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Original Article

Hepatic Ischemia-reperfusion Injury in Mice was Alleviated by Rac1 Inhibition – More Than Just ROS-inhibition

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Abstract

Background and Aims: Reducing reactive oxygen species (ROS) production has proven an effective way for alleviating oxidative stress during ischemia-reperfusion injury (IRI). Moreover, inhibition of Rac1 could reduce ROS production and prevent oxidative stress injury. Previous studies have suggested a positive interactivation feedback loop between Rac1 and hypoxia-inducible factor (HIF)-1α, the latter being up-regulated early during ischemia. The positive inter-activation between Rac1 and HIF-1α would aggravate ROS production, thereby promoting IRI. This study was designed to verify the effects of Rac1 inhibition on hepatic IRI both at animal and cellular levels and to explore the interaction between Rac1 and HIF-1α during hepatic IRI. ***Methods:*** C57B/6 mice and AML-12 cells were used for the construction of hepatic IRI animal and cell models. Rac1 inhibition was achieved by NSC23766 (a specific Rac1 inhibitor). Lentiviral vectors were used for Rac1 knockdown. At designated time points, serum and liver tissues were collected from the mice and treated cells were collected for further analysis. ***Results:*** NSC23766 treatment significantly alleviated the hepatic IRI in mice, manifesting as lower vacuolation score and less apoptosis cells, lower ROS and serum/liver alanine aminotransferase/aspartate aminotransferase levels, and fewer activated inflammatory cells. IRI of AML-12 was also alleviated by 50 μM NSC23766 or Rac1-knockdown, manifesting as reduced cell apoptosis, less extensive interruption of mitochondrial membrane potential, down-regulation of apoptosis, and effects on DNA damage-related proteins. Interestingly, Rac1 knockdown also down-regulated the expression level of HIF-1α. ***Conclusions:*** Our study supports a protective effect of Rac1 inhibition on hepatic IRI. Aside from the classic topics of reducing ROS production and oxidative stress, our study showed an interaction between Rac1 and HIF-1α signaling during hepatic IRI.

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Introduction

Ischemia-reperfusion injury (IRI) includes two interacting phases of local ischemia injury and inflammation-mediating reperfusion injury.[1] As a major risk factor for post-operative complication and mortality, hepatic IRI not only leads to liver dysfunction and failure but also affects other remote organs,[2] and in severe cases, causing multiple organ dysfunction syndrome or systemic inflammatory response syndrome[3] which severely affects patient survival and quality of life. Since liver transplantation is still the only effective treatment for patients with terminal liver diseases and primary hepatic carcinoma, its critical obstacle of organ shortage is aggravated by hepatic IRI-related organ damage, early dysfunction, and primary non-function of graft.[4] Therefore, seeking effective ways to alleviate hepatic IRI is of great importance.

The earliest change in hypoxic hepatocytes takes place in the mitochondria.[5] During IRI, large amount of Kuppffer cells (KCs) are activated by damage-associated molecular patterns (DAMPs) released by hepatocytes, leading to a significant increase in reactive oxygen species (ROS) production and the release of pro-inflammatory cytokines, including TNF-α and IL-1, which further aggravate KC activation and promote the recruitment of neutrophils,[5] forming a vicious circle that eventually leads to excessive inflammation injury, the key mechanism of reperfusion injury. As one of the most dangerous factors during hepatic IRI, ROS is produced both during ischemia and reperfusion phases.[6] Reducing ROS production has proven to be an effective way for alleviating oxidative stress during IRI. Rac1 is a small guanosine triphosphatase (GTPase) involved in oxidative

Keywords: Hepatic ischemia-reperfusion injury; Reactive oxygen species; Rac1; Hypoxia inducible factor.
Abbreviations: HIF, hypoxia inducible factor; HIRI, hepatic ischemia-reperfusion injury; ROS, reactive oxygen species.
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stress, the inhibition of which could reduce ROS production and prevent oxidative stress injury, as shown in several model systems.[7–9]

The up-regulation of hypoxia-inducible factor (HIF)-1α is an early event during IRI, which occurs before apoptosis[10] and can last until 24 h after hepatic resection.[11] HIF-1α can induce the transcription of many protective genes, reducing hepatic cell injury and death induced by IRI.[6] However, not all downstream effects of the HIFs are protective and its effects may change over time.[12] HIFs also exert pro-apoptotic effects.[12] More severe or long-term hypoxia can cause cell apoptosis through HIF signaling, in general.[12] Also, HIF-1α-dependent up-regulation of the transferrin gene promotes ROS production and participates in liver injury during reperfusion.[13] Previous studies have also suggested a cross-talk between Rac1 and HIF-1α which forms a positive feedback loop during pulmonary vascular remodeling.[14] Therefore, Rac1 and HIF-1α could activate each other and continuously increase ROS production. Inhibition of Rac1 could alleviate hepatic IRI not only by reducing ROS production but also by blocking this vicious positive feedback activation circle between Rac1 and HIF-1α.

In this article, we studied the role of Rac1 during hepatic IRI *in vitro* and *in vivo*, proving that Rac1 inhibition could effectively alleviate hepatic IRI in mice and reduce the IRI-induced mouse hepatocyte apoptosis. More importantly, we found that Rac1 inhibition down-regulated the expression level of HIF-1α, suggesting that there were more sophisticated mechanisms by which Rac1 inhibition exerts its protective effects.

Methods

Experimental animals

Six-to-eight-week-old male wild-type C57BL/6 mice were purchased from the Experimental Animal Center of Naval Medical University (NMU), and were raised under 12 h-12 h day-night cycle in a specific pathogen-free animal facility, with adequate food and water supply ensured. All animal operative procedures met the requirements of the ethics committee of NMU.

Hepatic IRI mouse model

Mice were randomly divided into six groups, namely Sham+phosphate-buffered saline (PBS), Sham+NSC23766 (NSC), Sham+N-acetylcysteine (NAC), IRI+PBS, IRI+NSC and IRI+NAC, of which the three IRI groups underwent the classic 70% warm hepatic IRI procedure, as previously described.[15] Anesthesia of mice was carried out with 1% pentobarbital sodium at a dose of 50 mg/kg. The middle and left branches of the portal vein were clamped for 1 h and then released for reperfusion. Mice from the three Sham groups underwent the same surgical treatment without vascular occlusion. Mice from the two NSC groups and the NAC groups were given intraperitoneal injection of 2.5 mg/kg NSC23766 (S8031; Selleck Corp., Ltd, Shanghai, China) and 50 mg/kg NAC (S1623; Selleck Corp.), respectively, on alternative days for 2 weeks[16] before undergoing the surgical operation; those from the PBS groups were given the same volume of PBS. At 6 and 24 h after reperfusion, three mice from each group were sacrificed by cervical dislocation after blood collection by eyeball removal, the left and middle lobes of liver were collected for hematoxylin-eosin (H&E) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNLE), immunohistochemistry (IHC) analysis, and ROS level detection.

Cell line and IRI cell model

The alpha mouse liver (AML-12) cell line (CRL-2254™; ATCC, Manassas, VA, USA) was cultured using DMEM/F12 1:1 (1×) (11320033; Gibco, Waltham, MA, USA) with 10% fetal bovine serum (SH30084.03; Hyclone, Logan, UT, USA), 1% antibiotic-antimycotic (100X) (15240112; Gibco) and 1% ITS cell culture supplement (100X) (ITSS-10201; Cyagen, Su Zhou, China) added. Cells were cultured in an incubator with air supply of 21% O_2 and 5% CO_2, at 37°C. For mimicking hepatic IRI condition, cells were put in an incubator containing 95% N_2 and 5% CO_2 at 37°C with culture medium of 1% FBS added, 24 h later, cells were put in normal condition with normal culture medium (10%FBS added) changed for reoxygenation.

