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KEAP1-NRF2 COMPLEX IN ISCHEMIA-INDUCED HEPATOCELLULAR DAMAGE OF MOUSE LIVER TRANSPLANTS

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Abstract

Background—The Keap1-Nrf2 signaling pathway regulates host cell defense responses against oxidative stress and maintains the cellular redox balance. Aims&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 (20h at 4 C) followed by orthotopic liver transplantation (OLT).

Results—The Keap1 hepatocyte-specific knock-out (HKO) in the donor liver ameliorated post-transplant IRI, evidenced by improved hepatocellular function and OLT outcomes (Keap1HKO Keap1HKO; 100% survival), as compared with controls (WT WT; 50% survival; $p<0.01$). In contrast, donor liver Nrf2 deficiency exacerbated IRI in transplant recipients (Nrf2KO Nrf2KO; 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, Keap1HKO increased Nrf2 levels, stimulated Akt phosphorylation, and enhanced expression of anti-oxidant Trx1, HIF-1 , and HO-1. Pretreatment of liver donors with PI3K inhibitor (LY294002) disrupted Akt/HIF-1 signaling and recreated hepatocellular damage in otherwise IR-resistant Keap1HKO 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.

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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-1 activity. HIF-1 -mediated overexpression of HO-1/CyclinD1 facilitates cytoprotection by limiting hepatic inflammatory responses, and hepatocellular necrosis/apoptosis in 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 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. Indeed, genetic disruption of Nrf2 augments the severity of ischemic/nephrotoxic acute kidney injury in mice [9]. In 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 activated Nrf2 and promoted cancer cell growth [18-20], whereas the loss of Keap1 activity led 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 PI3K-dependent manner.

Materials and Methods

Animals

Male Keap1 hepatocyte-specific knock-out (*Alb-Cre::Keap1* $^{flox/-}$; Keap1HKO) and Nrf2 knock-out (*Nrf2*$^{-/-}$; Nrf2KO) mice (BL/6; weighted 21-27g) 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, 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 20h were transplanted in the following experimental groups: WT WT; Keap-1 HKO Keap-1 HKO; and Nrf2KO Nrf2KO. Animals were sacrificed at 1h, 6h, and 24h 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; 0.5mg/kg) or vehicle [10% dimethyl sulfoxide (DMSO) and 90% PBS] at 1h prior to liver procurement.

Hepatocellular function assay

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

Histology and immunohistochemistry

Liver sections (5 m) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria on a scale from 0-4 [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 l, the following were added: 1 SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) cDNA and 10 M of each primer. Amplification conditions were: 50°C (2min), 95°C (5min), followed by 40 cycles of 95°C (15sec) and 60°C (30sec). Primers used to amplify specific gene fragments are shown (Supplementary Table).

Western blot analysis

Proteins (30 g/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 -actin (Cell Signaling Technology, Danvers, MA), polyclonal rabbit anti-mouse Nrf2, HIF-1 (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, 2μg/ml gentamycin, 15mM HEPES, 0.1μM dexamethasone, 4μg/ml insulin (Sigma) and 4mM 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 4h at 37°C and 5% CO_2; medium was then changed to WEM without FBS, and cultures continued for another 24h.

In vitro experiments

Primary Keap1 HKO hepatocytes (5×10^5 cells/well) were pretreated with PI3K inhibitor (LY294002, 10 M; Calbiochem) or DMSO (6.5 l/ml) for 1h. In some experiments, WT hepatocytes were transfected with Keap1 or Nrf2 siRNA (100nM; Santa Cruz Biotechnology) using lipofectamine 2000 reagent (Invitrogen) and incubated for 24h. Cells were then treated with HIF inhibitor (YC-1, 100 M; Calbiochem) for 1h, and supplemented with H_2O_2 (200 M) for additional 12h. Cell viability, assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Life Technologies) assay, was expressed as percentage of 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.

Statistical analysis

Data are expressed as mean SD. Statistical comparisons between groups were analyzed by Student's t-test. All differences were considered statistically significant at the p-value of <0.05.

Results

Hepatocyte deletion of the Keap1 gene confers resistance against IRI in OLTs

Donor livers were stored for 20h at 4 C prior to transplantation in WT WT; Keap-1 HKO Keap-1 HKO; and Nrf2KO Nrf2KO groups. The hepatocellular function in OLT recipients was assessed by sALT levels (IU/L) (Fig. 1A). Ablation of Keap1 signaling decreased sALT levels at 6h and 24h post-transplant, as compared with Keap1 proficient (WT) controls (6h: 3540±1059 and 8736±1339, respectively; $p<0.01$; 24h: 625±216 and 3956±432, respectively; $p<0.0005$). In contrast, Nrf2 deficiency exacerbated the hepatocellular damage, as evidenced by increased sALT levels at 6h (12467±1224; $p<0.05$) and 24h (7189±1009; $p<0.0005$) of reperfusion, as compared with controls.

These functional data correlated with Suzuki's histological grading of IR-mediated liver damage at 24h post-transplant (Fig. 1B, C). Unlike WT controls, which showed moderate to severe sinusoidal congestion, cytoplasmic vacuolization, and hepatocellular necrosis (Panel b; score=2.9 0.26), Keap1HKO liver transplants revealed minimal pathological changes (Panel c; score=0.93 0.09; $p<0.001$). In contrast, Nrf2KO liver transplants were characterized by widespread edema, profound sinusoidal congestion/cytoplasmic vacuolization, and extensive (30-50%) necrosis (Panel d; score=3.52 0.17; $p<0.001$).

