**Research Paper**

**IDH2 deficiency increases the liver susceptibility to ischemia-reperfusion injury via increased mitochondrial oxidative injury**

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**ABSTRACT**

Mitochondrial NADP\(^+\)-dependent isocitrate dehydrogenase 2 (IDH2) is a major producer of mitochondrial NADPH, required for glutathione (GSH)-associated mitochondrial antioxidant systems including glutathione peroxidase (GPx) and glutathione reductase (GR). Here, we investigated the role of IDH2 in hepatic ischemia-reperfusion (HIR)-associated mitochondrial injury using Idh2-knockout (Idh2\(^{-/-}\)) mice and wild-type (Idh2\(^{+/+}\)) littermates. Mice were subjected to either 60 min of partial liver ischemia or sham-operation. Some mice were administered with 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (mito-TEMPO, a mitochondria-targeting antioxidant). HIR induced severe histological and functional damages of liver in both Idh2\(^{+/+}\) mice and Idh2\(^{-/-}\) mice and those damages were more severe in Idh2\(^{-/-}\) mice than in wild-type littermates. HIR induces dysfunction of IDH2, leading to the decreases of NADPH level and mitochondrial GR and GPx functions, consequently resulting in mitochondrial and cellular oxidative injury as reflected by mitochondrial cristae loss, mitochondrial fragmentation, shift in mitochondrial fission, cytochrome c release, and cell death. These HIR-induced changes were greater in Idh2\(^{-/-}\) mice than wild-type mice. The mito-TEMPO supplement significantly attenuated the aforementioned changes, and these attenuations were much greater in Idh2\(^{-/-}\) mice when compared with wild-type littermates. Taken together, results have demonstrated that HIR impairs in the IDH2-NADPH-GSH mitochondrial antioxidant system, resulting in increased mitochondrial oxidative damage and dysfunction, suggesting that IDH2 plays a critical role in mitochondrial redox balance and HIR-induced impairment of IDH2 function is associated with the pathogenesis of ischemia-reperfusion-induced liver failure.

**1. Introduction**

Hepatic ischemia-reperfusion injury (HRI) is a key factor in the postoperative dysfunction of the liver and is a common clinical problem [1]. Studies have demonstrated that oxidation of intracellular molecules including proteins, lipids, and DNA, cause oxidative injury to various intracellular organelles eventually leading to cell death [1–5]. Among intracellular organelles, mitochondria are a major producer of reactive oxygen species (ROS), and are simultaneously a primary target of oxidative stress. Under normal conditions, mitochondrial redox status is balanced by mitochondrial antioxidant systems including manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx), and thioredoxin (Trx)/peroxiredoxin (Prx). However, pathological conditions such as ischemia-reperfusion induce dysfunction in these mitochondrial antioxidant systems, leading to the oxidation of mitochondrial entities and the subsequent mitochondrial dysfunction, structural disruption, and the activation of the intrinsic apoptosis pathway. Indeed, apoptosis is known to be a major form of cell death caused by HRI [6]. However, the role of the mitochondrial antioxidant systems in HIR-induced liver injury remains to be defined.

Glutathione is an important antioxidant in animals. Reduced glutathione (GSH) is capable of preventing oxidative damage to cellular components by directly neutralized free radicals and reactive oxygen compounds. In the mitochondrion, H\(_2\)O\(_2\) generated from superoxide by MnSOD is continuously converted to H\(_2\)O by GSH. During this process, GSH is oxidized form of oxidized glutathione (GSSG). This GSSG is recycled by glutathione reductase (GR) to form GSH, which can then continue in the H\(_2\)O\(_2\) removing process [7,8]. For the reduction of GSSG by GR, NADPH is required. Indeed, recent studies have demonstrated that mitochondrial NADPH levels are highly correlated with...
mitochondrial oxidative stress, which causes the dysfunction and structural damage to mitochondria [8,9]. Therefore, because neither NADPH nor GSH can penetrate the inner membrane of mitochondria [8,10,11], the NADPH producing system in the mitochondria may play a very important role in mitochondrial oxidative stress-related injuries including ischemia-reperfusion injury.