Rac1-knockdown AML-12 construction

Rac1 (Gene ID: 19353) knockdown and control lentiviral vectors were kindly provided by the Department of Radiation Medicine, Faculty of Naval Medicine, NMU. Virus were added at a concentration of MOI=20. Fluorescence microscope was used to verify the success of virus infection. Culture medium was changed with 5 μg/mL puromycin added. After 3 days of drug screening, all cells in the Blank group died and surviving cells from the control (Rac1-shNC) and Rac1-knockdown (Rac1-SH) groups were collected for infection efficiency detection by flow cytometry, qPCR and western blotting (WB).

Cell counting kit-8 detection

The Cell Counting Kit-8 (CK04: Dojindo, China) was used for drug cytotoxicity test according to manufacturer's instruction. A microplate reader was used for analyzing the results.

Apoptosis detection

The Annexin V-FITC/PI Cell Apoptosis Detection Kit (AD10) and Annexin V, 633 Apoptosis Detection Kit (AD11), all purchased from Dojindo Corp., Ltd, Shanghai, China, were used for apoptosis analysis of wild-type cell lines and virus-infected cell lines. An automated flow cytometry instrument was used for apoptosis ratio detection.

Mitochondrial membrane potential (MMP) detection

Rhodamine 123 (C2007; Beyotime, China) and MitoRed (R237; Dojindo Corp., Ltd) were used for MMP detection of wild-type and virus-infected AML-12 cells, respectively. An automated flow cytometry instrument was used for fluorescence intensity analysis.

WB

7.5% (PG111) and 12.5% (PG113) PAGE Gel Fast Preparation Kits were purchased from Epizyme Biotech Corp., Ltd (Shanghai, China). Gels were prepared according to the manufacturer's instruction. Beta tubulin antibody (66240-1-Ig), BAX antibody (50599-2-Ig) and BCL2 antibody (12789-

1-AP) were purchased from Proteintech (Wuhan, China). Anti-caspase-3 antibody (ab13847), anti-ATR (phospho T1989) antibody (ab227851), anti-Chk1 (phospho S345) antibody (ab47318) and anti-gamma H2A.X (phospho S139) antibody (ab11174) were purchased from Abcam (Shanghai) Corp., Ltd (China). Anti-Rac1 monoclonal antibody (ARC03) and anti-active Rac1-GTP mouse monoclonal antibody (26903) were purchased from Cytoskeleton (Denver, CO, USA) and NewEast Biosciences (King of Prussia, USA), respectively. HIF-1α rabbit monoclonal antibody (36169), PAK1 antibody (2602), phospho-PAK1 (Ser144)/PAK2 (Ser141) antibody (2606), anti-rabbit IgG, HRP-linked antibody (7074) and anti-mouse IgG, HRP-linked antibody (7076) were purchased from Cell Signaling Technology (Shanghai) Corp., Ltd (China). Analysis of WB results was conducted by ImageJ software.

Statistical analysis

SPSS19.0 software was used for data analysis. Analysis of variance (ANOVA) was used for difference analysis among multiple groups. Further comparison between subgroups was done by SNK-q test. A difference was considered as statistically significant if p was <0.05.

Results

Hepatic IRI in a mouse model was eased by Rac1 inhibition

C57BL/6 mice were given PBS or NSC (2.5 mg/kg NSC23766) on alternative days for 2 weeks before undergoing sham or hepatic I/R surgery. Simultaneously, NAC (50 mg/kg), a scavenger for ROS, was applied as the positive control. First, ROS level was evaluated by fluorescence probe. Compared with mice in the Sham+PBS group, the ROS levels of the mice in the IRI+PBS group were significantly increased at 6 h (p<0.05) and 24 h (p<0.001) after surgery (Fig. 1A and C). At both time points, the ROS levels in the IRI+NSC group and the IRI+NAC group were significantly lower than those in the IRI+PBS group (all p<0.001), with little difference between themselves. Next, H&E staining showed that hepatocellular swelling and cytoplasmic prosity were induced by the I/R treatment (Fig. 1B). Vacuolation score analysis of liver injury showed that, compared with the Sham+PBS group, the numbers of hepatocytes under high vacuolation grades were significantly increased at both time points in the IRI+PBS group (Fig. 1 D–E). Compared with the IRI+PBS group, the hepatocytes with high vacuolation grade in the IRI+NSC group and the IRI+NAC group were significantly fewer at 6 h and 24 h after surgery (all p<0.001; Fig. 1D–E), suggesting a lessened liver injury with NSC23766 or NAC treatment. Except hepatic pathology, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also an important indicator of liver injury. Compared with the Sham+PBS group, the ALT content in liver tissue and serum were significantly increased in the IRI+PBS group at 6 h and 24 h after surgery (all p<0.001; Fig. 1F and H); similar results were seen for the AST content (all p<0.05; Fig. 1G–I). At both time points, the ALT and AST contents in the liver tissue and serum of the IRI+NSC group and IRI+NAC group were significantly lower than those of the IRI+PBS group (all p<0.05; Fig. 1F–I). Together, these results supported that hepatic IRI was eased by NSC23766 treatment.

Since inflammation injury and apoptosis are the important mechanisms of IRI, the activation of inflammatory cells and hepatocellular apoptosis was evaluated. The number of macrophages was significantly increased at 24 h after surgery in the IRI+PBS group (p<0.01; Fig. 2 A–B), and the numbers of neutrophils were significantly increased at 6 h (p<0.05) and 24 h (p<0.001) (Fig. 2C–D), compared with those in the Sham+PBS group. The numbers of macrophages (p<0.05) and neutrophils (p<0.001) in the IRI+NSC group were significantly reduced compared to those in the IRI+PBS group at 24 h after surgery (Fig. 2B and D), suggesting a reduction in the activation of inflammatory cells in response to the NSC23766 treatment. TUNEL staining showed that apoptosis of hepatocytes was significantly induced by IRI, as compared with that seen in the Sham groups (Fig. 2E). Compared with the IRI+PBS group, the percentages of positive stained cells were significantly lower in the IRI+NSC group at both 6 h (p<0.01) and 24 h (p<0.05) after surgery (Fig. 2F), suggesting a reduction in hepatocyte apoptosis after IRI in response to the NSC23766 treatment. Collectively, these results supported the notion that hepatic IRI of mice was eased by Rac1 inhibition.

IRI of AML-12 was alleviated by inhibition of Rac1

Based on the IC50 (50 μM) of NSC23766, AML-12 cells were pretreated with PBS or different doses of NSC23766 (25, 50 and 100 μM) for 24 h and collected for drug toxicity testing by CCK8. The cell proliferation ability was mildly increased while inhibition by 25 μM and 100 μM of NSC23766, respectively, and was not significantly affected by 50 μM NSC23766 (Fig. 3A). AML-12 cells were pretreated with PBS or 25 or 50 μM NSC23766 2 h before H_2O_2 treatment (500 μM or 1 mM) and collected for apoptosis detection 24 h later. Cell apoptosis induced by 1 mM H_2O_2 treatment was significantly reduced by 50 μM NSC23766 (p<0.001; Fig. 3B). Thus, the dose of 50 μM was chosen for further experiments. Production of lactate dehydrogenase (LDH) 24 h after the control/IRI process was reduced by 50 μM NSC23766 pretreatment in both the control group (p<0.05) and IRI (p<0.01) group (Fig. 3C). Consistent with results from the H_2O_2-induced injury model, IRI-induced cell apoptosis of AML-12 was significantly reduced by 50 μM NSC23766 (p<0.01; Fig. 3D–E). Different from cell apoptosis, changes of MMP occurred earlier. Thus, MMP detection was conducted 4 h after the IRI process by Rdh123 with flow cytometry. Decrease in MMP was represented as increase in the mean fluorescence intensity of Rdh123. The decrease in MMP of AML-12 cells induced by IRI was significantly alleviated by 50 μM NSC23766 (p<0.001; Fig. 3F).

