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Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease☆

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Abstract

Recent experimental data suggest that the idiosyncratic nature of drug-induced liver disease (DILD) may be due in part to a deficiency of one or more hepatoprotective factors. In this study we have investigated whether interleukin (IL)-6 may also be one of these factors. Following the induction of liver injury with acetaminophen (APAP), a time-dependent increase in liver mRNA expression of IL-6 and its family members IL-11, leukemia inhibitory factor, and oncostatin M was observed in wild type (WT) mice, suggesting a possible hepatoprotective role played by this cytokine family. Indeed, mice lacking IL-6 (*IL-6*$^{-/-}$) were more susceptible than were WT mice to APAP-induced liver injury. The increased susceptibility of the *IL-6*$^{-/-}$ mice was associated with a deficiency in the expression of hepatic heat shock protein (HSP)25, 32, and 40 as well as inducible HSP70 following APAP treatment. These results suggest that IL-6 and possibly other family members may protect the liver from injury, at least in part, by up-regulating the hepatic expression of several cytoprotective HSPs.

Keywords: Acetaminophen; Protein-adducts; Hepatotoxicity; Interleukin-6; Interleukin-11; Oncostatin M; Leukemia inhibitory factor; Acute-phase response; Heme-oxygenase; Heat shock proteins

Acute hepatic failure (AHF) is a relatively uncommon disease that requires liver transplantation or causes death in more than 50% of its cases [1]. Although AHF has numerous causes, the vast majority are either viral- or drug-related [1]. Drug-induced liver disease (DILD) reportedly accounts for 12% of AHF cases in the US [2], but the incidence of drug-induced AHF and other forms of DILD may be much higher based upon the limited reporting of adverse drug events (estimated at ~10%) to the US Food and Drug Administration [3]. Regardless, DILD is still the major reason for removal of drugs from clinical development and widespread use [4] making it a public health issue of considerable concern.

Clinical and laboratory studies of DILD are extremely difficult because of the idiosyncratic nature of these reactions and their unknown etiology. Recent experimental data suggest that the relatively low incidence of DILD may be due, at least in part, to the activities of varied factors that protect against DILD in most individuals but which may be deficient in susceptible patient populations. Part of these protective mechanisms include factors such as interleukin (IL)-10 [5], cyclooxygenase (COX)-2 [6], as well as a number of other factors [7–13]. However, the range of protective factors and/or

☆ *Abbreviations:* DILD, drug-induced liver disease; IL, interleukin; APAP, acetaminophen; WT, wild type; *IL-6*$^{-/-}$, IL-6 knockout mice; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; iHSP, inducible heat shock protein; HO, heme-oxygenase; AHF, acute hepatic failure; COX, cyclooxygenase; ALT, alanine aminotransferase; LIF, leukemia inhibitory factor; OSM, oncostatin M; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ssDNA, single stranded DNA; Cyp, cytochrome P450.

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mechanisms that may counteract DILD has not yet been fully established.

While exceptions do exist [14,15], IL-6 also appears to serve key hepatoprotective functions against at least some forms of acute and chronic liver injury. After partial hepatectomy [16] and liver ischemia [17], *IL-6*$^{-/-}$ mice show impaired liver regeneration characterized by liver necrosis and hepatic failure. Following single or multiple doses of the hepatotoxin, carbon tetrachloride (CCl_4), IL-6 seems to be a critical anti-injury, anti-fibrotic, and pro-regenerative factor within the liver [18]. The precise mechanistic role of IL-6 and its extended family of cytokines, however, is not well defined in the context of DILDs.

The studies described herein were aimed at delineating the role of IL-6 in a murine model of DILD caused by acetaminophen (APAP). The data substantiate the complexity of endogenous pathways that protect the liver from drug-induced injury and implicate IL-6 and possibly other members of this cytokine family as potential hepatoprotective factors.

