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Protective role of heme oxygenase-1 induction in carbon tetrachloride-induced hepatotoxicity

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Abstract

Reductive metabolism of carbon tetrachloride (CCl_4) is thought to cause lipid peroxidation which results in hepatic injury. Heme oxygenase-1 (HO-1) (EC 1.14.99.3), the rate-limiting enzyme in heme catabolism, is known to be induced by oxidative stress and to confer protection against oxidative tissue injuries. In this study, we examined the role of HO-1 induction in a rat model of CCl_4-induced acute liver injury. CCl_4 treatment (1 mL/kg, intraperitoneally) produced severe hepatic injury in rats as revealed by significant increases in serum alanine transaminase (ALT) (EC 2.6.1.2) activity and hepatic malondialdehyde (MDA) content, severe liver cell injury, and increases in hepatic tumor necrosis factor-α (TNF-α) mRNA expression and DNA binding activity of nuclear factor-κB (NF-κB). Following CCl_4 treatment, hepatic HO-1 expression was markedly increased both at transcriptional and protein levels in hepatocytes, especially around the central vein. HO-1 induction was mediated in part through a rapid increase in microsomal free heme concentration presumably derived from hepatic cytochrome P450. Inhibition of HO activity by tin-mesoporphyrin (Sn-MP), which resulted in a sustained increase in microsomal free heme concentration, exacerbated liver injury, as judged by the sustained increase in serum ALT activity, extensive hepatocytes injuries, a more pronounced expression of hepatic TNF-α mRNA and an enhanced NF-κB activation. These findings indicate that induction of HO-1 is an adaptive response to CCl_4 treatment, and it may be critical in the recovery of hepatocytes from injury. Our findings also suggest that HO-1 induction may play an important role in conferring protection on hepatocytes from oxidative damage caused by free heme.

Keywords: Acute liver injury; Carbon tetrachloride; Heme oxygenase-1; Oxidative injury; Cytochrome P450; Heme

HO-1 is not only a key enzyme in heme catabolism [1,2], but also a heat shock protein (HSP 32) in the rat [3]. HO-1 is induced by its substrate heme [1,2], as well as by various oxidative stresses [4–6], and is thought to play an important protective role against oxidative injuries [5,7–11]. Treatment of animals with CCl_4 is known to cause severe hepatic injury [12]. It is generally thought that CCl_4 toxicity is due to a reactive intermediate, which is generated by its reductive metabolism by hepatic cytochrome P450. The reactive intermediate is believed to cause lipid peroxidation, and the breakdown of cellular membranes [13]. It has been reported that HO-1 is induced in the liver of rats treated with CCl_4 [14–16]. However, the role of HO-1 in the CCl_4-induced liver injury has not been fully defined. With this view in mind, we examined expression of hepatic HO-1 and nonspecific δ-aminolevulinate synthase (ALAS-N) (EC 2.3.1.37), the rate-limiting enzyme in heme catabolism [1,2] and biosynthesis [17], respectively, in hepatic injury induced by CCl_4 treatment. The degree of hepatic injury and inflammation was assessed by measurements of serum ALT activity, cytological examination of hepatic histology, determination of hepatic MDA concentration, the level of TNF-α mRNA, and DNA binding activity of

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Abbreviations: ALAS-N, nonspecific δ-aminolevulinate synthase; ALT, alanine transaminase; CCl_4, carbon tetrachloride; EMSA, electrophoretic mobility shift assay; HO, heme oxygenase; HO-1, heme oxygenase-1; HSP 70, heat shock protein 70; MDA, malondialdehyde; NF-κB, nuclear factor-κB; Sn-MP, tin-mesoporphyrin; TNF-α, tumor necrosis factor-α.

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NF-κB [18,19]. Microsomal cytochrome P450 and heme concentration were also determined and their relationship to oxidative injury and HO-1 induction was evaluated. We report here that CCl_4 treatment caused marked HO-1 induction in hepatocytes around the central vein which was mediated through an increase in microsomal heme concentration presumably derived from the destruction of hepatic cytochrome P450. Pretreatment of rats with Sn-MP, a specific competitive inhibitor of HO activity [20], produced a marked inhibition of HO activity, a sustained increase in microsomal heme concentration, aggravation of hepatic injury and exacerbation of tissue inflammation. Thus, HO-1 induction appears to be critical in the protection of the liver cells from an oxidative injury caused by free heme in the CCl_4-induced hepatic injury.

1. Materials and methods

1.1. Animals and treatments

Animal experiments were approved by the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with National Institutes of Health guidelines. Male Sprague–Dawley rats, weighing 200–260 g, were purchased from Charles River (Yokohama, Japan). They were housed in a temperature-controlled (25°) room with alternating 12 hr/12 hr light/dark cycles, and were allowed free access to water and chow diet until the start of experiments. They were injected intraperitoneally (i.p.) with CCl_4 (1 mL/kg, b.wt.; Sigma Chemical Co.) dissolved in an equal volume of corn oil. Control rats received the same volume of corn oil. After the injection, animals were returned to cages and allowed free access to food and water. CCl_4-treated animals were further divided into the following two groups; one group with pretreatment with Sn-MP (1 μmol/kg, b.wt.; Frontier Scientific, Inc.) i.v. via tail vein. Sn-MP had been dissolved in a small volume of 0.1 N NaOH solution, then pH was adjusted to 7.6 with 0.01 M sodium phosphate buffer [10]. The other group of animals was pretreated with the vehicle alone. Both pretreatments were made 1 hr prior to CCl_4 treatment. These animals were pair-watered and -fed to ensure that there were no differences in extracellular fluid volume, caloric intake, and other factors. In another set of experiments, rats were administered with hemin (ferriprotoporphyrin IX chloride) subcutaneously (30 or 60 μmol/kg, b.wt.), which had been dissolved in an alkaline solution (0.1 N NaOH) and then reconstituted in physiological saline [11]. To examine the effect of hepatic drug metabolizing enzyme cytochrome P450 on CCl_4-induced hepatic injury, some rats had been given 0.1% (w/v) phenobarbital solution instead of plain water for 5 days and were fasted on day 5 for 24 hr before CCl_4 treatment [11]. Under light anesthesia with ethyl ether, animals were sacrificed by decapitation at each defined time point (0–72 hr). Blood was collected for serum isolation, and the livers were excised. Livers were frozen immediately in liquid nitrogen and stored at −80° until use for the preparation of RNA and nuclear extracts, and measurement of MDA concentration. For the preparation of hepatic microsomal fraction, livers were perfused *in situ* with physiological saline until the venous effluent became clear, and then they were removed.

1.2. cDNA probes

Template cDNAs used were rat pRHO-1 [21] and rat pKRA2cA [22] for rat HO-1 and rat ALAS-N, respectively. Template cDNA for heat shock protein 70 (HSP 70) was rat HSP 70 cDNA corresponding to 163–1230 bp [23], which was cloned from the rat fetal brain library and constructed in pGEM-4Z Vector (Promega) with *Sma*I and *Pst*I sites [10,11]. Template cDNA for TNF-α was rat TNF-α cDNA corresponding to 27–668 bp [24], which was cloned from lipopolysaccharide-treated rat ileum library (lipopolysaccharide; *Escherichia coli*, 0127:B8; Sigma Chemical Co.; 10 mg/kg, b.wt., i.p. [6]) and constructed in pGEM-T Easy Vector (Promega). All probes used for Northern blot analysis were [α-^{32}P]dCTP-labeled (Amersham Pharmacia Biotech, Inc.) cDNA probes prepared according to the manufacturer's instructions by using a random primer DNA labeling system (Amersham Pharmacia Biotech, Inc.) [9].

1.3. RNA isolation and Northern blot analysis

Total RNA was isolated from the tissues according to the method of Chomczynski and Sacchi [25]. Twenty micrograms of total RNA was subjected to electrophoresis in a 1.2% (w/v) agarose gel containing 6.5% (v/v) formaldehyde. After blotting on to a sheet of BIO-RAD Zeta-Probe® membrane (Bio-Rad Laboratories), RNA samples were hybridized with [α-^{32}P]dCTP-labeled cDNA probe followed by washing under stringent conditions. The membrane was exposed to a sheet of Fuji Medical X-rays film with an intensifying screen at −70°, and autoradiographs were quantified using an image scanner (GelPrint® 2000i, Genomic Solutions, Inc.) and a computerized image analysis software (Basic Quantifier® Ver. 3.0, Genomic Solutions, Inc.) [9].

1.4. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from frozen liver sections as described by Dignam *et al.* [26], with some modifications. Briefly, tissues were homogenized in 2.5 vol. of ice-cold PBS using a glass handled homogenizer and centrifuged at 1200 *g* for 5 min at 4°. The pellet was lysed in lysis buffer containing 10 mM HEPES, pH 7.8, 10 mM KCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40, and

centrifuged at 2100 *g* for 5 min at 4° to obtain the nuclear fraction as a pellet. The resultant pellet was resuspended in the extraction buffer containing 20 mM HEPES, pH 7.8, 0.42 M KCl, 0.2 mM EDTA, and 10% glycerol followed by the centrifugation at 12,000 *g* for 5 min at 4°, and the supernatant was obtained as nuclear extracts. The double-stranded NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) [27] was end-labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Inc.) according to the manufacturer's instructions (Promega). Nuclear extracts were incubated with [γ-^{32}P]ATP-labeled NF-κB consensus oligonucleotide probe in a total volume of 10 μL of binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.5 μg poly dI:dC) at room temperature for 20 min according to the manufacturer's instructions (Promega). Following the incubation, the reaction mixture was subjected to electrophoresis in nondenaturing 5% polyacrylamide gel. The gels were vacuum dried and exposed to X-ray film at −70°. Self-competitions were carried out under the same condition using 100-fold excess of the unlabeled NF-κB oligonucleotide probe. Nonspecific competitions were similarly performed using an unlabeled oligonucleotide probe encompassing an OCT1 transcription factor binding site (5′-TGTCGAATGCAAATCACTAGAA-3′) [28].

