IL-6-deficient Mice Are Susceptible to Ethanol-induced Hepatic Steatosis: IL-6 Protects against Ethanol-induced Oxidative Stress and Mitochondrial Permeability Transition in the Liver

Osama El-Assal[1], Feng Hong[1], Won-Ho Kim[1], Svetlana Radaeva[1] and Bin Gao[1, 2]

Interleukin-6 (IL-6)-deficient mice are prone to ethanol-induced apoptosis and steatosis in the liver; however, the underlying mechanism is not fully understood. Mitochondrial dysfunction caused by oxidative stress is an early event that plays an important role in the pathogenesis of alcoholic liver disease. Therefore, we hypothesize that the protective role of IL-6 in ethanol-induced liver injury is mediated *via* suppression of ethanol-induced oxidative stress and mitochondrial dysfunction. To test this hypothesis, we examined the effects of IL-6 on ethanol-induced oxidative stress, mitochondrial injury, and energy depletion in the livers of IL-6 (-/-) mice and hepatocytes from ethanol-fed rats. Ethanol consumption leads to stronger induction of malondialdehyde (MDA) in IL-6 (-/-) mice compared to wild-type control mice, which can be corrected by administration of IL-6. *In vitro*, IL-6 treatment prevents ethanol-mediated induction of reactive oxygen species (ROS), MDA, mitochondrial permeability transition (MPT), and ethanol-mediated depletion of adenosine triphosphate (ATP) in hepatocytes from ethanol-fed rats. Administration of IL-6 *in vivo* also reverses ethanol-induced MDA and ATP depletion in hepatocytes. Finally, IL-6 treatment induces metallothionein protein expression, but not superoxide dismutase and glutathione peroxidase in cultured hepatocytes. In conclusion, IL-6 protects against ethanol-induced oxidative stress and mitochondrial dysfunction in hepatocytes *via* induction of metallothionein protein expression, which may account for the protective role of IL-6 in alcoholic liver disease. *Cellular & Molecular Immunology*. 2004;1(3):205-211.

Key Words: IL-6, alcoholic liver injury, oxidative stress, mitochondria, metallothionein

Introduction

One of the most common liver disorders worldwide, alcohol-induced liver injury is the direct consequence of alcohol-induced hepatotoxicity and cross-interactions between alcohol metabolism, multiple hepatic cells, and immunity (1-3). Although alcohol-induced hepatotoxicity has been extensively investigated, the underlying molecular and cellular mechanisms are still not fully understood. There is growing evidence suggesting that oxidative stress and mitochondrial injury induced by ethanol consumption play key roles in ethanol-induced liver injury. In short, ethanol metabolized by alcohol dehydrogenase and cytochrome p450 produces reactive oxygen species (ROS) that causes mitochondrial membrane depolarization and mitochondrial permeability transition (MPT), leading to hepatic apoptosis and necrosis (1-5). In addition to direct hepatotoxicity, alcohol consumption triggers inflammation and elevation of various cytokines that also play important roles in alcohol-induced liver injury (4-6). For example, serum levels of tumor necrosis factor-α (TNF-α) are elevated in alcoholic patients and in animals chronically fed ethanol (7-9). Liver injury induced by alcohol consumption can be abolished by administration of TNF-α antibody (10) or in TNF-α type I receptor knock-out mice (11), suggesting that TNF-α is critically involved in ethanol-induced liver injury. Serum levels of IL-6 are also increased in alcoholic patients and animals chronically fed ethanol (12-15); however the role of IL-6 in alcoholic liver disease is less clear. We have previously demonstrated that interleukin-6 (IL-6)-deficient mice are susceptible to ethanol-induced apoptosis in the liver and that IL-6 protects against ethanol-induced hepatocyte death *in vitro* (16). Here the molecular mechanism underlying the protective effect of IL-6 is further explored. We demonstrated that IL-6-deficient mice are prone to ethanol-induced lipid peroxidation and steatosis. IL-6 protects

[1]Section on Liver Biology, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892, USA.

[2]Corresponding to: Dr. Bin Gao, Section on Liver Biology, NIAAA/NIH, Park Bldg Rm 120, 12420 Parklawn Drive, MSC 8115, Bethesda, MD 20892. Tel: +01-301-443-3998, Fax: +01-301-480-0257, E-mail: bgao@mail.nih.gov.