Materials and methods

Animals and treatment. Male C57Bl/6J (WT) mice and *IL–6*$^{-/-}$ mice on a C57Bl/6J background were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were acclimated in autoclaved, microisolator cages for at least 6–7 days to a 12-h light-dark cycle in a humidity- and temperature-controlled, specific-pathogen-free environment according to National Institutes of Health standards. Mice were allowed free access to autoclaved food and water until initiation of each study. Before experimental use, mice were fasted for 16–18 h overnight and were then injected intraperitoneally with 20 ml/kg saline or various doses of APAP (Sigma) in warm saline, whereupon food supplies were restored. Blood was drawn at several time points after APAP treatment for serum collection and select mice were euthanized to obtain liver tissues for histological, protein, and mRNA analyses.

Assessment of hepatotoxicity. Serum alanine aminotransferase (ALT) activities were assayed as a marker of APAP-induced hepatotoxicity using a microtiter plate adaptation of a commercially available kit (Sigma Diagnostics). Liver injury was confirmed histopathologically in each study using liver sections that had been fixed in buffered formalin and then embedded in paraffin, mounted onto glass slides (5-μm thick sections), and stained with hematoxylin and eosin (H&E; American Histolabs, Gaithersburg, MD).

RNA isolation and inflammatory mediator analysis by reverse-transcription polymerase chain reaction. Approximately 60 mg samples of liver tissue (snap frozen and stored at −80 °C) were homogenized in 1.2 mL RLT buffer (Qiagen) containing 1% mercaptoethanol, and total cellular RNA was extracted according to manufacturer's instructions (Qiagen). Isolated RNA was quantified by spectrophotometric analysis at 260 nm and checked for integrity by electrophoresis. Expression of mRNA was assessed semi-quantitatively using total RNA (2.0 μg) in conjunction with Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech), and specific cDNA primer sets for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), IL-6, IL-11, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [5], COX-2 [6], leukemia inhibitory factor (LIF) [19], and oncostatin M (OSM) [20]. Following amplification, PCR products were resolved within 2% agarose gels in Tris–borate buffer and visualized using Vistra Green nucleic acid dye. Results of densitometric analysis (FluorImager 595, Molecular Dynamics) of each PCR product were normalized for the corresponding G3PDH expression within a given sample.

Preparation of liver homogenate and immunoblot analysis. Liver tissues (stored at −80 °C) were homogenized with a Tekmar tissuemizer (Tekmar) in ice-cold 100 mM Tris–acetate buffer (pH 7.5) containing 250 mM sucrose, 1 mM EDTA, and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche). Protein concentrations of homogenates were determined using the BCA Protein Assay kit (Pierce Chemical). Liver proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted as previously reported [21]. Membranes were immunochemically probed with specific antibodies directed against APAP-bound protein adducts (anti-sera from Drs. Neil R. Pumford and Jack A. Hinson, University of Arkansas, Little Rock, AK), and a variety of HSPs (StressGen). After incubation with an appropriate peroxidase-conjugated secondary antibody, immunoreactivity was visualized using enhanced chemiluminescent detection according to manufacturer's instructions (Amersham Pharmacia Biotech). Exposed X-ray films were scanned with a Personal Laser Densitometer (Molecular Dynamics).

Apoptosis determination. Paraffin embedded sections of liver from WT and *IL-6*$^{-/-}$ mice, 6 h after APAP treatment, were stained for single stranded (ss)DNA, a specific measure of apoptotic cells, with the use of anti-ssDNA Mab F7-26 (Chemicon) following previously published methods [22,23].

Statistical analysis. Experimental groups were compared by one-way analysis of variance followed by Student–Newman–Keuls multiple comparison test to determine significant differences between group means. Statistically significant differences were accepted when $p < 0.05$.

