Research Article

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Mindin deficiency protects the liver against ischemia/reperfusion injury

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Background & Aims: Hepatic ischemia/reperfusion (I/R) injury often occurs during liver surgery and may cause liver failure. Our previous studies revealed that Mindin is involved in the pathogenesis of ischemic stroke. However, the function of Mindin in hepatic I/R injury remains unknown.

Methods: Partial hepatic warm ischemia was induced in parallel in global Mindin knockout mice (*Mindin* KO), hepatocyte-specific Mindin knockdown mice, hepatocyte-specific Mindin transgenic mice (Mindin TG), myeloid cell-specific Mindin TG mice (LysM-Mindin TG), and their corresponding controls, followed by reperfusion. Hepatic histology, serum aminotransferase, inflammatory cytokines, and hepatocyte apoptosis and proliferation were examined to assess liver injury. The molecular mechanisms of Mindin function were explored *in vivo* and *in vitro*.

Results: *Mindin* KO and hepatocyte-specific Mindin knockdown mice exhibited less liver damage than controls, with smaller necrotic areas and lower serum transaminase levels. Mindin deficiency significantly suppressed inflammatory cell infiltration, cytokine and chemokine production, and hepatocyte apoptosis, but increased hepatocyte proliferation following hepatic I/R injury. In contrast, the opposite pathological and biochemical changes were observed in hepatocyte-specific Mindin TG mice, whereas no significant changes in liver damage were found in LysM-Mindin TG mice compared to non-transgenic controls. Mechanistically, Akt signaling was activated in livers of Mindin KO mice but was suppressed in Mindin TG mice. Most importantly, Akt inhibitor treatment blocked the protective effect of Mindin deficiency on hepatic I/R injury.

Conclusions: Mindin is a novel modulator of hepatic I/R injury through regulating inflammatory responses, as well as hepatocyte apoptosis and proliferation via inactivation of the Akt signaling pathway.

Introduction

Hepatic ischemia/reperfusion (I/R) injury frequently occurs as a result of liver transplantation, major resection of the liver or hemorrhagic shock and can lead to liver failure or even multi-organ system failure [1]. I/R injury causes up to 10% of early graft dysfunction, as well as both acute and chronic rejection [2]. Hepatic I/R injury is composed of two phases: the ischemic phase, which is characterized by oxidative stress and hepatocyte damage, and the subsequent reperfusion phase, which is characterized by cell death and inflammation. Hepatocyte apoptosis is initiated in response to hepatic I/R injury and is followed by the activation of Kupffer cells (KCs), the liver resident macrophages, which leads to the release of pro-inflammatory cytokines and chemokines. These inflammatory mediators recruit neutrophils and in turn induce hepatocyte death, which exacerbates local tissue injury [3,4]. Based on these observations, targeting inflammatory responses and cell death is a promising strategy for the treatment of hepatic I/R injury [5,6]. Thus, a thorough understanding of the mechanisms involved in the inflammatory response and hepatocyte damage during I/R injury is essential for designing therapies that will improve the outcome of liver surgery.

Mindin/spondin 2, an extracellular matrix (ECM) protein, is a critical regulator in the innate immune network, several factors

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Abbreviations: I/R, ischemia/reperfusion; ECs, endothelial cells; TNF-α, tumor necrosis factor α; DAMPs, danger-associated molecular patterns; TLRs, toll-like receptors; IRF, interferon regulatory factor; TRAF, TNF receptor-associated factors; ECM, extracellular matrix proteins; FS, F-spondin; TSR, thrombospondin-type 1 repeat; PPARα, peroxisome proliferation activating receptor-α; H/R, Hypoxia/Reoxygenation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; KC, Kupffer cell; NKT, natural killer T; MCAO, middle cerebral artery occlusion; ROS, reactive oxygen species; PCNA, proliferating cell nuclear antigen; LDH, lactate dehydrogenase; TUNEL, terminal dexynucleotidyl transferase(TdT)-mediated dUTP nick-end labeling; MCP-1, chemokine (C-C motif) ligand; GSK3β, glycogen synthase kinase 3 beta; IL-1β, interleukin-1 beta; CXCL, chemokine (C-X-C motif) ligand; FOXO1, forkhead box O1.

(*e.g.*, TLRs and IRFs) have been found to play dominant roles during hepatic I/R injury [7–11]. Due to its N-terminal F-spondin (FS) domains, Mindin holds the capacity to mediate the interaction with integrin and to participate in inflammatory cell recruitment and T cell priming [12,13]. Additionally, the C-terminal thrombospondin-type 1 repeat (TSR) domain of Mindin can recognize pathogen-associated molecular patterns (PAMPs) and initiate innate immune responses [14,15]. We previously reported that Mindin regulates cardiac hypertrophy [16], neointima formation [17], and several metabolic diseases, including hepatic steatosis and obesity [18]. Notably, we recently showed that Mindin contributes to cerebral I/R injury by regulating inflammation and neuronal apoptosis, suggesting a role of Mindin in I/R insult [19]. However, the function of Mindin in hepatic I/R injury has not been previously described.

In the current study, we hypothesize that Mindin is involved in the pathogenesis of hepatic I/R injury according to previous reports. To evaluate our hypothesis, genetic approaches were used to generate global or hepatocyte-specific *Mindin* KO mice and hepatocyte-specific or myeloid cell-specific Mindin TG mice. Upon hepatic I/R injury, Mindin-deficient mice exhibited strongly suppressed inflammatory responses, decreased hepatocyte apoptosis and increased hepatocyte proliferation, whereas hepatocyte-specific Mindin overexpression had the opposite effect. Furthermore, investigations into the underlying mechanisms highlighted Akt signaling to be crucial during Mindin-regulated hepatic I/R injury.