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Abbreviations: IL-6, interleukin-6; ROS, reactive oxygen species; MDA, malondialdehyde; MPT, mitochondrial permeability transition; SOD, superoxide dismutase; MT, metallothionein; ATP, adenosine triphosphate; GPXase, glutathione peroxidase.

against ethanol-induced ROS and MPT in steatotic hepatocytes from ethanol-fed rats. Significant induction of metallothionein protein expression may contribute to the protective role of IL-6 in ethanol-induced ROS, MPT, and steatosis.

Materials and Methods

Materials
Recombinant human IL-6 (hIL-6) was produced through recombinant DNA technology, and purified by solubilization, gel filtration chromatography, refolding, and cation exchange chromatography. The purified preparation had the expected amino acid sequence, as confirmed by compositional analysis and had the correct pI range of 7.0-7.1. The biological activity of the recombinant protein was measured by a proliferation assay employing 7TD1 cells, which demonstrated that the hIL-6 was active at $>2 \times 10^8$ units/mg, which is similar to the range estimated for the native cytokine. Sheep anti-Mn-SOD polyclonal antibody was purchased from Calbiochem-Novobiochem Corp (San Diego, CA). Mouse anti-MT monoclonal antibody was purchased from Stressgen Biotech (Victoria, BC Canada).

Chronic ethanol consumption model
Male C57BL/6J background IL-6 (-/-) mice and control C57BL/6J mice weighing 20-25 g were purchased from the Jackson Laboratory (Bar Harbor, Maine). IL-6 (-/-) and wild-type mice were divided into 2 groups based on the type of diet. One group of IL-6 (-/-) mice and control mice (5 of each) were fed ethanol-containing liquid diets (BioServ, Inc., Frenchtown, NJ), and the other group of IL-6 (-/-) mice and control mice (5 of each) were fed similar control diets whose ethanol content was substituted isocalorically with dextrin maltose (BioServ, Inc.). Both diets were dispensed in glass liquid diet feeding tubes (BioServ, Inc.). The ethanol diet was introduced gradually by increasing the ethanol content of the diet by 1% (v/v) every 2 days until the mice were consuming diets containing 4% (v/v) ethanol. During the entire feeding period, IL-6 (-/-) and control mice were observed to consume similar volumes of ethanol or control diets respectively on a daily basis, and that the ethanol-fed and pair-fed IL-6 (-/-) and control mice gained weight similarly. Male Sprague-Dawley rats weighing 300-350 g were purchased from Harlan (Madison, Wisconsin) and fed a nutritionally adequate liquid diet containing 7% ethanol (Bioserv, Frenchtown, USA) for 4 weeks as described by Leiber and DeCarli (17). Zucker rats (8-10 weeks), which develop severe steatosis by the age of 8 weeks, and lean littermates (9-11 weeks) were purchased from Charles River Laboratories (Kingston, New York).

Primary rat hepatocyte isolation and culture
Rats weighing 300-350 g were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally), and the portal vein was cannulated under aseptic conditions. The liver was perfused with hepatocyte perfusion medium (Gibco BRL, Gaithersburg, MD) and digested with a collagenase solution. The isolated hepatocytes were then cultured in Hepato-ZYME-SFM media (Gibco, BRL) in rat-tail collagen coated plates.

Hematoxylin-eosin (H&E) and oil red O staining of liver sections
Following fixation of the livers with 4% formalin/phosphate-buffered saline (PBS), livers were sliced and stained with H&E for histological examination. Frozen liver sections were stained with oil red O to determine hepatic lipids.

Measurement of reactive oxygen species
Intracellular ROS generation was quantified using flow-cytometric measurement of the cellular metabolism of 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Inc., Eugene, OR) to 2'7'-dichlorofluorescein. ROS produced since the cells oxidize DCFH-DA into the highly green fluorescent compound (2'7'-dichlorofluorescein) whose fluorescence intensity is directly proportional to ROS production (18). Cultured hepatocytes or freshly isolated hepatocytes (2×10^6) were loaded for 1 h with DCFH-DA at a final concentration of 5 μmol/L. Fluorescence was measured by flow cytometry. Results were expressed as relative fluorescence intensity and normalized to control cells.