Isocitrate dehydrogenase 2 (IDH2) is localized in mitochondria and reduces NADP⁺ to NADPH during the decarboxylation of isocitrate to α-ketoglutarate [9,10]. Given this reaction, IDH2 is recognized as a key generator of NADPH in mitochondria [9,10]. Recent studies have demonstrated that IDH2 is critical for the maintenance of mitochondrial redox balance [9,12–15]. Therefore, in this study, we investigated mitochondrial oxidative stress, which causes the dysfunction and structural damage to mitochondria [8,9]. Therefore, because neither NADPH nor GSH can penetrate the inner membrane of mitochondria [8,10,11], the NADPH producing system in the mitochondria may play a very important role in mitochondrial oxidative stress-related injuries including ischemia-reperfusion injury.

Isocitrate dehydrogenase 2 (IDH2) is localized in mitochondria and reduces NADP⁺ to NADPH during the decarboxylation of isocitrate to α-ketoglutarate [9,10]. Given this reaction, IDH2 is recognized as a key generator of NADPH in mitochondria [9,10]. Recent studies have demonstrated that IDH2 is critical for the maintenance of mitochondrial redox balance [9,12–15]. Therefore, in this study, we investigated
whether IDH2 is involved in the HIR-induced liver injury. Here, we report, for the first time, that HIR induces IDH2 dysfunction along with a reduction of mitochondrial NADPH levels and that Idh2 gene deletion exacerbates HIR-induced mitochondrial oxidative injury and liver cell death. These results suggest that IDH2 could be a potential target for therapeutics against HIR-induced liver failure.

2. Materials and methods

2.1. Animal preparation

All experiments were conducted using 8- to 10-week-old male Idh2 knockout (Idh2<sup>−/−</sup>) and wild-type (Idh2<sup>+/+</sup>) littermates weighing 22–24 g [16]. The study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University, Republic of Korea, and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 2011). The mice were provided with free access to water and standard chow. Some mice were treated with 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (mito-TEMPO [17], 5 mg/kg body weight; Sigma, St. Louis, MO), via intraperitoneal (i.p.) injection, twice, at 17 h and 1 h before surgery. The mice were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg body weight; Sigma) before surgery. Partial liver ischemia was performed by occlusion of the portal triad for 60 min. Briefly, the mouse abdomen was opened using scissors, beginning at the mid-abdomen and ending at the xiphoid process. Reperfusion was confirmed visually by the change in the liver color from reddish brown to cream. To protect fluid loss during surgery, sterilized gauze was put on the retracted intestines and 500 μL of sterilized saline was added to the gauze. After 60 min of ischemia, the clamp was removed carefully and 500 μL of saline was added to the abdominal cavity. Reperfusion was confirmed visually by observing the liver color return to reddish brown. The intestines were replaced in the abdominal cavity following reperfusion. Reperfusion was confirmed visually by observing the liver color return to reddish brown. The muscle layer and skin were sutured with 5-0 silk. Body temperature was maintained at 36.5–37 °C throughout all the surgical procedures using a temperature-controlled heating device (FHC, Bowdoin, ME). A sham surgery was performed that was equivalent to the operation for ischemia except that the portal triad was not clamped. The ischemic lobes were excised 5 h after surgery and were either snap-frozen in liquid nitrogen for western blot analysis or perfusion-fixed in PLP (4% paraformaldehyde, 75 mM L-lysine, and 10 mM sodium periodate; Sigma) for histological studies.
2.2. Histology

Liver paraffin sections were stained with Periodic acid–Schiff (PAS) stain according to a standard protocol. To score hepatocyte morphological damage, the injured areas were determined in five fields per liver.

