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BASIC RESEARCH

Heme oxygenase-1 overexpression increases liver injury after bile duct ligation in rats

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CONCLUSION: Hepatic fibrosis due to BDL is not reduced by the HO-1 inducer CoPP. In contrast, HO-1 overexpression increases liver injury in rats under conditions of experimental chronic cholestasis.

Key words: Heme oxygenase-1; Bile duct ligation; Chronic cholestasis; Liver fibrosis; Serum alanine transaminase; Transforming growth factor-β; Tumor necrosis factor- Ⅰα; Type Ⅰ collagen

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Abstract

AIM: To investigate the effects of heme oxygenase-1 (HO-1) against oxidant-induced injury caused by bile duct ligation (BDL).

METHODS: Either cobalt protoporphyrin (CoPP), a HO-1 inducer, or saline were injected intraperitoneally in male SD-rats. Three days later, BDL or sham-operations were performed. Rats were sacrificed 3 wk after BDL and livers were harvested for histology. Fibrosis was evaluated by sirius red staining and image analysis. Alpha-smooth muscular actin, which indicates activation of stellate cells, was detected by immunohistochemical staining, and cytokine and collagen- Ⅰα (Col- Ⅰα) mRNA expression was detected using RNase protection assays.

RESULTS: Serum alanine transaminase increased 8-fold above normal levels one day after BDL. Surprisingly, enzyme release was not reduced in rats receiving CoPP. Liver fibrosis was evaluated 3 wk after BDL and the sirius red-positive area was found to be increased to about 7.8%. However, in CoPP pretreated rats sirius red-positive areas were increased to about 11.7% after BDL. Collagen- Ⅰα and TGF-β mRNA increased significantly by BDL. Again, this effect was increased by HO-1 overexpression.

INTRODUCTION

Chronic cholestatic liver diseases are one of the leading indications for liver transplantation in children and adults[1,2]. Various drugs, total parenteral nutrition, chronic liver transplant rejection, and graft-*vs*-host disease can also produce chronic cholestasis[3-5]. This leads to liver injury and will finally progress to portal fibrosis, cirrhosis and end-stage liver disease necessitating liver transplantation. Ursodeoxycholic acid is currently the most promising therapy for chronic cholestatic liver diseases[6]; however, it cannot prevent fibrosis[7]. How cholestasis induces liver injury and fibrosis remains unclear. One possible mechanism is that accumulation of hydrophobic bile acids causes oxidative stress in the liver[8]. Previous studies showed that hepatic mitochondria generate reactive oxygen species when isolated hepatocytes are exposed to hydrophobic bile acids[8,9]. This mitochondrial free radical production may be an important mechanism of cholestatic liver injury. However oxypurinol, an inhibitor of xanthine oxidase, decreases hepatocelluar injury without decreasing lipid peroxidation in mitochondria and microsomes, suggesting that mitochondrial oxidative stress does not play an important role in cholestatic liver injury[10]. Therefore, the major source of free radicals remains unclear. In biliary obstructed rats, hepatic glutathione levels and activities of

antioxidant enzymes decrease, whereas 4-hydroxynonenal and malondialdehyde levels increase[9,11,12]. These findings also support the involvement of oxidative stress in cholestatic stress.

Heme oxygenases (HOs) are ubiquitous enzymes that catalyze the initial and rate-limiting steps in heme catabolism[13,14] yielding equimolar amounts of biliverdin, carbon monoxide, and free divalent iron[15-18]. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase, and the free iron is used in intracellular metabolism or sequestered into ferritin. The HO-system consists of three isoforms (HO-1, HO-2, and HO-3) as the products of separate genes[14,19,20]. HO-1, also known as heat shock protein 32, is an inducible isoform activated by most oxidative stress inducers and cytokines[17]. Constitutively expressed HO-2 is unresponsive to any of the known HO-1 inducers[17,20,21]. The HO-3 isozyme has 90% homology in the amino acid sequence with HO-2 and is nearly devoid of catalytic activity, serving mainly as heme-sensing/binding protein[19]. The mechanism by which HO-1 confers protection against oxidative stress has not yet been fully understood. It has been suggested that HO-1 possesses antioxidant activity deriving from the elimination of prooxidant heme and from the biological activities of its reaction products: biliverdin, bilirubin and iron[22-24]. Both biliverdin and bilirubin possess antioxidant properties[25], whereas iron released during heme catabolism can stimulate ferritin synthesis[26].

This study was designed to test whether an increased HO-1 expression by the HO-1 inducer cobalt protoporphyrin (CoPP) would minimize oxidative stress caused by experimental cholestasis and thereby decrease liver fibrosis.

