Acute hepatic ischemic-reperfusion injury induces a renal cortical “stress response,” renal “cytoresistance,” and an endotoxin hyperresponsive state

Richard A. Zager, Ali C. M. Johnson, and Kirsten B. Frostad
The Fred Hutchinson Cancer Research Center, Seattle, Washington; and University of Washington, Seattle, Washington
Submitted 3 July 2014; accepted in final form 25 July 2014

Zager RA, Johnson AC, Frostad KB. Acute hepatic ischemic-reperfusion injury induces a renal cortical “stress response,” renal “cytoresistance,” and an endotoxin hyperresponsive state. Am J Physiol Renal Physiol 307: F856–F868, 2014. First published July 30, 2014; doi:10.1152/ajprenal.00378.2014.—Hepatic ischemic-reperfusion injury (HIRI) is considered a risk factor for clinical acute kidney injury (AKI). However, HIRI’s impact on renal tubular cell homeostasis and subsequent injury responses remains ill-defined. To explore this issue, 30–45 min of partial HIRI was induced in CD-1 mice. Sham-operated or normal mice served as controls. Renal changes and superimposed injury responses (glycerol-induced AKI; endotoxemia) were assessed 2–18 h later. HIRI induced mild azotemia (blood urea nitrogen ~45 mg/dl) in the absence of renal histologic injury or proteinuria, implying a “prerenal” state. However, marked renal cortical, and isolated proximal tubule, cytoprotective “stress protein” gene induction (neutrophil gelatinase-associated lipocalin, heme oxygenase-1, hemopexin, hepcidin), and increased Toll-like receptor 4 (TLR4) expression resulted (protein/mRNA levels). Ischemia caused release of hepatic heme-based proteins (e.g., cytochrome c) into the circulation. This corresponded with renal cortical oxidant stress (malondialdehyde increases). That hepatic derived factors can evoke redox-sensitive “stress protein” induction was implied by the following: peritoneal dialysate from HIRI mice, soluble hepatic extract, or exogenous cytochrome c each induced the above stress protein(s) either in vivo or in cultured tubule cells. Functional significance of HIRI-induced renal “preconditioning” was indicated by the following: 1) HIRI conferred virtually complete protective morphologic protection against glycerol-induced AKI (in the absence of hyperbilirubinemia) and 2) HIRI-induced TLR4 upregulation led to a renal endotoxin hyperresponsive state (excess TNF-α/MCP-1 gene induction). In conclusion, HIRI can evoke “renal preconditioning,” likely due, in part, to hepatic release of pro-oxidant factors (e.g., cytochrome c) into the systemic circulation. The resulting renal changes can impact subsequent AKI susceptibility and TLR4 pathway-mediated stress.

hepatic ischemic-reperfusion injury; acute kidney injury; hepatic renal preconditioning

There is a growing literature to suggest that the acutely injured kidney can negatively impact extra-renal organs, such as heart, lung, and brain, and by so doing, contribute to acute kidney injury (AKI)-associated multi-organ failure (e.g., Refs. 1, 11, 13, 26, 33). Pathogenic mechanisms for this phenomenon, widely known as “organ cross talk,” are believed to involve the release of inflammatory mediators, such as cytokines, chemokines, and damage-associated molecular pathway molecules (DAMPs) from the kidney into the systemic circulation (10, 14). After binding to their respective receptors at extra-renal sites (e.g., TNFR1, CCR2, TLRs, RAGE, TREM-1), intracellular signaling (e.g., via NF-κB or MAPK pathways) evokes an inflammatory response, culminating in organ damage.

By analogy, it seems equally plausible that extra-renal tissue injury could secondarily influence the kidney via the above noted pathways. Given the well-recognized clinical association between liver injury and the development of AKI (3, 5, 6, 8, 17, 29, 35), we questioned with acute hepatic damage, such as induced by ischemia, secondarily impacts the kidney, thereby inducing renal tubular homeostatic changes that subsequently alter renal injury responses. Of particular interest in this regard is whether this hypothesized hepatic renal “preconditioning” alters renal inflammatory responses (e.g., due to endotoxemia) or superimposed ischemic or toxic AKI.

To address these issues, we utilized a model of partial hepatic ischemic-reperfusion injury (HIRI) that affects three of five hepatic lobes (2). After a 4- or 18-h recovery period, the presence of an acute renal “stress response” [e.g., neutrophil gelatinase-associated lipocalin (NGAL) expression], renal function, histology, endotoxin responsiveness, and susceptibility to superimposed tubule damage were assessed. The results of these studies indicate that HIRI can, indeed, evoke homeostatic, and functionally significant, changes within the kidney. The data that support this conclusion form the basis of this report.

METHODS

Model of Partial HIRI

All experiments were conducted using male CD-1 mice (30–45 g) obtained from Charles River Laboratories (Wilmington, MA). They were maintained under routine vivarium conditions with free food and water access. The employed HIRI protocols were approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

Following induction of deep anesthesia (40–50 mg/kg ip pentobarbital sodium), a midline abdominal incision was performed, exposing the liver. The liver was tilted upward, exposing the portal triad. The left branches of the hepatic artery and portal vein were occluded using anatraumatic microvascular clamp (2), and it was left in place for either 30 or 45 min. Following its removal, reperfusion was confirmed by the reestablishment of normal hepatic color. Body temperature was maintained at 36–37°C throughout with a heating lamp. The abdominal incisions were then sutured in two layers with 3–0 silk sutures and the mice were allowed to recover from anesthesia. Either 4 or 18 h post hepatic reperfusion, the mice were reanesthetized, the abdominal cavities were reopened, a blood sample was obtained from the inferior vena cava, and then the kidneys were resected and placed on ice. Renal cortical samples were extracted for either protein or total RNA for subsequent analyses. A terminal urine sample was also obtained from the urinary bladder.

