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CCAAT/Enhancer-binding Protein Homologous Protein Promotes ROS-mediated Liver Ischemia and Reperfusion Injury by Inhibiting Mitophagy in Hepatocytes

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Background. Liver ischemia and reperfusion (IR) injury represent a major risk factor in both partial hepatectomy and liver transplantation. CCAAT/enhancer-binding protein homologous protein (CHOP) is a key regulator of cell death, its precise molecular basis in regulating hepatocyte death during liver IR has not been delineated. **Methods.** Hepatocellular *CHOP* deficient mice were generated by bone marrow chimera models using global *CHOP* knockout mice. Liver partial warm ischemia model and hypoxia/reoxygenation model of primary hepatocytes were applied. Liver injury and mitophagy-related signaling pathways were investigated. IR-stressed patient liver tissues and serum samples were analyzed as well. **Results.** Mice with hepatocellular *CHOP* deficiency exhibited alleviated cell death, decreased reactive oxygen species (ROS) expression, and enhanced mitophagy in hepatocytes after IR, confirmed by in vitro studies of hepatocytes after hypoxia/reoxygenation. Mitochondria ROS scavenge by Mito TEMPO effectively attenuated hepatocyte death and liver IR injury of wild-type mice, whereas no significant effects were observed in hepatocellular *CHOP*-deficient mice. *CHOP* depletion upregulated dynamin-related protein 1 and Beclin-1 activation in the mitochondria of hepatocytes leading to enhanced mitophagy. Following IR, increased CHOP expression and impaired mitophagy activation were observed in the livers of patients undergoing hepatectomy. N-acetyl cysteine pretreatment significantly improved the liver function of patients after surgery. **Conclusions.** IR-induced CHOP activation exacerbates ROS-mediated hepatocyte death by inhibiting dynamin-related protein 1–Beclin-1–dependent mitophagy.

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INTRODUCTION

Liver ischemia and reperfusion (IR) injury, associated with various liver surgeries including partial hepatectomy and liver transplantation, significantly impairs postoperative liver function and patient recovery. The initial hepatocellular injury caused by oxygen and adenosine triphosphate (ATP) depletion during ischemia as well as the subsequent intrahepatic innate immune inflammatory response is the major cause of liver IR injury. However, the molecular mechanisms underlying hepatic IR injury remain unclear.

The endoplasmic reticulum (ER) plays a pivotal role in the synthesis, folding, trafficking, and maturation of proteins. Under conditions of stress, cells activate adaptive responses, including the unfolded protein response (UPR), to restore normal cellular function.[1] However, in the case of unrelieved or sustained ER stress, UPR eventually leads

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H.Z. and S.Z. were involved in study design, conducted the experiments, and drafted the article; S.Z., Z.R., Q.W., and Z.L. performed the experiments; H.Z. and F.C. provided material support; P.W., Y.X., and F.C. commented on the article; H.Z. and S.Z. designed the study, supervised the study, and wrote the article.

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to apoptosis, which involves caspase activation, calcium leakage, and mitochondrial damage.[2] The liver being a major site of protein synthesis is rich in ER, which, under stress, has been reported to play an important role in hepatocyte apoptosis triggered by various liver injuries.[3] Although apoptosis as well as necrosis are known to occur following liver IR, recent evidence suggests that apoptosis is the primary mechanism of hepatocyte death.[4]

CCAAT/enhancer-binding protein homologous protein (CHOP), a transcriptional repressor that is activated downstream of the protein kinase R-like endoplasmic reticulum kinase and inositol requiring enzyme-1 pathways of the UPR, is one of the major elements in the ER stress–mediated apoptosis program. Increasing evidence has demonstrated that CHOP is involved in diverse types of liver injuries.[5,6] Upon stimulation, it translocates into the nucleus, leading to anti-apoptotic gene inhibition and proapoptotic gene activation.[7,8] *CHOP* deficiency protects mice from various liver-specific challenges, such as diet-induced steatohepatitis or bile duct ligation.[9-11]

Previous studies have revealed the crosstalk between autophagy, ER stress, and cell death.[12-14] Autophagy inhibition leads to the accumulation of damaged mitochondria and reactive oxygen species (ROS), resulting in apoptosis.[15] It has been demonstrated that ER stress-dependent autophagy in hepatocytes plays an important role in liver IR injury.[16] Mitophagy, the selective removal of damaged mitochondria using autophagic machinery, is essential for maintaining mitochondrial quality and homeostasis.[17] Emerging evidence has demonstrated the critical role of mitophagy in the regulation of liver IR injury. Mitochondrial DNA from IR-stressed hepatocytes contributes to liver inflammation and injury by activating stimulator of interferon genes signaling in aged macrophages.[18] Mesenchymal stem cells ameliorate hepatocellular apoptosis during liver IR injury by promoting mitophagy activation.[19] Although attenuation of liver IR injury in *CHOP*-deficient mice has been reported,[20] the role as well as underlying mechanism of CHOP signaling in the regulation of hepatocellular mitophagy remains unclear.

In this study, we established bone marrow chimeras using wild-type (WT) and *CHOP* knockout (KO) mice to investigate the contribution of CHOP to liver IR injury in parenchyma cells by using a liver partial warm IR model as well as in vitro hypoxia/reoxygenation (H/R) model of primary hepatocytes.

MATERIALS AND METHODS

Animals

Male C57BL/6 (WT) and *CHOP* KO (C57BL/6 background) mice were purchased from Gempharmatech Co, Ltd (China). *CHOP* KO mice were generated by CRISPR/Cas9 technology to modify *Ddit3* gene. All mice were housed in constant environmental conditions under a standard rodent diet and water. All animals received humane care and all animals' procedures met the relevant legal and ethical requirements according to a protocol (number NMU08-092) approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Chimeric Mice Generation

The chimeric mice were generated as previously described.[21] The details are provided in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Liver IR Injury Model

A mouse model of segmental (70%) warm hepatic IR model was performed as previously described.[22] The details were provided in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Laboratory Methods

Details on serum biochemical analysis, histopathology, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining, ATP production assay, Western blot, Beclin-1 knockdown, ROS detection, Caspase-3 activity assay, transmission electron microscopy (TEM), immunofluorescence staining, and myeloperoxidase (MPO) detection can be found in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Primary Human and Murine Hepatocytes Isolation

Primary human hepatocytes were isolated and cultured according to previously described methods.[23] Primary murine hepatocytes were isolated from 8-wk-old WT and *CHOP* KO mice using a 2-stage collagenase perfusion method as described.[24] The details were provided in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Cell Treatment

H/R treatment was performed as previously described.[25] The details were provided in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Extraction of Mitochondrial Fraction

Mitochondrial proteins of the liver tissues were extracted using the Mitochondria Extraction Kit for Tissues (Beyotime, Shanghai, China) according to the manufacturer's instructions. The details were provided in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Patients' Study and Ethics Approval

This study was approved by the Research Ethics Committee of Animal and Human Experimental Action of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China (Institutional Review Board approval number 2020-SR-511). Signed consent forms were obtained from all patients, and all the experiments adhered to the tenets of the Helsinki Declaration. Details of grouping and sample collection can be found in Supplemental Materials and Methods (**SDC,** http://links.lww.com/TP/C504).

