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Induction of heme oxygenase-1 protects mouse liver from apoptotic ischemia/reperfusion injury

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Abstract

Ischemia/reperfusion (I/R) injury is the main cause of primary graft dysfunction of liver allografts. Cobalt-protoporphyrin (CoPP)–dependent induction of heme oxygenase (HO)-1 has been shown to protect the liver from I/R injury. This study analyzes the apoptotic mechanisms of HO-1-mediated cytoprotection in mouse liver exposed to I/R injury. HO-1 induction was achieved by the administration of CoPP (1.5 mg/kg body weight i.p.). Mice were studied in in vivo model of

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hepatic segmental (70 %) ischemia for 60 min and reperfusion injury. Mice were randomly allocated to four main experimental groups ($n = 10$ each): (1) A control group undergoing sham operation. (2) Similar to group 1 but with the administration of CoPP 72 h before the operation. (3) Mice undergoing in vivo hepatic I/R. (4) Similar to group 3 but with the administration of CoPP 72 h before ischemia induction. When compared with the I/R mice group, in the I/R+CoPP mice group, the increased hepatic expression of HO-1 was associated with a significant reduction in liver enzyme levels, fewer apoptotic hepatocytes cells were identified by morphological criteria and by immunohistochemistry for caspase-3, there was a decreased mean number of proliferating cells (positively stained for Ki67), and a reduced hepatic expression of: C/EBP homologous protein (an index of endoplasmic reticulum stress), the NF- κ B's regulated genes (CIAP2, MCP-1 and IL-6), and increased hepatic expression of I κ Ba (the inhibitory protein of NF- κ B). HO-1 overexpression plays a pivotal role in reducing the hepatic apoptotic IR injury. HO-1 may serve as a potential target for therapeutic intervention in hepatic I/R injury during liver transplantation.

Keywords

Heme oxygenase-1; Cobalt-protoporphyrin; Nuclear factor-kappaB (NF- κ B); Ischemia reperfusion injury; Liver

Introduction

Ischemia–reperfusion (I/R) injury is the main cause of both primary graft dysfunction (occurring in 10–30 % of grafts) and primary nonfunction of liver allograft (occurring in 5 % of grafts) [1]. The latter is responsible for 81 % of retransplantations during the first week after surgery [1, 2]. Therefore, minimizing the adverse effects of I/R injury could increase the number of both suitable transplantation grafts and patients who successfully recover from liver transplantation. Although it is widely accepted that innate immunity holds a crucial role in the pathogenesis of liver I/R injury, the exact molecular mechanisms that induce I/R injury-related innate immune cell activation have not been fully elucidated to date [3, 4]. The upregulation of heme oxygenase (HO-1) also known as a heat shock protein (hsp) 32, provides a protective response from cellular stress after ischemia and inflammation, preventing deleterious effects of heme as well as mediating anti-inflammatory and anti-apoptotic functions [5–7]. Indeed, HO-1 overexpression by pharmacological means or via genetic engineering exerts potent cytoprotective effects in hepatic I/R injury transplant models, as evidenced by the preservation of tissue architecture, organ function, and prolonged survival [8]. Moreover, proinflammatory responses remain profoundly diminished in HO-1 overexpressing liver transplants [9–11]. However, some studies indicate that the protection might be restricted to a narrow threshold of HO-1 overexpression [12]. High levels of HO-1 may even sensitize the cell to oxidative stress, e.g., through release of reactive iron. Transcriptional activation of the HO-1 gene is an integral part of the cellular response to oxidative stress, but its induction seems to be neither exclusively cytoprotective nor exclusively cytotoxic [12]. Indeed, several studies have shown that excessive overexpression of HO-1 is directly related to increased injury [12, 13].

This study analyzes the antiapoptotic mechanisms of HO-1-mediated cytoprotection by using a HO-1 inducer in mouse liver exposed to I/R injury.

Materials and methods

All male mice were maintained in a pathogen-free facility and were fed pellet food and water ad libitum, until the start of the experiment (at 12 weeks old). All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Felsenstein Medical Research Center and Tel Aviv University (Tel Aviv, Israel).