Two sets of controls were used for the above experiments. These consisted of either normal mice, or mice that had undergone sham surgery. The latter group was used to determine whether surgical stress, per se, independent of HIRI, impacted the assessed renal
homeostatic parameters. A sufficient number of mice was used to obtain an $n$ of 5–8 determinations for each assessment (total of 60 mice subjected to the HIRI protocol; equal numbers of controls).

**Post HIRI Assessments**

Blood urea nitrogen, plasma creatinine, and urine protein concentrations. Terminal blood samples were assayed for blood urea nitrogen (BUN; Biochain #Z5030016; Newark, NJ) and creatinine concentrations (Biochain #Z5030020; Newark, CA). Total urine protein concentrations were determined by the pyrogallous red method (7) and plasma samples were also assayed for MDA.

Plasma bilirubin concentrations. Terminal (18 h) blood samples from 45-min HIRI mice and sham-operated mice were assayed for total bilirubin concentrations by autoanalyzer (Alliance Laboratories, Seattle, WA).

As markers of hepatic injury, hepatic release of LDH, cytochrome c, and total heme protein (e.g., cytochrome c) were measured by the pyrogallous red method (7) and plasma samples were also assayed for MDA.

Renal histology. Cross sections of kidneys, obtained from sham-operated mice and 45-min HIRI mice, were fixed in 10% buffered formalin at 18 h post HIRI or sham surgery. Two-micrometer paraffin-embedded sections were cut and stained with either hematoxylin & eosin (H&E) or periodic acid Schiff (PAS) and examined under light microscopy.

mRNA analysis. Renal cortical samples, obtained from the control, sham-operated, and HIRI mice at either 4 or 18 h postsurgery, were immediately iced and extracted for total RNA (RNeasy; Qiagen; Hpx, and Hep (see RESULTS), and given that each of these genes is homeostatic parameters. A sufficient number of mice was used to obtain an $n$ of 5–8 determinations for each assessment (total of 60 mice subjected to the HIRI protocol; equal numbers of controls).

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mRNA analysis. Renal cortical samples, obtained from the control, sham-operated, and HIRI mice at either 4 or 18 h postsurgery, were immediately iced and extracted for total RNA (RNeasy; Qiagen; Alameda, CA). The samples were assayed by RT-PCR for the following mRNAs: 1) NGAL (40), 2) hemopexin (Hp) (41), 3) heme oxygenase-1 (HO-1) (41), 4) hepcidin (Hep), and 5) Toll-like receptor 4 (TLR4) (47). The primers used are presented in Table 1. Additional samples were analyzed for TNF-α and monocyte chemoattractant protein-1 (MCP-1) mRNAs, as described below (endotoxin response experiments). Results were factored by simultaneously obtained values for GAPDH, used as a reference housekeeping gene.

Renal cortical test protein concentrations. To assess whether observed mRNA changes correlated with their respective protein levels, renal cortical protein extracts, prepared in the presence of protease inhibitors, were subjected to ELISAs for HO-1 (Enzo Life Sciences #ADi-960–071; Farmingdale, NY), NGAL (R&D Systems #DY8157), Hp (Alpha Diagnostics #600–700-HPX; San Antonio, TX), and Hep (Intrinsinc LifeSciences, La Jolla, CA). Renal cortical TLR4 was assessed by immunohistochemistry, as described below.

Isolated proximal tubule segment assessments. To determine whether renal cortical stress responses were also expressed in proximal tubules, RNA and protein extracts were prepared from proximal tubules that had been isolated from normal, sham-operated, and 18-h post 45-min HIRI mice ($n = 5–6$ each) and immediately assayed for NGAL, Hp, HO-1, and Hep mRNAs and protein levels. The tubules were isolated by a process of cortical mincing, collagenase digestion, and Percoll centrifugation, as previously described in detail (44).

Renal cortical oxidative stress, as assessed by malondialdehyde content. Given that HIRI increased renal expression of NGAL, HO-1, Hp, and Hep (see RESULTS), and given that each of these genes is redox responsive, the potential impact of HIRI on renal cortical oxidative stress was assessed. To this end, renal cortical malondialdehyde (MDA) levels (thiobarbituric acid method) (7) were measured in tissue samples obtained from control, sham-operated, and 45-min HIRI mice ($n = 5$ each) at the 18-h time point. To assess whether potential renal MDA increases might have reflected MDA efflux from the postischemic liver with subsequent renal entry, matching hepatic and plasma samples were also assayed for MDA.

### Table 1. Primers used to determine test mRNAs in mouse cortex and in human-derived proximal tubule (HK-2) cells

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer Sequences</th>
<th>Product size, bp</th>
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<tr>
<td><strong>Mouse Primers for RT-PCR</strong></td>
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<td></td>
</tr>
<tr>
<td>NGAL</td>
<td>5'-AACATTGTGTTCTACGAGCTGACTG3'-3'</td>
<td>224</td>
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<tr>
<td>Hemopexin</td>
<td>5'-ACCTGCTGTTCACTGAGCTGACTG3'-3'</td>
<td>854</td>
</tr>
<tr>
<td>HO-1</td>
<td>5'-GACAAAGAGAGGAGCTGACTGACTG3'-3'</td>
<td>288</td>
</tr>
<tr>
<td>Hecidin</td>
<td>5'-CAGAGAGAGAGGAGCTGACTGACTGACTG3'-3'</td>
<td>218</td>
</tr>
<tr>
<td>TLR4</td>
<td>5'-ACGTCAGAGTGGAGAAATGTG3'-3'</td>
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<tr>
<td>GAPDH</td>
<td>5'-GCTGACATTCAGGCTGAGGAATGGTG3'-3'</td>
<td>347</td>
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<tr>
<td><strong>Human Primers for RT-PCR</strong></td>
<td></td>
<td></td>
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<tr>
<td>NGAL</td>
<td>5'-CCAGAAGAAAGAAGGCAGGTC3'-3'</td>
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<tr>
<td>Hemopexin</td>
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<td>HO-1</td>
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<td>GAPDH</td>
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Primers that were used to measure mouse or human mRNAs by RT-PCR during the course of these investigations. NGAL, neutrophil gelatinase-associated lipocalin; HO-1, heme oxygenase-1; TLR4, Toll-like receptor 4.

