IRF3 by repressing CaMKIIα. However, whether miR-148a knockout mice are more sensitive to liver I/R injury requires further study. Moreover, recent reports indicate that miR-148a, which is derived from hepatocytes and released into the serum during hepatocellular injury in humans [26, 27], could be chosen as a marker of liver function. Additional research should comprehensively explain the exact role of miR-148a during hepatic I/R.

Our experiments have also further elucidated the crosstalk between the CaMKIIα and TLR4 pathways *in vitro*. CaMKIIα can reduce the activity of proinflammatory proteins like NF-κB and MAPK/ErK, and purinergic receptors such as TLR4 and P2RX7. In addition to their involvement in inflammation, proteins like NF-κB and MAPK/ErK in the TLR4 signaling pathway are known to reduce synaptic activity of CaMKIIα by increasing its phosphorylation [28-31]. Further, TAK1 and IRF3 are two key cytoplasmic protein kinases in TLR4 signaling. Considerable research has shown that IRF3 is the key transcription factor activated through the TRIF-dependent pathway, and that it mediates the production of type I IFN in TLR3 and TLR4 signaling [32]. A recent report showed that downregulation of phosphorylated TAK1 could protect against hepatic I/R injury [33]. CaMKIIα can phosphorylate these kinases and, conversely, these kinases can also upregulate the expression of CaMKIIα, which is essential to fully activate the TLR4 signaling pathway and enhance the production of proinflammatory cytokines including TNF-α and IL-6. We found that knockdown of TAK1 and IRF3 significantly reduced the protein expression of TAK1, IRF3 and its phosphorylated form p-IRF3, and the release of inflammatory mediators in KCs following H/R *in vitro*. Moreover, transfection of KCs with siCaMKIIα decreased the expression of p-TAK1 and p-IRF3 and the secretion of TNF-α and IL-6, but did not affect the quantity of TAK1 or IRF3. Finally, there was no statistical difference in the expression of p-TAK1 and p-IRF3 or in the release of inflammatory cytokines in the siCaMKIIα + H/R group compared with the siTAK1 + siIRF3 + H/R group. These data suggest that p-TAK1 and p-IRF3 are important targets of CaMKIIα for reducing proinflammatory cytokine release in H/R treated KCs. Our findings were consistent with previous research on RAW264.7 treated with LPS by Liu et al. [11]. The TLR4 signaling pathway activates different intrahepatic cell types, including dendritic cells [34], sinusoidal endothelial cells [35], and hepatocytes [36], and the effects of miR-148a and CaMKIIα on these other cell types requires further investigation.

Conclusion

In conclusion, our results indicate that exogenous miR-148a can mitigate liver I/R injury in an *in vivo* liver I/R model and an *in vitro* H/R model. The mechanism of protection involves inactivation of the Ca^{2+}/CaMKII pathway and decrease in proinflammatory factor production directly through repression of CaMKIIα and indirectly through dephosphorylation of TAK1 and IRF3 involved in TLR4 signaling. These findings demonstrate that therapeutic modulation of the Ca^{2+}/CaMKII and TLR4 signaling pathways in the liver by administering miR-148a could be a promising biological strategy in humans for protecting the liver from I/R injury.

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Disclosure Statement

The authors declare that they have no conflicts of interest regarding the contents of this article.

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