Measurement of mitochondrial permeability transition
Tetramethylrhodamine ethyl ester (TMRE) is electrophoretically redistributed across the mitochondrial membrane according to the membrane potential. Cultured hepatocytes or freshly isolated hepatocytes (2×10^6) were incubated for 1 h at 37°C in DMEM medium containing 100 nM TMRE. Cells were then washed, re-suspended in DMEM medium. The MPT was analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. A minimum of 10^4 cells per sample were analyzed through a FL3-H channel. Data were acquired in list mode and analyzed using the CellQuest software.

Biological analysis of hepatic lipids
For measurements of hepatic lipids, the liver was homogenized at 4°C in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 0.5% Na DOC. Lipids in the total liver homogenate were extracted using the chloroform/methanol method (2:1), evaporated and dissolved in 2-propanol. Amounts of triglyceride, total cholesterol, and phospholipids were assayed enzymatically using kits obtained from Wako Pure Chemicals Co. (Richmond, VA).

Measurement of malondialdehyde (MDA)
Hepatic and cellular levels of malondialdehyde, the final product of lipid peroxidation, were determined by the spectrophotometric method. Hepatic tissues or isolated hepatocytes were homogenized in ice-cold 1.15% KCl. A mixture containing 8.1% SDS (0.2 ml), 0.9% thiobarbituric acid (1.5 ml) and 20% acetic acid (1.5 ml) was added to 0.2 ml of the homogenate, and the final volume was adjusted with distilled water to 4 ml. This mixture was incubated at 95°C for 1 hour and allowed to cool under cold water. To this mixture, an additional 1 ml of distilled water plus 5 ml n-butanol/pyridine (15:1, v/v) was added, then altogether

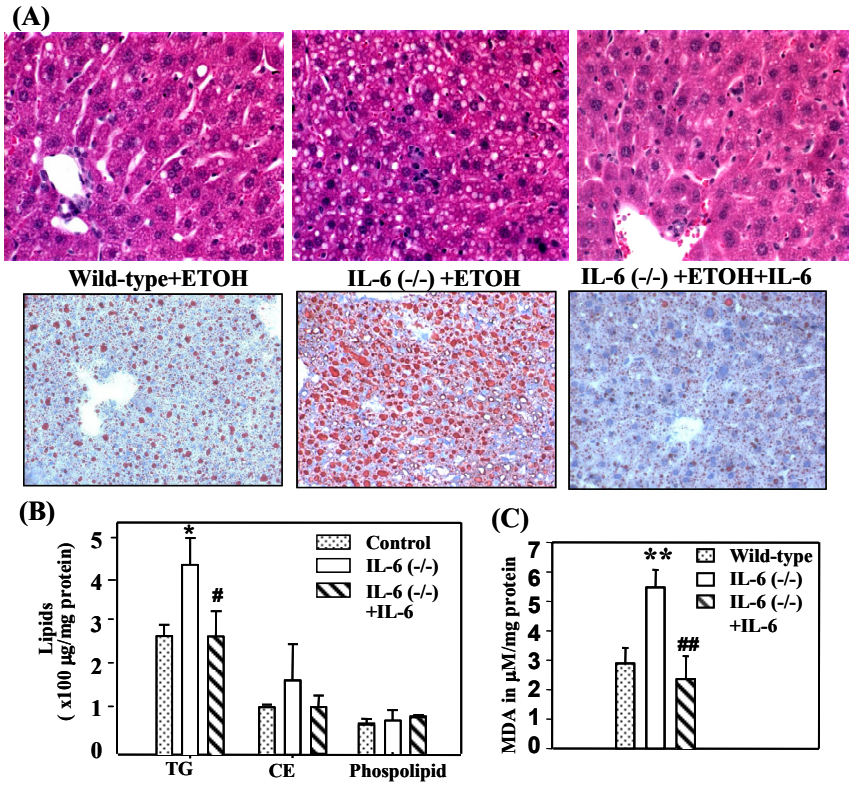

Figure 1. IL-6-deficient mice are susceptible to ethanol-induced fatty liver and MDA: protection by IL-6 administration. Wild type and IL-6 (-/-) mice were fed a nutritionally adequate Leiber-DeCarli liquid diet containing 4% ethanol for 4 weeks. Some IL-6 (-/-) mice were subcutaneously injected with IL-6 (1 μg/g) for the last 5 days of the feeding period. At the end of 4 weeks, mice were sacrificed and liver sections were stained with H&E or oil red O. (A) hepatic lipids (triglyceride, total cholesterol, and phospholipids) (B) and MDA (C) were determined as described in "Materials and Methods". Representative H&E staining (upper panel A) and oil red O staining (lower panel A) of liver sections are shown. Values in panels B and C are shown as means ± SEM from 3 independent experiments. * $p<0.05$, ** $p<0.01$, significant differences from corresponding wild-type control; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, significant differences from corresponding IL-6 (-/-) mice fed with the ethanol diet.

mixed and centrifuged at 4000 rpm for 10 min. The absorption peak of the organic phase was measured at 532 nm. For comparison, 1, 1, 3, 3-tetramethoxypropane was used as the standard (Sigma Chemicals Co).

