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Basal Rather Than Induced Heme Oxygenase-1 Levels Are Crucial in the Antioxidant Cytoprotection

Sei-ichiro Tsuchihashi,2,* Masha Livhits,2† Yuan Zhai,* Ronald W. Busuttil,* Jesus A. Araujo,3,4† and Jerzy W. Kupiec-Weglinski4*‡

Heme oxygenase-1 (HO-1) overexpression protects against tissue injury in many inflammatory processes, including ischemia/reperfusion injury (IRI). This study evaluated whether genetically decreased HO-1 levels affected susceptibility to liver IRI. Partial warm ischemia was produced in hepatic lobes for 90 min followed by 6 h of reperfusion in heterozygous HO-1 knockout (HO-1+/−) and HO-1−/− wild-type (WT) mice. HO-1+/− mice demonstrated reduced HO-1 mRNA/protein levels at baseline and postreperfusion. This corresponded with increased hepatocellular damage in HO-1−/− mice, compared with WT. HO-1−/− mice revealed enhanced neutrophil infiltration and proinflammatory cytokine (TNF-α, IL-6, and IFN-γ) induction, as well as an increase of intralobular apoptotic TUNEL+ cells with enhanced expression of proapoptotic genes (Bax/cleaved caspase-3). We used cobalt protoporphyrin (CoPP) treatment to evaluate the effect of increased baseline HO-1 levels in both WT and HO-1+/− mice. CoPP treatment increased HO-1 expression in both animal groups, which correlated with a lower degree of hepatic damage. However, HO-1 mRNA/protein levels were still lower in HO-1+/− mice, which failed to achieve the degree of antioxidant hepatoprotection seen in CoPP-treated WT. Although the baseline and postreperfusion HO-1 levels correlated with the degree of protection, the HO-1 fold induction correlated instead with the degree of damage. Thus, basal HO-1 levels are more critical than the ability to up-regulate HO-1 in response to the IRI and may also predict the success of pharmacologically induced cytoprotection. This model provides an opportunity to further our understanding of HO-1 in stress defense mechanisms and design new regimens to prevent IRI.

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I

schemia/reperfusion injury (IRI),5 an Ag-independent inflammatory component of organ procurement, remains an important problem in clinical transplantation. In the case of the liver, IRI causes up to 10% of early transplant failures and can lead to a higher incidence of acute and chronic rejection (1). Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme groups to biliverdin, CO, and free iron (2).

Biliverdin is converted into bilirubin, and free iron is used in intracellular metabolism or sequestered into ferritin. It is believed that the products derived from this catalytic reaction can mimic the physiological effects of HO. HO-1, also known as a heat shock protein 32, is the only inducible isofrom of HO. HO-1 has been shown to provide an important protective response from cellular stress following ischemia, inflammation, or radiation, preventing the deleterious effects of heme as well as mediating anti-inflammatory and antiapoptotic functions via its products (3–5), but the relationship of this cytoprotection with its kinetics of expression is a matter of controversy. It is unclear whether baseline HO-1 levels before the injury or the degree of HO-1 up-regulation following the injury is important to confer cytoprotection (6–8). Thus, HO-1 overexpression by pharmacological means or via genetic engineering has been shown to exert potent cytoprotective effects in hepatic IRI transplant models, where both proinflammatory and apoptotic responses remain profoundly diminished in HO-1-overexpressing liver transplants (9–11). In contrast, the lack or inhibition of HO-1 which generally accelerates host inflammatory responses, as shown in HO-1-deficient mice (12) as well as in HO-1 deficiency reported in humans (13), has yielded contrasting evidence in the liver IRI (11, 14).

In this study, to mimic a clinically relevant scenario, we evaluated the mechanisms by which genetically decreased basal HO-1 levels, rather than its total absence, affected the liver IRI cascade. For this purpose, we used genetically engineered mice, heterozygous for a targeted disruption of the HO-1 gene (HO-1+/−). When compared with HO-1+/− wild-type (WT) counterparts, HO-1−/− mice were significantly more susceptible to hepatic IRI, as evidenced by the severity of hepatocellular damage, enhanced neutrophil infiltration, marked proinflammatory cytokine expression, and proapoptotic responses. Although the baseline and postreperfusion HO-1 levels correlated with the degree of protection, in support of HO-1’s cytoprotective role, the HO-1 fold induction correlated instead with the degree of damage. Thus, basal HO-1 levels are more critical than the ability to up-regulate HO-1 in response to the IRI.

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findings underscore the key role of basal HO-1 levels in proinflammatory and apoptotic mechanisms during hepatic IRI.

