Research Article

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Inhibition of glycogen synthase kinase 3 ameliorates liver ischemia/reperfusion injury via an energy-dependent mitochondrial mechanism

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Background & Aims: The mechanisms of glycogen synthase kinase-3 (GSK-3)-mediated cytoprotection during liver ischemia/reperfusion (I/R) remain controversial, particularly in older organs. This study explores the role and potential mechanisms of GSK-3 in young and aging livers.

Methods: A rodent partial warm I/R model was used to evaluate the therapeutic potential of GSK-3 modulation during hepatic I/R in young and aging Sprague-Dawley rats.

Results: GSK-3 inhibition through IPC or SB216763 (SB21) preconditioning protected young rats from I/R-induced liver injury. This protection was absent in old animals but could be restored by glucose infusion prior to the I/R insult. The protection conferred by GSK-3 inhibition depended on mitochondrial metabolism regulation. Indeed, the inhibition of GSK-3 suppressed mitochondrial permeability transition pore (MPTP) opening, triggering mitohormesis in young animals, whereas insufficient fuel suppressed mitochondrial metabolism and inactivated the GSK-3-related protection in old animals. SB21 and glucose reactivated the mitochondrial F_0F_1-ATPase and subsequent protective cascades in the senescent liver. These effects were antagonized by an ATPase inhibitor and by an MPTP opener.

Conclusions: The protection conferred by GSK-3 inhibition during hepatic I/R insult is energy dependent, particularly in senescent livers. These findings demonstrate a key role for GSK-3-related mitochondrial energy homeostasis, which may shed new light on the clinical use of GSK-3 inhibitors to protect liver function in surgical settings, particularly for older patients.

Keywords: Mitochondria; Signal transduction; Ischemia/reperfusion injury; Reactive oxygen; Energy metabolism.

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Abbreviations: I/R, ischemia/reperfusion; IPC, ischemia preconditioning; SB21, SB216763; Oli, oligomycin; Atr, atractyloside; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MPTP, mitochondrial permeability transition pore; Akt, protein kinase B; GSK-3, glycogen synthase kinase-3; phospho-GSK-3, phosphorylated glycogen synthase kinase-3; ATP, adenosine triphosphate; PI3K, phosphoinositide 3′-OH kinase; ERK1/2, extracellular regulated protein kinases; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; H-E, hematoxylin-eosin; CRC, calcium retention capacity; ANT, adenine nucleotide translocase; CyP-D, cyclophilin D.

Introduction

Ischemia/reperfusion (I/R) injury is a common clinical problem in liver transplantation, partial hepatic resection and trauma settings [1,2]. Unfortunately, there are few effective therapies aimed at preventing or treating this devastating syndrome. Recent studies have consistently demonstrated that glycogen synthase kinase-3β (GSK-3β) inhibition can protect the heart and liver against I/R-induced injury [3–6]; however, the underlying molecular mechanisms remain unknown. GSK-3 is a serine/threonine kinase with two ubiquitously expressed homologs, GSK-3α (51 kDa) and GSK-3β (46 kDa). GSK-3 is normally active in unstimulated cells, and GSK-3 inactivation is regulated by serine phosphorylation [7]. Several studies have suggested that GSK-3β inhibition by small molecules or peptide inhibitors induces GSK-3β phosphorylation at serine 9, which then protects the myocardium against I/R-induced injury [4,5] by regulating the induction of mitochondrial permeability transition pore (MPTP) opening, a key step in triggering mitochondria-mediated cell death [8]. More recently, GSK-3 inhibition was also shown to provide hepatoprotection against I/R injury in mice [3,6,8]. We recently demonstrated that flurbiprofen, a COX inhibitor, protected mice from I/R-induced liver injury by increasing GSK-3β phosphorylation and inhibiting MPTP opening [6]. Verola *et al.* reported a similar effect using indirubin-3′-oxime, a GSK-3β inhibitor [3]. In contrast, Ren *et al.* suggested that an immune regulatory mechanism plays a role in the same model [8]. Therefore, the exact mechanisms underlying this hepatoprotective effect during the course of I/R remain unclear.

Given the dramatic increase in the number of older patients undergoing hepatic surgery because of liver diseases [9,10], an effective strategy to ameliorate I/R-induced injury in senescent livers is desirable. Importantly, older patients appear to be more sensitive to I/R injury, likely because of the aging-related decrease in mitochondrial function, including alterations in mitochondrial membrane potential and impairments in oxidative function, in several tissues [11,12]. Ischemia preconditioning (IPC) is usually considered an endogenous protective mechanism that preserves mitochondrial function and bioenergetics in the liver [13]. However, IPC aggravates I/R injury in senescent livers [14]. Recently, decreased GSK-3 phosphorylation was found to be accompanied by I/R-induced liver injury in our study on human allograft biopsy specimens, and increased vacuolization was observed in aging allografts (Supplementary Fig. 1). Therefore, the present study was conducted in a rat model to fully demonstrate the impact of GSK-3 signaling on I/R injuries in both young and aging livers.