General experimental protocols

In vivo I/R injury model—A model of segmental (70 %) hepatic ischemia was used as previously described [14]. Adult C57BL male mice weighing 25–28 g were anesthetized by intra-peritoneal injection of chloral hydrate (10 mg/100 g body weight). After a midline laparotomy, all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded for 60 min with a vascular a traumatic clamp. This method of partial hepatic ischemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reperfusion was initiated by removal of the clamp. Mice were randomly allocated to four main experimental groups ($n = 10$ each): (1) Control group undergoing sham operation. (2) Similar to group 1 with the administration of Cobalt-protoporphyrin (CoPP), an HO-1 inducer (1.5 mg/kg body weight i.p.) 72 h before the operation. (3) Mice undergoing in vivo hepatic I/R (4) Similar to group 3 with the administration of CoPP 72 h before the induction of ischemia.

Liver enzyme levels—Serum (0.5 ml) was collected at 6 h post reperfusion and kept on ice until processed. Levels of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase and gamma glutamyl transpeptidase (GGT) were determined in serum, in duplicate, using commercial kits, according to the manufacturer's protocols.

Pathological evaluation

Specimens from the ischemic liver in all groups were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin. Pathological findings were assessed by one of the author (OP) blinded to the group allocations. For each method used for detection of apoptosis, the number of apoptotic cells was calculated using high power microscopy in 50 ± 5 fields (original magnification 10). Apoptotic cells were identified by morphological criteria (cell shrinkage, chromatin condensation, margination, apoptotic bodies).

Immunohistochemistry for caspase-3—Apoptotic hepatocytes were identified using caspase-3 immunohistochemistry. For activated caspase-3 immunostaining, 5 μ m sections were dewaxed and hydrated through graded ethanols, cooked in 25 mM citrate buffer at pH 6.0 in a pressure cooker at 115 °C for 3 min (decloaking chamber; Biocare Medical), transferred into boiling deionized water, and let to cool for 20 min. After 5 min of treatment in 3 % H_2O_2 , the slides were incubated with rabbit polyclonal activated caspase-3 (Cell Signaling) antibodies diluted 1:100 in CAS-Block (Zymed) for 3 h at room temperature or overnight at

4 °C, washed three times with Optimax (HK583; Biogenex), incubated for 30 min with antirabbit Envision+ (K4007, Dako, Copenhagen, Denmark), and developed with 3,30-diaminobenzidine or 3-amino,9-ethylcarbazole.

Immunohistochemistry for Ki67—Immunostaining for Ki67, a marker for cell proliferation, was performed to evaluate the proliferation of hepatocytes. The primary antibody was a mouse monoclonal anti-human Ki67 antigen (MIB-1 clone). Immunohistochemistry on paraffin-embedded sections with Ki67 was performed using a fully automated system (Ventana Benchmark) as previously described. Briefly: The heat retrieval was standard for 60 min. Sections were incubated with Ki67 (DAKO, Glostrup, Denmark M7240), diluted 1; 100 in Zymed antibody diluent (Zymed, California, USA 00-3118).

Western blot analysis of liver tissue—Liver tissue samples (20 mg) were homogenized in RIPA lysis buffer and quantified for protein levels using a commercial assay (Bio-Rad). Liver extracts (40 µg protein/lane) were electrophoresed and subjected to SDS-PAGE under reducing conditions using 12.5 % polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with the appropriate antibodies. Rabbit anti-IκBa (the inhibitory protein of NF-κB) (Sigma Chemical Co. St. Louis, MO) and rabbit monoclonal pan actin, used as loading control (Abcam, Burlingame, CA). Rabbit polyclonal antibodies directed against heme oxygenase-1 (HO-1) purchased from Enzo Life Science (Farmingdale, NY), mouse monoclonal antibodies directed against GADD 153, CHOP (ER stress marker) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and beta-tubulin (used as loading control) purchased from Abcam. Detection was carried out by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Peroxidase-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and peroxidase-conjugated goat anti-rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO). Quantification of enhanced chemiluminescence was carried out by VersaDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

Hepatic expression of NF-κB regulated genes: CIAP2, MCP-1 and IL-6—Total RNA was extracted from liver samples using TRI-Reagent (Sigma), followed by treatment with 1 U of RNase-free DNase (Roche). Reverse transcription was performed on 2 µg total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. qPCR was performed for: CIAP2, MCP-1 IL6 and hprt as a reference control in an ABI Prism 7900 SDS (Applied Biosystems), using SYBR Green Real-Time PCR Kit (Applied Biosystems) according to manufacturer's specifications. All reactions were executed in a total volume of 20 µl containing 50 ng DNA and 1× SYBR Green. All the reactions were done in duplicates and relative gene expression values determined using the 2^{-ddCt} method with ABI Prism 7000 SDS (Applied Biosystems).