Having shown that hepatocyte Keap1-deficiency ameliorates, whereas Nrf2 deficiency enhances hepatocellular damage, we analyzed animal survival at day 14 in IR-stressed OLT groups (Fig. 1D). Unlike disruption of hepatocyte Keap1 signaling in donor livers, which led to 100% animal survival (10/10), Nrf2 deficiency diminished the survival to 40% (4/10), comparable with 50% survival (5/10) seen in unmodified WT controls ($p<0.01$).

Keap1HKO reduces macrophage/neutrophil trafficking and regulates inflammatory program in IR-stressed OLTs

To determine whether hepatocyte Keap1 deficiency may have influenced macrophage/ neutrophil trafficking, we stained IR-stressed OLTs for CD68 and Ly6G infiltrating cells at 24h of reperfusion (Fig. 2). Indeed, disruption of Keap1 signaling decreased accumulation of CD68+ macrophages (Fig. 2A, Panel b; 6.5 3.2), as compared with WT controls (Panel a: 28.1 4.6, $p<0.0001$). In contrast, liver transplants devoid of Nrf2 signaling showed increased macrophage sequestration (Panel c: 37.4 6.4, $p<0.005$). Consistent with our immunostaining data (Fig. 2B), reduced mRNA levels coding for TNF- , IL-1 and CXCL-10 were recorded in Keap1HKO but not Nrf2KO OLTs (Fig. 2C). Furthermore, Keap1 deficiency decreased Ly6G+ neutrophil accumulation in OLTs (Fig. 2D, Panel b: 8.6 3.1), as compared with WT controls (Panel a: 37.3 4.4, $p<0.0001$); Nrf2KO liver transplants showed increased Ly6G+ infiltrate (Panel c: 48.6 8.1, $p<0.001$). Consistent with the immunostaining data (Fig. 2D, E), MPO assay (Fig. 2F), reflecting hepatic neutrophil activity (U/g), was decreased in Keap1-deficient OLTs (6h: 1.67 0.3, $p<0.05$; 24h: 1.17 0.28, $p<0.05$), as compared with WT controls (6h: 4.34 0.31; 24h: 3.37 0.29). Enhanced MPO activity found in Nrf2KO transplants (6h: 5.1 0.26, $p<0.01$; 24h: 4.1 0.52, $p<0.01$) was accompanied by increased CXCL-1 mRNA, a major neutrophil chemoattractant (Fig. 2G).

Keap1HKO promotes anti-apoptotic functions and reduces apoptosis in IR-stressed OLTs

By 24h of reperfusion, Keap1 hepatocyte deficiency downregulated Western-assisted expression (AU) of cleaved caspase-3 (Fig. 3A, 0.3-0.4), yet upregulated Bcl-2, and Bcl-xL (1.8-2.0 and 1.6-1.7, respectively) in OLTs. In contrast, Nrf2 deficiency diminished Bcl-2 and Bcl-xL (0.1-0.3 and 0.1-0.2) but increased cleaved caspase-3 (1.5-1.7) expression. These results were confirmed by decreased caspase-3 activity (U) in Keap1HKO transplants (Fig. 3B: 6h: 0.37 0.29, $p<0.005$; 24h: 0.29 0.09, $p<0.0005$, respectively), compared with WT (6h: 3.54 0.34; 24h: 2.84 0.39). We then employed TUNEL assay to analyze liver cell apoptosis (Fig. 3C, D). OLTs devoid of hepatocyte-specific Keap1 signaling showed decreased ($p<0.001$) frequency of $TUNEL^+$ cells (Panel b: 4.6 1.1), as compared with WT (Panel a: 21.6 4.4) or Nrf2- deficient (Panel c: 37.2 5.1) OLTs. Moreover, Keap1 knockdown decreased total percentage of dead cells (apoptotic+necrotic cells=4.4%) in OLTs, as compared with WT controls (32.6%, $p<0.0001$; Suppl. Fig. 2B). Nrf2 deficiency, however, further increased liver transplant frequency of dead cells (41.8%, $p<0.0001$).

Keap1HKO activates Nrf2-mediated Trx1 and Akt/HIF-1 signaling in IR-stressed OLTs

We next investigated the role of Keap1 in the regulation of Nrf2 and other anti-oxidant molecules. By 24h of reperfusion, disruption of hepatocyte Keap1 augmented Western-assisted expression (AU) of Nrf2 and anti-oxidant Trx1 (Fig. 3E, 2.8-3.0 and 3.0-3.2), compared with Nrf2-deficient (0.2-0.4) or WT (0.5-0.7) OLTs. In parallel, Keap1HKO enhanced hepatic p-Akt, and HIF-1 /HO-1 expression (2.9-3.1, 2.8-2.9, and 2.4-2.5, respectively). Furthermore, Keap1 but not Nrf2 knock-out increased mRNA levels coding for anti-oxidant Trx1, Nqo1 and Gclc at both 6h and 24h post-OLT, as compared with WT controls (Fig. 3F).

