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ORIGINAL ARTICLE

Increased oxidative stress caused by impaired mitophagy aggravated liver ischemia and reperfusion injury in diabetic mice

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ABSTRACT

Aims/Introduction: Emerging evidence has suggested the detrimental role of oxidative stress in aggravating ischemia and reperfusion (IR) injury in diabetic livers. Interplay between oxidative stress and mitophagy has been shown. However, the role and mechanism of mitophagy in regulating oxidative stress and IR injury in diabetic livers remain unclear.

Materials and Methods: Wild-type and *db/db* (DB) mice were subjected to a partial warm liver IR model. Liver injury, oxidative stress, mitophagy and related molecular pathways were analyzed.

Results: Here, we found that increased liver IR injury was observed in DB mice, as evidenced by higher levels of serum alanine aminotransferase and serum aspartate, worsened liver architecture damage and more hepatocellular death. DB mice also showed increased mitochondrial oxidative stress. Mitochondrial reactive oxygen species scavenge alleviated liver IR injury in DB mice. Mechanistic analysis showed that 5′ adenosine monophosphate-activated protein kinase-mediated mitophagy was suppressed in DB mice post-IR. Pharmacological activation of 5′ adenosine monophosphate-activated protein kinase by its agonist effectively restored mitophagy activation, leading to decreased mitochondrial oxidative stress and attenuated liver IR injury in DB mice.

Conclusions: Our findings showed that diabetes increased oxidative stress to exacerbate liver IR injury by impairing 5′ adenosine monophosphate-activated protein kinase-mediated mitophagy. Strategies targeting oxidative stress and mitophagy might provide a promising approach to ameliorate liver IR injury in diabetes patients.

INTRODUCTION

In recent decades, the incidence of diabetes mellitus has rapidly increased worldwide. Diabetes mellitus is a major risk factor for various diseases and multiple organ dysfunction in patients[1]. Liver ischemia and reperfusion (IR) injury has been found to significantly impair the postoperative recovery of patients undergoing liver resection or liver transplantation[2]. Aggravated liver ischemic injury has been shown in diabetic mice by recent studies[3,4]. However, the underlying pathogenesis is unclear, and clinical interventions are lacking.

Excessive oxidative stress contributes to hepatocyte death and liver IR injury. Mitochondria, as the main source of cellular reactive oxygen species (ROS), play a critical role in energy metabolism and cell survival. Mitochondrial homeostasis also functions in regulating liver IR injury[5]. Block of the opening of the mitochondrial permeability transition pore attenuated liver IR injury[6]. Augmentation of liver regeneration protected steatotic hepatocytes against IR injury by anti-oxidation and mitochondrial preservation[7]. The mitochondrial pathway also mediated the protective role of ischemic postconditioning in liver IR injury[8]. Although mitochondrial dysfunction has been shown in diabetes[9], the role and mechanism of mitochondrial injury in regulating diabetic liver IR injury remain largely unknown.

Mitophagy selectively removes dysfunctional mitochondria through specific sequestration and engulfment of mitochondria

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for subsequent lysosomal degradation[10]. Mitophagy plays an essential role in re-establishing mitochondrial homeostasis and treating mitochondrial diseases[11]. Diabetes represses PINK1/Parkin-mediated mitophagy, resulting in increased renal IR injury[12]. In addition, 5′ adenosine monophosphate-activated protein kinase (AMPK) activation has been reported to promote mitophagy[13]. Interestingly, AMPK has been suggested as an attractive therapeutic target for both diabetes mellitus and liver IR injury[14,15].

Herein, we hypothesized that diabetes suppresses AMPK-mediated mitophagy, leading to aggravated mitochondrial injury and liver damage post IR. The present findings suggest that strategies targeting AMPK or mitophagy would be beneficial for alleviating liver IR injury in diabetes patients.

MATERIALS AND METHODS

Animals

The present study was approved by the Animal Care Committee of Changzhou No. 2. People's Hospital. Eight-week-old male C57BL6 wild-type (WT; control) and *db/db* (DB) mice were purchased from GemPharmatech Co., Ltd (Nanjing, China). The animals were housed in a standard environment.

Hepatic IR model

A murine warm partial hepatic IR injury model was established. Briefly, after successful anesthesia and laparotomy, 70% of the hepatic portal blood supply was blocked by a special vascular clamp for 90 min followed by 6 h of reperfusion. Mice in the sham group received the same procedure, except with no vascular occlusion. 5-aminoimidazole-4-carboxamide ribonucleotide (100 mg/kg; Tocris, Bristol, UK) or mitoTEMPO (5 mg/kg; MedChemExpress, Monmouth Junction, NJ, USA) vehicle control was administered intraperitoneally 1 h before surgery.