2.3. Western blot analysis

Western blotting was performed as described previously [18]. Western blots were performed using the following antibodies; IDH2 [9], Ly6G (eBioscience, San Diego, CA), COX IV (Cell Signaling, Danvers, MA), Optic atrophy 1 (Opa1; BD bioscience, San Diego, CA), Fission 1 (Fis1; Sigma), Bax (EMD Millipore, Billerica, MA), Bcl-2 (EMD Millipore), Bcl-xL (BD bioscience), cleaved caspase-3 (Cell Signaling), β-actin (Sigma), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NOVUS, Littleton, CO) antibodies.

2.4. Immunohistochemical staining

Immunohistochemical staining was performed using anti-NADPH (1:100; Biorbyt, Cambridge, UK), anti-8-hydroxy-2′-deoxyguanosine (8-OHdG; Abcam, Cambridge, MA), and anti-F4/80 (Serotec, Oxford, UK) antibodies, as described previously [19].

2.5. Measurement of hydrogen peroxide and lipid peroxidation in the liver

Hydrogen peroxide levels in liver tissues were measured using the ferric sensitive dye, xylene orange, as described previously [20]. To determine the extent of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS, Sigma) were used as described previously [21].

2.6. Preparation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions were prepared as described previously [22]. These fractions were confirmed by western blotting using antibodies against GAPDH for the cytosolic fraction and COX IV for the mitochondrial fraction (Fig. 3C).
2.7. Measurement of the oxidized glutathione (GSSG) to total glutathione ratio in the liver

The ratio GSSG to total glutathione (GSH + GSSG) was measured using an enzymatic recycling method, as described previously[23,24].

2.8. Measurement of enzyme activities and ATP levels

The activities of IDH2, glucose-6-phosphate dehydrogenase (G6PD), and catalase were measured as described previously[25]. The ATP levels were measured using an ATP Colorimetric/Fluorometric Assay Kit (Abcam), according to the manufacturer’s instructions.

2.9. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The TUNEL assay was performed using an in situ cell death detection kit (Fluorescein, Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s instructions. TUNEL-positive cells were counted in 10 fields per liver.

2.10. Transmission electron microscopy (TEM)

One and five hours after ischemia or sham operations, livers were perfusion-fixed via the abdominal aorta with 2.5% glutaraldehyde, and then stored overnight in the fixative at 4 °C. Samples were cut into 1 mm² cubes, washed in 0.1 M phosphate buffer, and then post-fixed in aqueous 2% OsO₄ for 1.5 h. After three 0.1 M phosphate buffer washes, the samples were dehydrated through a graded series of 50–100% ethanol and 100% propylene oxide. The samples were then infiltrated with 1:1, 1:2, and 1:3 mixtures of propylene oxide:Epon® Resin 828 (Polysciences Inc, Warrington, PA) for 1 h each. After samples had been incubated in 100% Epon® Resin 828 for over 8 h, they were embedded in molds, and cured at 35 °C and 45 °C for 12 h, followed by additional hardening at 60 °C for 2 days. Ultrathin (60 nm) sections were double stained with 2% uranyl acetate and 1% lead citrate. Sections were photographed under a transmission electron microscope (H-7000, Hitachi, Japan) at 75 kV. Electron micrographs of mitochondria were captured from hepatocytes near the central vein. The mitochondrial aspect ratio [(major axis)/(minor axis)], the extent of mitochondrial abnormal enlargement, and the loss of cristae were determined using 30 mitochondria per cell.

2.11. Statistical analysis

Results are expressed as means ± SEM. Statistical differences among groups were calculated using Student’s t-test and two-way analysis of variance (ANOVA) for comparison between two groups. A p value of < 0.05 was regarded as statistically significant.