Materials and methods

Animals and surgery

The experiments were performed using 6-week-old (young group, 200–220 g) and 72-week-old (old group, 450–520 g) male Sprague–Dawley rats (Sino-British SIPPR/BK Lab Animal Ltd., Shanghai, China). The animals received humane care, and all experiments were conducted in accordance with the guidelines of the local Institutional Animal Care and Use Committee. The experimental design was as follows (see Supplementary Fig. 2): young or old rats were randomly divided into sham, I/R, IPC, and GSK-3 inhibitor (SB216763 [SB21]) preconditioning groups. All animals were fasted for 12 h prior to surgery and subsequently anaesthetized with pentobarbital (40 mg/kg) intraperitoneally. The left femoral vein was exposed and cannulated to infuse 0.9% saline and drugs. A model for partial warm I/R in the liver was established as described previously [15]. The sham-operated rats underwent the same surgical procedures but without vascular occlusion. The IPC rats were subjected to 10 min of ischemia and 10 min of reperfusion before the 40-min ischemic insult. To inhibit GSK-3 kinase activity, rats were intravenously administered a single dose of SB21 (0.6 μg/g; Sigma, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO) 15 min prior to the onset of liver ischemia. Additionally, to enhance GSK-3-related glycogen synthesis, a single dose of D-glucose (0.7 mg/g) was slowly infused intravenously 20 min before ischemia and stopped prior to reperfusion in the old rats ("glc"). To inhibit F_0F_1-ATPase activity or increase MPTP susceptibility, rats were intraperitoneally administered a subtoxic dose of oligomycin (0.06 μg/g, Sigma) dissolved in 45% ethanol or atractyloside (10 μg/g, Enzo Life Sciences, Plymouth Meeting, PA, USA) dissolved in PBS, respectively, at the beginning of the reperfusion and every 6 h thereafter. The animals were euthanized 4 h or 24 h after the reperfusion; liver and serum samples were collected for further analysis.

Western blot analysis and GSK-3 kinase assays

The phospho-GSK-3α (Ser21) and phospho-GSK-3β (Ser9) (Cell Signaling Technology, Boston, MA, USA) levels were determined in the rat liver lysates using Western blot analysis, as previously reported [6]. GSK-3 activity was analysed in the rat tissue extracts after 4 or 24 h of reperfusion, as previously described [16].

Histochemical analysis

Liver samples were fixed with formalin and embedded with paraffin. Hepatocellular necrosis was determined in hematoxylin and eosin (H&E)-stained tissue using a semiquantitative scale by a point counting method as previously described [15]. Briefly, a total of 30 random fields were counted for each H&E-stained tissue sample under high power magnification (400×) in a blinded fashion to determine the percentage of necrotic cells. In this study, only grade 3 injury with disintegration of hepatic cords was counted as necrosis.

Plasma biochemical assays

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured after 4 h and 24 h of reperfusion with an AutoAnalyzer (H-7600, Hitachi Ltd., Tokyo, Japan).

Mitochondria isolation, calcium retention capacity (CRC) assessment and reactive oxygen species (ROS) generation analysis

Mitochondria were isolated through gradient centrifugation as previously described [6]. The CRC was determined as described by Ichas *et al.* [17]. CRC was defined as the amount of Ca^{2+} required to trigger massive Ca^{2+} release by isolated liver mitochondria. The extramitochondrial Ca^{2+} concentration was recorded using a fluorescence microplate reader controlled by SOFTmax PR software (Molecular Devices, Sunnyvale, CA, USA) in the presence of 1 μmol/L Calcium Green-5N (Invitrogen, Carlsbad, CA, USA); the excitation and emission wavelengths were 500 and 530 nm, respectively, as previously described [6]. Mitochondrial ROS production was determined fluorometrically using the same equipment; detailed information is available in the Supplementary materials and methods. The results are expressed as relative fluorescence units (RFUs).

Tissue SOD and CAT activity assay

SOD activity was assayed using commercially available kits (Sigma) and processed according to the manufacturer's instructions. CAT activity was determined according to Johansson *et al.* [18]. One unit of CAT was defined as the amount of enzyme that was capable of degrading 1 μl of H_2O_2 per min.

ATP concentration and F_0F_1-ATPase (complex V) activity assay

Liver tissue ATP concentrations were measured using luciferase-luciferin assays with a clarity luminescence microplate reader (Bio-Tek, Winooski, VT, USA), as previously described [14]. Mitochondrial F_0F_1-ATPase activity was measured using a MitoProfile® Rapid Microplate Assay Kit (Mitosciences, Eugene, OR, USA) according to the manufacturer's instructions. The relative enzyme activity was expressed in proportion to that of the sham controls in the same age group.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The statistical analysis was performed using a 2-way analysis of variance (ANOVA), and the tested groups were compared using Least Significant Difference (LSD) tests, with $p < 0.05$ considered statistically significant.

Results

Inhibition of GSK-3 by IPC and SB21 preconditioning and D-glucose infusion ameliorates I/R-induced liver injury in old rats

Given that inhibition of GSK-3, by IPC or the GSK-3 inhibitor SB21, has been suggested to protect animals against I/R-induced liver injury and the protective effect of IPC is more efficient in the rat model of 40 min of ischemia with reperfusion [19–21], we compared the impact of IPC and SB21 pretreatment on I/R-induced liver injury in young (6 weeks old) and old (72 weeks old) rats. As shown in Fig. 1, IPC or SB21 pretreatment markedly reduced GSK-3 kinase activity (Fig. 1A) and enhanced GSK-3α/β (Ser21/9) phosphorylation (Fig. 1B) in both young and old rats. A perceptible difference between young and old sham-operated rats was not detected via H&E staining, suggesting that aging did not affect hepatocyte morphology. Notably, pretreating young rats with IPC or SB21 significantly attenuated I/R-induced liver necrosis over 24 h of reperfusion (Fig. 2A and B) and decreased serum ALT and AST (Fig. 2C and D; Supplementary Fig. 3) over 4 h and 24 h of reperfusion. Remarkably, this protective effect