Original Paper

Rictor Deficiency Aggravates Hepatic Ischemia/Reperfusion Injury in Mice by Suppressing Autophagy and Regulating MAPK Signaling

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Key Words

Hepatic ischemia/reperfusion injury • MTORC2 • Rictor • Autophagy

Abstract

Background/Aims: The role of Rictor in hepatic ischemia/reperfusion (I/R) injury remains unknown. Here, we comprehensively examined the role of Rictor in hepatic I/R injury. **Methods:** We studied the expression of Rictor during hepatic I/R injury. The regulatory effects of Rictor on inflammatory responses, cytokine and chemokine release, apoptotic and anti-apoptotic responses, and autophagy induction during hepatic I/R injury were identified via the shRNA-mediated knockdown of Rictor. Subsequently, we collected the liver and blood samples of these mice to evaluate liver injury, mRNA and protein levels. Additionally, the signaling pathways induced by Rictor were investigated. Furthermore, the extent of activation of MAPKs in response to Rictor deficiency was investigated in lipopolysaccharide (LPS)-treated RAW264.7 cells. The mRNA expression levels were analyzed by real-time PCR, and the protein expression levels were analyzed using Western blot, immunofluorescence staining and enzyme-linked immunosorbent assay (ELISA). **Results:** The expression of Rictor was increased during hepatic I/R injury *in vivo* and hypoxia/reoxygenation (H/R) injury *in vitro*. Rictor deficiency enhanced the extent of liver injury by increasing macrophage and neutrophil infiltration, promoting cytokine and chemokine release, aggravating hepatocyte apoptosis, suppressing anti-apoptotic responses, and inhibiting autophagy induction during both hepatic I/R and H/R injury. Rictor was associated with the activation of hepatic I/R injury-induced MAPK signaling. In addition, Rictor deficiency affected MAPK activation in LPS-treated RAW264.7 cells. **Conclusion:** Rictor can substantially ameliorate I/R-induced liver injury. Therefore, our findings strongly suggest a therapeutic value of the Rictor/mTORC2 axis in hepatic I/R injury.

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Introduction

Hepatic ischemia/reperfusion (I/R) injury is a major concern after hepatic surgical treatments, including resection and transplantation, and comprises local ischemic insult and inflammation-derived reperfusion injury [1]. In patients undergoing liver transplantation, hepatic I/R has been found to be an unfavorable prognostic factor that significantly affects graft survival, resulting in hepatic failure, tumor recurrence, or organ rejection [2, 3]. Although a few mechanisms associated with the amelioration of I/R injury have been described, such as the generation of mitochondrial reactive oxygen species (ROS) by the ischemia-mediated accumulation of succinate, approaches to substantially reduce liver injury remain a major concern in the improvement of prognosis in patients undergoing liver transplantation [4].

Mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol-3-OH kinase-related kinase family that plays important regulatory roles in cellular metabolism, proliferation, growth, aging, survival, and protein synthesis [5, 6]. Previous studies have demonstrated that mTOR signals via two distinct protein complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [7]. It is well known that the regulatory protein of mTOR, mainly Raptor, has a significant role in various kidney diseases [8, 9]. Furthermore, mTORC1 signaling has been shown to affect fibrogenic processes by promoting fibroblast activation [10]. More recently, the activation of mTORC1 has been shown to enhance oxidative stress by increasing NADPH oxidase (Nox) expression and activity [11, 12].

In contrast to the functions of mTORC1, little is known regarding the functions of mTORC2. Rictor is the core component of mTORC2 and Rictor depletion has been demonstrated to disrupt mTORC2 activity [13, 14]. Previous studies have indicated the role of mTORC2 in cellular survival and actin cytoskeleton organization [15-17]. Moreover, Rictor/mTORC2 signaling has recently been shown to play a crucial role in ameliorating cisplatin-induced tubular epithelial cell death [14]. However, the role of Rictor in hepatic I/R remains unknown.

In the present study, we investigated the functions and mechanisms of Rictor after hepatic I/R injury both *in vivo* and *in vitro*. We observed an increase in the expression of Rictor after hepatic I/R injury. Furthermore, when Rictor was knocked down, the extent of liver injury was significantly increased.

