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Role of necroptosis in autophagy signaling during hepatic ischemia and reperfusion

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ABSTRACT

Ischemia and reperfusion (I/R) is a complex phenomenon involving massive inflammation and cell death. Necroptosis refers to a newly described cell death as "programmed necrosis" that is controlled by receptor-interacting protein kinase (RIP) 1 and RIP3, which is involved in the pathogenesis of several inflammatory diseases. Autophagy is an essential cytoprotective system that is rapidly activated in response to various stimuli and involves crosstalk between different modes of cell death and inflammation. In this study, we investigated pattern changes in necroptosis and its role in autophagy signaling during hepatic I/R. Male C57BL/6 mice were subjected to 60 min of ischemia followed by 3 h reperfusion. Necrostatin-1 (Nec-1, a necroptosis inhibitor; 1.65 mg/kg) was administered intraperitoneally 5 min before reperfusion. Hepatic I/R significantly increased the level of RIP3, phosphorylated RIP1 and RIP3 protein expression, and RIP1/RIP3 necrosome formation, which were attenuated by Nec-1. I/R also significantly increased serum levels of alanine aminotransferase, tumor necrosis factor-α, and interleukin-6, which were attenuated by Nec-1. Meanwhile, hepatic I/R activated autophagy and mitophagy, as evidenced by increased LC3-II, PINK1, and Parkin, and decreased sequestosome 1/p62 protein expression. Nec-1 attenuated these changes and attenuated the increased levels of autophagy-related protein (ATG) 3, ATG7, Rab7, and cathepsin B protein expression during hepatic I/R. Moreover, hepatic I/R activated the extracellular signal-regulated kinase (ERK) pathway, and Nec-1 attenuated this increase. Taken together, our findings suggest that necroptosis contributes to hepatic damage during I/R, which induces autophagy via ERK activation.

1. Introduction

Hepatic ischemia and reperfusion (I/R) injury is an important cause of liver damage that occurs in a variety of clinical conditions, including hypovolemic shock, liver transplantation, liver resection surgery, and trauma. I/R injury occurs due to not only oxygen and ATP depletion during hypoxia, but excessive inflammatory response after reperfusion, which leads to cell death including apoptosis, and necrosis, and ultimately organ dysfunction (van Golen et al., 2013). Although the nature of hepatic I/R has been extensively studied, the molecular mechanisms involved in hepatic cell death are not fully understood.

Necroptosis is a novel mode of cell death known as "programmed necrosis" controlled by receptor-interacting protein (RIP)1 and RIP3. Necroptosis is activated by a common pathway with apoptosis including pro-death ligand but morphologically resembles necrosis, marked by cell swelling and membrane rupture (Linkermann and Green, 2014). The execution of necroptosis requires formation of necrosomes depending on RIP1 and RIP3 kinase activity. Accumulating evidence indicates that necroptosis plays a crucial role in the pathogenesis of inflammatory diseases. In a mouse model of tumor necrosis factor (TNF)-induced systemic inflammation, kinase-inactive mutant RIP1 form and RIP3 deficiency protected mice from mortality (Duprez et al., 2011; Polykratis et al., 2014). Moreover, RIP3 increased in inflamed tissues of inflammatory bowel disease patients, indicating that necroptosis is strongly associated with intestinal inflammation (Pierdomenico et al., 2014). Recently, Dvoriantchikova et al. (2014) reported that treatment with necrostatin-1 (Nec-1), a selective necroptosis inhibitor, suppresses induction of pro-inflammatory responses in retinal I/R.

Autophagy is an evolutionarily conserved self-degradative process in which intracellular proteins or organelles are sequestered by

Abbreviations: I/R, ischemia and reperfusion; RIP, receptor-interacting protein; Nec-1, necrostatin-1; ATG, autophagy-related protein; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; ROS, reactive oxygen species; CQ, chloroquine; Rapa, rapamycin; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmunoprecipitation assay; TBS/T, Tween-20 in 1 × Tris-buffered saline; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy; MLKL, mixed-lineage kinase domain-like protein; PINK1, PTEN induced putative kinase 1; p62, sequestosome 1/p62.

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double-membraned autophagosomes and delivered into lysosomes for degradation. Autophagy is a dynamic process that can be dramatically induced or impaired by diverse stimuli. Several studies have demonstrated that reactive oxygen species (ROS) can activate starvation-induced autophagy and autophagic cell death (Huang et al., 2011). In this regard, autophagy has been reported to be increased by reperfusion (Sadoshima, 2008). Recent studies have uncovered critical links between autophagy and cell death. Inhibition of autophagy by chloroquine (CQ) exacerbated necrosis in an acetaminophen-induced hepatotoxicity model (Ni et al., 2012). Down-regulation of autophagy-related gene (ATG) 7 increased hepatocyte apoptosis in D-galactosamine and lipopolysaccharide-induced liver injury (Amir et al., 2013). In endothelial cells, inhibition of autophagy with either pharmacological inhibitors or genetic knockdown rescued palmitic acid-induced necroptosis (Khan et al., 2012). Meanwhile, Nec-1 inhibited autophagy in a TNF-α-treated mouse embryonic fibroblast cell line (Degterev et al., 2005). Dong et al. (2014) also reported that Nec-1 inhibited autophagy in an experimental retinal detachment model.

Therefore, we investigated the role of necroptosis in hepatic I/R injury, focusing on the autophagy signaling pathway.