1.5. Measurement of hepatic MDA concentration

Frozen liver tissues were homogenized in 10 vol. of 20 mM Tris–HCl pH 7.4 (w/v) with hydroxytoluene and centrifuged at 3000 *g* for 10 min at 4°. The supernatant was used for biochemical assay. The assay was carried out by using a commercial colorimetric kit (Lipid peroxidation assay kit, Calbiochem-Novabiochem Corporation) according to the manufacturer's instructions. The concentration of MDA was expressed as nmol/mg protein. The supernatant protein was determined by the method of Lowry *et al.* [29].

1.6. Histological study

For histological examination, liver tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4–6 μm thickness. After deparaffinization and dehydration, sections were stained with hematoxylin and eosin for microscopic examination. For immunohistochemical examination, the enzymatic activity of endogenous peroxidases in the liver section was first blocked with 3% hydrogen peroxide, followed by incubation with rabbit polyclonal anti-rat HO-1 (StressGen Biotechnologies) at 37° for 3 hr. The antigen–antibody reaction was detected using a secondary anti-rabbit antibody and an avidin-biotin immunoperoxidase staining kit (DAKO) [11]. The positive reaction was visualized as brown stain following treatment with 3,3′-diaminobenzidine. Normal rabbit serum was used as control for nonspecific staining. Sections were counterstained with hematoxylin.

1.7. Measurement of hepatic microsomal heme concentration

Livers were homogenized in 3 vol. of 1.15% (w/v) KCl and centrifuged at 10,000 *g* for 30 min at 4°, followed by 104,000 *g* centrifugation of the supernatant for 60 min at 4°, to obtain the microsomal fraction as a pellet. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 10% (v/v) glycerol and used for the measurement of heme concentration by the pyridine hemochromogen method [10,30]. Heme concentration was expressed as nmol per mg of protein. Microsomal protein was determined by the method of Lowry *et al.* [29].

1.8. Measurement of hepatic cytochrome P450 content

Livers were homogenized in 3 vol. of 1.15% KCl. Microsomal fractions were prepared as described above. Microsomal cytochrome P450 content was determined from a carbon monoxide (CO)-difference spectrum, using an EmM of 91, as described by Omura and Sato [11,31].

1.9. Hepatic HO activity

Livers were homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and protease inhibitor (Complete®, Boehringer Mannheim GmbH). Microsomal fractions were prepared as described above, and HO activity was measured spectrophotometrically as described previously [11,32]. The cytosolic fraction prepared from the livers of adult untreated rats served as a source of biliverdin reductase. HO activity was expressed as picomoles of bilirubin formed per milligram of protein per 60 minutes.

1.10. Western blot analysis

Livers were homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. Microsomal fractions were prepared as described above. Samples equivalent to 50 μg of protein were applied to a 12% (w/v) polyacrylamide–SDS gel. After electrophoretic separation, proteins were transferred to Nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with Tris-buffered saline (TBS) containing 10% (w/v) skim milk at 4° overnight, followed by incubation with a rabbit anti-HO-1 polyclonal antibody (StressGen Biotechnologies) diluted at 1:1000 with TBS containing 3% (w/v) skim milk. Then the membrane was treated with horseradish peroxidase-labeled goat anti-rabbit IgG (KPL). Antigen–antibody complexes were visualized with an ECL® chemiluminescence system (Amersham Pharmacia

Biotech, Inc.) and short exposure of the membrane to X-ray films. The obtained signals were quantified as described above. The transfer efficiency and an equal amount of loading per lane were verified by staining nitrocellulose membranes using Amido Black solution [33].

1.11. Assay of serum ALT activity

Serum was separated from whole blood by centrifugation at 1600 *g* for 10 min at 4°, and serum ALT activity was measured using an automatic biochemical analyzer calibrated with quality control standards (E.I. Du Pont de Nemours).

1.12. Statistical analysis

Statistical evaluation was performed with unpaired Student's *t*-test, or when multiple comparisons were made, with ANOVA followed by Scheffé's *F*-test, using Statview software (Abacus Concepts). Differences were considered as significant at $P < 0.01$. Data are presented as means ± SEM.

2. Results

2.1. CCl_4-induced hepatic injury

We examined serum ALT activity, histological changes of the liver 24 hr after i.p. injection of CCl_4. Serum ALT activity increased markedly in CCl_4-treated rats as compared with control rats (Fig. 1A). Histological examination of livers in the CCl_4-treated rats revealed massive and severe hepatocyte necrosis at the centrilobular zone with influx of inflammatory cells. Additional vacuolar degeneration was also observed around the portal tracts (Fig. 1B, top and middle) [15]. In control rats, liver injury was not observed (Fig. 1B, bottom). Since CCl_4-induced hepatic

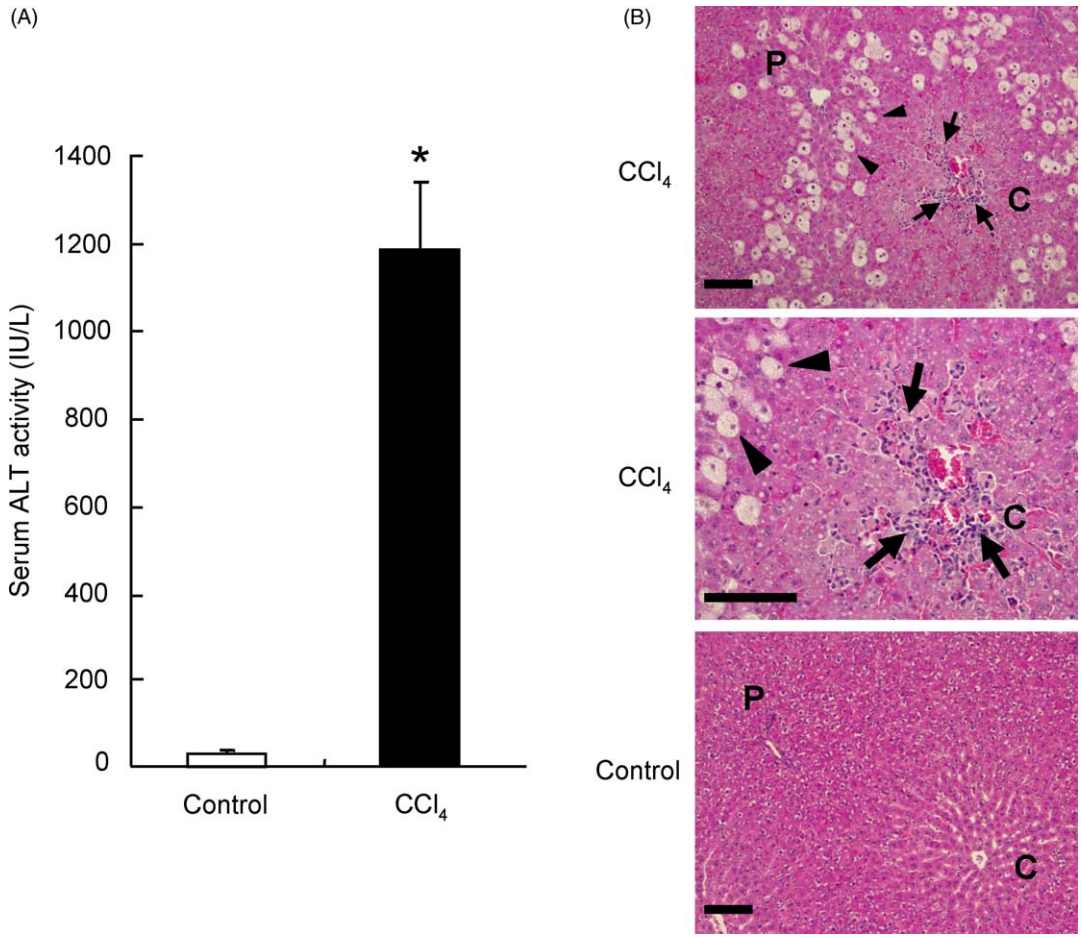

Fig. 1. Histological changes in the liver and changes in serum ALT activity after CCl_4 treatment. Rats were injected i.p. with CCl_4 (1 mL/kg), and were killed at 24 hr after the injection. Livers were removed for histological examination and whole blood was collected for determination of serum ALT activity as described in Section 1. (A) Serum ALT activities are shown as means ± SEM (N = 10). Control, the vehicle-treated control rats; CCl_4, CCl_4-treated rats. Statistical analysis by unpaired Student's *t*-test. $^*P < 0.01$ vs. control. (B) Representative light microscopy of livers after CCl_4 (top and middle) and vehicle (bottom) treatment from at least three independent experiments. P and C denote portal tract and central vein, respectively. Arrows and arrowheads indicate influx of inflammatory cells and ballooned liver cells with vacuolation in their cytoplasm, respectively. The bar represents 100 μm. Liver sections were stained with hematoxylin and eosin.