Materials and Methods

Animals

Wild-type (WT) male C57BL/6 mice (8 to 10 weeks old) were purchased from Shanghai SLAC Co. Ltd. (Shanghai, China). All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University School of Medicine (approval no. SYKX-2008-0050).

Hepatic I/R injury

The model for hepatic I/R injury has been previously described in one of our publications [18]. An atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad lobes of the liver for 90 min. Sham controls underwent the same procedure but without vascular occlusion. The number of animals in sham and I/R group is 4-6.

In vivo knockdown of RICTOR

RICTOR-targeting shRNA plasmids (sc-61479-SH) and control shRNA plasmids (sc-108060) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). To inhibit RICTOR expression in the mouse liver, we used a hydrodynamics-based transfection method to deliver naked shRNAs to the mice as previously described [19-21]. In brief, after the mice were anesthetized with sevoflurane, shRNA plasmids (10 µg in 2 ml of Ringer's solution) were injected into the dorsal veins of the mouse penis within 5 s. Approximately 72 h after shRNA injection, the mice were subjected to hepatic I/R injury.

Biochemical measurements

Blood samples were collected by direct puncture of the arterial aorta and then centrifuged at 3,000 g for 5 min. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured by a standard clinical automatic analyzer (Dimension Xpand; Siemens Dade Behring, Munich, Germany). The levels of malonaldehyde (MDA), superoxide dismutase (SOD), total nitric oxide synthase (TNOS) and induced nitric oxide synthase (iNOS) in the liver tissues were measured using corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Cell viability was assessed using a Cell Counting Kit (Obio, Shanghai, China). Hepatocyte cytotoxicity was determined via a lactate dehydrogenase (LDH) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histopathology

Paraffin-embedded liver tissues were cut into 5- μ m-thick sections. The sections were stained with hematoxylin and eosin. Suzuki's criteria were employed to histologically evaluate the severity of liver injury [18]. In brief, sinusoidal congestion, hepatocyte necrosis and ballooning degeneration were blindly graded from 0 to 4.

Immunohistochemistry (IHC)

Liver sections were rehydrated and processed for antigen retrieval. The sections were incubated with primary antibodies against myeloperoxidase (MPO) (Cell Signaling Technology, Boston, MA) and cleaved caspase-3 (Cell Signaling Technology, Boston, MA), F4/80 (AbD Serotec, Kidlington, UK) and Rictor (Cell Signaling Technology) overnight at 4 °C, and signals were detected using horseradish peroxidase-conjugated secondary antibodies.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The TUNEL assay was performed on liver sections using an In-Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). For each stained section, ≥ 3 images were captured, and the data from at least three mice per group were analyzed. Image-Pro Plus 6.0 was used for image analyses.

Transmission electron microscopy (TEM) analysis

Liver tissues were fixed with 2.5% glutaraldehyde and cut into ultrathin sections that were then double stained with uranyl acetate and lead citrate. To quantify autophagic vacuoles, 10 micrographs (primary magnification $\times 15,000$) were randomly obtained for each sample, and the total number of autophagic vacuoles was quantified.

Isolation, culture, and treatment of hepatocytes and RAW264.7 cells

Primary hepatocyte isolation and culture were performed as previously described [19]. RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in 6-well plates (2×10^5 cells per well) with or without lipopolysaccharide (LPS; 100 ng/ml) treatment. For Western blot analysis, cells were harvested at 0 min, 15 min, 30 min and 60 min after LPS treatment. To measure the mRNA levels or secreted protein levels of inflammatory cytokines, cells were harvested at 0 h, 6 h and 12 h after LPS treatment. The isolated hepatocytes were plated on 6-cm dishes (3×10^6 cells per dish), 6-well plates (2×10^5 cells per well) or 24-well plates (8×10^4 cells per well).

To simulate hypoxia/reoxygenation (H/R) *in vitro*, hepatocytes were cultured for 4 h at 37 °C in a modular incubator chamber (Biospherix, Lacona, NY, USA) with 5% CO₂, 90% N₂ and 5% O₂ for 4 h. For reoxygenation, hepatocytes were returned to a normoxic incubator for 0 h or 2 h. 100 nM of Rapamycin (Sigma, St. Louis, MO) was administered 3 h before H/R injury. 10 μ M of SB203580 (TOCRIS, USA) was administered 1 h before H/R injury. To assess cell injury, LDH production was measured using the LDH Release Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Optical density (OD) was measured at 490 nm with a reference wavelength of 630 nm. The Obio Cell Counting Kit (Obio, Shanghai, China) was used to evaluate cellular viability according to the manufacturer's protocols, and OD was measured at 450 nm using a multi-mode microplate reader after incubating the cells for 2 h.

