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Inhibition of Heme Oxygenase-1 Protects Against Tissue Injury in Carbon Tetrachloride Exposed Livers

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Background/Aims. **During the metabolism of the hepatotoxin carbon tetrachloride (CCl_4) by cytochrome P450, heme, and free radicals are released. Heme oxygenase (HO-1) is an enzyme that is induced by heme as well as oxidative stress and has been reported to be involved in mediating protection against toxic liver injury. The purpose of the present study was to specify the role of HO-1 in CCl_4-hepatotoxicity.**

Methods and Results. **We could demonstrate an up-regulation of HO-1 protein in CCl_4-exposed liver tissue that reaches its maximum after 6 to 12 h, along with intrahepatic leukocyte accumulation and tissue injury. When animals were pretreated with hemin for augmentation of HO-1 expression, CCl_4-exposure was associated with a reduction of intrahepatic leukocyte accumulation, while inhibition of CCl_4-induced HO-1 expression by tin protoporphyrin-IX (SnPP-IX) enhanced leukocytic response. Of interest, however, liver morphology, transaminases, and bile flow as parameters of hepatocellular integrity and excretory function did not concur with reduced leukocyte numbers in the hepatic microcirculation, and revealed best organ function and tissue preservation in case of HO-1 inhibition by SnPP-IX. In contrast, hemin-treated CCl_4-exposed livers demonstrated pathologic enzyme release and cholestasis.**

Conclusions. **Taken together, inhibition of HO-1 in CCl_4-hepatotoxicity protected the liver, while higher HO-1 activity harmed liver tissue, most probably due to interference of the HO-1 pathway with CCl_4-dependent metabolism via cytochrome P450 and heme overload-associated toxicity.**

Key Words: **carbon tetrachloride; heme oxygenase; intravital fluorescence microscopy; bile flow.**

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INTRODUCTION

Reductive metabolism of the hepatotoxin CCl_4 by cytochrome P450 includes the production of the trichloromethyl free radical, which impairs crucial cellular processes leading finally to severe hepatic injury [1]. In particular, the reaction of trichloromethyl radical with oxygen leads to the formation of trichloromethylperoxy radical [2], a highly reactive species initiating the chain reaction of lipid peroxidation leading to destruction of phospholipid-associated fatty acids and breakdown of cellular membranes [3]. High oxidative stress has been considered as a reason for the up-regulation of HO-1, as this enzyme is known to be highly inducible upon such stressors [4]. HO-1 is a rate limiting enzyme in the catabolism of heme [5] and a heat shock protein in the rat [6]. By equimolar production of the antioxidant bilirubin, free iron, and vasodilative CO, HO-1 represents a cytoprotective enzyme and, when expressed, produces therapeutic benefits in a number of different conditions and diseases, such as sepsis, inflammation, and ischemia/reperfusion injury [7–9]. In line with this, HO-1 induction has been shown to confer protection in CCl_4-induced hepatotoxicity, as assessed by measurements of liver transaminase levels and cytological examination of liver histology [10]. However, there is accumulating evidence that the suppression of HO-1 can also lead to cell protection [11, 12].

In the present study, we report that exposure of the liver to CCl_4 is associated with inflammatory cell accumulation, which was attenuated in hemin-pretreated CCl_4-exposed animals. Nevertheless, the animals pretreated in the latter way exhibited reduced choleresis and a 3- to 4-fold increase of liver transaminase release upon CCl_4-exposure. On the contrary, pretreatment of animals with the HO-1 inhibitor SnPP-IX aggravated intrahepatic leukocyte accumulation, but allowed maintenance of hepatobiliary excretion and prevention

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of hepatocellular integrity. Thus, inhibition of HO-1 turns out to be beneficial in CCl_4-exposed livers, most probably due to limitation of heme toxicity.

MATERIALS AND METHODS

Animal Model and Surgical Preparation

Sprague Dawley rats of either gender (body weight (bw) 250 to 300 g; Charles River Laboratories, Sulzfeld, Germany) were used for the experiments. Animals were kept on water and standard laboratory chow *ad libitum*. The experiments were conducted according to the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Twenty-four animals received a single subcutaneous injection of CCl_4 (2.5 mL/kg bw) dissolved in an equal volume of olive oil [13]. Control animals ($n = 8$) received equivalent volumes of olive oil only.

Intravital fluorescence microscopic analysis of the hepatic microcirculation was performed at 12 h after CCl_4-exposure. For this purpose, animals anesthetized with pentobarbital (50 mg/kg bw ip) were tracheotomized to facilitate spontaneous respiration (room air) and placed on a heating pad in a supine position for maintenance of body temperature at 36 to 37°C. Polyethylene catheters (i.d. 0.58 mm; Portex, Hythe, United Kingdom) both in the right carotid artery and jugular vein allowed the assessment of systemic hemodynamics and injection of fluorescent dyes as well as for permanent infusion of isotonic saline solution at a rate of 2 $ml \cdot kg^{-1} \cdot h^{-1}$. After transverse laparotomy the animals were positioned on their left side and the left liver lobe was exteriorized and covered with a glass slide for intravital fluorescence microscopy [14–16].

