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# Ischemic Preconditioning protects hepatocytes from ischemia-reperfusion injury via TGR5-mediated anti-apoptosis

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ABSTRACT

Ischemic preconditioning (IP) has been shown to protect hepatic tissue from liver ischemia-reperfusion injury (IRI). TGR5, as a new-type bile acid receptor, has been shown protective roles in several liver diseases. However, the relationship between TGR5 and IP is still unknown. This study investigated effects of IP on TGR5 as well as the roles of TGR5 on hepatic tissue lesions and apoptosis in liver IRI. We showed that TGR5 was significantly upregulated in liver tissues after IP. To further analyzed effects of the TGR5 on liver IRI, wild type and TGR5 knockout mice were used to establish the liver IRI model. IP effectively alleviated liver IRI, but TGR5 deficiency significantly neutralized IP-related liver protection, as evidenced by serum alanine aminotransferase levels, histological liver damage, hepatocellular apoptosis and cytokines expressions. In addition, molecules related to apoptosis were detected by Western Blot, which showed that activation of TGR5 by IP increased expression of Bcl-2, and inhibited expressions of IRAK4 and cleaved caspase-3, but TGR5 deficiency abolished IP-induced expressions of anti-apoptosis molecule. In vitro, effects of TGR5 on hepatocytes were further analyzed by TGR5 agonist (INT-777) and hypoxia/reoxygenation (H/R), which displayed that INT-777 markedly attenuated H/R-induced hepatocellular apoptosis. In conclusion, our study indicates that IP alleviates hepatocellular apoptosis, and reduces liver IRI through TGR5-mediated anti-apoptosis functions.



## 1. Introduction

TGR5 displays various levels of expression in many different organs [1]. Expressions of TGR5 were found in macrophages, cholangiocytes, hepatocytes, etc [2–4]. Many studies have shown that TGR5 can regulate BA homeostasis, cholangiocyte proliferation, and inflammatory responses [5–7]. In addition, TGR5 holds a powerful anti-apoptosis effect in cholangiocytes, hepatocytes, etc [4,6,7]. Study demonstrated that activation of TGR5 by its agonist INT-777 effectively decreased the LPS-triggered inflammatory response and apoptosis in hepatic tissue [8]. The latest study has suggested that Kupffer cell function may be suppressed by TGR5 in response to BA treatment [1,8]. Although the protective role of TGR5 in the inflammatory response has been studied, the specific mechanisms of its anti-apoptosis functions are still unclear.

Ischemia-reperfusion injury (IRI) is regarded as an important influencing factor in liver transplantation [9–14]. IRI can induce about 10% of failure of early graft liver transplantation [15]. To inhibit the destructive effect of IRI, several strategies have been developed to protect the liver from IRI. Ischemic preconditioning (IP) is the most studied method of mechanical preconditioning. Using this method, the liver is more effective in resisting a longer ischemic and reperfusion period [16,17].

As described above, TGR5 can protect liver tissues injury from liver diseases and IP can effectively alleviate liver impairment in liver IRI. However, the relationship between TGR5 and IP is still unclear. This study investigated effects of IP on TGR5 as well as the roles of TGR5 on hepatic tissue lesions and apoptosis in liver IRI.

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Our results demonstrate that IP activates TGR5 expression, which inhibits hepatocellular apoptosis, and attenuates liver IRI through increasing expression of Bcl-2, and inhibiting expressions of IRAK4 and cleaved caspase-3.

## 2. Materials and methods

### 2.1. Animals

Eight-week-old WT male mice (C57BL/6J; the Laboratory Animal Resources Center of Nanjing Medical University, China) and TGR5$^{-/-}$ C57BL/6J male mice were housed in constant environmental conditions under a standard condition of light-dark cycle in 12 h and free access to standard rodent diet and water. Procedures were carried out in accordance with the guidelines for the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care & Use Committee (IACUC) of Nanjing Medical University (Protocol Number NJMU08-092).