Apoptosis-related proteins and DNA damage-related proteins were further analyzed by WB. After incubation with PBS or 50 μM NSC23766 for 2 h, AML-12 were cultured under the control or IRI condition. Proteins were collected at designated time points (namely 0, 2, 6, 12 and 24 h after IRI). At 12 and 24 h after the IRI, the relative ratio of Bax/Bcl-2 and the expression level of cleaved caspase-3 were significantly reduced by 50 μM NSC23766, compared with that detected in the PBS group (all p<0.001; Fig. 4A–C), suggesting that the cell apoptosis induced by IRI was reduced by 50 μM NSC23766 pretreatment. At several time points after IRI, the up-regulation of p-ATR (12 and 24 h, p<0.05 and 0.001, respectively), p-CHK1 (0 and 24 h, p<0.01 and 0.05, respectively) and γ-H_2AX (6~24 h, all p<0.001) were significantly reduced by 50 μM NSC23766, compared with that detected in the PBS group (Fig. 4D–G), suggesting the DNA damage induced by IRI was alleviated by the 50 μM NSC23766 pretreatment. Collectively, these results supported that the I/R injury of AML-12 was alleviated by Rac1 inhibition.

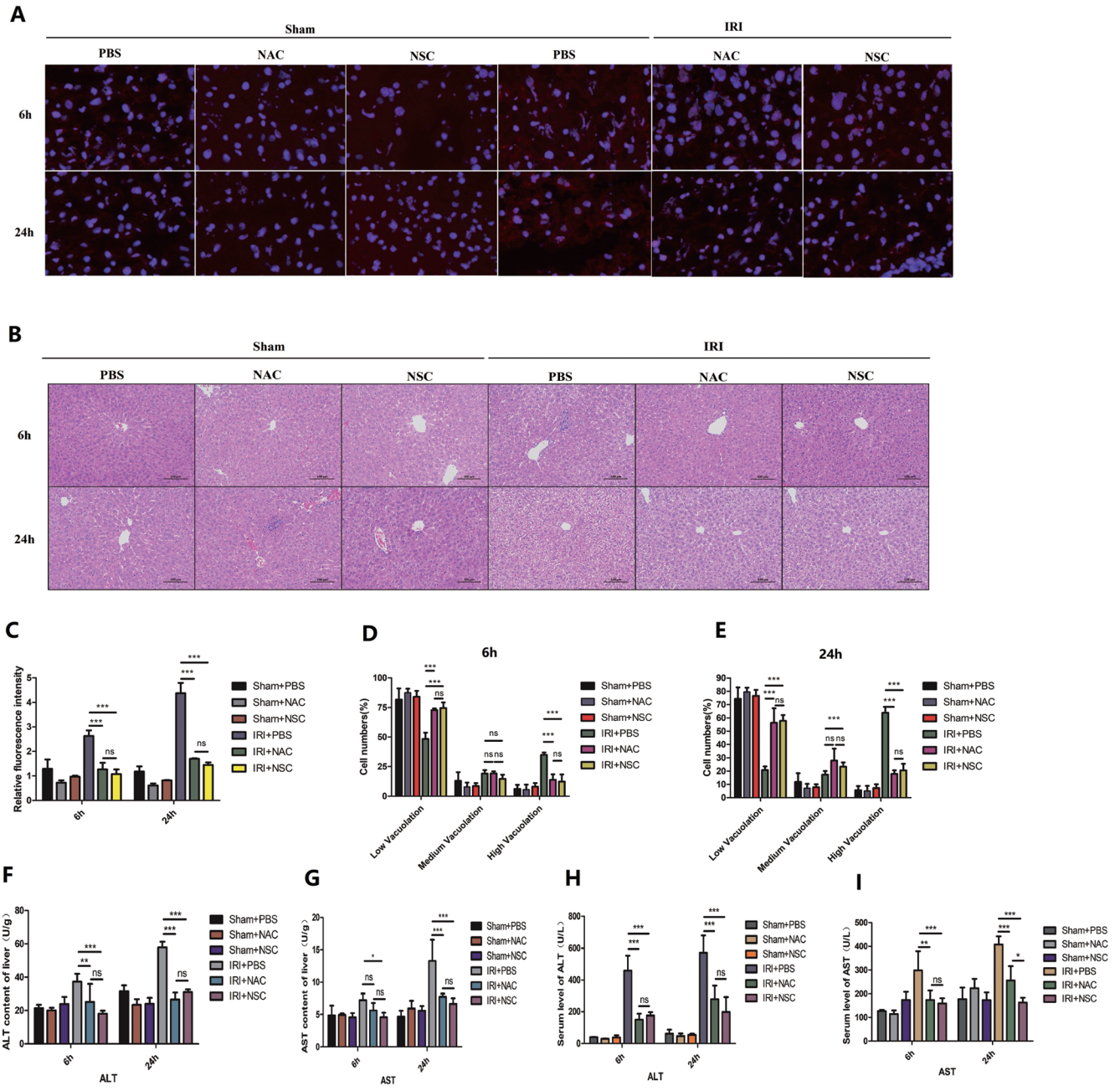

Fig. 1. Hepatic IRI in a mouse model was alleviated by Rac1 inhibition. Mice were randomly divided into six groups, namely the Sham+PBS, Sham+NSC, Sham+NAC, IRI+PBS, the IRI+NSC and IRI+NAC groups. Mice were intraperitoneally injected with PBS, 2.5 mg/kg NSC23766 or 50 mg/kg NAC on alternative days for 2 weeks before undergoing surgical operation. At 6 h and 24 h after surgery, mice from each group (n=3) were sacrificed, liver samples were collected for ROS level detection (A), H&E staining (B), and ALT/AST content analysis (F–G). (C) Statistical analysis of (A). (D, E) Quantitative analysis of (B). Serum samples were collected for AST and AST analysis (H-I). ns represents $p \geq 0.05$ between the corresponding groups. *, ** and *** represent $p<0.05$, 0.01 and 0.001 between the corresponding groups, respectively. Data are mean±standard error of the mean (SEM). Hepatic IRI, hepatic ischemia-reperfusion injury; AST, glutamic oxalacetic transaminase; ALT, glutamic-pyruvic transaminase.