Inhibition of PI3K disrupts Akt/HIF-1 signaling and recreates IRI in Keap1HKO OLTs

As Keap1HKO triggered activation of Nrf2-mediated Trx1 and Akt/HIF-1 (Fig. 3E, F), we then investigated cross-regulation between PI3K/Akt and HIF-1 signaling in our model. Keap1HKO donor mice were treated with PI3K inhibitor (LY294002) prior to liver procurement. At 6h and 24h post-OLT, sALT levels (IU/L) were increased in Keap1HKO transplant group given adjunctive PI3K inhibitor (Fig. 4A, 6h: 6244±1098, $p<0.05$; 24h: 3428±1430, $p<0.01$), compared with Keap1HKO controls given DMSO (6h: 3448±437, 24h: 1068±206). OLTs from Keap1HKO donors treated with DMSO showed minimal liver sinusoidal congestion without edema, vacuolization or necrosis (Fig. 4C, Panel c; score= 1.2 0.63, $p<0.0005$). In contrast, those given PI3K inhibitor in WT or Keap1HKO groups revealed significant edema, sinusoidal congestion, cytoplasmic vacuolization, and necrosis (30-50%; Fig. 4C, Panel c; score=3.2 0.42; Panel d; score=2.5 0.71). Although Keap1HKO increased Western blot-assisted expression (AU) of p-Akt (Fig. 4B, 1.9-2.1) and HIF-1 (1.8-2.0), adjunctive PI3K inhibition decreased p-Akt (0.5-0.7) and HIF-1 expression (0.6-0.8) in IR-stressed OLTs.

Activation of Keap1-dependent Nrf2 promotes anti-oxidant Trx1 and Akt/HIF-1 signaling in hypoxic hepatocytes

Our *in vivo* data has implicated the role of hepatocyte-specific Keap1 in mediating Nrf2 activation in IR-stressed OLTs. To further elucidate the regulatory mechanism of Keap1-Nrf2 signaling, we used primary hepatocyte culture system. Hepatocyte Keap1 deficiency or Keap1 siRNA transfection augmented Western–assisted expression (AU) of Nrf2, Trx1, and p-Akt (Fig. 4D, 3.2-3.4, 3.3-3.5, 3.1-3.3; and Suppl. Fig. 3A, Fig. 3A, 3.0-3.2, 2.7-2.9, 2.6-2.8, respectively) in H_2O_2-stressed hepatocytes, as compared with WT controls (0.5-0.9). In contrast, Nrf2 deficiency or siRNA silencing reduced hepatocyte expression of Trx1 and p-Akt (0.2-0.4) under hypoxia *in vitro* conditions. Furthermore, Keap1 deficiency or Keap1 siRNA silencing, but not Nrf2 ablation, increased HIF-1 expression (supplementary Fig. 3A, 2.6-2.8 vs 0.4-0.6). PI3K inhibition diminished p-Akt (Fig. 4E, 0.5-0.7) and HIF-1 (0.4-0.6) expression in Keap1-deficient hepatocyte cultures. Consistent with these findings, PI3K inhibition has led to decreased mRNA levels coding for HIF-1 , HO-1, and Cyclin D1 in H_2O_2-stressed hepatocytes (Fig. 4F). Keap1 HKO or Keap1 siRNA silencing increased cell viability (Suppl. Fig. 3B, 72.8 9.2%, $p<0.005$) and decreased LDH release (U/L; Fig. 4G, 6.4±1.35; $p<0.0005$). In contrast, Nrf2 deficiency or PI3K inhibition reversed hepatoprotection seen otherwise in Keap1-deficient hepatocyte cultures, evidenced by decreased cell viability (27.65.2%), and increased LDH levels (20.6±1.93). Moreover, HIF-1 inhibition (YC-1 pretreatment) decreased survival of Keap1-siRNA-transfected hypoxic hepatocytes (Suppl. Fig. 3C, 23.5 9.1, $p<0.005$), as compared with DMSO controls (70.4 8.9).

Discussion

Although the importance of Nrf2 transcription factor as a master switch of redox homeostasis in a variety of liver pathologies has been established [26], its role in liver IRI remains to be elucidated. In this study, we have identified a novel cytoprotective regulatory mechanism of Keap1-Nrf2 complex in a clinically-relevant mouse model of IR-stressed OLT damage (Suppl. Fig. 1). Although hepatocyte-specific disruption of Keap1 signaling in livers subjected to cold storage imposed IR-resistance and improved post-transplant survival, Nrf2 knock-out exacerbated hepatic IRI, implicating Nrf2 is tightly negatively -regulated by Keap1, which is essential for promoting Nrf2-mediated cytoprotection in IR-stressed OLTs.

Since Nrf2 activation orchestrates an array of cell defensive functions, the regulatory mechanism of Keap1-Nrf2 complex may involve multiple intercellular signaling pathways. We found that hepatocyte disruption of Keap1 activated Nrf2, which then increased induction of Trx1, a key redox regulation component, whereas Nrf2 HKO abolished Trx1 expression. Indeed, Trx1 may activate a number of transcription factors that contribute to cell growth/survival [27]. For instance, inhibition of cardiac Trx1 increased myocardial oxidative stress leading to heart failure [28], suggesting an important anti-oxidant regulatory role of Trx1. Our study has revealed that Keap1-dependent Nrf2 activation enhanced Akt phosphorylation and HIF-1 signaling, leading to increased transcription of Nqo1/Gclc genes. This indicates that Keap1-Nrf2 positively regulates the cellular anti-oxidant network in IR-

stressed OLTs. Consistent with the ability of Trx1 to stimulate cell survival by PI3K/Akt [29] and HIF-1 signaling in cancer cells [30], we now show that Keap1-dependent Nrf2 augmented Trx1, as well as promoted PI3K/Akt and HIF-1 activation.