Experimental Groups

To assess the kinetics of HO-1 expression in CCl_4-hepatotoxicity, we studied HO-1 protein levels over a period of 24 h upon CCl_4-exposure. In additional sets of experiments, CCl_4-treated animals were divided into the following groups: one group with pretreatment of the HO-1 inductor hemin (40 μmol/kg ip 6 h prior to CCl_4-exposure; hemin/CCl_4; $n = 8$) and another group with pretreatment of the HO-1 inhibitor SnPP-IX (50 μmol/kg ip 18 h prior to CCl_4-exposure; SnPP-IX/CCl_4; $n = 8$). Control animals received equivalent volumes of vehicle solutions mentioned below at the respective time points prior to CCl_4-exposure (CCl_4; $n = 8$).

Chemicals

The HO-1 inductor hemin (Fluka, Steinheim, Germany) was dissolved in dimethyl sulfoxide to a final concentration of 5 μmol/mL. The HO-1 inhibitor SnPP-IX (Frontier Scientific, Lancashire, United Kingdom) [17] was dissolved in 8.4% sodium-bicarbonate and PBS (PBS) to achieve a final concentration of 5 μmol/mL. The solutions were stored at a maximal temperature of 8°C in the dark and used within the next hour. All solutions were freshly prepared on the day of the experiment according to the manufacturers' directions. Dose and application mode of drugs were chosen in accordance to previously published work of our group [18, 19].

Intravital Fluorescence Microscopy

After the surgical procedure and a 20 min stabilization period, *in vivo* microscopy was performed using a modified fluorescence microscope with a 100-W mercury lamp (Eclipse E600-FN; Nikon, Tokyo, Japan). In epi-illumination technique, the microscopic images were taken by water immersion objectives (x20/0.75W; 40x/0.8W, Nikon), recorded by a charge-coupled device video camera (FK 6990-IQ; Pieper, Berlin, Germany), and transferred to a video system (S-VHS Panasonic AG 7350-E; Matsushita, Tokyo, Japan).

After tissue contrast enhancement by sodium fluorescein (2 μmol/kg iv; Merck, Darmstadt, Germany) sinusoidal perfusion failure was quantitatively analyzed using blue light epi-illumination (450–490 nm/>520 nm, excitation/emission wavelength) [20]. Leukocyte-endothelial cell interaction was assessed after *in vivo* white blood cell staining by rhodamine-6G (1 μmol/kg iv; Merck) using the green filter system (530–560/>580 nm) [15, 21, 22].

Quantitative Video Analysis

Assessment of hepatic microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images at a magnification of ×332- and ×583-fold using a computer-assisted image analysis system with a 19 in. monitor (CapImage; Zeintl, Heidelberg, Germany). Within 10 lobules per animal, sinusoidal perfusion failure was determined by the number of nonperfused sinusoids (in percent of all visible sinusoids) [20]. Leukocyte-endothelial cell interaction was analyzed within 10 hepatic lobules and 10 postsinusoidal venules per animal, including (1) the number of stagnant leukocytes, located within sinusoids (given as cells/mm^2 observation field) and not moving during an observation period of 20 s, and (2) the number of adherent leukocytes, located within postsinusoidal venules (given as cells/mm^2 endothelial surface, calculated from diameter and length of the vessel segment studied, assuming cylindrical geometry), and not moving or detaching from the endothelial lining during an observation period of 20 s [21, 22]. The number of rolling leukocytes within 10 postsinusoidal venules, moving with less than two-fifth of the centerline velocity, was calculated within an observation period of 20 s and given in percent of nonadherent leukocytes [22].

Sampling and Assays

After *in vivo* microscopy of the liver, arterial blood samples were taken for the spectrophotometric determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum activities, which served as an indicator for hepatocellular disintegration. Moreover, blood samples served for analysis of plasma bilirubin (Hitachi 704; Boehringer-Mannheim, Mannheim, Germany). Liver tissue was sampled for Western blot analysis, histology, and immunohistochemistry.