Statistical Analysis

Data are shown as the mean ± SD and are representative of at least 3 independent experiments from triplicate assays. Statistical analysis was carried out using Student's test between 2 groups and one-way analysis of variance followed by Bonferroni's post hoc test was used among

multiple groups. All analyses were performed using Stata software (version 11.0). $P > 0.05$ (2-tailed) were considered statistically significant.

RESULTS

CHOP Deficiency Alleviates Liver IR Injury in Hepatocytes

The liver consists of various types of cells; hepatocytes, endothelial cells, Kupffer cells, and neutrophils. We first measured the expression of CHOP in the IR-stressed livers of WT mice using double immunofluorescence staining. CHOP was significantly upregulated in hepatocytes that were labeled with albumin (Figure 1A). Then we created bone marrow chimeras as described in the Materials and Methods section. Chimeric properties were confirmed by Western blot analysis of CHOP expression in hepatocytes as well as intrahepatic macrophages isolated from reconstituted mice (Figure 1B). The chimeric mice were then subjected to partial warm liver IR. As compared with WT→WT mice, WT→KO mice exhibited attenuated IR-induced liver injury, as evidenced by improved preservation of liver architecture (Figure 1C) with reduced Suzuki's scores (Figure 1D) and decreased serum levels of alanine aminotransferase (ALT) (Figure 1E). *CHOP* deficiency also significantly inhibited hepatocellular apoptosis in IR-stressed WT→KO livers, as manifested by reduced TUNEL-positive cells (Figures 1F and G) and decreased Caspase-3 activity (Figure 1H). These results indicate that CHOP signaling is involved in IR-induced liver injury and hepatocellular apoptosis.

CHOP Deficiency Attenuates Liver IR Injury by Reducing ROS Production

ROS and oxidative stress are the most common mechanisms underlying liver IR injury.[26] Using dihydroethidium (DHE) staining and ROS assays, we found that IR-induced ROS production was significantly reduced in WT→KO livers as compared with WT→WT livers (**Figures S1A–1C, SDC,** http://links.lww.com/TP/C504). Since mitochondria are a major source of ROS during IR injury,[27] the mitochondria-targeted antioxidant, Mito TEMPO (MT), was used to scavenge mitochondrial ROS (mtROS) in vivo. MT treatment markedly attenuated liver IR injury in WT→WT mice, as evidenced by improved histological architecture, lowered Suzuki's scores, and reduced serum ALT levels (Figures 2A–C). IR-induced hepatocellular apoptosis in WT→WT mice, as evaluated by TUNEL staining and Caspase-3 activity, was also inhibited by MT treatment (Figures 2D–F), whereas no notable effects of MT treatment were observed in IR-induced WT→KO mice. These results suggest that CHOP activation in IR-stressed livers increases ROS production, leading to aggravated liver injury.

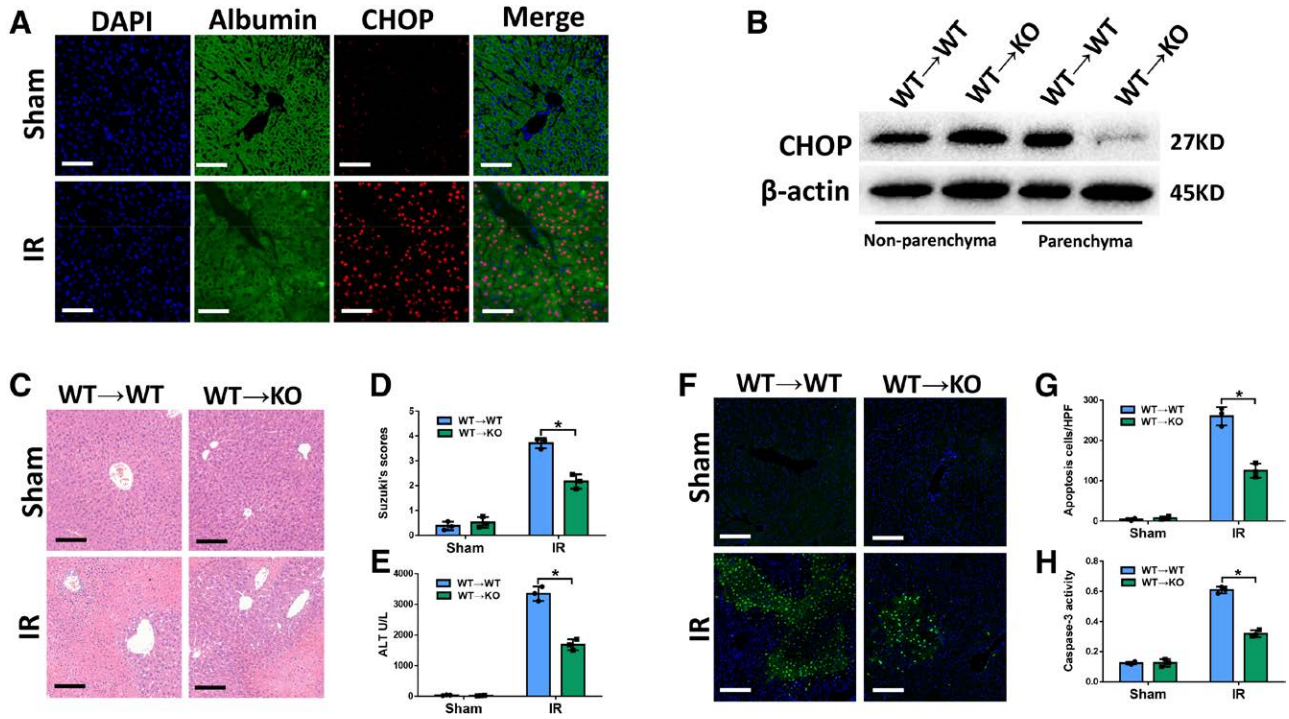

FIGURE 1. *CHOP* deficiency alleviates liver IR injury in hepatocytes. WT mice were subjected to 90 min of partial warm liver ischemia, followed by 6 h of reperfusion. (A) Immunofluorescence staining of albumin (green), CHOP (red), and DAPI (blue) in Sham and IR livers. Chimeric mice were established as described in the part of Materials and Methods (scale = 100 μm). (B) Western blot analysis of CHOP and β-actin expression in nonparenchymal cells and parenchymal cells of the 2 groups. WT→WT and WT→KO mice were subjected to 90 min of partial warm liver ischemia, followed by 6 h of reperfusion. (C) Representative histological staining (H&E) of ischemic liver tissue (scale = 100 μm). (D) Liver damage was evaluated by Suzuki's histological score. (E) Liver function was evaluated by ALT serum levels. (F) Immunofluorescence staining of TUNEL+ cells in ischemic livers (scale = 100 μm). (G) Quantification of TUNEL+ cells per high power field. (H) Liver Caspase-3 activity. n = 6 mice/group. All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; IR, ischemia and reperfusion; KO, knockout; ROS, reactive oxygen species; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