IRI of AML-12 was eased by Rac1-knockdown

For further validation of the effects of Rac1 inhibition, Rac1 knockdown (Rac1-SH) AML-12 and negative control (Rac1-NC) AML-12 were constructed using lentiviral vectors. Successful infection of viruses was determined by flow cytometry detection of mean fluorescence intensity. The infection efficiencies of Rac1-NC and Rac1-SH AML-12 were 98.87% and 99.13%, respectively (Fig. 5A–B). Successful knockdown of Rac1 was determined by qPCR and WB analysis. The relative mRNA and protein expression levels of Rac1 were significantly down-regulated in Rac1-SH AML-12 compared with those in Rac1-NC AML-12 (both $p<0.01$; Fig. 5C–E). At 2 h after IRI, up-regulation of the mRNA levels of IL-6, TNF-α, CXCL-1 and CXCL-2 were significantly decreased

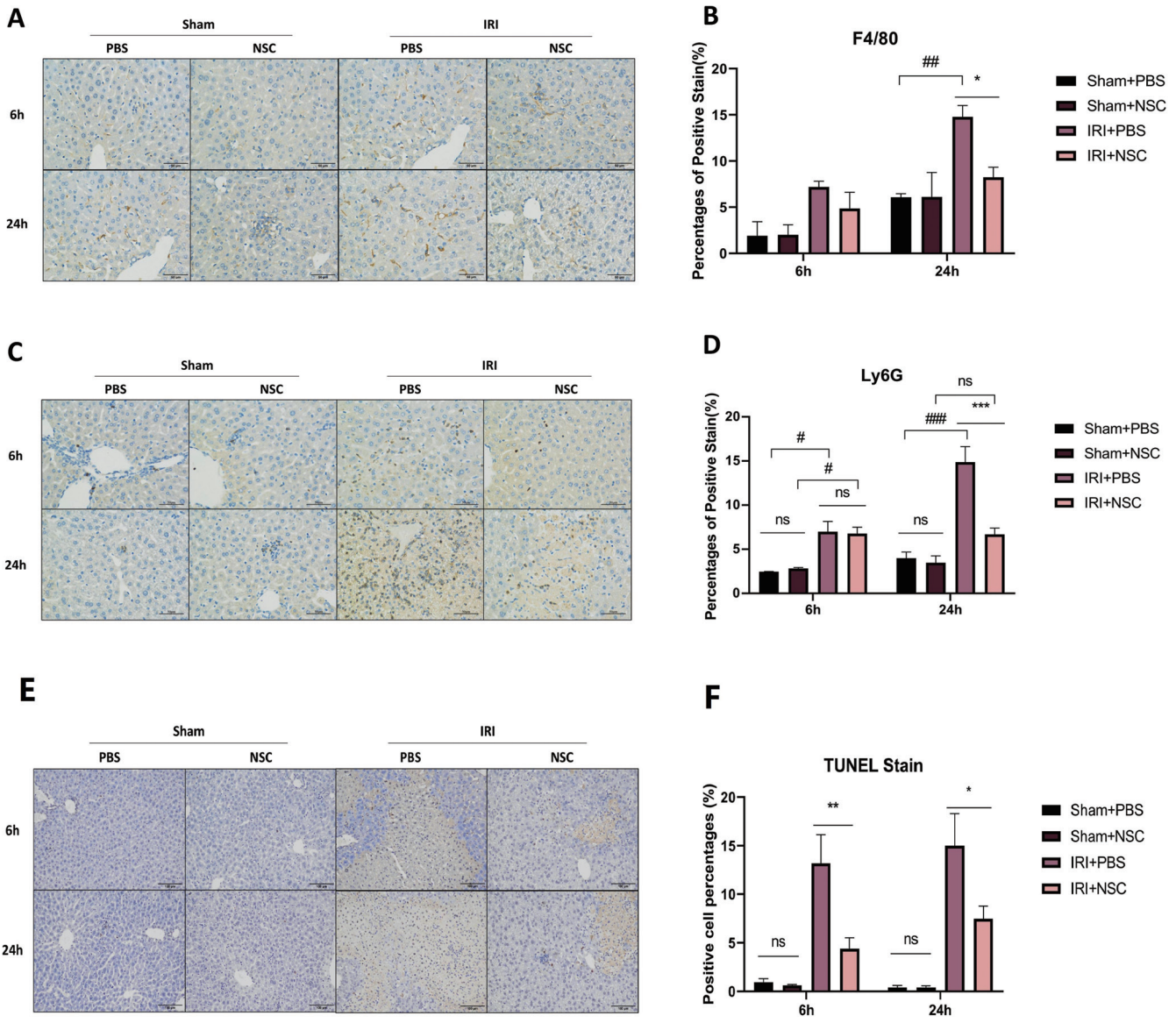

Fig. 2. Inflammatory cell activation and hepatocellular apoptosis induced by IRI was alleviated by Rac1 inhibition. Samples from the same mouse model presented in Fig. 1 were collected for immunohistochemistry analysis of F4/80 (A) and Ly6G (C), markers of macrophages and neutrophils, respectively, and apoptosis detection with TUNEL staining (E). (B), (D) and (F) show the statistical analyses of (A), (C) and (E), respectively. ns represents $p \geq 0.05$ between the corresponding groups. * and *** represent $p<0.05$ and 0.001 between the corresponding groups, respectively. #, ## and ### represent $P<0.05$, 0.01 and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Hepatic IRI, hepatic ischemia-reperfusion injury

in the Rac1-SH group compared with those in the Rac1-NC group ($p<0.01$ for IL-6 and CXCL-1, $p<0.001$ for TNF-α and CXCL-2; Fig. 5F), suggesting an alleviation in inflammation response mediated by Rac1 knockdown. Consistent with previous results from Rac1 inhibition, the cell apoptosis induced by 1 mM H_2O_2 ($p<0.001$) and IRI ($p<0.001$) were both reduced in the Rac1-SH group compared with those in the Rac1-NC group (Fig. 5G–H), similarly, the decrease in MMP induced by IRI was significantly reduced by Rac1-knockdown ($p<0.001$; Fig. 5I).

Apoptosis-related proteins and DNA damage-related proteins were also analyzed by WB. Rac1-NC/SH AML-12 cells under control or IRI conditions were collected at designated time points. At multiple time points after IRI, the relative ratio of Bax/Bcl-2 and the expression level of cleaved caspase-3 was significantly reduced in Rac1-SH cells compared with those in the Rac1-NC cells (Fig. 6A–C), suggesting the cell apoptosis induced by IRI was reduced by Rac1 knockdown. Likewise, the up-regulation of p-ATR, p-CHK1 and γ-H_2AX at multiple time points was significantly reduced in Rac1-SH cells compared with those in the Rac1-NC cells (Fig. 6D–G), suggesting the DNA damage induced by IRI was also alleviated by Rac1 knockdown. Collectively, these results showed that the I/R injury of AML-12 was eased by Rac1-knockdown.

