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Nuclear Factor Erythroid 2–Related Factor 2 Regulates Toll-Like Receptor 4 Innate Responses in Mouse Liver Ischemia-Reperfusion Injury Through Akt-Forkhead box Protein O1 Signaling Network

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Background. Nuclear factor erythroid 2–related factor 2 (Nrf2), a master regulator of the antioxidant host defense, maintains the cellular redox homeostasis.

Methods. This study was designed to investigate the role and molecular mechanisms by which Nrf2 regulates toll-like receptor (TLR)4-driven inflammation response in a mouse model of hepatic warm ischemia (90 min) and reperfusion (6 hr) injury (IRI).

Results. Activation of Nrf2 after preconditioning of wild-type mouse recipients with cobalt protoporphyrin ameliorated liver IRI, evidenced by improved hepatocellular function (serum alanine aminotransferase levels), and preserved tissue architecture (histology Suzuki's score). In marked contrast, ablation of Nrf2 signaling exacerbated IR-induced liver inflammation and damage in Nrf2 knockout hosts irrespective of adjunctive cobalt protoporphyrin treatment. The Nrf2 activation reduced macrophage and neutrophil trafficking, proinflammatory cytokine programs, and hepatocellular necrosis or apoptosis while increasing antiapoptotic functions in IR-stressed livers. At the molecular level, Nrf2 activation augmented heme oxygenase-1 expression and Stat3 phosphorylation and promoted PI3K-Akt while suppressing forkhead box O (Foxo)1 signaling. In contrast, Nrf2 deficiency diminished PI3K-Akt and enhanced Foxo1 expression in the ischemic livers. In parallel in vitro studies, Nrf2 knockdown in lipopolysaccharide-stimulated bone marrow-stimulated bone marrow–derived macrophages (BMMs) decreased heme oxygenase-1 and PI3K-Akt yet increased Foxo1 transcription, leading to enhanced expression of TLR4 proinflammatory mediators. Moreover, pretreatment of bone marrow–derived macrophages with PI3K inhibitor (LY294002) activated Foxo1 signaling, which in turn enhanced TLR4-driven innate responses in vitro.

Conclusion. Activation of Nrf2 promoted PI3K-Akt, and inhibited Foxo1 activity in IR-triggered local inflammation response. By identifying a novel integrated Nrf2-Akt-Foxo1 signaling network in PI3K-dependent regulation of TLR4-driven innate immune activation, this study provides the rationale for refined therapeutic approaches to manage liver inflammation and IRI in transplant recipients.

Keywords: Ischemia-reperfusion injury, Nrf2, TLR4, Foxo1, Innate immunity.

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Hepatic ischemia-reperfusion injury (IRI), an innate immunity-dominated local inflammation response, remains the leading cause of organ dysfunction and in liver transplantation. Ischemia-reperfusion activates Kupffer cells, which generate reactive oxygen species to facilitate proinflammatory cytokine-chemokine release, and cell apoptosis (*1*). We and others have documented toll-like receptor (TLR4) dependence of cytokine-chemokine programs required for inflammation and ultimate tissue damage in liver IRI immune cascade (*2, 3*).

The nuclear factor erythroid 2–related factor 2 (Nrf2), a master regulator of the antioxidant cell defense system (*4*), is held under normal conditions in the cytoplasm through binding to Kelch-lke ECH-associated protein 1 (Keap1) and leads to its degradation by the ubiquitin proteasome pathway (*5*). Activation of Nrf2 promotes cell growth and survival and contributes to cytoprotection in oxidative stress-induced injury, whereas disruption of Nrf2 enhances hyperoxia-induced acute lung injury and cell damage in oxidative stress-mediated neuroinflammation (*6, 7*). Moreover, disruption of Nrf2 signaling impairs angiogenic capacity of endothelial cells and antioxidant gene expression, leading to cardiac hypertrophy, myocardial fibrosis, and apoptosis in response to hemodynamic stress (*8*). Thus, Nrf2 is fundamental to the defense against oxidative stress and may be crucial in regulating inflammatory responses against oxidant stress-induced organ injury.

Forkhead box O (Foxo) family regulates multiple transcriptional targets in cell cycle, proliferation, survival, and apoptosis (*9*). Indeed, Foxos are essential in resistance to oxidative stress through regulation of cell survival (*10*). The phosphorylation of Foxo by Akt blocks Foxo DNA binding domain, leading to inhibition of Foxo1 transcriptional activity (*11, 12*). In turn, dephosphorylation of Foxo increases its nuclear accumulation or activity, which enhances target gene expression and apoptosis (*13, 14*). Increasing Foxo1 activity negatively regulated cardiomyocyte proliferation (*15*), whereas phosphorylation of Foxo1 increased cell survival and inhibited cell apoptosis in response to oxidative stress (*16*). Moreover, disruption of Foxo1 reduced the expression of innate immune factors, such as antimicrobial peptides and proinflammatory cytokines (*17*). Although these studies suggest an essential regulatory role of Akt-Foxo1 in a variety of cell functions, little is known about molecular mechanisms by which Nrf2-mediated Akt-Foxo1 signaling may regulate innate immune responses in liver IRI pathology.