Materials and Methods

Animals

Eight- to 10-wk-old HO-1 heterozygous (−/+ ) null mice were obtained from the mouse colony of Drs. J. A. Araujo and A. J. Lusis (University of California, Los Angeles, CA (UCLA)), originally developed by Poss and Tonegawa (15). This mouse colony was maintained on a mixed C57BL/6 background and genotyping was performed by PCR of tail DNA using specific primers to amplify the targeted HO-1 allele (5'-GCT TGG GTG GAG AGG CTA TTC-3' and 5'-CAA GGT GAT AGG GAA GAT C-3') and the WT HO-1 allele (5'-GTA CAC TGA CTG GTG GGG GAG-3' and 5'-AGG GCC GAG TAG ATG TGG TAC-3'). PCR conditions were as follows: 94°C for 10 min, 35 cycles of 94°C for 15 s, 54°C for 15 s, 72°C for 30 s, and a final 2 cycles of 72°C for 10 min. All reactions were performed on a PerkinElmer 9700 machine. Age-matched HO-1−/− littermates were used as controls as well as age-matched wild-type C57BL/6 mice from The Jackson Laboratory, which in preliminary trials were shown to exhibit the phenotypes of HO-1−/− littermates.

Mice were housed in the UCLA animal facility under specific pathogen-free conditions. All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (publication 86-23, revised 1985).

Hepatic warm IRI model

We used an established mouse model of partial warm hepatic IRI, as described (16). Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.), injected with heparin (100 U/kg), and an atraumatic clip was used to interrupt the artery and portal venous blood supply to the left and middle lobes of the liver. After 90 min of partial hepatic warm ischemia, the clamp was removed, initiating hepatic reperfusion. Mice were sacrificed at 6 h of reperfusion, and liver/blood samples were collected. The extent and severity of hepatic IRI were assessed in groups of 1) WT mice (HO-1+/+ ; n = 18); 2) heterozygous HO-1 knockout (KO) mice (HO-1−/− ; n = 15); 3) WT mice that were pretreated with cobalt protoporphyrin (CoP; Prophyrin Products), an HO-1 inducer (n = 8); and 4) HO-1−/− mice that were pretreated with CoP (n = 8). CoP, dissolved in 0.2 M lithium hydroxide (pH 7.4) was administered (5.0 mg/kg i.p) 24 h before the onset of ischemia. To determine the levels of basal hepatic HO-1 expression, liver biopsy was taken before the placement of the atraumatic clip on the vascular supply.

Histopathological evaluation

Liver paraffin sections (5-μm thick) were stained with H&E. The severity of IRI was blindly graded using modified Suzuki’s criteria (17). In this classification, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded on a scale of 0–4. No necrosis, congestion, or centrilobular ballooning are given a score of 0, whereas severe congestion/ballooning and >60% lobular necrosis are given a value of 4.

Neutrophil infiltration

The activity of myeloperoxidase (MPO), an enzyme specific for polymorphonuclear neutrophils (PMNs), was used as an index of hepatic neutrophil accumulation (16). Briefly, the frozen tissue was thawed and placed in iodinated 0.5% hexadecyltrimethyl-ammonium bromide and 50 mM potassium phosphate buffer solution (pH = 5.0). Each sample was homogenized and centrifuged at 15,000 rpm for 15 min at 4°C. Supernatants were then mixed with hydrogen peroxide-sodium acetate and tetramethyl-benzidine solutions. The change in absorbance was measured by spectrophotometry at 655 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μM peroxide/min at 25°C/g of tissue.

RNA extraction and real-time RT-PCR

Baseline and post-I/R liver tissue were preserved at −70°C after harvesting. Total RNA was extracted from liver tissue using the guanidine isothiocyanate/phenol method (18). To exclude potential genomic DNA contamination, the samples were treated with DNase I (amplification grade DNase I; Invitrogen Life Technologies). Reverse transcription was performed using 2 μg of RNA with the first-strand cDNA Synthesis Kit (iScript cDNA synthesis kit; Bio-Rad) using oligo(dT) and random hexamers.

Quantitative real-time PCR was used to measure intraintraparenchymal mRNA expression of HO-1, TNF-α, IFN-γ, IL-6, IL-10, and β-actin as a housekeeping control. PCR primers were designed from the corresponding cDNA sequences from the Ensembl database (19) using the Primer3 program (Table I). Each set of primers was designed to span exons. Specific PCR products were assessed by agarose gel electrophoresis to rule out the possibility of any nonspecific amplification. Each reaction was performed in duplicate in a total reaction volume of 25 μl, with 1 μM of primers and 1–2 μg of cDNA template. The conditions of the reaction were as follows: 95°C for 3 min, 40 cycles of 95°C for 10 s, 58–64°C for 30 s (see Table I for annealing temperature of each primer set), and 72°C for 20 s, performed on an ABI Prism 7000 (Applied Biosystems) using SYBR Green (iQ SYBR Green Supermix; Bio-Rad). Annealing temperature was optimized for each primer pair (Table I). A standard curve was created from serial dilutions of a pooled sample of cDNA. Gene expression was normalized to β-actin. PCR levels were displayed as levels of arbitrary units.