Western Blot Analysis

For Western blot analysis of HO-1, proliferating cell nuclear antigen (PCNA), and cleaved caspase-3, liver tissue was homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN_3, and 0.2 mM PMSF), incubated for 30 min on ice, and centrifuged for 15 min at 10,000 *g*. Prior to use, all buffers received a protease inhibitor cocktail (1:100 vol/vol; Sigma-Aldrich, Munich, Germany). Protein concentrations were determined by using the bicinchoninic protein assay (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as standard; 20 μg protein/lane were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (12% SDS-PAGE), and transferred to a polyvinyldifluoride membrane (Immobilon-P; Milipore, Eschborn, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 h at room temperature with rabbit polyclonal anti-HO-1 (1:5,000; Stress-Gen Biotech, San Diego, CA), rabbit polyclonal anti-PCNA (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anticleaved caspase-3 (1:1,000; Cell Signaling Technology, Frankfurt, Germany), followed by peroxidase-conjugated goat anti-rabbit IgG antibody (1:40,000; Cell Signaling Technology) as secondary antibody. β-actin signals served as equal loading controls (mouse monoclonal anti-β-actin antibody, 1:20,000; Sigma-Aldrich).

Protein expression was visualized by means of Luminol enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and by exposure of the membrane to a blue light

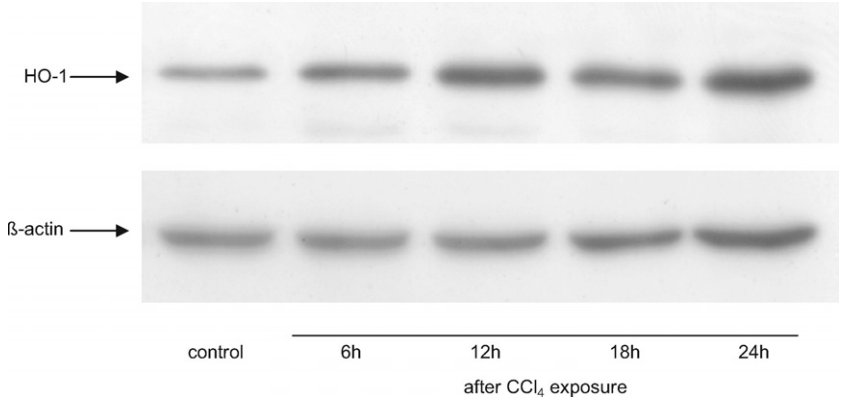

FIG. 1. Representative Western blot of HO-1 protein expression in liver tissue of animals prior to (control) and over 24 h after CCl_4-exposure. β-actin was used as loading control.

sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France).

Histology and Immunohistochemistry

At the end of each experiment, liver tissue was fixed in 4% phosphate buffered formalin for 2 to 3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4 μm sections were cut and stained with hematoxylin-eosin (HE) for histological analysis of liver injury. Leukocytes were stained by the chloroacetate esterase (CAE) technique and were identified by positive staining and morphology in 50 high power fields (HPF; $\times$400 magnification) using a conventional light microscope (Axioskop 40; Zeiss, Goettingen, Germany) [15].

For immunohistochemical demonstration of HO-1, liver sections collected on poly-L-lysine-coated glass slides were treated by microwave for antigen unmasking. Rabbit polyclonal anti-HO-1 (1:200; StressGen Biotech) was used as primary antibody and incubated for 90 to 120 min at room temperature, followed by an alkaline-phosphatase conjugated goat anti-rabbit antibody (1:25; Cell Signaling Technology) and development using new fuchsin as chromogen. The sections were counterstained with hematoxylin and examined by light microscopy (Axioskop 40; Zeiss).

Statistical Analysis

All data are expressed as means $\pm$ SEM. After proving the assumption of normality and equal variance across groups, differences between groups were assessed using one way ANOVA followed by the Holm-Sidak test for post-hoc comparison. Statistical significance was set at $P < 0.05$. Statistics were performed using the software package SigmaStat (Jandel Corporation, San Rafael, CA).

RESULTS

Western Blot Analysis and Immunohistochemistry of HO-1 Expression in CCl_4-Exposed Livers

As illustrated by Western blot analysis of rat liver tissue, CCl_4 caused an increase in HO-1 protein expression with peak levels within the first 6 to 12 h after intoxication, followed by a slight decrease at 18 and 24 h; however, without reaching baseline pre-exposition levels (Fig. 1). Hemin pretreatment of animals prior to CCl_4-exposure enhanced HO-1 protein expression, while application of the HO-1 inhibitor SnPP-IX decreased HO-1 protein levels (Fig. 2).

Parallel to kinetics of HO-1 protein expression, immunoreactivity of CCl_4-exposed and in particular hemin/CCl_4-exposed liver tissue displayed marked increase in immunoreactivity of HO-1 in parenchymal cells preferentially located around central veins (Fig. 3B and C), compared with minimal, if any, cellular immunoreactivity of HO-1 in nonexposed control and SnPP-IX/CCl_4-exposed liver tissue (Fig. 3A and D).