ATP content of living hepatocytes

ATP released from a suspension of living hepatocytes was measured using a bioluminescent somatic assay kit (Sigma chemical Co.).

Cell extraction, SDS-PAGE, and Western blotting

Cells were lysed in lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol) for 15 min at 4°C, vortexed and centrifuged at 16,000 rpm at 4°C for 10 min. Laemmli running buffer was added to the supernatant, then boiled for 4 min and finally subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against anti-metallothionein antibody. Membranes were washed with TPBS (0.05% [v/v] Tween 20 in phosphate-buffered saline [pH 7.4]) and incubated in a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

Superoxide dismutase and glutathione peroxidase enzyme activity kinetic assays

Superoxide dismutase enzyme activity was determined using a superoxide dismutase assay kit (Calbiochem-Novobiochem Corp., CA) according to the recommendation of the manufacturer. The test utilizes 5, 5, 6a-tetrahydro-3, 9, 10-trihydroxybenzoflourene, whose auto-oxidation is accelerated by superoxide dismutase activity, yielding a chromophore which is absorbed maximally at 525 nm. Superoxide dismutase from bovine erythrocytes was used as the standard for the reaction (Calbiochem).

Glutathione peroxidase activity was indirectly determined using a cellular glutathione peroxidase assay kit (Calbiochem) according to the recommendations of the manufacturer. The test involves two enzymatic steps and ending with the reduction of the NADPH produced by glutahtione peroxidase into NADP, as observed by decreased absorbance at 340 nm. The rate of reduction provides spectrophotometric means for monitoring glutathione peroxidase activity. Cellular glutahione peroxidase supplied by the manufacturer was used as the standard. The rate of reduction in absorption of NADPH at 340 nm was measured and the amount of NADPH consumed was calculated using the extinction coefficient for NADPH (0.00622 in $\mu M^{-1}cm^{-1}$).

Statistical analysis

For comparing values obtained in three or more groups, one-factor analysis of variance (ANOVA) was used, followed by Tukey's post hoc test. $p<0.05$ was taken to imply statistical significance.

Results

IL-6-deficient mice are susceptible to ethanol-induced steatosis and lipid peroxidation in the liver: protection by administration of IL-6