The question arises as to whether Nrf2-induced Akt can mediate HIF-1 signaling in our model? It has been reported that disruption of prolyl-hydroxylases (PHD1), a hydroxylase leading to degradation of HIF-1, increased hypoxia tolerance by reducing oxidative stress and reprogramming hepatocellular metabolism [31]. In addition, activation of HIF-1 promoted hepatocyte survival via Wnt- -catenin - HIF-1 interaction [32]. Our data suggests that HIF-1 activity was mediated by Akt pathway, as PI3K/Akt blockade in the donor liver impeded HIF-1 expression, resulting in IR-damage in both WT and Keap1HKO transplants.

Next, we used mouse H_2O_2-stressed primary hepatocyte cultures to analyze downstream molecular mechanisms by which Keap1-Nrf2 may regulate PI3K/Akt signaling in liver IRI. First, we found that Keap1 deficiency or siRNA silencing resulted in enhanced hepatocyte expression of Nrf2, Trx1, p-Akt, and HIF-1α, as well as increased cell survival (Suppl. Fig. 3A/B). In marked contrast, disruption of Nrf2 or knockdown of both Keap1 and Nrf2 diminished expression of these genes and decreased hepatocyte viability, suggesting that Keap1-dependent Nrf2 induced Trx1, and promoted PI3K/Akt signaling under hypoxia conditions. Second, blocking PI3K/Akt suppressed HIF-1 , which is critical for cell survival, as evidenced by decreased cell viability after pretreatment of Keap1-silenced hepatocytes with HIF inhibitor (Suppl. Fig. 3C). Third, inhibition of PI3K/Akt decreased mRNA levels coding for HO-1, one of HIF-1 target genes known to exert multiple regulatory functions in oxidative stress, inflammation, and apoptosis [3, 4]. Consistent with our findings, HO-1 induction in hepatocytes increased the resistance to cell death during endotoxemia [33], whereas hepatocyte-specific HO-1 deletion disrupted redox homeostasis in basal and oxidative conditions [34]. We have shown that HO-1 overexpression induced Stat3-mediated -catenin, which in turn inhibited TLR4 innate inflammatory response via a negative feedback regulation [35]. Although in the present study, the hepatocellular damage by 1h post-OLT was comparable in all animal groups, a sharp increase in sALT levels at 6h and 24h correlates with enhanced inflammatory cell infiltration in OLTs. It is likely that Keap1-deficient cells are more resistant to oxidative stress generated by inflammatory cells. Indeed, HIF-1 induced HO-1 in Keap1-deficient cells diminished both macrophage/ neutrophil trafficking, and proinflammatory cytokines in IR-stressed OLTs. In addition, PI3K/Akt blockade reduced HO-1 expression, leading to increased LDH release in hepatocyte cultures. Hence, HIF-1 -induced HO-1 upregulation represents one of the important cytoprotective mechanisms against hepatic IRI.

We found that Keap1-Nrf2 mediated Akt activation enhanced Cyclin D1 expression, accompanied by increased cell survival (Suppl. Fig. 3B) and decreased LDH release in hypoxic hepatocyte cultures. However, blocking PI3K/Akt has led to decreased Cyclin D1 transcription. Indeed, Akt, also known as Protein Kinase B, promotes cell survival by phosphorylating Bcl-2/ Bcl-xL–associated death promoter (BAD), a pro-apoptotic protein of the Bcl-2 family, and inhibiting caspase-mediated cell death program [36]. Moreover, Akt regulates Cyclin D1 during cell proliferation and apoptosis [37]. Overexpression of Cyclin D1 was also shown to increase tolerance to apoptosis and to promote cancer cell survival

[38]. Consistent with *in vitro* data, our *in vivo* results support the regulatory role of Keap1-Nrf2 mediated PI3K/Akt axis in hepatocyte death. Keap1HKO upregulated Bcl-2/Bcl-xL but downregulated cleaved caspase-3 expression/ activity, which in turn decreased apoptotic cell death in IR-stressed OLTs. Our results highlight the regulatory function of Keap1-Nrf2 to trigger PI3K/Akt signaling and prevent IR-induced liver cell apoptosis. In agreement with our finding, active PI3K-Akt pathway enabled Nrf2 to promote metabolic activities that support cell proliferation in addition to enhancing cytoprotection [39].