Systemic Hemodynamics

Animals of the experimental groups did not differ with respect to MAP and heart rate (data not shown). Moreover, there were no differences in hemoglobin,

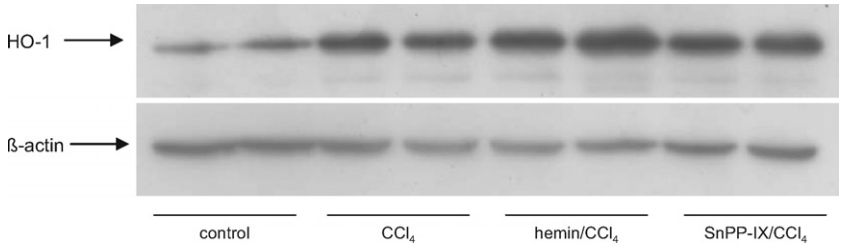

FIG. 2. Representative Western blot of HO-1 protein expression in animals upon CCl_4-exposure. β-actin was used as loading control. Animals were either pretreated with the vehicle solution (CCl_4), the HO-1 inductor hemin (hemin/CCl_4), or the HO-1 inhibitor SnPP-IX (SnPP-IX/CCl_4). Olive oil-treated animals served as control for CCl_4-exposed animals (control).

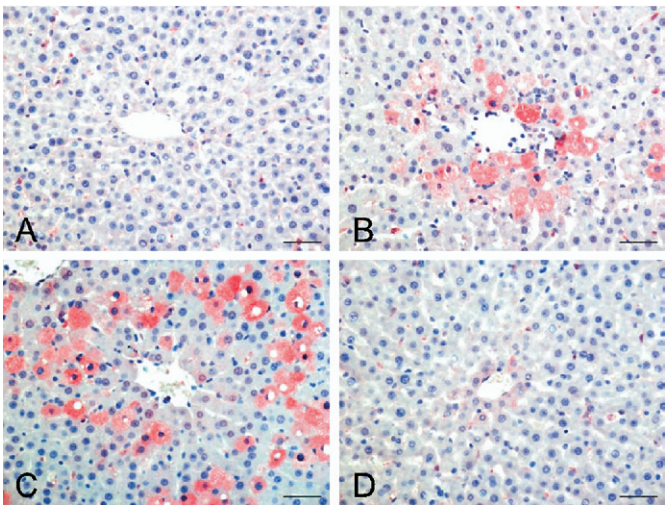

FIG. 3. Representative images of HO-1 immunohistochemistry in CCl_4-exposed liver tissue. Animals were either pretreated with the vehicle solution [CCl_4 (B)]; the HO-1 inductor hemin [hemin/CCl_4 (C)]; or the HO-1 inhibitor SnPP-IX [SnPP-IX/CCl_4 (D)]. Olive oil-treated animals served as control for CCl_4-exposed animals [control; (A)]. Note the marked immunoreactivity of parenchymal cells located preferentially around central veins in the CCl_4 (B), but in particular in the hemin/CCl_4-treated animal, while there was almost no positive staining in the control and HO-1 inhibited animals (A) and (D). Bars represent 50 μm.

hematocrit, systemic leukocyte count, and electrolytes between the groups (data not shown).

Hepatic Microcirculation in CCl_4-Exposed Liver Tissue

CCl_4-exposure was characterized by an inflammatory cell response with significant ($P < 0.05$) increases of leukocyte numbers, both rolling along (Fig. 4A) and firmly attaching to the venular endothelium (Fig. 4B). Hemin-enhanced induction of HO-1 was accompanied by with reduced numbers of rolling leukocytes (Fig. 4A), but in particular adherent leukocytes (Fig. 4B). In contrast, SnPP-IX-mediated HO-1 inhibition markedly aggravated intrahepatic leukocyte-endothelial cell interaction with an almost 2-fold increase of the number of adherent leukocytes when compared with solely CCl_4-exposed livers (Fig. 4B).

In line with *in vivo* fluorescence microscopic analysis of intrahepatic leukocyte accumulation, histology of CCl_4-exposed liver tissue revealed inflammatory cell infiltration (CCl_4: 2.4 ± 0.2 cells/HPF *versus* controls: 1.5 ± 0.2 cells/HPF; $P < 0.05$), which was found slightly reduced in hemin-pretreated animals toward values found in controls (hemin/CCl_4: 1.7 ± 0.4 cells/HPF). On the contrary, SnPP-IX pretreatment in CCl_4-exposed animals tends to further enhance leukocyte tissue infiltration (2.7 ± 0.4 cells/HPF).

CCl_4-exposure impaired nutritive perfusion with an average value of 9% ± 2% of nonperfused sinusoids (Fig. 5). Overexpression of HO-1 by hemin pretreatment slightly reduced perfusion failure (Fig. 5), while CCl_4-exposed animals pretreated with the HO-1 inhibitor SnPP-IX exhibited an extent of sinusoidal perfusion failure comparable to that seen in solely CCl_4-exposed animals (Fig. 5).