Sample collection and analysis

Serum and liver tissues were collected after 6 h of reperfusion. Serum aspartate aminotransferase and alanine aminotransferase levels were detected by an AU5400 automated chemical analyzer. Liver sections were cut and stained with hematoxylin–eosin. The severity of liver IRI was blindly assessed and graded according to Suzuki's criteria on a scale from 0 to 4. A fluorescence detection kit (Roche, Mannheim, Germany) was used for terminal deoxynucleotidyl transferase staining. A staining solution (Biosharp, Anhui, China) was used for Oil Red O staining. Malondialdehyde, glutathione and superoxide dismutase levels

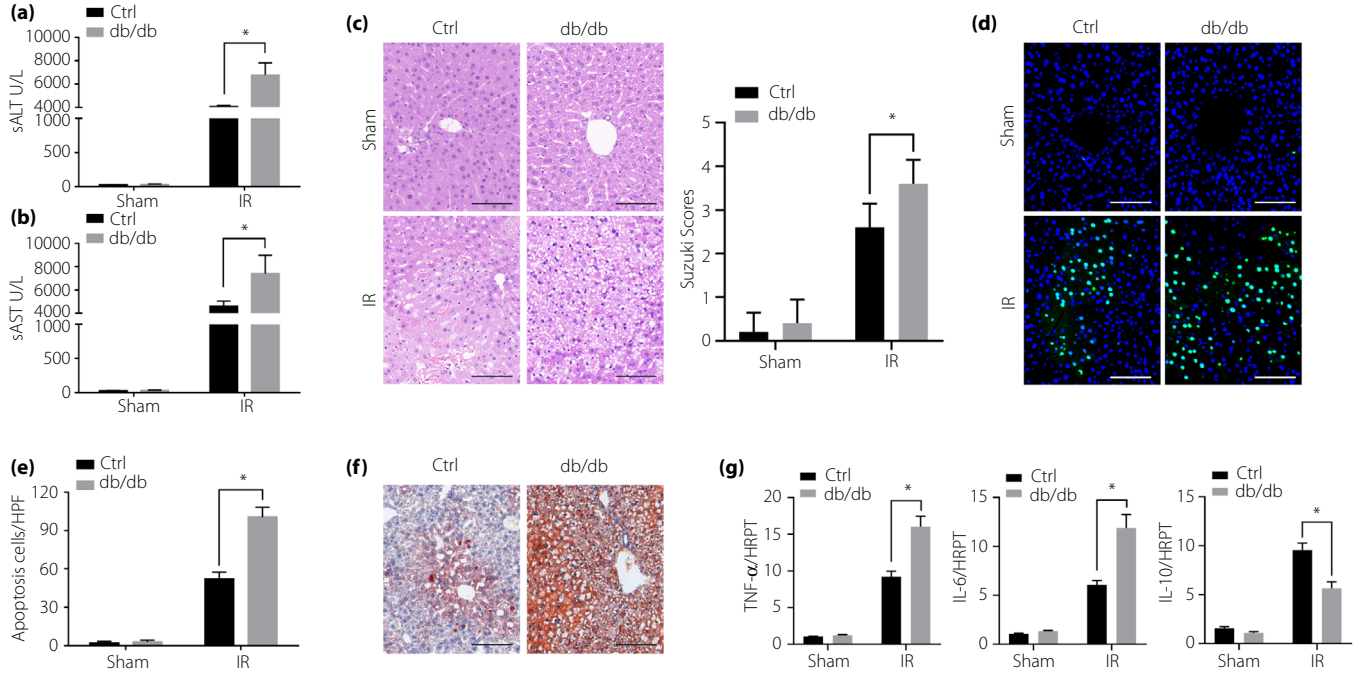

Figure 1 | Aggravated hepatic ischemia and reperfusion (IR) injury in *db/db* (DB) mice. DB and wild-type (control [Ctrl]) mice were subjected to liver partial warm IR or a sham procedure. Serum and liver samples were collected at 6 h post-reperfusion. (a) Serum alanine aminotransferase (sALT); (b) serum aspartate (sAST); (c) liver histopathology (×100 magnification, scale bars 100 μm) and Suzuki's scores; (d) terminal deoxynucleotidyl transferase staining of liver sections (×100 magnification, scale bars 100 μm); (e) terminal deoxynucleotidyl transferase-positive cell percentage evaluated by ImageJ software; (f) Oil Red O staining of liver sections (×100 magnification, scale bars 100 μm); and (g) quantitative reverse transcription polymerase chain reaction analysis of tumor necrosis factor-α, interleukin-6 and interleukin-10 in livers. The average target gene/hypoxanthine guanine phosphoribosyl transferase ratios of different experimental groups are presented. $n = 6$ mice/group. *$P < 0.05$.

in livers were measured using commercial enzyme-linked immunosorbent assay kits (Beyotime, Shanghai, China). Liver ROS levels were measured by dihydroethidium (Beyotime) staining based on the manufacturer's instructions.

