SPANDIDOS PUBLICATIONS

Highly liver-specific heme oxygenase-1 induction by interleukin-11 prevents carbon tetrachloride-induced hepatotoxicity

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Abstract. Heme oxygenase (HO)-1, the rate-limiting enzyme in heme catabolism, can be induced in response to various oxidative stimuli, and its induction is thought to be critical in the cellular defense against oxidative tissue injuries. Carbon tetrachloride (CCl_4) treatment of rats causes lipid peroxidation of cell membranes and produces massive hepatic injury. We previously demonstrated that HO-1 induction following CCl_4 treatment is an essential part of the cellular defense against the CCl_4-inducible toxic changes. As recombinant human interleukin-11 (rhIL-11) has been shown to induce HO-1 in cultured hepatoma cells, we examined the effect of rhIL-11 *in vivo* in rats on the CCl_4-induced tissue injury. rhIL-11 treatment of animals by itself markedly induced HO-1 mRNA and its functional protein principally in the liver. rhIL-11 treatment (150 μg/kg) of the CCl_4-administered (1 ml/kg) animals led to a further increase in HO-1 mRNA, while it markedly suppressed CCl_4-induced serum alanine transaminase, hepatic malondialdehyde formation, tumor necrosis factor-α mRNA, nitric oxide synthase mRNA, nuclear factor-κB DNA-binding activity, as well as inflammatory changes of hepatocytes. In contrast, inhibition of HO activity by tin-mesoporphyrin, a competitive specific inhibitor of HO, entirely abolished the cytoprotective effect of rhIL-11. These findings thus demonstrate that rhIL-11 confers significant protection against CCl_4-induced hepatic injury by virtue of its liver-specific HO-1 induction.

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Introduction

Heme oxygenase (HO)-1, the rate-limiting enzyme in heme catabolism (1), is also a heat shock protein (HSP 32) (2) as well as a stress-inducible protein (3). HO-1 is induced by various oxidative stresses, and is thought to play an important cytoprotective role against oxidative cellular injuries (4-6). Treatment of animals with CCl_4 elicits severe hepatic injury (7,8), which is due to a highly reactive intermediate of CCl_4 generated by cytochrome P450. The reactive CCl_4 metabolite causes lipid peroxidation, and breaks down cellular membranes (9). We have previously demonstrated that there is also HO-1 induction in the liver of CCl_4-treated rats, and it represents the fundamental protective response against oxidative tissue damage (7). Recombinant human interleukin-11 (rhIL-11) is a pleiotropic cytokine that induces megakaryocytic cell differentiation (10). It also induces HO-1 in cultured human hepatoma cells (11), and suppresses the production of pro-inflammatory mediators from activated macrophages (12). However, it remains unclear whether rhIL-11 may ameliorate CCl_4-induced hepatic injury *in vivo* in animals. In the present study, we examined the effect of rhIL-11 on HO-1 expression and CCl_4-induced hepatic injury in rats. The degree of hepatic injury and inflammation was assessed by measurements of serum alanine transaminase (ALT) activity, hepatic histology, malondialdehyde (MDA) content, tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) mRNAs, as well as DNA binding activity of nuclear factor (NF)-κB. In this study, we report that rhIL-11 treatment of rats induces both HO-1 mRNA and its protein highly specifically in the liver. rhIL-11 treatment also markedly ameliorated CCl_4-induced oxidative hepatic injury and inflammation. In contrast,

administration of tin-mesoporphyrin (SnMP), a specific competitive inhibitor of HO activity (13), to rhIL-11-treated animals completely abolished the cytoprotective effect of rhIL-11. Our findings indicate that treatment with rhIL-11 highly protective against the CCl_4-induced hepatic injury by virtue of its ability to induce HO-1 in the liver. Induction of hepatic HO-1 by rhIL-11 treatment thus may be a useful therapeutic means against oxidative liver injuries, including CCl_4 intoxication.

Materials and methods

Animals. The studies reported herein conform to guidelines for the care and use of laboratory animals established by Animal Use and Care Committee of the Okayama University Medical School. Male Sprague-Dawley rats weighing 200-260 g were purchased from CLEA Japan. They were housed in a temperature-controlled (25˚C) room with alternating 12 h/12 h light/dark cycles and were allowed free access to water and chow diet until the start of experiments.

Experimental design. Rats were administered with various doses of rhIL-11 (0-500 μg/kg) intravenously via the tail vein. rhIL-11 was generously provided by Astellas Pharmaceutical Co. (Tokyo, Japan). rhIL-11 had been dissolved in endotoxin-free Dulbecco's phosphate-buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO, USA) containing 0.1% (w/v) bovine serum albumin (Sigma Chemical Co.) as described previously (rhIL-11 group) (14). Control rats received the same volume of the vehicle (Vehicle group). Some rats were also treated with SnMP (0.5 μmol/kg; Frontier Scientific, Logan, UT, USA), a competitive inhibitor of HO activity (13), at 1 h before rhIL-11 (SnMP/rhIL-11 group) or vehicle treatment (SnMP group). SnMP was prepared as described below, immediately before use. SnMP was dissolved in a small volume of 0.1 N NaOH, and then pH of the solution was adjusted to 7.6 with 0.01 M sodium phosphate buffer (7). To induce hepatic injury, animals were injected intraperitoneally with CCl_4 (Sigma Chemical Co.) (1 ml/kg) dissolved in an equal volume of corn oil (7). Control rats received the same volume of corn oil (Control group). To test the effect of rhIL-11 on CCl_4-induced hepatic injury, CCl_4-treated animals were divided into two groups: 1) treatment with rhIL-11 (CCl_4/rhIL-11 group), and 2) treatment with the vehicle (CCl_4/Vehicle group) immediately after CCl_4 treatment. In addition, the third group of CCl_4/rhIL-11-treated rats was also treated with SnMP (0.5 μmol/kg) 1 h prior to CCl_4 treatment (SnMP/CCl_4/rhIL-11 group). After the various treatments, animals were returned to cages and allowed free access to food and water. Following light anesthesia with ethyl ether, animals were sacrificed by decapitation at each defined time point (0-24 h). Blood was collected for serum isolation, and the livers were excised. Livers were then frozen immediately in liquid nitrogen and stored at -80˚C until use for the preparation of RNA and nuclear extracts, and measurement of MDA concentration. For the preparation of hepatic microsomal fraction, livers were removed after perfusion *in situ* with physiological saline until the venous effluent became clear, and microsomal pellets were prepared as described in Western blot analysis.

cDNA probes. Template cDNAs for HO-1, TNF-α and iNOS were prepared as described previously (14,15). All probes used for Northern blot analysis were [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA)-labeled cDNA probes prepared by using a random primer DNA labeling system (Amersham Pharmacia Biotech) according to the manufacturer's instructions (14,15).