Results

Expression of IL-6 family of cytokines in APAP-induced hepatotoxicity

Significant increases in serum ALT activity following APAP (300 mg/kg) administration to male C57Bl/6 mice (Fig. 1A) were accompanied by an increase in liver mRNA expression of IL-6, as well as other IL-6 family members including IL-11, LIF, and OSM (Fig. 1B). IL-6 mRNA expression levels within the liver reached a maximum within 4 h, notably more rapid than peak levels of serum ALT activity and the hepatic mRNA expression of its related cytokine family members.

Increased APAP-induced hepatotoxicity in IL-6$^{-/-}$ *mice*

In order to evaluate the role of IL-6 in APAP-induced liver injury, hepatotoxicity studies were repeated comparing WT to *IL-6*$^{-/-}$ mice. It was observed that *IL-6*$^{-/-}$ mice were significantly more susceptible to the hepatotoxic effects of APAP as compared with their WT counterparts (Fig. 2). The increased susceptibility of *IL-6*$^{-/-}$ mice was not due to enhanced generation of the toxic and reactive metabolite of APAP [24] because the qualitative and quantitative nature of APAP-protein adducts in the liver of *IL-6*$^{-/-}$ and WT mice 2 h after APAP treatment was similar (data not shown).

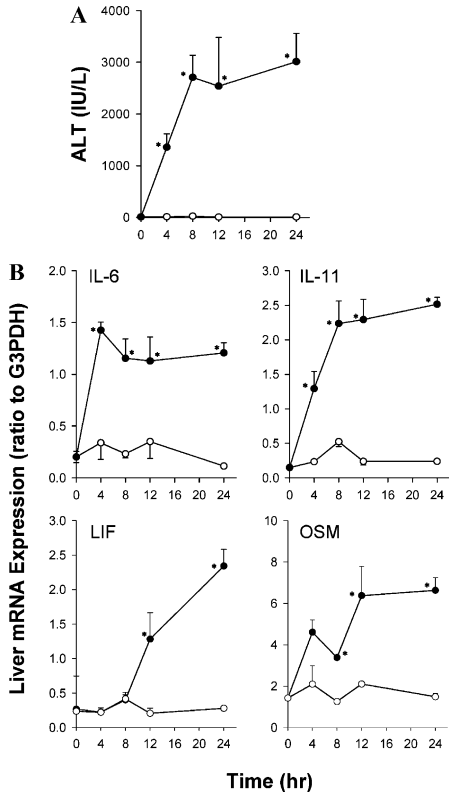

Fig. 1. Time course of hepatotoxicity and liver mRNA expression of the IL-6 family of cytokines after administration of APAP. C57Bl/6 mice were treated with APAP (300 mg/kg) or saline and (A) serum levels of ALT activity and (B) hepatic mRNA expression of IL-6, IL-11, LIF, OSM, and G3PDH was determined at times over 24 h. RT-PCR data were derived by densitometric analysis of stained cDNA fragments and expressed relative to the accompanying G3PDH expression within each sample. Filled circles are APAP treated animals and open circles are saline treated animals. Results shown the means ± SEM of 3–4 mice per group. *, significantly different from saline control at each respective time point ($p < 0.05$).