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Differences between groups were assessed using the analysis of variance (ANOVA) with repeated measurements using the multiple comparison option of Duncan.

Results

Liver enzymes

Liver enzyme levels remained within the normal range in the control, sham operated group for the duration of the study. A statistically significant difference was noted in mean serum ALT/AST levels following the induction of I/R, compared with sham operated mice ($p = 0.0001$, $p = 0.0001$, respectively). In CoPP pretreated mice following the induction of I/R, the increase in serum liver enzymes level was significantly less compared with non-treated mice ($p = 0.026$, $p = 0.033$, respectively). Serum LDH level increased significantly following the induction of I/R compared with sham operated mice ($p = 0.0001$). In CoPP pretreated mice, the increase in serum LDH level was significantly less compared with non-treated mice ($p < 0.05$) (Fig. 1). There was no significant difference in serum alkaline phosphatase and GGT levels (data not shown.)

Hepatic expression of HO-1 and CHOP protein following induction of I/R

Hepatic HO-1 expression was similar following the induction of I/R compared with sham operated mice. Pretreatment with CoPP increased significantly hepatic HO-1 expression ($p = 0.032$) (Fig. 2a). ER stress pathway was up-regulated following I/R as demonstrated by the significant increase in the hepatic expression of the CHOP protein $p = 0.05$. Pre-treatment with CoPP decreased CHOP protein expression $p < 0.05$ (Fig. 2b).

Western blot analysis of apoptotic pathways

I κ B (inhibitory proteins of NF- κ B) hepatic expression following induction of I/R—I κ B (inhibitory proteins of NF- κ B) hepatic expression was significantly lower following I/R ($p = 0.05$) compared with sham operated mice. Liver pretreated with CoPP had significantly higher hepatic expression of I κ B ($p = 0.01$) (Fig. 3).

Hepatic expression of NF- κ B targeted genes following induction of I/R—NF- κ B pathway was stimulated by the induction of hepatic ischemia, as is demonstrated by the increase in the hepatic expression of the three targeted NF- κ B's genes: CIAP2, MCP-1 and IL-6. CoPP pre-treatment significantly decreased CIAP2 ($p = 0.00001$), MCP-1 ($p = 0.0001$) and IL-6 hepatic ($p = 0.0005$) expression (Fig. 4).

Histological studies

Histologic examination of the liver tissue sections using hematoxylin-eosin staining showed damage in the I/R untreated livers (group 2), manifested by marked diffuse micro-steatosis and hydropic degeneration. Several apoptotic bodies were noted, appearing as round or oval masses of intensely eosinophilic cytoplasm with dense nuclear chromatin fragments. In addition, we detected focal spotty necrosis and, rarely, mitotic figures (Fig. 5a). In the CoPP-

pretreated group 3, the liver structure was better preserved, with mild hydropic degeneration and mild steatosis. Rare apoptotic bodies and were noted. (Fig. 5b).

Immunohistochemistry for caspase-3

Sections from I/R livers in group 2 were highly positively stained for the activated form of caspase-3. Scattered positive nuclei and cytoplasmic staining were noted in hepatocytes as well as sinusoidal cells (Fig. 6a). In the I/R pretreated-CoPP group, only rare cells stained for caspase-3 activity (Fig. 6b). I/R livers showed a significant increase in the mean number of apoptotic cells (positively stained for caspase-3) (50.0 ± 19.5) compared with livers pretreated with CoPP (2.33 ± 0.47) ($p = 0.013$).

Immunohistochemistry for Ki67

Sections from I/R livers were highly positively stained for Ki67 as a proliferation index (6D). In livers pretreated with CoPP, fewer hepatocytes stained for Ki67 (6E). Although I/R livers showed an increase in the mean number of proliferating cells (positively stained for Ki67) (58.0 ± 4.9) compared with livers pretreated with CoPP (41.6 ± 12.7), this was not significantly different (6F) ($p = \text{NS}$).