2. Materials and methods

2.1. Animals

All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health (NIH publication No. 86-23, revised 1985) and guidelines of the Sungkyunkwan University Animal Care Committee. Male C57BL/6 mice (7 weeks, 21–23 g; Orient Bio, Korea) were adapted to laboratory conditions at Sungkyunkwan University for at least 1 week before experiments. Mice were kept in a temperature- and humidity-controlled environment (25 ± 1 °C and 55 ± 5%, respectively) with a 12 h light-dark cycle.

2.2. Liver I/R procedure

The mice were fasted for 18 h prior to experiments but were provided with tap water ad libitum. Mice were anesthetized intraperitoneally with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and body temperature was maintained at 37 °C using heating pads. The left branches of the portal vein, hepatic artery, and bile duct were clamped with a micro serrefine clip (Fine Science Tools Inc., Vancouver, Canada) to induce complete ischemia of the median and left hepatic lobes. The right lobes remained perfused to prevent venous congestion of the intestine. After 60 min of ischemia, the clamp was removed to allow reperfusion. Sham-operated animals underwent the same procedure but without vessel occlusion. Under anesthesia, blood samples and liver tissues were collected at 3 h of reperfusion. Serum was separated by centrifugation at 10,000 rpm for 10 min at 4 °C. Liver tissues were stored in liquid nitrogen at −75 °C for later analysis; part of the left lobe was used for histological staining.

2.3. Drug treatment

Nec-1 (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 1.65% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) and administered (1.65 mg/kg, i.p.) 5 min before reperfusion. CQ was dissolved in PBS and injected (60 mg/kg, i.p.) 1 h prior to ischemia. Rapamycin (Rapa) was dissolved in DMSO and diluted with PBS to a final concentration of 1% DMSO in PBS. Rapa was injected (1 mg/kg, i.p.) 1 h prior to ischemia. The dosage and timing of Nec-1, CQ and Rapa administration were determined based on previous studies and preliminary investigations in our laboratory (Smith et al., 2007; Kang et al., 2014; Zhu et al., 2015). In vehicle-treated sham or I/R mice, 1.65% DMSO-PBS, PBS, or 1% DMSO-PBS was administered in the same volume and route as the respective drug treatment. Animals were randomly divided into six groups as follows: (a) vehicle-treated sham (sham), (b) Nec-1-treated sham (Nec-1), (c) vehicle-treated I/R (I/R), (d) Nec-1-treated I/R (I/R + Nec-1), (e) CQ-treated I/R (CQ + I/R), and (f) Rapa-treated I/R (Rapa + I/R). As there were no differences in any of the parameters between vehicle-treated sham and Nec-1-treated sham groups, the results of these groups were pooled and referred to as sham. To investigate the role of ERK in autophagic flux during I/R, PD98059 (Calbiochem, La Jolla, CA, USA) was dissolved in 9.09% DMSO in saline and administered (1 mg/kg, i.p.) 20 min before ischemia. The dosage and timing of PD98059 administration were selected based on previously published paper (Park et al., 2010) and our preliminary study. In vehicle-treated sham or I/R mice, 9.09% DMSO-saline was administered in the same volume and route as the respective drug treatment. Mice were divided into four groups: (a) vehicle-treated sham (sham), (b) PD98059-treated sham (PD98059), (c) vehicle-treated I/R (I/R), and (d) PD98059-treated I/R (PD98059 + I/R).

2.4. Serum aminotransferase activity

Serum alanine aminotransferase (ALT) activity was assayed at 37 °C by monitoring the decrease in absorbance at 340 nm for 1 min by standard spectrophotometric procedures using a ChemiLab ALT assay kit (IVDLab Co., Ltd., Suwon, Korea).

2.5. Histological analysis

Liver tissues were sampled from a portion of the left lobe and fixed immediately in 10% neutral buffered formalin at room temperature, embedded in paraffin, and cut serially into 5-μm sections. Hematoxylin and eosin (H&E)-stained sections were evaluated in a blind manner at ×200 magnification using an optical microscope (Olympus Optical CO., Tokyo, Japan).

2.6. Serum cytokine levels

The serum levels of TNF-α and interleukin (IL)-6 were quantified with an enzyme-linked immunosorbent assay (ELISA) with a commercial mouse TNF-α and IL-6 ELISA kit (BD Biosciences, San Diego, CA, USA), respectively, according to the manufacturer's instructions.

2.7. Protein extraction

Isolated liver tissues were homogenized in PRO-PREP™ protein extraction solution (iNtRON Biotechnology Inc., Seongnam, Korea) with phosphatase inhibitor mixture (Calbiochem, La Jolla, CA, USA) for Western blot or radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, pH 7.4) with protease and phosphatase inhibitor mixture for immunoprecipitation. Briefly, homogenates were incubated in 4 °C for 30 min and centrifuged at 13,000 rpm for 5 min. The supernatant was collected, and the protein concentrations of the lysates were determined using the BCA Protein Assay kit (Pierce Biotechnology, IL, USA).

2.8. Western blot analysis

Samples of protein (16–20 μg) were loaded onto polyacrylamide gels and then separated by SDS/PAGE and transferred to polyvinylidene fluoride membranes (Millipore) using the Semi-Dry Trans-Blot Cell (Bio-rad Laboratories, Hercules, CA, USA). After transfer, the membranes were blocked for 1 h at room temperature with 5% (w/v) skim milk powder in 0.1% Tween-20 in 1 × Tris-buffered saline (TBS/T). The blots were then incubated overnight at 4 °C with primary antibody. After washing each in TBS/T, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature and detected using West-Q Pico ECL Solution (GenDEPOT, Barker, TX, USA)