We have previously shown that significant hepatic steatosis

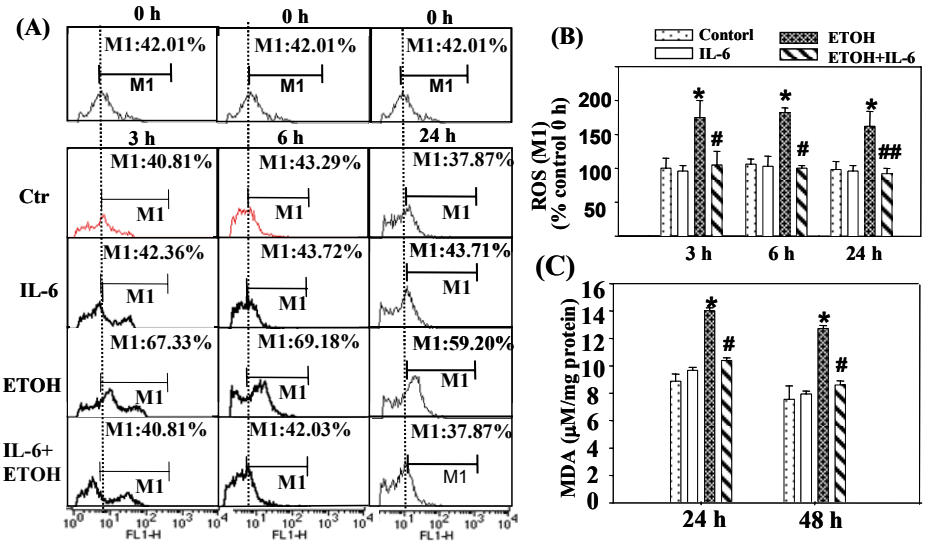

Figure 2. IL-6 protects against ethanol-induced ROS and MDA in hepatocytes from ethanol-fed rats. Hepatocytes were isolated from ethanol-fed rats and incubated with IL-6 (100 ng/mL) and/or ethanol (100 mM) for various time points as indicated. ROS and MDA were measured. Panel A, representative histograms of ROS are shown. Values shown in panels B and C are means ± SEM from 3 independent experiments. * $p<0.01$, significant differences from corresponding control groups; # $p<0.01$, ## $p<0.05$, significant differences from ethanol-treated groups.

was induced in both wild type and IL-6 (-/-) mice fed a diet containing ethanol for 8 weeks (16). Here we observed that feeding with an ethanol-containing diet for 4 weeks induced significant hepatic steatosis in IL-6 (-/-) mice but only minor steatosis in wild-type mice as demonstrated by both H&E staining (Figure 1A: upper panel) and oil red O staining (Figure 1A: lower panel). IL-6 treatment for the last 5 days completely reversed ethanol-induced hepatic steatosis in IL-6 (-/-) mice. Hepatic lipids in ethanol-fed wild type and IL-6 (-/-) mice were also measured. As shown in Figure 1B, ethanol feeding induced much higher levels of hepatic triglyceride in IL-6 (-/-) mice than in wild-type mice, which was reversed by IL-6 treatment. Ethanol feeding also increased slightly, but not significantly, total cholesterol levels in IL-6 (-/-) mice compared to wild-type mice. Similar levels of phospholipids were detected in ethanol-fed wild type and ethanol-fed IL-6 (-/-) mice. Moreover, MDA, the final product of lipid peroxidation, was also compared in ethanol-fed wild type and IL-6 (-/-) mice. As shown in Figure 1C, chronic ethanol consumption induced higher levels of MDA in the liver of IL-6 (-/-) mice than wild-type mice, which was suppressed by IL-6 injection.

IL-6 protects against ethanol-mediated ROS, MDA, and MPT induction in steatotic hepatocytes from ethanol-fed rats

To understand the underlying mechanism by which IL-6 protects against alcohol-induced liver injury, we examined the effect of IL-6 on ethanol-induced oxidative stress, lipid peroxidation, and MPT in hepatocytes *in vitro*. Exposure to 100 mM ethanol for 3, 6, and 24 h did not cause ROS induction in hepatocytes from pair-fed rats (data not shown), but enhanced ROS production in ethanol-fed rat hepatocytes (ROS peak shifted to the right) (Figure 2A and 2B), which was prevented by IL-6 co-treatment (Figure 2A and 2B). Similarly, ethanol treatment up to 48 h did not enhance MDA production in hepatocytes from pair-fed rats