3. Results

3.1. Idh2 gene deficiency worsens HIRI

In order to evaluate whether Idh2 gene deletion affects HIR injury, Idh2 knockout (Idh2−/−) and wild-type (Idh2+/+) littermates were subjected to 60 min of partial hepatic ischemia followed by 5 h of reperfusion (HIR). HIR induced severe histological and functional damage to the livers of both Idh2+/+ and Idh2−/− mice, as reflected by the disruption in liver structure (Fig. 1A, B) and increased plasma AST.
Fig. 1C and ALT concentrations (Fig. 1D). Furthermore, HIR significantly increased the number of F4/80-positive cells (a marker protein for monocytes/macrophages) in the interstitial area of the liver (Fig. 1E, F). Moreover, HIR increased the expression of Ly6G, a marker protein for neutrophils, in the liver (Fig. 1G, H). These functional and histological changes and the increased inflammation after HIR were greater in the Idh2+/− mice than those in the Idh2+/+ mice (Fig. 1A–H). These results indicate that Idh2 gene deletion increased the susceptibility of liver to HIR insult.

3.2. HIR impairs the mitochondrial IDH2-NADPH producing system in the liver

To determine whether HIR affects the mitochondrial IDH2-NADPH producing system, we evaluated the expression and activity of IDH2 in the liver after HIR. First, we determined the effects of the Idh2 knockout and HIR on the expression and activity of IDH2 in the liver. Idh2 gene deletion reduced IDH2 expression to nearly non-detectible levels (Fig. 2A, B). Similarly, Idh2 knockout reduced IDH2 activity to
negligible levels in the whole lysate and mitochondrial fraction of the liver of sham-operated
Idh2\(^{-/-}\) mice and Idh2\(^{+/+}\) mice (Fig. 2C). The presence of both the mitochondrial and cytosolic fractions was
confirmed by COX IV and GAPDH expression, respectively; COX IV was detected in the mitochondrial fraction, whereas GAPDH was detected in the cytosolic fraction (Fig. 2D). HIR significantly decreased the IDH2 expression and activity in Idh2\(^{+/+}\) mice, but IDH2 expression and activity was almost non-detectible in Idh2\(^{-/-}\) mice (Fig. 2A–C). Next, we examined whether Idh2 gene deletion and HIR affect NADPH production in the liver. NADPH production was significantly reduced in the post-HIR livers of both Idh2\(^{-/-}\) and Idh2\(^{+/+}\) mice compared with that in livers of the sham-operated mice (Fig. 2E, F). This HIR-induced decrease in NADPH was much greater in the Idh2\(^{-/-}\) mice than in the wild-type littermates (Fig. 2E, F). The NADPH level in the liver of sham-operated Idh2\(^{+/+}\) mice was lower than that in the liver of sham-operated Idh2\(^{-/-}\) mice (Fig. 2E, F). Taken together, these data indicate that HIR causes IDH2 dysfunction and that IDH2 deficiency exacerbates HIR-induced impairments including a decrease in NADPH production.

3.3. HIR impairs the mitochondrial GSH antioxidant system

NADPH plays an important role in the GSH-associated antioxidant system by helping to reduce GSSG to GSH, and given this role, it is considered a critical agent in a cell’s fight against oxidative stress. Given this information, we first investigated the effects of Idh2 gene deletion and HIR on the mitochondrial GSH-associated antioxidant system. The ratio of GSSG to total glutathione (tGSH, GSSG + GSH) in the mitochondria increased after HIR and this increase was greater in the Idh2\(^{-/-}\) mice than the Idh2\(^{+/+}\) mice (Fig. 3A). However, there were no differences in the ratio of GSSG to tGSH between sham-operated Idh2\(^{-/-}\)
Liver sections were stained with PAS reagent and the damaged areas were evaluated. The results are expressed as the mean ± SEM. *, p < 0.05 vs. respective sham-operated mice.