Western blot analysis

Protein was extracted from livers with ice-cold PBSTDS buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in PBS). Protein samples (40 μg/sample) in SDS-loading buffer were subjected to 10–20% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% dry milk and 0.1% Tween 20 (USB). Polyclonal rabbit anti-mouse HO-1 (StressGen Biotech), cleaved caspase-3 (Cell Signaling Technology), Bax (Santa Cruz Biotechnology), and monoclonal mouse anti-Bax (Abcam) were used. Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software) and presented in comparison to β-actin expression.

Table I. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequencea</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>F CAGTGTAAGTCGAGAGGA</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>R CAGTGTAAGTCGAGAGGA</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F GGCTGATTCCCCTCCATC</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>R ATGCCAGTGTCAAGGGTGA</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>F AGG GAT GAG AAG TCC CCA AA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R CAC TTT TTC GCC TAC GAC</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F GCC AAC TGT GTC AAG AAC AA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R TTC TAA GGG CAA TCT CTP CC</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F CCA GAG ATC CAA AGA AAT GAT GG</td>
<td>58</td>
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<tr>
<td></td>
<td>R ACT CCA GAA GAC CAG AGG AAA T</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>F GTG GAG CAG GTG AAG AGT GA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>R CGA GTT TTT CCA AGG AGT TG</td>
<td></td>
</tr>
</tbody>
</table>

a F, Forward; R, reverse.
Apoptosis assay

Apoptosis in cryostat liver sections was detected by the TUNEL method using the ApopTag Peroxidase kit (Chemicon International). The peroxidase activity was visualized with diaminobenzidine substrate, yielding a brown oxidation product; methyl green was used for counterstaining. The results were scored semiquantitatively by averaging the number of TUNEL$^+$ cells/field at a magnification of 400. Six-fields/tissue sample were evaluated.

Statistical analysis

All data are expressed as mean ± SEM. Differences between experimental groups were analyzed using one-way ANOVA/Tukey’s test or Student’s $t$ test for unpaired data. All differences were considered statistically significant at the $p$ value of $<0.05$.

Results

Basal liver HO-1 levels

To assess HO-1 expression in experimental livers, we performed real-time PCR and Western blot analyses. As shown in Fig. 1A, the baseline liver HO-1 mRNA levels in HO-1$^{-/-}$ mice were on average 32% of HO-1$^{+/+}$ counterparts (0.07 ± 0.01 vs 0.22 ± 0.03, respectively, $p < 0.005$). CoPP treatment increased HO-1 mRNA expression in HO-1$^{-/-}$ livers by 13-fold (0.91 ± 0.15, $p < 0.001$).

FIGURE 1. Comparative analysis of basal liver HO-1 levels in WT and HO-1$^{-/-}$ mice with or without CoPP treatment. Basal and post I/R HO-1 mRNA/protein levels are significantly decreased in HO-1$^{-/-}$, as compared with WT. After CoPP treatment, HO-1 mRNA/protein remained depressed in HO-1$^{-/-}$ as compared with WT. A, Real-time RT-PCR-assessed HO-1 mRNA levels. Means ± SEM are shown; $n = 7–8$ /group. B, Western blot-assessed HO-1 protein levels. Means ± SEM are shown; $n = 4$ /group. C, Real-time RT-PCR-assessed HO-1 mRNA levels at 6 h of reperfusion following 90 min of warm ischemia. Means ± SEM are shown; $n = 8–15$ /group. D, Western blot-assessed HO-1 protein levels post-I/R. Means ± SEM are shown; $n = 4$ /group. * $p < 0.0001$; ** $p < 0.001$; # $p < 0.05$; ## $p < 0.005$.