Table 1
Effect of CCl_4 treatment on MDA level in the liver and its modulation by administration of Sn-MP

Experimental group	MDA level (nmol/mg protein)
Vehicle-treated 24 hr	0.17 ± 0.01
CCl_4 24 hr	0.36 ± 0.05*
Sn-MP treated 48 hr	0.18 ± 0.01
CCl_4 48 hr	0.34 ± 0.04**
Sn-MP + CCl_4 48 hr	0.51 ± 0.04***

Livers were removed after various treatments for measurement of hepatic MDA concentrations as described in Section 1. Data are shown as means ± SEM (N = 6). Vehicle-treated 24 hr, the vehicle-treated control rats at 24 hr after the treatment; CCl_4 24 hr, CCl_4-treated rats at 24 hr after the treatment; Sn-MP treated 48 hr, control rats at 48 hr after Sn-MP treatment; CCl_4 48 hr, CCl_4-treated rats at 48 hr after the treatment; Sn-MP + CCl_4 48 hr, CCl_4-treated rats with Sn-MP at 48 hr after the treatment. Statistical analysis by unpaired Student's *t*-test or ANOVA with Scheffé's *F*-test.

* $P < 0.01$ vs. vehicle-treated 24 hr.

** $P < 0.01$ vs. Sn-MP treated 48 hr.

*** $P < 0.01$ vs. CCl_4 48 hr or Sn-MP treated 48 hr.

injury is thought to be due to CCl_4-mediated free radical production which causes membrane lipid peroxidation, we determined hepatic MDA level at 24 hr after CCl_4 treatment. Only low levels of MDA were seen in the untreated control rats and these values were considered normal (Table 1) [34]. In contrast, a significant increase in MDA production was found in the liver of CCl_4-treated rats (Table 1) [34]. We also examined changes in hepatic gene expression of TNF-α, a pro-inflammatory cytokine, after CCl_4 treatment. Hepatic TNF-α mRNA was not detectable in control rats (Fig. 2). Following CCl_4 treatment, however, its expression started to increase at 3 hr, increased in a linear fashion, reached a maximum at 12 hr and maintained its high level for 12 hr followed by the decrease to the 20 % of the maximal level at 48 hr (Fig. 2) [35]. Since activation of promoters of TNF-α gene is mediated in part by the transcription factor NF-κB [36,37], we further examined the effect of CCl_4 treatment on the DNA binding activity of hepatic NF-κB at 12 hr after the treatment. DNA biding activity was barely detectable in untreated control rat and vehicle-treated rat (Fig. 3, lanes 2 and 3). In contrast, EMSA revealed a significant NF-κB binding activity in CCl_4-treated rats (Fig. 3, lanes 5–7) [38]. The activity was specific to the NF-κB DNA binding motif since the binding activity could be completely competed away with unlabeled NF-κB oligonucleotide (Fig. 3, lane 14) but not with an unlabeled oligonucleotide sequence that binds to OCT1 transcription factor (Fig. 3, lane 15).

2.2. Expression of hepatic HO-1 mRNA

Since CCl_4 treatment caused significant hepatic injury and inflammation, we examined changes in hepatic HO-1 and HSP 70 mRNA levels after CCl_4 treatment. HO-1 mRNA as well as HSP 70 mRNA was barely detectable in the untreated control liver (Fig. 4). Following CCl_4 treatment, however, HO-1 mRNA started to increase at 3 hr after the treatment, reached a maximum at 6 hr, and then rapidly returned to the almost basal level by 9 hr (Fig. 4) [14]. The maximum level of HO-1 mRNA was ~3-fold of the spleen of normal rats, in which HO-1 is known to be constitutively expressed [1,2]. In contrast to HO-1 mRNA, HSP 70 mRNA significantly increased at 3 hr, reached a maximum at 6 hr, then remained at this level for about 3 hr, followed by a rapid decrease to ~30% of the maximal level by 12 hr, and by a gradual decrease to the basal level by 48 hr (Fig. 4) [14].

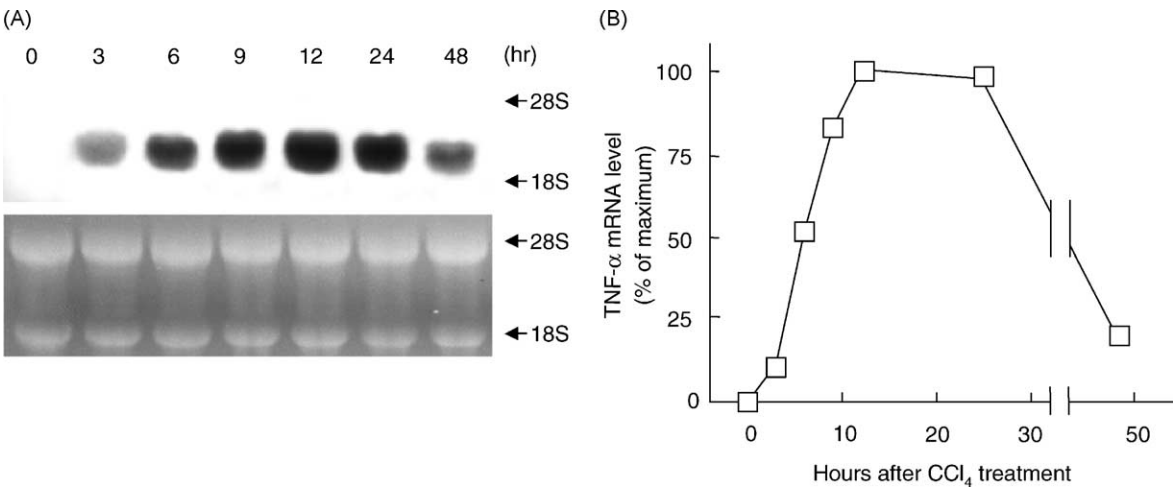

Fig. 2. Effect of CCl_4 treatment on TNF-α gene expression in the liver. Rats were killed at 0, 3, 6, 9, 12, 24, and 48 hr following i.p. injection of CCl_4 (1 mL/kg). Livers were removed for Northern blot analysis as described in Section 1. (A) Twenty micrograms of total RNA was subjected to Northern blot analysis. Shown are the autoradiographic signals of RNA blot hybridized with [α-^{32}P]dCTP-labeled TNF-α cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. (B) The concentrations of TNF-α mRNA are expressed as relative values to the maximal concentration.

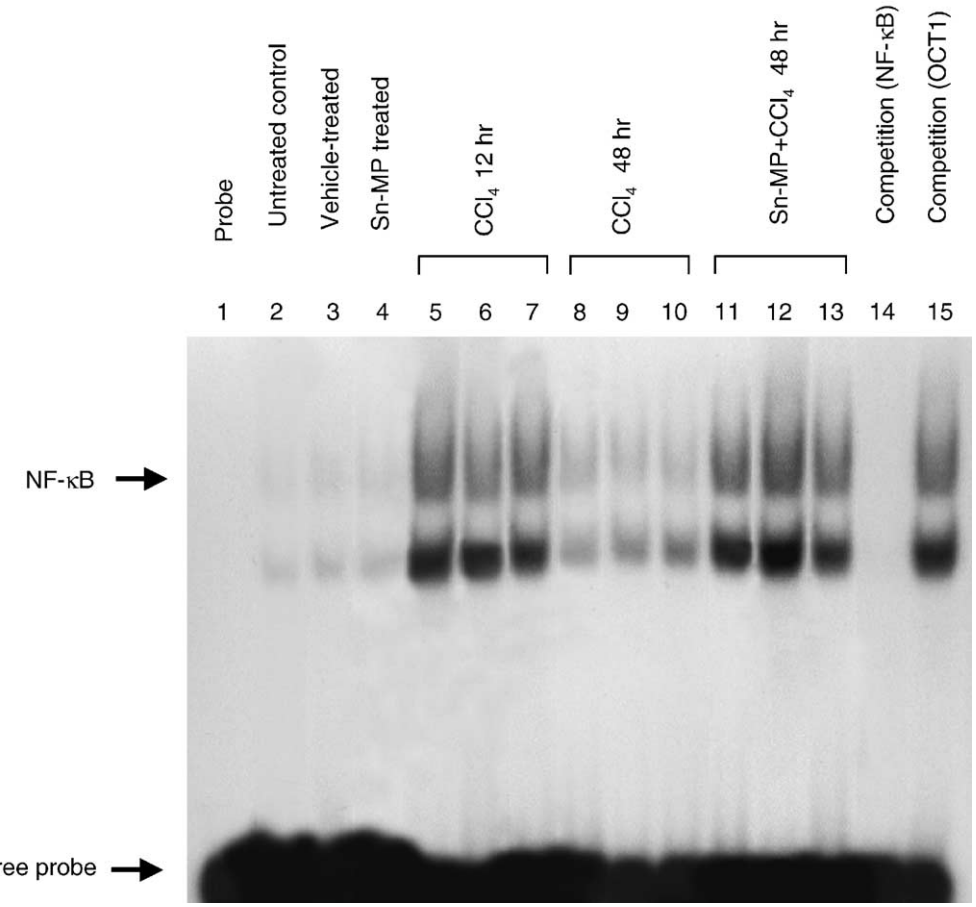

Fig. 3. Activation of NF-κB binding by CCl_4 treatment and its modulation by administration of Sn-MP. Nuclear extracts were prepared from the liver after various treatments. DNA binding activity of NF-κB was analyzed by EMSA as described in Section 1. Three independent experiments showed similar results, and a typical example is shown in the figure. Lane 1, a probe without nuclear extract; lane 2, untreated control rat; lane 3, vehicle-treated control rat; lane 4, control rat with Sn-MP, lanes 5–7, CCl_4-treated rats at 12 hr after the treatment; lanes 8–10, CCl_4-treated rats with vehicle at 48 hr after the treatment; lanes 11–13, CCl_4-treated rats with Sn-MP at 48 hr after the treatment; lane 14, CCl_4-treated rats at 12 hr after the treatment with the addition of 100-fold excess of unlabeled NF-κB oligonucleotide; lane 15, CCl_4-treated rats at 12 hr after the treatment with the addition of unlabeled OCT1 oligonucleotide.