Western blots

Proteins extracted from liver tissues were prepared for western blot analysis. Antibodies against LC3B, p62, PINK1, phospho-AMPK (Thr172), AMPK and β-actin were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Transmission electron microscopy

Transmission electron microscopy was applied to monitor autophagy and autophagic vacuoles. Liver samples were prepared and photographed using a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan).

Statistics

Data are expressed as the mean ± standard error of the mean. Student's *t*-test was applied to compare results between two groups, and one-way ANOVA followed by Bonferroni's post-hoc test was applied for comparisons of results among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

Aggravated hepatic IR injury in DB mice

We first compared liver injury in WT and DB mice post-IR. DB mice showed elevated serum levels of alanine aminotransferase and aspartate aminotransferase (Figure 1a,b). Histological examination of liver tissues with Suzuki's scores further confirmed increased liver damage in the DB mice post-IR (Figure 1c). More terminal deoxynucleotidyl transferase-positive cells were observed in IR-stressed livers from diabetes mellitus mice (Figure 1d,e). In addition, liver steatosis was found in DB mice, as shown by positive staining of Oil Red O (Figure 1f). DB mice also showed increased gene induction of pro-inflammatory tumor necrosis factor-α and interleukin-6, but decreased anti-inflammatory interleukin-10, showing enhanced inflammation in diabetic livers post-IR (Figure 1g). These data confirmed that diabetes aggravated hepatocellular death and liver injury post-IR.

Increased oxidative stress contributed to aggravated liver IR injury in DB mice

The mitochondria-related oxidative response plays an important role in regulating liver IR injury[5]. The mitochondrial-related oxidative response was evaluated in the livers of WT and DB