RNA isolation and Northern blot analysis. Total RNA was isolated from rat tissues using Tri-Reagent™ (Sigma Chemical Co.) according to the manufacturer's protocol. Northern blotting was performed as described previously (16). Total RNA (20 μg) were subjected to electrophoresis in a 1.2% (w/v) agarose gel containing 6.5% (v/v) formaldehyde. After blotting on a sheet of Bio-Rad Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA, USA), RNA samples were hybridized with [α-^{32}P]dCTP-labeled cDNA probes followed by washing under stringent conditions. The membrane was exposed to a sheet of Fuji Medical radiograph film with an intensifying screen at -70˚C, and autoradiographs and 18S ribosomal RNA were quantified by using an image scanner (GelPrint™ 2000i, Genomic Solutions, Ann Arbor, MI, USA) and a computerized image analysis software (Basic Quantifier™ version 3.0, Genomic Solutions). Relative amounts of radiolabelled cDNA that hybridized to the blots were normalized to 18S ribosomal RNA levels for loading errors.

Western blot analysis. Livers were homogenized in 3 volumes of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and centrifuged at 10,000 g for 30 min at 4˚C, followed by 104,000 g centrifugation of the supernatant for 60 min at 4˚C, to obtain the microsomal fraction as a pellet. The pellet was resuspended in 0.02 M Tris-HCl (pH 7.4) containing 20% (v/v) glycerol and microsomal protein content was determined by the method of Lowry *et al* (17). Western blotting was performed using a rabbit anti-HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, BC, Canada) as described previously (7). Chemiluminescent 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.

Histologic study. For histological examination, liver 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. Immunohistochemical analysis was performed using a rabbit polyclonal anti-rat HO-1 (Stress-Gen Biotechnologies) as described previously (7). Normal rabbit serum was used as control for non-specific staining. Sections were counterstained with hematoxylin.

Hepatic HO activity. Livers were homogenized in 3 volumes of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. Microsomal fractions were prepared as described above, and HO activity was measured spectrophotometrically as described previously (7). The cytosolic fraction prepared from the livers of adult untreated rats served as a source of

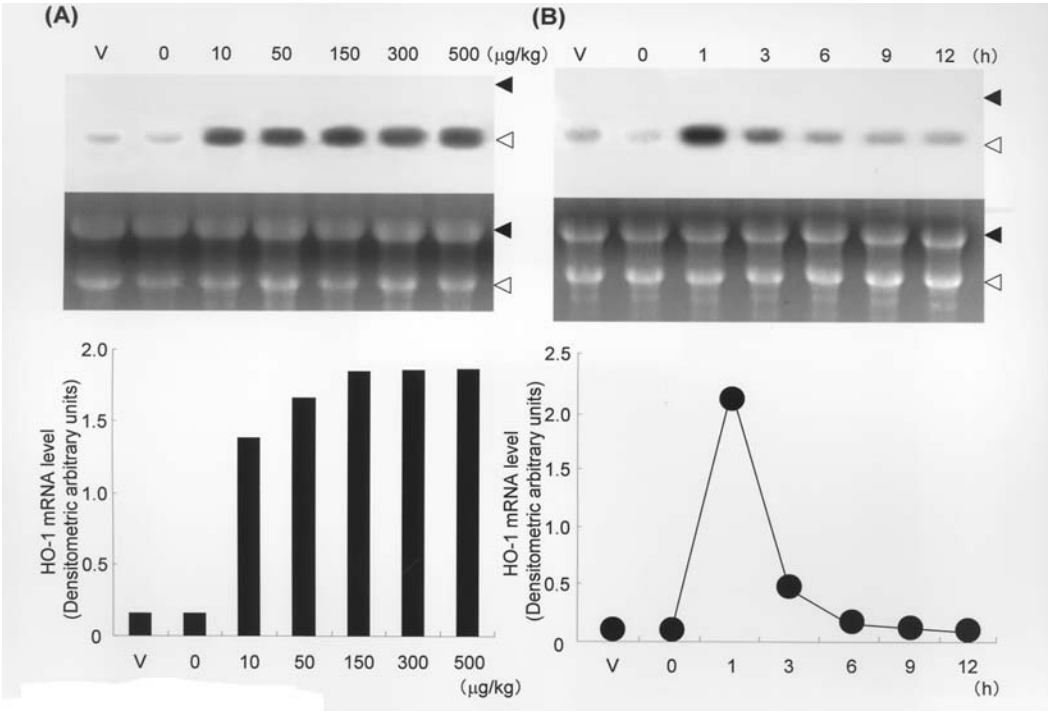


Figure 1. Effect of recombinant human interleukin-11 (rhIL-11) treatment on heme oxygenase-1 (HO-1) gene expression in the rat liver. (A) Dose-response relationship of hepatic HO-1 gene expression after rhIL-11 treatment. Rats were injected intravenously with rhIL-11 doses of 0, 10, 50, 150, 300, 500 μg/kg, or vehicle, the volume of which was identical to that of 500 μg/kg of rhIL-11 solution, were sacrificed at 1 h after the injection. Livers were removed for Northern blot analysis. (B) Time course of hepatic HO-1 gene expression after rhIL-11 treatment. Rats injected intravenously with rhIL-11 (150 μg/kg) were sacrificed at 0, 1, 3, 6, 9, and 12 h after injection. Vehicle-treated rats were also sacrificed at 1 h after injection. Livers were removed for Northern blot analysis. Top panel, 20 μg 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 cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA; V, vehicle-treated control animal. Three independent experiments showed similar results, and a typical example is shown. Bottom panel, levels of HO-1 mRNA are expressed as densitometric arbitrary units.