RNA interference in primary hepatocytes and RAW264.7 cells

siRNAs against the mouse RICTOR gene and scramble siRNAs were purchased from GenePharma (Shanghai). In brief, primary mouse hepatocytes maintained in medium without serum or antibiotics were plated in 24-well or 60-mm culture plates and incubated for 3 h at 37 °C with 5% CO₂ to enable cell adherence. Then, the cells were transfected with siRNA duplexes at a final concentration of 20 nM in Opti-MEM (Life Technologies, Grand Island, NY, USA) using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, USA) for 48 h, followed by H/R or LPS treatment.

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using 1,000 ng of total RNA via first-strand cDNA synthesis with a PrimeScript RT reagent Kit (Takara, Tokyo, Japan). RT-PCR was performed using the CFX 96 q-PCR system (Bio-Rad, California, USA). A SYBR Green RT-PCR Kit (Takara, Tokyo, Japan) was used for quantitative RT-PCR analyses. All reactions were performed in a 20 µl reaction volume in triplicate. The relative expression levels of target genes were normalized against the level of β-actin. The following primers were used for RT-PCR:

Il-6 forward: 5'- GCTACCAAACTGGATATAATCAGGA

Il-6 reverse: 5'- CCAGGTAGCTATGGTACTCCAGAA

Tnf forward: 5'- TTCTATGGCCAGACCCTCA

Tnf reverse: 5'- TTTGCTACGACGTGGGCTAC

Cxcl-10 forward: 5'- GCTGCCGTCATTTCTGC

Cxcl-10 reverse: 5'- TCTCACTGGCCCGTCATC

Cxcl-1 forward: 5'- TGGCTGGGATTACCTCAAGAACA

Cxcl-1 reverse: 5'- TTTCTGAACCAAGGGAGCTTCAGG

Il-1b forward: 5'- TGTAATGAAAGACGGCACACC

Il-1b reverse: 5'- TCTTCTTTGGGTATTGCTTGG

Il-10 forward: 5'- ACTGCACCCACTTCCCAGT

Il-10 reverse: 5'- TGTCCAGCTGGTCCTTTGTT

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels (TNF-α and IL-6) in mouse serum were measured by ELISA kits (NeoBioscience Technology, Shenzhen, China) according to the manufacturer's instructions.

Western blotting

Western blot analyses were performed as previously described [19]. After being blocked for non-specific binding, membranes were incubated with primary antibodies against Rictor, Raptor, mTOR, P-S6 kinase (S6K), S6K (Cell Signaling Technology), Bcl-xl (Abcam), Bcl-2 (Abcam), Atg-5 (Abcam), LC3BII (Novus Bio), P-AKT (Cell Signaling Technology), AKT (Cell Signaling Technology), P-ERK (Cell Signaling Technology), ERK (Cell Signaling Technology), P-JNK (Cell Signaling Technology), GAPDH (Abcam), and β-actin (Sigma-Aldrich) at 4 °C with gentle shaking overnight. Then, the membranes were developed with ECL Western Blot Substrate (GE Healthcare) and exposed to high-sensitivity films (GE Healthcare) for autoradiography.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by two-tailed, unpaired or paired Student's *t* tests. *P* values < 0.05 indicated statistically significant differences.

Results

Rictor deficiency aggravates liver injury

To identify the role of Rictor in hepatic I/R injury, we first analyzed the expression of Rictor protein in ischemic livers at different time points after reperfusion. As shown in Fig. 1A and B, the protein levels of Rictor substantially increased after 90 min of ischemia and 0 h, 2 h, and 4 h of reperfusion in mouse livers. Rictor expression was also enhanced after 0 h, 1 h, and 2 h of H/R injury in primary hepatocytes (Fig. 1C and D). Then, Rictor expression was