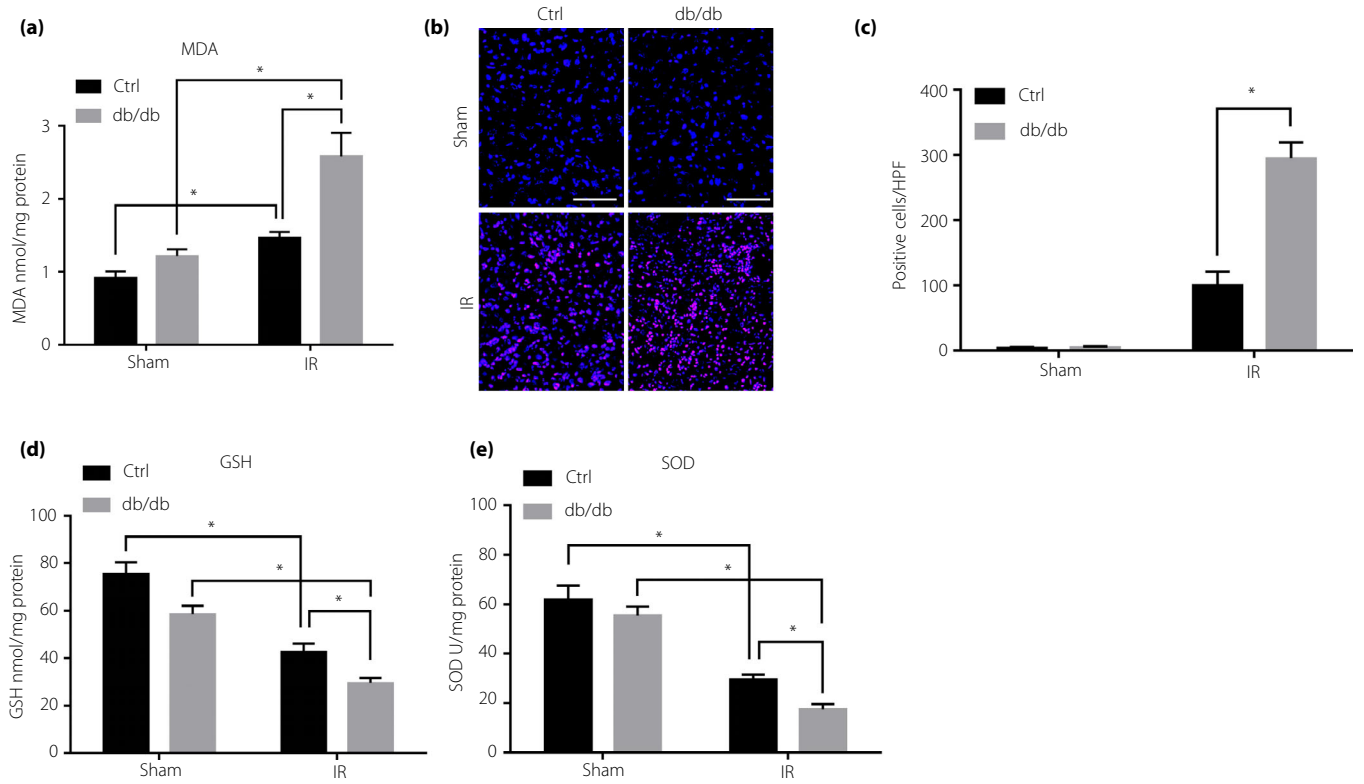

Figure 2 | Increased oxidative stress in *db/db* mice post-ischemia and reperfusion (IR). *db/db* and wild-type (control [Ctrl]) mice were subjected to liver partial warm IR or a sham procedure. Liver samples were collected at 6 h post-reperfusion. (a) Malondialdehyde (MDA); (b) reactive oxygen species (×100 magnification, scale bars 100 μm); (c) dihydroethidium-positive cell percentage evaluated by ImageJ software; (d) glutathione (GSH); and (e) superoxide dismutase (SOD). $n = 6$ mice/group. *$P < 0.05$. HPF, highpowerfield.

mice post-IR. Significantly increased MDA and ROS expression was found in DB mice compared with WT mice (Figure 2a–c). Meanwhile, the expression levels of anti-oxidative GSH and SOD were markedly decreased in DB mice post-IR (Figure 2d,e).

To further determine the role of increased oxidative stress in aggravating liver IR injury in DB mice, mitoTEMPO was used to scavenge mitochondrial ROS (Figure 3a,b). Interestingly, mitoTEMPO treatment abrogated the detrimental effect of diabetes on exacerbating liver IR injury (Figure 3c–e).

Impaired hepatocellular mitophagy in diabetic livers post-IR

Mitophagy functions in preserving mitochondrial homeostasis[16]. We next tested whether mitophagy was influenced by diabetes during liver IR injury. Indeed, DB mice showed decreased expression of LC3BII and increased p62 in their livers post-IR (Figure 4a). Analysis of signaling pathways involved in mitophagy showed decreased activation of PINK1 and phospho-AMPK in IR-stressed livers from DB mice (Figure 4b). DB mice also showed lower numbers of autophagosomes containing mitochondria in livers post-IR (Figure 4c). The results showed that IR induced liver mitophagy, which was inhibited by diabetes.

Mitophagy inhibition regulated by AMPK signaling in IR-stressed diabetic livers

Critical roles of AMPK signaling in regulating both liver IR injury and mitophagy have been reported. We tested whether impaired mitophagy in diabetic livers post-IR was dependent on AMPK inhibition. Interestingly, AMPK activation by its agonist, 5-aminoimidazole-4-carboxamide ribonucleotide, significantly increased LC3B II and PINK1, but decreased p62 expression in the livers of DB mice (Figure 5a). Electron microscopy analysis further confirmed that AMPK activation promoted mitophagy activation in DB mice post-IR (Figure 5b). AMPK activation also inhibited ROS expression (Figure 5c, d) in diabetic livers post-IR. These findings showed that defective mitophagy caused by AMPK inhibition contributed to excessive mitochondrial oxidative stress in diabetic livers post IR.

Pharmacological activation of AMPK alleviated IR injury in diabetic livers

Finally, we investigated the role of pharmacological activation of AMPK in regulating liver IR injury in DB mice. AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide pretreatment reversed the detrimental effect of diabetes on