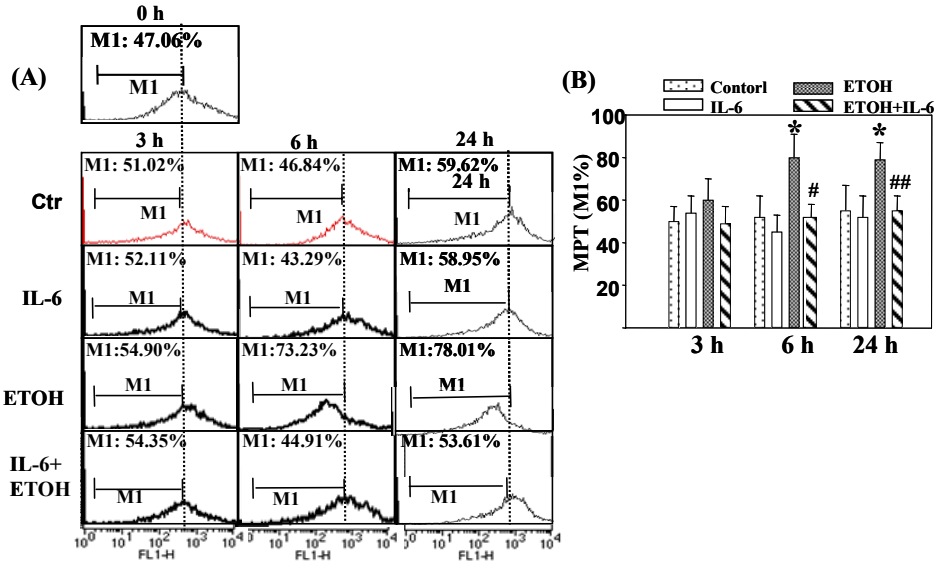

Figure 3. IL-6 protects against ethanol-mediated induction of MPT in hepatocytes from ethanol-fed rats. Hepatocytes were isolated from ethanol-fed rats and incubated with IL-6 (100 ng/mL) and/or ethanol (100 mM) for various time points as indicated. MPT was measured. Panel A, representative histograms of MPT are shown. Values shown in panel B are means ± SEM from 3 independent experiments. * $p<0.01$, significant differences from corresponding control groups; # $p<0.01$, ## $p<0.05$, significant differences from ethanol-treated groups.

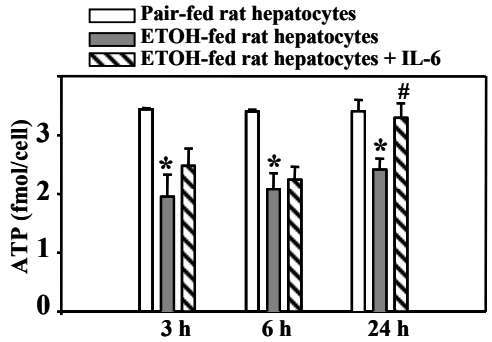

Figure 4. IL-6 restores ethanol-induced ATP depletion in steatotic hepatocytes from ethanol-fed rats. Hepatocytes were isolated from ethanol-fed rats or pair-fed rats and incubated with IL-6 (100 ng/mL) for various time points as indicated. ATP was measured. Values shown are means ± SEM from 3 independent experiments. *p<0.01, significant differences from corresponding hepatocytes from pair-fed control groups; $^{\#}$ p<0.01, significant differences from corresponding hepatocytes from ethanol-treated groups.

(data not shown), but treatment for 24 h or 48 h enhanced MDA production in ethanol-fed rat hepatocytes, which was completely suppressed by IL-6 co-treatment (Figure 2C). Exposure to ethanol for 6 and 12 h did not cause induction of MDA in ethanol-fed rat hepatocytes (data not shown).

The effects of ethanol on MPT induction was also examined by flow cytometry with TMRE (Figure 3). Exposure to ethanol for 6 and 24 h but not for 3 h caused an induction of MPT in ethanol-fed rat hepatocytes (MPT peak shifted to the left, indicating that fewer cells retained TMRE in their mitochondria). Pretreatment with IL-6 did not affect basal levels of MPT but blocked ethanol-induced MPT (Figure 3A and 3B). Similarly, our data also showed that IL-6 treatment protected against ethanol-mediated ROS, MDA, and MPT induction in steatotic hepatocytes from Zucker rats (data not shown).

IL-6 restores ethanol-induced depletion of ATP in steatotic hepatocytes from ethanol-fed rats

It has been shown that chronic ethanol consumption causes ATP depletion in hepatocytes (19, 20), which is implicated in the pathogenesis of alcoholic liver injury. Here we also confirmed that hepatocytes isolated from ethanol-fed rats contained lower levels of ATP than pair-fed rat hepatocytes (Figure 4). Treatment with IL-6 for 24 h, but not 3 and 6 h restored ATP levels in hepatocytes from ethanol-fed rats. Similarly, steatotic hepatocytes from Zucker rats contained lower levels of ATP than hepatocytes from lean controls, which were restored by IL-6 treatment for 24 h (data not shown).

In vivo administration of IL-6 attenuates ethanol-induced MDA, and restores ethanol-induced ATP depletion in rats

The above data clearly show that IL-6 protects against ethanol-induced oxidative stress and MPT in steatotic rat hepatocytes *in vitro*. We then sought to determine whether IL-6 also suppresses ethanol-induced MDA and restores ethanol-induced ATP depletion *in vivo*. To test this hypothesis, the effects of IL-6 injection on MDA and ATP levels in ethanol-fed rats were examined (Figure 5). Higher levels of MDA were detected in the livers of rats fed an ethanol diet for 4 weeks compared to rats fed a control diet. IL-6 treatment was able to reduce hepatic MDA levels in ethanol-fed rats (Figure 5A). Hepatocytes from IL-6-injected ethanol-fed rats contained higher ATP levels than hepatocytes from saline-injected ethanol-fed rats (Figure 5B, * p<0.01). Exposure to ethanol reduced ATP levels in hepatocytes from saline-injected ethanol-fed rats ($^{\#}$ p<0.01) but not from IL-6-injected ethanol-fed rats.