### HK-2 Cell mRNA Assessments: Impact of Liver Extract, Peritoneal Dialysate, and Cytochrome c

The purpose of the following three experiments was to test whether potential release of liver factors into the systemic circulation might gain renal access and thus induce a renal cortical stress response.

**Exposure of culture HK-2 cells to soluble liver extract.** Pieces of normal liver were homogenized in cell culture medium [keratinocyte serum-free medium (K-SFM)] (25) and then centrifuged to remove particulate material. The soluble homogenate was added to cultured human proximal tubule derived (HK-2) (25) cells that were plated in 12-well Costar plates (liver homogenate concentrations of 0, 10, or 35 µg/ml). After an overnight incubation, the cells were recovered by scraping with a rubber policeman, RNA was extracted (RNeasy), and assayed for human HO-1, Hpx, Hep, NGAL, and TLR4 mRNAs using the primers shown in Table 1 ($n = 5$ wells/treatment).

**Exposure to peritoneal dialysate from sham-operated and HIRI mice.** This experiment assessed whether circulating factors post HIRI can induce a tubule “stress” protein response. To this end, sham-operated and 18-h post HIRI mice were anesthetized and injected intraperitoneally with 2 ml of cell culture medium (K-SFM). After 1 h (allowing time for dialysate equilibrium with plasma) (48), the abdominal cavities were opened, the dialysate was removed, and the mice were killed by vena cava transection. HK-2 cells, cultured in 12-well plates, had the initial K-SFM replaced with either fresh K-SFM, or K-SFM containing 10% dialysate recovered from sham or HIRI mice. After an overnight incubation, the cells were recovered by scraping with a rubber policeman, total RNA was extracted, and then assayed for human HO-1, Hpx, Hep, NGAL, and TLR4 mRNA.

AJP-Renal Physiol. doi:10.1152/ajprenal.00378.2014 www.ajprenal.org
Results were factored by simultaneously determined GAPDH product (primers listed in Table 1).

Cytochrome c injection. Five mice were injected with 2 mg of cytochrome c (from bovine heart; C3131; Sigma Chemicals; in 0.1 ml saline). Five saline-injected mice served as controls. Four hours later, the mice were anesthetized, the kidneys were removed, and RNA extracts were assayed for HO-1, Hpx, NGAL, and Hep mRNAs. The results were compared between the two groups.

Renal Cortical Responses to Endotoxemia

Diverse forms of AKI evoke renal hyperresponsiveness to TLR4 ligands, most notably LPS (19, 45, 46, 49). The following experiment assessed whether HIRI-induced “renal preconditioning” also evokes this LPS hyperresponsive state. Six groups of mice (n = 5 each) were established as follows: 1) normal mice; 2) sham-operated mice; 3) 18-h post 45-min HIRI mice; 4) normal mice + LPS injection; 5) sham-operated mice + LPS injection; and 6) HIRI mice + LPS injection. LPS was injected via the tail vein at the 18-h time point (10 mg/kg of Escherichia coli LPS; 0111:B4; L-2630; Sigma Chemicals; stock solution, 4 mg/ml saline). Non-LPS-treated groups received saline vehicle injection. Two hours postinjection, the mice were anesthetized, the abdominal cavities were opened, a blood sample was obtained from the inferior vena cava, and then the kidneys and areas of injured liver were resected. As an index of LPS responsiveness, renal and hepatic RNA extracts were assayed for TNF-α and MCP-1 mRNAs (RT-PCR) (45). Plasma TNF-α and MCP-1 protein levels were measured by ELISA (TNF-α, BD Biosciences #555268; San Diego, CA; MCP-1, BD Biosciences #555260) (45).

Renal cortical TLR4 expression. Because the above experiment demonstrated that HIRI evoked renal LPS hyperresponsiveness, the impact of hepatic injury on renal expression of TLR4 (the LPS receptor) was assessed. To this end, TLR4 mRNA levels were measured in renal cortex and isolated proximal tubules from control, sham-operated, and 18-h post HIRI mice (47). To ascertain whether changes in TLR4 mRNA were associated with changes in TLR4 protein expression, formalin-fixed, paraffin-embedded, 2-μm kidney cross sections were cut and subjected to TLR4 immunohistochemical analysis, as previously described (47). Isotype-negative IgG served as a negative control.

Renal Responses to Glycerol-Induced AKI

To determine the potential impact of HIRI “renal preconditioning” on renal AKI susceptibility, mice were subjected to either sham surgery or 45 min of HIRI (n = 6 per group). Four hours later, all mice were injected with hypertonic glycerol (50%; 8 ml/kg), administered in equally divided doses into the hind limbs. Eighteen hours later, the mice were anesthetized with pentobarbital sodium, the abdominal cavities were reopened, a blood sample was obtained from the vena cava for BUN and creatinine analysis, and the kidneys were removed. The severity of glycerol-induced myolyis/hemolysis was assessed by plasma LDH assay, and total plasma free heme protein assay (400-nm optical density). Because renal cortical LDH loss is a highly sensitive and reliable quantitative marker of in vivo AKI severity (42), the percent loss of renal cortical LDH was assessed [(normal cortical LDH values – post-glycerol renal cortical LDH values)/normal values] × 100%. Finally, kidney cross sections were fixed in 10% formalin and 2-μm paraffin-embedded sections were cut and stained with H&E for histologic injury severity. The sections were blind coded, and injury was graded semiquantitatively on a 1+ to 5+ scale (least to most severe injury observed in individual kidney sections). Injury severity was based on the extent of tubular necrosis and cast formation. Scores were compared by Wilcoxon Rank Sum test.

Calculations and Statistics

All values are given as means ± 1 SE. Statistical comparisons were made by unpaired Student’s t-test unless stated otherwise. If multiple comparisons were made, the Bonferroni correction was applied.