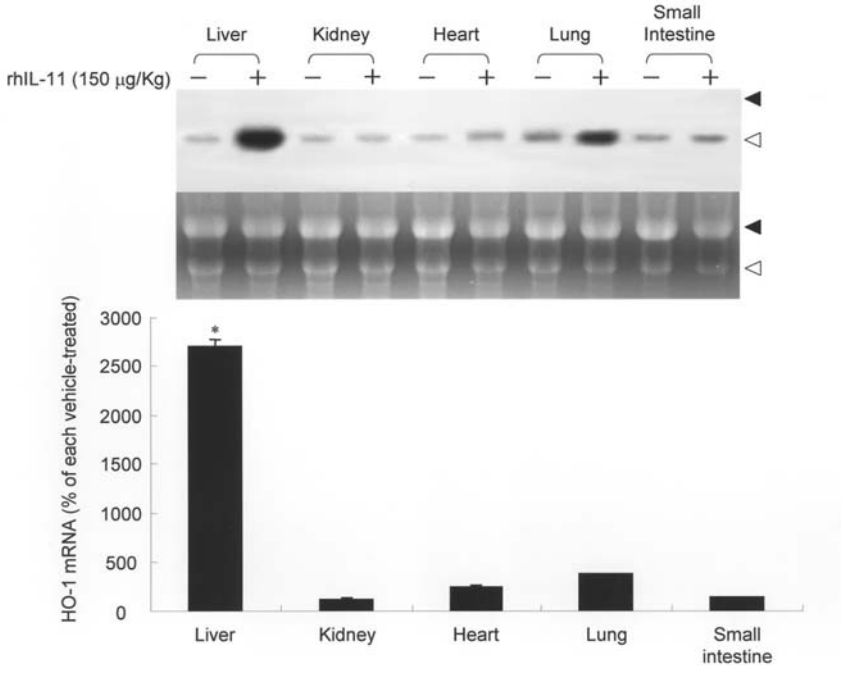


Figure 2. Effect of recombinant human interleukin-11 (rhIL-11) treatment on heme oxygenase-1 (HO-1) gene expression in various organs of rats. Rats injected intravenously with rhIL-11 (150 μg/kg) or vehicle were sacrificed at 1 h after injection. Liver kidney, heart, lung, and small intestine were removed for Northern blot analysis. Top panel, 20 μg of total RNA were subjected to Northern blot analysis. Shown are the autoradiographic signals of the RNA blot hybridized with [α-^{32}P]dCTP labeled HO-1 cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA; Bottom panel, the levels of HO-1 mRNA are expressed as the percentage of each vehicle-treated animal. Data are presented as means ± SEM (n=3 per each organ). Statistical analysis by analysis of variance with the Scheffé F-test. $^{*}p<0.01$ vs. kidney, heart, lung, or small intestine.

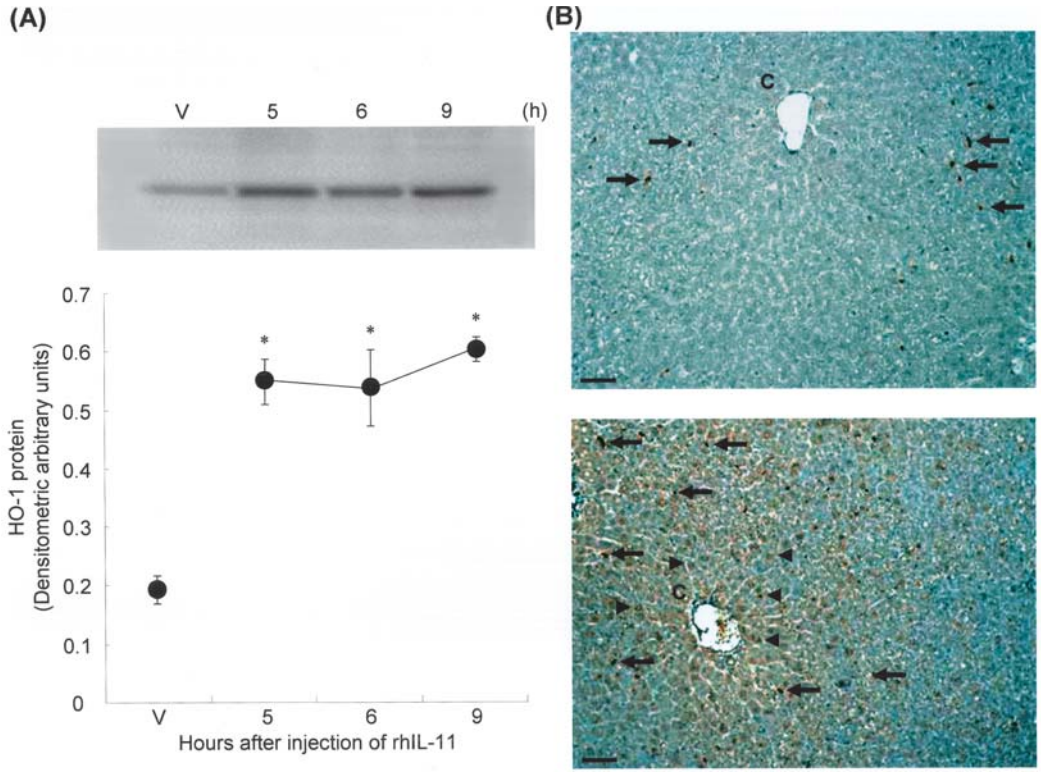


Figure 3. Effect of recombinant human interleukin-11 (rhIL-11) treatment on heme oxygenase-1 (HO-1) protein expression in the rat liver. (A) Top panel, rats injected intravenously with rhIL-11 (150 μg/kg) or vehicle were sacrificed at 5, 6, and 9 h after injection. Livers were removed for Western blot analysis as described in Materials and methods. 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. V, vehicle-treated control animal. Bottom panel, levels of HO-1 protein are expressed as densitometric arbitrary units. Data are presented as means ± SEM (n=3 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. $^*p<0.01$ vs. vehicle-treated control animals. (B) Immunohistochemistry of HO-1 in the liver of rhIL-11 treated rats. Liver sections from rats 6 h after the injection intravenously with rhIL-11 (150 μg/kg) or vehicle were used for immunohistochemical detection of HO-1 by using rabbit polyclonal anti-rat HO-1 as a primary antibody. Immunohistochemical staining was carried out as described in Materials and methods. Each photograph represents at least three independent experiments. Top panel, vehicle-treated control liver; bottom panel, rhIL-11-treated liver. Arrow and arrowheads indicate HO-1 positively stained Kupffer cells and hepatocytes, respectively. The bars, 100 μm.

biliverdin reductase. HO activity was expressed as nmol of bilirubin formed per mg of protein per 60 min.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared from frozen liver sections as described by Dignam and colleagues (18), with some modifications (7). Nuclear extracts were incubated with [γ-^{32}P]ATP labeled NF-κB consensus oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') (19) 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, Madison, WI). Following the incubation, the reaction mixture was subjected to electrophoresis in non-denaturing 5% polyacrylamide gel. The gels were vacuum-dried and exposed to X-ray film at -70˚C. 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') (20).