Discussion

In our study we demonstrated that HO-1 higher-expression (achieved by the administration of CoPP) prior to the induction of hepatic I/R injury was associated with the attenuation of I/R hepatic parenchymal and apoptotic injury. A significant reduction was noted in liver enzyme levels, fewer apoptotic hepatocytes cells were identified by morphological criteria and by immunohistochemistry for caspase-3. We used also Ki67 immunostaining as a cell proliferation marker to determine if HO-1 has relevant functions in promoting cell proliferation of liver cells following I/R injury. Although I/R livers showed an increase in the mean number of proliferating cells compared with livers pretreated with CoPP no significant difference was observed between the two groups. In addition, we have noted decreased hepatic expression of CHOP protein and the NF- κ B's target genes and the increased hepatic expression of the inhibitor protein of NF- κ B (I κ B α). The possible mechanism by which HO-1 overexpression protected the liver in our study from the apoptotic injury following I/R seems to involve the pro-apoptotic NF- κ B pathway and the endoplasmic reticulum stress transcriptional induction C/EBP homologous protein (CHOP).

I/R injury is still one of the major obstacles affecting prognosis of liver transplantation [1, 2]. HO-1 is up-regulated in response to oxidative stress and catalyzes the degradation of pro-oxidant heme to carbon monoxide (CO), iron and bilirubin. The upregulation of HO-1 provides a protective response from cellular stress after ischemia, preventing deleterious effects of heme as well as mediating anti-inflammatory and antiapoptotic functions [5–11]. Indeed, HO-1 overexpression by pharmacological means or via genetic engineering exerts potent cytoprotective effects in hepatic IR injury transplant models, as evidenced by the preservation of tissue architecture, organ function, and prolonged survival. Moreover, proinflammatory responses remain profoundly diminished in HO-1 overexpressing liver transplants models [9–11]. The byproducts of HO-1, CO, mediates a cytoprotective effect

through downregulating the proapoptotic Bax and upregulating anti-apoptotic Bcl-2 [15]. Biliverdin, another byproduct, has been demonstrated to have potent antioxidant properties that protect cells from oxidative injury after hepatic I/R [16]. Given the potential importance of HO-1 in attenuating hepatic I/R injury, a special effort has been focused on induction of HO-1. Preconditioning with nodosin, a traditional Chinese herbal medicine, provided protective effect through inducing HO-1 expression and attenuated I/R injury in liver transplantation [17]. Glutamine protected tissue against oxidative injury during rat hepatic I/R, by induction of HO-1 to fulfill antioxidative and antiapoptotic effects [18] and HO-1 induction by pegylated hemin led also to significantly improved hepatic I/R injury by exerting antioxidative effect [19]. Moreover, the administration of the HO-1 inducer, CoPP, prevented I/R injury resulting from prolonged storage of liver transplants as measured by the AST level and analyzed by histology (H&E staining) [11]. CoPP-induced HO-1, down-regulated the activation of STAT1 via the type-1 IFN pathway downstream of TLR4, which in turn decreased CXCL-10 production thus leading to cytoprotection against liver I/R injury as analyzed by serum ALT levels and liver histology [20]. CoPP administration - or gene therapy-induced (adenoviral HO-1) HO-1, prevented I/R injury in steatotic rat livers [8]. Extended animal survival from 40 % in untreated controls to about 80 % after CoPP or adenoviral HO-1 therapy has been noted [9]. This effect correlated with preserved hepatic architecture, improved liver function, and depressed infiltration by T cells and macrophages [9]. However, some studies indicate that the protection of HO-1 might be restricted to a narrow threshold of HO-1 overexpression [12]. Cytoprotection was achieved with low (less than fivefold) HO-1 activity whereas high levels of HO-1 expression (greater than 15-fold) were associated with significant oxygen cytotoxicity [12, 21]. Froh et al. [22] also reported that CoPP-induced HO-1 overexpression increases liver injury after bile duct ligation in rats, as demonstrated by the overexpression of hepatic ALT, aggravation of cell necrosis, and fibrosis. They suggested that high levels of HO-1 may sensitize cells to oxidative stress due to an accumulation of free divalent iron, thereby increasing oxidative injury. In our study we have found that the increased hepatic expression of HO-1 was associated with a significant reduction in liver enzyme levels, fewer apoptotic hepatocytes cells were identified by morphological criteria and by immunohistochemistry for caspase-3. Thus our findings support the beneficial role of HO-1 overexpression in hepatic I/R injury. It could be suggested that there is a beneficial threshold of HO-1 overexpression as reported by Suttner and Dennery [12], however we have not tested different levels of HO-1 activity in our study.