Materials and methods

Animals and treatment

Male wild-type (WT) (C57BL/6J) mice, *Mindin* knockout (*Mindin* KO) mice (on a C57BL/6J background), and hepatocyte-specific Mindin transgenic (Mindin TG) mice were bred at our facility. Mindin-specific shRNA or control shRNA adenoviruses were generated as previously described [18]. For hepatocyte-specific gene knockdown, mice were injected with adenovirus (5×10^9 pfu) through the jugular vein 4 h prior to ischemia. To generate myeloid cell-specific Mindin overexpression (LysM-Mindin TG) mice, full-length mouse Mindin cDNA was ligated into a vector containing a myeloid-specific lysozyme M (LysM) promoter and an SV40 polyA signal. This construct was then microinjected into fertilized mouse embryos (C57BL/6J background). *Mindin* KO and Mindin TG male mice used for experiments were confirmed to possess the desired genotype by standard genotyping techniques [18]. In experiments to block the activation of Akt signaling, the Akt Inhibitor IV (SC-203809, Santa Cruz Biotechnology, CA, USA; 0.5 mg/kg) or an equal volume of 0.5% DMSO/PBS was administered intraperitoneally 30 min prior to the ischemic insult [20,21]. Only 8- to 10-week-old male mice (24–27 g) were used. Animal protocols were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University, and experiments were conducted in adherence with the National Institutes of Health Guidelines for the Use of Laboratory Animals (NIH publication 86–23, revised 1985).

Mouse liver ischemia/reperfusion (I/R) injury model

We used an established mouse model of segmental (70%) hepatic I/R injury [11]. Briefly, mice were injected with pentobarbital sodium (50 mg/kg), and an atraumatic microvascular clamp (Fine Science Tools, North Vancouver, British Columbia, Canada) was used to interrupt the arterial/portal venous blood supply to the left/middle liver lobes under aseptic conditions. The clamp was removed after 60 min of ischemia, thereby initiating hepatic reperfusion. Mice were sacrificed after the indicated time courses of reperfusion, and serum and tissue samples were collected. Sham-operated mice underwent the same surgical procedure without vasculature occlusion.

Serum sample assays

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) levels were measured using an ADVIA 2400 Chemistry System analyzer (Siemens, Tarrytown, NY, USA). The cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein (MCP)-1 and the chemokines CXCL-1 and CXCL-2 were measured in the serum using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Peprotech, Rocky Hill, NJ, USA; Invitrogen, Carlsbad, CA, USA; R&D Systems, Minneapolis, MN, USA).

Histology and immunofluorescence staining

Liver sections (5 μm) were stained with H&E to analyze necrotic areas using Image Pro Plus software (version 6.0, Media Cybernetics, Rockville, MD, USA). All slides were blindly quantified in more than 15 high-power fields to assess the necrotic area in each section. The data were then averaged to calculate the necrotic area for each mouse. The necrotic area ratio was expressed as a percentage of the total area examined as previously described [11]. Immunofluorescence staining was performed as previously described [11]. Primary antibodies against mouse CD3 (ab16669, Abcam), MAC1 (ab75476, Abcam) and Ly6G (551459, BD Biosciences) were used on paraffin-embedded liver sections. Proliferating cell nuclear antigen (PCNA) staining was performed with an anti-PCNA antibody (2586, Cell Signaling Technology). The secondary antibodies used were goat anti-rabbit IgG, anti-rat IgG, and anti-mouse IgG (Invitrogen, Carlsbad, CA). Liver sections were evaluated blindly by counting labeled cells/10 high-power field.

Quantitative real-time PCR

High quality total RNA was extracted from frozen livers using TRIzol Reagent (15596-026, Invitrogen). Two micrograms of total RNA was reverse-transcribed into complementary DNA using a Transcriptor First Stand cDNA Synthesis Kit (04896866001, Roche). Quantitative real-time PCR was performed as previously described [22]. Primers used to amplify specific gene fragments are shown in Supplementary Table 1.

Western blot

Protein expression levels were determined in mouse liver lysates using Western blot analysis, as previously reported [22]. A ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA) was used for signal detection. Protein expression levels were quantified with Image Lab™ Software and normalized to the loading control GAPDH. All of the antibodies used in this study are listed in Supplementary Table 2.

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the ApopTag® Plus *In Situ* Apoptosis Fluorescein Detection Kit (EMD Millipore Corporation, S7111) according to the manufacturer's protocol.

Hepatocytes and non-parenchymal cells (NPCs) isolation and culture

Hepatocytes and hepatic NPCs were isolated from the liver by non-recirculating collagenase perfusion through the portal vein. First, mice were anesthetized with pentobarbital sodium, and livers were then perfused *in situ* with 45 ml Liver Perfusion Medium (Life Technologies, 17701-038) followed by Liver Digest Medium (Life Technologies, 17703-034). Then, the liver was excised, minced, and strained through a steel mesh. Hepatocytes and NPCs were separated by 50% Percoll solution and centrifuged for 15 min at 500 x g at room temperature. The NPC-enriched supernatant was collected and washed twice for further experiments. Hepatocytes were obtained by low-speed centrifugation (3 times at 50 g for 5 min each) and washed twice with Dulbecco's modified Eagle's medium (DMEM). The hepatocytes were counted, and their viability was determined by trypan blue exclusion. The hepatocytes were cultured in DMEM/F12 supplemented with 10% fetal calf serum under normoxic conditions (air/5% CO_2) for further experiments.