knocked down in cells using naked shRNAs, and hepatic I/R injury was induced as described in Fig. 1E. As shown in Fig. 1G and H, Rictor-shRNA/siRNA could sufficiently knock down Rictor protein levels, whereas other mTOR components, such as Raptor and mTOR, were not significantly affected (Fig. 1G and H). We further confirmed this by IHC for Rictor in liver sections after shRNA injection into mice (Fig. 1F). The extent of hepatic I/R injury was assessed by biochemical measurements and histopathological analyses. As shown in Fig. 1I, serum levels of both ALT and AST were significantly increased in mice injected with Rictor-shRNA. Consistent with the biochemical index, hepatic necrotic areas were also significantly increased in mice injected with Rictor-shRNA (Fig. 1J and K). Therefore, Rictor deficiency aggravated the detrimental effects of hepatic I/R injury.

Rictor deficiency increases macrophage/neutrophil infiltration and cytokine/chemokine release during hepatic I/R injury

Since inflammatory responses could be activated to promote hepatic I/R injury, we assessed the hepatic infiltration of inflammatory cells using MPO and F4/80 staining. As

Fig. 1. Rictor deficiency increases liver injury induced by IR. (A-B) Rictor protein levels in the livers of mice subjected to 90 min of ischemia and subsequent reperfusion for 0, 2, 4, 6h. β -actin served as a loading control. (n=3 per group). (C-D) Western blot analysis (left) and quantification (right) of Rictor expression in primary hepatocytes subjected to H/R injury. (n=2-4 replicates per experiment group). (E) Schematic presentation of the experimental procedures. (F) Representative sections from Rictor staining (original magnification, 400 $\times$) after treatment with Rictor shRNA for 72 h, n=3 per group. (G) Western blot indicating expression of Rictor, Raptor, mTOR protein levels after treatment with Rictor shRNA for 72 h. β -actin served as a loading control. n=4-6 per group. (H) Western blot indicating expression of Rictor protein after treatment with Rictor siRNA for 48 h. β -actin served as a loading control. n=3 replicates per experiment group. (I) Serum ALT and AST levels at 6 h post-reperfusion; mean $\pm$ SEM, n=4-6 per group. (J-K) Representative hematoxylin and eosin(HE) stained sections(original magnification, 100 $\times$) and relevant average Suzuki score at 6 h post-reperfusion or sham controls. (mean $\pm$ SEM, n=4-6 per group, ** P<0.01, *P<0.05).

Fig. 2. Rictor deficiency increases macrophage/neutrophil infiltration and cytokine/chemokine release during hepatic I/R injury. (A-B) Representative sections from MPO staining and F4/80 staining (C-D) at 6h after reperfusion(original magnification, 400 $\times$) and the numbers of MPO and F4/80 positive cells that infiltrated the livers were determined. n=4-6 per group. (E) mRNA levels of cytokines and chemokines were determined by quantitative real-time PCR, and (F) Serum cytokine levels measured by ELISA. n=4-6 per group. All data shown as the mean $\pm$ SEM, **P<0.01, *P<0.05.

shown in Fig. 2A-D, the number of MPO- and F4/80-positive cells was significantly increased after I/R injury. The number of MPO- and F4/80-positive cells was even higher in mice injected with Rictor-shRNA (Fig. 2A-D). In line with the reduced hepatic infiltration of innate immune cells, the mRNA levels of IL-6, TNF- α , CXCL-10 and CXCL-1 significantly increased in the Rictor-shRNA group after I/R injury (Fig. 2E). Serum levels of TNF- α and IL-6 were also significantly increased after I/R injury in mice injected with Rictor-shRNA (Fig. 2F). Therefore, Rictor deficiency enhanced inflammatory responses following hepatic I/R injury.

Rictor deficiency aggravates apoptosis and inhibits anti-apoptotic responses during hepatic I/R injury

Apoptosis is an important mechanism of cell death following I/R injury in the liver [22]. We performed the TUNEL assay and Western blotting to detect the expression of anti-apoptotic proteins in ischemic livers to further identify the effects of Rictor on hepatocellular apoptosis induced by I/R. As shown in Fig. 3A and B, the number of TUNEL-positive