Inhibition/knockdown of Rac1 down-regulated HIF-1α

Findings from other studies[14,17] have supported a cross-talk

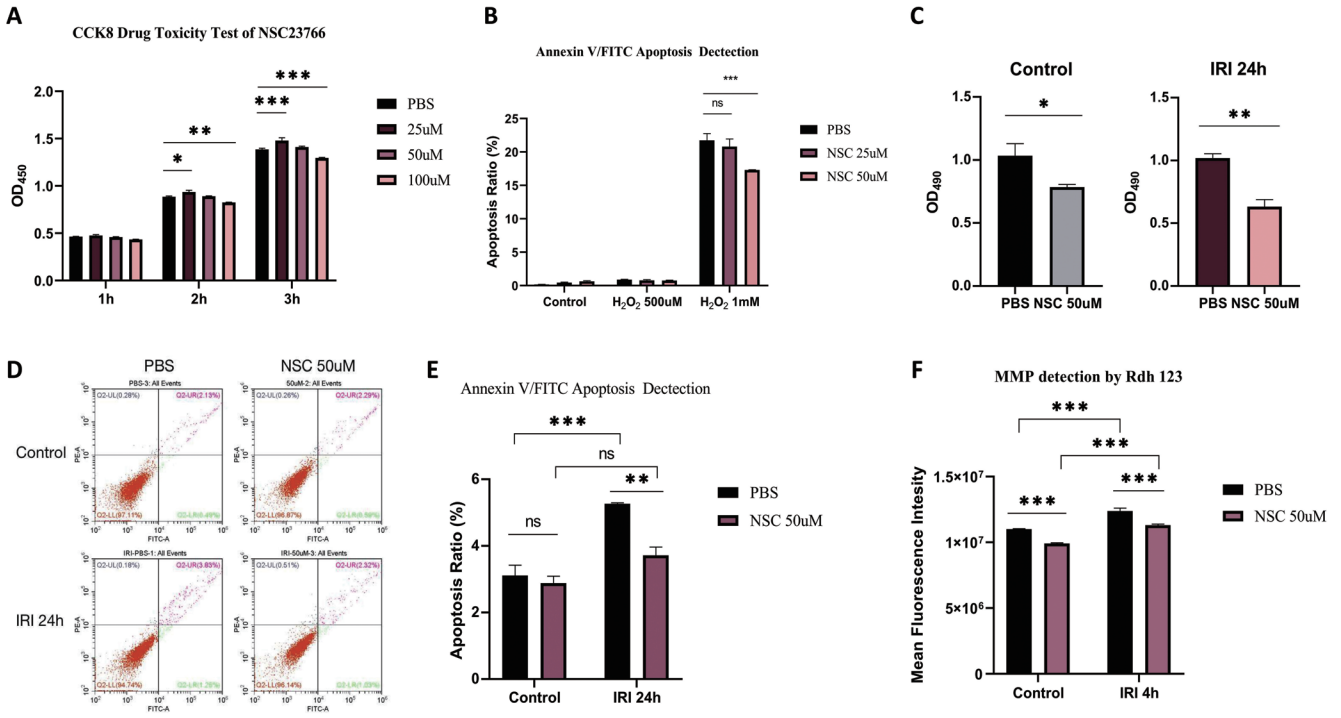

Fig. 3. IRI of AML12 was alleviated by inhibition of Rac1 with NSC23766. AML-12 cells were treated with PBS or different doses of NSC23766 (namely 25, 50 and 100bμM) for 24 h and then tested for drug toxicity by the CCK8 method (n=6) (A). After pretreatment with PBS or 25 μM/50 μM NSC23766 for 2 h, AML-12 cells were stimulated with PBS or 500 μM/1 mM H_2O_2 and the cell apoptosis ratio was detected 24 h later by flow cytometry (n=3) (B). The cell apoptosis induced by 1 mM H_2O_2 treatment was significantly reduced by 50 μM NSC23766 pretreatment. Thus, the dose of 50 μM was selected for further experiments. AML-12 cells were pretreated with PBS or 50 μM NSC23766, and 2 h later cultured under control or IRI condition (specific procedures are provided in the Materials and Methods section). Cells were collected 24 h later for LDH cytotoxicity assay (n=5) (C) and apoptosis analysis (n=3) (D). (E) Statistical analysis of (D). MMP detection (F) by Rhd123 was conducted 4 h after subject to control or IRI condition. ns represents $p \geq 0.05$ between the corresponding groups. *, ** and *** represent $p<0.05$, 0.01 and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Hepatic IRI, hepatic ischemia-reperfusion injury; LDH, lactate dehydrogenase; MMP detection, mitochondrial membrane potential detection;

between Rac1-signaling and HIF-1α signaling pathways. Rac1 could stimulate the expression of HIF-1α through PAK-1 and, in return, HIF-1α could bind to the promoter of Rac1, thereby enhancing its transcription.[14] Thus, Rac1 and HIF-1α may form a positive-feedback during ROS injury. Therefore, the interaction between Rac1 and HIF-1α was further investigated under the IRI condition. The expression levels of active Rac1, total Rac1, HIF-1α, p-PAK1/2 and PAK1 were analyzed by WB. Inhibition of Rac1 by 50 μM NSC23766 significantly reduced the level of active Rac1 under control and IRI conditions (all $p<0.001$; Fig. 7A–B). Interestingly, compared with the control condition, the expression levels of HIF-1α were significantly up-regulated in both PBS and NSC treated AML-12 cells by ischemia treatment but decreased dramatically at 2 h after reperfusion while being up-regulated again at 6~24 h later (Fig. 7A and C). Compared with the PBS group, the up-regulation of HIF-1α at 0 h and 6~24 h after IRI was significantly reduced by Rac1 inhibition (Fig. 7C). At 12 h and 24 h after IRI, the relative ratios of p-PAK1/PAK were significantly decreased by Rac1 inhibition (Fig. 7A and D). Similar results were achieved by Rac1-knockdown. The expression level of Rac1 was significantly down-regulated in the Rac1-SH cells at every time point compared with those in the Rac1-NC cells (all $p<0.001$; Fig. 7E–F). Under the control condition, Rac1 knockdown significantly down-regulated the expression level of HIF-1α ($p<0.001$; Fig. 7G). The up-regulation of HIF-1α at 6~24 h after IRI was down-regulated by Rac1-knockdown (Fig. 7G). The relative ratios of p-PAK1/PAK at every time point, except 24 h, were significantly decreased by Rac1 knockdown (Fig. 7H).

In conclusion, these results supported that Rac1-inhibition/knockdown could down-regulate HIF-1α signaling through its downstream effector PAK1. In addition, *in vivo* experiments were also performed. The results demonstrated that the expression of Rac1 and HIF-1α were all significantly up-regulated at 24 h after IRI; however, the IRI-induced up-regulation of HIF-1α was inhibited by NSC23766, without change of Rac1 expression (Fig. 7I).

Discussion

One of the most important mechanisms of IRI is oxidative stress injury. ROS produced by hepatocytes initiates the process of liver IRI. Apart from triggering a local sterile immune response, ROS could also recruit KCs and neutrophils to the injury site, which further produce large amounts of ROS, inducing apoptosis and necrosis of liver tissue.[3] ROS production is induced during the ischemia phase and aggravated during the reperfusion phase.[6] As a component of the activated NADPH oxidase in monocytes and neutrophils,[18] Rac1 is crucial to ROS production. Previous studies showed that inhibition of Rac1 could reduce ROS production and alleviate oxidative stress injury.[7,8] However, few studies investigated its role in hepatic IRI. One paper[19] reported a protective effect of Rac1 inhibition on a liver IRI mouse model with little research on elucidating the possible mechanisms. Consistent with their result, our data supported that inhibition of Rac1 could alleviate hepatic IRI.