Tissue Injury and Regeneration in CCl_4-Exposed Livers

Analysis of cleaved caspase 3 protein revealed higher expression in CCl_4-exposed and in particular hemin-

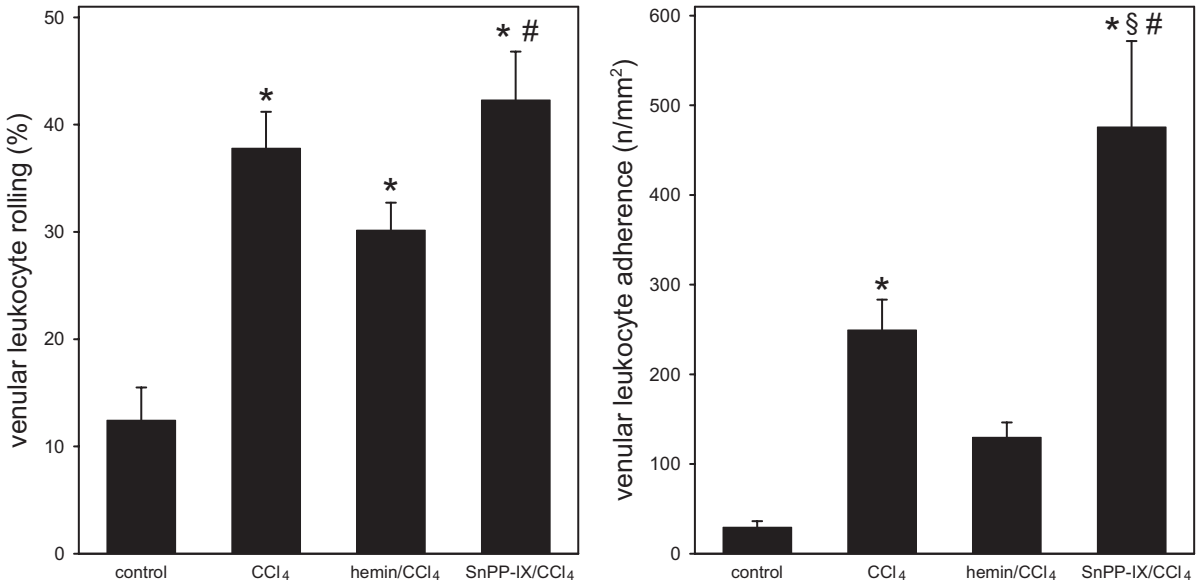

FIG. 4. Rolling and firm adherence of leukocytes in hepatic postsinusoidal venules in CCl_4-associated liver injury. Animals were randomly assigned to the following groups: one group was pretreated with the HO-1 inductor hemin (40 μmol/kg ip 6 h prior to CCl_4-exposure; hemin/CCl_4; $n = 8$) and one group was pretreated with the HO-1 inhibitor SnPP-IX (50 μmol/kg ip 18 h prior to CCl_4-exposure; SnPP-IX/CCl_4; $n = 8$). Control animals received equivalent volumes of vehicle solutions at the respective time points prior to CCl_4-exposure (CCl_4; $n = 8$). Olive oil-treated animals served as controls for CCl_4-exposed animals (controls, $n = 8$). Values are given as means ± SEM; *$P < 0.05$ *versus* control; §$P < 0.05$ *versus* CCl_4; #$P < 0.05$ *versus* hemin/CCl_4.

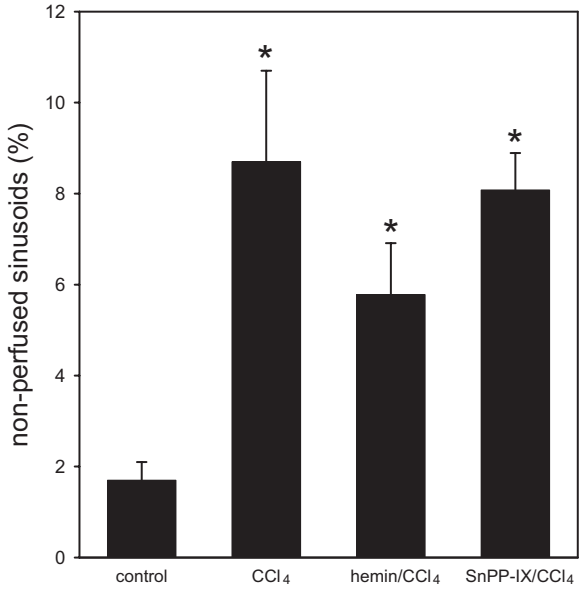

FIG. 5. Hepatic sinusoidal perfusion failure in CCl_4-associated liver injury. Animals were randomly assigned to the following groups: one group was pretreated with the HO-1 inductor hemin (40 μmol/kg ip 6 h prior to CCl_4-exposure; hemin/CCl_4; $n = 8$) and one group was pretreated with the HO-1 inhibitor SnPP-IX (50 μmol/kg ip 18 h prior to CCl_4-exposure; SnPP-IX/CCl_4; $n = 8$). Control animals received equivalent volumes of vehicle solutions at the respective time points prior to CCl_4-exposure (CCl_4; $n = 8$). Olive oil-treated animals served as controls for CCl_4-exposed animals (controls, $n = 8$). Values are given as means ± SEM; $^*P < 0.05$ *versus* controls.