Fig. 3. Rictor deficiency aggravates apoptosis and inhibits antiapoptotic function in hepatic I/R injury. (A–B) Representative sections of TUNEL-staining at 6 h after reperfusion. TUNEL-positive hepatocytes were expressed as a percentage of the total hepatocytes. (n=4–6 per group). (C–D) Representative sections from cleaved caspase-3 staining (original magnification, 200×), the cleaved caspase-3 positive area that infiltrated the livers were determined. (n=4–6 per group). (E–F) Western blot analysis of Bcl-xl and cleaved caspase-3 in the livers of Vehicle and Rictor shRNA pretreatment mice at 6 h after I/R or a sham operation. β -actin served as a loading control. (n=4 per group). (G–H) Effects of the Rictor deficiency on the levels of SOD, MDA, TNOS and iNOS in mice livers at 6 h after reperfusion. (n=4–6 per group). All data shown as the mean $\pm$ SEM, ** P <0.01, * P <0.05.

hepatocytes was significantly increased after I/R injury in mice injected with Rictor-shRNA. Furthermore, the expression of cleaved caspase-3 was significantly higher (Fig. 3C and D), whereas the expression of Bcl-xl was significantly lower (n = 4 to 6 per group; Fig. 3E and F) in the Rictor-shRNA group than in the vehicle group. Given that elevated ROS production can lead to energetic and metabolic imbalances affecting cell survival, we speculated that Rictor deficiency affects oxidative stress during hepatic I/R injury. Moreover, Rictor deficiency was significantly associated with increased levels of MDA, TNOS, and iNOS in mouse livers at 6 h after reperfusion (Fig. 3G). However, the level of SOD was significantly decreased.

Rictor deficiency aggravates apoptosis and inhibits anti-apoptotic responses during H/R injury

To further confirm the role of Rictor in hepatocyte apoptosis, we isolated primary hepatocytes from WT mice, pretreated with or without Rictor-siRNA *in vitro* and subjected to H/R to mimic *in vivo* I/R injury. As shown in Fig. 1H, Rictor-siRNA could efficiently knock down Rictor expression. Cellular viability and damage in response to H/R were determined by CCK-8 and LDH assays, respectively. LDH is released by cells in response to various injuries. The average cytotoxicity and extent of cellular injury were significantly increased (Fig. 4A), whereas cellular viability was significantly decreased in primary hepatocytes infected with Rictor-siRNA (Fig. 4B). As shown in Fig. 4C–F, the number of TUNEL- and cleaved caspase-3-positive hepatocytes was increased at 4 h after H/R injury following infection with Rictor-siRNA. Additionally, compared to that in control siRNA-infected hepatocytes, the expression of BCL-xl was significantly inhibited after H/R injury in Rictor-siRNA-infected hepatocytes (Fig. 4G and H). Consistent with the results of the TUNEL assay, Western blotting showed that the expression of cleaved caspase-3 was increased after H/R injury in hepatocytes infected with Rictor-siRNA (Fig. 4G and H). Therefore, Rictor deficiency enhanced I/R- and H/R-induced apoptosis.

Rictor deficiency reduced the induction of autophagy during hepatic I/R injury

Our previous study demonstrated that the induction of autophagy and activation of mTORC2 by rapamycin could play an important protective role in hepatic I/R injury [18]. Therefore, we assessed whether Rictor affects the induction of autophagy during I/R injury. As shown in Fig. 5A-D, Western blot analyses revealed that, in accordance with the increased protein levels of Rictor, P-Akt (Ser473) was also upregulated by I/R or H/R injury, whereas Rictor deficiency decreased the abundance of P-AKT. The increased levels of ribosomal p70 S6K in the liver showed that the mTORC1 signaling pathway could also be activated by I/R injury. Rictor deficiency increased the protein levels of P-S6K both *in vivo* and *in vitro*. Furthermore, during both hepatic I/R and H/R injury, Rictor deficiency was found to be associated with significantly decreased levels of Atg-5 and LC3BII, which are standard indicators of autophagic activity. Correspondingly, transmission electron micrographs also showed that at 6 h after reperfusion, the number of autophagosomes in the ischemic lobes was significantly decreased in the groups pretreated with Rictor-shRNA before I/R injury (Fig. 5E). To validate whether the enhancement of autophagy could ameliorate the detrimental effects of Rictor deficiency, we employed rapamycin to induce autophagy before the induction of H/R injury in the Rictor-siRNA pretreatment group. As shown in Fig. 5F, rapamycin could significantly enhance autophagy, as shown by the markedly increased protein levels of LC3B. Furthermore, Western blot analyses showed that the expression pattern of P-S6K and P-AKT was reversed by rapamycin. Accordingly, the enhancement of autophagy ameliorated the extent of cell injury, represented by reduced LDH levels (Fig. 5G). Therefore, Rictor deficiency negatively regulated autophagy during hepatic I/R injury, and enhancement of autophagy could selectively ameliorate these detrimental effects.

