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Scientific Article

Small Interfering RNA Targeting Heme Oxygenase-1 (HO-1) Reinforces Liver Apoptosis Induced by Ischemia–Reperfusion Injury in Mice: HO-1 Is Necessary for Cytoprotection

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Abstract

We have shown that overexpression of heme oxygenase-1 (HO-1) prevents the liver inflammation response leading to ischemia and reperfusion injury (IRI). This study was designed to explore the precise function and mechanism of HO-1 cytoprotection in liver IRI by employing a small interfering RNA (siRNA) that effectively suppresses HO-1 expression both *in vitro* and *in vivo.* Using a partial lobar liver warm ischemia model, mice were injected with HO-1 siRNA/nonspecific control siRNA or Ad-HO-1/Ad-β-gal. Those treated with HO-1 siRNA showed increased serum glutamic-oxaloacetic transaminase levels, significant liver edema, sinusoidal congestion/cytoplasmic vacuolization, and severe hepatocellular necrosis. In contrast, Ad-HO-1-pretreated animals revealed only minimal sinusoidal congestion without edema/vacuolization or necrosis. Administration of HO-1 siRNA significantly increased local neutrophil accumulation and the frequency of apoptotic cells. Mice treated with HO-1 siRNA were characterized by increased caspase-3 activity and reduced HO-1 expression, whereas those given Ad-HO-1 showed decreased caspase-3 activity and increased HO-1/Bcl-2/Bcl-x_L, data confirmed by use of an *in vitro* cell culture system. Thus, by using an siRNA approach this study confirms that HO-1 provides potent cytoprotection against hepatic IRI and regulates liver apoptosis. Indeed, siRNA provides a powerful tool with which to study gene function in a wide range of liver diseases.

Overview Summary

Heme oxygenase-1 (HO-1), a stress-responsive molecule, exerts potent cytoprotective effects in liver ischemia and reperfusion injury. We explored the precise function and mechanism of HO-1 function by using small interfering RNA (siRNA) that effectively suppresses HO-1 expression *in vitro* and *in vivo.* Using a partial lobar liver warm ischemia model, HO-1 siRNA-treated mice revealed significant hepatocellular damage and necrosis, whereas Ad-HO-1-conditioned animals exhibited diminished hepatic injury. HO-1 siRNA increased local neutrophil accumulation and the frequency of apoptotic cells. Moreover, mice treated with HO-1 siRNA revealed increased caspase-3 activity and reduced HO-1 expression, whereas Ad-HO-1 decreased caspase-3 activity and increased HO-1/Bcl-2/Bcl-x_L, data confirmed by *in vitro* studies. This study highlights the importance of the siRNA approach to study gene function in various disease states.

Introduction

Ischemia and reperfusion injury (IRI), an antigen-independent component of organ procurement, represents a complex series of processes that result in tissue damage, microcirculatory failure, followed by necrosis and ultimate cell death (Vardanian *et al.*, 2008). Studies of hepatic IRI have provided some etiologic factors, including activation of Kupffer cells, release of proinflammatory cytokines, increased expression of vascular cell adhesion molecules, and neutrophil influx (Teoh and Farrell, 2003). Although innate immunity might trigger and mediate liver IRI cascade (Zhai *et al.*, 2004; Shen *et al.*, 2007), the exact mechanisms and mediators involved remain to be elucidated.

Apoptosis, or programmed cell death, occurs in various organs exposed to IR-induced damage (Jaeschke and Lemasters, 2003), and represents a critical mechanism of IRI (Jha *et al.*, 2008). Blockade of the Fas–Fas ligand interaction

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results in the inhibition of hepatocyte apoptosis during IRI (Nakajima *et al.*, 2008). Indeed, activation of liver apoptotic signaling needs a variety of mediators, including tumor necrosis factor (TNF)-α, Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Binding to their respective receptors results in triggering the extrinsic pathway to apoptosis (Jaeschke and Lemasters, 2003). Moreover, the apoptotic pathway activated during the early phase of reperfusion after liver ischemia is involved in sinusoidal endothelial cell (SEC) damage during organ preservation (Gao *et al.*, 1998). As more than 50% of hepatocytes and SECs undergo apoptosis via caspase-3 activation during the first 24 hr of reperfusion (Gujral *et al.*, 2001), treatment with caspase inhibitors completely prevented Fas antibody- or TNF-induced apoptosis (Bajt *et al.*, 2000). Conversely, overexpression of Bcl-2 in hepatocytes protected liver against IRI (Bilbao *et al.*, 1999). Thus, inhibition of apoptosis represents a rational strategy to reduce the risk of IRI in liver transplants.

Heme oxygenase-1 (HO-1), a rate-limiting enzyme in heme catabolism, generates biliverdin, free iron, and carbon monoxide (CO) (Maines, 1997). It has been shown that HO-1 is a stress-responsive protein and has a cytoprotective defense response against oxidative injury (Otterbein *et al.*, 2003). We have documented that HO-1 overexpression exerts potent adaptive antiinflammatory and antiapoptotic effects in several transplantation models (reviewed in Katori *et al.*, 2002a). HO-1 induction was cytoprotective in rat liver transplant (Ke *et al.*, 2002) and extended organ cold ischemia (Amersi *et al.*, 1999; Katori *et al.*, 2002b) models. Indeed, adenovirus (Ad)-mediated HO-1 gene expression significantly increased allograft survival. Further studies have shown that Ad-HO-1 (recombinant adenovirus encoding the heme oxgenase-1 gene) gene transfer decreased macrophage infiltration in the portal areas and inducible nitric oxide synthetase (iNOS) expression (Coito *et al.*, 2002), while increasing the expression of antiapoptotic Bcl-2/Bcl-x_L and Bag-1 genes (Ke *et al.*, 2002). Although the exact mechanism of protection remains unclear, the antiapoptotic genes, such as Bcl-2/Bcl-x_L and Bag-1, may contribute to prevention of hepatocyte apoptosis.