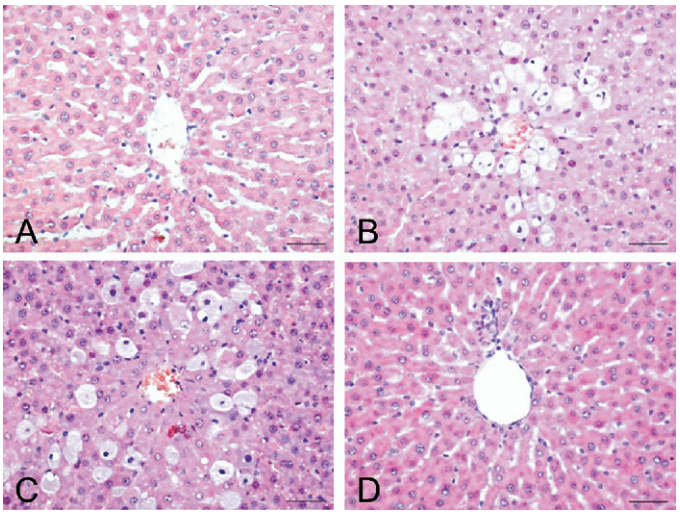

FIG. 7. Representative light microscopic images of HE-stained liver tissue. Animals were either pretreated with CCl_4 (B), the HO-1 inductor hemin [hemin/CCl_4 (C)], or the HO-1 inhibitor SnPP-IX [SnPP-IX/CCl_4 (D)]. Olive oil-treated animals served as control for CCl_4-exposed animals [control, (A)]. Please note severe pericentral damage in liver tissue of CCl_4- and hemin/CCl_4-treated animals (B) and (C) in contrast to the regular histological appearance of liver tissue in controls (A) and SnPP-IX/CCl_4-treated animals (D). Bars represent 50 μm.

pretreated CCl_4-exposed livers when compared with that of SnPP-IX-pretreated CCl_4-exposed animals and controls (Fig. 6A). Moreover, PCNA protein levels, serving as regeneration parameter in response to injury, were found to be slightly higher in the hemin-pretreated CCl_4-exposed and CCl_4-exposed animals when compared with SnPP-IX/CCl_4-treated animals (Fig. 6B). Concomitantly, HE staining of CCl_4-exposed liver tissue showed severe pericentral damage (Fig. 7B), in contrast to the regular histological appearance of liver tissue from control animals (Fig. 7A). Histology of hemin-pretreated liver tissue did not markedly differ from CCl_4-exposed ones (Fig. 7C), while upon SnPP-IX/CCl_4-exposure the tissue revealed almost absence of cell injury (Fig. 7D).