RESULTS

Hepatic Injury

Placement of a vascular clamp on the hepatic vasculature produced an immediate blanching of the three cephalad-affected liver lobes, while the caudal lobes appeared normal, as previously reported (2). After clamp removal, there was prompt recovery of normal hepatic color in the affected lobes. At 18 h postsurgery, extensive injury was apparent, with the surface of involved liver lobes manifesting gross macroscopic signs of tissue necrosis (see Fig. 1, *). The caudal lobes continued to appear normal (Fig. 1, *).

As expected, 30–45 min of partial hepatic necrosis produced stepwise increases in 18-h plasma LDH concentrations (Fig. 1, right). That the LDH increments were a manifestation of liver injury was indicated by relatively trivial plasma LDH increases following sham surgery. Liver injury also caused an approximately sixfold rise in plasma cytochrome c levels at 4 h postsurgery, and the latter remained elevated, albeit at lesser levels, at the 18-h time point (Fig. 1, right). Because cytochrome c is a heme-based intracellular protein that is released in response to injury (e.g., Ref. 39), we also assessed total heme protein release, and progressive plasma increases were observed (sham surgery 1.4 ± 0.1; 4 h post HIRI 2.0 ± 0.2; 18 h post HIRI 2.8 ± 0.2 OD U/ml; P < 0.05 vs. sham controls).

[Of note, the rise in plasma-free heme did not arise from muscle injury, given that CPK levels in sham-operated and HIRI mice did not significantly differ (95 ± 22 vs. 101 ± 35 U/ml, respectively)]. Despite the presence of severe liver injury, only a 0.18-mg/dl increase in total bilirubin concentrations was observed (sham surgery 0.2 ± 0.03; HIRI 0.38 ± 0.05 mg/dl).

Renal Functional and Morphologic Assessments

Sham surgery did not alter either the BUN or plasma creatinine concentrations (vs. controls). In contrast, 30 and 45 min of HIRI produced stepwise azotemia (Fig. 2). However, renal histologic assessments, performed on both H&E- and PAS-stained sections, revealed morphologically normal kidneys. In particular, there was no evidence of tubule necrosis, cast formation, vascular clot formation, or glomerular abnormalities (Fig. 3). Particularly noteworthy was that PAS staining (Fig. 3, A and B) revealed a lush, intact brush border. Of note, brush-border blebbing with luminal shedding is considered a highly sensitive histologic marker of acute proximal tubule damage (4, 31). Consistent with the absence of glomerular abnormalities were normal urine protein concentrations in HIRI mice (3.5 ± 0.4 vs. control values of 3.8 ± 0.5; mg/mg urine creatinine).

Renal Cortical NGAL mRNA Assessments

Because NGAL is a highly sensitive marker of a renal cortical stress response (22, 30, 42), its mRNA within renal cortex was assessed at both 4 and 18 h after either 30 or 45 min of HIRI. As shown in Fig. 4, stepwise NGAL mRNA increases...
were observed, both over time (4 h, 18 h) and with increasing lengths of hepatic ischemia. At 18 h post 45 min of HIRI, ~30-fold NGAL mRNA elevations were observed (vs. sham-operated controls).

Renal Cortical HO-1 and Hpx mRNA Levels

HO-1 and Hpx, like NGAL, are renal cortical stress markers (40, 41). As shown in Fig. 5, both HO-1 and Hpx mRNAs manifested marked increases in response to HIRI. These increases were as great, or greater, than those seen for NGAL mRNA (Fig. 5, left), underscoring their robust nature. Part of the HO-1 mRNA response was due to surgery, not HIRI, given that sham surgery (S) alone also increased HO-1 mRNA; however, this increase was significantly less than that seen with HIRI \( (P < 0.01) \).

Renal Cortical Hep mRNA Levels

In addition to its primary role as a Fe regulatory protein, Hep is also a hepatic acute stress reactant (16, 24). However, its induction within the kidney in response to stress has not previously been ascertained. As shown in Fig. 5, right, HIRI induced ~10-fold renal cortical Hep mRNA increases. That

![Graph showing plasma LDH and Cyt C levels](image)

Fig. 1. Liver injury 18 h following 45 min of partial hepatic ischemia. As depicted by the (+) signs, 3 of 5 resected lobes appeared pale and grossly necrotic, whereas the remaining liver lobes (*) appeared grossly normal. Right: correlate of this hepatic injury was a marked increase in plasma LDH concentrations with increasing values observed with 30 and 45 min of hepatic ischemia, as observed at 18 h of reperfusion. In addition, an increase in cytochrome c (cyt c) release into plasma was observed, with the most dramatic elevation seen at 4-h post hepatic ischemia-reperfusion injury (HIRI). By 18 h post HIRI, cyt c levels were still significantly elevated, albeit at a reduced level vs. the 4-h values. All LDH and cyt c values were statistically significant compared with values in time-matched sham-operated controls (S). The cyt c values were analyzed by Wilcoxon rank sum test as the 4-h values had a nonparametric distribution.

![Graph showing BUN and plasma creatinine](image)

Fig. 2. Blood urea nitrogen (BUN) and plasma creatinine concentrations measured at 18 h following either sham surgery or hepatic ischemia (either 30 or 45 min in length). Sham surgery did not alter either BUN or creatinine concentrations compared with normal [control (C)] mouse values. Conversely, stepwise azotemia was induced by increasing hepatic ischemia times \( (P < 0.01 \text{ vs. sham surgery or control values}) \).
this was induced by HIRI, not surgical stress, was indicated by normal Hep mRNA levels in sham-operated animals.

Renal Cortical NGAL, HO-1, Hpx, and Hep Protein Levels

To assess whether the observed NGAL, HO-1, Hpx, and Hep mRNA increases were associated with increases in their respective proteins, each was measured by ELISA. As shown in Fig. 6, 3- to 10-fold NGAL, HO-1, Hpx, and Hep protein increases were observed in renal cortex.