MATERIALS AND METHODS

Animal husbandry and treatment

Adult male Sprague-Dawley rats (200-250 g) were housed four to a cage in a facility approved by the Association for the Accreditation and Assessment of Laboratory Animal Care International. Three days before surgery, rats were randomly assigned to two experimental groups and received either 0.5 mL of lactated Ringer's solution or CoPP (Frontier Scientific Inc., Logan, UT, USA) at a concentration of 5 mg/kg diluted in 0.5 mL of lactated Ringer's solution intraperitoneally. After surgery, rats were allowed free access to standard laboratory chow and tap water. All animals received humane care in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Bile duct ligation

Rats underwent bile duct ligation (BDL) and transection or sham operation under ether anesthesia, as described elsewhere[27].

Clinical chemistry and histology

Blood samples were collected from the tail vein at times indicated in Figure 1. Serum enzymes were measured using analytic kits from Sigma (St. Louis, MO, USA). For histology, the liver was rinsed with normal saline, followed by slow infusion of 5 mL 10% buffered formaldehyde. After 48 h in fixative, paraffin sections were prepared and stained with hematoxylin-eosin or 0.1% Sirius red and Fast green FCF (Sigma)[28]. Areas in sections stained for collagens by Sirius red were quantified by image analysis using an Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, PA, USA) incorporating an Axioskop 50 microscope. Detection thresholds were set for the red color of stained collagen based on an intensely labeled point and a default color threshold range that was assigned. The degree of labeling in each section was determined from the area within the color range divided by the total cellular area.

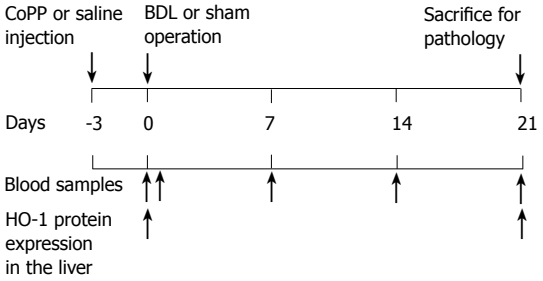


Figure 1 Experimental design. Either CoPP or saline were injected intraperitoneally in male rats. Three days later, bile duct ligation (BDL) or sham operation was performed. Some animals were killed 3 d after CoPP injection or 3 wk after BDL to assess HO-1 protein expression in liver tissue. Blood samples were taken just before and at 0, 1, 7, 14, and 21 d after BDL to assess for alanine transaminase (ALT), aspartate aminotransaminase (AST), serum alkaline phosphatase (ALP), and bilirubin. Liver sections were taken from rats 3 wk after BDL to evaluate pathological changes.

Immunohistochemistry for α-smooth muscle actin

Immunohistochemistry for α-smooth muscle actin (α-SMA) was performed on 6 μm sections of formalin-fixed, paraffin-embedded liver-sections according to the supplier's instructions. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in phosphate-buffered saline with 1% Tween 20. Staining was performed with monoclonal primary antibodies (DAKO, Carpinteria, CA, USA) against α-SMA and followed by peroxidase labeling using an EnVision kit (DAKO). The primary antibody was diluted 1:200 with 1% bovine albumin (Sigma) in phosphate-buffered saline. After immunohistochemistry, samples were lightly counterstained with hematoxylin.

Protein isolation and Western blotting

Whole liver tissue was homogenized in lysis buffer with protease inhibitors at 4℃. The supernatant was collected and used as the source of protein for Western blots, as described elsewhere[29,30]. Protein was analyzed using a 1:1000 dilution of anti-HO-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a 1:5000 dilution of horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

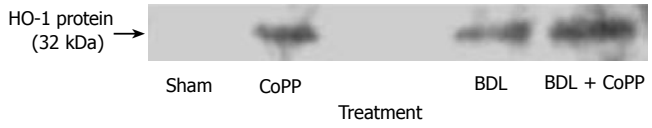


Figure 2 Immunodetection of HO-1 in liver tissues 3 wk after BDL or sham operation. The expression of HO-1 using an anti-HO-1 polyclonal antibody was determined by Western blot. Data are representative of 4 to 6 animals per group.

AP-1 and NF-κB determination using EMSA

Measurement of AP-1 and NF-κB by EMSA was performed, as described in detail elsewhere[29-31].

RNase protection assay for collagen-Iα, TNF-α, and transforming growth factor-β mRNA

Total RNA was isolated from liver tissue using RNA STAT 60 (Tel-Test, Friendswood, TX, USA). The RNA was extracted and purified following standard phenol/chloroform extraction and ethanol precipitation. RNase protection assays were performed using the RiboQuant multiprobe assay system (Pharmingen, San Diego, CA, USA) or an individual probe[29,30]. Gels were visualized by autoradiography[32]. Bands corresponding to the protected labeled fragment were quantitated by scanning densitometry software (ImageQuant 5.0), and where statistical data are given, they were normalized to the level of GAPDH as the housekeeping gene.

Statistical analysis

Data are presented as mean ± SD. ANOVA and the Student-Newman-Keuls post hoc tests were used to determine statistical significance between treatment groups. A P value less than 0.05 was selected before the study as the level of significance.