Measurement of hepatic MDA concentration. In order to estimate the extension of lipid peroxidation, hepatic MDA concentration was measured according to methods of Ohkawa *et al* (21) with some modifications (22). Frozen liver tissues were homogenized with 30 (w/v) % of cold 1.15% KCl-0.1 M phosphate buffer pH 7.4. The protein contents of liver homogenates as determined by the method of Lowry *et al* (17). In a test tube, each 0.1 ml of homogenate was mixed with 0.7 ml of water, 50 μl of 0.8% butylated hydroxytoluene solution in glacial acetic acid, 0.2 ml of 8.1% SDS solution, 1.5 ml of 20% acetic acid solution adjusted at pH 3.5 with 10 N NaOH, 0.1 ml of water and 1.5 ml of 0.8% thiobarbituric acid solution. The mixture was kept at 5˚C for 60 min and then heated 100˚C for 60 min. After cooling, the mixture was extracted with 1.0 ml of water and 5.0 ml of n-butanol:pyridine (15:1, v/v). The mixture was centrifuged at 1600 g for 10 min, and the absorbance at 532 nm of the organic phase was measured. The calibration curve of an accurately prepared standard MDA solution was also run for quantification. The concentration of MDA was expressed as nmol per mg protein.

Assay of serum ALT activity. Serum was separated from whole blood by centrifugation at 1600 g for 10 min at 4˚C, and serum ALT activity was measured using an automatic biochemical analyzer calibrated with quality control standards (DADE Dimension AR Clinical Chemistry System™, GMI, Inc. Ramsey, MN, USA).

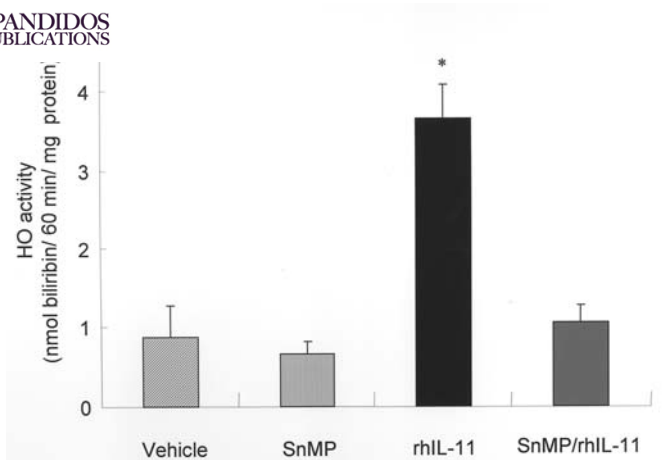


Figure 4. Effect of recombinant human interleukin-11 (rhIL-11) treatment on heme oxygenase (HO) activity in the rat liver. Rats were injected intravenously with rhIL-11 (150 μg/kg) or vehicle via tail vein. Some rats were additionally administered with tin mesoporphyrin (SnMP; 0.5 μmol/kg) intravenously 1 h prior to the treatment with rhIL-11 or vehicle. The liver was removed at 5 h after rhIL-11 or vehicle administration, and HO activity was measured as described in Materials and methods. Vehicle, vehicle-treated control animals; SnMP, SnMP-administered animals; rhIL-11, rhIL-11-treated animals; SnMP/rhIL-11, rhIL-11 treatment with SnMP administration. Data are presented as means ± SEM (n=8 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. *p<0.05 vs. Vehicle, SnMP or SnMP/rhIL-11.

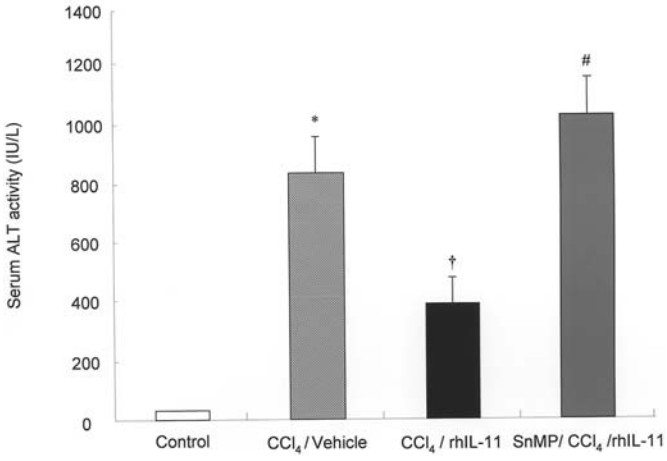


Figure 5. Effect of recombinant human interleukin-11 (rhIL-11) treatment on serum alanine transaminase (ALT) activity in CCl_4-induced liver injury. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Tin mesoporphyrin (SnMP, 0.5 μmol/kg) was administered to rats intravenously 1 h before CCl_4 treatment. Twenty-four hours after CCl_4 treatment, whole blood was collected for determination of serum ALT activity as described in Materials and methods. Control, control animals; CCl_4/Vehicle, CCl_4 with vehicle treatment; CCl_4/rhIL-11, CCl_4 with rhIL-11 treatment; SnMP/CCl_4/rhIL-11, CCl_4/rhIL-11 treatment with SnMP administration. Serum ALT activities are shown as means ± SEM (n=20 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. *p<0.01 vs. Control; †p<0.05 vs. CCl_4/Vehicle; #p<0.01 vs. CCl_4/rhIL-11.

Statistical analysis. Statistical evaluation was performed with analysis of variance followed by the Scheffé F-test, by using Statview software (Abacus Concepts, Berkeley, CA). Differences were considered as significant at p<0.05. Data are presented as means ± SEM.

Results

Effect of rhIL-11 treatment on HO-1 expression. As HO-1 mRNA was shown to be inducible by rhIL-11 in cultured human hepatoma cells (11), we examined the effect of rhIL-11 on HO-1 expression in the liver of rats treated with the compound *in vivo*. While HO-1 mRNA was hardly detectable in vehicle-treated control animals, HO-1 mRNA levels showed marked increases in the liver beyond 10 μg/kg of rhIL-11 treatment, and the increase was dose-dependent through 150 μg/kg where it reached the maximum (Fig. 1A). Since we found previously that this dose of rhIL-11 (150 μg/kg) significantly ameliorated LPS-induced hepatic injury (14), we used 150 μg of rhIL-11/kg in the following experiments in this study. We also examined time courses of the level of hepatic HO-1 mRNA following rhIL-11 treatment (150 μg/kg). While hepatic HO-1 mRNA was barely detectable in the vehicle-treated control animals, it increased markedly and reached a maximum 1 h after the treatment (Fig. 1B), then rapidly declined to ~20% of the maximal level by 3 h, followed by a decrease to the control level by 12 h (Fig. 1B). Using the dose which induced the maximum effect in the liver (150 μg/kg), HO-1 gene expression was examined in liver, kidney, heart, lung, and small intestine 1 h after rhIL-11 treatment. Although HO-1 mRNA level was low in the liver of vehicle-treated animals, it was markedly increased following rhIL-11 treatment (>2,500-fold) (Fig. 2). In contrast to the liver, HO-1 mRNA levels were only slightly increased in lung, or hardly increased in kidney, heart, and small intestine (Fig. 2). These findings indicate that the strong rhIL-11-mediated HO-1 induction occurred exclusively in the liver. HO-1 protein levels examined by Western blot analysis showed barely detectable levels in the vehicle-treated control liver, while it increased significantly (>2-fold) 5 h after rhIL-11 treatment and the induced level was maintained for 9 h (Fig. 3A). By immunochemical analysis, the HO-1 specific positive stain was detected in Kupffer cells, but not in hepatocytes in the vehicle-treated control liver (Fig. 3B, top panel) (7). In contrast, the positive stain was markedly increased in hepatocytes following rhIL-11 treatment (Fig. 3B, bottom panel). A section of the same liver gave no signal when incubated with non-immune rabbit serum (data not shown). Hepatic HO activity was also examined 5 h after rhIL-11 treatment. HO activity was ~4-fold compared with that of the vehicle-treated control level. Administration of SnMP, a competitive inhibitor of HO activity, had little effect on HO activity in the vehicle-treated control liver, while the same treatment entirely abolished the increase in the rhIL-11-induced HO activity (Fig. 4). These results indicate that rhIL-11 treatment markedly and specifically increased HO-1 in the liver, and that administration of SnMP completely inhibited the increased HO activity induced by rhIL-11 treatment.