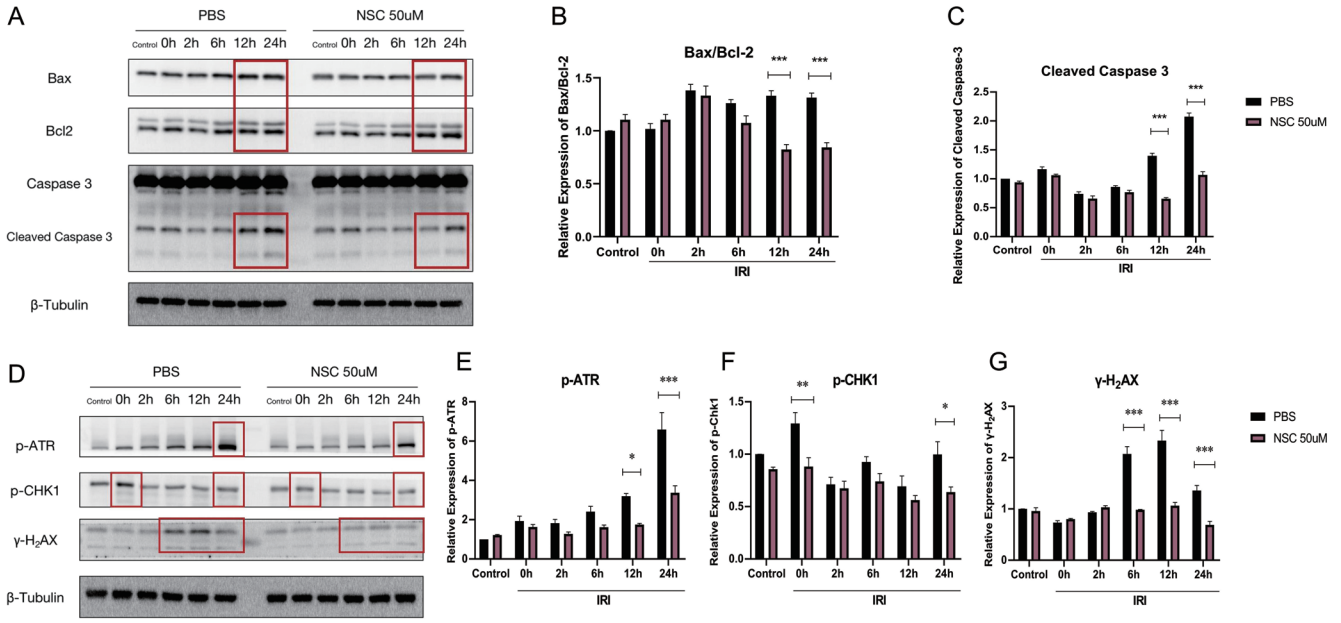

Fig. 4. IRI-induced cell apoptosis and DNA damage were alleviated by Rac1 inhibition. AML-12 cells were pretreated with PBS or 50 μM NSC23766 for 2 h before subject to control/IRI condition and collected at designed time points for WB analysis of apoptosis-related proteins (A) and DNA damage-associated proteins (D). The relative expression level of Bax/Bcl-2 (B), cleaved caspase-3 (C), p-ATR (E), p-CHK1 (F) and γ-H2AX (G) were calculated. *, ** and *** represent $p<0.05$, 0.01 and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Experiments were repeated in triplicate. Hepatic IRI, hepatic ischemia-reperfusion injury

Meanwhile, we also compared the effect of oxidation resistance and hepatic protection between the Rac1 inhibitor and a classical ROS scavenger, NAC. Results demonstrated that both Rac1 inhibition and NAC reduced IRI-induced ROS production and remitted hepatic injury, while there was no significant difference between the effects of the two drugs, suggesting that the inhibitory effect on ROS-production by the Rac1 inhibitor and the ROS scavenger was equivalent and that ROS-reduction was the main mechanism by which Rac1-inhibition mediated its protective effect on hepatic IRI, which was consistent with previous studies.[7,8] However, as a long-acting molecule with multiple functions, Rac1 may also interact with other molecules other than inhibiting ROS.

Next, our study not only investigated the influence of Rac1 inhibition/knockdown on hepatic IRI both at animal and cellular levels but also made more efforts in investigating the possible mechanism. MMP and apoptosis detection by flow cytometry was conducted, and apoptosis and DNA damage-related proteins were analyzed. First, the vacuolation score (H&E staining), the positive TUNEL-stained cells, and the serum and liver AST/ALT levels (markers of liver injury) after IRI were reduced by Rac1 inhibition, supporting an alleviation of liver injury. Immunofluorescence detection showed that the activation of neutrophils and macrophages, and the ROS production after IRI were reduced by Rac1 inhibition, supporting an alleviation in immune response and oxidative stress injury. Effects of Rac1 inhibition on hepatic IRI animals were further validated using the AML-12 IRI model. Flow cytometry study showed that the IRI-induced cell apoptosis and the decrease in MMP induced by IRI of AML-12 were significantly reduced by 50 μM NSC23766. WB analysis showed that the up-regulation of apoptosis-related and DNA damage-related proteins induced by IRI were reduced by 50 μM NSC23766. These series studies were further repeated using Rac1-knockdown/control AML-12 cells. Collectively, by using Rac1 inhibitor and Rac1 knockdown cell line, our cell studies supported the protective effect of Rac1 inhibition on hepatic IRI.

Interestingly, we found that Rac1 inhibition down-regulated the expression of HIF-1α, an important early response factor during IRI. Although HIF-1α may exert protective effects on IRI-induced hepatic cell injury and death by inducing the transcription of many protective genes; however, it also has pro-apoptotic effects.[20] According to previous studies, up-regulation of HIF-1α could mediate harmful effects by several mechanisms (Fig. 8). First, HIF-1α could stabilize p53,[21] and more severe or long-term hypoxia can cause cell apoptosis through the interaction of HIF and p53.[20] Second, HIF-1α could induce the transcription of several pro-apoptosis genes, including HGTD-P and BNIP3,[22] inhibition of which could exert a protective effect on hepatic IRI. Third, HIF-1α-dependent up-regulation of the transferrin gene promotes ROS production and participates in liver injury during reperfusion.[13] Also, HIF-1α could activate macrophages[23] and regulate Th_{17}/T_{reg} balance,[24] and up-regulation of HIF-1α could lead to an excessive inflammatory response. More importantly, previous studies[14,17] suggested a cross-talk between Rac1 and HIF-1α, which forms a positive feedback loop; therefore, Rac1 and HIF-1α could activate each other and continuously increase ROS production. Inhibition of Rac1 could alleviate hepatic IRI not only by reducing ROS production but also by blocking this vicious positive feedback activation cycle between Rac1 and HIF-1α. In support of this, we found that the expression level of HIF-1α was shortly decreased (at 2 h) after reperfusion while being up-regulated again (at 6 h later) and lasting until 24 h after the reperfusion; a similar trend was seen for the levels of active Rac1. Thus, the prolonged activation of Rac1 and up-regulation of HIF-1α may aggravate ROS production and excessive inflammation and induce the transcription of pro-apoptosis genes, eventually leading to cell apoptosis.

Aside from the classic topics of reducing ROS production and oxidative stress, our study showed an interaction between Rac1 and HIF-1α signaling. By down-regulating HIF-1α, Rac1 inhibition could interrupt the positive interactivation loop formed by Rac1 and HIF-1α during reperfusion. This is the first study investigating the positive interactivation loop of Rac1 and HIF-1α within IRI. Although