RESULTS

Expression of HO-1 in the liver

Experimental design is depicted in Figure 1. To confirm that CoPP pretreatment induced HO-1 overexpression, HO1 protein expression in whole livers was studied by immunoblotting analysis. As expected, HO-1 protein was identified in saline pretreated rats 3 wk after BDL (Figure 2), confirming previously published data[33]. Almost no HO-1 protein signal was detected in sham-operated rats pretreated with saline (Figure 2). In contrast, CoPP pretreatment produced a strong HO-1 protein signal in sham-operated and bile duct-ligated rats, indicating an increased HO-1 expression thru CoPP (Figure 2).

Serum enzymes and histology

In untreated rats that were fed a standard chow diet, serum alanine aminotransaminase (ALT) levels averaged 70 U/L (Figure 3) and were not significantly altered by CoPP pretreatment and sham operation. After BDL, ALT increased to 386 U/L after 1 d (Figure 3), indicating liver injury. ALT levels decreased afterward and reached a new steady-state level of about 80 U/L after 1 wk (Figure 3).

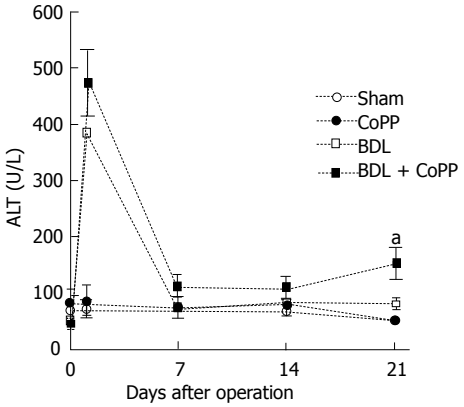


Figure 3 Time course of alanine transaminase (ALT) release after BDL or sham operation. BDL or sham operation were performed 3 d after CoPP or saline injection, and blood samples were collected at time points indicated in the figure. Serum ALT activity was determined using commercial kits from Sigma. Values are means ± SD (n = 5-6 in each group). $^aP < 0.05$ *vs* bile duct-ligated group receiving saline.

When rats were pretreated with the HO-1 inducer CoPP, ALT levels increased to 472 U/L one day after BDL (Figure 3). ALT levels decreased also afterward and reached a steady-state level of about 110 U/L after 1 wk before they significantly increased to 148 U/L after 3 wk (Figure 3).

Normal liver architecture was observed after saline or CoPP pretreatment and sham operation (Figure 4A). Three weeks after BDL characteristic morphology of obstructive biliary cirrhosis, including bile duct proliferation, diffuse micronodules, focal necrosis and white blood cell infiltration were observed in livers from rats receiving saline pretreatment (Figure 4B). These pathological changes were increased by rats receiving CoPP pretreatment (Figure 4C).

Fibrogenesis in the liver

To evaluate the effects of HO-1 overexpression on liver fibrosis after BDL, liver sections were stained with Sirius red for collagen. Independently of pretreatment (saline or CoPP), no fibrosis was observed in livers from sham-operated rats (Figure 5A). In rats receiving normal saline, hepatic fibrosis developed within 2 wk after BDL (data not shown) and was severe after 3 wk (Figure 5B). When rats pretreated with CoPP were subjected to BDL, histology revealed increased fibrosis (Figure 5C). Image analysis revealed that Sirius red stained an area of about 0.7% of liver sections from sham-operated rats, independently of pretreatment (Figure 6). Sirius red staining increased to 7.8% after 3 wk following BDL (Figure 6). Pretreatment with CoPP raised this increase in Sirius red staining significantly after BDL to 11.7% of the measured areas (Figure 6).

Collagen gene expression was evaluated by RNase protection assay for collagen-Iα mRNA and was barely detectable in livers from sham-operated rats, independently of pretreatment (Figure 7A). Three weeks after BDL, however, collagen-Iα mRNA expression increased to about 2000% compared with sham-operated groups