2.3. *Effects of CCl_4 treatment on ALAS-N mRNA expression, microsomal heme concentration, and cytochrome P450 content in the liver*

We also examined levels of ALAS-N mRNA, the rate-limiting enzyme in heme biosynthesis, which is the target of the feed back control by heme [17,22,39,40]. Following CCl_4 treatment, hepatic ALAS-N mRNA levels decreased markedly at 6 hr, and reached a minimum at 9 hr (∼10% of the level in the untreated control liver), followed by a gradual increase and exceeded the basal level at 24 hr, then returned almost to the basal level again at 48 hr (Fig. 5) [16]. This oscillating pattern of ALAS-N mRNA was very similar to that reported for ALAS activity in the liver of rats treated with an i.v. injection of hematin [41]. Since it is known that HO-1 is up-regulated [1,2], while ALAS-N is down-regulated by heme [22,39,40], we determined hepatic microsomal heme concentration after CCl_4 treatment. There was a significant increase in microsomal heme concentration in the liver at 4 hr after the treatment, in comparison to that in the control animals (Fig. 6A). Since microsomal cytochrome P450 is the major protein that accounts for ∼60% of hemeproteins in the liver, and is known to turn over very rapidly [42], we also examined its level in the liver after CCl_4 treatment. In contrast to a significant increase in microsomal heme concentration in the liver, hepatic cytochrome P450 content decreased significantly at 4 hr after the treatment (Fig. 6B) [43]. These findings suggest that the principal portion of the increase in microsomal heme concentration must reflect an increase in free heme which is derived from the destruction of cytochrome P450 [44].

2.4. *Expression of hepatic HO-1 protein*

Although Northern blot analysis indicated that CCl_4 treatment markedly induced HO-1 mRNA in the liver, it is yet to be determined whether it accompanies an increase in HO-1 protein or not. Thus, we examined the level of HO-1 protein by Western blot analysis as well as HO activity 12 hr after CCl_4 treatment. Hepatic HO-1 protein was barely detectable both in the vehicle-treated, and

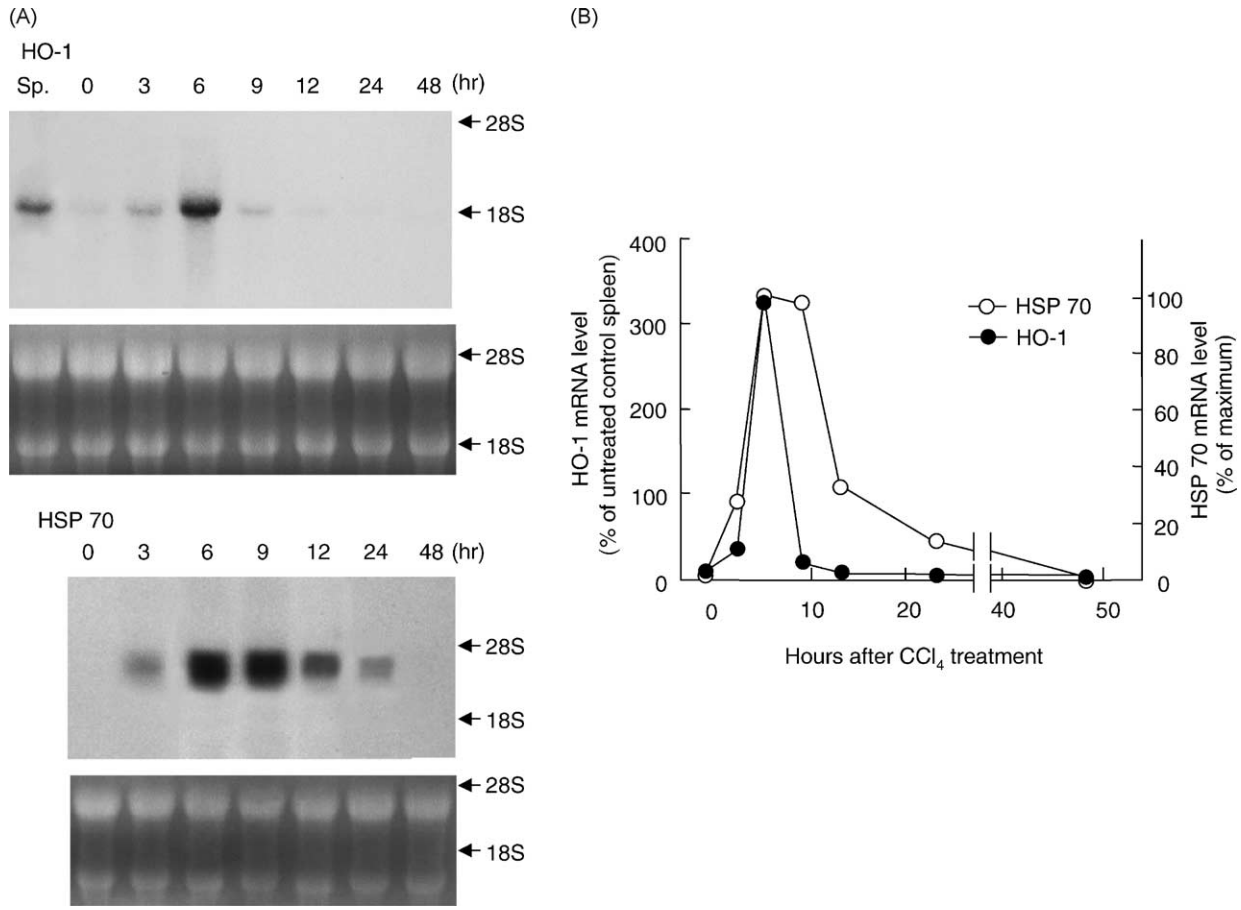

Fig. 4. Effect of CCl_4 treatment on HO-1 and HSP 70 gene expression in the liver. Rats were killed at 0, 3, 6, 9, 12, 24, and 48 hr following i.p. injection of CCl_4 (1 mL/kg). Livers were removed for Northern blot analysis as described in Section 1. (A) Twenty micrograms of total RNA was subjected to Northern blot analysis. Shown are the autoradiographic signals of RNA blot hybridized with [α-^{32}P]dCTP-labeled HO-1 (top) and HSP 70 (bottom) cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. ''Sp.'' denotes untreated control spleen. (B) The concentrations of HO-1 mRNA (●) are expressed as relative values to the concentration of untreated control spleen, and the concentrations of HSP 70 mRNA (○) are expressed as relative values to the maximal concentration.

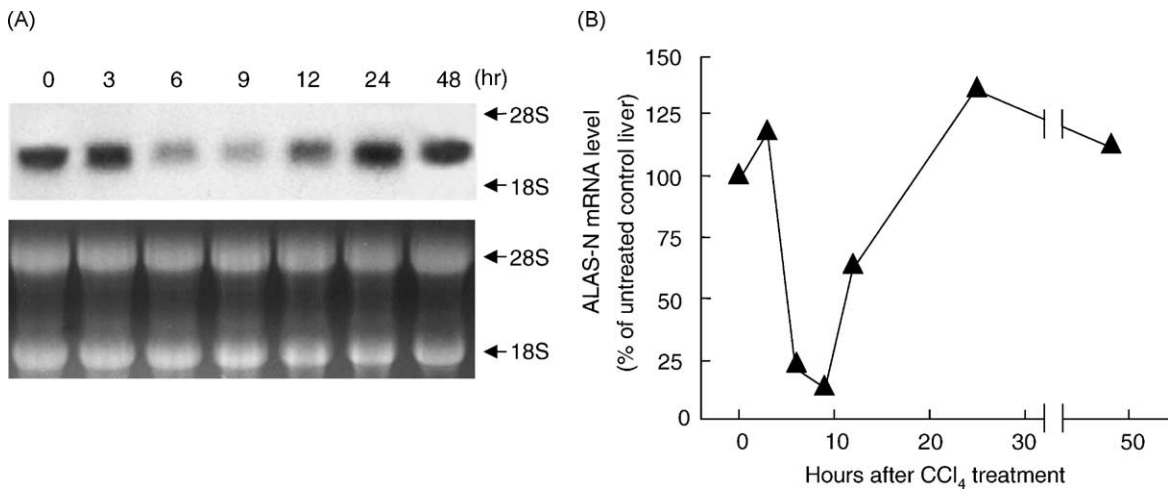

Fig. 5. Effect of CCl_4 treatment on ALAS-N gene expression in the liver. Rats were killed at 0, 3, 6, 9, 12, 24, and 48 hr following i.p. injection of CCl_4 (1 mL/kg). Livers were removed for Northern blot analysis as described in Section 1. (A) Twenty micrograms of total RNA was subjected to Northern blot analysis. Shown are the autoradiographic signals of RNA blot hybridized with [α-^{32}P]dCTP-labeled ALAS-N cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. (B) The concentrations of ALAS-N mRNA are expressed as relative values to the concentration of untreated control liver.