RNA-mediated interference (RNAi), an emerging technique, can target specific mRNA via duplex RNA, silencing the corresponding gene. RNAi has been demonstrated as a highly specific and effective gene inhibitor. As the second generation of therapeutic RNA, small interfering RNA (siRNA) is more specific and stable and has become a promising candidate for therapeutic gene targeting (Soutschek *et al.*, 2004; Takabatake *et al.*, 2005). A number of reports on siRNA, using systemic delivery, have demonstrated the beneficial effects as a therapeutic strategy for a variety of organ diseases (Sato *et al.*, 2005; Zheng *et al.*, 2006). Studies have showed that gene silencing with transforming growth factor (TGF)-β_1 siRNA suppressed tubulointerstitial fibrosis in the kidney (Hwang *et al.*, 2006). Inhibition of connective tissue growth factor by siRNA prevents liver fibrosis in rats (Li *et al.*, 2006). siRNA targeting caspase-3/8 reduced hepatic IRI in mice (Contreras *et al.*, 2004). Moreover, inhibition of HO-1 with HO-1 siRNA resulted in striking increases in apoptosis in TNF-α-treated cells, consistent with HO-1-mediated cytoprotection (Chae *et al.*, 2006). In the present study, we used the RNAi technique to design and synthesize effective siRNAs based on HO-1 mRNA sequences. We aimed to examine the inhibitory effect of siRNA on the expression of HO-1 *in vitro* and *in vivo*, and explore the function and potential mechanism of HO-1 in a murine model of liver IRI.

Materials and Methods

siRNA design/preparation

The siRNAs against HO-1 were designed with the siRNA Selection Program (Ambion Inc., Austin, TX). The sense and antisense strands of murine HO-1 siRNA for sequence 1 were as follows: 5′-UGAACACUCUGGAGAUGAC-3′ (sense) and 5′-GUCAUCUCCAGAGUGUCCA-3′ (antisense); and for sequence 2 they were 5′-GCCACACAGCACUAUGUAA-3′ (sense) and 5′-UUACAUAGUGCUGUGUGGC-3′ (antisense), as described (Reynolds *et al.*, 2004). The murine nonspecific siRNA scrambled duplex (sense, 5′-GCGCGCUUUGUAGG AUUCG-3′; antisense, 5′-CGAAUCCUACAAAGCGCGC-3′) and the nonsilencing siRNA (NS siRNA: sense, 5′-UUCUC CGAACGUGUCACGU-3′; antisense, 5′-ACGUGACACGU UCGGAGAA-3′) were also synthesized (Qiagen, Chatsworth, CA), and served as negative controls. The siRNAs were synthesized in 2′-deprotected, duplexed, desalted, and purified siRNA form by Qiagen.

Generation of recombinant adenovirus

Ad-HO-1 was generated as described previously (Shibahara *et al.*, 1985). Briefly, the 1.0-kbp rat HO-1 cDNA flanked by *Xho*I–*Hin*dIII sites was cloned into plasmid pAC-CMVpLpA. The resulting pAC-HO-1 plasmid was cotransfected with plasmid pJM17 into 911 cells. Homologous recombination resulted in a replication-defective Ad-HO-1. Recombinant Ad-HO-1 clones were screened by Southern blots. Ad carrying the *Escherichia coli* β-galactosidase gene (Ad-β-gal) has been described (Ke *et al.*, 2000). Isolation and propagation were carried out, and the viral titer was assessed by plaque assay (Graham and van der Eb, 1973).

Cell cultures

YPEN-1 endothelial cells and RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA). YPEN-1 cells were maintained in Eagle's minimal essential medium (EMEM; ATCC) supplemented with 5% fetal bovine serum (FBS) and heparin (0.03 mg/ml). RAW 264.7 macrophages were maintained in Dulbecco's modified Eagle's high-glucose medium (DMEM) supplemented with 2 m*M* L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA), and 10% fetal calf serum (FCS) (Gemini Bio-Products, Sacramento, CA). Both YPEN-1 and RAW 264.7 cells were incubated with 5% CO_2 and 95% air at 37°C, and used for *in vitro* transfection.

YPEN-1 cells and RAW 264.7 cells were seeded into 6- or 12-well plates and cultured overnight. After washing, Ad-HO-1 or Ad-β-gal (at a multiplicity of infection [MOI] of 10) was added to YPEN-1 cells and RAW 264.7 macrophages, respectively, and incubated for 1 hr. The medium was removed and changed to EMEM + 2% FBS or DMEM + 2% FBS. After 24 hr, cells were transfected with siRNA, using Lipofectamine 2000 reagent (Invitrogen), and then incubated for 18–24 hr. For the RAW 264.7 macrophage cultures, cells were transfected with HO-1 siRNA sequence 1 or sequence 2.

After washing, cells were treated with 50 μM etoposide (Calbiochem, San Diego, CA) in YPEN-1 cultures for 2 hr or cells were treated with cobalt protoporphyrin (CoPP, an HO-1 inducer, 10 μg/ml; Porphyrin Products, Logan, UT).

Animals

Male C57BL/6 wild-type mice (6–8 weeks of age) were used (Jackson Laboratory, Bar Harbor, ME). Mice were housed in the University of California Los Angeles (UCLA) animal facility under specific pathogen-free conditions. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Mouse liver IRI model/treatment

We have employed a well-defined mouse model of warm hepatic IRI followed by reperfusion (Zhai *et al.*, 2004; Shen *et al.*, 2007). Briefly, mice anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal) were injected with heparin (100 mg/kg), and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad lobes of the liver. After 90 min of partial hepatic ischemia, the clip was removed, initiating hepatic reperfusion. Mice were killed 6 hr of reperfusion.

In the treatment groups, each mouse was injected via the tail vein with Ad-HO-1 or Ad-β-gal (2.5×10^9 plaque-forming units [PFU]) 24–48 hr before the onset of warm ischemia. The HO-1 siRNA, and nonspecific or scrambled control siRNAs (2 mg/kg), were infused intravenously 4 hr before warm ischemia.

Hepatocellular damage assay

Serum glutamic-oxaloacetic transaminase (sGOT) levels, an indicator of hepatocellular injury, were measured in blood samples with an AutoAnalyzer (ANTECH Diagnostics, Los Angeles, CA).

Histology

Tissue samples were harvested and sliced into small pieces, preserved in 10% neutral-buffered formalin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E). The histological severity of I/R injury was graded according to Suzuki's classification, in which sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4 (Suzuki *et al.*, 1993). No necrosis or congestion/centrilobular ballooning is given a score of 0, whereas severe congestion/degeneration and >60% lobular necrosis is given a value of 4.