Fig. 4. Rictor deficiency aggravates apoptosis and inhibits antiapoptotic function in hepatic H/R injury. (A-B) Cell death of isolated primary hepatocytes and the cell viability after H/R injury were measured by LDH assays and CCK-8. Average cytotoxicity (% cell death) in relevant groups were plotted. 3-5 replicates per experiment group performed on different days. (C-D) Representative sections of TUNEL-staining after H/R stimuli. TUNEL-positive hepatocytes were determined. (original magnification, 200×, n=3-5 replicates per experiment group performed on different days, mean ± SEM, **P<0.01, *P<0.05). (E-F) Representative sections from cleaved caspase-3 staining (original magnification, 200×), the cleaved caspase-3 positive area that infiltrated were determined. (n=3-5 replicates per experiment group performed on different days, mean ± SEM, **P<0.01, *P<0.05). (G-H) Bcl-xl, cleaved caspase-3 and Rictor protein expression were analyzed by Western blotting in isolated hepatocytes from WT with or without Rictor siRNA pretreatment subjected to H/R for 4 h. (n=3-5 replicates per experiment group performed on different days, mean ± SEM, **P<0.01, *P<0.05).

Rictor deficiency mediates I/R-induced activation of MAPK signaling

Since the mitogen-activated protein kinase (MAPK) pathway plays important roles in cellular apoptosis and immune responses in response to various injuries [23, 24], we next assessed the activation of MAPKs in our model. As shown in Fig. 6A, the protein levels of phosphorylated Erk and Jnk were increased during H/R injury, which was in line with the expression of Rictor. The protein levels of Rictor decreased in response to Rictor-shRNA pretreatment before I/R injury or H/R injury (Fig. 6B and C). Western blot analyses showed that the MAPK pathway was activated by I/R injury, as the levels of P-ERK and P-JNK were increased. In addition, the expression of P-ERK and P-JNK was significantly enhanced in response to Rictor-shRNA pretreatment before I/R injury or H/R injury (Fig. 6D and E). To validate whether the inhibition of MAPK could ameliorate the detrimental effects of Rictor deficiency, we adopted SB203580 to inhibit MAPK pathway before H/R injury in Rictor siRNA pretreatment group. As is shown in Fig. 6F, MAPK inhibition resulted in lessened extent of cell injury, which was presented by decreased LDH levels. Therefore, Rictor likely regulates the MAPK pathway.

Rictor deficiency affects the activation of MAPKs in LPS-treated RAW264.7 cells

To further determine the role of Rictor in regulating MAPKs, we analyzed the effect of Rictor on LPS-stimulated RAW264.7 cells. Western blotting revealed that the expression of P-JNK and P-ERK was enhanced in cells treated

Fig. 5. Rictor deficiency lessens liver Autophagy induction in hepatic I/R injury. (A–B) Western blot analysis of Rictor, P-S6K, S6K, P-AKT, AKT, LC3B and ATG5 protein expression in the Vehicle and Rictor shRNA pretreatment group at 6 h after reperfusion. β -actin served as a loading control. Corresponding densitometric analysis of Rictor, P-S6K, P-AKT, LC3B-II and ATG5 expression. $n = 4$ –6 per group. (C–D) Western blot analysis of Rictor, P-S6K, S6K, P-AKT, AKT, LC3B and ATG5 protein expression in the Vehicle and Rictor siRNA pretreatment primary hepatocytes isolated from WT which subjected to H/R injury. β -actin served as a loading control. Corresponding densitometric analysis of Rictor, P-S6K, P-AKT, LC3B-II and ATG5 expression. $n = 3$ –5 replicates per experiment group performed on different days. (E) Representative transmission electron micrographs showing autophagosomes in the ischemic lobes at 6 h of reperfusion. Autophagosomes are indicated by arrows. Scale bars 1 μ m. The numbers of autophagosomes were determined. $n = 4$ –6 per group. (F) Immunoblots indicating expression of P-AKT, AKT, P-S6K, S6K and LC3B protein after treatment with Rictor siRNA in the presence and absence of rapamycin (100 nM) subjected to H/R injury and densitometric analysis of P-AKT, P-S6K, LC3B-II expression. (G) Quantification of average cytotoxicity (% cell death) in different groups were plotted. $n = 3$ –5 replicates per experiment group performed on different days. The data are shown as the mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