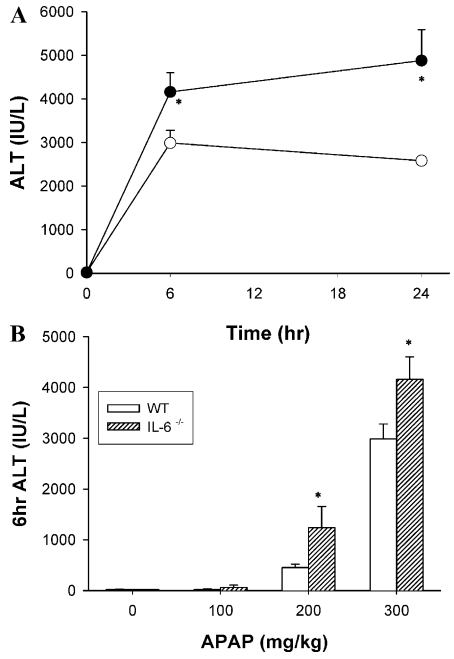

Fig. 2. Time- and dose-dependent APAP-induced liver injury in *IL-6*$^{-/-}$ and WT mice. (A) Following treatment with APAP (300 mg/kg), serum ALT activity was determined in *IL-6*$^{-/-}$ (filled circles) and WT (open circles) mice at 0, 6, and 24 h. Results shown are means ± SEM of 4–7 mice per group. (B) Additional mice were treated with varied doses of APAP (100, 200, or 300 mg/kg) or saline and serum ALT activity was determined 6 h after treatment. Results shown are means ± SEM of 6–7 mice per group. *, significantly different from WT mice ($p < 0.05$) at the corresponding dose of APAP.

To determine whether hepatocytes from *IL-6*$^{-/-}$ mice might be more susceptible than WT mice to apoptotic cell death following APAP treatment, liver slices were immunochemically stained for ssDNA, a procedure that has been reported to be a specific and sensitive detection method for apoptotic cells, including hepatocytes [22,23]. However, no differences in susceptibility of *IL-6*$^{-/-}$ and WT mice to APAP-induced apoptotic cell death were found (results not shown).

Expression of inflammatory mediators in WT versus IL-6$^{-/-}$ *mice*

Liver mRNA expression analyses of the inflammatory mediators, TNF-α, IFN-γ, IL-10, and COX-2, which have been shown to have roles in various models of APAP-induced liver injury [5,6,23,25], failed to show any significant associations that might explain the increased susceptibility of *IL-6*$^{-/-}$ mice to APAP (data not shown). Subsequent analysis of the mRNA expression of IL-6 related cytokines, however, demonstrated a marked increase in IL-11 and LIF in *IL-6*$^{-/-}$ mice after APAP administration as compared to WT mice (Fig. 3, IL-11 and LIF), but no difference in OSM expression (Fig. 3, OSM).

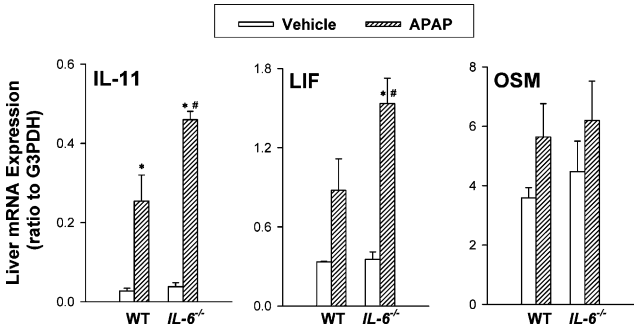

Fig. 3. Liver IL-11, LIF, and OSM mRNA expression in *IL-6*$^{-/-}$ and WT mice. IL-11, LIF, OSM, and G3PDH mRNA expression was determined by RT-PCR 6 h following treatment of *IL-6*$^{-/-}$ and WT mice with APAP (300 mg/kg) or saline. Results shown represent the ratio of densitometric analyses of each cytokine relative to the G3PDH expression within a given sample and are means ± SEM of 3–4 mice per group. *, significantly different from saline controls ($p < 0.05$); #, significantly different from WT mice treated with APAP ($p < 0.05$).