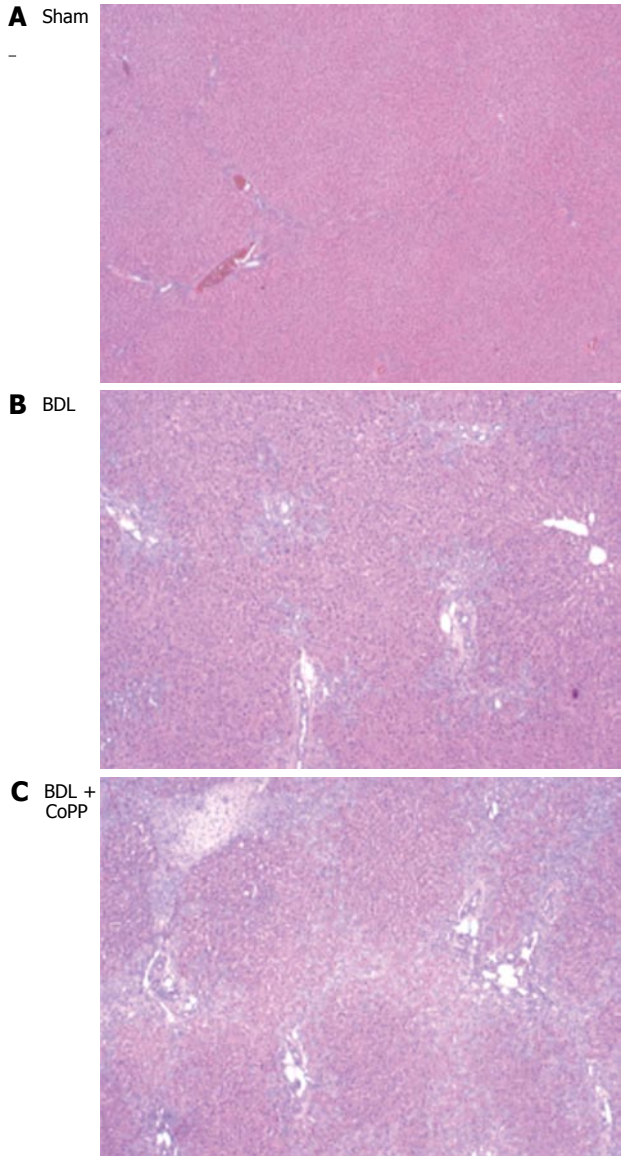


Figure 4 Effects of HO-1 overexpression on hepatic pathology changes caused by BDL. Rats were pretreated with CoPP or saline. Livers were harvested 3 wk after BDL, and hematoxylin and eosin staining was performed to evaluate pathological changes. Shown are representative images (x 40). **A**: sham operation with saline pretreatment; **B**: BDL with saline pretreatment; **C**: BDL with CoPP pretreatment.

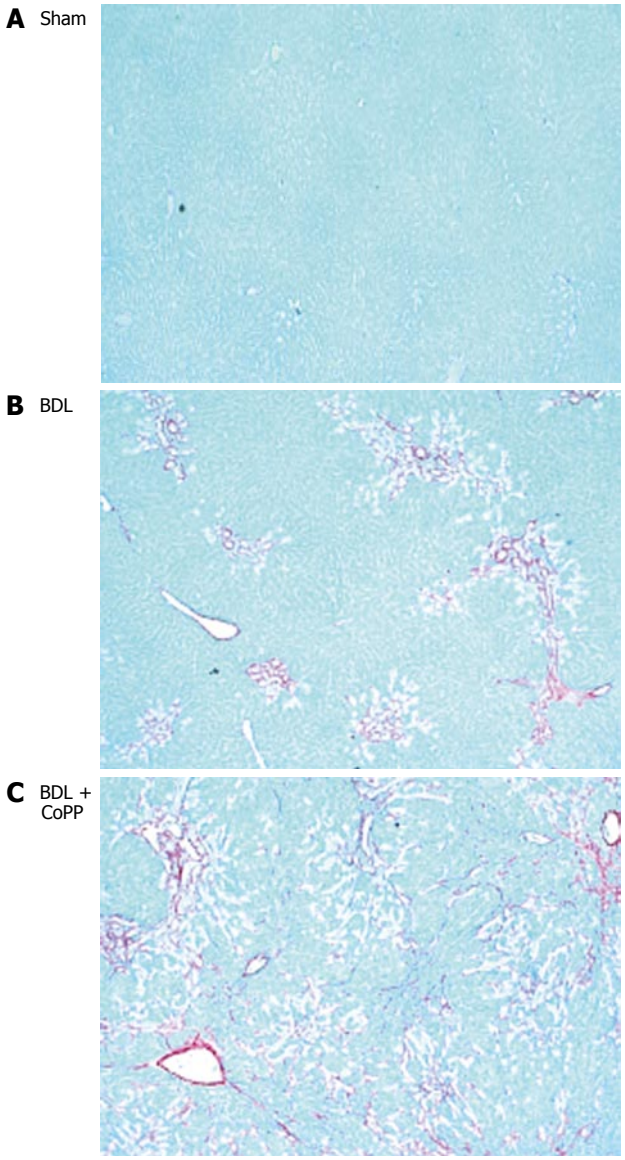


Figure 5 Effects of HO-1 overexpression on hepatic fibrosis after BDL. Rats were pretreated with CoPP or saline. Livers were harvested 3 wk after BDL, and Sirius red staining of sections was performed. Shown are representative images (x 40). **A**: sham operation with saline pretreatment; **B**: BDL with saline pretreatment; **C**: BDL with CoPP pretreatment.

(Figure 7A and B). Pretreatment with CoPP worsen the increase in collagen- I α mRNA expression to about 3000% (Figure 7A and B).