Endoplasmic reticulum (ER) is the site of synthesis and folding of secretory proteins. Perturbations of ER homeostasis affect protein folding and cause ER stress. I/R injury induces ER stress by modulating liver inflammatory immune response in experimental animal models [23] and by initiating cell death mechanisms. We have demonstrated in a previous study that ATP deficiency caused by hypoxia can promote escape of Ca^{2+} from ER into cytosol, triggering several downstream pathways that promote cell death [24]. One of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) [25]. Several agents that strongly affect ER function are identified to be strong inducers of CHOP/GADD153, such as thapsigargin which depletes ER calcium stores, tunicamycin which blocks protein glycosylation, and dithiothreitol which disrupts disulfide bond

formation [26], suggesting that induction of CHOP/GADD153 is highly responsive to ER stress. In our current study we have shown that HO-1 over-expression was associated with hepatoprotective and antiapoptotic properties by down-regulating the hepatic expression of CHOP protein. I/R induces the down-regulation of HO-1 causing ER stress, which is characterized by the increase of CHOP/GADD153 expression and apoptotic signaling. At the same time, the induction of HO-1 was associated with decreased CHOP/GADD153 hepatic expression and much reduced apoptotic injury. Bailly-Maitre et al. [27] have also reported that bi-1 (Bax Inhibitor-1), an evolutionarily conserved ER protein that suppresses cell death, provided endogenous protection of liver and kidney from ER stress and IR injury. Compared to wild-type, bi-1 knockout mice subjected to hepatic I/R injury exhibited increased necrotic and apoptotic markers together with greater increases in the expression of ER stress proteins C/EBP homologous protein and spliced XBP-1 protein [27]. Given that oxidative stress is important in IR injury and based on findings linking oxidative injury to ER stress we can conclude that *HO-1* provides endogenous protection of liver from ER stress in IR injury.

We and others have previously demonstrated the role of the transcriptional factors nuclear factor kappa B (NF- κ B) as an important mediator of the apoptotic hepatic I/R injury [28–30]. Activation of NF- κ B is tightly controlled by its endogenous inhibitor I κ B, which complexes NF- κ B in the cytoplasm. Phosphorylation and proteolytic degradation of I κ B, allows the release and nuclear translocation of NF- κ B, followed by transcription of many proinflammatory genes. As a proinflammatory transcription factor, NF- κ B may trigger upregulation of cytokines (e.g., TNF- α and IL-1) [28] and adhesion molecules (e.g., ICAM-1), thereby initiating inflammatory responses. In our current study, we hypothesized that inhibition of NF- κ B activation could contribute to the prevention of the apoptotic hepatic I/R injury. Our data lend support to this hypothesis, since HO-1 induction resulted in blocking NF- κ B activation and thus antiapoptotic signaling. As revealed by our results, the increased hepatic expression of NF- κ B in the I/R group was associated with many apoptotic hepatocyte cells that were identified by immunohistochemistry for caspase-3, but decreased hepatic expression of NF- κ B following HO-1 induction, significantly decreased the mean number of apoptotic hepatocytes in the I/R group. Moreover, we have also shown that the down-regulation of NF- κ B following HO-1 induction decreased the hepatic expression of the NF- κ B regulated genes (CIAP2, MCP-1 and IL-6) and was associated with greatly reduced I/R apoptotic injury. All of these genes have pro-inflammatory properties and have been implicated in hepatic inflammation in liver injury induced by toxins and ischemia reperfusion and in cell cultures [31–33]. HO-1 induction effectively prevented NF- κ B activation and the associated up-regulation of the pro-inflammatory genes CIAP2, MCP-1 and IL-6. This was associated with the reduced severity of hepatic apoptotic liver cell injury, indicating that recruitment of an inflammatory reaction and injury to liver cells in I/R is, at least partly, driven by NF- κ B. Our findings are also confirmed by others: Xu et al. [34] reported that NF- κ B decoy oligodeoxynucleotides protected against I/R injury in liver graft by suppressing NF- κ B activation and subsequent expression of proinflammatory mediators and Giakoustidis et al. [35] showed that the administration of (–)-epigallocatechin-3-gallate (green tea extracts) attenuates the expression of NF- κ B, c-Jun, and caspase-3 in a model of severe hepatic I/R. In addition, c-Jun terminal kinase-2 gene-deleted mice overexpress HO-1

and are protected from hepatic ischemia reperfusion injury [36]. The vascular HO system involves mechanisms that decrease apoptosis mediated by oxidative stress-inducing factors, including hypoxia inflammatory molecules, such as TNF [37–39].