Isolated Proximal Tubule NGAL, HO-1, Hpx, and Hep Protein and mRNA Levels

As shown in Table 2, isolated proximal tubules from HIRI mice had dramatically elevated NGAL, HO-1, Hpx, and Hep protein levels. NGAL, HO-1, and HPX mRNA levels were also elevated. [Of note, however, is that Hep mRNA levels could not be quantified in isolated tubules due to its degradation during tubule isolation.] That isolated tubule protein and mRNA values paralleled those seen in whole renal cortex indicated that the cortical increases reflected changes in proximal tubules.

Assessment of Renal Cortical Oxidative Stress

Given that NGAL, HO-1, Hpx, and Hep are each redox-sensitive stress proteins, a potential mechanism for their up-regulation could be liver injury-initiated renal oxidant stress (e.g., due to hepatic release of heme proteins such as cytochrome c). To address this possibility, renal cortical lipid peroxidation was assessed by MDA tissue concentrations. At 18 h postinduction of 45 min of liver ischemia, a 65% increase in renal cortical MDA levels was observed (Fig. 7; \( P < 0.005 \)). The HIRI protocol also induced hepatic oxidant stress, as indicated by a fourfold increase in hepatic MDA levels. To ascertain whether the renal increases could have been secondarily affected by MDA release from the liver into plasma with subsequent renal uptake, plasma MDA levels were measured. No plasma MDA increases were observed. Finally, sham surgery did not affect renal, hepatic, or plasma MDA concent-
trations. Thus, these data imply that HIRI caused renal lipid peroxidation, perhaps due to hepatic heme protein release, and the resultant oxidant stress caused, or contributed to, the upregulation of redox-sensitive proteins in the kidney.

**HK-2 Cell mRNA Assessments: Impact of Liver Extract and Peritoneal Dialysate Addition**

*Liver extract addition.* As shown in Table 3, addition of liver extract caused dramatic, and in general dose-dependent, increases in HO-1, NGAL, Hpx, and Hep mRNAs. The most sensitive was HO-1 mRNA, which increased 10-fold over control values. Conversely, TLR4 mRNA was not induced.

*Peritoneal dialysate addition.* When peritoneal dialysate from HIRI mice was added to HK-2 cells, a tripling of HO-1 mRNA resulted, compared with values observed under normal or control dialysate incubations. The HIRI dialysate did not change the levels of the other test mRNAs (data not shown), possibly because they were less sensitive to HO-1 induction (see liver extract addition experiments).

**In Vivo Cytochrome c Effects**

Cytochrome c injection significantly increased each of the four tested mRNAs, as follows: HO-1: $0.52 \pm 0.03$ vs. $0.33 \pm 0.02, P < 0.05$; NGAL: $0.69 \pm 0.28$ vs. $0.03 \pm 0.00, P < 0.01$;...
Renal Cortical Responses to Endotoxemia

Renal cortical TNF-α/MCP-1 mRNAs. AKI is known to elicit TLR4 pathway/LPS hyperresponsiveness (19, 45, 46, 49), as assessed by renal TNF-α and MCP-1 expression. To determine whether HIRI might secondarily induce this phenomenon, TNF-α and MCP-1 mRNA levels were assessed in the following groups of mice: 1, 2) normal mice ± 2 h post LPS; 3, 4) 18 h postsham surgery ± 2 h post LPS; 5, 6) 18-h post HIRI mice ± 2 h post LPS injection. As shown in Fig. 8, baseline levels of renal cortical TNF-α and MCP-1 mRNA manifested only trivial elevations at 18 h post HIRI. All mice showed dramatic TNF-α/MCP-1 mRNA increases in response to LPS. These responses were approximately twice as high in HIRI mice vs. the normal mice or sham-operated controls (note the different y-axis scales in the two Fig. 8 panels).

Plasma TNF-α/MCP-1 protein levels. Plasma TNF-α and MCP-1 levels at 2 h post LPS quantitatively mirrored the renal cortical TNF-α/MCP-1 mRNA changes. Specifically, HIRI mice manifested twice the plasma TNF-α and MCP-1 increases in response to LPS vs. control or sham-operated mice (Fig. 9).

Not depicted, in the absence of LPS injection, barely detectable TNF-α and MCP-1 protein levels were observed.

Hepatic responses to LPS. To assess whether the injured liver hyperresponded to LPS, hepatic TNF-α and MCP-1 mRNA levels were measured at baseline or 2-h post LPS injection. As shown in Fig. 10, left panel, the injured liver had slight increases in baseline TNF-α and MCP-1 mRNA levels. Unlike kidney, no hepatic LPS hyperresponsiveness was observed, given comparable hepatic TNF-α and MCP-1 mRNA increases in response to LPS in the control, sham-operated, and HIRI groups.

HIRI effects on renal TLR4 expression. As a possible reason for HIRI-induced renal hyperresponsiveness to LPS, an increase in TLR4 expression was sought. As shown in Fig. 11, left, at 18 h postinduction of liver ischemia, renal cortical TLR4 mRNA levels were two- to threefold higher in HIRI mice vs. control or sham-operated animals. That proximal tubules were involved in this response was indicated by the fact that TLR4 mRNA levels in isolated proximal tubules matched those observed in whole renal cortex (Fig. 11, middle). Finally, the mRNA increases were associated with increased proximal tubule TLR4 density, as assessed by immunohistochemistry (Fig. 11: A, isotype negative control; B, sham surgery at 18 h; C, 45-min HIRI mice at 18 h).

Table 2. Isolated proximal tubule mRNA and protein concentrations: tubules harvested from control (normal) mice, sham-operated mice, and mice subjected to 45 min of hepatic ischemia + 18 h of reflow

<table>
<thead>
<tr>
<th>mRNA protein</th>
<th>Control</th>
<th>Sham</th>
<th>HIRI</th>
</tr>
</thead>
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<tr>
<td>NGAL</td>
<td>0.27±0.27</td>
<td>0.6±0.11</td>
<td>14.1±3.2*</td>
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<td>1.5±0.08</td>
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<td>Hpx</td>
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<td>Protein</td>
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</tr>
<tr>
<td>NGAL</td>
<td>0.19±0.05</td>
<td>0.25±0.02</td>
<td>15±2</td>
</tr>
<tr>
<td>Hpx</td>
<td>1.23±0.05</td>
<td>1.8±0.1</td>
<td>3.9±2.0*</td>
</tr>
</tbody>
</table>

mRNA values are expressed as a ratio to simultaneously obtained GAPDH values. XXX, not measured due to hepcidin mRNA degradation during tubule isolation. Protein concentrations are given as ng/mg total protein (hepcidin, hemopexin (hpx), HO-1) or µg/mg NGAL protein extracted from proximal tubules.