Immunohistochemical staining for α-SMA

Activated stellate cells are the major source of matrix proteins in diseased liver[34]. Accordingly, we evaluated α-SMA, an indicator of stellate cell activation, by immunohistochemical staining after BDL. In livers from sham-operated rats (independently of pretreatment), small amounts of α-SMA were detected in the smooth muscle and endothelium of blood vessels (Figure 8A). After BDL, α-SMA increased markedly in perisinusoidal cells consistent with stellate cell activation in cholestatic livers (Figure 8B). Again, HO-1 overexpression by CoPP treatment before BDL significantly increased the amount of α-SMA-positive areas, indicating an increase of stellate cell activation (Figure 8C).

Transcription factors and cytokine expression in the liver

NF-κB/DNA complexes were barely detectable in livers from sham-operated rats (independently of pretreatment) but increased around 3.5-fold after BDL, consistent with our previous studies[29,30]. CoPP pretreatment did not suppress this activation (data not shown). A small amount of AP-1/DNA complex was detected in livers from sham-operated rats. Three weeks after BDL, however, AP-1/DNA complex levels increased around 2-fold, indicating activation of AP-1[29,30]. Again, HO-1 overexpression thru CoPP pretreatment did not suppress this activation (data not shown).

Minimal amounts of TGF-β1 mRNA were detected in

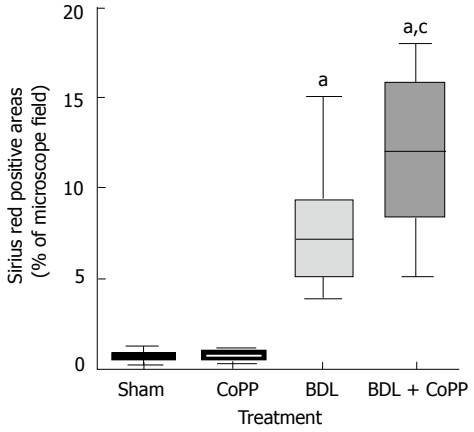
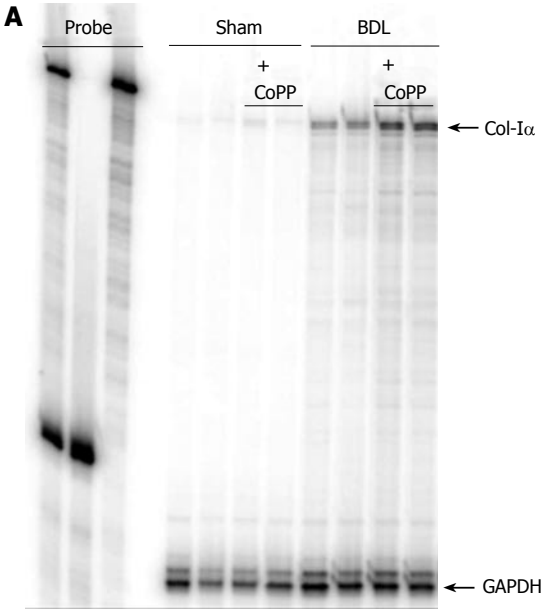


Figure 6 Quantification of hepatic fibrosis after BDL. Liver sections were stained with Sirius red, as described in Figure 4. Five microscope fields (x 40 objective) were selected randomly, and fractional areas that were Sirius red positive were determined by image analysis. Values are means ± SD (n = 5-6 in each group). $^{a}P < 0.05$ *vs* sham-operated groups, $^{c}P < 0.05$ *vs* bile duct-ligated group receiving saline.



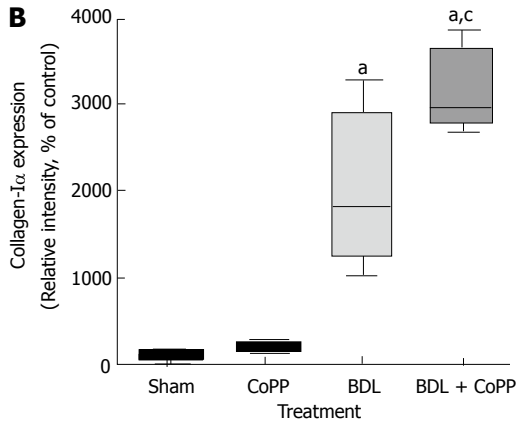


Figure 7 Effects of cholestasis and HO-1 overexpression on collagen-Iα mRNA expression. Livers were harvested 3 wk after BDL, perfused with normal saline, and frozen in liquid nitrogen. RNase protection assays were performed using an individual probe. Values are means ± SD (n = 4 in each group). Specific bands were quantitated by scanning densitometry and normalized to the signal of GAPDH. **A**: representative autoradiogram of collagen-Iα mRNA expression; **B**: densitometric analysis of collagen-Iα mRNA expression. $^{a}P < 0.05$ *vs* sham-operated groups, $^{c}P < 0.05$ vs bile duct-ligated group receiving saline.

livers from rats after sham operation. After BDL, TGF-β1 mRNA expression significantly increased by around threefold (Figure 9A and B). CoPP pretreatment did not suppress these increases in TGF-β expression. In contrast, TGF-β1 mRNA expression was even more increased in rats receiving CoPP before BDL (Figure 9B). TNF-α mRNA was barely detectable in livers from sham-operated rats (Figure 9A and C) but was increased around twofold after BDL. HO-1 overexpression thru CoPP pretreatment did not suppress this increase in TNF-α mRNA expression (Figure 9C).