In conclusion, HO-1 plays an important role in the regulation of hepatic IR injury. HO-1 overexpression is associated with reduced hepatic apoptotic IR injury. The possible mechanism by which HO-1 overexpression protected the liver from apoptotic injury following I/R seems to involve the pro-apoptotic NF- κ B pathway and the endoplasmic reticulum stress transcriptional induction CHOP. HO-1 may serve as a potential target for therapeutic intervention in hepatic I/R injury during liver transplantation, liver resection and trauma. The controversial role of HO-1 expression in human liver allografts of either cytoprotection or increased cytotoxicity ought to be investigated in more detail in the future.

References

1. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation*. 1992; 53:957–978. [PubMed: 1585489]
2. Jaeschke H. Preservation injury: mechanisms, prevention and consequences. *J Hepatol*. 1996; 25:774–780. [PubMed: 8938560]
3. Kojima Y, Suzuki S, Tsuchiya Y, Konno H, Baba S, Nakamura S. Regulation of pro-inflammatory and anti-inflammatory cytokine responses by kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia. *Transpl Int*. 2003; 16:231–240. [PubMed: 12730803]
4. Nathan C. Points of control in inflammation. *Nature*. 2002; 420:846–852.
5. Geuken E, Buis CI, Visser DS, et al. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. *Am J Transpl*. 2005; 5:1875.
6. Katori M, Busuttill RW, Kupiec-Weglinski JW. Heme oxygenase-1 system in organ transplantation. *Transplantation*. 2002; 74:905. [PubMed: 12394829]
7. Shi W, Haberland ME, Jien ML, Lusis AJ. Endothelial responses to oxidized lipoproteins determine genetic susceptibility to atherosclerosis in mice. *Circulation*. 2000; 102:75. [PubMed: 10880418]
8. Sass G, Barikbin R, Tiegs G. The multiple functions of heme oxygenase-1 in the liver. *Z Gastroenterol*. 2012; 50(1):34–40. [PubMed: 22222796]
9. Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest*. 1999; 104:1631. [PubMed: 10587527]
10. Coito AJ, Buelow R, Shen XD, Amersi F, Moore C, Volk HD, et al. Heme oxygenase-1 gene transfer inhibits inducible nitric oxide synthase expression and protects genetically fat Zucker rat livers from ischemia–reperfusion injury. *Transplantation*. 2002; 74:96. [PubMed: 12134106]
11. Kato H, Amersi F, Buelow R, Melinek J, Coito AJ, Ke B, et al. Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. *Am J Transpl*. 2001; 1:121.
12. Suttner DM, Dennery PA. Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. *FASEB J*. 1999; 13:1800–1809. [PubMed: 10506583]
13. Suttner DM, Sridhar K, Lee CS, Tomura T, Hansen TN, Dennery PA. Protective effects of transient HO-1 overexpression on susceptibility to oxygen toxicity in lung cells. *Am J Physiol*. 1999; 276:L443–L451. [PubMed: 10070108]
14. Selzner N, Selzner M, Jochum W, Clavien PA. Ischemic preconditioning protects the steatotic mouse liver against reperfusion injury: an ATP dependent mechanism. *J Hepatol*. 2003; 39:55–61. [PubMed: 12821044]
15. Nakao A, Kimizuka K, Stolz DB, Neto JS, Kaizu T, Choi AM, Uchiyama T, et al. Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. *Am J Pathol*. 2003; 163:1587–1598. [PubMed: 14507665]