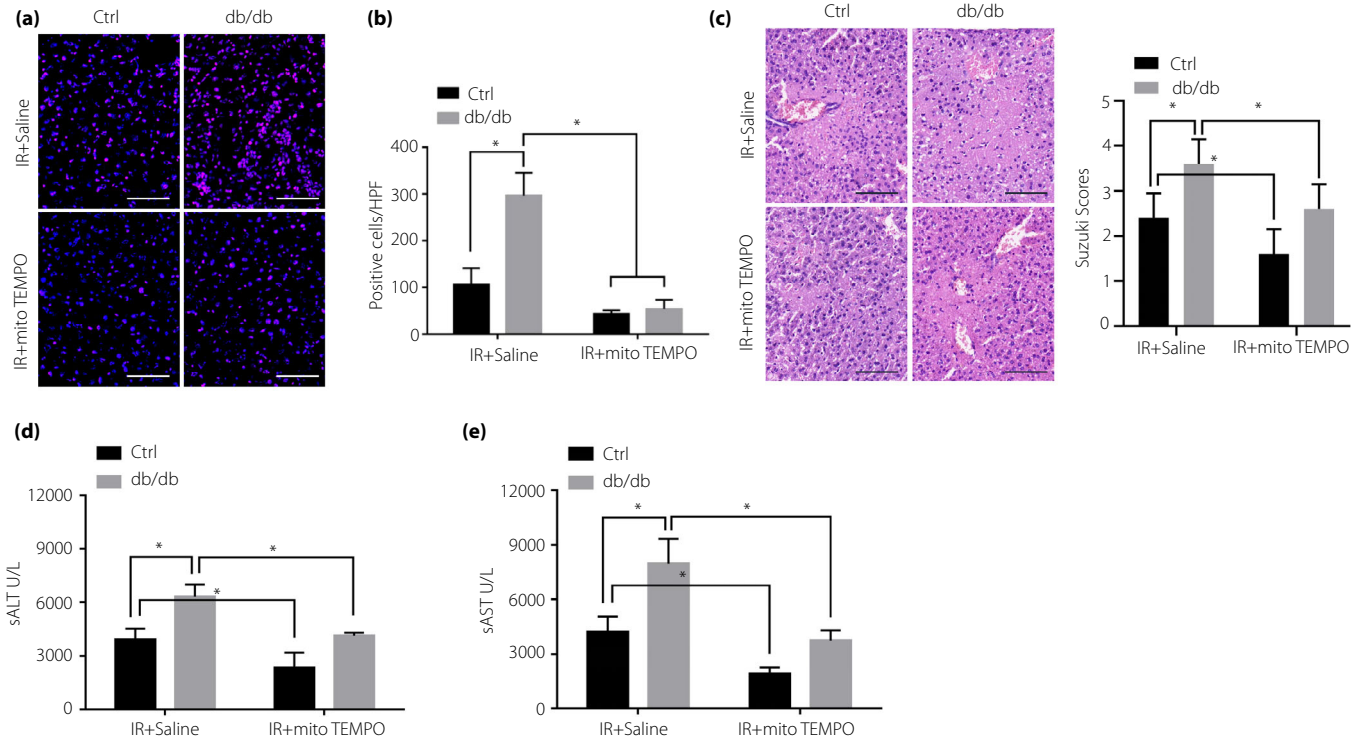

Figure 3 | Reactive oxygen species scavenge alleviated hepatic ischemia and reperfusion (IR) injury in *db/db* mice. *db/db* and wild-type (control [Ctrl]) mice were pretreated with mitoTEMPO or saline control and then subjected to liver partial warm IR or a sham procedure. Serum and liver samples were collected at 6 h post-reperfusion. (a) Reactive oxygen species (×100 magnification, scale bars 100 μm); (b) dihydroethidium-positive cell percentage evaluated by ImageJ software; (c) liver histopathology (×100 magnification, scale bars 100 μm) and Suzuki's scores; (d) serum alanine aminotransferase (sALT); and (e) serum aspartate (sAST). $n = 6$ mice/group. *$P < 0.05$. HPF, highpowerfield.