Fig. 7. Effect of Mito-TEMPO treatment on the survival rate, and functional and morphological damage to the livers of idh2−/− and idh2+/+ mice after HIR. Mice were treated with mito-TEMPO (mito-T), twice, 17 h and 1 h before either hepatic ischemia or sham-operation and livers were harvested 5 h after surgery. (A) Cumulative survival rates were determined until 5 h after HIR. Data are expressed as the cumulated survival rates (B, C) Five hours after surgery, plasma concentrations of AST (B) and ALT (C) were determined. (D, E) Liver sections were stained with PAS reagent and the damaged areas were evaluated. The results are expressed as the mean ± SEM. †, p < 0.05 vs. respective vehicle-I/R. #, p < 0.05. NS, no significance.

3.4. Idh2 gene deletion augments mitochondrial oxidative stress after HIR

To further confirm that the impairment in the NADPH-GSH antioxidant system affects hepatocyte oxidative stress, we measured 1) the H2O2 level in the whole lysate, 2) protein oxidation by oxidized peroxiredoxin (Prx-SO3, a well-known indicator of oxidative damage) [28] in the mitochondrial fraction, 3) lipid oxidation, as indicated by MDA production in the whole lysate, and 4) DNA oxidation by the intracellular expression pattern of 8-hydroxy-2′-deoxyguanosine (8-OHdG). Here, we found that HIR led to the increases in H2O2 levels (Fig. 4A), 8-OHdG-positive signals (Fig. 4B–D), Prx-SO3 expression (Fig. 4E, F), and MDA levels (Fig. 4G) in both idh2−/+ and idh2−/− mouse liver, and these increases were greater in the idh2−/− mice than those in the wild-type littersmates (Fig. 4A–E). Taken together, these data indicate that a defect in IDH2 exacerbates HIR-induced oxidative injuries including increasing the oxidation of lipids, proteins, and DNA.

3.5. Idh2 knockout exacerbates mitochondrial injury after HIR

To confirm whether defects of the IDH2-NADPH-GSH-GPx system after HIR and IDH2 knockout affect mitochondrial injury, we evaluated the morphology of mitochondria by TEM at 1 and 5 h after HIR. TEM images indicated swelling, cristae loss, and fragmentation of mitochondria in the hepatocytes of both idh2−/+ and idh2−/− mice at both time points, with the condition worsening at 5 h post-reperfusion (Fig. 5A–D). Furthermore, the TEM images revealed that mitochondrial damage was most severe in the idh2−/− mice as compared to that in the idh2−/+ mice (Fig. 5A–D), indicating that mitochondria of idh2−/− mouse hepatocytes are more susceptible to HIR than those of the idh2−/+
Here, we also found that HIR shifted mitochondria into fission (Fig. 5A, D). This shift was greater in the Idh2−/− mice than in the Idh2+/+ mice (Fig. 5A, D). In addition, we found that HIR decreased the expression of mitochondrial dynamin like GTPase (Opa1, a mitochondrial fusion protein), whereas it increased the expression of fission 1 (Fis1, a mitochondrial fission protein) (Fig. 5E–G), and these HIR-induced changes were greater in the Idh2−/− mice than in the Idh2+/+ mice (Fig. 5E–G). However, we did not find any significant differences in the morphology, aspect ratio, and expression of Opa1 and Fis1 between sham-operated Idh2−/− and Idh2+/+ mouse livers (Fig. 5A–G).

When mitochondrial ATP production was assessed, we found that HIR decreased ATP levels significantly in both Idh2+/+ and Idh2−/− mice (Fig. 5H). These decreases were greater in the Idh2−/− mice than in the Idh2+/+ mice (Fig. 5H). However, we did not find any significant differences in the ATP levels between sham-operated Idh2−/− and Idh2+/+ mouse livers (Fig. 5H). These data indicate that Idh2 gene deletion exacerbates liver susceptibility to HIR.