We identify the Nrf2-Akt-Foxo1 axis as a regulator of inflammation response in IR-stressed liver. Activation of Nrf2 decreased macrophage or neutrophil sequestration, enhanced antiapoptotic functions, and increased heme oxygenase (HO)-1-Stat3 while inhibiting Foxo1 signaling, with resultant amelioration of liver IRI. This study reveals a novel PI3K-dependent crosstalk between integrated Nrf2-Akt-Foxo1 signaling network and TLR4-driven innate responses.

RESULTS

We used an established model of hepatocellular damage in mouse livers subjected to 90 min of warm ischemia

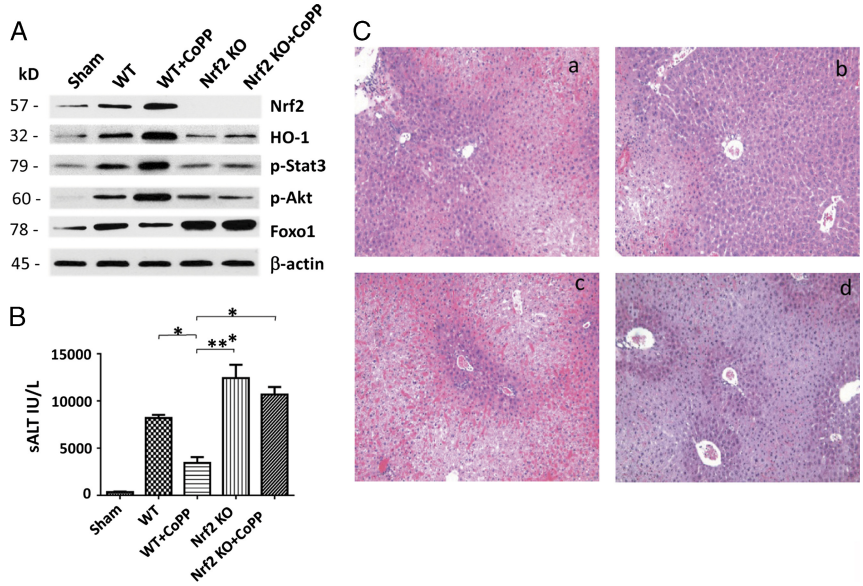

FIGURE 1. Activation of Nrf2 promotes Akt-Foxo1 signaling, and ameliorates liver IRI (6 hr of reperfusion after 90 min of warm ischemia). A, Western-assisted expression of Nrf2, HO-1, phosphorylated Stat3 (p-Stat3), p-Akt, and Foxo1 in CoPP-preconditioned WT, Nrf2-deficient and CoPP-preconditioned Nrf2-deficient livers. β-actin served as an internal control. Data representative of three experiments. B, The hepatocellular function in distinct animal groups evaluated by sALT (IU/L) levels. Mean±SD; n=4–6 mice per group. *P<0.01, **P<0.005. C, Representative H&E staining (magnification ×100) of IR-stressed livers. (a) WT; (b) WT+CoPP; (c) Nrf2 KO; (d) Nrf2 KO+CoPP; Mean±SD; n=4–6 mice per group. Symbols used: sham (▩); WT (▩); WT+CoPP (▤); Nrf2 KO (▥); Nrf2 KO+CoPP (▨). sALT, serum alanine aminotransferase; p-Stat3, phosphorylated Stat3; Nrf2, nuclear factor erythroid 2–related factor 2; HO, heme oxygenase; CoPP, cobalt protoporphyrin; WT, wild-type; SD, standard deviation; IRI, ischemia-reperfusion injury; Foxo1, Forkhead box O; H&E, hematoxylin-eosin.

and 6 hr reperfusion (*18*). First, as shown in Figure 1(A), we found deletion of Nrf2 in IR-stressed livers decreased HO-1 expression (absorbance units [AU]) as compared with wild-type (WT) mice (0.28±0.05 vs. 0.84±0.07, $P<0.0005$). Unlike in WT controls, Nrf2 deficiency reduced the Stat3 and Akt phosphorylation (0.35±0.02 vs. 0.81±0.04, $P<0.0005$ and 0.51±0.04 vs. 0.83±0.08, $P<0.01$) but increased Foxo1 expression (1.79±0.11 vs. 1.26±0.08, $P<0.005$). Moreover, we confirmed that preconditioning of WT mice with cobalt protoporphyrin (CoPP) enhanced the expression of Nrf2 and HO-1 in IR-stressed livers as compared with untreated WT mice (1.08±0.04 vs. 0.46±0.12, $P<0.005$ and 1.28±0.07 vs. 0.84±0.07, $P<0.0005$). In contrast, Nrf2 deficiency, irrespective of adjunctive CoPP treatment, depressed HO-1 expression (0.42±0.11 vs. 1.08±0.04, $P<0.0005$) in IR-stressed livers. Furthermore, preconditioning with CoPP increased p-Stat3 (1.5±0.05 vs. 0.52±0.13, $P<0.0005$) and p-Akt (1.64±0.06 vs. 0.25±0.05, $P<0.0001$) but diminished nuclear total Foxo1 (0.78±0.06 vs. 1.94±0.09, $P<0.0001$) expression, as compared with Nrf2 deficiency with concomitant CoPP in IR-stressed livers (Fig. 1A).