FIGURE 2. Sex differences in HO-1 expression compared with hepatocellular damage. A, Basal HO-1 mRNA levels measured by real-time RT-PCR were greater in WT males ($n = 6$) compared with females ($n = 4$) and HO-1$^{-/-}$ males ($n = 3$) compared with females ($n = 4$; $p = NS$). B, SGOT levels were greater in WT females ($n = 8$) compared with males ($n = 10$) and HO-1$^{-/-}$ females ($n = 8$) compared with males ($n = 11$). # $p < 0.05$.
and in WT livers by 14.4-fold (3.17 ± 0.47, \( p < 0.0001 \)). However, despite CoPP treatment, HO-1 mRNA remained depressed in HO-1\(^{-/-}\) as compared with WT livers (\( p < 0.0001 \)). As shown in Fig. 1B and consistent with mRNA data, Western blot-assessed basal HO-1 protein levels were significantly decreased in HO-1\(^{-/-}\) livers as compared with WT (0.09 ± 0.01 vs 0.22 ± 0.01; \( p < 0.005 \)). Although CoPP treatment significantly up-regulated HO-1 protein in both animal groups (\( p < 0.0001 \)), its levels in HO-1\(^{-/-}\) livers were still markedly lower, as compared with WT (0.72 ± 0.07 vs 2.02 ± 0.09; \( p < 0.0001 \)). Interestingly, analysis of HO-1 levels by sex revealed that WT males exhibited baseline HO-1 expression that was 2-fold greater than in WT females (0.28 ± 0.03 vs 0.14 ± 0.04, respectively; \( p = 0.015 \), Fig. 2A), with a similar yet not significant trend in the HO-1\(^{-/-}\) animals (0.10 ± 0.01 vs 0.08 ± 0.02, respectively; \( p = NS \)).

**Hepatocellular function in liver IRI**

We used our well established mouse model of partial liver warm IRI (16, 20, 21) to assess the effects of HO-1 expression on hepatocellular function. As shown in Fig. 3A, sGOT/GPT levels (IU/L) at 6 h of reperfusion following 90 min of warm ischemia were significantly increased in HO-1\(^{-/-}\) mice in comparison to WT (16,600 ± 1,930/15,700 ± 2,120 vs 4,380 ± 660/3,300 ± 470; \( p < 0.0001 \)). There was a trend toward sex differences in transaminase levels (Fig. 2B). Indeed, sGOT/GPT levels were increased in females compared with male mice for both WT (5,280 ± 784 vs 3,656 ± 973/3,887 ± 620 vs 2,835 ± 682, \( p = NS \)) and HO-1\(^{-/-}\) groups (19,247 ± 2,536 vs 12,700 ± 2,286, \( p = 0.04 \), 13,254 ± 2,636 vs 12,688 ± 3,820, \( p = NS \)), opposite to the most complete preservation of lobular architecture (score 0.10 ± 0.01 vs 0.08 ± 0.02, respectively; \( p = NS \)).

**PMN infiltration**

We performed MPO assay in livers at 6 h of reperfusion after 90 min of warm ischemia, as a surrogate marker for PMN infiltration (Fig. 4). The MPO activity (units per gram) was significantly higher in the HO-1\(^{-/-}\) group, as compared with WT (9.50 ± 0.66 vs 4.99 ± 0.36; \( p < 0.0005 \)). Although CoPP treatment diminished MPO activity in WT (2.01 ± 0.37, \( p < 0.0005 \)), the corresponding MPO levels remained highly elevated in HO-1\(^{-/-}\) mice (5.58 ± 0.64, \( p < 0.001 \) compared with CoPP-treated WT).

**IRI-induced liver HO-1 levels**

IRI-induced HO-1 levels were decreased in HO-1\(^{-/-}\) mice, as compared with WT counterparts (Fig. 1C; mRNA: 0.93 ± 0.14 vs 2.04 ± 0.36; \( p < 0.05 \); Fig. 1D; protein: 0.38 ± 0.06 vs 0.83 ± 0.09; \( p < 0.005 \), respectively; \( p < 0.005 \)). Although CoPP treatment improved liver function in HO-1\(^{-/-}\) mice (sGOT: 4.350 ± 2,560/sgPT: 5.620 ± 2,450 IU/L; \( p < 0.01 \)), it remained deteriorated and comparable with untreated WT controls.

The hepatocellular damage was also evaluated by liver histology and graded using a modified Suzuki’s criteria (17) (Fig. 3B). The ischemic lobes in HO-1\(^{-/-}\) livers showed a more severe hepatocyte necrosis and sinusoidal congestion (score = 10.33 ± 0.98), as compared with WT (score = 6.75 ± 1.71; \( p < 0.01 \)). CoPP-treated WT mice revealed minimal necrosis/sinusoidal congestion and almost complete preservation of lobular architecture (score = 1.43 ± 0.98; \( p < 0.001 \) compared with untreated WT). In contrast, CoPP treatment did not prevent hepatocellular damage in HO-1\(^{-/-}\) mice (score = 7.00 ± 0.70; \( p = NS \)). Taking of a liver biopsy before the occlusion of the vascular supply to the liver did not alter the degree of hepatic IRI (data not shown).