3.6. Idh2 gene deletion exacerbates apoptosis

Typically, mitochondrial damage stimulates apoptotic signaling pathways [29]. Therefore, we evaluated whether IDH2 deficiency and HIR influence the activation of apoptosis. We found that the expression of cytochrome c in the mitochondrial fraction of liver lysate decreased in both Idh2+/+ and Idh2−/− mice after HIR, whereas the expression of...
cytochrome c in the cytosolic fractions increased in both \textit{idh2}+/- and \textit{idh2}–/– mice (Fig. 6A–C). Furthermore, we found that the release of cytochrome c was greater for the \textit{idh2}–/– mice than the \textit{idh2}+/- mice (Fig. 6A–C). This indicates that HIR induces cytochrome c release from the mitochondria into the cytosol and \textit{idh2} gene deletion augments this process. In addition, we found that HIR increased pro-apoptotic factors, Bax and cleaved-caspase 3 (c-casp. 3), in both \textit{idh2}+/- and \textit{idh2}–/– mice (Fig. 6D–I). Conversely, HIR suppressed the expression of anti-apoptotic factors Bcl-2 and Bcl-xl in both \textit{idh2}+/- and \textit{idh2}–/– mice (Fig. 6D–I). These post-HIR increases in pro-apoptotic factors and decreases in anti-apoptotic factors were greater in the \textit{idh2}–/– mice than in the \textit{idh2}+/- mice after HIR injury (Fig. 6D–I). Additionally, HIR increased TUNEL-positive cells in the livers of both \textit{idh2}+/- and \textit{idh2}–/– mice, and these increases were greater in the \textit{idh2}–/– mice than in the \textit{idh2}+/- mice (Fig. 6D, K). These results indicate that \textit{idh2} gene deletion exacerbates HIR-induced apoptosis.

3.7. \textit{mito-TEMPO}, a mitochondria-targeted antioxidant, mitigates HIR-induced liver injury and this mitigation is greater in \textit{idh2} gene-deleted mice than in \textit{idh2} wild type mice

In order to confirm that the increased susceptibility of \textit{idh2} knockout mice to HIR injury is due to a reduction in mitochondrial antioxidant capacity, we tested whether \textit{mito-TEMPO}, a mitochondria-targeted antioxidant molecule [17], mitigates the increased susceptibility due to \textit{idh2} deletion. \textit{Mito-TEMPO} treatment dramatically increased survival rates in both mice and this survival rate increase was greater in the \textit{idh2}–/– mice than in the \textit{idh2}+/- mice. Specifically, the survival rates increased from ~42% to ~85% for \textit{idh2}+/- mice and from ~93% to 100% for \textit{idh2}–/– mice (Fig. 7A). Moreover, \textit{mito-TEMPO} treatment mitigated post-HIR increases in AST and ALT concentrations but also defects in the antioxidant systems, leading to increased oxidative stress, but further studies would be needed to define the exact molecular mechanisms as to how HIR induces IDH2 dysfunction.

In addition, \textit{mito-TEMPO} significantly inhibited HIR-induced increases in mitochondrial \textit{H2O2} levels (Fig. 8A), Prx-SO3 expression (Fig. 8B, C), and \textit{8-OHdG}-positive cells (Fig. 8D, E) in both \textit{idh2}+/- and \textit{idh2}–/– mice. Again, these inhibitory effects of \textit{mito-TEMPO} were significantly greater in the \textit{idh2}+/- mice than in the \textit{idh2}–/– mice (Fig. 8A–E); Reductions in cisplatin-induced mitochondrial \textit{H2O2} production by \textit{mito-TEMPO} treatment were about 30% in the \textit{idh2}+/- mice and about 17% in the \textit{idh2}–/– mice (Fig. 8A); \textit{Prx-SO3} expressions after \textit{mito-TEMPO} were also dramatically reduced (about 64% in the \textit{idh2}+/- mice and about 38% in the \textit{idh2}–/– mice) (Fig. 8B). Furthermore, changes were more dramatically inhibited by \textit{mito-TEMPO} treatment in the \textit{idh2}+/- mice than in the \textit{idh2}–/– mice (Fig. 8F, G). These data indicate that the increased susceptibility of \textit{idh2} gene-deleted mice to hepatic injury is associated with the increased mitochondrial oxidative stress due to impairments in the mitochondrial antioxidant system. In conclusion, we suggest that HIR-induced hepatic cell damage is associated with impairments in the IDH2-NADPH-GSH axis and that IDH2 is critical for the maintenance of the mitochondrial redox state.