Effect of rhIL-11 treatment on CCl_4-induced hepatic injury. The effect of rhIL-11 administered immediately after CCl_4 treatment on hepatic injury was then examined. Serum ALT activity of the CCl_4/Vehicle-treated rats was markedly

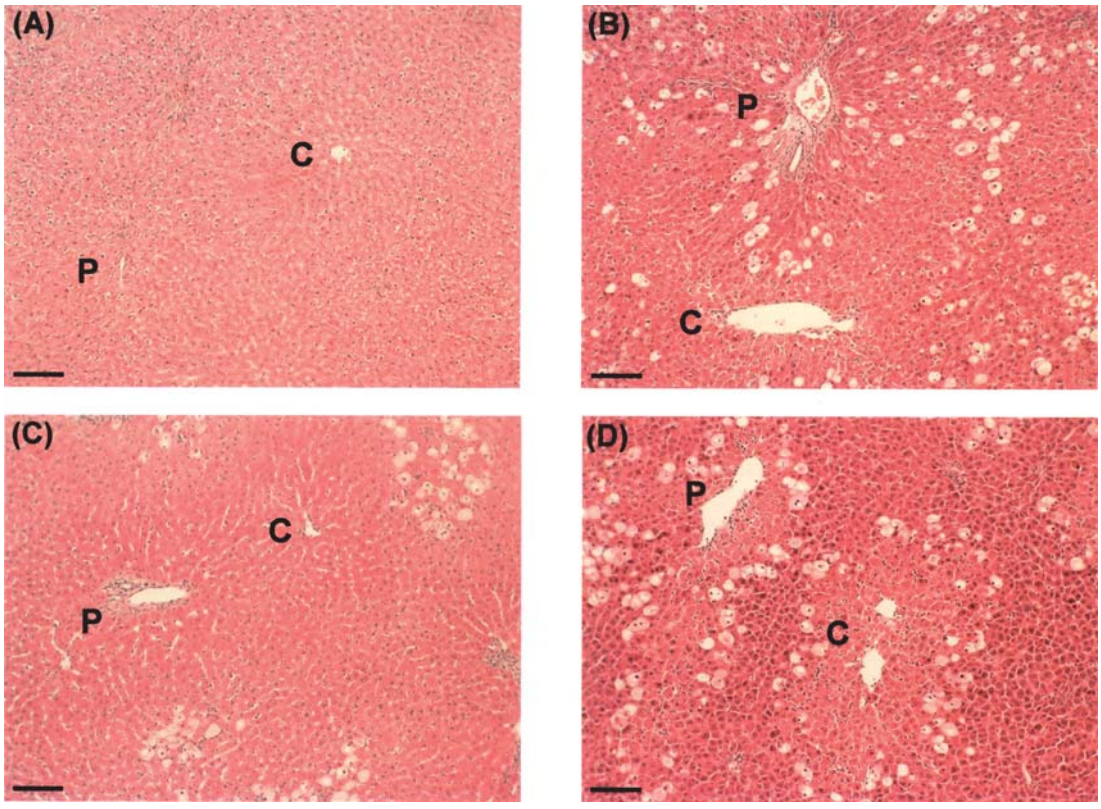


Figure 6. Effect of recombinant human interleukin-11 (rhIL-11) treatment on histological change in CCl_4-induced liver injury. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Tin mesoporphyrin (SnMP, 0.5 μmol/kg) was administered to rats intravenously 1 h before CCl_4 treatment. Twenty-four hours after CCl_4 treatment, livers were removed for histological examination as described in Materials and methods. (A), control animals; (B) CCl_4 with vehicle treatment; (C), CCl_4 with rhIL-11 treatment; (D), CCl_4/rhIL-11 treatment with SnMP administration. Representative light microscopy of livers after each treatment from at least three independent experiments. P and C denote portal tract and central vein, respectively. The bar, 100 μm. Liver sections were stained with hematoxylin and eosin.

increased compared with that of untreated control rats 24 h after the chemical treatment (Fig. 5). In contrast, CCl_4/rhIL-11-treated animals showed a significantly lesser ALT activities than the CCl_4/Vehicle-treated animals, suggesting that CCl_4-induced hepatotoxicity can be suppressed by rhIL-11 treatment (Fig. 5). Histological changes of the CCl_4/Vehicle-treated liver showed massive and severe necrosis of hepatocytes in the centrilobular zone with additional vacuolar degeneration around the portal tracts (Fig. 6B). In contrast, only few necrotic cells were observed around the central vein with minor vacuolization of hepatocytes around portal tracts when animals were additionally treated with rhIL-11 (Fig. 6C). However, additional SnMP treatment to the CCl_4/rhIL-11-treated animals entirely abolished the cytoprotective effect of rhIL-11 (Figs. 5 and 6D).