**FIGURE 3.** Serum transaminase levels (IU/L) in WT and HO-1\(^{-/-}\) mice with or without CoPP treatment. A. Significantly higher sGOT/GPT levels are seen in HO-1\(^{-/-}\) mice as compared with WT mice. The therapeutic effect of CoPP treatment upon liver IRI in WT mice was more pronounced than in HO-1\(^{-/-}\) mice. Means ± SEM are shown; \( n = 8–15/\)group. *, \( p < 0.0001 \); #, \( p < 0.05 \); ##, \( p < 0.01 \). B. Representative photomicrographs of mouse livers following 90 min of warm ischemia and 6 h reperfusion. Livers in HO-1\(^{-/-}\) mice with even more severe hepatocyte necrosis/sinusoidal congestion even more pronounced than in WT mice. CoPP-treated WT livers with good preservation of lobular architecture without edema, congestion, and absence of centrilobular necrosis compared with untreated WT. CoPP-treated HO-1\(^{-/-}\) livers with extensive hepatocyte necrosis/sinusoidal congestion. Original magnification, \( \times 100 \); H&E stain; \( n = 6–8/\)group.
0.18; p < 0.005). We assessed the HO-1 induction as the ratio between postreperfusion and basal mRNA levels for each individual mouse. The HO-1 heterozygous KO mice underwent a greater fold of hepatic HO-1 induction (13.3-fold), compared with WT mice (9.3-fold; p < 0.05). CoPP treatment increased HO-1 expression in both HO-1+/−/ and WT recipients subjected to hepatic I/R insult (p < 0.0001), although levels remained decreased in HO-1+/- mice as compared with WT (mRNA: 2.19 ± 0.40 and 6.56 ± 1.00, respectively; p < 0.0005; protein: 1.05 ± 0.12 and 3.52 ± 0.46, respectively; p < 0.0001).

Similar to the differences in basal HO-1 expression, there was a trend toward greater post-I/R levels in males, as compared with females both in WT (2.42 ± 0.52 vs 1.58 ± 0.44, respectively; p = 0.12) and HO-1+/- mice (1.30 ± 0.31 vs 0.95 ± 0.17, respectively; p = 0.17). This yielded a trend toward a greater fold of HO-1 induction in the female compared with male mice (WT: 21.44 ± 10.73 vs 9.84 ± 4.56, p = 0.19; HO-1+/-: 11.62 ± 3.11 vs 7.86 ± 1.86, p = 0.17).

**Correlation of HO-1 expression with hepatocellular damage**

Analysis of baseline HO-1 expression and the degree of hepatocellular damage assessed by sGPT for individual samples revealed that basal HO-1 levels correlated strongly (p < 0.001) with the degree of cytoprotection in WT and HO-1+/- mice (Fig. 5, A and B). A threshold effect was apparent at greater levels of HO-1, after which a further increase in HO-1 expression did not translate into greater protection. Postreperfusion HO-1 levels had a similar negative correlation with sGPT levels for both WT compared with HO-1+/- mice (data not shown; p = 0.02) and all groups analyzed together (Fig. 5C; p < 0.0001). In contrast, HO-1 fold induction correlated directly with the degree of I/R-induced damage (Fig. 5D; p < 0.0001), which was irrespective of CoPP treatment because it was also evident in untreated animals (data not shown, p = 0.03).

**Cytokine expression**

We used real-time RT-PCR to analyze cytokine expression in the liver during hepatic IRI and calculated the ratio between post-I/R and basal mRNA levels in each animal (Fig. 6). HO-1+/- mice showed significantly increased induction ratios of TNF-α, IL-6, IFN-γ, and IL-10, as compared with WT (Fig. 6). There was a differential induction of IL-4, with a significant up-regulation in the WT that was completely absent in HO-1+/- mice (induction ratio 6.9 vs 0.65; p = 0.01). CoPP treatment either completely abrogated (IL-6 and IFN-γ) or substantially decreased (TNF-α and IL-10) the induction of cytokines in both HO-1+/- and WT (p < 0.05). Interestingly, baseline cytokine mRNA levels in WT compared with HO-1+/- mice were on average 2.1× greater for TNF-α, 4× greater for IL-6, 6.2× greater for IFN-γ, and 5.5× greater for IL-10 (p < 0.01) (data not shown).
Apoptosis

We evaluated hepatocyte apoptosis by TUNEL staining of livers subjected to 90 min of warm ischemia and 6 h reperfusion (Fig. 7). Increased hepatocyte apoptosis (TUNEL+ cells per field) was readily detectable in HO-1−/− (Fig. 7, B and F; 51.7 ± 2.5), as compared with WT (Fig. 7, A and E; 22.6 ± 2.2; p < 0.001). CoPP treatment profoundly decreased the frequency of apoptotic cells in WT (Fig. 7, C and G; 3.3 ± 0.5; p < 0.001) and in HO-1−/− (Fig. 7, D and H; 25.1 ± 2.2; p < 0.001). However, livers in HO-1−/− mice stained densely for apoptotic cells despite CoPP treatment (Fig. 7, D and H; 25.1 ± 2.2; p < 0.001) as compared with WT: 1) significantly reduced HO-1 gene and protein expression at baseline as well as postreperfusion; 2) greater IRI-induced HO-1 relative up-regulation; 3) more severe hepatocellular damage; 4) enhanced intrahepatic neutrophil infiltration; 5) increased proinflammatory cytokine induction, and 6) augmented apoptosis and proapoptotic (Bax/caspase-3) pathways. Unlike in WT, HO-1 induction with CoPP largely failed to reduce the cardinal features of hepatocellular damage in HO-1−/− mice to the same degree as it did in WT mice.