DISCUSSION

Cholestasis leads to liver injury and will finally progress to fibrosis necessitating liver transplantation[1,35]. Current therapy for cholestasis, such as ursodeoxycholic acid, does not prevent fibrosis[7]. Therefore, new strategies to prevent cholestasis-induced liver injury and fibrosis are needed. Previous work suggest that oxidative stress occurs during cholestasis and probably plays a role in cholestasis-induced liver injury[8,9,36]. Accordingly, antioxidant therapy represents a potential strategy to prevent liver injury and fibrosis.

There are several reports suggesting protective effects of HO-1 against oxidant-induced injury and the induction of HO-1 as an adaptive response against oxidative damage. In detail, HO-1 overexpression can protect steatotic rat liver from ischemia reperfusion injury[37], prolong cardiac allograft[38], prevent arteriosclerosis and cardiac fibrosis in cardiac transplantation[39], be neuroprotective[40], attenuate chronic rejection of renal transplantation[41], and protect animals from endotoxic shock[42]. Based on this data, we assumed that HO-1 overexpression leading to less oxidative damage reduced cholestasis-induced liver injury and fibrosis. Accordingly, we assessed the effect of CoPP, a HO-1 inducer, leading to a stable HO-1 overexpression[37] in a rat model of cholestasis induced by BDL.

CoPP-induced HO-1 overexpression increases liver injury and hepatic fibrosis due to BDL

In confirmation of previous work from our and several other laboratories[11,12,27,29,30], BDL caused hepatic ALT release (Figure 3), cell necrosis (Figure 4) and fibrosis (Figures 5-7) as expected. Surprisingly, enzyme release and histopathological changes were not reduced in rats receiving CoPP. Moreover, a significant increase in ALT levels (Figure 3) and pathology (Figure 4) could be demonstrated 3 wk after BDL for CoPP pretreated vs bile duct-ligated rats receiving saline. Furthermore, HO-1 overexpression significantly deteriorated fibrosis caused by cholestasis, as shown by Sirius red staining and collagen-Iα mRNA expression (Figures 5-7).

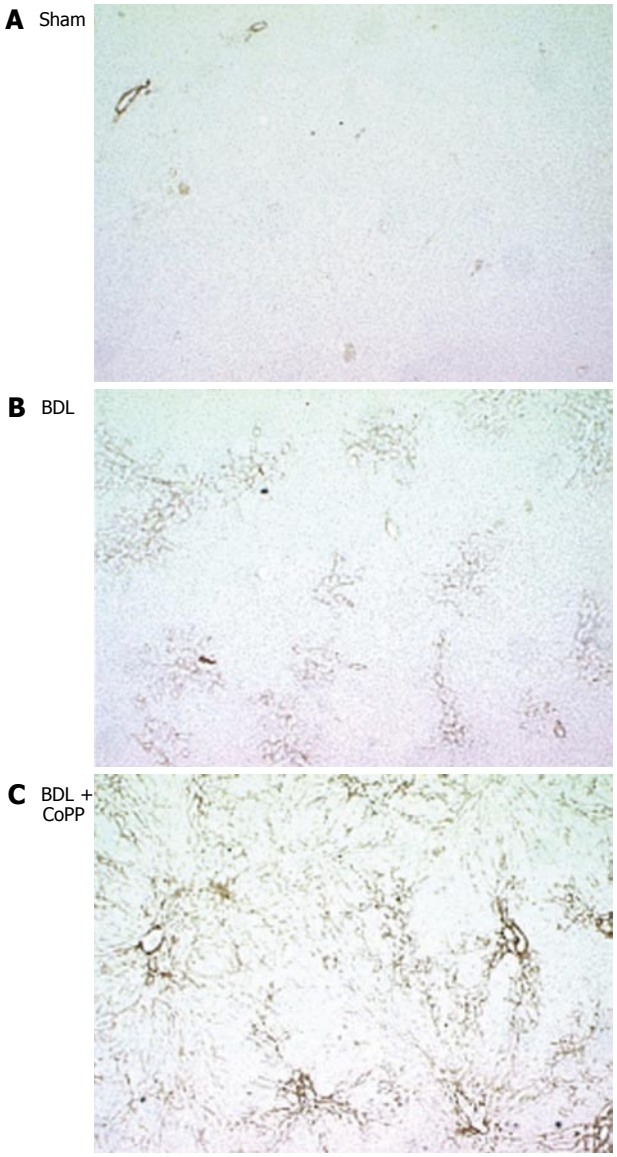


Figure 8 Effects of HO-1 overexpression on α-smooth muscle actin (α-SMA) formation after BDL. Livers were harvested 3 wk after BDL, and sections were stained immunohistochemically to assess α-SMA. Shown are representative images (x 40). **A**: sham operation with saline pretreatment; **B**: BDL with saline pretreatment; **C**: BDL with CoPP pretreatment.