Cobalt protoporphyrin-preconditioning decreased serum alanine aminotransferase levels (IU/L), compared with untreated WT controls (3450±605 vs. 9195±425, $P<0.01$), Nrf2 knockout (KO) or Nrf2 KO+CoPP–treated mice (11929±1378 and 10679±807, $P<0.005$; Fig. 1B). We then evaluated the severity of hepatic IRI by Suzuki's histologic grading (Fig. 1C). Unlike WT controls, which showed moderate to severe sinusoidal congestion, cytoplasmic vacuolization, and hepatocellular necrosis (a; score=3.33±0.33), those pretreated with CoPP had well-preserved hepatic architecture (b; score=1.5±0.29, $P<0.01$). In marked contrast, Nrf2-deficient IR-stressed livers showed significant edema, severe sinusoidal congestion or cytoplasmic vacuolization, and extensive (30%–50%) necrosis (c; score=3.75±0.25, $P<0.005$). Similar findings were recorded in Nrf2-deficient IR-stressed livers despite CoPP preconditioning (d; score=3.4±0.25, $P<0.01$).

Nrf2 Activation Regulates Macrophage-Neutrophil Trafficking in Liver IRI

To determine whether Nrf2 activation may regulate cell trafficking, we performed immunohistochemical staining of CD68+ macrophages and Ly6G+ neutrophils in livers at 6 hr of reperfusion after 90 min ischemia. Indeed, Nrf2 activation decreased macrophage infiltration (Fig. 2A and C, b; 7±0.91) as compared to WT controls (a: 18.33±3.53, $P<0.05$), whereas Nrf2 deficiency augmented hepatic cell sequestration (c: 26.33±1.86, $P<0.001$; d: 23.67±2.03, $P<0.001$). Furthermore,