We also analyzed the expression of proapoptotic Bax and caspase-3 proteins by Western blots. The relative expression levels were determined by densitometry and expressed as ratios to β-actin, the housekeeping gene. As shown in Fig. 8, both Bax and caspase-3 were strongly up-regulated in HO-1−/− livers (0.96 ± 0.15 and 1.27 ± 0.15, respectively), as compared with WT (0.45 ± 0.14 and 0.65 ± 0.03, respectively; p < 0.01). CoPP treatment profoundly suppressed their expression in WT (0.22 ± 0.03 and 0.23 ± 0.03, respectively; p < 0.001), but had a less pronounced effect in the HO-1−/− group (Bax: 0.49 ± 0.02 and caspase-3: 0.63 ± 0.13, p < 0.001), as compared with WT.

Discussion

In this study, we have evaluated the mechanisms by which genetically decreased basal HO-1 levels affected hepatocellular damage in mouse livers subjected to partial warm ischemia followed by reperfusion. Our findings led us to conclude that the basal HO-1 levels are more important than the degree of HO-1 up-regulation in the antioxidant cytoprotection against IRI as HO-1−/− mice revealed when compared with WT: 1) significantly reduced HO-1 gene and protein expression at baseline as well as postreperfusion; 2) greater IRI-induced HO-1 relative up-regulation; 3) more severe hepatocellular damage; 4) enhanced intrahepatic neutrophil infiltration; 5) increased proinflammatory cytokine induction, and 6) augmented apoptosis and proapoptotic (Bax/caspase-3) pathways. Unlike in WT, HO-1 induction with CoPP largely failed to reduce the cardinal features of hepatocellular damage in HO-1−/− mice to the same degree as it did in WT mice.

The effect of decreased HO-1 expression in HO-1−/− mice on the susceptibility to IRI has been addressed in studies that have used pharmacological competitive inhibitors of HO activity (e.g., SnPP or ZnPP). However, the latter have yielded quite conflicting results, with worsening of liver function in some (11) and improved liver function in others (14). Although the basis of these disparate findings is uncertain, it is possible that dose-dependent effects and nonspecific actions of HO inhibitors may have played
scored semiquantitatively by averaging the number of TUNEL+/H11001 cells, as compared with WT (A and E). CoPP-treated WT livers (C and G) had a decreased frequency of TUNEL+ cells as compared with CoPP-treated HO-1+/−/− livers (D and H). The results were scored semiquantitatively by averaging the number of TUNEL+ cells (mean ± SEM) per microscopic field at ×400 magnification. Minimum of six fields were evaluated per sample in four different experiments. *p < 0.001 vs WT or HO-1+/−/− + CoPP; #p < 0.001 vs WT or HO-1+/−/− + CoPP.

FIGURE 7. Representative TUNEL-assisted detection of intrahepatic apoptosis. The HO-1+/−/− group (B and F) had a higher number of TUNEL+ cells (dark brown spots), as compared with WT (A and E). CoPP-treated WT livers (C and G) had a decreased frequency of TUNEL+ cells as compared with CoPP-treated HO-1+/−/− livers (D and H). The results were scored semiquantitatively by averaging the number of TUNEL+ cells (mean ± SEM) per microscopic field at ×400 magnification. Minimum of six fields were evaluated per sample in four different experiments. *p < 0.001 vs WT or HO-1+/−/− + CoPP; #p < 0.001 vs WT or HO-1+/−/− + CoPP.

FIGURE 8. Western blot-assisted analysis of Bax and caspase-3. A, Bax and cleaved caspase-3 protein was detected by polyclonal rabbit anti-mouse Abs. Ab against β-actin was used as internal control. B, Each bar graph shows the ratio of protein to β-actin expression. There was increased Bax and cleaved caspase-3 expression in HO-1+/−/− as compared with WT mice. Although CoPP treatment suppressed Bax/cleaved caspase-3 in both animal groups, Bax/cleaved caspase-3 expression remained increased in HO-1+/−/− as compared with WT mice despite CoPP treatment. These data represent the mean ± SEM of four different experiments. *p < 0.001; #p < 0.01.

a role. Hence, pharmacological competitive HO inhibitors may exert cytoprotective functions despite HO-1 inhibition. In this study, we used HO-1+/−/− mice, which allowed us to study the effect of genetically reduced basal HO-1 levels without the nonspecific component seen otherwise with pharmacological HO inhibitors. The use of HO-1+/−/− instead of HO-1−/− mice allowed us to address the mechanisms by which decreased HO-1 expression affected the liver IRI cascade, a much more physiologically relevant scenario than totally absent HO-1 expression as seen in HO-1-homozygous KO mice.