Myeloperoxidase activity assay

The presence of myeloperoxidase (MPO) was used as an index of neutrophil accumulation in the liver (Mullane *et al.*, 1985). Briefly, frozen tissue was thawed and weighed and placed in 4 ml of iced 0.5% hexadecyltrimethyl-ammonium bromide and 50 mmol of potassium phosphate buffer solution with the pH adjusted to 5. Each sample was then homogenized for 30 sec and centrifuged at 15,000 rpm for 15 min at 4°C. Supernatants were mixed with hydrogen peroxide–sodium acetate and tetramethylbenzidine solutions. The change in absorbance was measured spectrophotometrically at 655 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 25°C per gram of tissue.

Detection of apoptosis

A commercial *in situ* histochemical assay (Klenow FragEL; Oncogene Research Products, Cambridge, MA) was performed to detect DNA fragmentation characteristic of apoptosis in formalin-fixed paraffin-embedded liver sections. In this assay, Klenow binds to exposed ends of DNA fragments generated in response to apoptotic signals and catalyzes the template-dependent addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected with a streptavidin–horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells. The results were scored semiquantitatively by averaging the number of apoptotic cells per microscopic field at ×200 magnification. Six fields were evaluated per tissue sample.

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from frozen liver samples, using an RNase mini kit (Qiagen), and RNA concentration was determined with a spectrophotometer. A total of 2.5 μg of RNA was reverse-transcribed into cDNA (SuperScript III first-strand synthesis system; Invitrogen). Primer sequences used for the amplification of HO-1 and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were as follows: HO-1, 5′-TCAGTCCCAAACGTCGCGGT-3′ (forward) and 5′-GCT GTGCAGGTGTTGAGCC-3′ (reverse); HPRT, 5′-TCAACGG GGGACATAAAAGT-3′ (forward) and 5′-TGCATTGTTTTA CCAGTGTCAA-3′ (reverse).

Quantitative real-time polymerase chain reaction (PCR) was performed with the DNA Engine with Chromo4 detector (MJ Research/Bio-Rad, Waltham, MA). In a final reaction volume of 25 μl, the following were added: 1× SuperMix (Platinum SYBR Green qPCR kit; Invitrogen), cDNA, and a 10 μM concentration of each primer. Amplification conditions were as follows: 50°C (2 min), 95°C (5 min), followed by 50 cycles of 95°C (15 sec) and 60°C (30 sec).

Western blot analysis

Protein was extracted from liver tissue, CoPP-treated macrophages, or transfected YPEN-1 cells with PBSTDS buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, and 1% Triton X-100, pH 7.2). Proteins (30 μg/sample) in SDS-loading buffer (50 mM Tris [pH 7.6], 10% glycerol, 1% SDS) were subjected to SDS–12% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The gel was stained with Coomassie blue to document protein loading. The membrane was blocked with 3% dry milk +0.1% Tween 20 (USB, Cleveland, OH). Polyclonal rabbit anti-mouse HO-1 (StressGen Biotech, Victoria, BC,

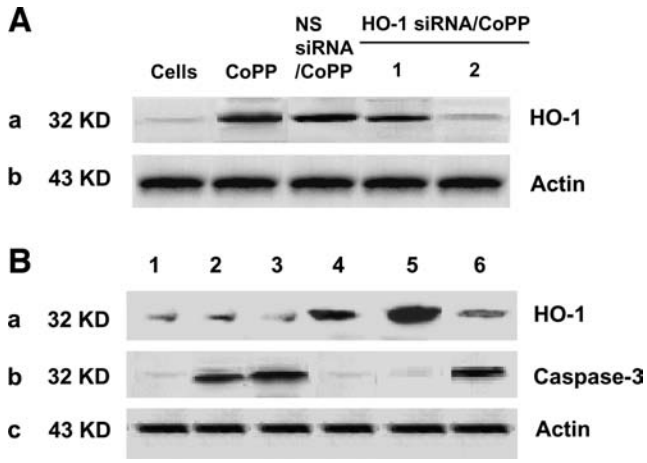

FIG. 1. (**A**) Western blot analysis of HO-1 protein expression after HO-1 siRNA addition to CoPP-treated macrophages. RAW 264.7 macrophages were transfected with two different HO-1 siRNA sequences (1 and 2) and nonspecific control siRNA before CoPP treatment. Lanes 1 and 2 represent HO-1 siRNA sequences 1 and 2, respectively. *Note:* HO-1 siRNA sequence 2 inhibited CoPP-induced HO-1 protein induction, whereas sequence 1 and nonspecific siRNA had no effect on HO-1 expression. (**B**) Western blot analysis of HO-1 and caspase-3 gene products in Ad-HO-1-transfected YPEN-1 cells. The expression of HO-1 and caspase-3 was probed with rabbit anti-mouse HO-1 (**a**) and caspase-3 (**b**) antibodies. Lane 1, YPEN-1 cells alone; lane 2, YPEN-1 cells plus etoposide (50 μM); lane 3, YPEN-1 cells transfected with Ad-HO-1 and HO-1 siRNA plus etoposide (50 μM); lane 4, YPEN-1 cells transfected with Ad-HO-1 and nonspecific siRNA plus etoposide (50 μM); lane 5, YPEN-1 cells transfected with Ad-HO-1; lane 6, YPEN-1 cells transfected with Ad-β-gal. *Note* the selectively inhibited expression of HO-1 in HO-1 siRNA-treated YPEN-1 cells (lane 3a), as compared with nonspecific siRNA and Ad-HO-1 (lanes 4a and 5a). In contrast, the expression of caspase-3 increased in cells treated with 50 μM etoposide (lane 2b) or after HO-1 siRNA (lane 3b) or Ad-β-gal (lane 6b), as compared with nonspecific siRNA (lane 4b) or Ad-HO-1 (lane 5b). Anti-β-actin antibody was used to ensure equal protein amounts between the samples. Data shown are representative of three separate experiments.

Canada), caspase-3, Bcl-2, Bcl-x_L, and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The membranes were incubated with antibody and relative quantities of proteins were determined with a densitometer and expressed as absorbance units (AU).

Caspase-3 activity

Caspase-3 activity was determined with an assay kit (Calbiochem). Briefly, transfected YPEN-1 cells or liver tissue samples were resuspended with ice-cold cell lysis buffer (50 m*M* HEPES, 5 m*M* dithiothreitol [DTT], 0.1 m*M* EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [CHAPS], and 0.1% Triton X-100), and incubated for 10 min on ice. After centrifugation for 10 min at 10,000×*g*, protein supernatant was transferred to a fresh tube and hold on ice until use. For measuring caspase-3 activity, protein (30 μg/sample) was incubated with 200 μM enzyme-specific colorimetric caspase-3 substrate I, acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA) at 37°C for 2 hr. Caspase-3 activity was assessed by measuring absorbance at a wavelength of 405 nm with a plate reader (Bio-Tek Instruments, Winooski, VT). To determine cellular activity, inhibitor-treated protein extracts and purified caspase-3 (as a standard) were used.