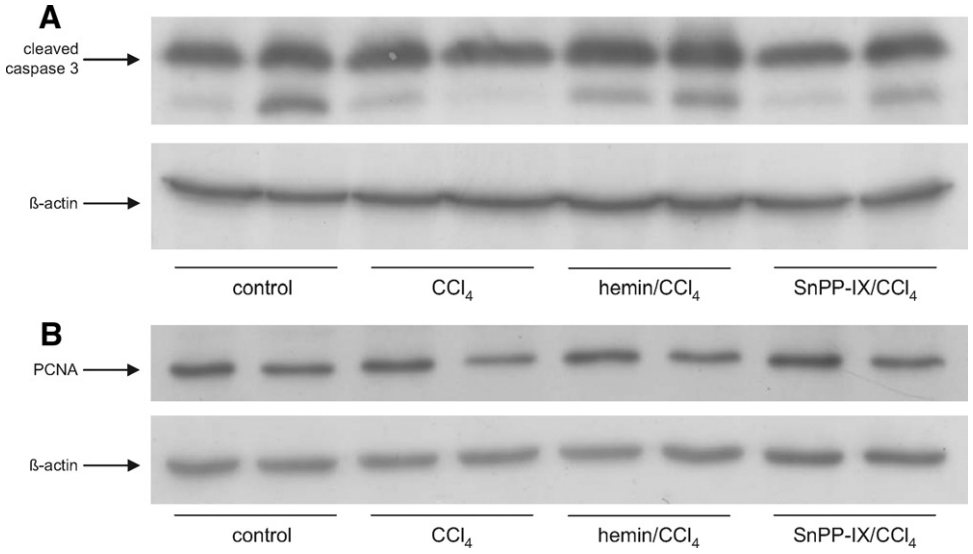

FIG. 6. Representative Western blots of cleaved caspase 3 (A) and PCNA (B) protein expression in animals upon CCl_4-exposure. β-actin was used as loading control. Animals were either pretreated with the vehicle solution (CCl_4), the HO-1 inductor hemin (hemin/CCl_4), or the HO-1 inhibitor SnPP-IX (SnPP-IX/CCl_4). Olive oil-treated animals served as control for CCl_4-exposed animals (control).

TABLE 1

Liver Enzyme Release and Bile Flow in CCl_4-Associated Liver Injury

	Control	CCl_4	Hemin/CCl_4	SnPP-IX/CCl_4
ALT (U/L)	54 ± 5	115 ± 26	289 ± 82*§	68 ± 5
AST (U/L)	138 ± 21	263 ± 95	683 ± 110*§	139 ± 8
Bile flow (μL/min × g)	1.85 ± 0.23	1.72 ± 0.24	1.06 ± 0.06*§	1.68 ± 0.15#

Animals were randomly assigned to the following groups: one group was pretreated with the HO-1 inductor hemin (40 μmol/kg ip 6 h prior to CCl_4-exposure; hemin/CCl_4; $n = 8$) and one group was pretreated with the HO-1 inhibitor SnPP-IX (50 μmol/kg ip 18 h prior to CCl_4-exposure; SnPP-IX/CCl_4; $n = 8$). Control animals received equivalent volumes of vehicle solutions at the respective time points prior to CCl_4-exposure (CCl_4; $n = 8$). Olive oil treated animals served as controls for CCl_4-exposed animals (controls, $n = 8$). Values are given as means ± SEM.

* $P < 0.05$ *versus* control.

§ $P < 0.05$ *versus* CCl_4.

# $P < 0.05$ *versus* hemin/CCl_4.

In accordance with the results of the liver histology, analysis of liver enzymes revealed a 2-fold increase in serum ALT activity in CCl_4-exposed animals and a 10-fold increase upon hemin/CCl_4-exposure, while SnPP-IX/CCl_4-exposed animals exhibited almost physiological transaminase levels (Table 1). Moreover, hemin pretreatment caused a significant ($P < 0.05$) elevation in bilirubin levels (8.2 ± 0.6 μmol/L) when compared with the mean values of all other groups (control: 6.2 ± 0.4 μmol/L; CCl_4: 6.6 ± 0.4 μmol/L; SnPP-IX/CCl4: 5.7 ± 0.2 μmol/L). Concomitant to liver enzyme release and bilirubin levels, bile flow as functional parameter was only slightly lower in both CCl_4- and SnPP-IX/CCl_4-exposed animals when compared with controls, but was found markedly altered in hemin/CCl_4-exposed animals (Table 1). Regression analysis revealed a significant negative correlation between serum enzyme release and bile flow (Fig. 8).

DISCUSSION

Herein, we inform about the following new findings: (1) augmentation of HO-1 expression reduced intrahepatic leukocyte accumulation in CCl_4-exposed livers, however, did not ameliorate liver injury, as assessed by high hepatic enzyme release and low excretory biliary function. (2) *Vice versa*, SnPP-IX induced HO-1 inhibition enhanced inflammatory cell accumulation in CCl_4-exposed livers, but was capable of preserving hepatocellular integrity and function.

These findings underline the accumulating evidence that the suppression of HO-1 can lead to cell protection [11, 12], although there are numerous reports on the beneficial effects of HO-1 in a variety of experimental conditions, including CCl_4-exposure of the liver [10, 23–26]. Several mechanisms whereby HO-1 could provide this protection have been suggested, including production of the antioxidants biliverdin and bilirubin, release of vasodilative CO, depletion of the oxidant heme, and elevation of intracellular free iron levels to facilitate ferritin synthesis [5, 23]. In rats displaying a marked HO-1 expression in mesenteric microvessels, it has been shown that H_2O_2 superfusion of these microvessels neither induced rolling nor exhibited adherent responses of leukocytes. This antiadhesive effect of HO-1 induction could be mimicked by bilirubin, but not CO, indicating that the HO reaction product bilirubin mainly mediates attenuation of inflammatory responses [27]. In line with this, the current observation that HO-1 induction down-regulates intrahepatic leukocyte accumulation, while HO-1 inhibitor SnPP-IX restores decreased leukocyte adhesive responses, might be ascribable to bilirubin, which was actually elevated in the plasma of hemin-pretreated CCl_4-exposed animals. When compared with untreated CCl_4-exposed animals, it