There is ample evidence in the literature that up-regulation of HO-1 provides potent cytoprotection against IRI (3, 5, 9–11, 22–24). In contrast, the lack of HO-1 uniformly increases susceptibility to a myriad of inflammatory conditions (12, 13, 25–27). Yoshida et al. (25) observed an increased susceptibility to oxidative stress in HO-1+/−/− mice including significantly reduced ventricular recovery, increased creatine kinase release and increased infarct size in a myocardial IRI model. This is similar to Liu et al. (26), who have reported larger infarct areas and increased mortality in HO-1−/− mice subjected to myocardial IRI as compared with HO-1+/−/− mice. Likewise, the only human subject reported to have complete absence of HO-1 expression exhibited increased sensitivity to oxidative injury in the context of increased tissue iron deposition, increased iron binding capacity, anemia, and growth failure (28). All these findings confirm that HO-1 expression represents an important defense mechanism against oxidative stress.

In this study, we introduced a novel modification to the model of murine liver warm IRI: a liver biopsy before the onset of ischemia. This allowed us to assess the true basal levels of HO-1 and cytokines in each animal and correlate it with their levels after IRI and the degree of hepatocellular damage. We showed that livers from HO-1+/−/− mice exhibited significantly decreased baseline HO-1 gene and protein levels, which resulted in greater I/R-induced hepatocellular damage as compared with WT mice. Although CoPP treatment increased baseline HO-1 expression levels in both WT and HO-1−/−, resulting in lowering the degree of hepatic damage in both groups, the degree of protection was of significantly lesser magnitude in the HO-1−/− mice. These results support the idea that basal HO-1 levels rather than the ability to up-regulate HO-1 expression are crucial in the antioxidant cytoprotection against IRI. This is consistent with our previous studies in which disruption of the TLR4-signaling pathway (21), STAT-4 transcription (16), or CD154-CD40 T cell costimulation (20) prevented hepatic warm IRI in the context of significant up-regulation of HO-1 expression. Likewise, Ad-IL-13-transfected rat livers were resistant to hepatic IRI with a parallel HO-1 induction after transplant (29). Collectively, these results support the idea that HO-1 functions as a “therapeutic funnel” required for the action of several therapeutic molecules and/or interventions (4).

Although absolute HO-1 levels both at baseline and postreperfusion corresponded with protection against injury, the fold of IRI-related HO-1 induction correlated with hepatocellular damage instead. This is in agreement with the findings of Shi et al. (7) in the atherosclerosis model, where the use of recombinant strains allowed them to establish that a higher degree of HO-1 protein fold induction in endothelial cells in culture directly correlated with the development of atherosclerotic lesions, while basal HO-1 levels in endothelial cells from different strains (C57BL6 vs C3H) inversely correlated with susceptibility to atherosclerosis.

Geuken et al. (6) recently reported that higher HO-1 levels before transplantation correlated with greater I/R injury and worse hepatobiliary function, and that higher HO-1 induction during transplantation resulted in greater protection instead. Although these results seem to contradict ours, the latter authors acknowledged that HO-1 expression was already up-regulated in their liver donors, from brain-dead subjects, in comparison with control livers. They could not ascertain what the true basal HO-1 levels of the liver donors were and whether liver donors which exhibited greater damage could have had lower true baseline levels with higher pretransplant up-regulation. Here, we described a clear negative correlation of basal HO-1 and positive correlation of HO-1 fold induction with hepatocellular damage. This study benefits from the detection of true baseline HO-1 levels before the I/R and from the
Kapturczak et al. (12) have also reported that HO-1 proinflammatory cytokine (TNF-α) as compared with HO-1 model (36) and inhibited TNF-α cytokines exhibited increased TNF-α. Here, we show that livers in Under normal conditions, hepatocytes are believed to be resistant to injury. The protective response of HO-1 may be overwhelmed at some level of damage, at which point the induction of HO-1 is insufficient to decrease injury, as evidenced by an apparent threshold effect at greater levels of HO-1, after which a further increase in HO-1 expression did not translate into greater protection.