Statistical analysis

All data are expressed as means ± SD. Statistical comparisons between groups were analyzed by Student *t* test. All differences were considered statistically significant at the $p < 0.05$.

Results

HO-1 siRNA inhibits HO-1 protein expression in vitro

To delineate the role of HO-1 in the pathophysiology of liver IRI, we first sought to knock down HO-1 induction by using an HO-1 siRNA approach, and then test its effects in a well-defined *in vitro* system, that is, CoPP (HO-1 inducer)-treated macrophages and Ad-HO-1-transfected YPEN-1 cells. We designed and selected two HO-1 siRNA murine sequences (1 and 2), followed by transfection into RAW 264.7 macrophages (Fig. 1A). siRNA sequence 2 was shown to diminish HO-1. We determined that sequence 2 was the most effective in inhibiting CoPP-induced HO-1 protein induction, whereas the nonspecific siRNA had no measurable effect on HO-1 expression.

We then used HO-1 siRNA sequence 2 to analyze HO-1 expression in Ad-HO-1-transfected YPEN-1 cells. As shown in Fig. 1B, HO-1 siRNA inhibited HO-1 (0.6 AU, lane 3a) in Ad-HO-1-transfected YPEN-1 cells, as compared with nonspecific siRNA (1.5 AU, lane 4a). In contrast, Ad-HO-1-transfected YPEN-1 cells alone showed markedly increased HO-1 (2.5 AU, lane 5a), compared with HO-1 siRNA or Ad-β-gal groups (0.9 AU, lane 6a).

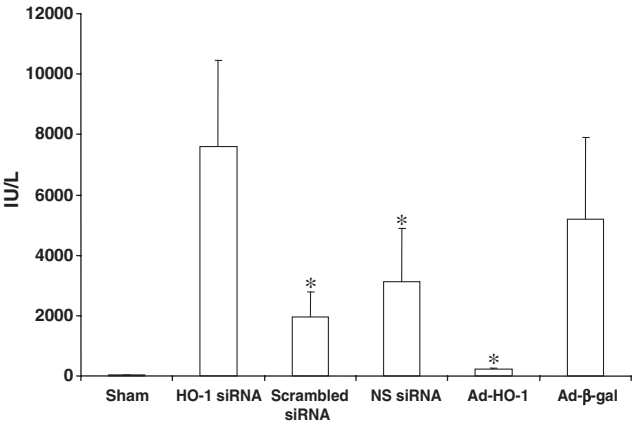

FIG. 2. Hepatocellular damage, as analyzed by sGOT level (IU/liter), in mice that underwent 90 min of hepatic warm ischemia followed by 6 hr of reperfusion. *Note:* sGOT levels were significantly increased in recipients treated with HO-1 siRNA, as compared with control scrambled siRNA, nonspecific siRNA, or Ad-HO-1 (*$p < 0.05$). In contrast, Ad-HO-1 decreased sGOT levels, as compared with Ad-β-gal controls (*$p < 0.05$). Mean and SD are shown ($n = 4$–6 per group).

HO-1 siRNA augments IR-induced hepatocellular damage in vivo

On the basis of the results with the *in vitro* cell culture system (Fig. 1A), we chose HO-1 siRNA sequence 2 for our *in vivo* liver IRI studies. Liver function, as assessed by sGOT levels (IU/liter), after 90 min of hepatic warm ischemia followed by 6 hr of reperfusion, markedly deteriorated in mice treated with HO-1 siRNA, as compared with those conditioned with scrambled siRNA, nonspecific siRNA or Ad-HO-1 (7593 ± 2859 vs. 1957 ± 824, 3104 ± 1777, and 211.5 ± 40, respectively; $p < 0.05$; Fig. 2). Consistent with the cytoprotective function of HO-1 overexpression, local intraliver Ad-HO-1 gene transfer significantly decreased sGOT levels (IU/liter), as compared with Ad-β-gal control (211.5 ± 40 vs. 5199 ± 2711, $p < 0.05$).

We then evaluated the severity of IRI on the basis of the Suzuki histological classification of liver damage. Indeed, mice treated with HO-1 siRNA or Ad-β-gal revealed significant edema, severe sinusoidal congestion, cytoplasmic vacuolization, and extensive hepatocellular necrosis (30–50%; Fig. 3A and D; score, 3.3 ± 0.5 and 3.2 ± 0.4, respectively). In contrast, animals treated with nonspecific scrambled siRNA showed mild to moderate edema, sinusoidal congestion, and cytoplasmic vacuolization (Fig. 3B; score, 1.8 ± 0.7; $p < 0.005$), whereas livers in recipients given Ad-HO-1 showed good preservation, with no edema or necrosis (Fig. 3C; score, 1.2 ± 0.8; $p < 0.001$).

We analyzed local neutrophil sequestration by MPO assay. Indeed, treatment with HO-1 siRNA significantly increased MPO activity (U/g: 5.39 ± 0.24), as compared with nonspecific siRNA (4.14 ± 0.18, $p < 0.05$; Fig. 4). In contrast, Ad-HO-1 gene transfer reduced MPO (1.33 ± 0.13), as compared with HO-1 siRNA ($p < 0.05$) or Ad-β-gal (4.88 ± 0.16; $p < 0.005$).

HO-1 siRNA increases hepatocellular apoptosis in IRI

To determine the biological effect of HO-1 on apoptosis, we performed TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) staining on ischemic tissue samples. HO-1 siRNA significantly increased the number of TUNEL-positive cells (31.8 ± 5.5; Fig. 5A) as compared with nonspecific siRNA (18.5 ± 5.4, $p < 0.005$; Fig. 5B). In contrast, livers overexpressing HO-1 after Ad-HO-1 transfer exhibited significantly diminished TUNEL staining (3.5 ± 2.2; Fig. 5C), and contrasted with those after HO-1siRNA ($p < 0.0001$) or Ad-β-gal (27.2 ± 8.5, $p < 0.0005$; Fig. 5D) treatment.