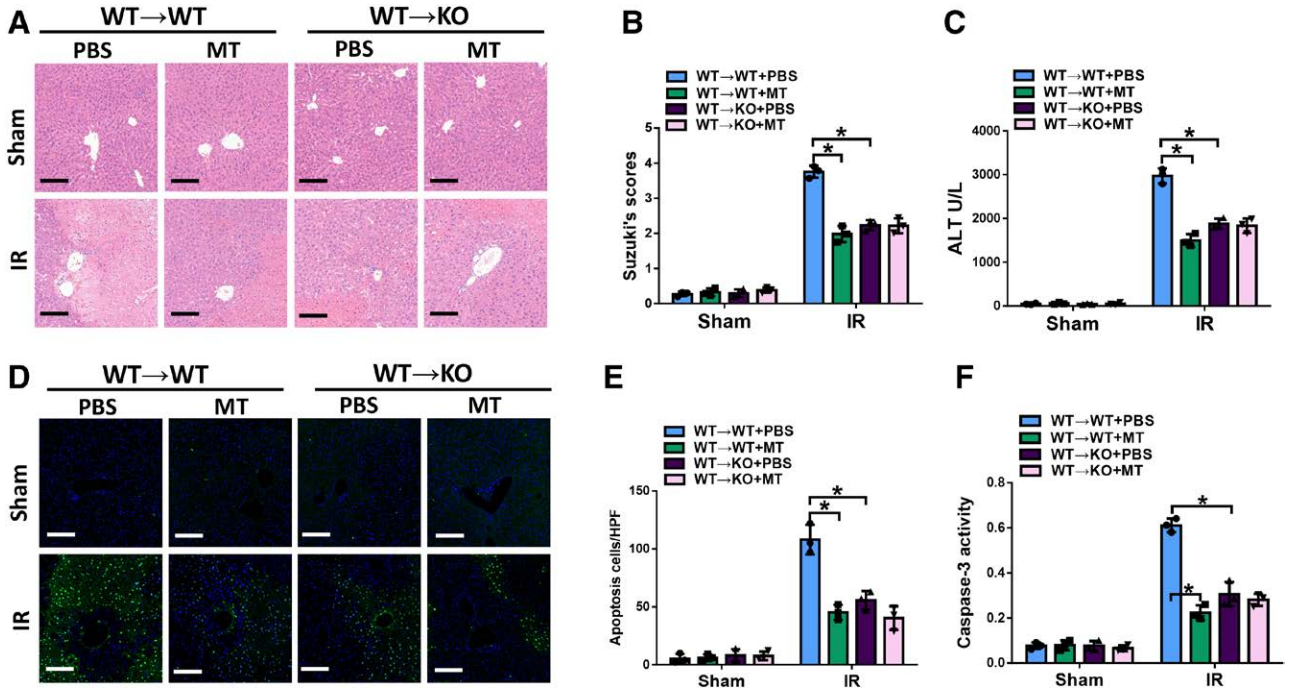

FIGURE 2. *CHOP* deficiency attenuates liver IR injury by reducing ROS production. WT→WT and WT→KO mice were pretreated with Mito TEMPO (MT, 3 mg/kg) or PBS 3 h before liver IR onset. Samples were procured 6 h after reperfusion. (A) Representative histological staining (H&E) of ischemic livers (scale = 100 μm). (B) Liver damage was evaluated by Suzuki's histological score. (C) Liver function was evaluated by serum ALT levels. (D) Immunofluorescence staining of TUNEL+ cells in ischemic livers (scale = 100 μm). (E) Quantification of TUNEL+ cells per high power field. (F) Liver Caspase-3 activity. n = 6 mice/group. All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; H&E, hematoxylin and eosin; IR, ischemia and reperfusion; KO, knockout; PBS, phosphate-buffered saline; ROS, reactive oxygen species; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

CHOP Deficiency Reduces Hepatocellular ROS Production and Prevents Liver IR Injury in a Mitophagy-dependent Manner

The removal of dysfunctional mitochondria by mitophagy has been suggested to have a protective effect in various models of organ and cell injury.[28-30] To determine whether CHOP is involved in regulating mitophagy, we evaluated mitochondrial autophagic flux in the livers of IR-induced WT→KO and WT→WT mice. The results indicated that autophagic flux was significantly enhanced by *CHOP* KO, as indicated by increased expression of the autophagosomal marker, light chain 3B-II (LC3B-II), but decreased expression of a receptor of autophagy, p62, in the mitochondria of IR-induced WT→KO livers, as compared with the WT→WT controls (Figure 3A). We also observed mitophagy directly, using TEM, the results demonstrated that post-IR, the number of isolation membranes as well as autophagosomes was significantly higher in the livers of IR-stressed WT→KO as compared with that in WT→WT, suggesting that CHOP deficiency enhanced hepatocellular mitophagy in response to IR (Figures 3B and C). Furthermore, mitochondrial quality was significantly improved in IR-induced WT→KO livers, as evidenced by increased ATP production and enhanced activities of mitochondrial complexes I, II+III, and IV (**Figure S2,** SDC, http://links.lww.com/TP/C504). Functionally, autophagic inhibitor 3-methyladenine (3-MA) treatment significantly exacerbated liver IR injury as well as hepatocellular apoptosis in WT→KO mice, as manifested by worsened liver architecture, higher serum ALT levels, increased number of TUNEL-positive cells, and higher Caspase-3 activity (Figures 3D–I). Additionally, 3-MA treatment augmented ROS production in IR-stressed WT→KO livers (**Figures S3A–3C, SDC,** http://links.lww.com/TP/C504), whereas MT treatment significantly reversed the detrimental role of 3-MA (**Figures S3 and S4, SDC,** http://links.lww.com/TP/C504). These results demonstrated that *CHOP* deficiency protected against liver IR injury by inhibiting hepatocellular ROS production in a mitophagy-dependent manner.

CHOP Deficiency Promotes Hepatocellular Mitophagy in Response to H/R In Vitro via Dynamin-related Protein 1–Beclin-1 Signaling

To further verify the in vivo findings, primary hepatocytes were isolated from WT as well as *CHOP* KO mice and subjected to H/R in vitro. First, we measured the levels of mitophagy in both H/R-induced WT and KO hepatocytes. Consistent with the in vivo results, *CHOP* deficiency markedly activated mitophagy in the mitochondrial fractions, as indicated by the increased and decreased expression of the autophagosomal marker, LC3B-II and p62, respectively (Figure 4A). The increased colocalization of mitochondria and lysosomes in H/R-stressed *CHOP* KO hepatocytes, as examined by immunofluorescence double-staining of the lysosomal marker, LAMP1 as well as pyruvate dehydrogenase (PDH), further confirmed that *CHOP* deficiency promoted mitophagy activation (Figure 4B).

Thereafter, we sought to explore the potential mechanisms underlying *CHOP* deficiency–mediated hepatocellular mitophagy in response to H/R. Emerging evidence has demonstrated that the phosphorylation of dynamin-related