HIRI, hepatic ischemic-reperfusion injury. *P < 0.01. †P < 0.05.

Hep: 1.16±0.57 vs. 0.17±0.05, P < 0.05; Hpx: 1.23±0.05 vs. 0.18±0.04, P < 0.03 (cytochrome c vs. controls, respectively).

Fig. 7. Malondialdehyde (MDA) levels in renal cortical, hepatic, and plasma samples taken from normal mice (C), sham-operated mice, and mice subjected to the 45-min liver ischemia (HIRI) protocol. The HIRI protocol induced significant increases in both renal cortical and hepatic MDA levels. These increases could not be ascribed to surgery alone, given that the values observed in sham-operated mice remained normal. Plasma MDA levels remained either at, or below, normal values (implying that the tissue MDA increases did not reflect tissue loading from plasma).
Table 3. HK-2 incubation with soluble liver extract or peritoneal dialysate: effect on HO-1, Hpx, Heparidin, NGAL, and TLR4 mRNAs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 μg/ml</th>
<th>35 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1 mRNA</td>
<td>1.2 ± 0.2</td>
<td>7.5 ± 1.3</td>
<td>11.9 ± 0.4*</td>
</tr>
<tr>
<td>Hpx mRNA</td>
<td>0.35 ± 0.07</td>
<td>0.73 ± 0.03</td>
<td>1.0 ± 0.08*</td>
</tr>
<tr>
<td>Heparidin mRNA</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.03</td>
<td>0.08 ± 0.02†</td>
</tr>
<tr>
<td>NGAL mRNA</td>
<td>1.5 ± 0.03</td>
<td>2.3 ± 0.13</td>
<td>2.0 ± 0.03*</td>
</tr>
<tr>
<td>TLR4 mRNA</td>
<td>0.2 ± 0.04</td>
<td>0.14 ± 0.04</td>
<td>0.23 ± 0.07 (NS)</td>
</tr>
</tbody>
</table>

HK-2 mRNA levels following the addition of normal culture medium or culture medium containing either 10 or 35 μg/ml of soluble liver extract (see text). *P < 0.001. †P < 0.025. ‡P < 0.0001.

Impact of HIRI on Renal Tubular Responses to Glycerol-Induced AKI

By 18-h postglycerol injection, both groups of mice manifested marked BUN and creatinine elevations (Fig. 12). However, given the fact that HIRI, in the absence of glycerol injection, induces modest azotemia (Fig. 2), the degree of BUN and creatinine increases in HIRI mice that could be attributable to glycerol, per se, was half as much as that observed in the sham-operated group (this is depicted by the vertical arrows rising above basal values within the bars in Fig. 12). Further supporting HIRI-induced protection against glycerol-induced injury was a significant decrease in the degree of renal cortical LDH loss (42) (Fig. 12). Finally, there was dramatically less histologic injury in the HIRI group. The latter demonstrated near normal histology, with only occasional heme-stained casts in the outer/inner medulla and essentially no tubule necrosis. Conversely, in the sham-operated controls, glycerol induced severe cast formation and widespread proximal tubule necrosis in both the cortex and outer medullary stripe (Fig. 13). The markedly decreased amount of injury in the HIRI/glycerol group could not be ascribed to less glycerol-induced muscle necrosis/hemolysis, given that terminal plasma LDH increases, and free heme levels were >twice as high in the HIRI vs. the sham-operated group (P < 0.01 for each).

DISCUSSION

There is strong clinical evidence suggesting that acute liver injury is a risk factor for AKI (3, 5, 6, 8, 17, 29, 35). However, the impact of liver injury on renal tubular cell homeostasis and injury responses remains ill-defined. Previous experimental studies underscore the complexity of this issue. For example, this laboratory previously demonstrated that a focus of necrotic liver sensitizes to gentamicin-induced acute renal failure (ARF), due in large part to markedly increased renal gentamicin uptake (36). In a different study, we found that liver contains soluble low molecular weight cytotoxic factors that, if released into the circulation, can potentially cause tubular necrosis and ARF (37). Other groups have utilized a total common bile duct ligation (CBDL) model in rodents to explore this issue. Using this model, Tajiri et al. (28) reported that CBDL sensitizes the kidney to ischemic AKI. Conversely, Leung et al. (12) found that CBDL × 1 wk, raising bilirubin levels ~50-fold, paradoxically protected rats against the glycerol model of rhabdomyolysis-induced ARF.

Undoubtedly, the most common form of liver injury that exists in critically ill patients is not CBDL but rather HIRI. Indeed, under conditions of decreased cardiac output, the liver experiences disproportionate reductions in blood flow (15, 21), leading to ischemic hepatic damage. That about two-thirds of hepatic blood flow is derived from poorly O2-saturated portal venous blood further predisposes to ischemic damage. Despite the frequency of HIRI in critically ill and/or hypotensive patients, its impact on renal homeostasis, and secondary injury responses, has not previously been assessed. Thus, in the

Fig. 8. Renal cortical TNF-α and monocyte chemoattractant protein-1 (MCP-1) mRNA levels under baseline conditions (left) and at 2-h post LPS injection (right). Under basal conditions, the liver ischemia (HIRI) protocol induced only quantitatively trivial increases in TNF-α and MCP-1 mRNA values (note different y-axis scales in the left vs. right panels). At 2-h post LPS injection, marked increases in both TNF-α and MCP-1 mRNAs were observed in all animals. However, the degrees of increase were approximately twice as great in the HIRI animals. This hypersensitivity to LPS was due to liver ischemia, not surgical stress, given that sham (S) surgery did not impact LPS-induced renal mRNA increases. NS, not significant.
present study, we pursued this issue with the following questions in mind: 1) does HIRI induce overt renal injury, or alternatively, trigger a secondary renal cortical “stress response”? 2) because acute ischemic or nephrotoxic tubular injury is known to increase renal sensitivity to TLR4 ligands (most notably endotoxin) (19, 45, 46, 49), might HIRI also lead to a renal TLR4 pathway/endotoxin hyperresponsive state? and 3) what is the direct impact of HIRI on renal tubular susceptibility to superimposed tubular necrosis and ARF?