HO-1 siRNA enhances hepatocellular apoptosis by activating caspase-3

To analyze putative cross-talk between HO-1 and apoptosis, we measured caspase-3 activity in our experimental system. As shown in Fig. 6A, caspase-3 activity increased in

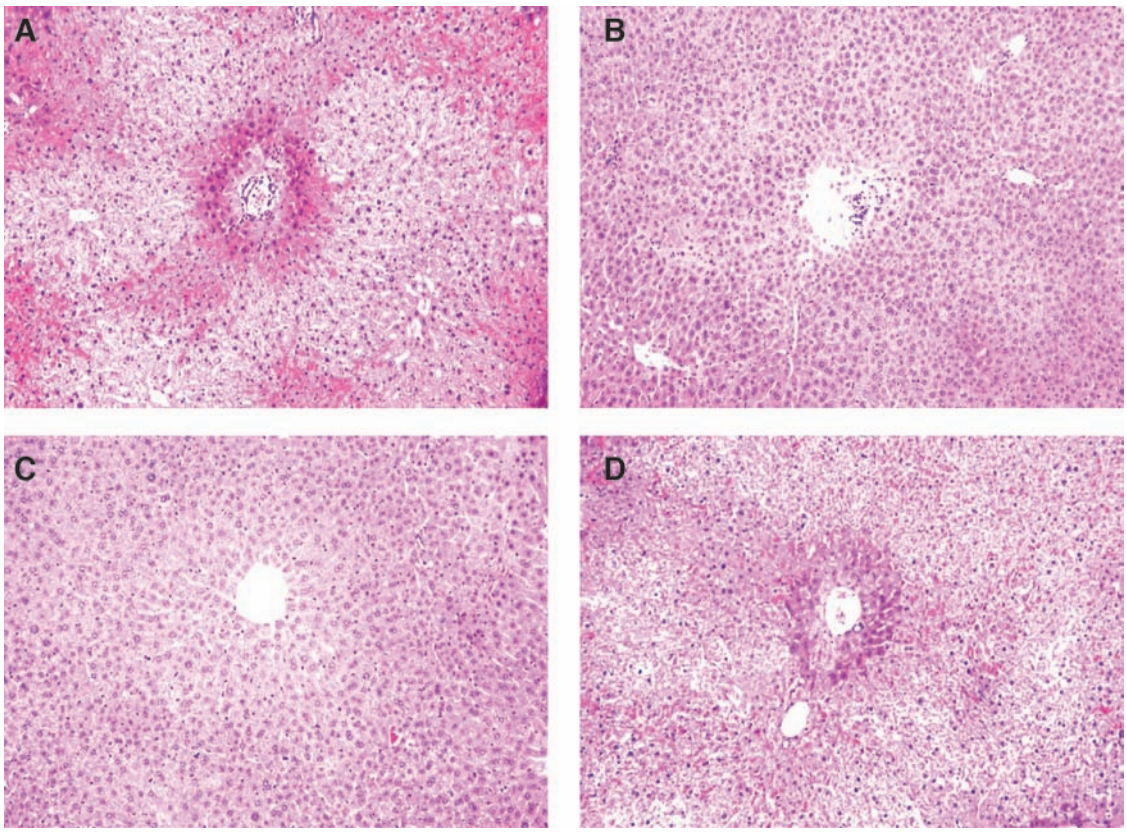

FIG. 3. Representative histological findings in mouse liver after 90 min of warm ischemia followed by 6 hr of reperfusion in mice treated with (**A**) HO-1 siRNA (Suzuki score, 3.3 ± 0.5); (**B**) nonspecific scrambled control siRNA (score, 1.8 ± 0.7); (**C**) Ad-HO-1 (score, 1.2 ± 0.8); (**D**) Ad-β-gal (score, 3.2 ± 0.4); Results are representative of four to six mice per group; original magnification, $\times 200$. Color images available online at www.liebertonline.com/hum.

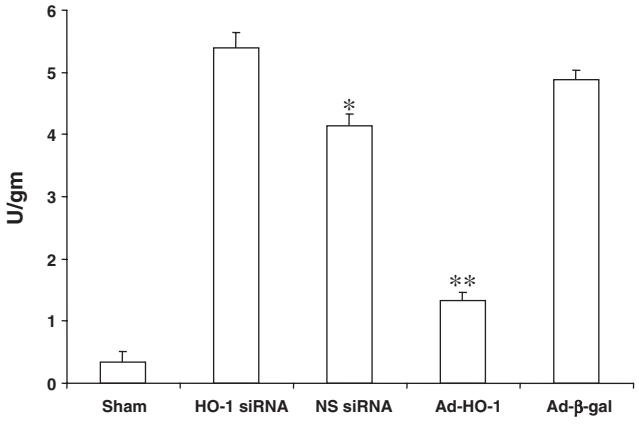

FIG. 4. Intragraft neutrophil accumulation at 6 hr of reperfusion after 90 min of warm ischemia, as analyzed by MPO enzymatic activity (U/g) in ischemic lobes. *Note:* MPO activity was significantly increased in the HO-1 siRNA group, as compared with those given nonspecific siRNA ($^*p < 0.05$) or Ad-HO-1 ($^*p < 0.05$). Administration of Ad-HO-1 reduced MPO activity, as compared with the Ad-β-gal ($^{**}p < 0.005$) group. Data represent four to six animals per group. Means and SD are shown.

mice treated with HO-1 siRNA (3.65 ± 0.2) as compared with those in the control siRNA group (1.76 ± 0.23; $p < 0.001$). In contrast, HO-1 overexpression decreased caspase-3 activity (0.76 ± 0.2) compared with that of HO-1 siRNA ($p < 0.0001$) or Ad-β-gal (2.95 ± 0.25; $p < 0.001$); data were confirmed by Western blots at the protein level (Fig. 7).

To further explore potential mechanisms of HO-1 in hepatocellular apoptosis, we investigated the effect of HO-1 siRNA on caspase-3 activity in Ad-HO-1-transfected YPEN-1 cells. As shown in Fig. 6B, HO-1 siRNA markedly increased etoposide-induced caspase-3 activity in Ad-HO-1-transfected YPEN-1 cells (4.54 ± 0.41) compared with control siRNA (2.41 ± 0.5; $p < 0.001$). In contrast, HO-1 induction by Ad-HO-1 decreased caspase-3 activity (1.15 ± 0.25) compared with that of HO-1 siRNA ($p < 0.005$) or Ad-β-gal (3.64 ± 0.35; $p < 0.005$).