As CCl_4-induced hepatic injury may be due to CCl_4-mediated free radical production (9), hepatic MDA content was also examined. Low levels of MDA were detected in the untreated control liver 24 h after CCl_4 treatment, while a significantly higher level of MDA was found in the CCl_4/Vehicle-treated liver (Fig. 7). In contrast, rhIL-11 treatment entirely abolished the increase in the CCl_4-induced MDA formation (Fig. 7). Conversely, SnMP treatment increased MDA content to a level similar to that in the CCl_4/Vehicle-treated liver (Fig. 7). Collectively, these findings indicate that rhIL-11 treatment markedly improves hepatocyte oxidative

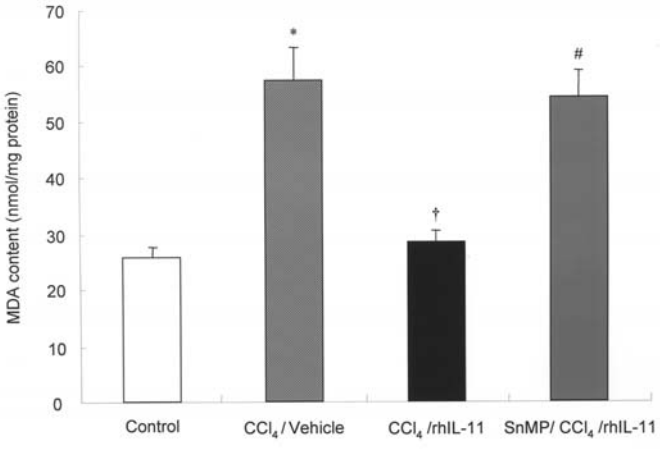


Figure 7. Effect of recombinant human interleukin-11 (rhIL-11) treatment on hepatic malondialdehyde (MDA) content in CCl_4-induced liver injury. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Tin mesoporphyrin (SnMP, 0.5 μmol/kg) was administered to rats intravenously 1 h before CCl_4 treatment. Livers were excised 24 h after CCl_4 treatment for measurement of hepatic MDA concentrations as described in Materials and methods. Control, control animals; CCl_4/Vehicle, CCl_4 with vehicle treatment; CCl_4/rhIL-11, CCl_4 with rhIL-11 treatment; SnMP/CCl_4/rhIL-11, CCl_4/rhIL-11 treatment with SnMP administration. Data are presented as means ± SEM (n=10 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. $^*p<0.01$ vs. Control; $^\dagger p<0.01$ vs. CCl_4/Vehicle; $^\#p<0.01$ vs. CCl_4/rhIL-11.

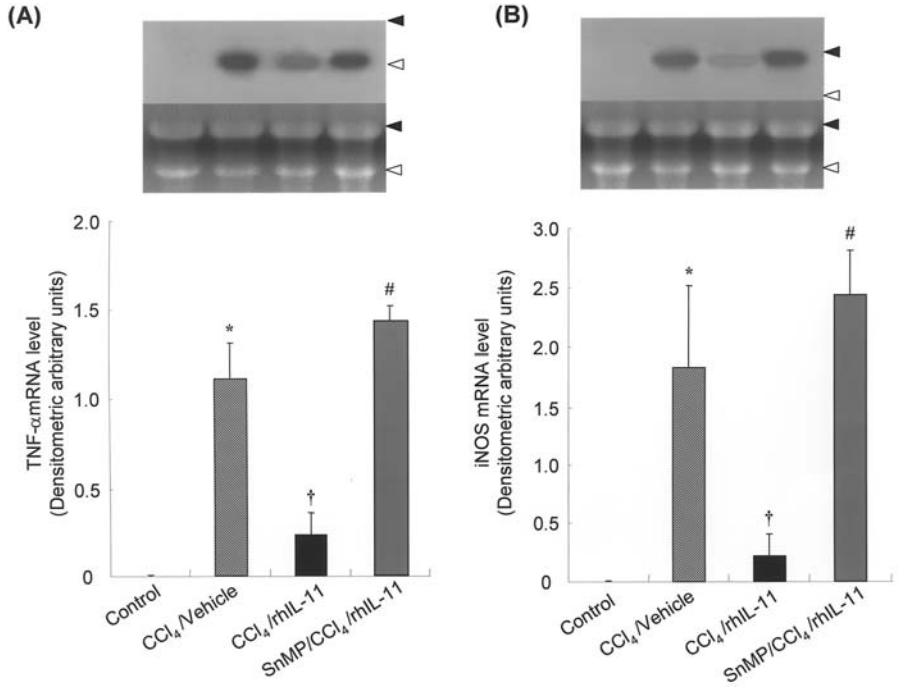


Figure 8. Effect of recombinant human interleukin-11 (rhIL-11) treatment on gene expression of hepatic tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) in CCl_4-induced liver injury. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Tin mesoporphyrin (SnMP, 0.5 μmol/kg) was administered to rats intravenously 1 h before CCl_4 treatment. Livers were excised 24 h after CCl_4 treatment for Northern blot analysis as described in Materials and methods. Top panel, 20 μg 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 (A) or iNOS (B) cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA. Three independent experiments showed similar results, and a typical example is shown. Control, control animals; CCl_4/Vehicle, CCl_4 with vehicle treatment; CCl_4/rhIL-11, CCl_4 with rhIL-11 treatment; SnMP/CCl_4/rhIL-11, CCl_4/rhIL-11 treatment with SnMP administration. Bottom panel, Levels of TNF-α (A) or iNOS (B) mRNA are expressed as densitometric arbitrary units. Data are presented as means ± SEM (n=3 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. *p<0.01 vs. Control; †p<0.05 vs. CCl_4/Vehicle; #p<0.01 vs. CCl_4/rhIL-11.

injury via removing free radicals induced by CCl_4 treatment, and intact HO activity is critical in this process.

Effect of rhIL-11 treatment on TNF-α and iNOS gene expression. As rhIL-11 treatment ameliorated the CCl_4-induced hepatic injury, we examined the effect of rhIL-11 on inflammatory indices, such as TNF-α and iNOS. Hepatic TNF-α mRNA was hardly detectable in untreated control animals (Fig. 8A), while it increased significantly 24 h after CCl_4 treatment (Fig. 8A). rhIL-11 treatment markedly decreased the CCl_4-induced TNF-α mRNA levels (Fig. 8A). Similarly, hepatic iNOS mRNA was significantly increased in the CCl_4/Vehicle-treated liver after the chemical treatment, while much of the increase was eradicated by rhIL-11 treatment (Fig. 8B). Additional SnMP treatment entirely abolished the effect of rhIL-11 on both indices (Fig. 8). These findings indicate that rhIL-11 treatment suppresses the CCl_4-induced inflammatory indices such as TNF-α and iNOS gene expression, and that intact HO activity is essential in the suppression of inflammatory changes.

Effect of rhIL-11 treatment on hepatic NF-κB activation. Next, the effect of rhIL-11 treatment on NF-κB activation was examined using DNA binding activity of NF-κB. While NF-κB DNA binding activity was barely detectable in the liver of untreated control rats (Fig. 9), it was markedly increased 6 h after CCl_4 treatment (Fig. 9), suggesting that increases in inflammatory indices such as TNF-α and iNOS may be mediated by NF-κB activation (7,23,24). rhIL-11 treatment largely prevented the CCl_4-induced NF-κB DNA binding activity (Fig. 9), while SnMP treatment abolished the effect of rhIL-11. These findings indicate that rhIL-11 treatment significantly suppressed the CCl_4-induced NF-κB activation, and that the increased HO activity by rhIL-11 is essential in this suppression.