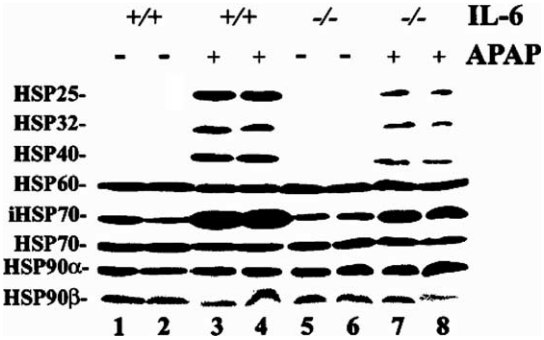

Fig. 4. Deficient HSP responsiveness in *IL-6*$^{-/-}$ mice following APAP treatment. WT and *IL-6*$^{-/-}$ mice were treated with saline (−) or APAP (+, 300 mg/kg) and then sacrificed 6 h after treatment. Liver homogenates were immunoblotted with anti-HSP25, 32 (HO-1), 40, 60, iHSP70, 70, 90α, and 90β antibodies. Shown are the results from two individual mouse liver homogenates within each treatment group.

Effect of IL-6 on HSP responsiveness

Because HSP expression can be induced by IL-6 in a variety of cell types [26–29] and because HSPs protect against apoptosis and necrosis [30,31] and toxin insult [7,12], we hypothesized that the IL-6-mediated protective effect against APAP-induced liver injury might be due, at least in part, to an effect on HSP responsiveness. This hypothesis was evaluated by comparing HSP expression in the liver of *IL-6*$^{-/-}$ mice with that in WT mice. Following APAP treatment, the induction of HSP25, 32, 40, and inducible HSP70 (iHSP70) in the liver of *IL-6*$^{-/-}$ mice was significantly impaired as compared to WT mice (Fig. 4).

Discussion

We have previously hypothesized that the relatively low incidence of DILD in patients and the difficulty in reproducing DILD in laboratory animals may be linked to the production of regulatory factors that normally protect against DILD [5,6]. In this regard, animal studies have shown that endogenous IL-10 [5], COX-2-derived mediator(s) [6], as well as a number of other factors [7–13] are capable of protecting against drug-induced liver injury. These data suggest that the regulation of DILD is extremely complex with no single protective factor being absolute. The redundancy in hepatoprotective mechanisms further suggests that additional factors might also play an important role in protecting the liver from drug-induced injury.

The recent development of *IL-6*$^{-/-}$ mice has led to the discovery that IL-6 also has protective functions in models of liver injury caused by CCl4 [18], concanavalin-A [32], and partial hepatectomy [16]. Our observation of increased hepatic expression of IL-6, IL-11, OSM, and LIF after a hepatotoxic dose of APAP (Fig. 1) suggested to us that IL-6 and possibly other family members, which all share the common signal transducer gp130 as part of their receptors [33], might protect the liver against DILD. Indeed, *IL-6*$^{-/-}$ mice were significantly more susceptible to APAP toxicity than WT mice (Fig. 2) implicating IL-6 as a hepatoprotectant in this model. Moreover, the significantly higher expression of IL-11 and LIF in *IL-6*$^{-/-}$ mice following APAP treatment (Fig. 3) suggested that members of this cytokine family may also compensate for the activity of one another under conditions where one or more of the adaptive, protective responses of its related family members are lacking.

The precise mechanism of liver protection by IL-6 as well as its related family members is still a matter of debate [16,18,19,34–38]. One hypothesis has focused upon their ability to affect bioactivation of hepatotoxicants. IL-6, IL-11, and OSM have been shown to down regulate cytochrome P450 (Cyp) expression [35,39,40], including those that metabolize APAP to its reactive metabolite (Cyp2e1, 1a2, 2a5, and 3a11) (review [24]). However, in our studies, effects of endogenous IL-6 on hepatic Cyp expression did not appear to be the mechanism by which IL-6 protects the liver because *IL-6*$^{-/-}$ mice were more susceptible to APAP-induced liver injury (Fig. 2), despite non-significant differences in protein adduct levels compared with WT mice (data not shown). These results are consistent with findings of other investigators who have previously shown that WT and *IL-6*$^{-/-}$ mice have comparable levels of Cyp2e1 [18,35], the major Cyp isoform that metabolizes APAP to its reactive and toxic *N*-acetyl-*p*-benzoquinoneimine metabolite [24]. Moreover, the protective effects of IL-6 and IL-11 against hepatic injury have also been observed in other models of liver injury where Cyp-dependent bioactivation was not necessary for toxicity [16,17,32,34,36]. These data suggest that IL-6, and possibly other family members, may play a hepatoprotective role against DILD by a mechanism(s) other than the direct effect on drug bioactivation.