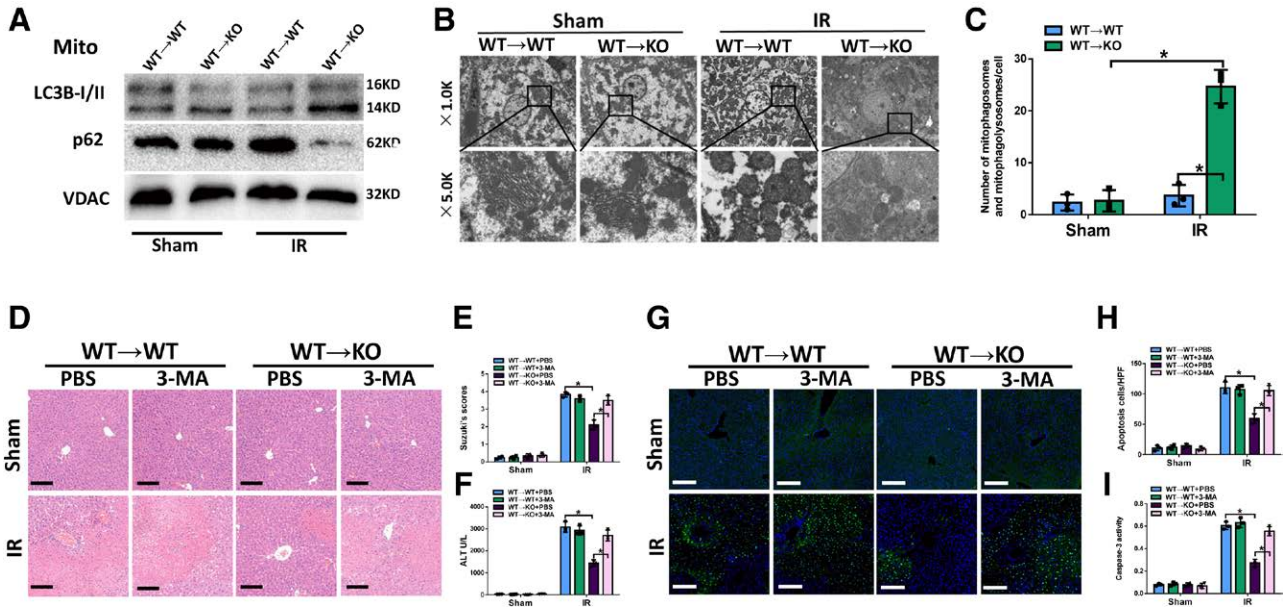

FIGURE 3. *CHOP* deficiency reduces hepatocellular ROS production and prevents liver IR injury in a mitophagy-dependent manner. WT→WT and WT→KO mice were subjected to 90 min of partial warm liver ischemia, followed by 6 h of reperfusion. (A) Western blot analysis of LC3B-I/II, p62, and VDAC in mitochondrial fraction of the livers. (B) Autophagosomes in the mice livers were observed using transmission electron microscopy (TEM). (C) Number counts of mitophagosomes and mitolysosomes based on the TEM images. WT→WT and WT→KO mice were administered intraperitoneally with 3-methyladenine (3-MA, 30 mg/kg) or PBS 3 h before liver IR onset. (D) Representative histological staining (H&E) of ischemic livers (scale = 100 μm). (E) Liver damage was evaluated by Suzuki's histological score. (F) Liver function was evaluated by serum ALT levels. (G) Immunofluorescence staining of TUNEL+ cells in ischemic livers (scale = 100 μm). (H) Quantification of TUNEL+ cells per high power field. (I) Liver Caspase-3 activity. n = 6 mice/group. All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; H&E, hematoxylin and eosin; IR, ischemia and reperfusion; KO, knockout; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

Protein 1 (Drp1) plays a critical role in maintaining mitochondrial homeostasis by facilitating mitophagy.[31] To determine whether Drp1 is involved in *CHOP* deficiency–mediated mitophagy in H/R-stressed hepatocytes, the protein levels of Drp1, phosphorylated Ser616 Drp1 (p-S616-Drp1), and autophagy-related proteins were analyzed. The results showed that post-H/R, the total protein levels of Drp1 were not changed in either WT or KO hepatocytes, but the expression of p-S616-Drp1 was significantly increased in H/R-stressed KO as compared with WT hepatocytes (**Figure S5, SDC,** http://links.lww.com/TP/C504). Notably, the expression of p-S616-Drp1 as well as Drp1 in the mitochondrial fractions of KO hepatocytes was significantly elevated in response to H/R, indicating mitochondrial translocation of Drp1 (Figure 4C). Moreover, Beclin-1 was markedly upregulated in the mitochondrial fractions of KO hepatocytes (Figure 4C). These findings prompted us to consider that *CHOP* deficiency might promote Drp1 mitochondrial translocation, resulting in Beclin-1–dependent mitophagy activation.

To verify our hypothesis, we first blocked Drp1 activation in H/R-stressed hepatocytes using mitochondrial division inhibitor-1 (Mdivi-1), an inhibitor of Drp1. We found that Mdivi-1 treatment significantly downregulated Beclin-1 as well as inhibited mitophagy in H/R-stressed KO hepatocytes (Figure 4D). Furthermore, immunofluorescence double staining showed that Mdivi-1 effectively inhibited the colocalization of mitochondria and lysosomes (Figure 4E). Furthermore, we used Beclin-1–siRNA to knockdown Beclin-1 in H/R-stressed hepatocytes, with nonspecific RNA (NS-siRNA) as a control. Beclin-1 knockdown did not influence the mitochondrial levels of Drp1 or p-S616-Drp1 but effectively inhibited hepatocellular mitophagy in H/R-stressed KO hepatocytes (Figure 4F and G). These findings indicate that CHOP–Drp1–Beclin-1 signaling plays a critical role in regulating mitophagy in H/R-stressed hepatocytes.

CHOP Deficiency Alleviates H/R-induced Hepatocellular Damage via Drp1–Beclin-1 Signaling–mediated Mitophagy

Consistent with our in vivo results, *CHOP* deficiency significantly alleviated H/R-induced hepatocellular injury, reduced ROS production, and improved mitochondrial quality (Figures 5A and B). We further explored the contribution of Drp1–Beclin-1 signaling to the protective effects of *CHOP* deficiency against H/R-induced hepatocellular injury. We found that Mdivi-1 and Beclin-1–siRNA significantly blunted the twin beneficial effects conferred by *CHOP* deficiency; reduction in ROS production as well as mitigation of hepatocellular injury (Figures 5C and D). In contrast, neither Mdivi-1 nor Beclin-1–siRNA had any significant effect on H/R-induced WT hepatocytes. Thus, these findings suggest that *CHOP* deficiency induces mitophagy via Drp1–Beclin-1 signaling post-H/R, leading to diminished ROS production and attenuated hepatocellular injury.

CHOP Deficiency Attenuates Liver IR Injury In Vivo via Drp1–Beclin-1 Signaling–mediated Mitophagy

We next investigated the role of CHOP–Drp1–Beclin-1 signaling in regulating mitophagy in vivo.