To address these issues, we employed a model of reversible HIRI, induced by partial occlusion of the portal triad. As shown in Fig. 1, 45 min of hepatic ischemia caused extensive hepatic necrosis, involving three of five liver lobes. Although progressive HIRI evoked stepwise azotemia (Fig. 2), renal morphologic assessments revealed completely normal histology. Specifically, neither tubular necrosis nor cast formation was observed. Furthermore, there was a complete absence of proximal tubule brush-border shedding, a highly sensitive morphologic marker of acute tubular damage (4, 31). Given this normal histology, plus the fact that liver injury activates a variety of renal vasoconstrictive mediators (e.g., the renin-angiotensin, endothelin, and sympathomimetic pathways) (27, 34), it appears all but certain that the observed post HIRI azotemia reflected a “prerenal” azotemic state. The involved
vasoactive mediators, and their resultant hemodynamic changes (e.g., potential decreases in renal blood flow, glomerular capillary pressure, possible activation of tubuloglomerular feedback), remain unknown at this time.

Despite the absence of histologic injury, HIRI induced abrupt dose- and time-dependent increases in renal cortical NGAL mRNA and protein levels. That these changes reflected proximal tubule events was indicated by parallel NGAL mRNA and protein increases in isolated proximal tubule preparations. Given that NGAL is widely regarded as a renal cortical “stress reactant” (22), these findings imply that although “prerenal” azotemia developed following HIRI, a concomitant renal tubular “stress response” also occurred. To access its potential broader reaching implications, HIRI’s impact on renal cortical HO-1 gene expression was assessed. As with NGAL, significant HO-1 mRNA and protein increases were observed in both renal cortex and in isolated proximal tubules. In a recent series of studies, we reported that a variety of liver-based “stress proteins,” most notably α-fetoprotein (32), α-1 antitrypsin (43), haptoglobin (50), and hemopexin (41), are unmasked within kidney during ischemic and diverse forms of nephrotoxic AKI, leading to increases in their respective proteins and RNAs. We dubbed this phenomenon the “renal hepatization” response (43). Given this backdrop, we

Fig. 11. Renal cortical and isolated proximal tubule Toll-like receptor 4 (TLR4) responses to HIRI. By 18 h postinduction of 45-min HIRI, marked increases in TLR4 mRNA were observed in renal cortex (left), compared with values in control renal cortical tissues or cortical tissues obtained from sham-operated (S) animals. These mRNA changes were paralleled by those observed in isolated proximal tubules (middle). Finally, increased TLR4 protein density was observed in proximal tubules, as assessed by immunohistochemistry (A: isotype antibody negative control; B: S mouse; C: 18 h post HIRI).

Fig. 12. Assessments of acute kidney injury (AKI) severity in sham-operated and HIRI mice 18 h following glycerol-induced AKI. As shown in the left 2 panels, the severity of glycerol-induced azotemia at 18-h postglycerol injection was comparable between the 2 groups. However, because HIRI induces significant azotemia in the absence of glycerol (see Fig. 2), the degree of increase over nonglycerol-injected values (shown by vertical arrows rising above baseline bars) was ~50% lower in the HIRI vs. the sham-operated group. Further suggesting protection was a significant reduction in LDH loss from renal cortex in the HIRI vs. sham group. Finally, and most dramatically, renal histology indicated strikingly less injury in the HIRI group (right; also see Fig. 13).
next tested whether HIRI would also induce this state. Indeed, this appeared to be the case, as denoted by the fact that HIRI-activated the renal cortical/isolated proximal tubule Hpx gene (mRNA/protein increases). Furthermore, we found that Hep, another liver-based “stress protein” (16, 24), was also induced in the kidney in response to HIRI. To our knowledge, this is the first demonstration of stress-induced Hep gene induction outside of the liver, and specifically, within the kidney. In sum, these findings indicate that although HIRI did not produce overt tubular damage (e.g., cell necrosis, cast formation), it was capable of causing a marked change in renal tubular phenotype with an upregulation of a variety of stress proteins.

It is noteworthy that all of the above documented HIRI-induced stress proteins (HO-1, Hpx, NGAL, Hep) have been reported to have renal cytoprotective effects (9, 18, 20, 23, 50). Thus, we posited that HIRI-induced “renal preconditioning” would confer cytoprotection upon proximal tubule cells. To address this issue, sham-operated and HIRI mice were subjected to the glycerol model of ARF, which is mediated by combined myohemoglobinuria and renal ischemia (38). A number of points indicate that HIRI did, indeed, confer a protective effect, as follows: First, although both groups of mice manifested substantial and approximately equal degrees of azotemia, given that HIRI independently causes substantial azotemia, the finding of comparable BUN and creatinine elevations postglycerol implies less glycerol-induced renal damage. Second, a 50% decrease in glycerol-induced renal cortical LDH loss was noted in the HIRI vs. sham-operated mice. Given that cortical LDH loss is a quantitative marker of AKI severity (42), this implies that less lethal tubular cell injury occurred; and third, renal histology confirmed remarkable protection, given the striking decreases in tubular necrosis, brush-border loss, and cast formation in the postglycerol HIRI vs. the glycerol-injected sham-operated controls. Indeed, the minimal injury in the postglycerol/HIRI mice suggests that most, if not all, of the azotemia in this group likely reflected a prerenal state (vs. necrosis/cast formation in the sham-operated/glycerol group). In sum, all three assessed parameters indicate that HIRI induced marked cytoprotection upon proximal tubules. It has previously been reported that profound cholestasis (~50-fold bilirubin increase), arising after 1 wk of CBDL, can confer renal protection against glycerol-induced ARF (12). In contrast, the currently observed protection was expressed within just hours of HIRI, and in the virtual absence of cholestasis (only a 0.2-mg/dl bilirubin increase). This suggests that in the CBDL model, it may be direct hepatocyte injury triggered by CBDL, rather than cholestasis, per se, that triggered the previously reported renal cytoprotected state (12).