In conclusion, Keap1-Nrf2 complex ameliorated hepatic IRI in OLTs through Keap1 negatively regulating Nrf2 activity (Suppl. Fig. 1). Hepatocyte Keap1 deficiency facilitated Nrf2 nuclear translocation and activated Trx1, an ARE targeting gene. In turn, Trx1 promoted PI3K/Akt, crucial for HIF-1 signaling. HIF-1 -mediated antioxidant HO-1 and CyclinD1 expression resulted in cytoprotection by downregulating hepatic inflammation and apoptosis. By identifying new molecular pathways of Keap-1-Nrf2 regulation, the novel therapeutic strategy could be established to develop Nrf2 pharmacological inducers in the management of inflammatory injury in liver transplant recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

ARE	anti-oxidant response element
HIF-1	hypoxia inducible factor-1
HKO	hepatocyte knock-out
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

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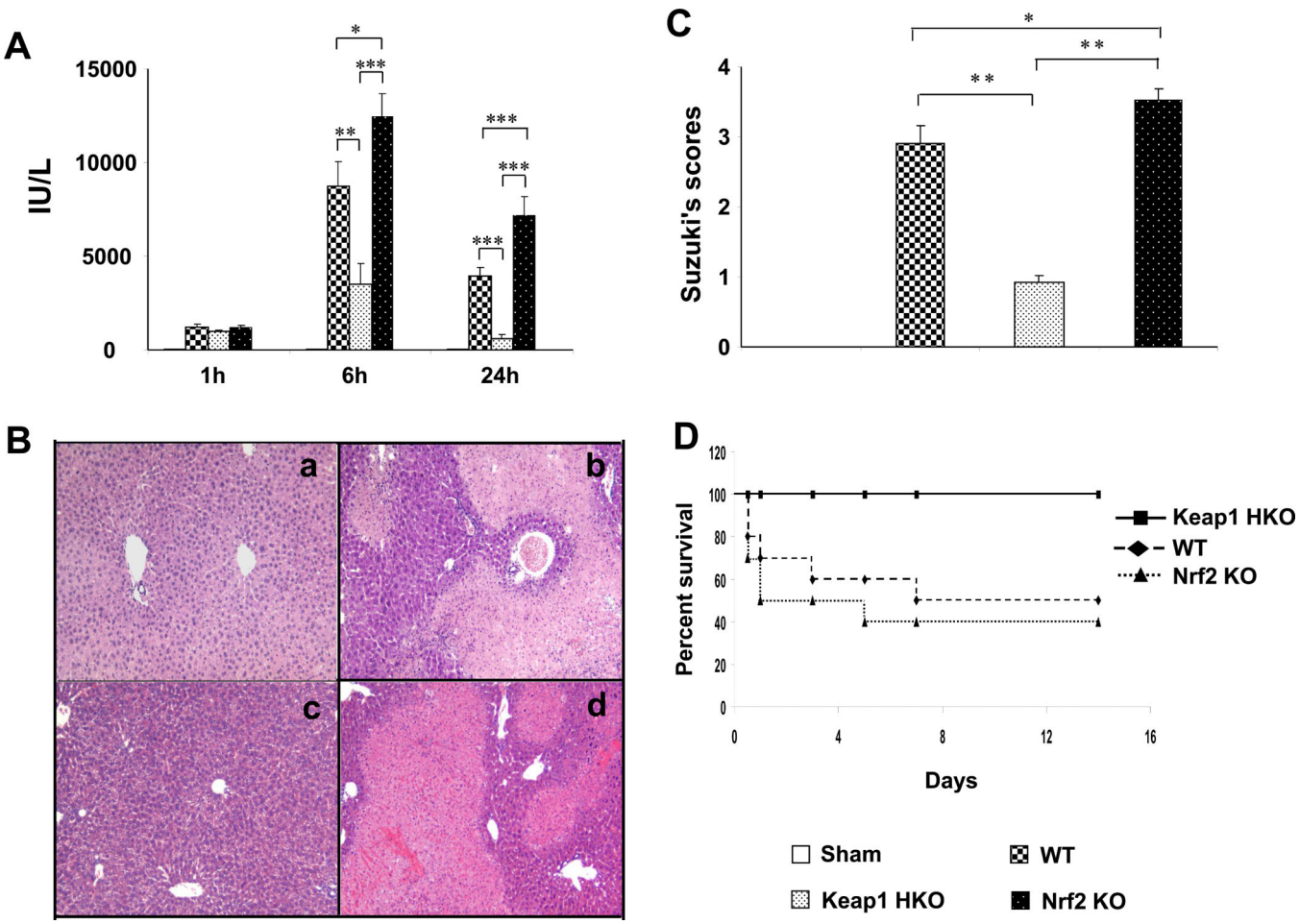

Figure 1.

Hepatocyte-specific Keap1 deficiency (HKO) ameliorates hepatic IRI in OLTs. Donor livers were stored in UW solution (4 C) for 20h prior to the transplant. Experimental groups: (a; □) sham; (b; ⊠) WT WT; (c; ⊡) Keap1HKO Keap1HKO; (d; ■) Nrf2KO Nrf2KO. (A) sALT levels. Mean SD; n=4 mice/group. *p<0.05; **p<0.01; ***p<0.0005. (B) Representative OLT histology (H&E; magnification x100); (C) Suzuki's histological grading of IRI (24h). Mean SD; n=4-6 mice/group. *p<0.05, **p<0.001. (D) OLT survival: **(■)** Keap1HKO Keap1HKO (100%); **(◆)** WT WT (50%); **(▲)** Nrf2KO Nrf2KO (40%; p<0.01). N=10 mice/group.