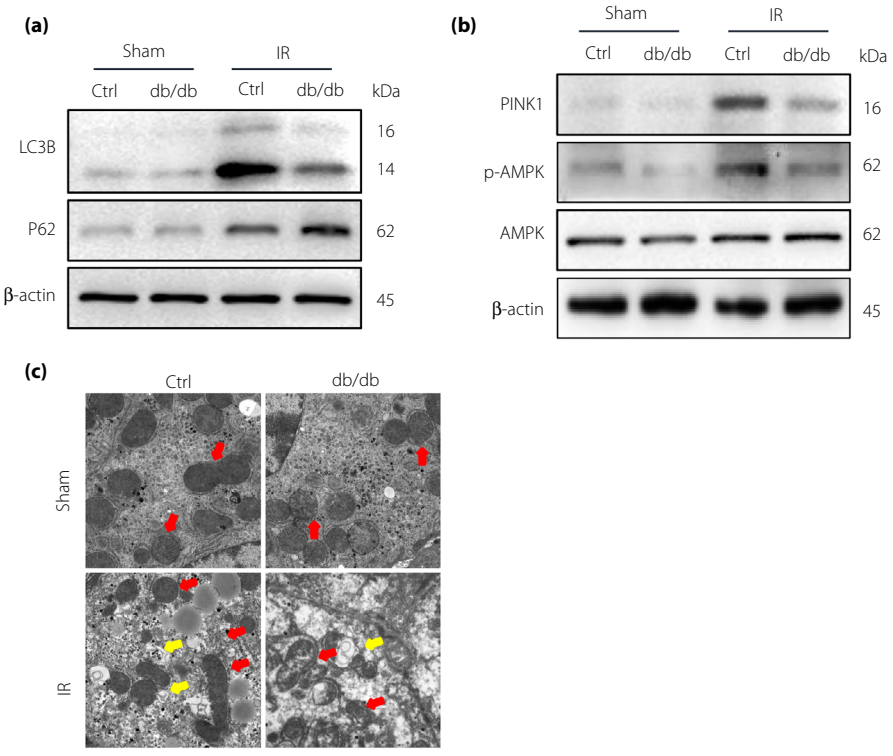

Figure 4 | Impaired activation of mitophagy in diabetic livers post-ischemia and reperfusion (IR). *db/db* and wild-type (control [Ctrl]) mice were subjected to liver partial warm IR or a sham procedure. Liver samples were collected at 6 h post-reperfusion. (a, b) Western bolt analysis of LC3B, p62, PINK1, phosphorylated 5′ adenosine monophosphate-activated protein kinase (p-AMPK), 5′ adenosine monophosphate-activated protein kinase (AMPK) and β-actin; and (c) mitophagy evaluated by transmission electron microscopy. Red arrows: mitochondria; yellow arrows: mitophagy. $n = 6$ mice/group.

aggravating liver IR injury, as shown by lower levels of alanine aminotransferase and aspartate aminotransferase (Figure 6a, b), and reduced liver pathological injury (Figure 6c). Together, these results suggested that diabetes exacerbated liver IR injury by impairing AMPK-mediated mitophagy.

DISCUSSION

Diabetes and its complications are among the leading causes of organ dysfunction worldwide. Although increased IR injury has been found in diabetic livers, the underlying mechanism is largely unclear. The present study showed that diabetes impaired AMPK-mediated mitophagy, resulting in increased oxidative stress and liver damage post-IR. Mitochondrial ROS scavenge or pharmacological restoration of mitophagy by activating AMPK signaling effectively protected the diabetic liver against IR injury.

Liver IR injury refers to partial liver resection with intraoperative hepatic blood inflow occlusion, leading to hepatocellular injury and impaired patient recovery. During the ischemia stage, oxygen and nutrition depletion directly causes hepatocellular metabolism dysfunction and cell injury. Reperfusion triggers oxidative responses, including ROS production, which aggravates cell damage. Furthermore, the damage-associated molecular patterns derived from injured/stressed liver parenchymal cells can activate various immune cells through pattern recognition receptors, resulting in further inflammatory injury. Increased oxidative stress and intrahepatic inflammation have been reported to contribute to aggravating IR injury in diabetic livers[17,18].

Mitochodria play important roles in cellular energy metabolism and cell survival, and a key role in adenosine triphosphate generation through oxidative phosphorylation[19]. Mitochondria can not only generate adenosine triphosphate, but also control various types of cell death, such as apoptosis, pyroptosis, necroptosis and ferroptosis. Mitochondrial dysfunction has been implicated in multiple diseases. Drugs targeting the mitochondria to regulate cell death have been shown to be effective in many trials.

Critical roles of mitochondria in regulating liver IR injury have been reported[5]. Mitochodria-derived ROS are the hallmark of liver IR injury. On liver IR stress, mitochondrial oxidative phosphorylation is inhibited, and oxidative respiratory chain damage results in massive adenosine triphosphate consumption and large amounts of ROS. Excessive ROS induce different types of cell death, including apoptosis, ferroptosis and autophagic cell death[20]. Mitochondrial outer