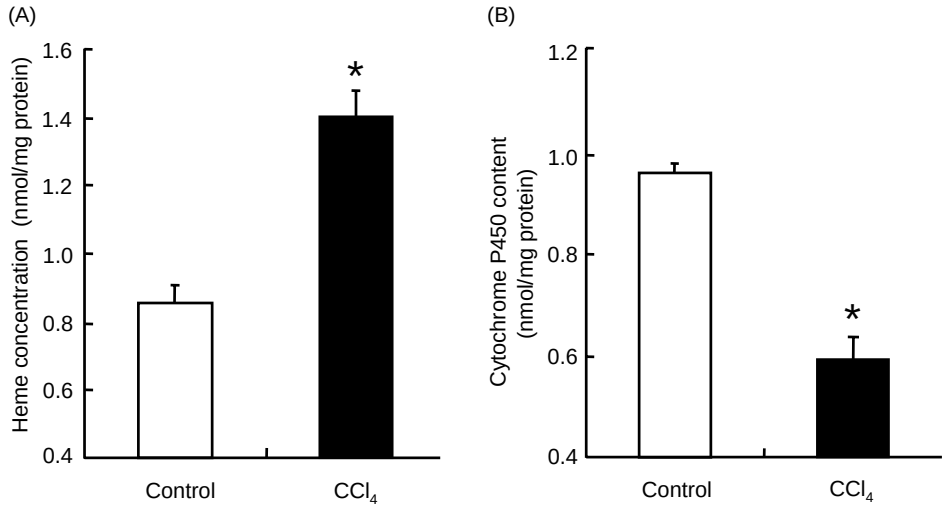

Fig. 6. Effect of CCl_4 treatment on hepatic microsomal heme concentration and cytochrome P450 content. Rats were killed at 4 hr after i.p. injection of CCl_4 (1 mL/kg). Livers were perfused *in situ*, then removed for measurement of microsomal heme concentration (A) and cytochrome P450 content (B) as described in Section 1. Control, the vehicle-treated control rats; CCl_4, CCl_4-treated rats. Statistical analysis by unpaired Student's *t*-test. $^*P < 0.01$ vs. control. Data are shown as means ± SEM (N = 8).

in the untreated control liver, while its level markedly increased in the CCl_4-treated animals (Fig. 7A). Hepatic HO-1 protein induced by CCl_4 reached almost the same level as in the rat liver treated with hemin, the potent inducer of HO-1 [1,2]. Consistent with marked elevation of HO-1 protein, HO activity increased by ~5-fold compared to the control level 12 hr after CCl_4 treatment (Fig. 7B) [16].

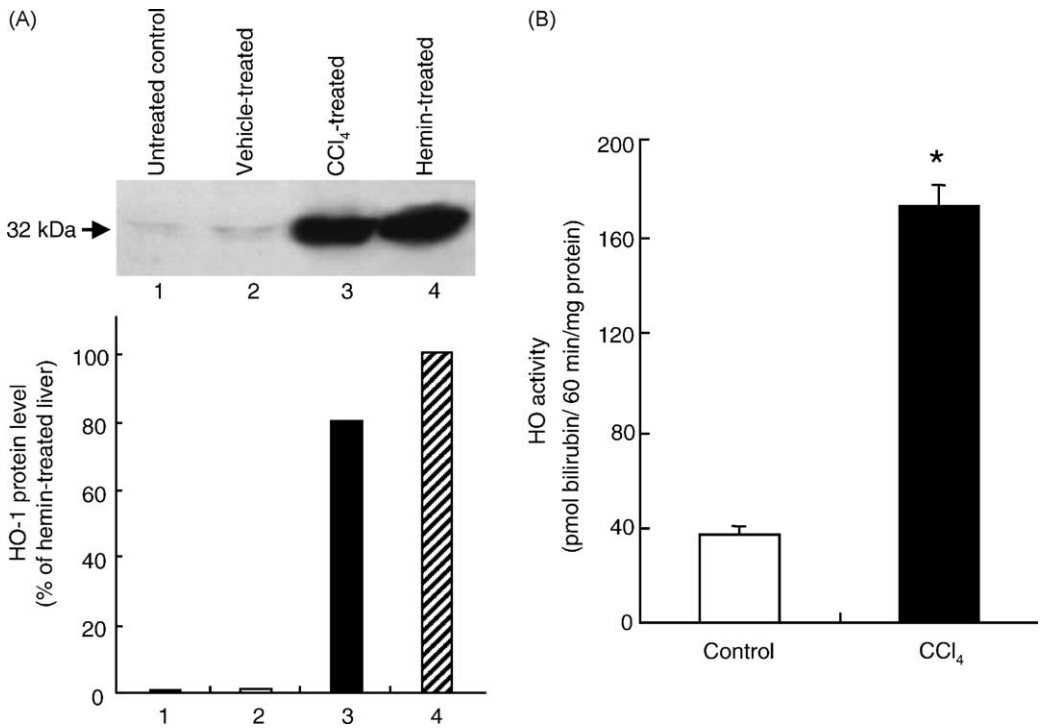

Fig. 7. Effect of CCl_4 treatment on hepatic HO-1 protein expression and HO activity. Rats were killed at 12 hr after i.p. injection of CCl_4 (1 mL/kg) or subcutaneous injection of hemin (30 μmol/kg). Livers were removed for Western blot analysis and HO activity measurement as described in Section 1. (A, Top) Fifty micrograms of microsomal protein was subjected to Western blot analysis. Shown are the chemiluminescent signals of protein blots reacted with a rabbit polyclonal anti-rat HO-1 antibody. Three independent experiments showed similar results, and a typical example is shown in the figure. Lane 1, untreated control rat; lane 2, the vehicle-treated control rat; lane 3, CCl_4-treated rat; lane 4, hemin-treated rat. (A, Bottom) The concentrations of HO-1 protein are expressed as relative values to the concentration of hemin-treated liver. Lane numbers correspond to the number showing treatment in top panel. (B) HO activities are shown as means ± SEM. (N = 8). Control, the vehicle-treated control rats; CCl_4, CCl_4-treated rats. Statistical analysis by an unpaired Student's *t*-test. $^*P < 0.01$ vs. control.

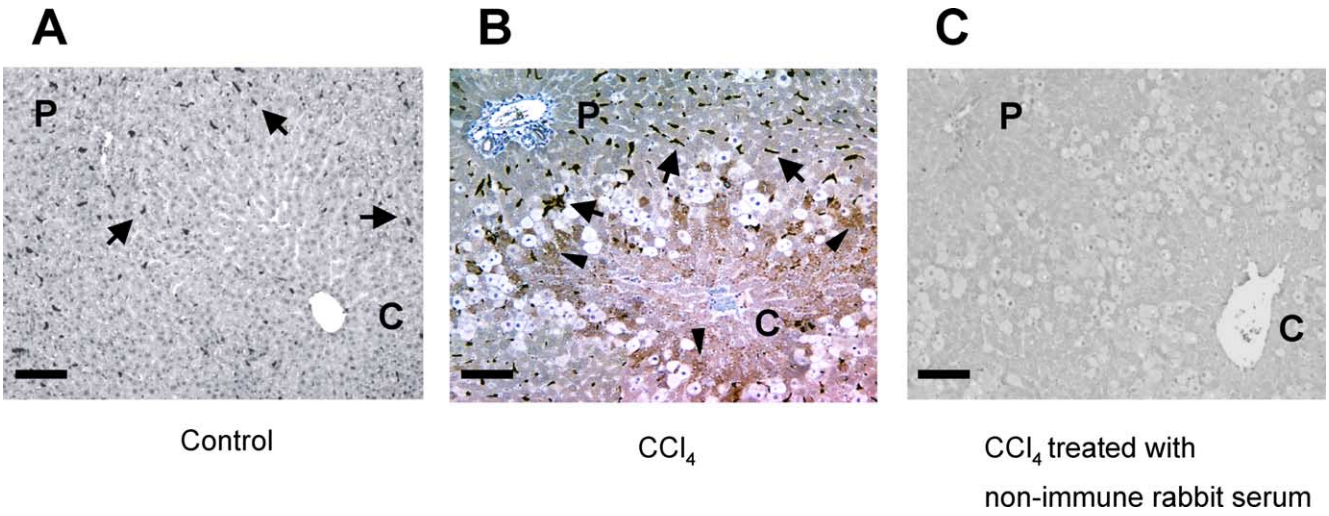

Fig. 8. Immunohistochemistry of HO-1 in the liver of CCl_4-treated rats. Livers at 12 hr after CCl_4 treatment or vehicle were used for immunohistochemical analysis of HO-1, using rabbit polyclonal anti-rat HO-1 antibody as a primary antibody. Immunohistochemical staining was carried out as described in Section 1. Three independent experiments showed similar results, and a typical example is shown in the figure. P and C denote portal tract and central vein, respectively. Arrows and arrowheads indicate positively stained Kupffer cells and hepatocytes, respectively. The bar represents 100 μm. (A) The vehicle-treated control rat. (B) CCl_4-treated rat. (C) CCl_4-treated rat using normal rabbit serum as a primary antibody.