16. Fondevila C, Shen XD, Tsuchiyashi S, Yamashita K, Csizmadia E, Lassman C, et al. Biliverdin therapy protects rat livers from ischemia and reperfusion injury. *Hepatology*. 2004; 40:1333–1341. [PubMed: 15565657]
17. Wang CF, Wang ZY, Tao SF, Ding J, Sun LJ, Li JY, Quan ZW. Preconditioning donor liver with Nododin perfusion lessens rat ischemia reperfusion injury via heme oxygenase-1 upregulation. *J Gastroenterol Hepatol*. 2012; 27(4):832–840. [PubMed: 22098251]
18. Zhang SC, Shi Q, Feng YN, Fang J. Tissue-protective effect of glutamine on hepatic ischemia-reperfusion injury via induction of heme oxygenase-1. *Pharmacology*. 2012; 91(1–2):59–68. [PubMed: 23172166]
19. Fang J, Qin H, Seki T, Nakamura H, Tsukigawa K, Shin T, Maeda H. Therapeutic potential of pegylated hemin for reactive oxygen species-related diseases via induction of heme oxygenase-1: results from a rat hepatic ischemia/reperfusion injury model. *J Pharmacol Exp Ther*. 2011; 339(3):779–789. [PubMed: 21890508]
20. Tsuchihashi S, Zhai Y, Bo Q, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 mediated cytoprotection against liver ischemia and reperfusion injury: inhibition of type-1 interferon signaling. *Transplantation*. 2007; 83(12):1628–1634. [PubMed: 17589347]
21. Schmidt R. Cobalt protoporphyrin as a potential therapeutic agent? *FASEB J*. 2007; 21(11):2639–2640. [PubMed: 17766326]
22. Froh M, Conzelmann L, Walbrun P, Netter S, Wiest R, Wheeler MD, Lehnert M, Uesugi T, Scholmerich J, Thurman RG. Heme oxygenase-1 overexpression increases liver injury after bile duct ligation in rats. *World J Gastroenterol*. 2007; 13:3478–3486. [PubMed: 17659695]
23. Liu J, Ren F, Cheng Q, Bai L, Shen X, Gao F, et al. Endoplasmic reticulum stress modulates liver inflammatory immune response in the pathogenesis of liver ischemia and reperfusion injury. *Transplantation*. 2012; 94(3):211–217. [PubMed: 22790388]
24. Ben-Ari Z, Pappo O, Yitzhaki S, Cheporko Y, Shainberg A, Zinman T, et al. Uridine-5'-triphosphate protects against hepatic-ischemic/reperfusion injury in mice. *Transplantation*. 2009; 87(8):1155–1162. [PubMed: 19384161]
25. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, et al. Signals from the stressed endoplasmic reticulum induce C/EBP homologous protein (CHOP/GADD153). *Mol Cell Biol*. 1996; 16:4273–4280. [PubMed: 8754828]
26. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ*. 2004; 11:381–389. [PubMed: 14685163]
27. Bailly-Maitre B, Fondevila C, Kaldas F, Droin N, Luciano F, Ricci JE, et al. Cytoprotective gene bi-1 is required for intrinsic protection from endoplasmic reticulum stress and ischemia-reperfusion injury. *Proc Natl Acad Sci USA*. 2006; 103(8):2809–2814. [PubMed: 16478805]
28. Hochhauser E, Pappo O, Ribakovsky E, Ravid A, Kurtzwalld E, Cheporko Y, et al. Recombinant human erythropoietin attenuates hepatic injury induced by ischemia/reperfusion in an isolated mouse liver model. *Apoptosis*. 2008; 13(1):77–86. [PubMed: 18085440]
29. Ben-Ari Z, Avlas O, Fallach R, Schmilovitz-Weiss H, Chepurko Y, Pappo O, Hochhauser E. Ischemia and reperfusion liver injury is reduced in the absence of Toll-like receptor 4. *Cell Physiol Biochem*. 2012; 30(2):489–498. [PubMed: 22797797]
30. Arii S, Teramoto K, Kawamura T. Current progress in the understanding of and therapeutic strategies for ischemia and reperfusion injury of the liver. *J Hepatobiliary Pancreat Surg*. 2003; 10(3):189–194. [PubMed: 14605974]
31. Kaplowitz N, Tsukamoto H. Oxidative stress and liver damage. *Prog Liver Dis*. 1996; 14:131–159. [PubMed: 9055577]
32. Teoh NC, Farrell GC. Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection. *J Gastroenterol Hepatol*. 2003; 18:891–902. [PubMed: 12859717]
33. Muraoka K, Shimizu K, Sun X. Hypoxia, but not reoxygenation, induces IL-6 gene expression through NF- κ B activation. *Transplantation*. 1997; 63:466–470. [PubMed: 9039941]
34. Xu MQ, Shuai XR, Yan ML, Zhang MM, Yan LN. Nuclear factor-kappaB decoy oligodeoxynucleotides attenuates ischemia/reperfusion injury in rat liver graft. *World J Gastroenterol*. 2005; 11(44):6960–6967. [PubMed: 16437600]