The caspase-3 activity data correlated with *in vitro* expression of caspase-3 protein (Fig. 1B). Indeed, HO-1 siRNA increased etoposide-induced caspase-3 in Ad-HO-1-transfected YPEN-1 cells (1.8 AU, lane 3b) as compared with nonspecific siRNA (0.5 AU, lane 4b). HO-1 overexpression after Ad-HO-1 administration decreased caspase-3 expression (0.4 AU, lane 5b) as compared with that of the HO-1 siRNA or Ad-β-gal group (1.7 AU, lane 6b).

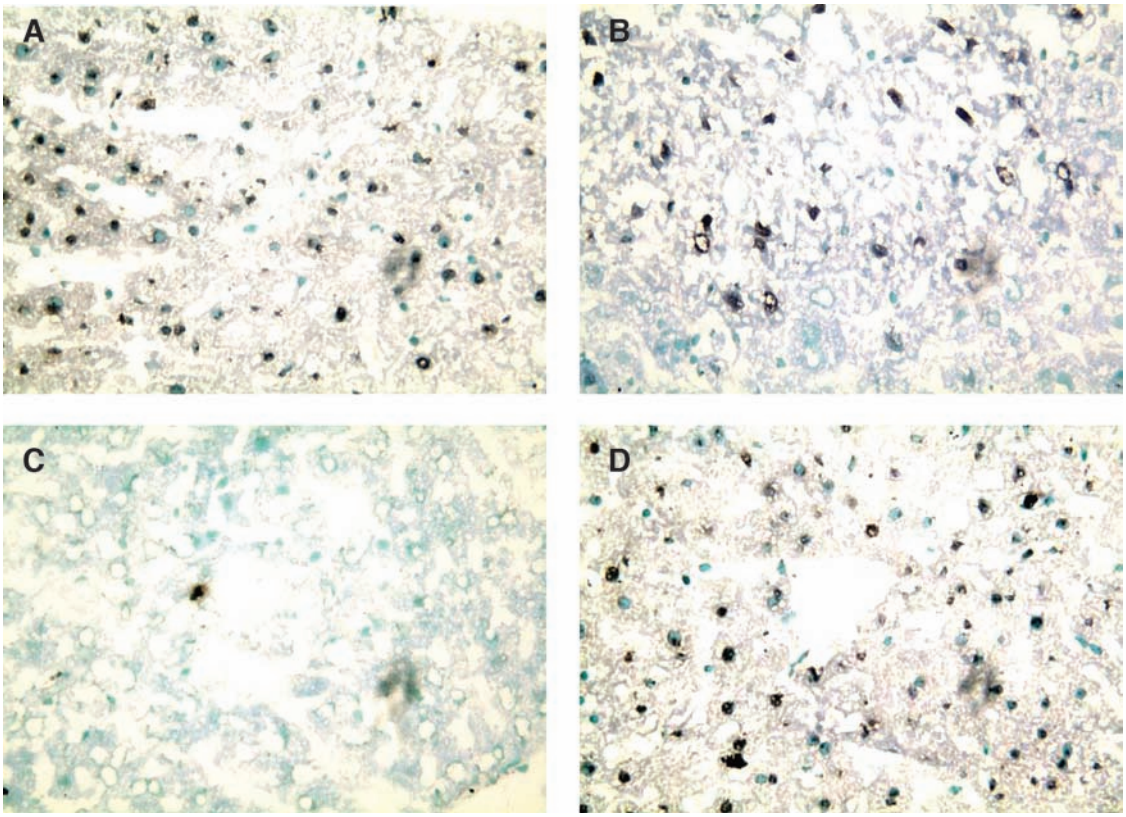

FIG. 5. TUNEL-assisted detection of apoptosis in mouse liver after 90 min of warm ischemia followed by 6 hr of reperfusion. *Note* the dense infiltration by apoptotic cells in ischemic liver tissue in mice treated with HO-1 siRNA (**A**) and Ad-β-gal (**D**), as compared with nonspecific siRNA treatment (**B**; $p < 0.0001$). The Ad-HO-1 gene transfer group showed a decreased frequency of apoptotic cells (**C**; $p < 0.0005$) as compared with HO-1 siRNA or Ad-β-gal (**D**). The results were scored semiquantitatively by averaging the number of apoptotic cells (mean $\pm$ SD) per field at $\times 200$ magnification. A minimum of six fields was evaluated per sample. Results shown are representative of three experiments. Color images available online at www.liebertonline.com/hum.

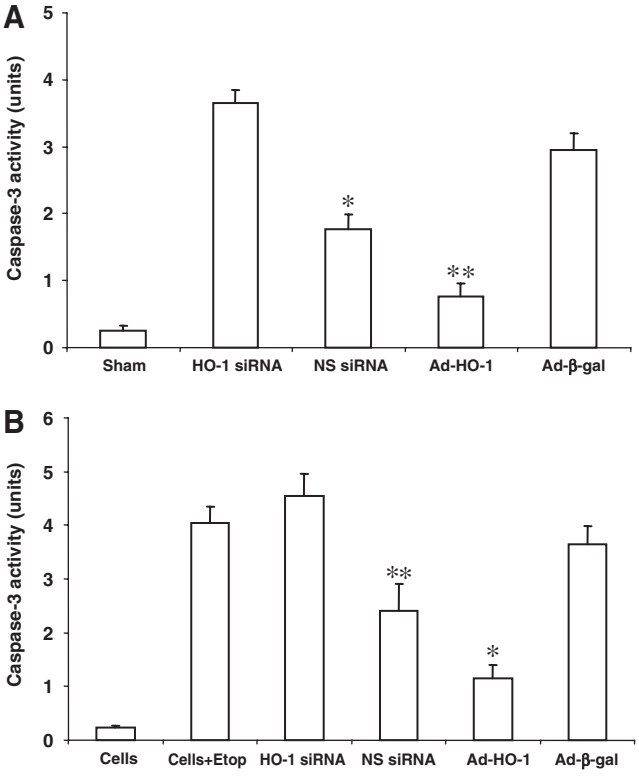

FIG. 6. (**A**) Activity of caspase-3 in IRI. Caspase-3 activity was markedly increased in mice treated with HO-1 siRNA, compared with those in control siRNA groups (*$p < 0.001$). In contrast, overexpression of HO-1 by Ad-HO-1 decreased caspase-3 activity, as compared with that of HO-1 siRNA (**$p < 0.0001$) or Ad-β-gal (*$p < 0.001$). (**B**) Activity of caspase-3 in Ad-HO-1-transfected YPEN-1 cells. HO-1 siRNA increased etoposide-induced caspase-3 activity, as compared with nonspecific control siRNA (**$p < 0.001$). In contrast, HO-1 overexpression decreased caspase-3 activity, compared with that of HO-1 siRNA (*$p < 0.005$) or Ad-β-gal (*$p < 0.005$). Data shown are representative of three separate experiments. Means and SD are shown.