Next, to determine what type of cell(s) in the liver expressed HO-1 protein, we carried out an immunohistochemical study of the liver 12 hr after CCl_4 treatment. In the liver of control rats, positive staining of HO-1 protein was observed only in Kupffer cells (Fig. 8A) [11]. In contrast, HO-1 expression was observed not only in Kupffer cells, but also in hepatocytes, and the level of its expression was markedly increased following CCl_4 treatment (Fig. 8B) [15]. A section of the liver from CCl_4-treated animals showed no signal when treated with nonimmune rabbit serum (Fig. 8C).

2.5. Effect of Sn-MP on hepatic HO activity, microsomal heme concentration, and liver injury

To investigate the functional role of HO-1 in the CCl_4-induced hepatotoxicity, we administered Sn-MP, a specific competitive inhibitor of HO activity [20], 1 hr prior to

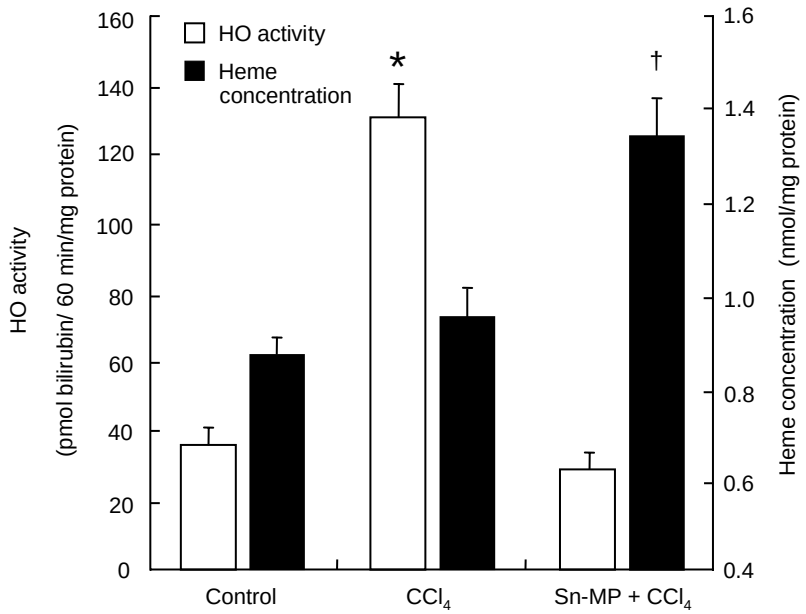

Fig. 9. Effect of Sn-MP administration on hepatic HO activity and microsomal heme concentration. Sn-MP (1 μmol/kg) was administered to rats i.v. 1 hr prior to i.p. injection of CCl_4 (1 mL/kg). Livers were removed at 48 hr after CCl_4 treatment and, microsomal heme concentration and HO activity were measured as described in Section 1. Microsomal heme concentrations (closed bars) and HO activities (open bars) are shown as means ± SEM (N = 8). Control, the vehicle-treated control rats; CCl_4, CCl_4-treated rats with vehicle; Sn-MP + CCl_4, CCl_4-treated rats with Sn-MP. Statistical analysis by ANOVA with Scheffé's *F*-test. $^{*}P < 0.01$ vs. control or Sn-MP + CCl_4; $^{\dagger}P < 0.01$ vs. CCl_4.

CCl_4 treatment, and examined its effect on hepatic HO activity, hepatic microsomal heme concentration, and liver injury. The degree of hepatic injury was assessed by serum ALT activity, hepatic MDA concentration, TNF-α mRNA expression, DNA binding activity of NF-κB, and histological examination. Following CCl_4 treatment, a marked increase in HO activity (∼5-fold of the control level) was observed at 48 hr, while it was significantly suppressed when rats were pretreated with Sn-MP (Fig. 9). Hepatic microsomal heme concentration in the vehicle-pretreated control rats returned to the untreated level by 48 hr after CCl_4 treatment, while it remained at a significantly higher level in Sn-MP-pretreated rats, consistent with the inhibition of HO activity by the metalloporphyrin (Fig. 9). Serum ALT activity in the vehicle-pretreated control rats increased and reached a maximum at 24 hr after CCl_4 treatment, and then returned almost to the control level by 72 hr (Fig. 10A). In contrast, Sn-MP pretreatment resulted in a further increase in serum ALT activity at 48 hr followed by a sustained increase, even at 72 hr (Fig. 10A). Thus, ALT activity both at 48 and 72 hr was significantly higher in the Sn-MP-pretreated rats compared with the vehicle-pretreated control rats, suggesting that hepatic function was worsened by Sn-MP pretreatment. Hepatic MDA levels increased at 48 hr after CCl_4 treatment [34], and its level further increased by ∼1.5-fold by Sn-MP treatment (Table 1). Thus, hepatic MDA level at 48 hr was significantly higher in the Sn-MP-pretreated rats compared with vehicle-pretreated control rats, suggesting that hepatic tissue damage was exacerbated by Sn-MP treatment.

Hepatic TNF-α mRNA levels also increased at 48 hr after CCl_4 treatment, and its level increased further by ∼2.5-fold by Sn-MP pretreatment (Fig. 11). Thus, both MDA and TNF-α mRNA levels was significantly higher in Sn-MP-pretreated animals than in control animals treated with CCl_4 alone. DNA binding activity of NF-κB in the liver also increased at 48 hr after CCl_4 treatment (Fig. 3, lanes 8–10) and its activity increased further by Sn-MP pretreatment (Fig. 3, lanes 11–13). These results suggest that tissue inflammation was significantly increased by

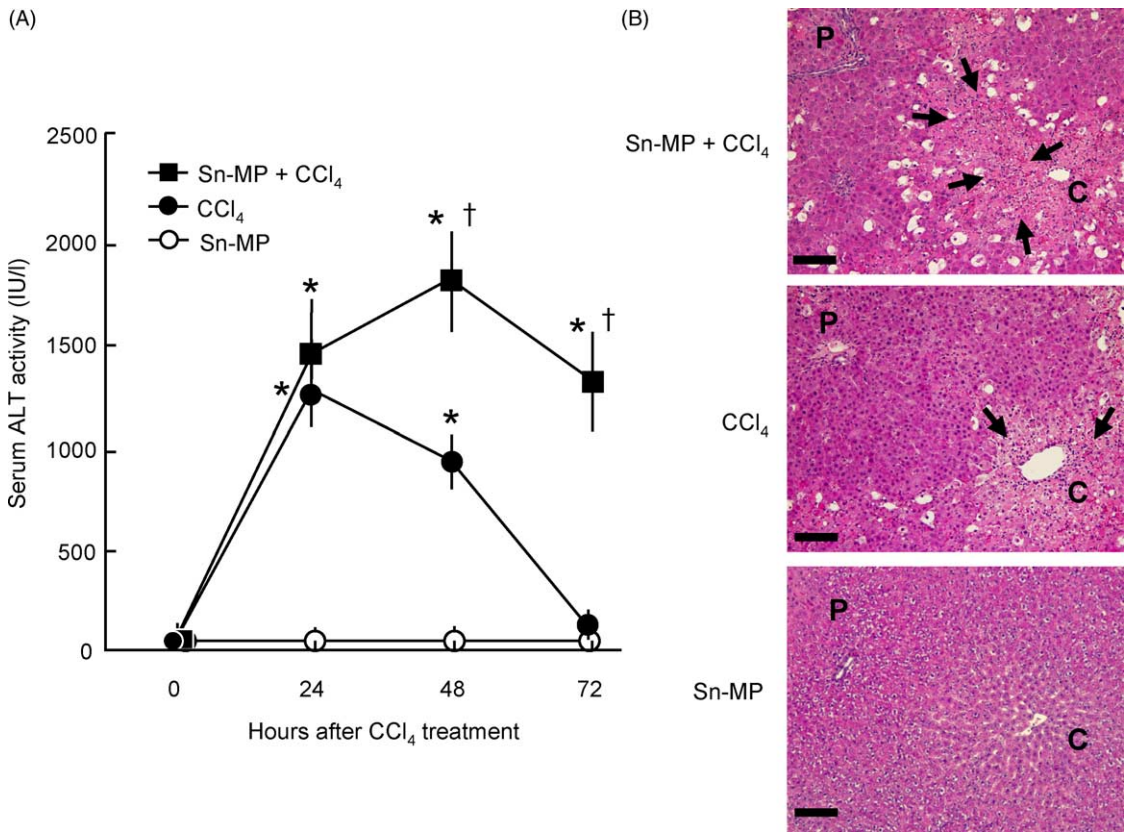

Fig. 10. Effect of Sn-MP administration on serum ALT activity and histological changes of the liver in rats with acute liver injury. Sn-MP (1 μmol/kg) was administered to rats i.v. 1 hr prior to i.p. injection of CCl_4 (1 mL/kg). (A) Rats were killed at 0, 24, 48, and 72 hr after CCl_4 treatment, and whole blood was collected for measurement of serum ALT activity as described in Section 1. Serum ALT activities are shown as means ± SEM (N = 10). (■), CCl_4-treated rats with Sn-MP; (●), CCl_4-treated rats with vehicle; (○), control rats with Sn-MP. Statistical analysis by ANOVA with Scheffé's F-test. $^{*}P < 0.01$ vs. time-matched Sn-MP; $^{\dagger}P < 0.01$ vs. time-matched CCl_4. (B) Forty-eight hours after CCl_4 treatment, livers were removed for histological examination. Three independent experiments showed similar results, and a typical example is shown in the figure. Liver sections were stained with hematoxylin and eosin. P and C denote portal tract and central vein, respectively. Arrows indicate influx of inflammatory cells. The bar represents 100 μm. Sn-MP + CCl_4, CCl_4-treated rat with Sn-MP; CCl_4, CCl_4-treated rat with vehicle; Sn-MP, control rat with Sn-MP.