### 2.2. Surgical procedure and treatment

The mice were divided into six groups (n = 6) as follows: Sham-WT group, Sham-TGR5$^{-/-}$ group, WT-IR group, TGR5$^{-/-}$-IR group, IP + WT-IR group and IP + TGR5$^{-/-}$-IR group. The mouse model of segmental (70%) warm hepatic tissue IR injury was used in this study [18]. Briefly, a midline laparotomy was performed under 10% chloral hydrate (0.3 g/kg, intraperitoneally) anesthesia. The mice were then injected with heparin (100 U/kg). After a midline laparotomy, the midline laparotomy, portal vein and bile duct in left and median liver lobes were interrupted with an atraumatic bulldog clamp. After 90 min of liver ischemia, the clip was relieved for hepatic tissue reperfusion. The abdomen was immediately closed with a continuous 4-0 silk suture. The mice were sacrificed after 6 h hepatic tissue reperfusion, and blood as well as liver tissue of mice samples were harvested for further analysis. In the sham-operated group, mice were given anesthesia and subjected to laparotomy as well as exposure of the portal triad without hepatic ischemia. IP: WT mice were subjected to 5 min of hepatic ischemia followed by 10 min of hepatic tissue reperfusion.

### 2.3. Biochemical analysis of serum

Blood samples were collected 6 h after hepatic tissue reperfusion and centrifuged to obtain serum for analysis. We used an automated chemical analyzer (Olympus Company, Tokyo, Japan) to detect the hepatic serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

### 2.4. Hematoxylin and eosin study

The liver specimens were fixed with 10% neutral formaldehyde and then embedded in paraffin. The specimens were sectioned in 4-μm slices and stained with hematoxylin and eosin (H&E). Using the Suzuki method, we scored the severity of liver damage in the different groups [19]. Suzuki score for the assessment of liver damage following hepatic ischemia/reperfusion is shown in Table 1.

### 2.5. Caspase-3 activity assay

Caspase-3 activity was analyzed in liver tissues 6 h after reperfusion. The frozen hepatic tissues were homogenized and centrifuged at 20,000 g for 20 min. Activity of Caspase-3 was tested using a caspase-3 activity assay kit (Tiangen Biotech, Beijing, China).

**Table 1**
Suzuki score for the assessment of liver damage following hepatic ischemia/reperfusion.

| Score | Congestion | Vacuolization | Necrosis |
|---|---|---|---|
| 0 | None | None | None |
| 1 | Minimal | Minimal | Single cell necrosis |
| 2 | Mild | Mild | −30% |
| 3 | Moderate | Moderate | −60% |
| 4 | Severe | Severe | >60% |

### 2.6. TdT-mediated duTP-biotin nick-end labeling (TUNEL) staining

Paraffin sections of Hepatic tissues (4 μm thickness) were deparaffinated in toluene and then dehydrated by a graded series of ethanol solutions. The hepatic tissues were carried on staining by TUNEL using cell death detection kit (Roche-Boehringer, Mannheim, Germany).

### 2.7. Immunohistochemistry

Liver tissues were harvested and fixed in 4% paraformaldehyde overnight at 4 °C for further processing. The liver samples were cut into 5 μm sections, placed on slides and stained immunohistochemically for TGR5 (Abcam Trading Shanghai Company, Shanghai, China).

### 2.8. Western blot analysis

Proteins were extracted from ischemic liver samples, and the concentrations in the different groups were determined by the Bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific Inc., Rockford, IL, USA). Approximately 30 μg protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Polyvinylidene Fluoride (PVDF) membrane. The following antibodies from Cell Signaling Technology (Trading Shanghai Company, Shanghai, China) were used: Tubulin and cleaved caspase-3; IRAK4 and Bcl-2 antibodies were from Abcam (Trading Shanghai Company, Shanghai, China). We used skim milk powder which was dissolved by phosphate buffered saline that contained 0.1% Tween 20 (PBS-T) to block these membranes at 4 °C overnight. Then, we used peroxidase-conjugated secondary antibody from Abcam (Trading Shanghai Company, Shanghai, China) to incubate the membranes at 37 °C for 2 h. The results were visualized with Kodak autoradiography film (Kodak XAR film, Shenzhen, China) and quantitated using ImageJ software (National institutes of Health, Bethesda, USA). Tubulin expression served as an internal control. Images representative of three experiments are shown.

### 2.9. Quantitative real-time polymerase chain reaction (PCR)

RNA was collected from liver tissues harvested using a simple total RNA kit following the manufacturer's instructions (Tiangen Biotech, Beijing, China). Complementary DNA was synthesized using RT-Master Mix (TaKaRa, Dalian, China). The complementary DNA product was then amplified by quantitative real-time polymerase chain reaction (PCR) using the SteponePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA). Expression of the target genes (TGR5, TNF-α, IL-6 and IL-10) (Invitrogen, Nanjing, China) was calculated based on the ratio of the gene of interest to the housekeeping gene β-actin. These primers were selected from a PubMed database and are shown in Table 2.

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