We also observed increased baseline HO-1 levels in male compared with female mice, associated with decreased HO-1 fold induction and hepatocellular damage, confirming a previously suggested trend for higher expression in male liver rats in comparison with female counterparts (30) but opposite to the relationship reported in rat adrenals (31), which raises the possibility that sex influences HO-1 expression in a tissue-specific manner. Our results also vary from those of Toth et al. (8) who had reported that female rats exhibited a greater relative HO-1 up-regulation in response to trauma and hemorrhagic shock that resulted instead in a mild attenuation of hepatocellular damage. However, their baseline HO-1 levels, which did not reflect a sex dependence, were not true basal levels because they were determined in sham-operated animals where the surgical stress may have already up-regulated HO-1.

The mechanism of hepatic IRI is largely attributed to accumulation of PMNs at the site of tissue injury, with subsequent Kupffer cell activation and release of proinflammatory mediators such as reactive oxygen species and cytokines. Proinflammatory cytokines, generated mainly by Kupffer cells, can also increase local PMN sequestration and activate CD4+ T cells (as shown in our present and other studies) (32–35). Here, we show that livers in HO-1+/− mice were characterized by significantly increased proinflammatory cytokine (TNF-α, IL-6, and IFN-γ) induction ratios as compared with WT, which is in agreement with our previous findings that the therapeutic benefit of Ad-HO-1 gene therapy depends on depression of intrahepatic TNF-α and IFN-γ (23). Kapturczak et al. (12) have also reported that HO-1+/− splenocytes exhibited increased TNF-α, IL-6, and IFN-γ induction ratios after LPS stimulation. The exact mechanisms of HO-1-based broad range of anti-inflammatory and cytoprotective functions have not been fully elucidated. HO-1 enzymatic products, particularly CO and biliverdin have been suggested as possible anti-inflammatory mediators. Indeed, exogenous CO effectively blocked early up-regulation of TNF-α, IL-1β, and IL-6 in a rat intestinal cold IRI model (36) and inhibited TNF-α expression in rat liver IR (37) and liver transplants (38). Likewise, we have shown that biliverdin treatment reduced the expression of TNF-α, IL-1, and IL-6 in a rat liver model of cold ischemia followed by transplantation (39). Here, we document that basal levels of HO-1 represent a key factor that modulates proinflammatory cytokine induction patterns during hepatic IRI.

Interestingly, there was an association of basal HO-1 levels with higher levels of TNF-α, IL-6, IFN-γ, and IL-10 seen noted in WT as compared with HO-1+/− mice (data not shown). Though there is no clear mechanism to explain this finding, it may be related to a protective effect such as seen with ischemic preconditioning. Under normal conditions, hepatocytes are believed to be resistant to the cytotoxic effects of TNF-α (40, 41). TNF-α has been reported to be important in cellular regeneration and prevention of apoptosis in the liver (42, 43), and has been shown to be a critical mediator of hepatoprotection induced by ischemic preconditioning (44). Thus, depressed baseline cytokine levels as seen in HO-1+/− mice may represent a diminished ability to protect against cellular stress.

Apoptosis has been shown to be an important event after reperfusion of liver grafts, the severity of which correlates with the degree of hepatic injury (45). In this study, HO-1+/− livers revealed significantly increased frequency of TUNEL+ apoptotic cells, accompanied by marked proapoptotic Bax/caspase-3 expression, as compared with WT which is consistent with our previous reports where HO-1 overexpression depressed apoptotic events and up-regulated antiapoptotic proteins (Bcl-2/Bag-1) in heart and liver grafts (10, 46). CO seems to be an important antiapoptotic mediator via activation of the p38 MAPK pathway. Indeed, CO exposure inhibited caspase-3 via p38 MAPK pathway during lung IRI (47, 48) in agreement with our studies in liver IRI (37). Biliverdin may also mediate antiapoptotic effects by up-regulating antiapoptotic Bcl-2/Bag-1 and down-regulating caspase-3 (39). Our results support the dependence of HO-1 on host apoptotic regulatory mechanisms during hepatic IRI.

In summary, basal HO-1 levels rather than the degree of HO-1 up-regulation are crucial in the antioxidant cytoprotection against liver IRI as they influence not only its severity, but may also predict the success of pharmacologically induced cytoprotection. Noteworthy, while basal HO-1 levels may determine the degree of HO-1-mediated cytoprotection, the IRI-related HO-1 fold induction correlated instead with the degree of hepatic damage and therefore, it could serve as a marker of the degree of injury. The use of genetically engineered mice with decreased rather than totally ablated HO-1 levels is of clinical relevance and allowed us to document that proinflammatory and apoptotic pathways are of major importance in HO-1-based antioxidant therapy. This model provides a unique opportunity to further our understanding of the role of HO-1 in stress defense pathogenic mechanisms and to design novel regimens to prevent IRI in transplant patients.

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Disclosures
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References