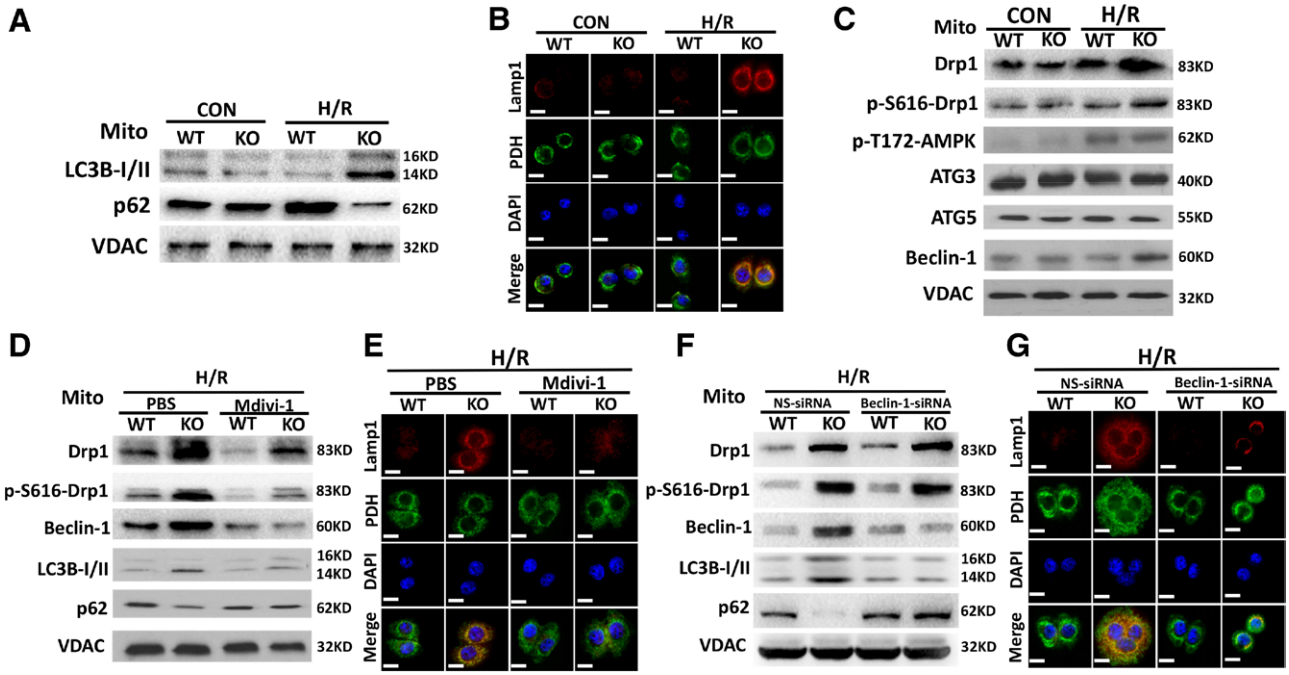

FIGURE 4. *CHOP* deficiency promotes hepatocellular mitophagy in response to H/R in vitro via Drp1–Beclin-1 signaling. Primary hepatocytes isolated from WT and *CHOP* KO mice were subjected to H/R. Total proteins and mitochondrial proteins of the primary hepatocytes were prepared for further studies. (A) Western blot analysis of LC3B-I/II, p62, and VDAC expression in mitochondrial fractions. (B) Immunofluorescence staining of PDH (green), Lamp1 (red), and DAPI (blue) of the primary hepatocytes (scale = 200 μm). (C) Western blot analysis of Drp1, p-S616-Drp1, p-T172-AMPK, ATG3, ATG5, Beclin-1, and VDAC expression in mitochondrial fractions of the primary hepatocytes. Primary hepatocytes isolated from WT and *CHOP* KO mice were pretreated with mitochondrial division inhibitor-1 (Mdivi-1, 50 μM) or PBS as control 2 h before H/R. (D) Western blot analysis of Drp1, p-S616-Drp1, Beclin-1, LC3B-I/II, p62, and VDAC expression in mitochondrial fractions of the primary hepatocytes. (E) Immunofluorescence staining of PDH (green), Lamp1 (red), and DAPI (blue) of the primary hepatocytes (scale = 200 μm). Primary hepatocytes isolated from WT and *CHOP* KO mice were transfected with Beclin-1–siRNA or NS-siRNA (control) 24 h before H/R. (F) Western blot analysis of Beclin-1, Drp1, p-S616-Drp1, LC3B-I/II, p62, and VDAC expression in mitochondrial fractions of the primary hepatocytes. (G) Immunofluorescence staining of PDH (green), Lamp1 (red) and DAPI (blue) of the primary hepatocytes (scale = 200 μm). All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; DAPI, 4',6-diamidino-2-phenylindole; Drp1, dynamin-related protein 1; H&E, hematoxylin and eosin; H/R, hypoxia/reoxygenation; IR, ischemia and reperfusion; KO, knockout; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

Beclin-1–siRNA complexed with Invivofectamine was used to specifically knock down Beclin-1 in WT→KO livers. Western blot analysis showed that Beclin-1 knockdown effectively inhibited mitophagy in WT→KO livers with no impact on Drp1 protein levels (Figure 6A). The number of autophagosomes that engulfed the contents of mitochondria with destroyed membranes in response to IR was drastically decreased in Beclin-1–siRNA–treated livers as compared with NS-siRNA-treated livers (Figure 6B). Furthermore, ROS production was significantly increased in IR-stressed livers of Beclin-1–siRNA–treated WT→KO mice (Figures 6C–E). Functionally, as compared with NS-siRNA-treated WT→KO mice, Beclin-1 knockdown markedly exacerbated liver IR injury in WT→KO mice as evidenced by worsened histological architecture, higher Suzuki's scores, and higher serum ALT levels (Figures 6F–H). Additionally, the results of TUNEL staining and Caspase-3 activity demonstrated that Beclin-1 knockdown aggravated hepatocellular apoptosis in IR-induced WT→KO livers (Figures 6I–K). Furthermore, the detrimental effects of Beclin-1 knockdown were abrogated by MT treatment (Figures 6C–K). Taken together, these results suggested that *CHOP* deficiency protects the liver against IR injury by facilitating the decrease of intrahepatic ROS production via Drp1–Beclin-1–dependent mitophagy.

CHOP Activation Is Related to Autophagy Inhibition and Increased ROS Levels in Human Ischemic Livers

To determine the clinical relevance of ROS modulation in CHOP-mediated mitophagy, we collected a series of peritumoral tissues during hepatic resection of 6 patients with hepatocellular carcinoma (HCC) after various durations of ischemia (0, 15, and 30 min). Liver ischemia upregulated CHOP activation, as shown by Western blot analysis (Figure 7A) and immunofluorescence staining (Figures 7B and C). In contrast, Beclin-1 and autophagy were inhibited by liver ischemia, as shown by the decreased expression of Beclin-1 and LC3B II as well as the increased expression of p62, postischemia (Figure 7A). Human primary hepatocytes were used to confirm the effect of CHOP on H/R-induced mitophagy in vitro. As shown in Figure 7D, 4-PBA treatment significantly activated Drp1–Beclin-1 axis and triggered mitophagy in response to H/R. Finally, 8 HCC patients, who were preparing for liver partial resection with portal triad clamping, were divided into 2 equal groups; an N-acetyl cysteine (NAC) group (with intravenous infusion of NAC before surgery) and a control (CON) group (without NAC infusion). The results showed that NAC pretreatment significantly decreased serum levels of MPO on the first day and ALT levels on the third day after surgery in patients in the NAC group as compared with that in the CON group (Figures 7E and F). These results indicate