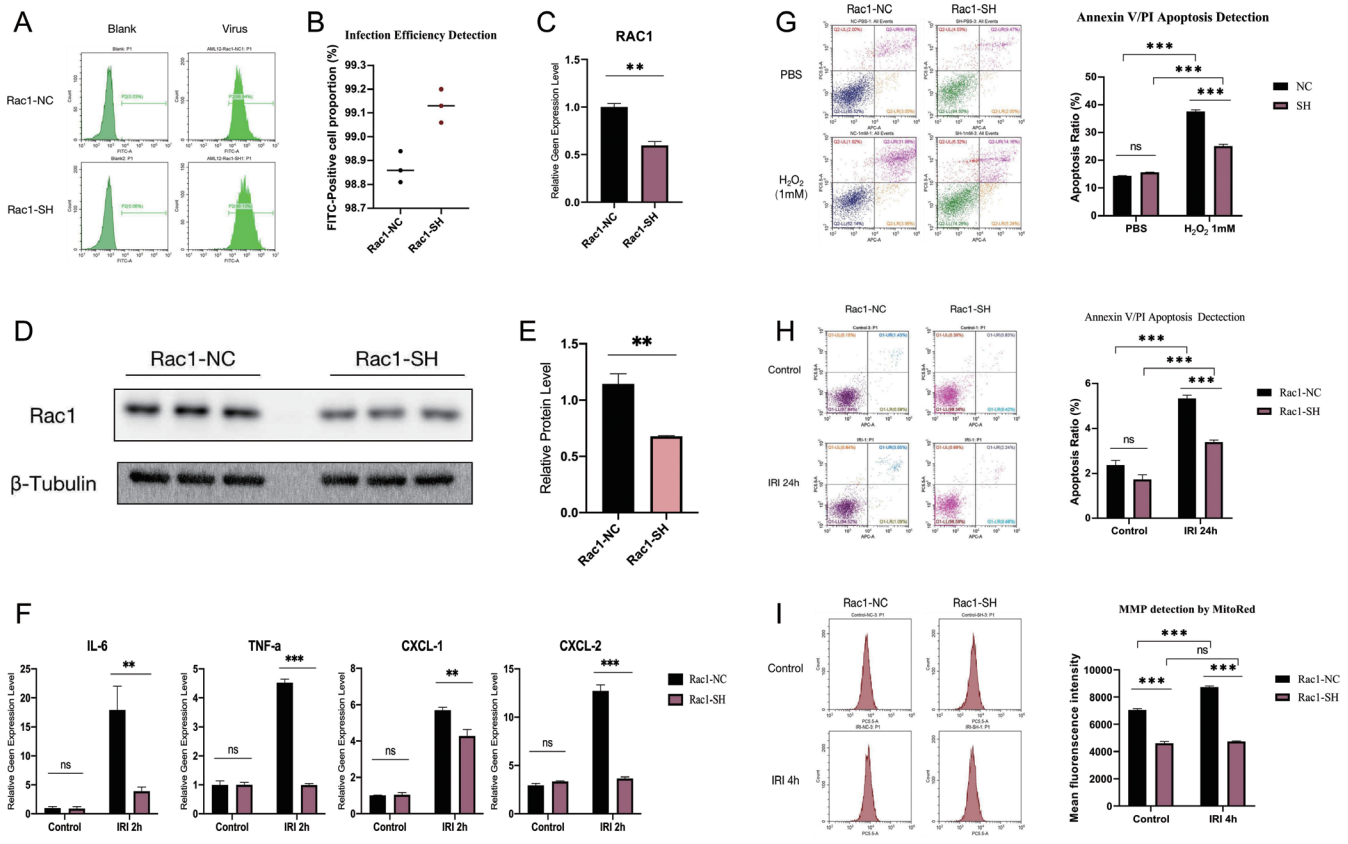

Fig. 5. IRI of AML12 was alleviated by knockdown of Rac1. AML-12 cells were infected with Rac1-NC (negative control) or Rac1-SH (Rac1 knockdown) viruses. Virus infection efficiency was detected by flow cytometry (A). The mean infection efficiencies of Rac1-NC and Rac1-SH were 98.87% and 99.13%, respectively (B). Successful knockdown of Rac1 was demonstrated by qPCR (C) and WB (D). (E) Statistical analysis of (D). Rac1-NC or Rac1-SH AML-12 cells were cultured under control or IRI condition and collected 2 h later for qPCR analysis of IL-6, TNF-α, CXCL-1 and CXCL-2 (F). Rac1-NC and Rac1-SH AML-12 cells were collected 24 h after PBS/1 mM H_2O_2 treatment (G) or control or IRI condition (H) for apoptosis ratio analysis by flow cytometry. At 4 hours later, the cells were collected for MMP detection by MitoRed (I). ns represents $p \geq 0.05$ between the corresponding groups. ** and *** represent $p < 0.01$ and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Hepatic IRI, hepatic ischemia-reperfusion injury; WB, western-blotting; MMP detection, mitochondrial membrane potential detection.

more research is required for deeper understanding of the specific means of action between these two important molecules, our study indeed points out an important yet neglected pathway during IRI, which might shed light on the treatment of hepatic IRI.

Conclusions

Our study supports a protective effect of Rac1 inhibition on hepatic IRI. Aside from the classic topics of reducing ROS production and oxidative stress, our study showed an interaction between Rac1 and HIF-1α signaling during hepatic IRI, suggesting a deeper thought of the effects of HIF-1α. Like the pros and cons of p53, maybe there is no absolute "good" molecule with the big picture considered. The temperate up-regulation of HIF-1α may help in cell survival during hypoxia and its prolonged up-regulation may participate more in activating Rac1 and ROS production, thereby tilting the balance to the "bad" end of the spectrum. After all, it's all about the balance.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study (SS, GD, HL), performed the experiments (ZS, YY, RL, HB, JD), analyzed the data (MG, YZ), wrote the paper (ZS, YY), performed the experiments for revised manuscript (HB), supported by providing funding (GD). All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 85-23, National Academy Press, Washington, DC, revised 1996), with the approval of the

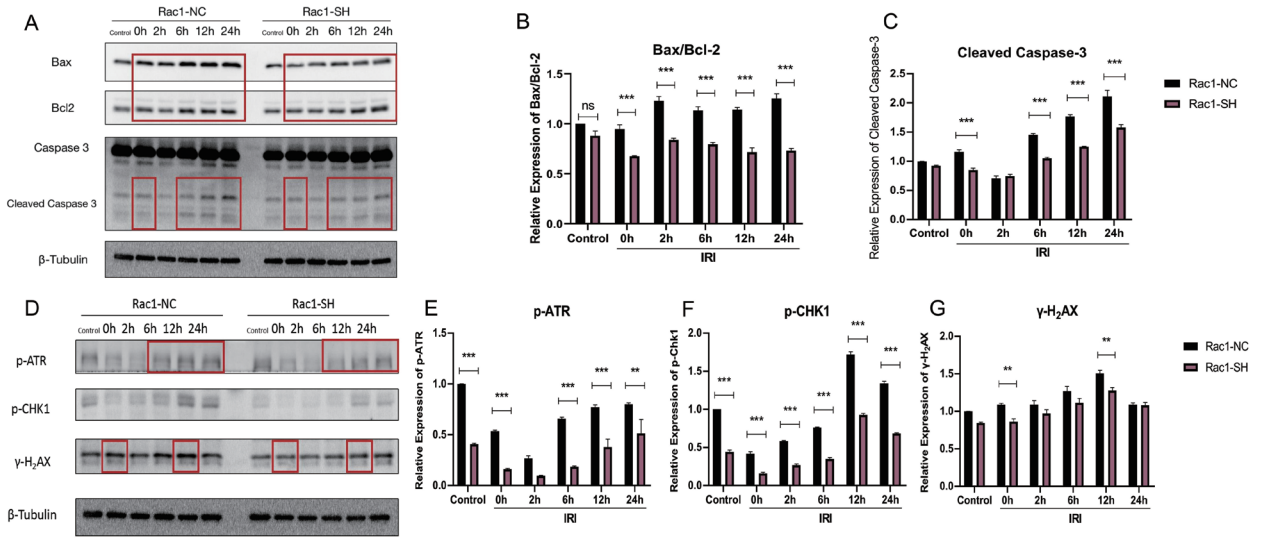

Fig. 6. IRI-induced cell apoptosis and DNA damage were alleviated by Rac1 knockdown. Rac1-NC and Rac1-SH AML-12 cells were cultured under the control or IRI condition and collected at designated time points for WB analysis of apoptosis-related proteins (A) and DNA damage-associated proteins (D). The relative expression level of Bax/Bcl-2 (B), cleaved caspase-3 (C), p-ATR (E), p-CHK1 (F) and γ-H2AX (G) were calculated. ** and *** represent $p<0.01$ and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Experiments were repeated in triplicate. Hepatic IRI, hepatic ischemia-reperfusion injury; WB, western-blotting.