IL-6 treatment induces metallothionein protein expression in cultured hepatocytes

To understand the molecular mechanism by which IL-6 protects against ethanol-induced ROS production and mitochondrial injury, we examined the effects of IL-6 on expression and activities of several anti-oxidant genes in cultured hepatocytes from ethanol-fed rats. These genes include superoxide dismutase, glutathione peroxidase, and metallothionein, which have been shown to protect against ethanol-induced liver injury (21-24). As shown in Figure 6, IL-6 treatment did not increase manganese superoxide dismutase enzyme activity nor manganese superoxide dismutase protein expression. In addition, glutathione peroxidase activity was not affected by IL-6 treatment. In contrast, treatment of hepatocytes with IL-6 enhanced metallothionein protein expression in cultured hepatocytes from ethanol-fed rats.

Discussion

Alcoholic liver disease is associated with elevated IL-6 in

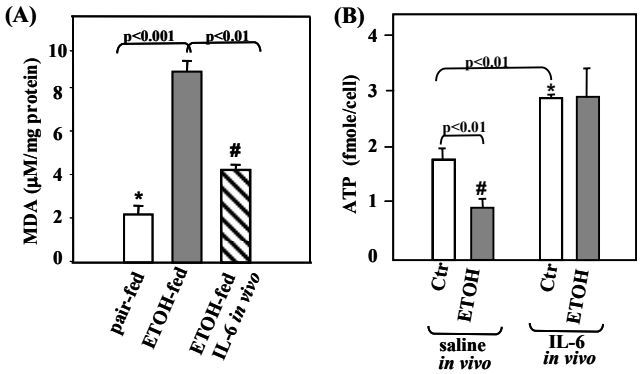

Figure 5. *In vivo* injection of IL-6 protects against ethanol-induced MDA and depletion of ATP in the livers of ethanol-fed rats. Male Sprague-Dawley rats (n = 6) were fed a nutritionally adequate liquid diet containing 7% ethanol for 4 weeks. Three of six rats were injected with IL-6 (1 μg/g) for the last 5 days of the feeding period. Hepatocytes were isolated from ethanol-fed rats and IL-6-treated ethanol-fed rats. (A) MDA levels were measured. Values shown are means ± SEM from 3 independent experiments. *p<0.001, significant differences from the liver of ethanol-fed rats; $^{\#}$ p<0.01, significant differences from the liver of ethanol-fed rats. (B) Hepatocytes from saline-injected or IL-6-injected ethanol-fed rats were cultured in the absence or presence of ethanol (100 mM) for 6 h. Levels of ATP were measured. Values shown are means ± SEM from 3 independent experiments. * p<0.01 and $^{\#}$ p<0.01, significant differences from control (Ctr) hepatocytes from saline-injected ethanol-fed rats.

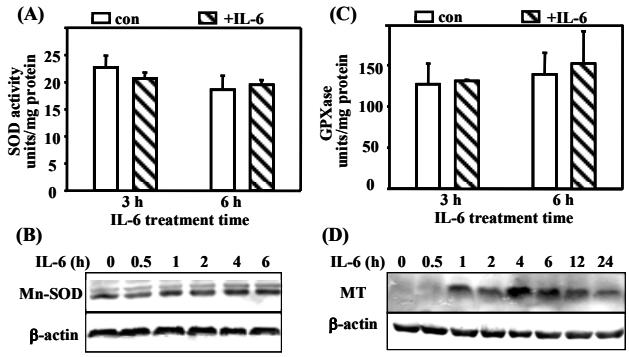

Figure 6. Effects of IL-6 on expression and activities of anti-oxidant genes. Hepatocytes from ethanol-fed rats were incubated with IL-6 (20 μg/ml) for various time points. (A) Total SOD activity was measured. (B) MnSOD protein and β-actin were analyzed by Western blotting. (C) GPXase activity was measured. (D) MT protein was analyzed by Western blotting. β-actin protein was also analyzed as loading controls.