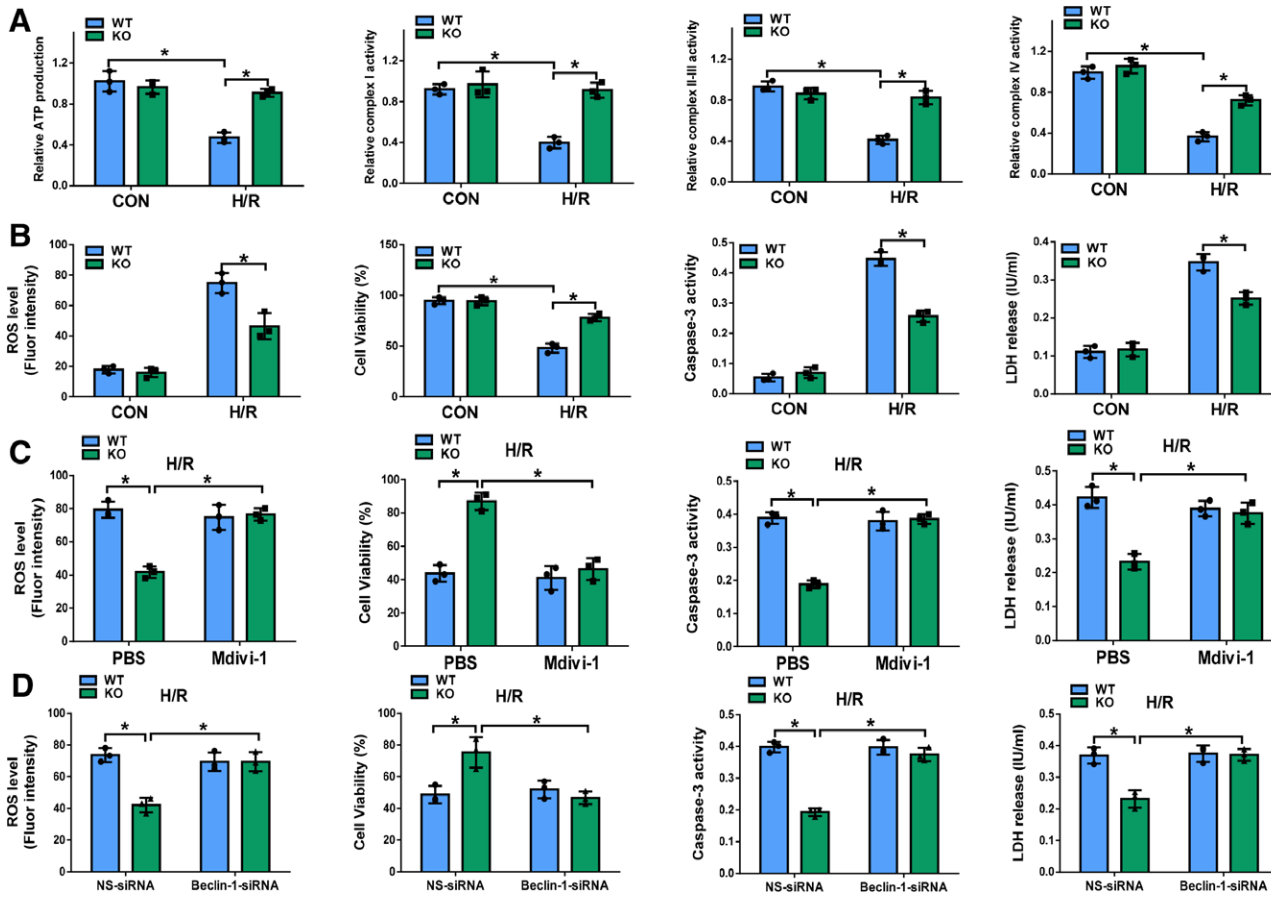

FIGURE 5. *CHOP* deficiency alleviates H/R-induced hepatocyte damage via Drp1–Beclin-1 signaling–mediated mitophagy. Primary hepatocytes isolated from WT and *CHOP* KO mice were subjected to H/R. (A) Relative mitochondrial ATP production, respiratory chain complex I activity, respiratory chain complex II+III activity and respiratory chain complex IV activity of the hepatocytes. (B) Hepatocellular ROS levels were measured by ROS detection assay; Hepatocyte viability was evaluated by CCK-8 assay; Caspase-3 activity was assessed by Caspase-3 activity assay; LDH production was measured by LDH release assay. Primary hepatocytes isolated from WT and *CHOP* KO mice were pretreated with Mdivi-1 (50 μM) or PBS (control) 2 h before H/R onset. (C) Hepatocellular ROS levels were measured by ROS detection assay; Hepatocyte viability was evaluated by CCK-8 assay; Caspase-3 activity was assessed by Caspase-3 activity assay; LDH production was measured by LDH release assay. Primary hepatocytes isolated from WT and *CHOP* KO mice were transfected with Beclin-1–siRNA or NS-siRNA (control) 24 h before H/R. (D) Hepatocellular ROS levels were measured by ROS detection assay; Hepatocyte viability was evaluated by CCK-8 assay; Caspase-3 activity was assessed by Caspase-3 activity assay; LDH production was measured by LDH release assay. All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; ATP, adenosine triphosphate; CHOP, CCAAT/enhancer-binding protein homologous protein; Drp1, dynamin-related protein 1; H&E, hematoxylin and eosin; H/R, hypoxia/reoxygenation; IR, ischemia and reperfusion; KO, knockout; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

that liver ischemia results in CHOP activation, leading to Beclin-1–mediated autophagy downregulation and subsequent reduction in ROS clearance, ultimately promoting oxidative hepatocellular injury.

DISCUSSION

This study analyzed the role of CHOP signaling in the regulation of oxidative hepatocyte cell death during liver IR injury. A bone marrow chimera mouse model was created to analyze the role of hepatocellular CHOP signaling depletion in vivo as well as in vitro. The results demonstrated that CHOP signaling controls oxidative hepatocellular injury by modulating mitophagy activation via the Drp1–Beclin-1 axis.

ER stress and autophagy have been reported to be associated with liver IR injury. We have reported previously that prolonged ischemia triggers a pathogenic ER stress response, leading to impaired liver autophagy and eventually to IR injury.[32] Although the protective role of *CHOP* deficiency in liver IR injury has been reported,[20] the underlying mechanism, especially the cell type-specific roles of CHOP signaling, required further study.

CHOP is a key component of ER stress–mediated apoptosis in hepatocytes and macrophages and has been implicated in various liver diseases and injuries.[33,34] However, studies have suggested that CHOP performs complex functions in different cells as well as in different liver diseases. Russell et al reported that *CHOP* deficiency did not alleviate but instead worsened methionine-choline-deficient–mediated liver disease,[35] whereas another study showed that *CHOP* deficiency attenuates apoptosis, inflammation, and fibrosis under fat-loading conditions in a methionine-choline–deficient diet.[36] Yet another study showed that CHOP-dependent macrophage apoptosis protects mice from steatohepatitis.[34]

We have previously demonstrated that CHOP promotes liver IR injury in hyperglycemic mice by inhibiting KC M2 polarization.[37] Consistent with the present one, a