35. Giakoustidis DE, Giakoustidis AE, Iliadis S, Koliakou K, Antoniadis N, Kontos N, et al. Attenuation of liver ischemia/reperfusion induced apoptosis by epigallocatechin-3-gallate via down-regulation of NF-kappaB and c-Jun expression. *J Surg Res.* 2010; 159(2):720–728. [PubMed: 19394642]
36. Devey L, Mohr E, Bellamy C, Simpson K, Henderson N, Harrison EM, et al. c-Jun terminal kinase-2 gene deleted mice overexpress hemoxygenase-1 and are protected from hepatic ischemia reperfusion injury. *Transplantation.* 2009; 88(3):308–316. [PubMed: 19667931]
37. Kushida T, Li Volti G, Quan S, Goodman A, Abraham NG. Role of human heme oxygenase-1 in attenuating TNF-alpha-mediated inflammation injury in endothelial cells. *J Cell Biochem.* 2002; 87:377–385. [PubMed: 12397597]
38. Petrache I, Otterbein LE, Alam J, Wiegand GW, Choi AM. Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *Am J Physiol Lung Cell Mol Physiol.* 2000; 278:L312–L319. [PubMed: 10666115]
39. Abraham NG, Kappas A. Heme oxygenase and the cardiovascular-renal system. *Free Radic Biol Med.* 2005; 39(1):1–25. [PubMed: 15925276]

Fig. 1.

A statistically significant difference was noted in mean serum ALT/AST/LDH levels following the induction of I/R, compared with sham operated mice. In CoPP pretreated mice following the induction of I/R, the increase in serum liver enzymes level was significantly less compared with non-treated mice

Fig. 2.

Pre-treatment with CoPP significantly increased hepatic HO-1 expression ($p = 0.032$) (a).

ER stress pathway was up-regulated following I/R as demonstrated by the significant increase in the hepatic expression of the CHOP protein ($p < 0.05$). Pre-treatment with CoPP decreased CHOP protein expression $p < 0.05$ (b)

Fig. 3.

a IκB (inhibitory proteins of NF-κB) hepatic expression was significantly lower following I/R ($p = 0.05$) compared with sham operated mice. **b** Liver pretreated with CoPP had significantly higher hepatic expression of IκB ($p = 0.01$)

Fig. 4.

NF- κ B pathway was stimulated by the induction of hepatic ischemia, as is demonstrated by the increase in the hepatic expression of the three targeted NF- κ B's genes: CIAP2, MCP-1 and IL-6. CoPP pre-treatment significantly decreased **a** CIAP2 ($p = 0.00001$), **b** MCP-1 ($p = 0.0001$), and **c** IL-6 hepatic ($p = 0.0005$) expression

Fig. 5. Histologic examination of sections from liver tissue stained with hematoxylin-eosin revealed damage in the I/R untreated livers manifested by many acidophilic bodies and groups of necrotic hepatocytes (*black line* delineating area of coagulative necrosis) (**a**). Following the pre-administration of CoPP only occasional apoptotic bodies were found (*arrows*) (**b**)

Fig. 6.

Sections from I/R livers were highly positively stained for the activated form of caspase-3. Scattered positive nuclei and cytoplasmic staining were noted in hepatocytes as well as sinusoidal cells (a). In the I/R pretreated-CoPP group, only rare cells stained for caspase-3 activity (b). I/R livers showed a significant increase in the mean number of apoptotic cells (positively stained for caspase-3) ($p = 0.013$) (c). Sections from I/R livers were highly positively stained for Ki67 as a proliferation index (d). In livers pre treated with CoPP, fewer hepatocytes stained for Ki67 (e). Although I/R livers showed an increase in the mean number of proliferating cells (positively stained for Ki67) compared with livers pre treated with CoPP ($p = \text{NS}$)