HO-1 siRNA decreases antiapoptotic Bcl-2/Bcl-x_L expression in mouse liver IRI

Antiapoptotic Bcl-2 and Bcl-x_L molecule expression plays an important role in liver cytoprotection during IRI. As shown in Fig. 7, Ad-HO-1 selectively increased the expression of Bcl-2 (2.2 AU, lane 4C) and Bcl-x_L (2.1 AU, lane 4D), and decreased the expression of caspase-3 (0.3 AU, lane 4B), compared with Ad-β-gal (0.3-0.4 AU, lanes 5C and D; 1.1 AU, lane 5B). In contrast, when we knocked down HO-1 with HO-1 siRNA, the expression of antiapoptotic Bcl-2 and Bcl-x_L markedly decreased (0.4 AU, lanes 2C and D), whereas the expression of caspase-3 increased (1.9 AU, lane 2B).

HO-1 siRNA inhibits HO-1 mRNA/protein expression in mouse liver IRI

Finally, to document the *in vivo* efficacy of HO-1 siRNA gene silencing, we first measured liver mRNA levels in our experimental model. As shown in Fig. 8, the expression of HO-1 markedly fell after treatment with HO-1 siRNA ($p < 0.05$) as compared with nonspecific siRNA or Ad-β-gal. In contrast, HO-1 expression significantly increased in the Ad-HO-1 group as compared with all other groups ($p < 0.01$).

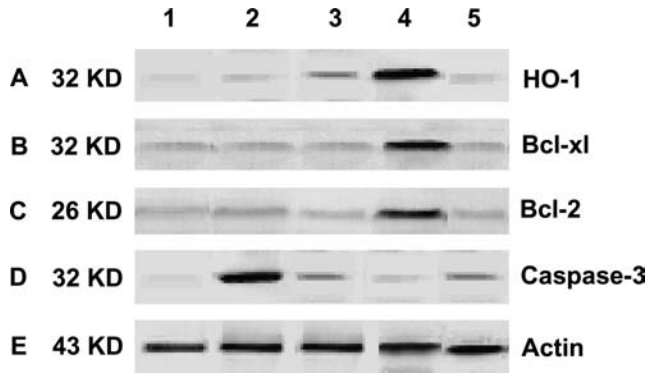

FIG. 7. Western blot analysis of HO-1, caspase-3, Bcl-2, and Bcl-x_L gene products in hepatic lobes 6 hr after 90 min of warm ischemia. Lane 1, sham; lane 2, HO-1 siRNA; lane 3, nonspecific siRNA; lane 4, Ad-HO-1; lane 5, Ad-β-gal. *Note* the selectively decreased expression of HO-1 and Bcl-2/Bcl-x_L, and markedly increased caspase-3, in mice treated with HO-1 siRNA as compared with those conditioned with nonspecific siRNA or Ad-HO-1. In contrast, Ad-HO-1 increased the expression of HO-1 and Bcl-2/Bcl-x_L, and decreased that of caspase-3. Data shown are representative of three separate experiments.

These data correlated with Western blot-assisted detection of HO-1 protein. Figure 7A shows that HO-1 siRNA inhibited HO-1 protein expression (0.3 AU, lane 2A), as compared with nonspecific siRNA (1.0 AU, lane 3A). In contrast, Ad-HO-1 gene transfer increased HO-1 (2.4 AU, lane 4A), as compared with HO-1 siRNA or Ad-β-gal (0.4 AU, lane 5A).

Discussion

The discovery of gene silencing in mouse liver by systemic delivery of siRNA targeting a specific gene has facilitated the

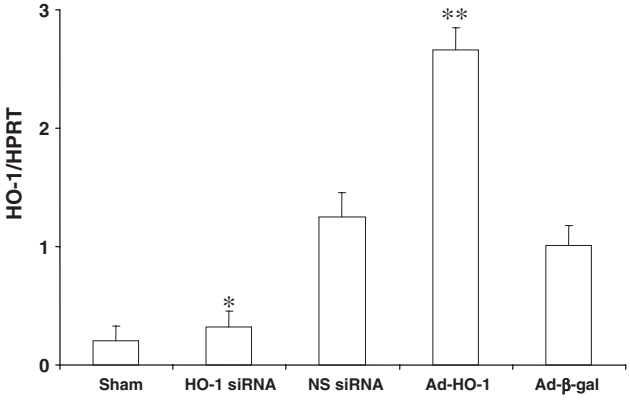

FIG. 8. Quantitative real-time PCR to measure HO-1 levels in mouse liver after 90 min of warm ischemia followed by 6 hr of reperfusion. *Note:* Expression of mRNA encoding HO-1 was markedly depressed after treatment with HO-1 siRNA (*$p < 0.05$), as compared with nonspecific siRNA or Ad-β-gal. In contrast, expression of HO-1 was significantly increased in the Ad-HO-1 group, as compared with other groups (**$p < 0.01$). Each column represents the mean $\pm$ SD ($n = 3$ or 4 samples per group).

possibility of regulating hepatic gene expression to prevent liver diseases (Soutschek *et al.*, 2004; Takabatake *et al.*, 2005). In the present study, we used specific siRNA targeting the HO-1 gene to investigate the function and mechanism of HO-1 *in vitro* and *in vivo*. The principal findings of this study are as follows: (1) HO-1 siRNA inhibits HO-1 expression and enhances liver apoptosis induced by IRI, and (2) HO-1 exerts cytoprotection against IRI by regulating liver apoptosis and inhibiting the caspase-3 activation pathway.