Fig. 6. Rictor deficiency mediates I/R-induced activation of MAPK signaling. (A) Western blot indicating expression of Akt, Erk and Jnk phosphorylation protein levels in primary hepatocytes subjected to H/R injury. GAPDH served as a loading control. n=2-4 replicates per experiment group. (B-C) Western blot analysis of Rictor, P-JNK, JNK, P-ERK, ERK in the Vehicle and Rictor shRNA pretreatment group at 6 h after reperfusion. GAPDH served as a loading control. Corresponding densitometric analysis of Rictor, p-JNK and p-ERK expression. n =4-6 per group. (D-E) Western blot analysis of Rictor, P-JNK, JNK, P-ERK, ERK in the Vehicle and Rictor siRNA pretreatment group after H/R stimuli. GAPDH served as a loading control. Corresponding densitometric analysis of Rictor, p-JNK and p-ERK expression. (F) Quantification of average cytotoxicity (% cell death) in different groups were plotted. n=3-5 replicates per experiment group. The data are shown as the mean $\pm$ SEM. **P<0.01, *P<0.05.

Fig. 7. Rictor deficiency affects MAPKs activation on LPS treatment RAW264.7 cell line. (A) Western blot analysis of Rictor, P-JNK, JNK, P-ERK and ERK in control and Rictor siRNA pretreatment of RAW264.7 at the indicated time points, upon LPS stimuli. (B) Supernatant cytokine levels of IL-6 and TNF- α after in LPS-stimulated at the indicated time points, measured by ELISA. (C) mRNA levels of cytokines and chemokines such as IL-6, TNF- α , IL-1 β and IL-10 were determined by quantitative real-time PCR. n=3-5 replicates per experiment group performed on different days. All data shown as the mean $\pm$ SEM, **P<0.01, *P<0.05.

with 100 ng/ml of LPS (Fig. 7A). The levels of IL-6 and TNF- α in the supernatant were significantly increased in Rictor-knockdown cells at both 6 h and 12 h post-treatment with LPS (Fig. 7B). Compared with the corresponding levels in negative control cells, the mRNA levels of cytokines and chemokines, including IL-6, TNF- α , and IL-1 β , were significantly increased, whereas the mRNA level of IL-10 was non-significantly decreased ($P = 0.058$) in Rictor-knockdown cells (Fig. 7C).

Discussion

In the present study, the expression of Rictor was found to be significantly increased in response to hepatic I/R injury. Additionally, Rictor deficiency was demonstrated to be associated with increased neutrophil and macrophage infiltration, enhanced production of cytokines and ROS, aggravated apoptosis, and suppressed anti-apoptotic responses. Mechanistically, the suppression of autophagy and the activation of MAPKs via the inhibition of mTORC2 activity may have contributed to these detrimental effects. Therefore, to the best of our knowledge, this study is the first to propose Rictor as a protective factor ameliorating the extent of hepatic I/R injury.