the serum (12-15). The significance of such an elevation, however, is unclear. We have previously shown that IL-6 can protect against ethanol-induced hepatocyte apoptosis *in vivo* and *in vitro* through the induction of anti-apoptotic Bcl-xL protein, suggesting that elevated IL-6 acts as a protective cytokine against alcoholic liver disease (16). In the present paper, we further extended this study to investigate additional molecular mechanisms underlying IL-6 protection against alcohol-induced liver injury. Emerging evidence suggests that mitochondrial dysfunction caused by oxidative stress is an early event that plays an important role in the pathogenesis of ethanol-induced apoptosis and steatosis (1-3, 5, 25-28). Thus, anti-oxidant based therapeutic approaches to prevent alcoholic liver disease are being actively sought. For example, gene therapies targeting several anti-oxidant genes (21, 22) and several anti-oxidant compounds (29-31) have been shown to protect against alcoholic liver injury. Here we demonstrate that IL-6-deficient mice are prone to ethanol-induced lipid peroxidation and steatosis, which are prevented by IL-6 injection. IL-6 treatment protects against ethanol-induced ROS and MPT, and restores ethanol-induced ATP depletion in the liver *in vitro* and *in vivo*. Taken together, our findings suggest that the protective role of IL-6 in alcoholic liver disease is mainly mediated through suppression of ethanol-induced ROS and mitochondrial injury.

The molecular mechanism underlying IL-6-mediated protection against ethanol induction of ROS was further studied in this paper. Several anti-oxidant genes, including superoxide dismutase, glutathione peroxidase, and metallothionein, have been shown to protect against ethanol-induced liver injury (21-24). IL-6 treatment did not affect the enzyme activity or protein expression of both superoxide dismutase and glutathione peroxidase (Figure 6), suggesting that IL-6 suppression of ethanol-induced ROS is not mediated *via* modulation of these two anti-oxidant enzymes. In contrast, IL-6 treatment markedly induced metallothionein protein expression in primary rat hepatocytes from ethanol-fed rats (Figure 6). Metallothionein is a highly conserved, low-molecular weight, thiol-rich protein, which has been shown to act as an antioxidant in a variety of organs and cells (32-34), including the liver (23, 24). Transgenic mice over-expressing metallothionein are protected from ethanol-induced oxidative stress, lipid peroxidation, and steatosis, suggesting that metallothionein protects against alcoholic liver injury through inhibition of oxidative stress (23). Thus, it is plausible that induction of metallothionein plays an important role in IL-6 protection against ethanol-induced oxidative stress, mitochondrial injury, and steatosis in the liver. STAT1 and STAT3 binding sites have been identified in the mouse metallothionein gene promoter, which may be responsible for IL-6-mediated induction of metallothionein gene expression (35). In addition to metallothionein, we have previously shown that IL-6 treatment induces expression of anti-apoptotic Bcl-xL protein in primary mouse hepatocytes, which may contribute to the anti-apoptotic effect of IL-6 in hepatocytes (16). Overexpression of Bcl-xL has been shown to suppress oxidative stress induced by staurosporine (36), indicating that induction of Bcl-xL may also play a role in IL-6 protection against ethanol-induced oxidative stress and mitochondrial injury in the liver.

IL-6-deficient mice are susceptible to ethanol-induced steatosis in the liver, and IL-6 injection can reverse alcoholic fatty liver (Figure 1), but the underlying mechanism is not clear. Since mitochondrial dysfunction caused by ethanol has been considered as one of the major mechanisms contributing to lipid metabolism changes in the liver leading to steatosis (37, 38), IL-6 protection against ethanol-induced mitochondrial damage may be one mechanism responsible for IL-6 amelioration of fatty livers. Recently, we have shown that IL-6 treatment alleviates steatosis in obesity-associated fatty livers in mice partly *via* down-regulation of TNF-α, stimulation of fatty acid β-oxidation, and induction of PPAR-α protein expression (39), which might also be involved in IL-6 protection against ethanol-induced steatosis in the liver.

In conclusion, our findings here, together with our previous findings (16), suggest that in alcoholic liver disease, elevated IL-6 levels act as a protective cytokine to protect against alcoholic liver injury through suppression of ethanol-induced ROS and MPT, and IL-6 could be used as an anti-oxidant cytokine to treat alcoholic liver disease.

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