Research Article

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KEAP1-NRF2 complex in ischemia-induced hepatocellular damage of mouse liver transplants

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Background & Aims: The Keap1-Nrf2 signaling pathway regulates host cell defense responses against oxidative stress and maintains the cellular redox balance.
Methods: We investigated the function/molecular mechanisms by which Keap1-Nrf2 complex may influence liver ischemia/reperfusion injury (IRI) in a mouse model of hepatic cold storage (20 h at 4 °C) followed by orthotopic liver transplantation (OLT).
Results: The Keap1 hepatocyte-specific knockout (HKO) in the donor liver ameliorated post-transplant IRI, evidenced by improved hepatocellular function and OLT outcomes (*Keap1* HKO→*Keap1* HKO; 100% survival), as compared with controls (WT→WT; 50% survival; p <0.01). By contrast, donor liver Nrf2 deficiency exacerbated IRI in transplant recipients (*Nrf2* KO→*Nrf2* KO; 40% survival). Ablation of Keap1 signaling reduced macrophage/neutrophil trafficking, pro-inflammatory cytokine programs, and hepatocellular necrosis/apoptosis, while simultaneously promoting anti-apoptotic functions in OLTs. At the molecular level, *Keap1* HKO increased Nrf2 levels, stimulated Akt phosphorylation, and enhanced expression of anti-oxidant Trx1, HIF-1α, and HO-1. Pretreatment of liver donors with PI3K inhibitor (LY294002) disrupted Akt/HIF-1A signaling and recreated hepatocellular damage in otherwise IR-resistant *Keap1* HKO transplants. In parallel *in vitro* studies, hydrogen peroxide-stressed Keap1-deficient hepatocytes were characterized by enhanced expression of Nrf2, Trx1, and Akt phosphorylation, in association with decreased release of lactate dehydrogenase (LDH) in cell culture supernatants.
Conclusions: Keap1-Nrf2 complex prevents oxidative injury in IR-stressed OLTs through Keap1 signaling, which negatively regulates Nrf2 pathway. Activation of Nrf2 induces Trx1 and promotes PI3K/Akt, crucial for HIF-1α activity. HIF-1α-mediated overexpression of HO-1/Cyclin D1 facilitates cytoprotection by limiting hepatic inflammatory responses, and hepatocellular necrosis/apoptosis in a PI3K-dependent manner.

Introduction

Ischemia/reperfusion injury (IRI) remains the major challenge in clinical liver transplantation, hepatic resection, trauma, and shock. This innate immune-dominated cascade includes reactive oxygen species (ROS) generation, which initiate tissue injury, and local inflammatory responses leading to endothelial and Kupffer cell activation, cytokine/chemokine release, and cell apoptosis [1]. It becomes recognized that oxidative stress-induced IR-damage involves multiple cell signaling pathways that result in liver failure or hepatoprotection and homeostasis [2]. Our group has pioneered the concept of cytoprotection by overexpression of heme-oxygenase-1 (HO-1) in IR-stressed organ transplants [3,4].

Keap1 (Kelch-like ECH-associated protein 1) has been shown to interact with Nrf2 (nuclear factor erythroid 2-related factor 2), a master regulator of intracellular redox homeostasis [5]. Under normal conditions, Nrf2 is anchored in the cytoplasm through binding to Keap1, and facilitates ubiquitination/proteolysis of Nrf2 [6]. Inactivation of Keap1 leads to stabilization of Nrf2, which in turn translocates into the nuclei to activate cytoprotective target genes through binding to the anti-oxidant response element (ARE) [7]. Modification of Keap1 may also damage the structural integrity of Keap1-Cul3 E3 ligase complex, decrease the ubiquitination activity and increase Nrf2 accumulation [8]. Nrf2-driven regulation of anti-oxidant and anti-inflammatory functions is important in cytoprotection.

Keywords: Liver ischemia/reperfusion injury; Liver transplantation; Keap1-Nrf2 redox system; HO-1.
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Abbreviations: ARE, anti-oxidant response element; HIF-1, hypoxia inducible factor-1; HKO, hepatocyte knockout; HO-1, heme-oxygenase-1; HRE, hypoxia response element; H_2O_2, hydrogen peroxide; IRI, ischemia/reperfusion injury; Keap1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; OLT, orthotopic liver transplantation; PI3K, phosphoinositide 3-kinase; sALT, serum alanine aminotransferase; Trx1, thioredoxin 1; TUNEL, terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling.

Indeed, genetic disruption of Nrf2 augments the severity of ischemic/nephrotoxic acute kidney injury in mice [9]. By contrast, activation of Nrf2 has been reported to protect against cerebral [10], retinal [11], cardiac [12] and intestinal [13] IR-tissue damage. Interestingly, human livers from older donors have lower levels of Nrf2, perhaps exposing them to increased IRI, and hence influencing the clinical outcomes [14]. While Nrf2 promotes cell growth/survival under oxidative stress conditions, its deletion reduces both constitutive and inducible expression of cytoprotective genes, and aggravates cellular damage. Moreover, disruption of Nrf2 signaling impairs angiogenic endothelial cell capacity and anti-oxidant gene expression, leading to cardiac hypertrophy, myocardial fibrosis, and apoptosis in response to hemodynamic stress [15]. The diverse Nrf2-mediated cell survival and protection phenotypes may progress through Keap1-Nrf2-ARE pathway [16]. Disruption of Keap1 signaling in the liver enhances Nrf2 activity and increases expression of ROS-detoxifying cytoprotective genes [17]. Moreover, dysfunction of *Keap1* gene activates Nrf2 and promotes cancer cell growth [18–20], whereas the loss of Keap1 activity leads to constitutive activation of Nrf2 and anti-oxidant genes [21]. Thus, Keap1 is one of the key molecules to negatively regulate Nrf2 during oxidative stress.