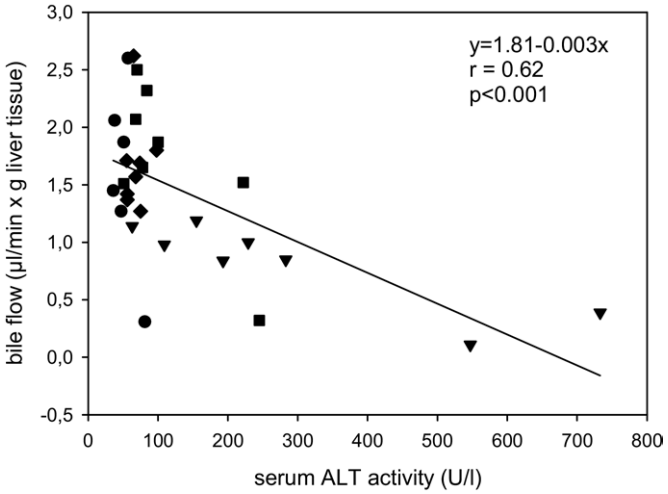

FIG. 8. Linear regression analysis between liver enzyme release and bile flow in animals with CCl_4-associated liver injury. Animals were randomly assigned to the following groups: one group was pretreated with the HO-1 inductor hemin (inverted triangle; $n = 8$) and one group was pretreated with the HO-1 inhibitor SnPP-IX (diamond; $n = 8$). Control animals received equivalent volumes of vehicle solutions at the respective time points prior to CCl_4-exposure (square; $n = 8$). Olive oil-treated animals served as controls for CCl_4-exposed animals (circle, $n = 8$). r, regression coefficient.

was found that SnPP-IX even increased inflammatory cell accumulation, supposedly due to inhibition of not only HO-1 but also HO-2, representing a constitutively expressed protective factor of the liver [28, 29].

Beside bilirubin, CO has also been considered as protective mediator [30, 31]. This HO-1 metabolite is reported to function as a vasorelaxing mediator and is necessary to reduce sinusoidal tone for maintenance of hepatic nutritive perfusion [32, 33]. Besides vasotonus control, properties such as activation of soluble guanylyl cyclase [34–36], inhibition of cytochrome P450 activity [37, 38], suppression of the PAI-1-mediated fibrin deposition [39], and p38 mitogen-activated protein kinase-dependent inhibition of cell apoptosis [40], may also allow CO to confer protection. In line with this, exogenous CO and CO-liberating methylene chloride were shown to protect mice from cytokine or CD95-induced apoptotic liver damage, whereas SnPP-IX even aggravated liver injury in these models of immune-mediated tissue damage [41]. This is in clear contrast to the amelioration of CCl_4-induced liver injury by SnPP-IX that we observed in the present study. The reason for these discrepant results could be due to the distinct metabolism of CCl_4, as hepatotoxicity of CCl_4 has been reported to be potentiated by the CO donor dichloromethane [42].

Meanwhile, the increasing understanding of the properties of the HO-1 pathway metabolites allows us to differentially consider their significant roles in various pathologies. Degradation of protoheme IX by HO-1 involves NADPH-cytochrome P450 reductase, which in turn constitutes a major source of heme undergoing degradation via HO-1. The complex interrelation of these systems seems to be of considerable importance for the net result of HO-1 activity in CCl_4-hepatotoxicity. Because the enzymatic reaction mediated by HO requires reducing equivalents supplied by NADPH via NADPH-cytochrome P450 reductase [43], generation of metabolites from the HO activity can be limited [44]. On the other hand, exposure to CCl_4 may increase turnover of cytochrome P450-associated heme, which further induces HO-1 [44]. Acute stress conditions, such as CCl_4-exposure in hemin-pretreated animals, might render the liver sensitive to acute heme overloading, as it has been hypothesized for the endotoxemic liver [9]. With respect to the fact that—besides biliverdin and CO—free iron is released in equimolar doses, and that excess iron is potentially harmful and can induce cell injury [45, 46], overloading with free iron could be responsible for enhanced tissue injury in the hemin-pretreated CCl_4-exposed animals. Although iron induces ferritin, which in turn prevents lipid peroxidation [45], we like to suppose that heme/iron overload might be a reason for the lack of advantage from HO-1 activity in hemin-pretreated CCl_4-exposed animals. This view is underlined by reports of others that ferritin overexpression by recombinant adenoviral gene transfer does not mimic the protective effect of HO-1 [41, 47].

In conclusion, our results show that CCl_4-exposed livers in the end benefit from HO-1 inhibition. However, although HO-1 mediates some anti-inflammatory effect, activity of the enzyme system interferes with the cytochrome P450-guided CCl_4-biotransformation supposedly resulting in heme/iron overload-associated harmful effects.

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