Original Article

Short-term starvation attenuates liver ischemia-reperfusion injury (IRI) by Sirt1-autophagy signaling in mice

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Abstract: Calorie restriction or starvation (fasting) has some beneficial effects in terms of prolonging life and increasing resistance to stress. It has also been shown that calorie restriction has a protective role during ischemia-reperfusion injury (IRI) in several organs, but the underlying mechanism has not been elucidated. In this study we investigated the effects and molecular mechanisms of short-term starvation (STS) on liver IRI in a mouse liver IRI model. We found that STS significantly attenuated liver IRI in this model, as evidenced by inhibition of serum aminotransferase levels, and decreased pathological damage and hepatocellular apoptosis, especially after 2- or 3-day starvation. Furthermore, we found that 2- or 3-day starvation induced expression of hepatocellular autophagy in vivo and in vitro. Further experiments provided support for the notion that STS-induced autophagy played a key role during starvation-regulated protection against liver IRI via autophagy inhibition with 3-methyladenine. Interestingly, the longevity gene Sirt1 was also significantly up-regulated in liver after STS. Importantly, inhibition of Sirt1 by sirtinol abolished STS-induced autophagy and further abrogated STS-mediated protection against liver IRI. In conclusion, our results indicate that STS attenuates liver IRI via the Sirt1-autophagy pathway. Our findings provide a rationale for a novel therapeutic strategy for managing liver IRI.

Keywords: Short-term starvation, autophagy, liver, ischemia reperfusion injury, Sirt1

Introduction

Temporary interruption of hepatic blood flow is usually required during liver resection or transplantation; however, this process, when accompanied by ischemia and subsequent reperfusion, ultimately leads to liver injury. Ischemia-reperfusion injury (IRI) is a common clinical problem associated with acute liver dysfunction and failure, acute graft rejection, and chronic liver dysfunction [1, 2], but no effective therapy is available to prevent or treat this clinical condition. The pathogenesis of IRI involves a two-stage process: an initial lack of blood flow leads to oxygen and nutrient deprivation in hepatic tissue that is chiefly characterized by ATP depletion. Blood reperfusion causes further damage via oxidative stress and then via inflammatory mediators in the reperfusion stage [3-5]. Liver IRI is characterized by progressive hepatocellular injury, hepatocellular apoptosis/necrosis, and acute inflammatory responses during pathogenesis.

Calorie restriction or starvation (fasting) has beneficial effects, the most remarkable of which is its impact on longevity. Many studies have demonstrated that calorie restriction extends lifespan in a variety of species ranging from yeast to primates [6-8]. The mechanisms underlying prolongation of life are thought to involve changes in energy production and utilization, handling of oxidative stress, insulin sensitivity, inflammatory responses, and alterations in the communication between cells and organs [9, 10]. In addition to extending lifespan, calorie restriction can increase resistance to multiple forms of acute stress. In rodents, calorie restriction enhances resistance to paraquat toxicity and IRI [11, 12]. Thus, calorie restriction
may effectively improve the outcome of IR-associated post-operative complications. Dietary restriction (DR) is commonly used as a calorie restriction method, but cannot be used in the clinical setting because of the length of time required. Fortunately, STS or fasting can rapidly induce similar benefits to long-term DR in terms of gene expression, physiology, and stress resistance [13]. Fasting for 3 days is as effective as 1 month of DR in reducing IRI. Importantly, STS or fasting may be a feasible strategy for reducing liver IRI in the clinical setting. Although previous studies using liver ischemic models have demonstrated some beneficial effects of pre-operative DR/fasting in liver IRI, the underlying mechanism is not clear and some findings are contradictory [13-15]. To provide further insight into the clinical significance of DR/fasting, the mechanisms underlying starvation-related protection against liver IRI need to be elucidated.

In the present study, we determined whether and how STS attenuated liver IRI. Using the classic model of warm ischemia in liver, we demonstrated that: 1) STS effectively attenuated liver IRI by increasing anti-apoptosis and inhibiting hepatocellular apoptosis; 2) starvation-induced autophagy played a critical role in STS-mediated protection against liver IRI; and 3) Sirt1 was a key molecule during STS-induced autophagy and STS-mediated protection against liver IRI.