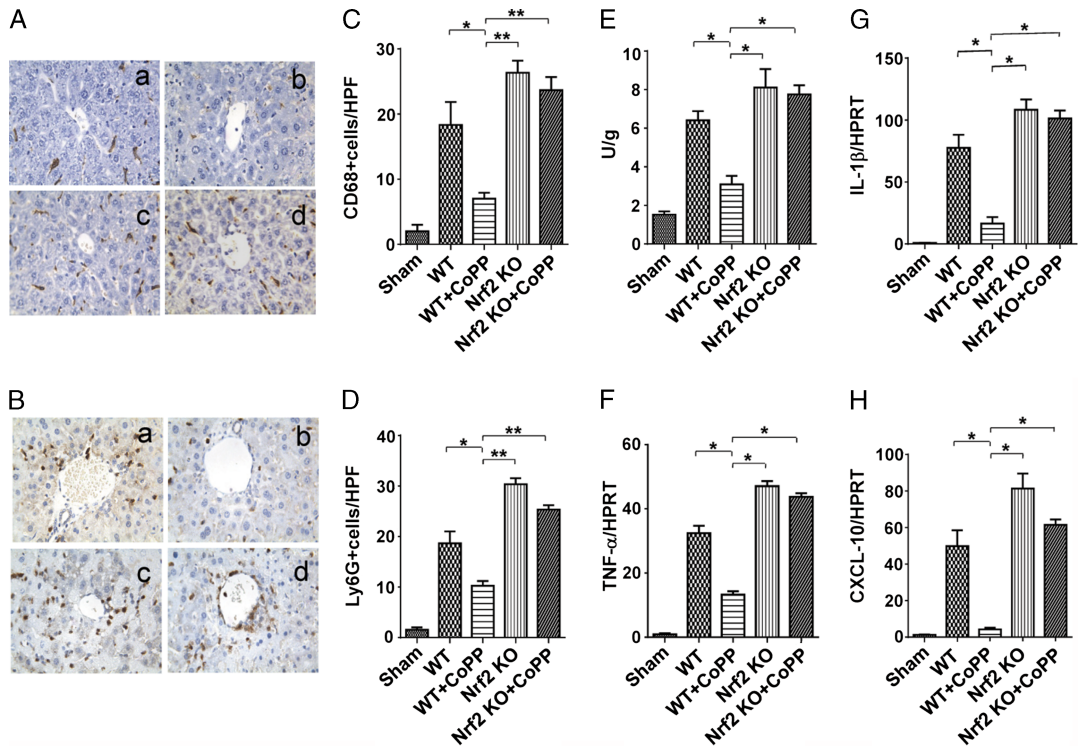

FIGURE 2. Activation of Nrf2 regulates intrahepatic macrophage-neutrophil trafficking and inflammatory programs in liver IRI (6 hr of reperfusion after 90 min of warm ischemia). Immunohistochemical staining for CD68+ cells (A–C) and LY6G+ cells (B–D): (a) WT; (b) WT+CoPP; (c) Nrf2 KO; (d) Nrf2 KO+CoPP; Mean±SD; Representative of 4 mice per group; magnification ×400; (C): *$P<0.05$; **$P<0.001$; (D): *$P<0.01$; **$P<0.001$. E, Neutrophil MPO activity (U/g). Mean±SD (n=4–6 samples/group). *$P<0.005$ (F–H) Quantitative RT-PCR-assisted detection of cytokine or chemokine gene expression; Mean±SD; n=3–4 per group; *$P<0.005$. Symbols used: sham (▩); WT (▩); WT+CoPP (▤); Nrf2 KO (▥); Nrf2 KO+CoPP (▨). Nrf2, nuclear factor erythroid 2–related factor 2; HO, heme oxygenase; CoPP, cobalt protoporphyrin; WT, wild-type; SD, standard deviation; IRI, ischemia-reperfusion injury; Foxo1, Forkhead box O; KO, knockout.

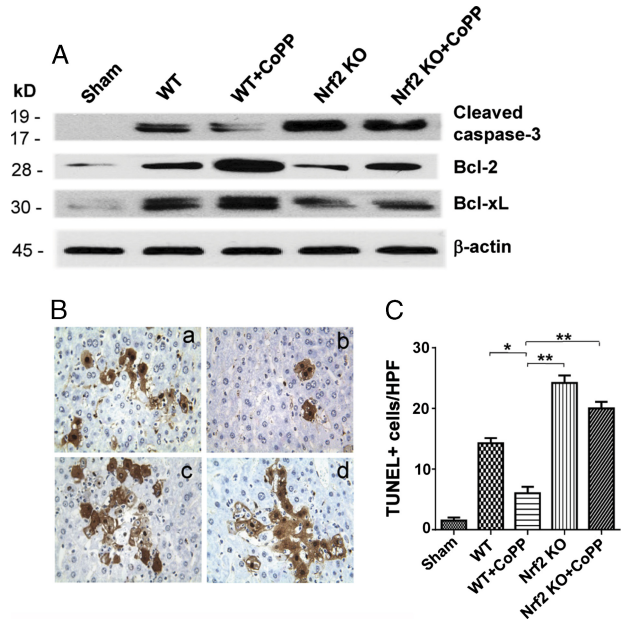

FIGURE 3. Activation of Nrf2 reduces hepatocellular apoptosis in liver IRI (6 hr of reperfusion after 90 min of warm ischemia). A, Western-assisted analysis of cleaved caspase-3, Bcl-2-Bcl-xl. Representative of three experiments. B and C, TUNEL staining. (a) WT; (b) WT+CoPP; (c) Nrf2 KO; (d) Nrf2 KO+CoPP; mean±SD; Representative of 4 mice per group; magnification ×400 *P<0.005; **P<0.001. Symbols used: sham (▩); WT (▩); WT+CoPP (▤); Nrf2 KO (▥); Nrf2 KO+CoPP (▨). Nrf2, nuclear factor erythroid 2–related factor 2; CoPP, cobalt protoporphyrin; WT, wild-type; SD, standard deviation; KO, knockout; TUNEL, terminal deoxynucleotide tranferase-mediated dUTP nick-end labeling.

CoPP-preconditioned livers showed decreased neutrophil accumulation (Fig. 2B and D, b; 11.25±0.85) compared to WT (a: 22±2.08, P<0.01) or Nrf2-deficient livers (c: 30.33±1.2, P<0.001; d: 25.33±0.88, P<0.001). The MPO assay (Fig. 2E) has revealed comparable hepatic neutrophil activity (U/g) pattern in CoPP-treated versus WT versus Nrf2 KO livers, respectively (3.09±0.44 vs. 6.41±0.47, P<0.005; vs. 8.11±0.06, P<0.005; vs. 7.75±0.48, P<0.005). Consistent with the immunostaining data, the mRNA levels coding for liver IRI signature genes, that is, IL-1β,TNF-α, and CXC chemokine ligand-10 (CXCL-10) were consistently reduced in Nrf2-overexpressing, as compared with untreated WT or Nrf2-deficient livers (Fig. 2F–H).

Nrf2 Activation Promotes Resistance to IR-Hepatocellular Apoptosis

We used Western blots to analyze whether Nrf2 signaling regulates cell apoptosis in IR-stressed livers (Fig. 3A). Indeed, CoPP-induced Nrf2 activation markedly increased hepatic expression (AU) of Bcl-2 and Bcl-xl, as compared with Nrf2-deficient (1.98±0.04 vs. 0.46±0.05, P<0.001 and 1.75±0.1 vs. 0.83±0.1, P<0.001) or Nrf2-deficient+CoPP (0.52±0.19 and 0.84±0.1, respectively, P<0.0005) groups. Moreover, Nrf2 deficiency, irrespective of adjunctive CoPP, consistently augmented the expression of cleaved caspase-3 (1.38±0.16 and 1.32±0.08), compared with WT controls (0.85±0.1, P<0.01).