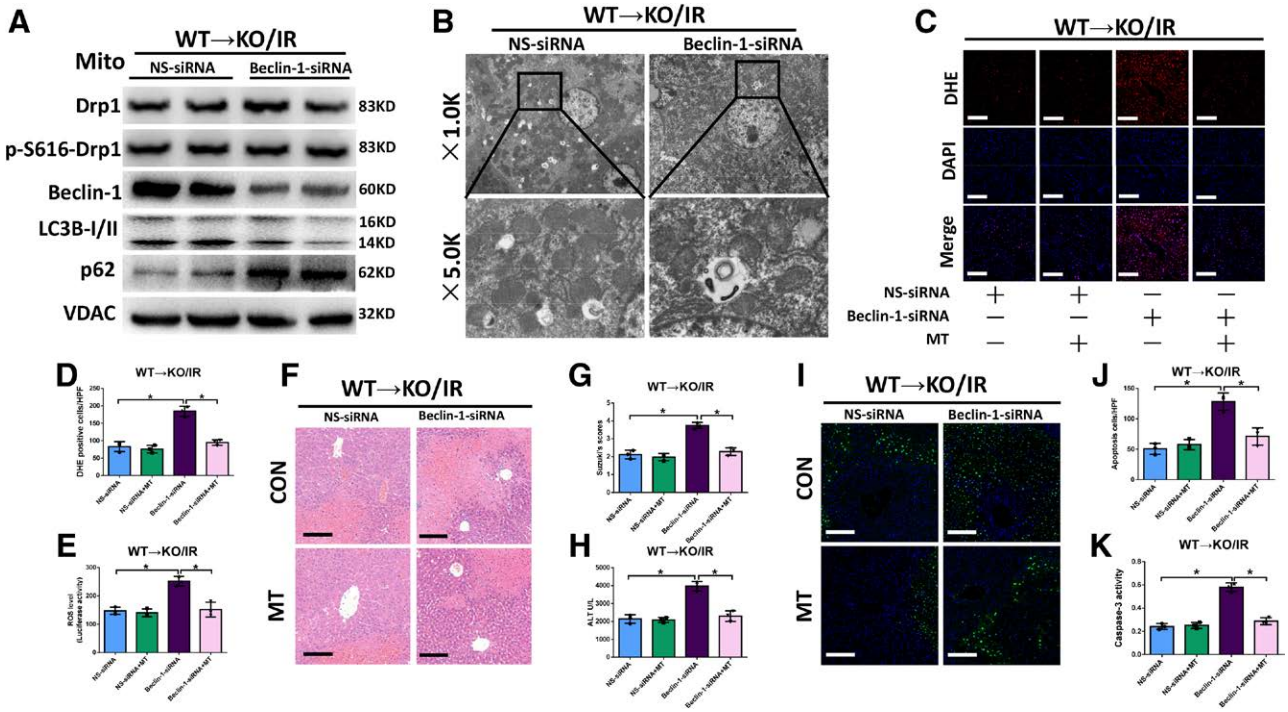

FIGURE 6. *CHOP* deficiency attenuates liver IR injury in vivo via Drp1–Beclin-1 signaling–mediated mitophagy. WT→KO mice were injected through tail vein with Beclin-1–siRNA–Invivofectamine complex or NS-siRNA-Invivofectamine complex daily for 3 d before liver IR onset. Mitochondrial proteins of the livers were prepared for further studies. (A) Western blot analysis of Drp1, p-S616-Drp1, Beclin-1, LC3B-I/II, p62, and VDAC expression in mitochondrial fractions of the livers. (B) Autophagosomes in the livers were observed using transmission electron microscopy (TEM). WT→KO mice treated with Beclin-1–siRNA or NS-siRNA were administered with MT or PBS 3 h before liver IR onset. (C) Immunofluorescence staining of dihydroethidium (DHE, red) and DAPI (blue) in liver tissues (scale = 100 μm). (D) Quantification of DHE+ cells per high power field. (E) ROS activity in liver tissues. (F) Representative histological staining (H&E) of ischemic livers (scale = 100 μm). (G) Liver damage was evaluated by Suzuki's histological score. (H) Liver function was evaluated by serum ALT levels. (I) Immunofluorescence staining of TUNEL+ cells in ischemic livers (scale = 100 μm). (J) Quantification of TUNEL+ cells per high power field. (K) Liver Caspase-3 activity. n = 6 mice/group. All results were representative of at least 2 independent experiments. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; DAPI, 4',6-diamidino-2-phenylindole; Drp1, dynamin-related protein 1; H&E, hematoxylin and eosin; IR, ischemia and reperfusion; KO, knockout; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

recent study showed that liver IR induces CHOP expression, whereas *CHOP* deficiency attenuates liver IR injury by inhibiting apoptosis,[20] although the underlying mechanism remains unclear.

Drp1, a cytosolic guanosine triphosphatase, has been well established as an essential promoter of mitochondrial fission. Recent studies have demonstrated that mitophagy initiation involves the recruitment of Drp1 to the surface of mitochondria.[38] However, the role of Drp1 as shown in various ischemic models has been contradictory. Mitophagy has been shown to be suppressed in *Drp1*-deficient cardiomyocytes, leading to enhanced myocardial injury in response to IR.[39] In response to ischemic stress, Ulk1-Rab9-Rip1-Drp1 forms a protein complex in cardiomyocytes, which phosphorylates Drp1 at S616, leading to alleviation of heart ischemic injury in a mitophagy-dependent manner.[40] It has been reported that a specific inhibitor of Drp1 rectifies cardiac IR injury by preventing mitochondrial fission.[41] The AKAP1/PKA complex as well as deletion of Bβ2 inhibited Drp1-dependent mitochondrial fission via the maintenance of Drp1 Ser637 phosphorylation, resulting in the attenuation of cerebral ischemic injury.[42,43] In contrast, inhibition of Drp1 by pharmacological inhibitors or siRNA exacerbated cerebral ischemic injury via mitophagy suppression.[44] These discrepancies might be because of the difference in approaches or varied sites of Drp1 inhibition. It is generally accepted that excessive mitochondrial fission is a detrimental adaptive mechanism, whereas mitophagy is a protective response.[45]

Since the ER and mitochondria are amongst the vital dynamic organelles involved in physiological as well as pathological processes, the association between them has received significant attention in the last decade. The mitochondria-associated ER membrane (MAM) is the site of mitochondrial fission as well as autophagosome formation.[46,47] The current study suggests that IR-induced CHOP activation in hepatocytes could inhibit the accumulation of mitophagy-related proteins at the MAM, partially by restraining Drp1 Ser616 phosphorylation; however, the precise mechanism requires further study.

Previous studies have suggested that Beclin-1 plays a pivotal role in mitophagy mediated by various stimuli.[48,49] It has been reported that Beclin-1 is engaged in Drp1-dependent mitophagy and protects against pressure overload-induced heart failure.[50] In this study, we showed that inhibition of Drp1 significantly suppressed Beclin-1 activation, thereby restraining mitophagy. Importantly, 3-MA or Beclin-1 siRNA significantly blocked the protective effects of *CHOP* deficiency in IR-induced hepatocellular injury in vivo as well as in vitro, suggesting that the CHOP–Drp1–Beclin-1 axis plays a critical role in modulating liver IR injury.