Materials and methods

Animal studies

Wild-type C57BL/6 mice were purchased from the Laboratory of Animal Resources of Nanjing Medical University (NMU). Male 8-week-old mice were used in the study. The animal protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (protocol number NMU08-092). All animal procedures were carried out in accordance with approved guidelines.

Warm liver IRI model

As previously described [4], an atraumatic clip was used to interrupt the artery/portal vein blood supply to the left and middle liver lobes for 90 min under isoflurane/O₂ inhalation anesthesia. Mice were sacrificed 6 h after reperfusion, and blood and liver tissue samples were harvested for analysis. Sham controls were subjected to the same procedure but without vascular occlusion. Some mice were starved for 1, 2, or 3 days prior to ischemia.

Isolation and culture of mouse hepatocytes

Mouse hepatocytes were isolated using a two-step in situ collagenase perfusion procedure [16]. Livers from C57BL/6 mice were perfused in situ through the portal vein with ethylene glycol tetraacetic acid (EGTA) buffer (0.5 mM EGTA, 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 0.65 mM MgSO₄, and 10.07 mM HEPES, pH 7.4) at a flow rate of 5 ml/min for 10 min, followed by collagenase buffer (67 mM NaCl, 6.7 mM KCl, 4.76 mM CaCl₂, 0.035% collagenase type II, and 10.07 mM HEPES, pH 7.6) at a flow rate of 5 ml/min for 15 min. After centrifugation, the hepatocytes were collected and seeded in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml).

Serum biochemical measurements

Serum levels of alanine aminotransferase (sALT) and aspartate aminotransferase (sAST) were measured with an AU5400 automated chemical analyzer (Olympus, Tokyo, Japan).

Histopathology

Liver specimens were fixed with 10% neutral formaldehyde and then embedded in paraffin. The specimens were sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (HE) for histopathologic analysis by light microscopy. Sections were scored on a scale from 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchyma, as described by Suzuki et al. [17]. LC3B was detected in liver specimens using immunohistochemistry staining as previously described [18].

Caspase-3 activity

Caspase-3 activity was measured in liver tissues using dedicated assay kit (Jiancheng Biotechnology, Nanjing, China) according to the manufacturer’s instructions.

TUNEL staining

Paraffin sections (4 μm) were stained via TUNEL using a commercially available kit (In Situ Cell
Death Detection kit, Roche-Boehringer, Mannheim, Germany).

Western blot analysis

Protein samples (30 μg) from cell culture or liver tissue were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Primary antibodies directed against BCL-2, BCL-xI, P-Akt, cleaved caspase-3, LC3B, P62, and β-actin (Cell Signaling Technology, San Diego, CA, USA), and Sirt1 (Abcam, Shanghai, China) were used. The blots were exposed to Kodak XAR autoradiographic film and then visualized using a chemiluminescent detection system (ECL Substrate Western Blot Detection system, Pierce, Rockford, IL, USA). β-Actin expression served as an internal control. Images representative of three experiments are shown.

Quantitative real-time PCR

Total RNA (2.5 μg) was reverse-transcribed to cDNA using a SuperScript III System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using SuperMix (Platinum SYBR Green qPCR kit, Invitrogen) in a DNA Engine system with Chromo 4 Detector (MJ Research, Waltham, MA, USA) as previously described [18]. Primer sets (sense and anti-sense sequences) for the genes were as follows: HPRT forward, 5’-TCA ACG GGG GAC ATA AAA GT-3’, reverse, 5’-TGC ATT GTT TTA CCA GTG TCA A-3’; Sirt1 forward, 5’-GCC TCT TCT CAT TCC TGC TGT T-3’, and reverse, 5’-TTG AGA TCC ATG CCG TTG-3’.
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**Statistical analysis**

Data are presented as the mean ± standard deviation (SD) for at least three independent experiments. The Mann-Whitney U test was used for comparison of two groups. All P values were two-sided, and P<0.05 was considered statistically significant.