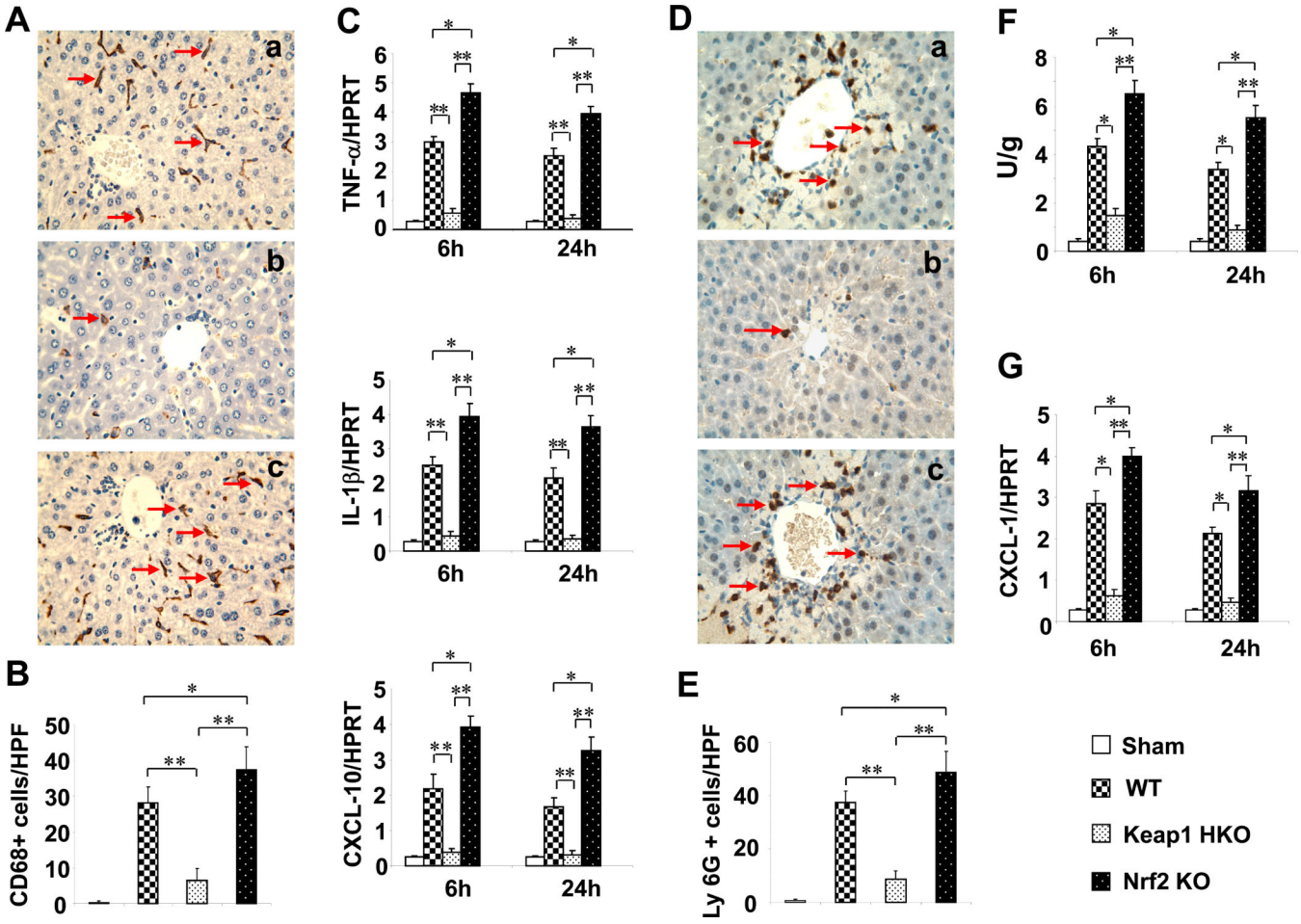

Figure 2.

Hepatocyte-specific Keap1 deficiency (HKO) reduces macrophage and neutrophil trafficking/activation in IR-stressed OLTs: (a; ▩) WT WT; (b; ⬚) Keap1HKO Keap1HKO; (c; ■) Nrf2KO Nrf2KO. (A/B) Immunohistochemical staining for CD68+ cells. Representative of 4 mice/group; magnification ×400; *p<0.005, **p<0.0001. (C) Quantitative RT-PCR-assisted cytokine/chemokine gene expression; Mean±SD; n=3-4/group; *p<0.05, **p<0.005. (D/E) Immunohistochemical staining for LY6G+ cells. Representative of 4 mice/group; magnification ×400; *p<0.001, **p<0.0001. (F) Neutrophil MPO activity (U/gm). Mean±SD; n=3-4/group; *p<0.05, **p<0.01. (G) Quantitative RT-PCR-assisted detection of CXCL-1. Mean±SD; n=3-4/group; *p<0.05, **p<0.01.