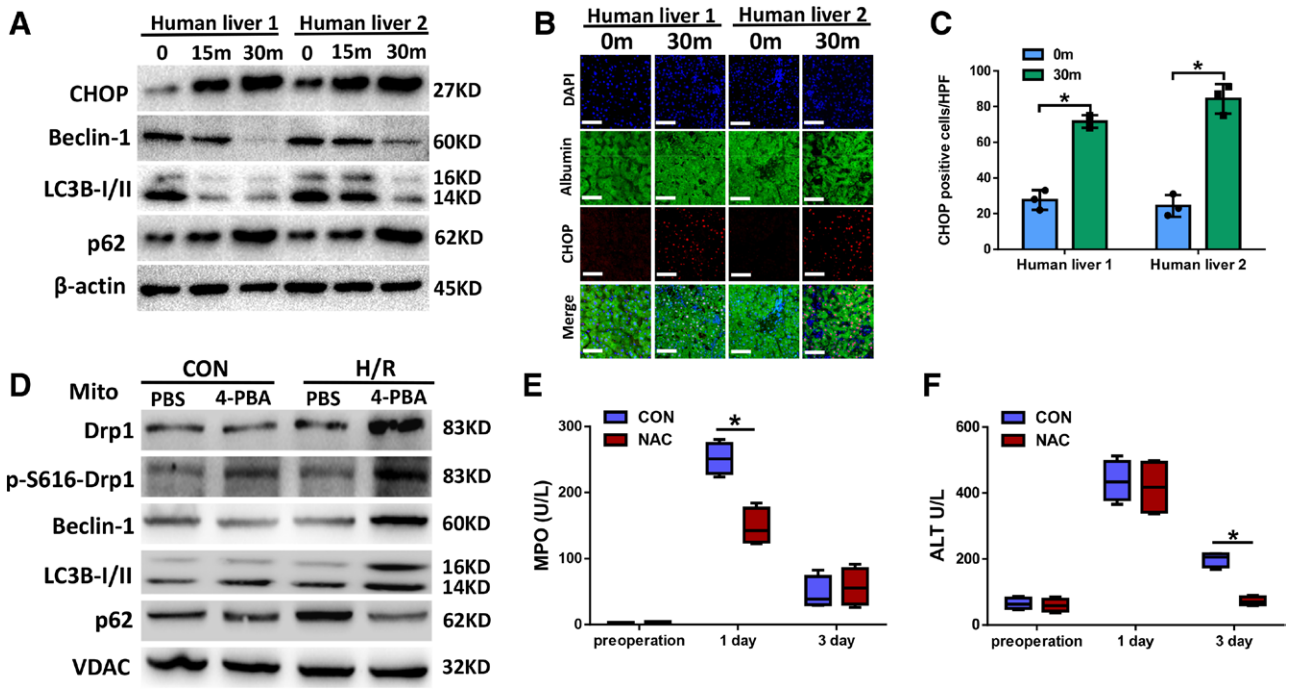

FIGURE 7. CHOP activation is related to autophagy inhibition and increased ROS levels in human ischemic livers. The pericarcinomatous liver tissues were collected from 6 patients who were undergoing hepatectomy with portal triad clamping before ischemia, 15 min and 30 min after ischemia. (A) Representative of Western blot analysis of CHOP, Beclin-1, LC3B-I/II, p62, and β-actin in the human livers (n = 6). (B) Immunofluorescence staining of Albumin (green), CHOP (red), and DAPI (blue) in the human liver tissues (scale = 100 μm). (C) Quantification of CHOP+ cells per high power field. Primary human hepatocytes were prepared from liver samples from 3 patients undergoing liver resection. Primary human hepatocytes were treated with 5 mmol/L 4-PBA or PBS as control for 2 h before H/R onset. (D) Representative of Western blot analysis of Drp1, p-S616-Drp1, Beclin-1, LC3B-I/II, p62, and VDAC expression in mitochondrial fractions of the cells (n = 3). Eight successive patients who were preparing for partial hepatectomy with portal triad clamping were randomly divided into NAC group or control (CON) group (4 in each group) according to the presence or absence of NAC (150 mg/kg/d for 3 d before the operation) administration. (E) Serum MPO levels of the patients. (F) Liver function was evaluated by serum ALT levels. *$P < 0.05$. Values were expressed as mean ± SD. ALT, alanine aminotransferase; CHOP, CCAAT/enhancer-binding protein homologous protein; DAPI, 4',6-diamidino-2-phenylindole; Drp1, dynamin-related protein 1; H&E, hematoxylin and eosin; H/R, hypoxia/reoxygenation; R, ischemia and reperfusion; MPO, myeloperoxidase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; WT, wild type; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

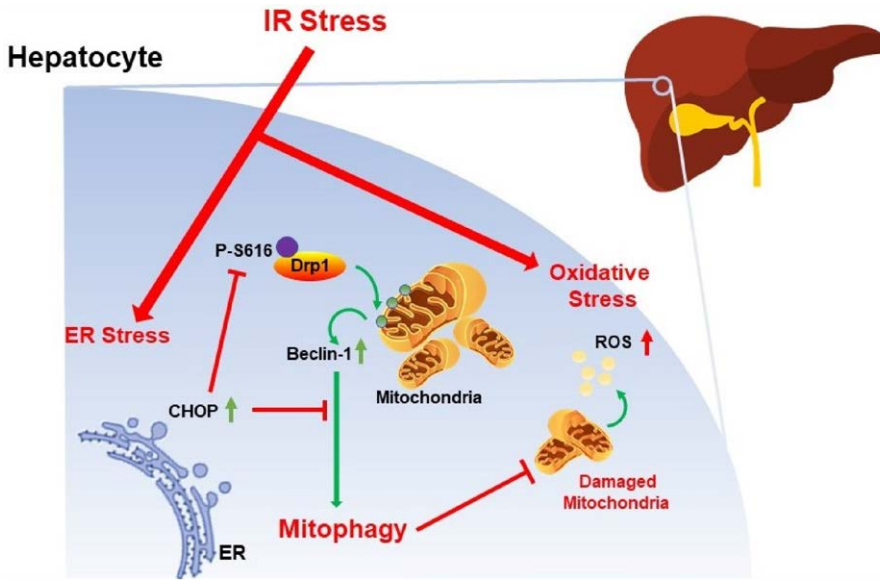

FIGURE 8. Proposed mechanism by which CHOP activation promotes liver IR injury. CHOP activation mediated by IR inhibits Drp1 mitochondrial translocation resulting in suppressed Beclin-1–dependent mitophagy and thereby exacerbates ROS-mediated liver injury. CHOP, CCAAT/enhancer-binding protein homologous protein; Drp1, Dynamin-related protein 1; IR, ischemia and reperfusion; ROS, reactive oxygen species.

Emerging evidences have shown the critical role of CHOP signaling in regulating mitophagy. CHOP was found to promote head kidney macrophage apoptosis by inhibiting mitophagy.[51] Carbon monoxide gas inhalation significantly suppressed CHOP expression in livers and dramatically increased hepatic HO-1 and Parkin expression, leading to alleviated APAP-induced liver damage.[52] Similarly, in the present study, whereas increased CHOP expression was found in IR-stressed livers, CHOP inhibition restored Drp1-mediated mitophagy activation in primary hepatocytes from both mice and humans. Figure 8 shows the potential mechanisms by which CHOP activation promotes liver IR injury by inhibiting Drp1–Beclin-1 signaling–mediated mitophagy and ROS clearance.

Emerging studies have shown the interplay between ER stress and ROS. Persistent ER stress–initiated ROS cascades plays an important role in the pathogenesis of multiple human disorders.[53] Meanwhile, ROS inhibition by NAC could inhibit CHOP activation in various disease models.[54-56]

In the present study, we found that although IR-induced CHOP activation promoted ROS production in hepatocytes, ROS inhibition by NAC alleviated hepatocyte injury. However, the effect of NAC on regulating CHOP activation was not determined in the current study. We focused on the direct role of NAC in ROS scavenge, but whether the protective role of NAC treatment depended on CHOP inhibition remains unclear. Mitochondria are not only the primary source of ROS production, but also the target organelles in oxidative stress. In this study, using animal and human systems, we demonstrated that *CHOP* depletion, mitophagy activation, or direct scavenging of ROS protect hepatocytes from cell death.

In conclusion, we identified a hitherto unrecognized role of CHOP signaling in regulating hepatocyte cell death in response to IR stress. IR-induced CHOP activation exacerbates ROS-mediated hepatocyte cell death by inhibiting Drp1–Beclin-1–dependent mitophagy.

Table S1, SDC, http://links.lww.com/TP/C504.

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