Effect of rhIL-11 treatment on HO-1 gene expression in CCl_4-administered rats. Effect of rhIL-11 on HO-1 mRNA expression in the CCl_4-treated animals was then examined. Hepatic HO-1 mRNA level in the CCl_4/Vehicle-treated animals was higher than that of untreated control animals (~10-fold) (Fig. 10). rhIL-11 treatment resulted in a further elevation in the CCl_4-induced HO-1 mRNA level (~50-fold) (Fig. 10). These findings demonstrate that rhIL-11 treatment further augments hepatic HO-1 mRNA expression induced by CCl_4 treatment, and suggest that this augmented HO-1 expression may be important in the rhIL-11-based tissue protection.

Discussion

Our study is the first to examine the effect of rhIL-11 on HO-1 expression *in vivo* in animals. The findings demonstrated that

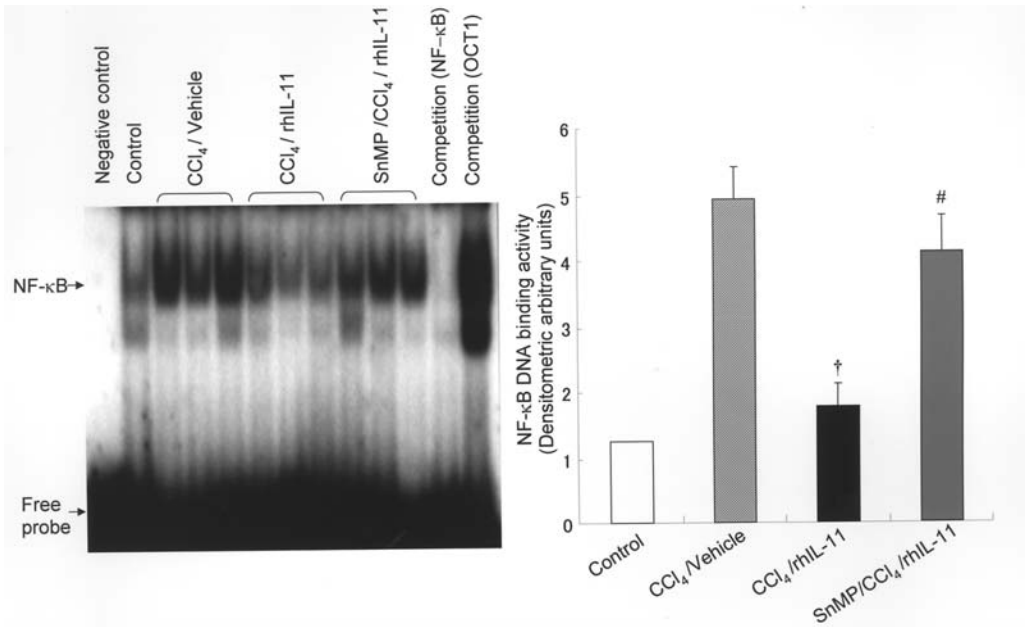


Figure 9. Effect of recombinant human interleukin-11 (rhIL-11) treatment on the activation of nuclear factor-κB (NF-κB) binding in CCl_4-induced liver injury. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Tin mesoporphyrin (SnMP, 0.5 μmol/kg) was administered to rats intravenously 1 h before CCl_4 treatment. Livers were excised 6 h after CCl_4 treatment for electrophoretic mobility shift assay as described in Materials and methods. Autoradiographs (left) were quantified by image analyzer, and the levels of NF-κB activity are expressed as densitometric arbitrary units (right). Negative control, a probe without nuclear extract; Control, control animals; CCl_4/Vehicle, CCl_4 with vehicle treatment; CCl_4/rhIL-11, CCl_4 with rhIL-11 treatment; SnMP/CCl_4/rhIL-11, CCl_4/rhIL-11 treatment with SnMP administration. Competition (NF-κB), CCl_4/Vehicle with the addition of 100-fold excess of unlabeled NF-κB oligonucleotide; Competition (OCT1), CCl_4/Vehicle with the addition of 100-fold excess of unlabeled OCT1 oligonucleotide. Data are presented as means ± SEM (n=3 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. $^{\dagger}p<0.01$ vs. CCl_4/Vehicle; $^{\#}p<0.05$ vs. CCl_4/rhIL-11.

rhIL-11 treatment elicits highly liver-specific induction of HO-1, and is protective against the hepatic injury induced by CCl_4 treatment in rats. Namely, rhIL-11 treatment markedly increased HO-1 expression in hepatocytes, and led to the attenuation of inflammation and oxidative tissue injury in the liver. On the other hand, inhibition of HO activity by SnMP, a specific competitive inhibitor of HO activity, completely abolished the cytoprotective effect of rhIL-11. These findings add further support to the concept that HO-1 plays a fundamental role in the protective response against oxidative injury (7,25,26).

Following rhIL-11 treatment, HO-1 mRNA level increased markedly in the liver, while it showed only marginal increases in the lung, or little to no increases in other organs such as kidney, heart, and small intestine (Fig. 2). It has been shown that rhIL-11 treatment increases HO-1 gene expression in cultured human HepG2 hepatoma cells (11). Our findings in this study also showed that HO-1 gene activation occurs *in vivo* in rats following rhIL-11 treatment and its induction is highly restricted to the liver. Hepatic HO-1 mRNA level increased in a dose-dependent manner in response to rhIL-11 treatment, and the maximum effect was observed at ≥150 μg/kg (Fig. 1A). Our previous study showed that the dose of 150 μg/kg did not influence serum ALT concentration, or liver histology, indicating that rhIL-11 treatment *per se* does not influence hepatic function, or histology (14). Consistent with augmented HO-1 gene activation, increased HO-1 protein level was also detected in hepatocytes following rhIL-11 treatment (Fig. 3B). This finding may be important in that hepatocytes are known to be the target cell of the CCl_4 toxicity (7). Hepatic HO activity was also significantly increased following rhIL-11 treatment (Fig. 4), indicating that functional HO-1 enzyme protein increased in the liver. Compared to a large increase in gene activation, the increase in HO-1 activity was only 4-fold, suggesting that the increase in the functional HO activity may take place much later than its gene activation.