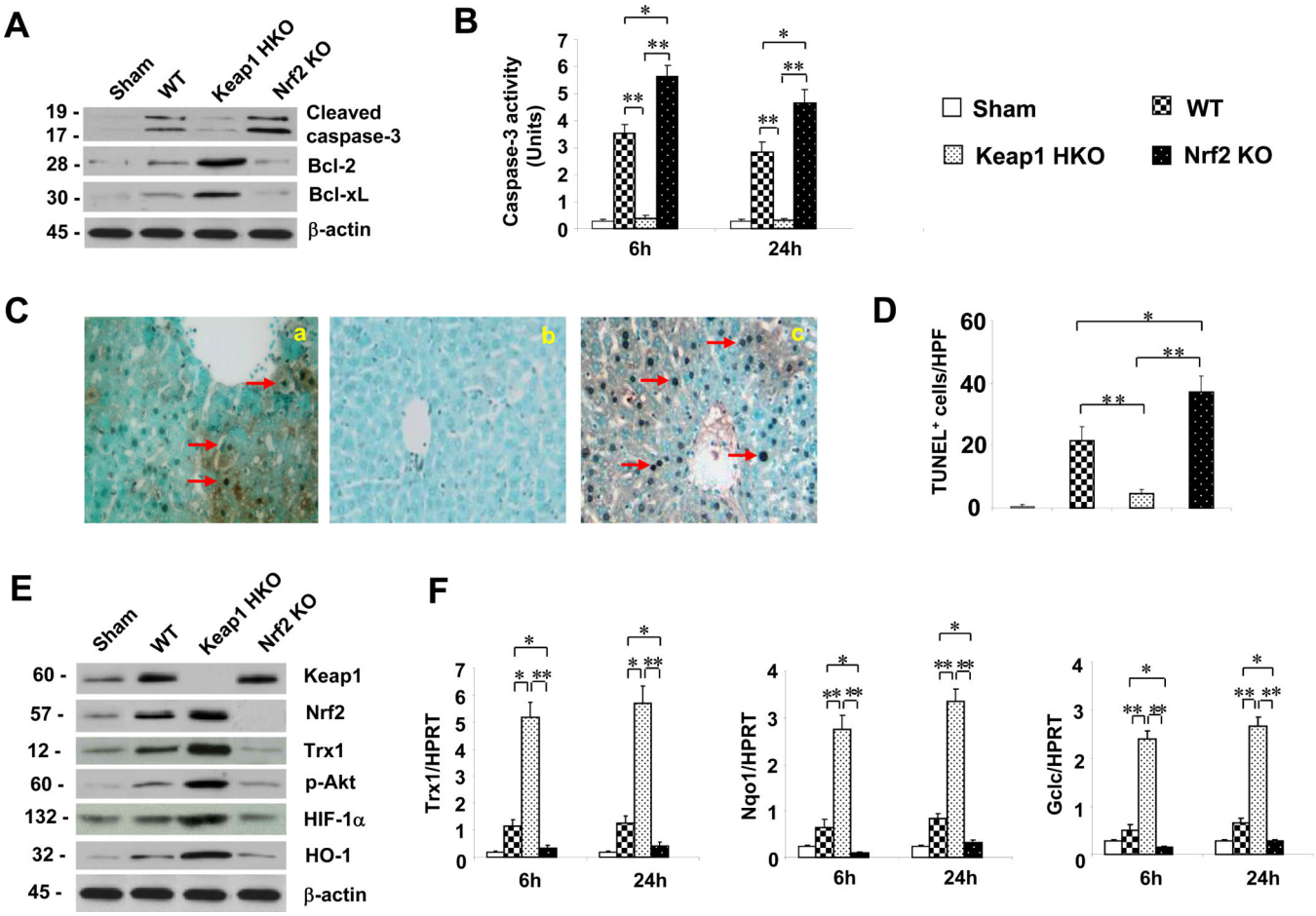

Figure 3.

Hepatocyte-specific Keap1 deficiency (HKO) promotes anti-apoptotic functions, reduces apoptosis and activates Nrf2-mediated Trx1/Akt/HIF-1 in IR-stressed OLTs. (A) Western analysis of cleaved caspase-3 and Bcl-2/Bcl-xl. Representative of three experiments. (B). Caspase-3 activity. Mean±SD; n=3-4/group; *p<0.005; **p<0.0005. (C/D) TUNEL staining: (a; ⊠) WT WT; (b; ⊡) Keap1HKO Keap1HKO; (c; ■) Nrf2KO Nrf2KO. Representative of 4 mice/group; magnification ×200 *p<0.05; **p<0.001. (E) Western analysis of Keap1, Nrf2, Trx1, p-Akt, HIF-1 , and HO-1 in OLTs. β-actin served as an internal control. Representative of three experiments. (F) Quantitative RT-PCR-assisted detection of mRNA coding for Trx1, Nqo1 and Gclc. Data were normalized to HPRT gene expression. Mean±SD; n=3-4/group; *p<0.05, **p<0.01.