The HO-1 system has been considered one of the major protective pathways in a variety of organ inflammatory diseases. The induction of HO-1 has provided potent cytoprotection against the development of transplant arteriosclerosis and chronic cardiac allograft rejection (Hancock *et al.*, 1998). Moreover, HO-1 overexpression resulted in long-term acceptance of cardiac xenografts (Soares *et al.*, 1998) and prevented hyperoxia-induced lung injury (Otterbein *et al.*, 2003), and the use of HO-1-inducing metalloporphyrins ameliorated injury induced by IR in rat hearts (Katori *et al.*, 2002b). Furthermore, Ad-based HO-1 gene transfer prevented CD95/FasL-mediated apoptosis, and significantly prolonged survival after allogeneic orthotopic liver transplantation (OLT) via the CO downstream signaling pathway (Ke *et al.*, 2002). The cytoprotective function of HO-1 depends on the ability to generate heme degradation and generate downstream mediators such as biliverdin, its metabolite bilirubin, along with CO and free iron (Maines, 1997). We have shown that HO-1 exhibits potent cytoprotective effects against hepatic IRI (Amersi *et al.*, 1999; Katori *et al.*, 2002a; Ke *et al.*, 2003). In a cold *ex vivo* rat liver perfusion model and in a syngeneic OLT model, treatment of normal or genetically obese Zucker rats with Ad-HO-1 improved portal venous blood flow, increased bile production, and decreased hepatocyte injury.

Despite well-described cytoprotective functions, the exact molecular mechanism of HO-1-mediated effects remains to be elucidated. Previous studies on HO-1 inhibition or deficiency have been limited due to the lack of specific HO-1 inhibitors and major limitations in generating HO-1-deficient mice. To determine the efficacy of siRNA to suppress HO-1 expression, we first designed two different murine siRNA sequences against HO-1 and transfected them into CoPP-treated macrophages. The most effective HO-1 siRNA was selected and then used in all our *in vivo* studies. In mice receiving HO-1 siRNA, the expression of HO-1 was inhibited by 80–90% in the liver, spleen, lung, and kidney (data not shown), as compared with that in mice receiving nonspecific or scrambled siRNA. Here, we have applied siRNA technology to analyze HO-1 function in a hepatic IRI model. Indeed, mice treated with HO-1 siRNA showed increased hepatic injury, as analyzed by sGOT levels, local neutrophil accumulation, and hepatocellular necrosis. In contrast, HO-1 overexpression by means of an Ad-based delivery system successfully rescued mice from hepatic IRI. These results are in agreement with our earlier reports in which HO-1 overexpression ameliorated liver damage in both warm and cold IRI models (Amersi *et al.*, 1999; Katori *et al.*, 2002a; Ke *et al.*, 2003).

A variety of mechanisms have been implicated in HO-1 cytoprotection. Liver IRI associates with hepatocellular apoptosis, mediated by death receptors such as Fas and tumor necrosis factor (TNF)-α. The activation of caspases, intracellular cysteine proteases, plays a critical role in the execution of apoptosis (Cohen, 1997; Goyal, 2001). Caspase-3 is a downstream caspase effector in the apoptotic pathway. In the present study, using gene transfer of Ad-HO-1 into YPEN-1 endothelial cells, specific knockdown of HO-1 expression with siRNA abolished cytoprotection seen otherwise in Ad-HO-1-transfected YPEN-1 cells, and significantly increased etoposide-induced apoptosis, as assessed by increased caspase-3 expression and activity. In contrast, HO-1 induction using a viral delivery system protected endothelial cells from etoposide-induced apoptosis. This result is consistent with the ability of HO-1 to prevent endothelial cell apoptosis and caspase-3 activation (Brouard *et al.*, 2000). Alternatively, HO-1 may inhibit SECs or hepatocellular apoptosis induced by IR via inhibition of caspase-3 (Sass *et al.*, 2005). Our current *in vivo* studies have shown that specific HO-1 siRNA delivery significantly increased caspase-3 activity and the frequency of $TUNEL^+$ cells in ischemic livers in mice, whereas Ad-HO-1 transfer markedly inhibited caspase-3 expression and the frequency of $TUNEL^+$ cells. These are consistent with enhances IR-induced lung apoptosis with HO-1 siRNA (Zhang *et al.*, 2004), and inhibition of apoptosis by siRNA targeting caspase-3 that provided protection against liver IRI (Contreras *et al.*, 2004). Collectively, HO-1 profoundly affects apoptosis in hepatic IRI via the caspase 3-dependent pathway.

Our present data also show that HO-1 overexpression by Ad-HO-1 gene transfer selectively upregulated the expression of antiapoptotic Bcl-2 and Bcl-x_L. The cellular and physiological mechanisms by which HO-1 exerts cytoprotective functions may involve the expression of antiapoptotic proteins in the ischemic organ. Indeed, preventing liver apoptosis in this study was accompanied by enhanced local expression of Bcl-2 and Bcl-x_L. Both are protective molecules and exert antiapoptotic functions, with Bcl-2 preventing the release of apoptogenic factors, such as cytochrome *c*, an apoptosis-inducing factor from mitochondria, into the cytosol (Susin *et al.*, 1996; Kluck *et al.*, 1997). Moreover, Bcl-2 overexpression blocks cell death by preserving mitochondrial integrity and promoting ATP generation (Saikumar *et al.*, 1998). Consistent with our findings, increased expression of Bcl-2 or Bcl-x_L prevented cell apoptosis in ischemic liver, whereas silencing of HO-1 with siRNA diminished Bcl-2 and Bcl-x_L expression. These are consistent with previous data on the role of antiapoptotic Bcl-2/Bcl-x_L in mouse liver IRI (Bilbao *et al.*, 1999).

Our study has shown that HO-1 siRNA has potent blocking efficacy both *in vitro* as well as *in vivo*. RNA interference with siRNA is a new and powerful technology that allows the silencing of selective genes. The exogenous administration of siRNA resulted in silencing of the messenger RNA in liver and resulted in decreased plasma levels of targeting gene. It has been shown that systemic delivery of caspase-8 and caspase-3 with siRNA prevents vascular endothelial cell injury in mice with endotoxic shock (Matsuda *et al.*, 2007), and protects against liver IRI (Contreras *et al.*, 2004). Moreover, administration of siRNA targeting complement 3 prevented renal IRI (Zheng *et al.*, 2006). Furthermore, silencing Fas expression with siRNA protected hepatocytes from fulminant hepatitis (Song *et al.*, 2003). Although transgenic mice as well as viral constructs have been used to deliver siRNA *in vivo* (Hasuwa *et al.*, 2002; Rubinson *et al.*, 2003), systemic siRNA delivery via transfection of chemicals or viral vectors might raise potential

toxicity concerns in the clinical setting. Our study demonstrates that siRNA can be used safely and with great potency. Hence, siRNA delivery to the liver, without mediation by viral vectors or transfection agents, is biologically effective and may be successfully used to study the function of individual genes.

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Author Disclosure Statement

No competing financial interests exist.

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