We further analyzed liver cell apoptosis by TUNEL staining. As shown in Figure 3(B–C), CoPP preconditioning decreased the frequency of TUNEL$^+$ cells in ischemic liver lobes (b: 6.00±1.08), as compared with WT (a: 14.33±1.2, P<0.005) or Nrf2-deficient groups, which consistently showed increased numbers of TUNEL+ cells (c: 22.67±1.45, P<0.001; and d: 20.01±1.08, P<0.001).

Nrf2 Activation Promotes Akt-Foxo1 to Suppress TLR4 Response In Vitro

Our in vivo data show that Nrf2 signaling activated hepatic Akt-Foxo1, which in turn diminished liver IR-inflammation. We then assessed lipopolysaccharide (LPS)-stimulated bone marrow–derived macrophage (BMM) cell cultures by Western blots to test a hypothesis that Nrf2 regulates TLR4 through an Akt-Foxo1 pathway (Fig. 4A). Indeed, compared with LPS-stimulated WT BMMs, CoPP treatment induced Nrf2 activation (1.24±0.08 vs. 0.52±0.09, P<0.005) and upregulated the expression (AU) of HO-1 (2.32±0.08 vs. 1.22±0.08, P<0.0001) and p-Akt (1.16±0.14 vs.

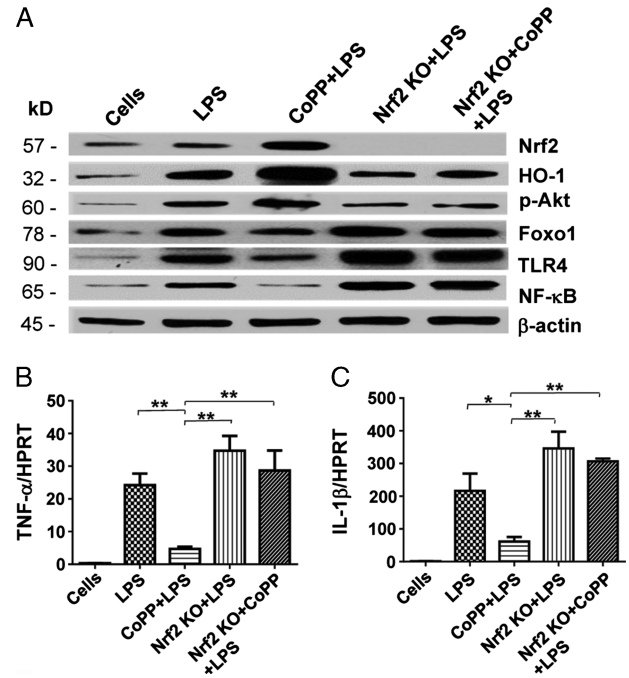

FIGURE 4. Activation of Nrf2 regulates Akt-Foxo1 signaling and TLR4-driven innate immune activation in vitro. Murine BMM cultures were supplemented for 4 hr with LPS (100 ng/ml) +/−CoPP (50μM). A, Western-assisted expression of Nrf2, HO-1, p-Akt, Foxo1, TLR4 and NF-κB. Representative of three experiments. B–C, Quantitative RT-PCR-assisted detection of mRNA coding for TNF-α and IL-1β. Data were normalized to HPRT gene expression. Mean±SD; n=3–4 per group; *P<0.05, **P<0.005. Symbols used: Cells (▩); LPS (▩); CoPP+LPS (▤); Nrf2 KO+LPS (▥); Nrf2 KO+CoPP+LPS (▨). Nrf2, nuclear factor erythroid 2–related factor 2; CoPP, cobalt protoporphyrin; SD, standard deviation; mRNA, messenger RNA; RT-PCR, reverse-transcriptase polymerase chain reaction; Foxo1, Forkhead box O; TNF, tumor necrosis factor; IL, interleukin; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LPS, lipopolysaccharide.

0.65±0.06, $P<0.005$) while depressing Foxo1 (0.87±0.05 vs. 1.18±0.03, $P<0.001$), TLR4 (0.49±0.03 vs. 1.2±0.14, $P<0.001$), and NF-κB (0.15±0.16 vs. 0.79±0.09, $P<0.0005$) levels. In contrast, Nrf2 deficiency, irrespective of adjunctive CoPP, depressed HO-1 (0.63±0.08 and 0.56±0.06, $P<0.005$), p-Akt (0.38±0.11 and 0.39±0.04, $P<0.05$) a yet enhanced Foxo1 (1.48±0.02 and 1.45±0.09, $P<0.05$), TLR4 (1.83±0.2 and 1.72±0.2, $P<0.05$) and NF-κB (1.21±0.01 and 1.19±0.04, $P<0.005$) as compared with LPS-stimulated WT BMMs. Furthermore, the expression of both TNF-α and IL-1β markedly increased in LPS-stimulated Nrf2-deficient as compared with CoPP-conditioned WT cells (Fig. 4B–C).