How does HO-1 overexpression increase cholestasis-induced liver fibrosis?

Beside protective effects of upregulation of the HO-1 pathway there is increasing evidence that HO-1 overexpression and activity is not exclusively cytoprotective. Recent studies indicate that the protection might be restricted to a narrow threshold of HO-1 overexpression[43-46]. High levels of HO-1 may even sensitize the cell to oxidative stress, as demonstrated in fibroblast cell cultures, where low HO-1 induction was shown to be cytoprotective, whereas excessive activation of HO-1 resulted in the accumulation of free divalent iron and increased oxidative injury[43]. Moreover, increased HO-1 expression was shown in human NASH patients, reflecting the severity of the disease and the progression of liver failure[44]. Furthermore, a dual role for HO-1

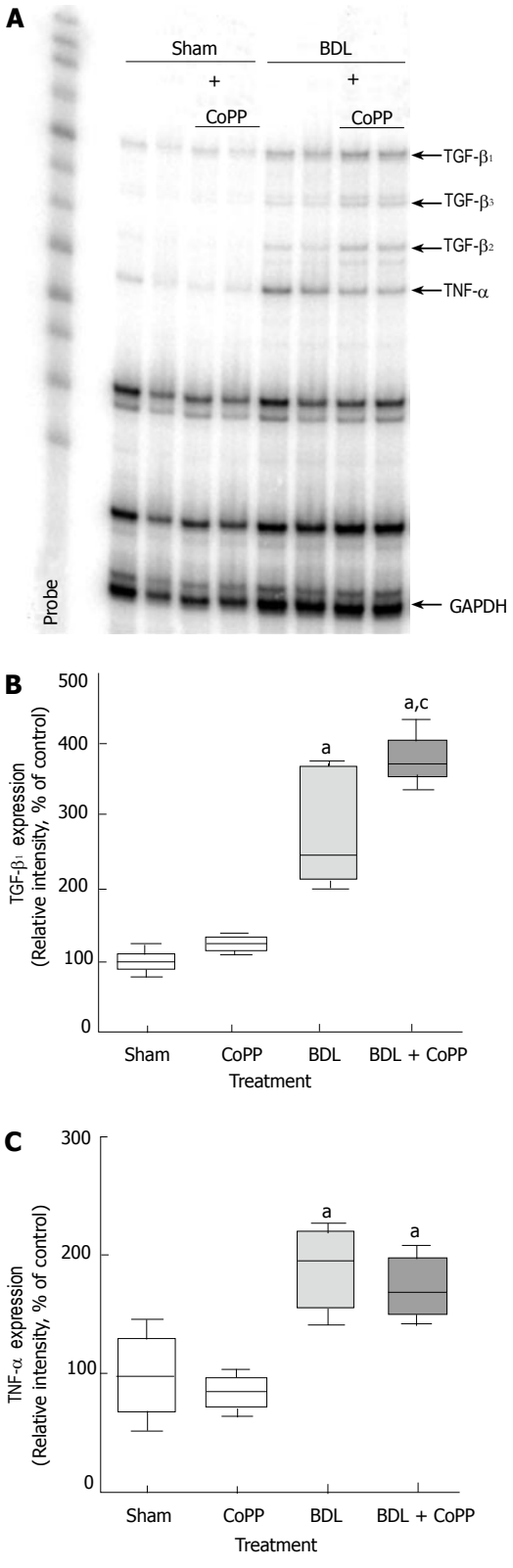


Figure 9 Effects of cholestasis and HO-1 overexpression on production of TGF-β and TNFα. Livers were harvested 3 wk after bile duct ligation and snap-frozen in liquid nitrogen. TGF-β and TNFα mRNAs were assessed by RNase protection assay using cytokine template sets, as described in MATERIAL AND METHODS. Values are means ± SD (n = 4 in each group). Specific bands were quantitated by scanning densitometry and normalized to the signal of GAPDH. **A**: representative autoradiogram of TGF-β and TNFα mRNA expression; **B**: densitometric analysis of TGF-β1 mRNA expression. [a]P < 0.05 *vs* sham-operated groups, [b]P < 0.05 *vs* bile duct-ligated group receiving saline; **C**: densitometric analysis of TNFα mRNA expression. [a]P < 0.05 *vs* sham-operated groups.

expression in human liver transplants, with either cytoprotection or increased cytotoxicity, depending on the initial level of overexpression could be demonstrated[45]. These results point towards the potentially different effects of HO-1, which may be neither exclusively cytoprotective nor exclusively cytotoxic.