As with ischemic and nephrotoxic AKI (19, 45, 46, 49), the present study demonstrates that HIRI-induced “renal preconditioning” also evokes an LPS hyperresponsive state. Thus, after a 2-h LPS challenge, a doubling of renal cortical TNF-α/MCP-1 mRNAs and of plasma TNF-α/MCP-1 protein levels was observed in the HIRI vs. the control groups. Surprisingly, this LPS hyperresponsiveness was not shared by injured liver, which showed no preferential LPS-initiated TNF-α/MCP-1 response. To explore a potential explanation for HIRI-initiated LPS hyperresponsiveness in the kidney, renal cortical, as well as isolated tubule, TLR4 mRNA levels were assessed, and in both instances, an approximate doubling was observed in the HIRI group. Furthermore, a corresponding increase in proximal tubule TLR4 density was observed, as assessed by immunohistochemistry. Thus, an attractive hypothesis is that HIRI increases TLR4 expression, and by so doing, it may lead to increased TLR4-LPS binding and thus, an LPS hyperresponsive state. Of note, this proposed mechanism stands in sharp contrast to the situation of ischemic and nephrotoxic AKI.
where LPS hyperresponsiveness is expressed in the setting of decreased, rather than increased, renal cortical TLR4 levels (47).

Finally, potential mechanisms by which HIRI might impact renal stress protein expression and injury susceptibility were sought. Given that HIRI leads to hepatic cytokine generation (TNF-α/MCP-1) (2), cytokine release into the systemic circulation with potential “downstream” renal changes were assessed. However, neither plasma TNF-α nor MCP-1 levels were significantly elevated in the employed HIRI model, seemingly negating this possibility. Next, we hypothesized that HIRI might release pro-oxidant factors into the circulation, inducing renal cortical oxidant stress with subsequent induction of redox-sensitive stress proteins. Three pieces of evidence support this concept. First, HIRI induced renal cortical lipid peroxidation, as indicated by increases in renal MDA content. Second, addition of peritoneal dialysate from HIRI mice induced an HO-1 response in HK-2 cells, as denoted by a threefold increase in its mRNA; and third, addition of a soluble hepatic extract induced HO-1, Hpx, Hep, and NGAL mRNAs in HK-2 cells. The hepatic-based molecular mediators that induced renal oxidant stress and redox-sensitive stress protein induction will require much future in-depth investigation. However, one potential group of candidates could be hepatic-based cytochromes, such as cytochrome c, which is known to enter plasma from injured tissues (39). Given that HIRI caused marked cytochrome c release into plasma, and given that it is a readily filtered (12 kDa) heme-based (pro-oxidant) protein, renal oxidant stress, lipid peroxidation, and redox-sensitive stress protein induction should result. That intravenous cytochrome c injection into normal mice induced a renal cortical HO-1, NGAL, Hpx, and Hep response clearly supports this hypothesis. It is also quite possible that other hepatic-based heme proteins, e.g., electron transporters or cytochrome P-450s, could be involved. In this regard, it is notable that total free heme protein levels in plasma were elevated post HIRI, and not simply cytochrome c.

Based on the above, we offer the following conclusions: 1) Experimental HIRI can acutely induce a renal cortical “stress response,” culminating in the upregulation of multiple cytoprotective proteins (HO-1, NGAL, Hpx, Hep). 2) Although HIRI can evoke modest azotemia, this appears not to result from overt renal damage, but rather from a “prerenal” state. 3) Despite suggestions that acute liver injury predisposes to AKI, the present study indicates that, at least in the setting of the glycerol-induced AKI, the converse may well be the case. 4) The observed cytoprotection seems likely to be mediated, at least in part, by broad-based “cytoprotective stress protein” induction. 5) Although Hep is considered to be an hepatocentric “stress protein,” the current study demonstrates, for the first time, that HIRI can secondarily induce renal Hep gene expression. 6) The above findings may arise, at least in part, via HIRI-initiated renal cortical oxidant stress. The available data indicate that the release of pro-oxidant factors from the damaged liver, e.g., cytochrome c, may well be involved. And 7) as with intrinsic AKI, HIRI-induced renal “preconditioning” leads to a TLR4/LPS hyperresponsive state. HIRI-induced renal cortical/proximal tubule TLR4 gene upregulation with enhanced LPS binding seems likely to be involved. Finally, it is noteworthy that sepsis syndrome is a leading cause of clinical AKI. Hence, renal hypersensitivity to TLR4 ligands, rather than increased tubular susceptibility to injury, may be a more likely contributor to the clinical association between acute liver disease and the risk of ARF.

REFERENCES

AJP-Renal Physiol doi:10.1152/ajprenal.00378.2014 www.ajprenal.org

ACKNOWLEDGMENTS
The authors thank Dr. Mark Westerman, Intrinsinc Life Sciences, La Jolla, CA, for kindly providing mouse hepcidin ELISAs for use in these experiments.

GRANTS
This work was supported by research grants from the National Institutes of Health (DK38432, DK68520).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: R.A.Z. conception and design of research; R.A.Z. and A.C.J. analyzed data; R.A.Z. and A.C.J. interpreted results of experiments; R.A.Z. prepared figures; R.A.Z. drafted manuscript; R.A.Z. edited and revised manuscript; R.A.Z., A.C.J., and K.B.F. approved final version of manuscript; A.C.J. and K.B.F. performed experiments.

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