Inhibition of PI3K Disrupts Akt-Foxo1 and Enhances TLR4 Response in vitro

Because Nrf2 activation promoted Akt-Foxo1 signaling in IR-stressed livers, we then asked as to whether and how PI3K-Akt modulates Foxo1-mediated TLR4 function in vitro. Lipopolysaccharide-stimulated BMMs were treated first with a PI3K inhibitor (LY294002) or dimethyl sulfoxide, followed by CoPP. As shown in Figure 5(A), Western blot analysis has revealed that unlike CoPP-conditioned WT cells, adjunctive inhibition of PI3K abolished the expression (AU) of p-Akt (0.1–0.2 vs. 0.5–0.6) while increasing Foxo1 (1.5–1.6 vs. 0.8–0.9), TLR4 (1.5–1.6 vs. 0.8–0.9) and NF-κB (0.8–1.0 vs. 0.2–0.3). These findings were consistent with increased TNF-α-IL-1β and monocyte chemoattractant protein-1 (MCP-1)-CXCL-10 levels in BMMs subjected to PI3K blockade (Fig. 5B–E).

DISCUSSION

In this study, we have identified molecular mechanisms by which Nrf2 signaling modulates innate immune responses in mouse livers subjected to 90 min warm ischemia and 6 hr reperfusion. Notably, our in vivo and in vitro findings document that CoPP-induced Nrf2 activation downregulated TLR4-mediated inflammation response through an integrated Akt-Foxo1 signaling network in PI3K-dependent manner.

Because liver IR triggers macrophage activation and neutrophil recruitment leading to local inflammation, Nrf2 regulatory mechanisms encompass multiple immune signaling pathways. Consistent with the ability of CoPP to upregulate Nrf2 protein in human liver cells by posttranscriptional site of action (*19*), we found that Nrf2 activation after CoPP conditioning in vivo, increased Stat3 and Akt phosphorylation but inhibited Foxo1, consistent with diminished Stat3-Akt yet enhanced Foxo1 protein levels in Nrf2-deficient livers. It is important to note that CoPP-mediated effects in our present study were Nrf2-dependent, as global knockdown of Nrf2 consistently triggered fulminant liver IRI phenotype irrespective of adjunctive CoPP conditioning.

Foxo1 signaling is known to promote proinflammatory cytokine gene programs (*20*), and to regulate innate immune functions in respiratory epithelial cells (*17*). Moreover, we have recently shown the ability of PTEN-mediated Foxo1 to regulate inflammatory response by enhancing TLR4 activation in liver IRI (*21*). Our current results show that activation of Nrf2 signaling, unlike its deficiency, promoted hepatic Akt-Foxo1 signaling while decreasing macrophage-neutrophil

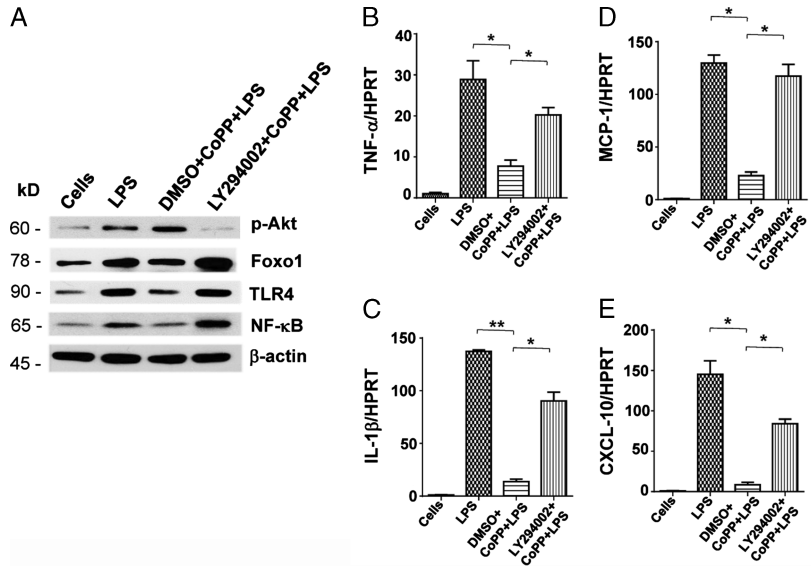

FIGURE 5. Inhibition of PI3K disrupts Akt-Foxo1 signaling and enhances TLR4-innate activation in vitro. BMMs from WT mice were pretreated with PI3K inhibitor LY294002 or DMSO, followed by LPS +/−CoPP. A, Western-assisted expression of Nrf2, p-Akt, Foxo1, TLR4 and NF-κB. β-actin served as an internal control. Representative of three experiments. B–E, Quantitative RT-PCR-assisted detection of TNF-α, IL-1β, MCP-1 and CXCL-10. Mean±SD; n=3–4 per group; *$P<0.05$, **$P<0.005$. Symbols used: Cells (▩); LPS (▩); DMSO+CoPP+LPS (▤); LY294002+CoPP +LPS (▥). BMMS, bone marrow–derived macrophages; DMSO, dimethyl sulfoxide; TLR, toll-like receptor; Nrf2, nuclear factor erythroid 2–related factor 2; Foxo, Forkhead box O; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; IL, interleukin; LPS, lipopolysaccharide.