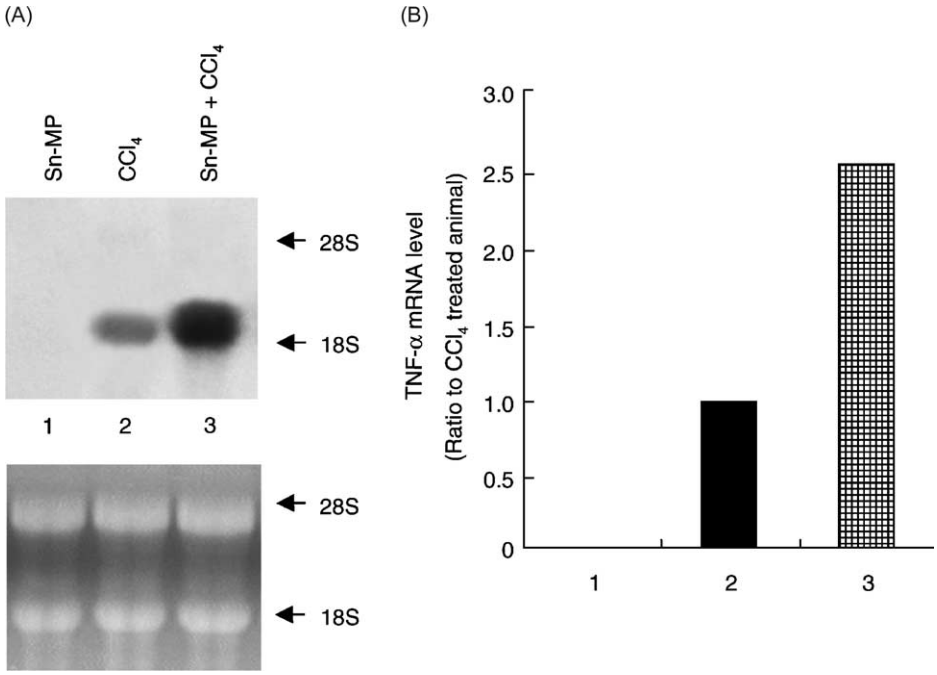

Fig. 11. Effect of Sn-MP administration on hepatic TNF-α gene expression in rats with acute liver injury. Sn-MP (1 μmol/kg) was administered to rats i.v. 1 hr prior to i.p. injection of CCl_4 (1 mL/kg). Rats were killed at 48 hr after CCl_4 treatment. Livers were removed for Northern blot analysis as described in Section 1. (A) Twenty micrograms of total RNA was subjected to Northern blot analysis. Shown are the autoradiographic signals of RNA blot hybridized with [α-^{32}P]dCTP-labeled TNF-α cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. Lane 1, control rat with Sn-MP; lane 2, CCl_4-treated rat with vehicle; lane 3, CCl_4-treated rat with Sn-MP. (B) Concentrations of TNF-α mRNA are expressed as the ratio to CCl_4-treated rat with vehicle. Lane numbers correspond to the number showing treatment in panel A.

Sn-MP. Consistent with the sustained increase in serum ALT activity, hepatic MDA levels, TNF-α mRNA levels and NF-κB activity, histological examination of the liver in Sn-MP-pretreated rats revealed severe and extensive liver cell injuries, especially around the central vein at 48 hr after CCl_4 treatment (Fig. 10B, top). In contrast, hepatocyte injuries were significantly less in the vehicle-pretreated control rats at 48 hr after CCl_4 treatment (Fig. 10B, middle). Sn-MP treatment in control rats had no effect on serum ALT activity (Fig. 10A), hepatic MDA concentration (Table 1), TNF-α mRNA expression (Fig. 11), NF-κB activity (Fig. 3, lane 4) and histological changes (Fig. 10B, bottom). All these findings indicate that hepatic injury by CCl_4 treatment was aggravated further by Sn-MP pretreatment, and suggest that the inhibition of heme catabolism worsened hepatic injury.

2.6. Effects of hemin treatment on DNA binding activity of NF-κB

To assess the contribution of increased hepatic free heme concentration on enhanced DNA binding activity of NF-κB after CCl_4 treatment, we examined the effect of hemin treatment of intact animals on DNA binding activity of NF-κB at 6 hr after hemin treatment. DNA binding activity was only barely detectable in untreated control rats and vehicle-treated rats (Fig. 12, lanes 2 and 3). In contrast, EMSA revealed a significant NF-κB binding activity in 30 μmol/kg of hemin-treated rats (Fig. 12, lanes 4 and 5). Its activity increased further in rats treated with 60 μmol/kg of hemin (Fig. 12, lanes 6 and 7). The activity was specific to the NF-κB since the binding activity was completely competed away with an unlabeled NF-κB oligonucleotide (Fig. 12, lane 8), but not with an unlabeled oligonucleotide sequence that binds to OCT1, a transcription factor unrelated to NF-κB (Fig. 12, lane 9).

2.7. Effects of phenobarbital pretreatment on CCl_4-induced hepatic injury

To assess the contribution of increased hepatic free heme concentration derived from cytochrome P450 to the CCl_4-induced hepatotoxicity, we examined the effect of phenobarbital pretreatment on serum ALT activity and histological findings in the liver after CCl_4 treatment. Serum ALT activity markedly increased in phenobarbital-pretreated animals as compared with that untreated control animals (2048 ± 205 IU/L vs. 1241 ± 213 IU/L, phenobarbital-pretreated rats vs. untreated control rats, respectively, $P < 0.01$) [45]. Histological examination of livers showed increased necrotic areas at the centrilobular zone with more pronounced influx of inflammatory cells in the phenobarbital-pretreated animals as compared with untreated control animals (data not shown) [45].

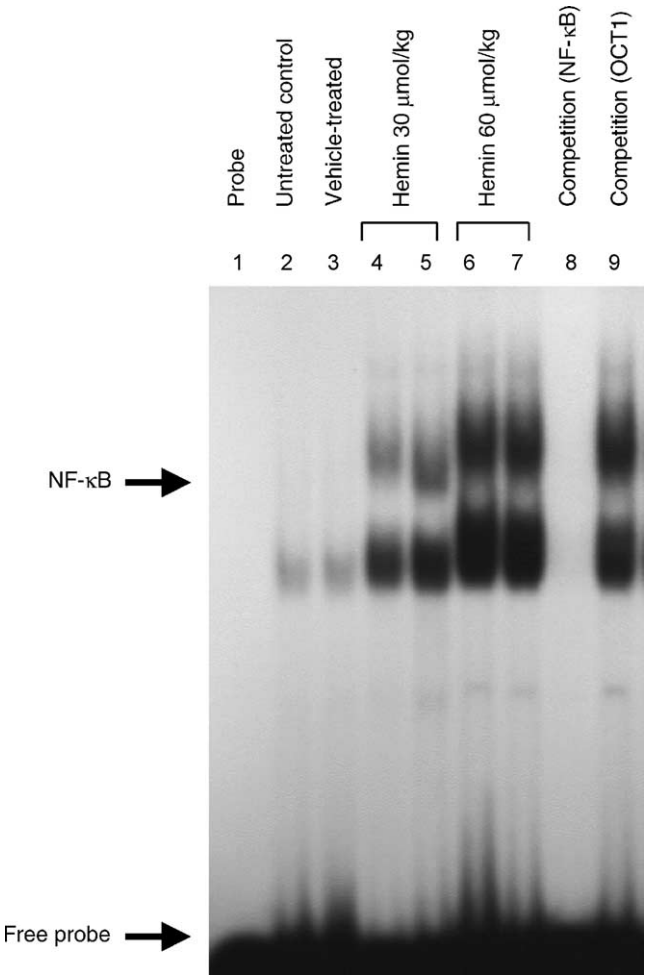

Fig. 12. Activation of hepatic NF-κB binding by hemin treatment. Nuclear extracts were prepared from the liver after various treatments. DNA binding activity of NF-κB was analyzed by EMSA as described in Section 1. Three independent experiments showed similar results, and a typical example is shown in the figure. Lane 1, a probe without nuclear extract; lane 2, untreated control rat; lane 3, vehicle-treated control rat; lanes 4 and 5, hemin (30 μmol/kg, b.wt.)-treated rats at 6 hr after the treatment; lanes 6 and 7, hemin (60 μmol/kg, b.wt.)-treated rats at 6 hr after the treatment; lane 8, hemin (60 μmol/kg)-treated rats at 6 hr after the treatment with the addition of 100-fold excess of unlabeled NF-κB oligonucleotide; lane 9, hemin (60 μmol/kg)-treated rats at 6 hr after the treatment with an unlabeled OCT1 oligonucleotide.