**Results**

**STS attenuates liver IRI**

To determine the effects of STS on liver IRI, we starved mice for 1 day (1d-ST), 2 days (2d-ST), or 3 days (3d-ST), when they had access to drinking water only, before establishing a warm ischemia liver model. Liver injury was then assessed in terms of hepatocellular function and histologic analysis after 6 h of reperfusion. As shown in Figure 1A and 1B, sALT and sAST levels were significantly lower in 1d-ST, 2d-ST, and 3d-ST mice compared to controls (P<0.05), especially in 2d-ST and 3d-ST mice (P<0.01). These serum transaminase changes were in agreement with liver pathology results. The control group showed marked liver sinusoidal congestion and vacuolization, severe edema, and extensive hepatocellular necrosis, all of which were significantly improved in the 1d-ST, 2d-ST, and 3d-ST groups (Figure 1C). The histologic parameters observed in the control, 1d-ST, 2d-ST, and 3d-ST groups were in accordance with Suzuki et al. [17], scored as 3.67±0.21, 2.50±0.43, 1.00±0.26, and 1.16±0.31, respectively (P<0.05; Figure 1D).

Figure 2. STS increases anti-apoptosis and inhibits hepatocellular apoptosis in liver subjected to ischemia-reperfusion stress. Groups of mice starved for 1 day (1d-ST), 2 days (2d-ST), or 3 days (3d-ST) were subjected to 90 min of partial liver ischemia, followed by a 6-h reperfusion. A: Western blot analysis of BCL-2, BCL-xl, P-Akt, Cleaved caspase-3 and β-actin; gels were run under the same experimental conditions. Data are representative of three independent experiments. B: Caspase-3 activity. C: Hepatocellular apoptosis was analyzed in terms of TUNEL staining. D: Apoptotic cells were quantified in six high-power fields (400×) and expressed as a percentage of total cells. (mean ± SD; **, P<0.01; *, P<0.05 vs. control group).
These results indicate that starvation for 1, 2, and 3 days effectively attenuates liver IRI, especially in 2d-ST and 3d-ST mice.

**STS increases anti-apoptosis and inhibits hepatocellular apoptosis in IR-stressed livers**

We determined the effects of STS on hepatocellular apoptosis induced by IR. Anti-apoptotic proteins (BCL-2, BCL-xl and P-Akt) were detected by western blot analysis. Figure 2A showed that the expression of BCL-2, BCL-xl and P-Akt was significantly higher in the starvation-treated groups than in the control group. In addition, 2d-ST and 3d-ST mice had higher expression of BCL-2, BCL-xl and P-Akt than 1d-ST mice, but there was no significant difference between 2d-ST and 3d-ST mice. In contrast to anti-apoptotic proteins, expression of cleaved capase-3, a pro-apoptotic protein, was effectively inhibited in the starvation-treated groups (Figure 2A). In addition to the western blots, Figure 2B showed that caspase-3 activity in ischemic liver was significantly inhibited by starvation treatment for 1, 2, and 3 days compared to the control group (2.97±0.59, 1.27±0.45, and
1.40±0.23 vs. 3.92±0.54). To further determine the status of hepatocellular apoptosis, ischemic livers were analyzed by TUNEL staining, which revealed that STS markedly decreased the frequency of TUNEL-positive cells compared to the control group (Figure 2C). The frequency of TUNEL-positive cells in total hepatocytes was 43.17±6.63%, 17.50±3.63%, 18.53±3.34%, and 67.17±7.80% in the 1d-ST, 2d-ST, 3d-ST, and control groups, respectively (Figure 2D), supporting the notion that hepatocellular apoptosis was significantly inhibited by STS treatment. These findings indicate that STS significantly increases anti-apoptosis and inhibits apoptosis in ischemic liver, especially in 2d-ST and 3d-ST mice.