Here, we report on novel regulatory mechanisms by which Keap1-Nrf2 complex prevents inflammation and exerts cytoprotection in a clinically-relevant mouse model of prolonged hepatic cold ischemia and orthotopic liver transplantation (OLT). Thus, Keap1-dependent Nrf2 activation enhanced anti-oxidant Trx1 and stimulated PI3K/Akt system, which in turn facilitated HIF-1α signaling to promote hepatoprotection in a PI3K-dependent manner.

Materials and methods

Animals

Male Keap1 hepatocyte-specific knockout (*Alb-Cre::Keap1*$^{flox/-}$; *Keap1* HKO) and Nrf2 knockout (*Nrf2*$^{-/-}$; *Nrf2* KO) mice (BL/6; weighted 21–27 g) were used (breeding pairs provided by Dr. T. Kensler, The Johns Hopkins University, Baltimore, MD). Wild-type (WT; C57BL/6) mice at 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in UCLA animal facility under specific pathogen-free conditions, and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985).

Mouse liver cold ischemia and transplantation model

We have developed a mouse model of *ex vivo* hepatic cold ischemia followed by OLT [22]. Donor livers stored in UW solution at 4 °C for 20 h were transplanted in the following experimental groups: WT→WT; *Keap-1* HKO→*Keap-1* HKO; and *Nrf2* KO→*Nrf2* KO. Animals were euthanized at 1 h, 6 h, and 24 h post-OLT or followed for survival at day 14. Separate groups of WT "sham" controls underwent the same procedures but without ischemia/OLT. In some experiments, donor mice were treated i.p. with PI3K inhibitor (LY294002; Calbiochem; 0.5 mg/kg) or vehicle [10% dimethyl sulfoxide (DMSO) and 90% PBS] at 1 h prior to liver procurement.

Hepatocellular function assay

Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by the IDEXX Laboratories (Westbrook, ME).

Histology and immunohistochemistry

Liver sections (5 μm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria on a scale from 0 to 4 [23]. Liver macrophages and neutrophils were detected using primary rat anti-mouse CD68 (AbD Serotec, Raleigh, NC) and Ly6G (BD Biosciences, San Jose, CA) mAb, respectively. The secondary, biotinylated goat anti-rat IgG (Vector, Burlingame, CA) was incubated with immunoperoxidase (ABC Kit, Vector). Positive cells were counted blindly in 10 HPF/section.

Caspase-3 activity and TUNEL assays

Caspase-3 activity was analyzed by an assay kit (Calbiochem, La Jolla, CA), as described [24]. The Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ) was used to detect DNA fragmentation characteristic of apoptosis in formalin-fixed paraffin-embedded liver sections [24]. Results were scored semi-quantitatively by averaging the number of TUNEL+ apoptotic cells/microscopic field at 200× magnification. Ten fields were evaluated per tissue sample.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25 μl, the following were added: 1×SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) cDNA, and 10 μM of each primer. Amplification conditions were: 50 °C (2 min), 95 °C (5 min), followed by 40 cycles of 95 °C (15 s) and 60 °C (30 s). Primers used to amplify specific gene fragments are shown in Supplementary Table 1.

Western blot analysis

Proteins (30 μg/sample) from cell cultures/liver samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Monoclonal rabbit anti-mouse Keap1, Trx1, p-Akt, Bcl-2, Bcl-xL, cleaved caspase-3, and β-actin (Cell Signaling Technology, Danvers, MA), polyclonal rabbit anti-mouse Nrf2, HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) and HO-1 (Stressgen Biotech, Victoria, BC, Canada) Abs were used. The relative quantities of proteins were determined by densitometer, and expressed in absorbance units (AU).

Mouse hepatocyte cultures

Primary hepatocytes from WT, *Keap1* HKO or *Nrf2* KO mice were isolated, as described [25]. Livers were perfused with warm (37 °C) saline, followed by a collagenase-buffer (collagenase type IV, Sigma, St Louis, MO), and William's E culture medium (WEM) containing 10% FBS, 2 μg/ml gentamycin, 15 mM HEPES, 0.1 μM dexamethasone, 4 μg/ml insulin (Sigma) and 4 mM glutamax (Invitrogen). Cells were purified by Percoll gradient centrifugation. Viable hepatocytes, suspended in WEM + 10% FBS, were added to 24-well dishes (1.5×10^5 cells/well). Cells were allowed to attach for 4 h at 37 °C and 5% CO_2; medium was then changed to WEM without FBS, and cultures continued for another 24 h.

In vitro experiments

Primary *Keap1* HKO hepatocytes (5×10^5 cells/well) were pretreated with PI3K inhibitor (LY294002, 10 μM; Calbiochem) or DMSO (6.5 μl/ml) for 1 h. In some experiments, WT hepatocytes were transfected with *Keap1* or *Nrf2* siRNA (100 nM; Santa Cruz Biotechnology) using lipofectamine 2000 reagent (Invitrogen) and incubated for 24 h. Cells were then treated with HIF inhibitor (YC-1, 100 μM; Calbiochem) for 1 h, and supplemented with H_2O_2 (200 μM) for additional 12 h. Cell viability, assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Life Technologies) assay, was expressed as percentage of the total number of cells. Cell death was screened by lactate dehydrogenase (Stanbio Laboratory) release, and expressed as LDH activity (U/L), according to the manufacturer's instructions.