4. Discussion

In the present study, we report, for the first time, that HIR impairs the function of IDH2, leading to defects in the mitochondrial NADPH-GSH antioxidant systems, eventually resulting in mitochondrial oxidative stress, mitochondrial dysfunction, mitochondrial damage, the activation of apoptosis, and cell death. In addition, \textit{idh2} gene deficiency exacerbates HIR-induced mitochondrial oxidative stress and hepatic dysfunction and cell death. Furthermore, a mitochondria-targeting antioxidant treatment attenuates HIR-induced liver damage and this attenuation was greater in the \textit{idh2} knockout mice than in wild-type littermates. These results indicate that HIR injury is associated with the impairment of the IDH2-NADPH-GSH antioxidant system and that IDH2 is a critical part of mitochondrial antioxidant defense. Given these results, IDH2 could be considered a novel therapeutic target for HIR-induced liver failure.

Mitochondria inevitably produce ROS due to leakage from the electron transport chain during oxidative phosphorylation, and consequently this increases the production of superoxide radicals. Under normal conditions, the superoxide radicals in mitochondria are converted into \textit{H2O}2 via MnSOD and \textit{H2O}2 is further degraded to \textit{H2O} by GPx (about 70–80% of \textit{H2O}2) [30]. However, pathological conditions such as ischemia-reperfusion induces not only excessive ROS production but also defects in the antioxidant systems, leading to increased oxidation of proteins, lipids, and nucleic acids. These effects invariably lead to mitochondrial dysfunction and damage [30]. Furthermore, \textit{H2O}2 generated in mitochondria affect other intracellular organelles and surrounding cells. In mitochondria, GPx removes most of the \textit{H2O}2 via oxidizing GSH to form GSSG, which is subsequently reduced by GR that uses NADPH as the electron donor. In mitochondria, IDH2 catalyzes the reduction of \textit{NADP}+ to NADPH and is therefore a major provider of NADPH to GPx, glutathione, and Trx/Ptx systems for peroxide detoxification [31,32]. It has been reported that IDH2 dysfunction impairs mitochondrial redox balance, leading to a shift toward a more oxidized state where there is an increase in oxidative damage to mitochondrial components, mitochondrial membrane disruption, activation of apoptosis, and eventually cell death [9,12–14]. Evidence has shown that the pathogenesis of HIR injury is associated with excessive mitochondrial ROS generation that overwhelms the mitochondria’s antioxidant defense [5,33].

In the present study, HIR causes a reduction in IDH2 expression and activity, leading to a decrease in NADPH levels, an increase in GSSG/GSH, a decrease of GPx activity, and an increase in \textit{H2O}2 levels. Overall, these changes increase oxidative stress within the mitochondria and increases lipid, protein, and DNA oxidation. Thus, it appears that HIR-induced liver damage is associated with reduced IDH2 function. Supporting this, in the present study, knockout of \textit{idh2} exacerbated HIR-induced mitochondrial oxidative stress and increased mitochondrial damage and dysfunction. Furthermore, treatment with the mitochondria-targeted antioxidant, \textit{mito-TEMPO}, 17 h prior to and at the beginning of HIR protected mitochondria and the liver cells, and this protection was greater in the \textit{idh2} gene-deleted mice than in wild-type littermates. A previous study by Okatani et al. supported these results, as they determined that ischemia-reperfusion in the liver decreases mitochondrial GPx activity and causes mitochondrial disorganization, and that antioxidant treatment prevents these HIR-induced injuries [34]. In addition, Mukhopadhyay et al. reported that mitochondrial antioxidants, such as Mito-Q and Mito-CP, ameliorated HIR-induced apoptotic cell death and liver dysfunction by reducing mitochondrial damage and inflammatory responses [5].