Autophagy generally exerts cytoprotective effects against multiple cellular stresses and is mediated by several responses, including the degradation of long-lived cytosolic and damaged proteins, the clearance of ROS, and the modulation of cell death [25, 26]. Previous studies have demonstrated that autophagy plays essential roles during hepatic I/R injury [27, 28]. Recently, Li et al. [29] demonstrated that high expression of microRNA-17 could upregulate autophagy to aggravate hepatic I/R injury by suppressing signal transducer and activator of transcription-3 (STAT-3) and that low expression of microRNA-17 was associated with increased levels of STAT-3 and reduced autophagy. In contrast, a study from Korea demonstrated that the everolimus-induced activation of autophagy could significantly protect against hepatic I/R injury and suggested that autophagy activation could be a potential therapeutic strategy for treating hepatic I/R injury [30]. Our previous study also showed that pretreatment with rapamycin partially alleviated hepatic I/R injury through the induction of autophagy and promotion of mTORC2 activity and that the inhibition of rapamycin-induced autophagy by chloroquine worsened liver injury [18]. In the previous study, to identify to key role of mTORC2 pathway, we adopted mTORC1 and 2 inhibitor Torin1, and found that though Torin1 pretreatment could also induce autophagy during I/R injury, but it failed to protect livers from I/R injury. Although rapamycin could directly target mTORC1, it also effects mTORC2 assembly and activities as well when used at high concentrations or for long term [31]. Therefore, we believe the mTORC2 pathway plays essential role in hepatic I/R injury. What's more, recently, Li et al. [14] showed that the inhibition of mTORC2 via Rictor knockdown increased cisplatin-induced injury by suppressing autophagic induction. In accordance with previous studies, we also found that the deficiency of Rictor could reduce autophagy induction during I/R injury, as the expression of Atg-5 and LC3BII and the number of autophagosomes were significantly decreased in cells pretreated with Rictor-shRNA. In addition, the induction of autophagy and activation of mTORC2 pathway via rapamycin pretreatment could alleviate cellular injury induced by H/R in the presence of Rictor deficiency, thereby supporting the protective effect of autophagy and mTORC2 against cell death. Our results indicate that the Rictor deficiency-mediated inhibition of autophagy is associated with increased I/R- and H/R-induced apoptosis and excessive ROS production. Thus, the Rictor/mTORC2 axis could be a potential therapeutic target in hepatic I/R injury.

Sub-acute inflammatory responses have been demonstrated to lead to the progression of liver damage during hepatic I/R injury through the release of proinflammatory cytokines and accumulation of neutrophils, which secrete oxidants and proteases [32]. However, approaches to reduce inflammation in hepatic I/R injury are still lacking. In the present study, we observed an increase in the number of MPO-positive and F4/80-positive cells in mice pretreated with Rictor-shRNA prior to hepatic I/R injury, indicating that the activation

or enhancement of Rictor expression may be a potential therapeutic strategy to substantially reduce inflammation in hepatic I/R injury. Previous studies have demonstrated that MAPK signaling is significantly associated with inflammatory responses and the functional and structural protection of the liver during hepatic I/R injury [23, 33]. In the present study, the levels of P-ERK and P-JNK were significantly increased in mice pretreated with Rictor-shRNA prior to the induction of hepatic I/R injury. SB203580 has been testified to efficiently inhibit the MAPKs pathway, specifically inhibit p38. In our study, MAPKs pathway inhibition by SB203580 resulted in lessened extent of cell H/R injury in Rictor siRNA pretreatment group. Furthermore, the results from LPS-stimulated RAW264.7 cells also confirmed that Rictor deficiency was associated with an increased expression of P-JNK and P-ERK and elevated release of cytokines, indicating that Rictor plays an important role in suppressing MAPKs to ameliorate liver damage induced by I/R injury. Therefore, we propose here that Rictor protects against hepatic I/R injury by regulating MAPK signaling.

Although our results suggest that the deficiency of Rictor, a core component of mTORC2, significantly aggravates hepatic I/R injury, the following limitations of the study remain to be addressed: First, this is an animal model- and hepatocyte-based study, and our results are thus not translatable to the clinic before comprehensive verification in clinical trials. Second, because of the animal-based nature of this investigation and limited resources, the number of samples was relatively small. Thus, our results need to be verified in larger sample sizes. What's more, to better identify the role of Rictor/mTORC2 in the immune cells during I/R injury, results of knocking down Rictor in primary macrophage or other cell types in the circulation or liver are needed. The more neutral further research will be needed to address these questions. Despite the above limitations, our study is the first to identify the protective effect of Rictor against hepatic I/R injury.

Conclusion

Rictor deficiency increases macrophage and neutrophil infiltration, promotes cytokine and chemokine release, inhibits anti-apoptotic responses, and reduces autophagy induction and MAPK pathway activation during hepatic I/R injury in mice. Therefore, our findings strongly suggest a therapeutic value of the Rictor/mTORC2 axis in hepatic I/R injury.

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Disclosure Statement

The authors have declared that no competing interest exists.

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