hepatic sequestration, which further implies that Nrf2 regulates innate immune functions in IR-stressed livers. This finding complements our recent report in which hepatocyte-specific Keap1 deficiency ameliorated IRI by facilitating Nrf2 nuclear translocation and activating panels of cytoprotective genes in cold-preserved mouse liver transplants (*22*).

We found that Nrf2 activation in ischemic livers decreased caspase-3 but increased Bcl-2-Bcl-xL expression. In fact, the emerging evidence suggests involvement of Foxo transcription factors in intracellular apoptosis pathways (*23*). As downstream target of the serine-threonine protein kinase B (PKB)-Akt, phosphorylation of Foxo1 by Akt reduces its DNA-binding capacity and exports Foxo1 from nucleus to cytoplasm, where it degrades after ubiquitinylation, leading to cell survival (*24*). Thus, by regulating Akt-Foxo1 transcription, activation of Nrf2 promotes hepatocellular survival. Our TUNEL assay provided further evidence for increased frequency of apoptotic cells in Nrf2-deficient livers (−/+CoPP), whereas decreased apoptotic cell death was readily detectable in Nrf2 overexpressing WT livers.

The innate immune response, initiated by means of pathogen-associated molecular patterns signaling through TLRs expressed on host innate immune cells (*25*) can be influenced by the cellular redox state (*26, 27*). The Nrf2 is critical for induction of hepatic glutathione S-transferase and NAD(P)H:quinine oxidoreductase in response to oxidative stress (*28*). Moreover, although increased Nrf2 activity was shown to ameliorate chronic kidney disease-associated inflammation (*29*), disruption of Nrf2 resulted in increased susceptibility to bacterial infection and enhanced inflammatory cytokine programs during acute lung injury (*30*). In agreement with the regulatory function of Nrf2 in reactive oxygen species–mediated TLR4 activation in sepsis (*31*), our current in vivo data highlight the essential role of Nrf2 in the modulation of TLR4-driven inflammatory responses in IR-stressed livers,

Given our findings on Nrf2-mediated regulation of inflammation in IR-stressed livers, we next turned to well-controlled cell culture system to explore putative molecular mechanisms by which Nrf2 signaling may affect innate immune activation. Indeed, we have confirmed that Nrf2 activation was critically required for increased HO-1 expression in CoPP-conditioned LPS-stimulated BMMs. The immunomodulatory role of HO-1 associates with cell type–specific functions in myeloid cells (macrophages or monocytes), pivotal for inflammatory responses (*32*). Because stress-dependent HO-1 induction is primarily mediated through the cap'n' collar Nrf2 (*33*), activation of the latter is controlled by the cytosolic inhibitor Keap1, which permits subsequent nuclear translocation of Nrf2 (*34*). We have recently shown the essential role of Keap1-Nrf2 axis in preventing hepatic IRI in mouse liver transplants subjected to prolonged cold storage (*22*). Thus, in agreement with others (*35*), we now document that Nrf2-dependent HO-1 induction represents an important component of anti-inflammatory innate immune network in IR-stressed livers.

Activation of nuclear Foxo1 increases the expression of antimicrobial peptide, an important class of innate effector molecules that modulate an array of defense inflammatory responses (*36*). Our data show that Nrf2 activation increased Akt phosphorylation and inhibited Foxo1, TLR4, and NF-κB expression. In contrast, ablation of Nrf2 signaling depressed p-Akt, yet enhanced Foxo1, TLR4, and NF-κB, along with proinflammatory cytokine programs. Indeed, increasing Nrf2 activation regulated antioxidant myeloid leukocyte functions and improved septic survival by balancing inflammatory responses by means of redox regulation of TLR4 signaling while preserving antibacterial defenses (*37*). Activation of Nrf2 was also found to attenuate pulmonary inflammation by regulating NF-κB activation and proinflammatory cytokine programs (*38*).