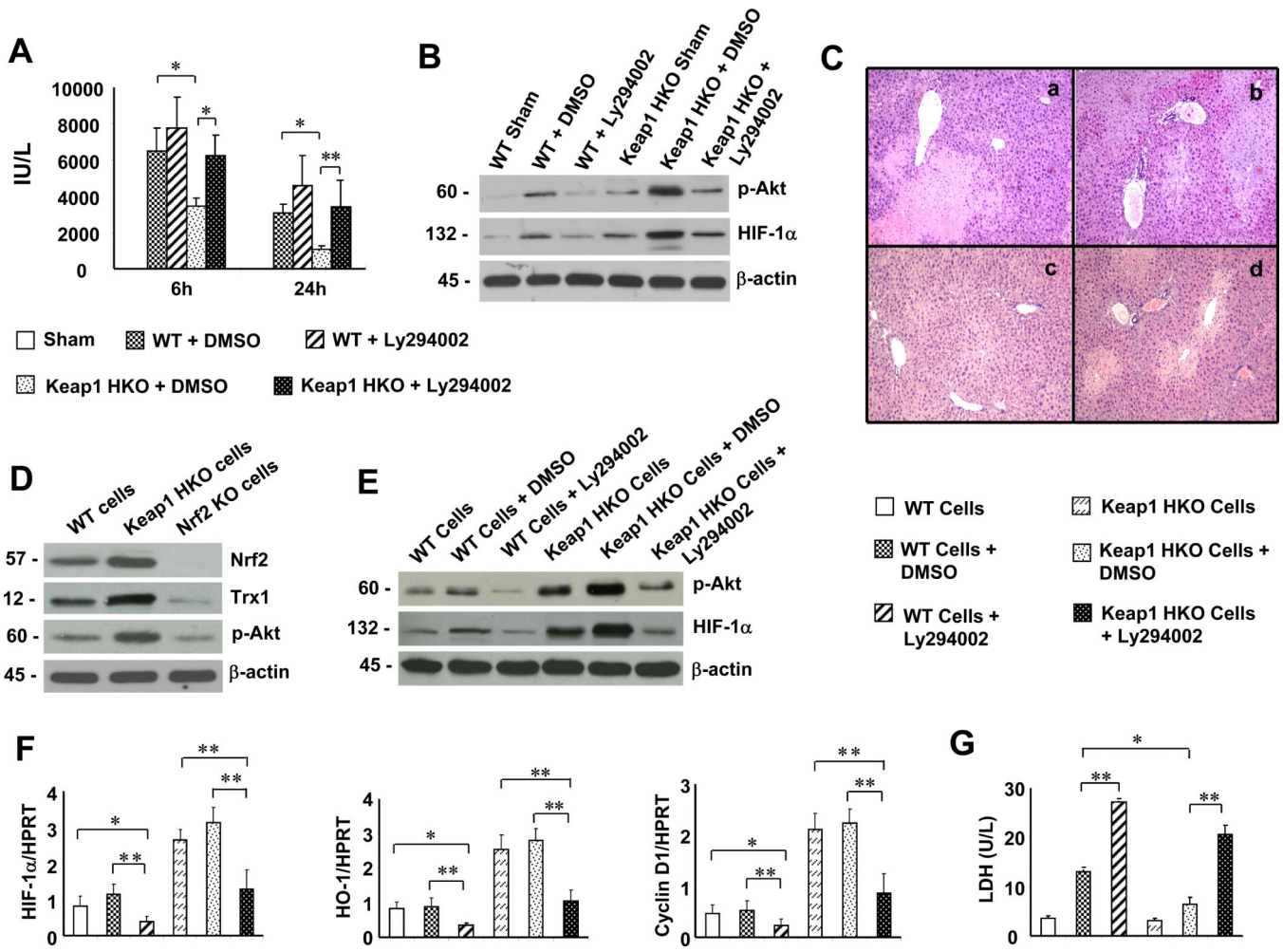

Figure 4.

Inhibition of PI3K disrupts Akt/HIF-1 signaling and recreates liver IRI in Keap1HKO OLTs. Groups of WT or Keap1HKO liver donor mice were pre-treated with Ly294002 or DMSO (-1 h). (A) sALT levels (IU/L): (□) sham; (▩) WT+DMSO; (▨) WT+ Lly294002; (▩) Keap1 HKO+DMSO; (■) Keap1 HKO+Ly294002. Mean±SD; n=4 mice/group; *p<0.05, **p<0.01. (B) Western analysis of p-Akt and HIF-1 in OLTs. β-actin served as an internal control. Representative of three experiments. (C) Representative H&E staining of OLTs (n=4) at 24h: Panel (a) WT+DMSO; (b) WT+Ly294002; (c) Keap1HKO+DMSO; (d) Keap1HKO+Ly294002 (magnification x100). Keap1-dependent Nrf2 activation promoted Trx1/Akt/HIF-1 signaling in mouse hepatocytes *in vitro*. (D) Western blot expression of Nrf2, Trx1 and p-Akt in primary H_2O_2-stressed hepatocyte (WT, Keap1HKO or Nrf2KO) cultures. Representative of three experiments. (E) Primary H_2O_2-stressed hepatocytes were pretreated with PI3K inhibitor (LY294002) or DMSO; p-Akt/HIF-1 expression was analyzed by Western blots. Representative of three experiments. (F) Quantitative RT-PCR-assisted detection of HIF-1 , HO-1, and Cyclin D1 in LY294002/DMSO-pretreated H_2O_2-stressed hepatocyte cultures. (G) LDH release (U/L) in LY294002/DMSO-treated hepatocytes. (F-G): (□) WT cells; (▩) WT cells+DMSO; (▨) WT cells+Ly294002; (▨)

Keap1 HKO cells; (▨) Keap1 HKO cells+DMSO; (■) Keap1 HKO cells+Ly294002. Mean ±SD; n=3-4/group; *p<0.005, **p<0.0005.