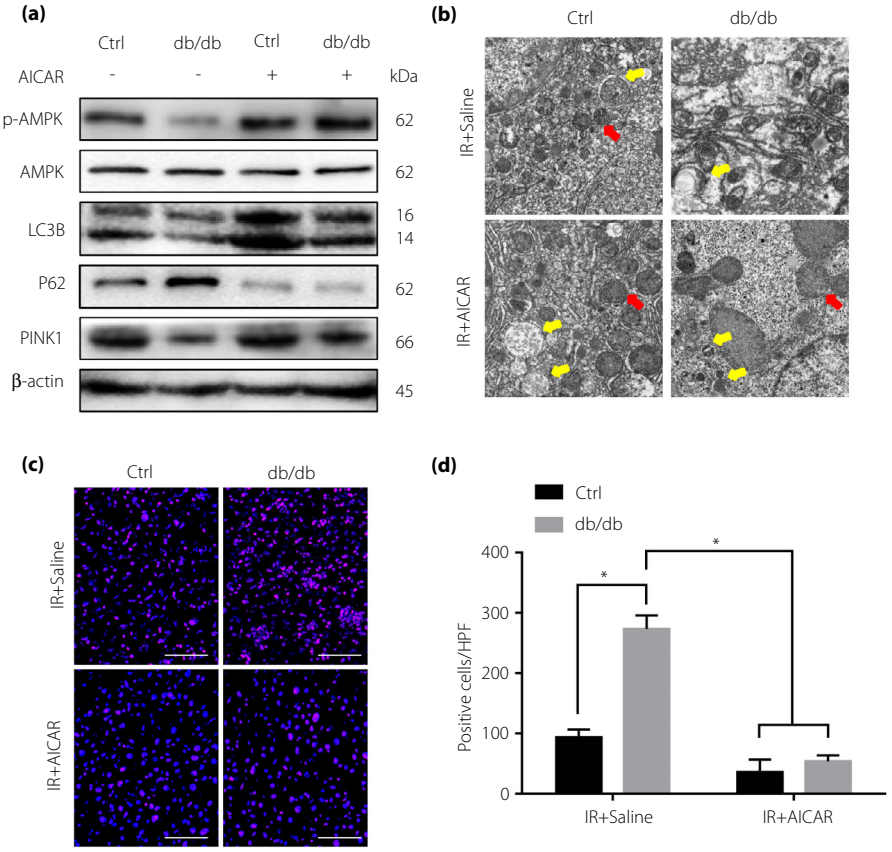

Figure 5 | 5-Aminoimidazole-4-carboxamide ribonucleotide treatment restored mitophagy activation in diabetic livers post-ischemia and reperfusion (IR). *db/db* and wild-type (control [Ctrl]) mice were pretreated with 5-aminoimidazole-4-carboxamide ribonucleotide or saline control and then subjected to liver partial warm IR or a sham procedure. Liver samples were collected at 6 h post-reperfusion. (a) Western bolt analysis of LC3B, p62, PINK1, phosphorylated 5′ adenosine monophosphate-activated protein kinase (p-AMPK), 5′ adenosine monophosphate-activated protein kinase (AMPK) and β-actin; (b) mitophagy evaluated by transmission electron microscopy. Red arrows: mitochondria; yellow arrows: mitophagy; (c) reactive oxygen species (×100 magnification, scale bars 100 μm); and (d) dihydroethidium-positive cell percentage evaluated by ImageJ software. $n = 6$ mice/group. *$P < 0.05$. HPF, highpowerfield.

membrane permeabilization has been well established to initiate a signaling cascade that leads to cell apoptosis[21]. Dysregulation of mitochondrial fusion and fission reduces the number of mitochondria, and changes the mitochondrial structure to form mitochondrial membrane permeable transport pores, leading to various types of cell death. Interestingly, studies have shown impaired mitochondrial function in diabetes[9,22].

Mitophagy, which refers to targeted engulfment and destruction of mitochondria by the cellular autophagy apparatus, is essential for maintaining mitochondrial homeostasis under physiological and pathological conditions[16]. Impaired mitophagy induces mitochondrial damage and dysfunction to promote cell death and tissue injury[10]. Interventions targeting mitophagy have shown therapeutic potential. A recent study found that mitophagy activation by augmenting liver regeneration protects against hepatic IR injury[23]. Hepatocellular Dj-1 deficiency significantly promoted damaged mitochondria clearance during liver IR injury by enhancing mitophagy[24]. Impaired mitophagy in aged mice results in the onset of mitochondrial permeability transition and mitochondrial dysfunction, and aggravates liver IR injury[25].

In the resting state, DB mice showed reduced autophagic vacuoles with increased protein levels of p62 in the tubular cells[26]. In livers, similar protein levels of p62 were found in DB mice[27]. The present study showed no significant changes of autophagy/mitophagy markers between WT and DB mice in the sham group, indicating that compared with WT mice, DB mice showed comparable mitophagy in the resting state. However, impaired mitophagy was found in DB mice post-IR.