To further elucidate the regulatory mechanisms of Nrf2-mediated Akt-Foxo1 signaling in innate inflammation in vitro, we blocked PI3K-Akt pathway in LPS-stimulated BMMs. We found that pretreatment of BMMs with Ly294002 enhanced Foxo1 and TLR4-NF-κB activation, leading to increased proinflammatory profile. Akt is a serine-threonine kinase that is regulated by means of activation of the second message PI3K (*39*). The PI3K-Akt signaling regulates cell proliferation or survival in part by phosphorylating Foxo (*24*). Increasing Foxo1 transcriptional activity enhances IL-1β–IL-2 production after LPS stimulation, whereas activation of NF-κB increases Foxo1 binding to IL-1β promoter (*20*), suggesting –NF-κB dependence of Foxo1-mediated regulation of proinflammatory cytokines. Moreover, by binding to multiple enhancer-like elements within the TLR4 gene, Foxo1 triggers macrophage TLR4 activation (*40*).

In conclusion, Nrf2 activation promotes PI3K-Akt and inactivates Foxo1 signaling, which in turn inhibits TLR4 activation in the mechanism of liver IRI (**Figure S1, SDC,** http://links.lww.com/TP/B34). By identifying a novel PI3K-dependent crosstalk between integrated Nrf2-Akt-Foxo1 signaling network and TLR4-driven innate immune activation, this study provides the rationale for refined therapeutic approaches to manage liver inflammation and IRI in transplant recipients. Of note, our present results are important in the context of conflicting recent findings on the biologic effects of Nrf2 induction in the liver, that is, benefits of Nrf2-dependent cytoprotection against IRI (*41*), as in our study; yet impaired hepatic regeneration in CCL4-injured livers despite constitutive Nrf2 hepatocyte overexpression (*42*).

MATERIALS AND METHODS

Animals

The WT male mice (C57BL/6) at 6–8 weeks of age were used (Jackson Laboratory, Bar Harbor, ME). The Nrf2-deficient ($Nrf2^{-/-}$; KO) breeding pairs (C57BL/6) were kindly provided by Dr. T. Kensler (Johns Hopkins University, Baltimore, MD). Animals were housed in University of California, Los Angeles 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 IRI Model

We used an established mouse model of warm hepatic ischemia followed by reperfusion (*18*). Groups of WT and Nrf2 KO mice were injected with heparin (100 U/kg), and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad liver lobes. After 90 min, the clip was removed, and mice were killed at 6 hr of reperfusion. Additional groups of WT and Nrf2 KO animals were treated with CoPP (5 mg/kg, intraperitoneally; Frontier Scientific Porphyrin Products, Logan, UT) 24 hr before the ischemia.

Hepatocellular Function Assay

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

Histology and Immunohistochemistry

Liver sections (5 μm) were stained with hematoxylin-eosin. The severity of IRI was graded using Suzuki's criteria on a scale from 0 to 4 (*43*). Liver macrophages and neutrophils were detected using primary rat anti-mouse CD68 (AbD Serotec, Raleigh, NC) and Ly6G (BD Biosciences, San Jose, CA) monoclonal antibodies. The secondary, biotinylated goat anti-rat immunoglobulin G (Vector, Burlingame, CA) was incubated with immunoperoxidase (ABC Kit, Vector). Positive cells were counted blindly in 10 high-power field per section (×400).

TUNEL Assay

The Klenow-FragEL DNA Detection Kit (EMD Chemicals, Gibbstown, NJ) was used to detect the DNA fragmentation characteristic of apoptosis in formalin-fixed paraffin-embedded liver sections (*44*). The results were scored semiquantitatively by averaging the number of apoptotic cells per high-power field (400×magnification). Ten fields were evaluated per tissue sample.

Quantitative RT-PCR Analysis

Quantitative real-time PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25μl, the following were added: 1× SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) complementary DNA 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 sec), and 60°C (30 sec). Primers used to amplify specific gene fragments are shown in (**Table S1, SDC,** http://links.lww.com/TP/B34).

Western Blot Analysis

Proteins (30 μg/sample) from cell cultures or liver samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Monoclonal rabbit anti-mouse phos-Akt, phos-Stat3, Foxo1, NF-κB, Bcl-2, Bcl-xl, cleaved caspase-3, and β-actin Abs (Cell Signaling Technology, Danvers, MA), Polyclonal rabbit anti-mouse Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), HO-1 (StressGen Biotech, Victoria, BC, Canada), and TLR4 (Imgenex, San Diego, CA) were used. Relative quantities of protein were determined using a densitometer and are expressed in AU.

Cell Isolation and In Vitro Cultures

Murine BMM were generated, as described (*44*). Bone marrow cells were removed from the femurs and tibias of WT and Nrf2 KO mice, cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS and 15% L929-conditioned medium. Cells (1×10^6/well) cultured for 7 days were treated with CoPP (50 μM) and LPS (100 ng/mL) for 4 hr. In some experiments, cells were pretreated with PI3K inhibitor (LY294002; 10 μM [Calbiochem, San Diego, CA]) or dimethyl sulfoxide (6.5μL/mL) for 1 hr.

Statistical Analysis

Data are expressed as mean±standard deviation. Statistical comparisons were analyzed by Student's *t* test. Differences were statistically significant at *P* value less than 0.05.

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