In one recent study, it was suggested that the protective capability of exogenous IL-11 against APAP-induced liver injury might be due to the inhibition of TNF-α secretion [37]. However, experiments using TNF-α knockout mice as well as an antibody against TNF-α indicated that TNF-α does not play a major role in APAP-induced liver injury in WT mice [41]. Similarly, the increased susceptibility of *IL-6*$^{-/-}$ mice to APAP-induced liver injury was not associated with an elevation of mRNA expression of pro-inflammatory cytokines such as TNF-α and IFN-γ, nor with a diminution of hepatoprotective factors including IL-10 and COX-2 (data not shown). Thus, IL-6 and possibly its related cytokine family members may play a different protective role in DILD compared with other

injury/inflammation models [32,42,43] where IL-6 appears to regulate TNF-α, IFN-γ, and other protoxicant factors.

In CCl4-, ethanol-, and anti-Fas antibody-induced models of liver injury, pretreatment with IL-6 reduced hepatocyte apoptosis and tissue damage and accelerated regeneration in the liver of both *IL-6*$^{-/-}$ and WT mice [18,36,38]. The protection against apoptosis by IL-6 has been associated with an induction of anti-apoptotic factors Bcl-2, Bcl-xL, and FLIP [36,38]. Nevertheless, in our model, *IL-6*$^{-/-}$ and WT mice did not appear to differ in their susceptibility to apoptotic-induced liver injury 6 h after APAP treatment. Consistent with these findings, *IL-6*$^{-/-}$ and WT mice showed similar rates of hepatocyte apoptosis in a model of acute liver injury following partial hepatectomy [16] and in a model of chronic liver injury model secondary to bile duct ligation [44]. Taken together, these results suggest that the hepatoprotective effects of IL-6 may be dependent on or independent of its effects on apoptosis, depending upon the mechanism of toxicity.

Because HSP expression can be induced by IL-6 in a variety of cell types [26–29] and because HSPs protect against cell death [30,31] and toxin insult, including APAP-induced liver injury [7,12], we hypothesized that IL-6 might also protect from liver injury by inducing HSP responsiveness. Indeed, the normal induction of HSP25, 32, 40, and iHSP70 following APAP treatment [6] was significantly lower in *IL-6*$^{-/-}$ mice compared to WT mice (Fig. 4). To the best of our knowledge, these data are the first to link a hepatoprotective role of IL-6 to HSP expression. However, IL-6 does not appear to regulate the synthesis of HSP60 and 90 in the liver (Fig. 4) as it has been shown to do in human astrocytes [27] and in human peripheral blood lymphocytes [28], respectively. These data suggest that IL-6 and possibly other family members may differentially regulate the expression of HSPs within different tissues. Although the mechanisms by which IL-6 induces HSP expression in the liver are still not clear, the transcription factors, nuclear factor-IL-6 (NF-IL-6) [45] and the signal transducer and activator of transcription protein 3 (STAT3) [26,45], may have regulatory roles in these processes.

Based upon current findings and past literature reports, it seems plausible that factors which affect hepatic expression of IL-6 may have some role in determining individual susceptibility to liver injury. In this regard, the antidiabetic drug troglitazone, which causes idiosyncratic liver injury in humans [46], has been shown to down-regulate IL-6 mRNA expression in mouse liver [47]. If this drug as well as others can also decrease hepatic IL-6 expression in humans, they may increase the risk of DILD or other forms of liver injury. Although it is unknown whether genetic polymorphisms in IL-6 expression might be associated with increased susceptibility to DILD, this possibility is worth exploring in the future.

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