Cells possess several mechanisms for mitophagy signaling cascades depending on different stimuli and distinct cellular contexts. Generally, mitophagy regulatory pathways are

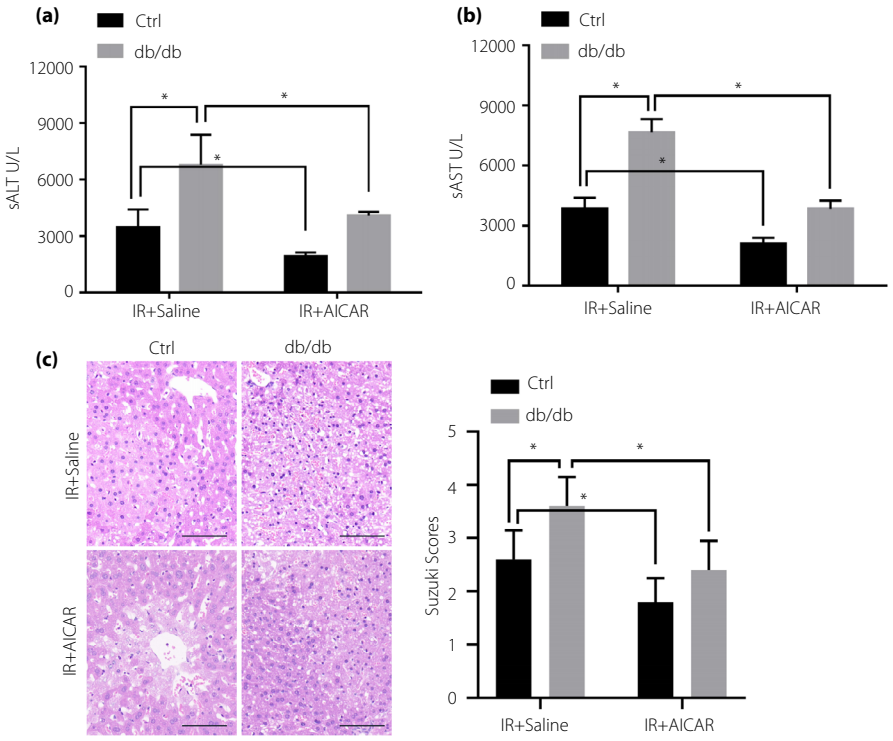

Figure 6 | Mitophagy restoration by 5-aminoimidazole-4-carboxamide ribonucleotide treatment alleviated hepatic ischemia and reperfusion (IR) injury in *db/db* mice. *db/db* and wild-type (control [Ctrl]) mice were pretreated with 5-aminoimidazole-4-carboxamide ribonucleotide or saline control, and then subjected to liver partial warm IR or a sham procedure. Serum and liver samples were collected at 6 h post-reperfusion. (a) serum alanine aminotransferase (sALT); (b) serum aspartate (sAST); and (c) liver histopathology (×100 magnification, scale bars 100 μm) and Suzuki's scores. $n = 6$ mice/group. *$P < 0.05$.

classified as ubiquitin-dependent or ubiquitin-independent. Although the PINK1–Parkin pathway regulates ubiquitin-dependent mitophagy, mitophagy receptors, such as BNIP3, NIX and FUNDC1, can mediate mitochondrial elimination by directly interacting with LC3[10]. AMPK, an energy sensor, functions in regulating metabolic homeostasis. Emerging evidence has shown the important role of AMPK signaling in protecting IR injury by regulating energy metabolism, oxidative response, mitochondrial function and autophagy[15]. In addition, AMPK was found to be critical in mediating the macrophage inflammatory response during liver IR injury[28]. Ischemia induces AMPK activation to further regulate liver autophagy[29]. Mesenchymal stem cells were found to protect against liver IR injury by upregulating PINK1-dependent mitophagy through AMPK activation[30]. Interestingly, therapeutic importance of AMPK activators have been indicated in diabetes[14,31]. In the present study, decreased AMPK activation was found in diabetic livers post-IR. AMPK activation restored mitophagy and attenuated liver IR injury.

In conclusion, the present study showed that diabetes impairs mitophagy by downregulating AMPK activation, resulting in increased oxidative stress and aggravated liver IR. Our findings suggest that modulation of oxidative stress or mitophagy might have potential therapeutic applications in liver IR injury in diabetes patients.

ACKNOWLEDGMENT

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DISCLOSURE

The authors declare no conflict of interest.
Approval of the research protocol: This study was approved by the Animal Care Committee of Changzhou No. 2. People's Hospital.
Informed consent: N/A.
Registry and the registration no. of the study/trial: N/A.
Animal studies: All animal procedures were carried out strictly according to the recommendations in the protocol (number NMU08-092) endorsed by the Institutional Animal Care and Use Committee of Nanjing Medical University.

DATA AVAILABILITY STATEMENTS

Data used to support the findings of this study are available from the corresponding author upon request.

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