**STS induces autophagy in liver**

It has been reported that calorie restriction or fasting/starvation can induce autophagy in various organs [19, 20]. Furthermore, autophagy may protect against IRI in heart, kidney, and liver [20-22]. We investigated whether STS induced autophagy in liver tissues. Because of the data reported above, we chose 2d-ST mice for this analysis. LC3B and P62 markers of autophagy in liver were measured by western blotting. As expected, starvation for 2 days significantly increased LC3B expression (2.38±0.23 vs. 1.00±0.13; P<0.01) and decreased P62 expression (0.50±0.07 vs. 1.00±0.15; P<0.05) compared to controls (Figure 3A). To confirm the western blotting results, autophagosomes were measured via LC3B staining, which revealed that starvation effectively increased LC3B staining compared to controls (Figure 3B). In addition, we determined whether STS induced autophagy in hepatocytes in vitro. Hepatocytes were cultured in complete medium or glucose-deprived (GD) medium for 2 days. Figure 3C showed that GD medium significantly increased LC3B expression (2.38±0.23 vs. 1.00±0.13; P<0.01) and decreased P62 expression (0.50±0.07 vs. 1.00±0.15; P<0.05). These data strongly support the notion that STS effectively induces autophagy in liver.
Autophagy is critical for STS-mediated protection against liver IRI

We determined whether STS-induced autophagy regulated STS-mediated protection against liver IRI. We injected 3-methyladenine (3-MA) to block autophagy expression in 2d-ST mice. Figure 4A showed that starvation for 2 days markedly attenuated IR-enhanced sALT (8824.2±2470.6 vs. 876.4±129.2; P<0.01), but the beneficial effect was effectively abolished by 3-MA treatment (4821.3±946.2 vs. 876.4±129.2; P<0.01). These data were consistent with HE staining, which showed that starvation for 2 days effectively reduced IR-induced edema, sinusoidal congestion, structural damage, and hepatocellular necrosis, but 3-MA treatment abolished these effects in ischemic liver (Figure 4B, 4D). Suzuki scores for the IR, ST+IR, and 3-MA+ST+IR groups were 3.6±0.3, 1.5±0.2, and 3.2±0.3, respectively. We further analyzed hepatocellular apoptosis in ischemic livers via TUNEL staining (Figure 4C, 4D), which revealed that 3-MA almost reversed the starvation-decreased frequency of TUNEL-positive cells in ischemic liver. The frequency of TUNEL-positive cells was 60.17±7.36%, 24.00±3.51%, and 54.33±5.89% in the IR, ST+IR, and 3-MA+ST+IR groups, respectively. Taken together, the above findings demonstrate that autophagy is critical for STS-mediated protection against liver IRI.

STS-induced autophagy is mediated by Sirt1 in the liver

Calorie restriction or fasting can induce expression of Sirt1 in some organs. Sirt1 may be an important molecule during starvation-induced autophagy [23]. We first determined whether STS increased Sirt1 expression in liver tissues. Sirt1 induction increased with starvation time, and Sirt1 mRNA expression was significantly up-regulated in the 2d-ST and 3d-ST groups. As shown in Figure 5A, Sirt1 mRNA expression was increased 1.6-fold after 1-day, 2.5-fold after 2-day, and 2.0-fold after 3-day starvation. Western blotting confirmed these data; Sirt1 protein expression was markedly enhanced after 2- and 3-day starvation (Figure 5B). To determine whether or not Sirt1 mediates STS-induced autophagy, we injected mice with sirtinol (a Sirt1 inhibitor) before and during starvation. Figure 5C showed that sirtinol effectively inhibited STS-induced LC3B expression and stabilized P62 expression in liver. Immunohistologic staining further confirmed that STS-induced autophagy was mediated by Sirt1 (Figure 5D).

STS-mediated protection is regulated by Sirt1 during liver IRI

We investigated whether Sirt1 regulated STS-mediated protection against liver IRI. We administrated sirtinol to inhibit STS-induced Sirt1 expression before and during starvation. As expected, sirtinol almost neutralized starvation-mediated protection against liver IRI. Figure 6A and 6B showed that sirtinol effectively restored IR-enhanced sALT (2949.0±920.9 vs. 8006.0±1291.0; P<0.01) and sAST (4060±965.3 vs. 8854.0±2279; P<0.01) from starvation treatment. Consistent with biochemical markers, HE staining showed that the sirtinol treatment group had similar histologic damage as the IR group. These results demonstrate that Sirt1 plays a vital role during STS-mediated protection against liver IRI.