3. Discussion

The present study demonstrates that hepatic HO-1 mRNA, HO-1 protein, and HO enzyme activity were all markedly increased in hepatocytes of CCl_4-induced acute hepatic injury. A rapid and significant induction of HO-1 mRNA took place following CCl_4 treatment, which may be partially mediated by a free radical mechanism, since hepatic MDA levels were significantly increased after CCl_4 treatment. HO-1 induction in this model also accompanied an increase in microsomal heme concentration which may have further contributed to the generation of free radicals (Fig. 6). In support of this hypothesis, inhibition of HO activity by Sn-MP, a specific competitive inhibitor of the enzyme, led to a significant increase in hepatic microsomal heme and resulted in a further aggravation of hepatic injury, augmented TNF-α mRNA levels and enhanced NF-κB activity. The increase in microsomal heme is thought to be due to an increase in free heme which was released from cytochrome P450 during its metabolism of CCl_4, since the increase in free heme coincided with the decline of cytochrome P450 at 4 hr after CCl_4 treatment. The inflammatory flare induced by Sn-MP is clearly due to the inhibition of CCl_4-induced HO activity, since Sn-MP administration to control animals showed no effect. These results suggest that a strong induction response of HO-1 by CCl_4 treatment is to protect liver cells from CCl_4-induced oxidative cellular injuries, and that its inhibition results in the aggravation of tissue injuries.

HO-1 mRNA increased and reached a maximum level at 6 hr after CCl_4 treatment, and then rapidly returned almost to the control level by 9 hr (Fig. 4) [14]. In contrast, HSP 70 mRNA increased significantly at 3 hr after CCl_4 treatment, reaching a maximum at 6 hr, maintained its concentration up to 9 hr, and then decreased to the control levels at 48 hr (Fig. 4) [14]. These results suggest that, although both HO-1 and HSP 70 are heat shock proteins, their gene expression is regulated separately, resulting in a different pattern of time courses [10,11].

In contrast to HO-1 induction, gene expression of ALAS-N, the rate-limiting enzyme in heme biosynthesis [17], was significantly suppressed after CCl_4 treatment (Fig. 5). Namely, ALAS-N mRNA level decreased drastically at 6 hr and reached the minimum at 9 hr after CCl_4 treatment (Fig. 5) [16]. This inverse kinetics between HO-1 and ALAS-N gene expression strongly suggests that there may be a significant increase in hepatic intracellular free heme after CCl_4 treatment, since it is known that HO-1 is up-regulated [1,2], while ALAS-N is down-regulated by free heme [22,39,40]. ‘‘Free heme’’ is considered as a protein-unbound heme which is either newly synthesized but not yet combined with its apoprotein, or free heme released from a hemeprotein but not yet destroyed by HO. Free heme is believed to play a critical role in regulating gene expression of ALAS-N and HO-1 [39,40]. Consistent with this hypothesis, prior to induction of HO-1 mRNA, there was a rapid decline in microsomal cytochrome P450 and a concurrent increase in microsomal heme at 4 hr after CCl_4 treatment (Fig. 6), suggesting that an increased free heme concentration contributed to the induction of HO-1 mRNA as well as to the repression of ALAS-N mRNA.

It should also be noted that ALAS-N mRNA levels showed an oscillating response up to 48 hr following CCl_4 treatment (Fig. 5). This pattern of oscillation is very similar to that reported for ALAS activity in the liver of rats after the administration of hematin [41]. The oscillatory response of ALAS activity was also thought to be due to an oscillatory change in free heme concentration [41]. It is known that hepatic cytochrome P450 undergoes

significant destruction during the metabolism of CCl_4 [43,44]. Thus, it is likely that heme may be released from cytochrome P450 when CCl_4 is metabolized, and is reflected in an increase in intracellular free heme concentration. Consistent with this hypothesis, it is known that there is a rapid increase in microsomal heme concentration following reperfusion in a rat model of ischemic acute renal failure, and in hepatic free heme concentration, which was measured as an increased enzymatic activity of tryptophan pyrrolase, a heme dependent enzyme, in a rat model of halothane-induced hepatic injury [10,11]. Both conditions also accompanied induction of HO-1 and suppression of ALAS-N, reflecting an increase in free heme concentration [10,11].

While heme is required as the prosthetic group for hemeproteins that are necessary for cellular viability [46], an excess amount of free heme is deleterious, since it acts as a potent pro-oxidant, leading to oxygen radical formation [47]. In the CCl_4-induced hepatic injury, CCl_4 is metabolized by cytochrome P450 reductively to a trichloromethyl free radical ($CCl_3^{\bullet}$) intermediate, which catalyzes lipid peroxidation [13]. Thus, an increase in intracellular heme concentration may contribute further to the free radical production by CCl_4 treatment, leading to additional cellular injuries [8–11].

Consistent with a marked elevation in HO-1 mRNA level, HO-1 protein was also increased in the liver (Fig. 7A). HO-1 induction occurred at 12 hr after CCl_4 treatment exclusively in hepatocytes around the central vein, where severe cell injuries were observed (Fig. 8). HO activity was also significantly increased at this time (Fig. 7B), indicating that the functional HO-1 protein was increased. HO-1 is known not only to act as a heme cleaving enzyme [1,2], but also behaves as a stress-inducible protein, a heat shock protein [3–5], and an acute phase reactant [48]. These findings suggest that, in the CCl_4-induced hepatic injury, HO-1 induction occurs in order to protect hepatic cells from an additional injury caused by free heme. This interpretation is also consistent with the finding that liver cell injury was aggravated when HO activity was inhibited by Sn-MP, a specific competitive inhibitor of HO (Fig. 10; Table 1) [20]. In addition, tissue inflammation, as judged not only by increased TNF-α mRNA expression but also by increased NF-κB activity, was markedly exacerbated by Sn-MP pretreatment (Figs. 3 and 11). Inhibition of the CCl_4-induced HO activity by Sn-MP was also confirmed directly by the assay of HO enzyme activity (Fig. 9). In contrast to the CCl_4-treated animals, Sn-MP did not influence TNF-α level (Fig. 11), NF-κB activity (Fig. 3, lane 4), and liver injury in untreated control animals (Fig. 10A and B, bottom; Table 1).

Our results also demonstrate that, following the initial rapid rise at 4 hr, microsomal heme concentration started to decrease after the induction of HO-1 at 6 hr, then returned to the control level at 48 hr after treatment (Figs. 6 and 9), which coincided with the amelioration of liver injury, as judged by both measurements of serum ALT activity and histological changes (Fig. 10). In the Sn-MP-pretreated rats, microsomal heme concentration remained elevated even at 48 hr after CCl_4 treatment (Fig. 9) and resulted in an exacerbation of liver injuries (Fig. 10; Table 1) as well as in a pronounced tissue inflammation (Figs. 3 and 11). These results suggest that the hepatotoxicity of CCl_4 is due not only to its reactive intermediate but also to free heme, a potent pro-oxidant [47], which may be largely released from cytochrome P450. This hypothesis was also supported not only by the fact that CCl_4-induced hepatotoxicity is worsened by phenobarbital pretreatment in comparison to the untreated control animals (2048 ± 205 IU/L vs. 1241 ± 213 IU/L, phenobarbital-pretreated rats vs. untreated control rats, respectively, $P < 0.01$) [45], but also by the fact that the DNA binding activity of NF-κB in hemin-treated animals significantly increased in a dose-dependent manner (Fig. 12). These data also suggest that HO-1 induction in the CCl_4-treated animals plays a significant protective role against oxidative liver injury by removing excessive intracellular free heme. Previously we have also described that HO-1 plays an important protective role against oxidative tissue injury in other models such as halothane-induced hepatic injury and ischemic acute renal failure [8–11]. Furthermore, it has been reported that hepatic HO-1 is significantly increased in acetaminophen-induced hepatic injury, another animal model of hepatotoxicity [49]. Our findings in the present study are in an excellent agreement with these previous studies, and extend the protective role of HO-1 to the CCl_4-induced hepatic injury.

HO-1 oxidatively cleaves heme, and yields CO, iron, and biliverdin IXα, which is then reduced to bilirubin IXα by biliverdin reductase [1,2]. In doing so, HO-1 reduces the cellular concentration of free heme, the pro-oxidant [47]. Although an excess free iron would be cytotoxic since it catalyzes Fenton reaction to generate free radicals [50], iron produced by HO is immediately inactivated by sequestration into ferritin [51]. In addition, bilirubin IXα functions as a potent endogenous anti-oxidant [52], which may further contribute to the cellular defense. Recently, it has been reported that CO has anti-inflammatory and anti-apoptotic properties [53,54]. Thus, CO may also have an important protective function against oxidative tissue injuries [55]. While it is unclear to what extent each mechanism may contribute, all these reactions may provide a fundamental protective milieu for hepatocytes in coping against CCl_4-induced hepatotoxicity, by favoring the removal of oxidants as well as by increasing the amount of anti-oxidants.

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