Our previous study demonstrated that HO-1 levels were markedly increased in hepatocytes following CCl_4 treatment, while inhibition of HO activity by SnMP exacerbated the CCl_4-induced tissue injury (7). Using the condition in the same model, we found that HO-1 mRNA expression was markedly increased in the CCl_4/rhIL-11-treated liver (Fig. 10). We also found that the additional rhIL-11 treatment markedly improved the CCl_4-induced hepatic injury. Specifically, rhIL-11 treatment markedly reduced serum ALT activity (Fig. 5), and improved hepatocyte histology, compared with those observed in the CCl_4/Vehicle animals (Fig. 6). Moreover, hepatic MDA content in the CCl_4/rhIL-11 animals was significantly lower than that in the CCl_4/Vehicle animals (Fig. 7), suggesting that rhIL-11 treatment significantly attenuated the CCl_4-mediated oxidant formation. In addition, the administration of SnMP (0.5 μg/kg), a specific competitive inhibitor of HO activity, to rhIL-11-treated animals entirely abolished the cytoprotective effect of rhIL-11 (Figs. 5-7). We previously demonstrated that this dose of SnMP did not influence serum ALT concentration, liver histology, or hepatic MDA content, indicating that SnMP treatment *per se* did not influence hepatic function, histology,

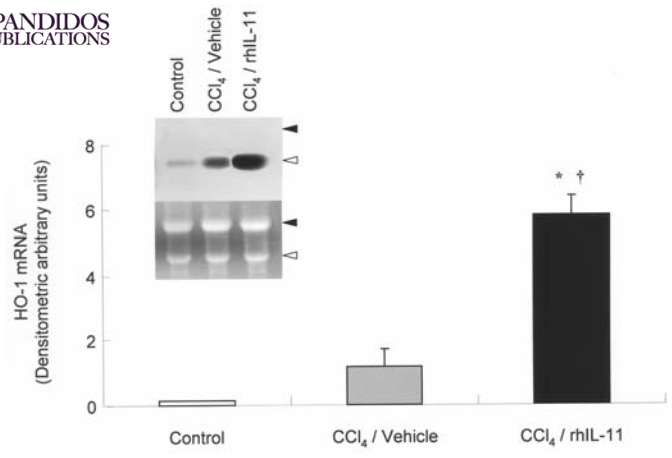


Figure 10. Effect of recombinant human interleukin-11 (rhIL-11) treatment on heme oxygenase (HO-1) gene expression in CCl_4-administered rats. rhIL-11 (150 μg/kg) or vehicle was administered to rats intravenously immediately after CCl_4 treatment (1 ml/kg, i.p.). Livers were excised 3 h after CCl_4 treatment for Northern blot analysis as described in Materials and methods. Insets: 20 μg 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 cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA. Three independent experiments showed similar results, and a typical example is shown. Control, control animals; CCl_4/Vehicle, CCl_4 with vehicle treatment; CCl_4/rhIL-11, CCl_4 with rhIL-11 treatment. Levels of HO-1 mRNA are expressed as densitometric arbitrary units. Data are presented as means ± SEM (n=3 per each group). Statistical analysis by analysis of variance with the Scheffé F-test. $^*p<0.01$ vs. Control; $^\dagger p<0.05$ vs. CCl_4/Vehicle.

or oxidant formation (7). Moreover, SnMP treatment by itself did not affect hepatic HO activity in otherwise untreated animals (Fig. 4). Collectively, these findings indicate that the cytoprotective effect of rhIL-11 can be attributed to its ability to increase hepatic HO-1 activity. We also reported previously that hemin treatment conferred protection against oxidative tissue injuries in halothane-induced hepatic injury (26). As hemin-induction of HO-1 occurred principally in the liver and protected the halothane-induced hepatic injury, the liver-specific HO-1 induction by rhIL-11 may also be important in the protection from the CCl_4-induced toxicity. While this organ-specific induction by hemin and rhIL-11 has been underappreciated, it merits a particular attention as the kidney-specific HO-1 induction by $SnCl_2$ has also been shown to protect ischemic renal failure in rats (27).

Consistent with the amelioration of hepatic injury, rhIL-11 treatment also suppressed the CCl_4-induced pro-inflammatory cytokine expression such as TNF-α (Fig. 8A) and iNOS (Fig. 8B). In addition, rhIL-11 treatment attenuated the CCl_4-mediated NF-κB activation, which plays a pivotal role in the gene transcription of TNF-α and iNOS (Fig. 9) (23,24). These findings indicate that rhIL-11 plays a pivotal role in the protection of tissues from oxidative tissue injury. Treatment of murine peritoneal macrophages with rhIL-11 *in vitro* has also been found to reduce the LPS-induced production of pro-inflammatory cytokine and NO, which was also due to the inhibition of the nuclear translocation of NF-κB (28,29). Treatment of animals with rhIL-11 also down-regulated inflammatory responses of acute hepatic injuries such as those induced by acetaminophen (30) or Concanavalin A (31). rhIL-11 treatment of rats was also found to markedly suppress LPS-induced hepatic tissue inflammation as well as TNF-α and iNOS gene expression, and NF-κB DNA binding (14). Of note, findings in the present study also showed that inhibition of HO activity by SnMP completely ablated the cytoprotective effect of rhIL-11 (Figs. 8 and 9), indicating that the anti-inflammatory effect of rhIL-11 is due to its increased HO activity. These findings indicate that the hepatocyte-specific HO-1 induction by rhIL-11 is the key for the improvement from the CCl_4-induced tissue inflammation.

The mechanism by which HO-1 mediates protection against the CCl_4-induced hepatic injury and inflammation may not be entirely clear. However, it probably involves at least mitigation of reactive oxygen species formation that is known to play a critical role in the CCl_4-induced tissue injury (9). Our previous findings also showed that free heme released from hepatic cytochrome P450, a potent pro-oxidant (32), may contribute to the CCl_4-induced hepatic injury and inflammation, while endogenously induced HO-1 plays a pivotal role against the CCl_4-induced tissue injury by reducing toxic free radical levels (7). Thus, our findings in this study suggest that HO-1 induced by rhIL-11 may degrade an excess 'free heme', leading to the amelioration of the CCl_4-induced tissue injury. HO-1 cleaves heme at the expense of molecular oxygen to release carbon monoxide (CO), iron, and biliverdin IXα, which is then rapidly reduced to bilirubin IXα by bilirubin reductase (1). The released iron, which is an oxidant, is directly sequestered and inactivated by co-induced ferritin (33). In addition, biliverdin IXα and bilirubin IXα have been shown to function as endogenous potent anti-oxidants (34). For instance, higher baseline serum bilirubin levels have been shown to correlate with a lower incidence of myocardial infarction in men, probably because of the inhibitory effect of bilirubin on the oxidation of low-density lipoproteins (35). Carbon monoxide is also known to exhibit anti-inflammatory function via the p38 and c-Jun N-terminal kinase-MAPK pathways (36,37). Accordingly, induction of HO-1 results not only in the reduction of oxidant stress by removing free heme, a potent pro-oxidant, but also in the increase in the level of anti-oxidants and anti-inflammatory substances. While it remains unclear to what extent each mechanism may contribute, these findings may be important in the host cellular defense against oxidative tissue injuries.

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