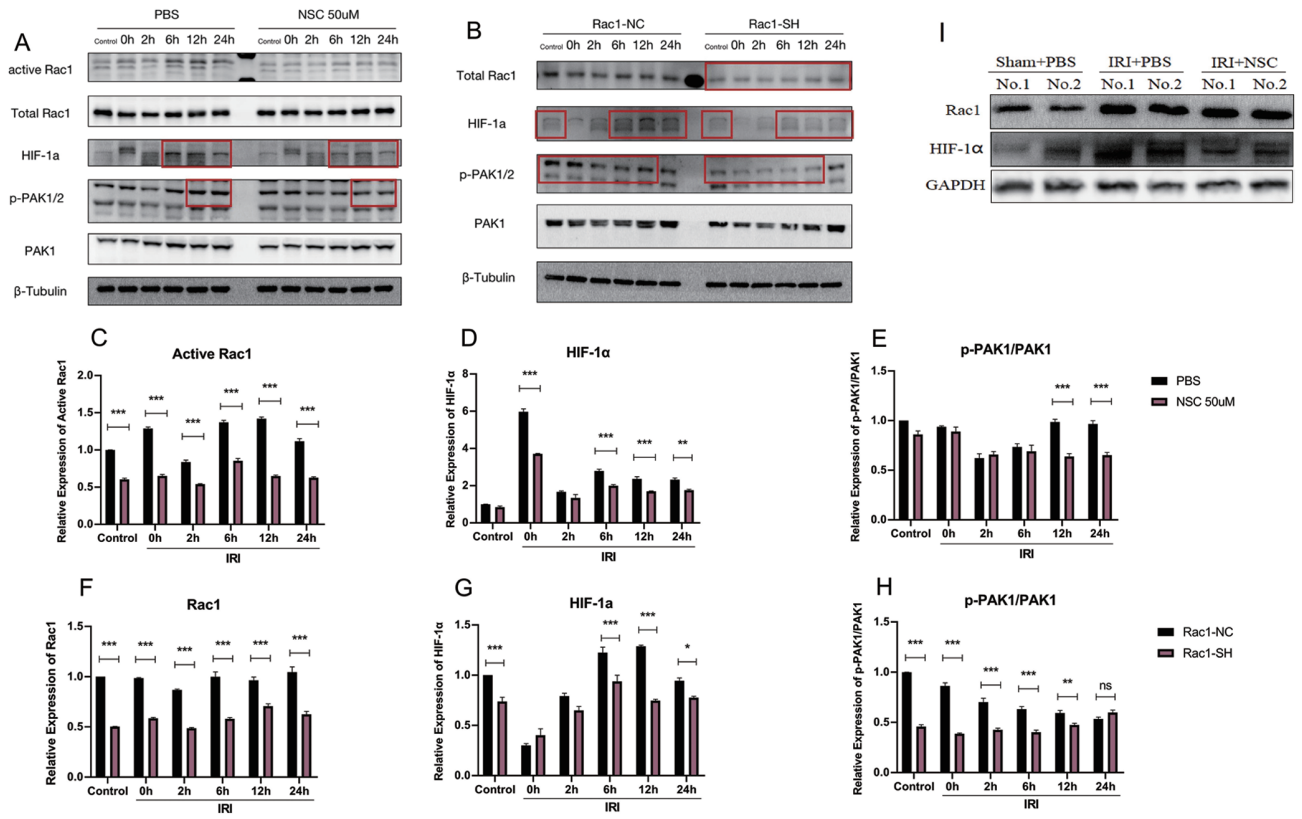

Fig. 7. Inhibition of Rac1 reduced the prolonged up-regulation of HIF-1α induced by reperfusion. AML-12 cells were pretreated with PBS or 50 μM NSC23766 for 2 h before subject to control or IRI condition and collected at designed time points for WB analysis of active Rac1, HIF-1α, p-PAK1 and PAK1 (A). The relative expression level of active Rac1 (C), HIF-1α (D) and p-PAK1/PAK1 (E) were calculated. Rac1-NC and Rac1-SH AML-12 cells were cultured under the control or IRI condition and collected at designed time points for WB of total Rac1, HIF-1α, p-PAK1 and PAK1 (B). The relative expression levels of total Rac1 (F), HIF-1α (G) and p-PAK1/PAK1 (H) were calculated. The proteins in liver tissues at 24 h after modeling in the Sham+PBS, IRI+PBS and IRI+NSC23766 groups were extracted, and the expression levels of Rac1 and HIF-1α were detected by WB (I). ns represents $p\geq0.05$ between the corresponding groups. *, ** and *** represent $p<0.05$, 0.01 and 0.001 between the corresponding groups, respectively. Data are mean±SEM. Experiments were repeated in triplicate. HIF-1α, hypoxia inducible factor-1 α; WB, western-blotting.

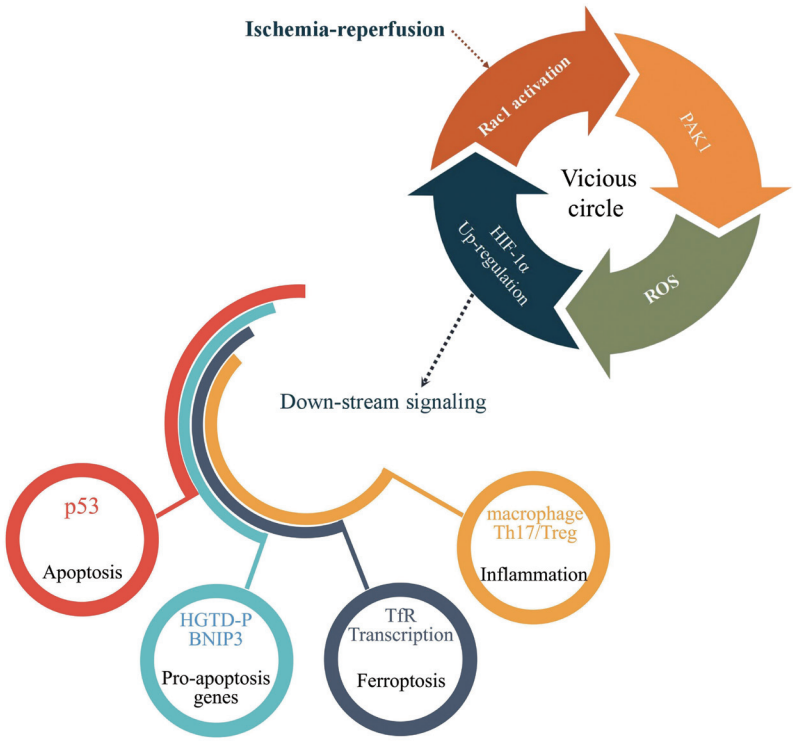

Fig. 8. Summary diagram of possible mechanisms. Our data showed that hepatic IRI in a mouse model was alleviated by Rac1 inhibition. IRI of AML-12 cells was alleviated by Rac1 inhibition/knockdown. This protective effect was achieved not only by reduction in ROS production but also Rac1 inhibition/knockdown down-regulated the expression of HIF-1α. According to previous studies, HIF-1α could activate Rac1 and form a vicious positive inter-activation circle, thereby magnifying ROS production. Besides, HIF-1α could directly bind with p53 and promote p53-mediated apoptosis, induce transcription of several pro-apoptosis genes and TfR which will lead to ferroptosis, and promote the activation of macrophage and regulate Th17/Treg balance. Therefore, prolonged up-regulation of HIF-1α could promote ROS production, cell apoptosis and an excessive inflammatory response. Aside from the classic topics of reducing ROS production and oxidative stress, our study showed an interaction between Rac1 and HIF-1α signaling during hepatic IRI, suggesting more sophisticated mechanisms by which Rac1 inhibition mediate its protective effects. Hepatic IRI, hepatic ischemia-reperfusion injury; ROS, Reactive oxygen species; HIF-1α, hypoxia inducible factor-1 α.

Laboratory Animal Center of the Second Military Medical University, Shanghai. The approval ID for this study was SMMU-20197629.

Data sharing statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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