Normally, factors of an increased HO-1 expression in a model of experimental cirrhosis may include NO and oxidative stress[9,47,48], as well as especially increased cytokine and endotoxin levels[49]. It is known, that HO-1 expression and activity is greatly enhanced in cirrhotic rat livers[33,50,51], suggesting an important role in cirrhosis development. In BDL livers, HO-1 was induced especially in hepatocytes but it was also enhanced in Kupffer cells[33,51]. Accordingly, the additionally exogenous induction of HO-1 in a model of hepatic fibrosis due to BDL could further increase an already elevated HO-1 expression, resulting in potentially detrimental effects instead of cytoprotection.

Oxidant-induced cell death may also cause exaggerated repair leading to fibrosis. Therefore, non-prevention of cell death may increase subsequent fibrosis. There are numerous reports indicating anti-apoptotic abilities of HO-1[39,52-54] by suppressing one or several signaling pathways of the pro-apoptotic stimuli. In contrast to these findings, we found that HO-1 overexpression increased ALT release and necrosis of parenchymal cells (Figures 3 and 4), as well as maximizing fibrosis (Figures 5-7) in this model of chronic cholestasis due to BDL.

Activation of hepatic stellate cells appears to be a critical step in hepatic fibrogenesis that is regulated by several factors, including cytokines and oxidative stress[34,55,56]. Previous studies could demonstrate HO-1 overexpression as a negative regulator in the control of fibrogenic activities of hepatic stellate cells[57,58]. Therefore it was hypothesized that HO-1 overexpression may prevent fibrosis by inhibiting oxidant-dependent activation and proliferation of stellate cells. In contrast to this hypothesis, α-SMA, a marker of stellate cell activation, dramatically increased after BDL (Figure 8), an effect that was significantly worsen by CoPP pretreatment. Activated stellate cells also produce TGF-β1, which causes further proliferation of stellate cells and strongly stimulates production of collagens[59,60]. Oxidative stress not only increases production of TGF-β1 but also activates latent TGF-β1[61,62]. Additionally, H_2O_2 activates transcriptional factors such as AP-1 and NF-κB[63] that are involved in stellate cell activation and synthesis of TGF-β1[55,63]. Consistent with these earlier findings, activation of AP-1, NF-κB, and increases in TGF-β1 mRNA were all observed in the present study after BDL (Figure 9), supporting the hypothesis that oxidative stress stimulates TGF-β1 production, leading to collagen gene and protein expression (Figure 7). But in contrast, CoPP pretreatment before BDL did not blocked activation of transcription factors and cytokine production. Furthermore, HO-1 overexpression induced TGF-β1 (Figure 9B) expression after BDL even more, leading to a significant increase in collagen- I α mRNA and α-SMA protein expression (Figures 7 and 8). These findings also point to the potential pro-oxidant consequences of an increased HO-1 expression and activity.

Another possibility is that elevated HO-1 overexpression aggravates the activation of Kupffer cells, thus increasing formation of inflammatory and fibrogenic mediators. Previous reports identified Kupffer cells as the main source of HO-1 expression in human livers[45,64] in contrast to controversial data from rat livers, where considerable expressions of HO-1 has also been found in hepatocytes[51,65]. Besides the large body of evidence demonstrating cytoprotective effects of HO-1 upregulation in transplant models in animals[37,66,67], Geuken *et al*[45] suggested that high HO-1 overexpression in Kupffer cells in human liver allografts may contribute to an increased cell injury. In general, the role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride[68]. By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis[69]. Kupffer cells release many mediators that activate stellate cells, including TNF-α, TGF-β, human growth factor, PDGF, and reactive oxygen species[60,70]. TNF-α production and NF-κB activation increase during cholestasis[71,72]. Activation of NF-κB, probably due to oxidative stress, could lead to expression of TNF-α. An inhibition of proinflammatory cytokine production as a result of HO-1 overexpression, as reported for several other liver injury models[73-75], could not be demonstrated in the present study. In addition, we have shown that activation of NF-κB and expression of TNF-α and TGF-β were increased by cholestasis and that these effects were not blocked by HO-1 overexpression (Figure 9). In contrast, TGF-β1 mRNA expression was significantly increased in rats receiving CoPP before BDL (Figure 9B) demonstrating an additional accumulation of one key mediator in fibrogenesis. However, in a previous study we could demonstrate that suppression of Kupffer cell function with $GdCl_3$, a treatment that blocks carbon tetrachloride-induced fibrosis[68], did not attenuate fibrosis caused by cholestasis[29]. This might indicate that Kupffer cells likely do not play a prominent role in cholestasis-induced fibrosis *in vivo* and that HO-1 up-regulation does not work exclusively by increasing Kupffer cell activation.

In conclusion, we could demonstrate that hepatic fibrosis due to BDL is not reduced by HO-1 overexpression. In contrast, CoPP dependent HO-1 overexpression increases liver injury in rats under conditions of experimental chronic cholestasis. In conjunction with previous reports, these findings indicate that increased HO-1 activity is not exclusively protective. Rather, the HO-1 induced cytoprotection might be restricted to a narrow threshold of overexpression in the injury model used.

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