Discussion

Calorie restriction has beneficial effects in terms of extending lifespan and increasing resistance to multiple forms of stress. The role of calorie restriction via fasting/starvation or DR in IRI has been investigated, but different groups have obtained conflicting results. Most studies confirm that fasting/starvation or DR plays a protective role by up-regulating baseline levels of the anti-oxidant enzymes SOD2, Gpx1, and GSR and the stress response gene HO-1, and down-regulating circulating HMGB-1 [13, 14, 23], but Domenicali et al. reported that food deprivation is associated with greater mitochondrial oxidative injury after warm IR [15]. We investigated the role of STS (1-, 2-, and 3-day starvation) and found that STS effectively attenuated liver IRI. STS markedly inhibited IR-increased sALT and sAST, and improved ischemic liver damage, especially in the 2d-ST and 3d-ST groups (Figure 1). Consistent with most published data, fasting for 2 and 3 days offered greater protection against liver IRI than starvation for 1 day [13, 15]. However, Clavien et al. [23] reported that fasting for 1 day, but not for 2 or 3 days, significantly decreased liver...
IRI. The discrepancy between our data and those reported by Clavien et al. [23] on the role of short-term fasting may be related to differences between the models used.
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Apoptosis/necrosis is a key mechanism for cell death in liver IRI and directly indicates the extent of liver damage. STS plays multiple roles in anti-apoptosis and pro-apoptosis functions in different models. In colon cancer models, STS down-regulates glycolysis and glutaminolysis, increases OXPHOS and OCR, reduces APT synthesis, enhances ROS production, and promotes cellular apoptosis [24]. By contrast, intermittent fasting may act directly on cardiac myocytes to increase resistance to apoptosis in myocardial infarction models [25, 26]. The question arises as to whether STS regulates anti-apoptotic and pro-apoptotic pathways during liver IRI. We showed that STS significantly increased expression of the anti-apoptotic protein BCL-2/BCL-xL/P-Akt, decreased protein expression of cleaved caspase-3, and inhibited caspase-3 activity (Figure 2). These data were further confirmed by TUNEL assays for ischemic livers, consistent with other studies demonstrating that starvation induces the expression of genes involved in anti-apoptosis [25, 26]. In addition, inflammatory responses play a key role in the pathogenesis of hepatocellular apoptosis during liver IRI, especially the innate...
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Several studies have reported that starvation, fasting, or DR may induce expression of autophagy in different organs, including liver, brain, heart, and kidney [19-22]. Consistent with literature data, we demonstrated that starvation for 2 days effectively increases hepatocellular autophagy in vivo and in vitro (Figure 3). Autophagy is a bulk degradation pathway responsible for degrading protein aggregates and damaged organelles. The cross-talk between autophagy, a pathway that functions primarily in cell survival, and apoptosis, a pathway that invariably leads to cell death, is complex. The pro-survival function of autophagy has been demonstrated at cellular and organism levels in different contexts, including nutrient and growth factor deprivation, endoplasmic reticulum stress, development, microbial infection, and diseases characterized by accumulation of protein aggregates [28-31]. We investigated whether autophagy regulates apoptosis and starvation-mediated protection against liver IRI. Our results show that 3-MA nearly abolished starvation-mediated protection and restored sALT and ischemic liver damage to IR-induced high levels (Figure 4). In addition, TUNEL staining directly reflected the induction of autophagy as an anti-apoptotic function (Figure 4). These data show that autophagy induction is critical for starvation-mediated protection against liver IRI.

In conclusion, our study demonstrated that STS attenuates hepatocellular death/apoptosis during liver IRI. Fasting activated Sirt1 signaling, induced autophagy, and promoted anti-apoptosis, resulting in local cytoprotection against multiple damaging factors. By identifying the mechanisms involved in starvation-mediated Sirt1-autophagy signaling in regulating liver IRI, our study provides a rationale for novel therapeutic management of hepatic damage triggered by IR.

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Disclosure of conflict of interest

None.

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