Although we cannot provide an exact answer as to how HIR decreases IDH2 function and expression, it is clear that the two are closely linked. A possible answer may stem from HIR-induced mitochondrial oxidative stress, but further studies would be needed to define the exact molecular mechanisms as to how HIR induces IDH2 dysfunction.

Mitochondrial damage such as alterations in mitochondrial membrane integrity, fragmentation, and dysfunction, activate the release of pro-apoptotic substances such as cytochrome c and AIF from the mitochondrial intermembrane space into the cytoplasm, and this leads to the activation of apoptosis signaling pathways [35,36]. Oishi et al. reported that HIR induces apoptosis of hepatocytes by producing oxygen free radicals, and antioxidants effectively inhibit apoptosis and protect the liver against HIR insult [37]. Moreover, Frank et al. reported that mitochondrial oxidative stress disrupts mitochondrial membrane integrity, inducing the release of cytochrome c, and activating apoptosis in hepatocytes [38]. Consistent with these reports, the present study demonstrated that HIR-induced mitochondrial structural injury and
cytochrome c release were exacerbated in Idh2 knockout mice. This indicates that IDH2 plays an important role in preventing mitochondrial-associated apoptotic signaling events via maintaining mitochondrial redox balance.

Recently it has been reported that mitochondrial oxidative stress by ROS and ischemia-reperfusion induces a shift in mitochondria toward fission, which eventually leads to fragmentation [39]. Under normal physiological conditions, the fission and fusion of mitochondria are tightly regulated [40]. Excessive fission of mitochondria precedes cytochrome c release and subsequent apoptosis. Zhang et al. reported that the inhibition of fission due to the inhibition of Drp-1, a fission inducing protein, protects liver cells against apoptosis after HIR [41]. Furthermore, it has been reported that Drp-1 assists Bax in assembling into large ring-like structures in the mitochondrial outer membrane to facilitate the release of pro-apoptotic proteins such as cytochrome c from the mitochondria into the cytoplasm [42,43]. In addition, Frank et al. reported that the release of cytochrome c further promotes mitochondrial fission [38]. Moreover, recently it has been reported that Drp-1 and ROS stress activate mitophagy, a selective form of autophagy in mitochondrial degradation; autophagy aggravates hepatic ischemia-reperfusion injury [44], whereas inhibition of autophagy alleviates hepatic ischemia-reperfusion injury [45]. In the present study, HIR shifts mitochondria towards fission (as evidenced by an increased aspect ratio, increased Fis1 expression, and decreased Opal1 expression), along with increased cytochrome c release into the cytosol, and apoptosis. These HIR-induced alterations were much greater in the Idh2−/− mice than in the wild-type littermates. It indicates that mitochondrial oxidative stress after HIR shifts mitochondrial dynamics into fission, which can activate apoptosis in hepatocytes. Further supporting this is the fact that a mitochondria-targeting antioxidant treatment prevents the increase of Fis1 expression and mitochondrial fission, which is accompanied by decreased liver dysfunction and mortality in mice. Furthermore, the protective effects of a mitochondria-targeting antioxidant were more prominent in the Idh2−/− mice than in the wild-type littermates.

Taken together, our results demonstrate that HIR impairs the IDH2-associated mitochondrial antioxidant system, resulting in mitochondrial oxidative stress, disruption of mitochondrial function, activation of apoptosis, and overall liver dysfunction. This suggests that IDH2 may be a critical enzyme for maintaining mitochondrial redox balance and may be a useful target protein for the development of therapeutics against liver injury.

Conflict of interest

None declared.

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