Biochemical Pharmacology 79 (2010) 1363–1371

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Susceptibility to acetaminophen (APAP) toxicity unexpectedly is decreased during acute viral hepatitis in mice

Yonas Getachew [a], Laura James [b], William M. Lee [a], Dwain L. Thiele [a], Bonnie C. Miller [a,*]

[a] Division of Liver and Digestive Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9151, United States
[b] Department of Pediatrics, University of Arkansas Medical Center, Little Rock, AR, United States

ARTICLE INFO

Article history:
Received 15 October 2009
Accepted 17 December 2009

Keywords:
Acetaminophen
Liver
DILI (drug induced liver injury)
Cytochrome P450
Adenovirus

ABSTRACT

Acetaminophen (APAP) hepatotoxicity results from cytochrome P450 metabolism of APAP to the toxic metabolite, n-acetyl-benzoquinone imine (NAPQI), which reacts with cysteinyl residues to form APAP adducts and initiates cell injury. As APAP is commonly used during viral illnesses there has been concern that APAP injury may be additive to that of viral hepatitis, leading physicians to advise against its use in such patients; this has not been investigated experimentally. We infected C57BL/6 male mice with replication-deficient adenovirus to produce moderately severe acute viral hepatitis and observed that APAP doses that were hepatotoxic or lethal in control mice produced neither death nor additional increase in serum ALT when administered to infected mice at the peak of virus-induced liver injury. Moreover, the concentration of hepatic APAP-protein adducts formed in these mice was only 10% that in control mice. Protection from APAP hepatotoxicity also was observed earlier in the course of infection, prior to the peak virus-induced ALT rise. Hepatic glutathione limits APAP-protein adduct formation but glutathione levels were similar in control and infected mice. Cyp1a2 (E.C. 1.14.14.1) and Cyp2e1 (E.C. 1.14.13.n7) mRNA expression decreased by 3 days post-infection and hepatic Cyp2e1 protein levels were reduced almost 90% at 7 days, when adduct formation was maximally inhibited. In vitro, hepatocytes from virally infected mice also were resistant to APAP-induced injury but sensitive to NAPQI. Rather than potentiating APAP-induced liver injury, acute viral hepatitis in this model resulted in selective down-regulation of APAP metabolizing P450s in liver and decreased the risk of APAP hepatotoxicity.

1. Introduction

The use of medications in the setting of acute or chronic liver disease is a major concern to many clinicians as the liver is a major site of drug metabolism and clearance. Of particular concern are drugs that are themselves associated with liver injury such as acetaminophen (APAP). The incidence of drug induced liver injury is rising worldwide and APAP induced liver injury is now a leading cause of acute liver failure in the U.S. and many other Western nations [1–4]. Although most cases of APAP induced liver injury are associated with overdose, others represent episodes of therapeutic misadventure [1–4]. Thus, it is common for clinicians to advise patients with acute or chronic viral hepatitis to limit or avoid APAP use [5–7] despite the absence of definitive data assessing the safety of APAP in such patients. Lack of clinically determined or scientifically based guidelines for the use of analgesics such as APAP in individuals with liver disease contributes to the significant challenge of pain management in these patients [8].

Consistent with the clinical experience, in vivo animal experiments have demonstrated that APAP liver injury is proportional to dose of APAP ingested and to the rate of production of the toxic reactive (electrophilic) metabolite, n-acetyl-benzoquinone imine (NAPQI) (reviewed in [9]). APAP is metabolized by multiple pathways in the liver with the predominant pathways of glucuronidation and sulfation producing non-toxic metabolites excreted in urine. In humans and other species that are particularly sensitive to APAP induced injury, however, a significant portion of APAP also is metabolized by specific cytochrome P450s to the reactive metabolite NAPQI. Hepatic glutathione reacts stably with NAPQI to form non-toxic conjugates but, when the detoxification capacity of hepatic glutathione levels is exceeded, NAPQI reacts with proteins and other cellular macromolecules to initiate multiple pathways of cell damage and oxidative stress [9].

Abbreviations: ALT, alanine aminotrasferase; APAP, acetaminophen, paracetamol; CYP, designation of specific cytochrome P450 isomers in humans, rats and most species other than mouse; Cyp, cytochrome isomer designation in mice; LDH, lactate dehydrogenase; NAPQI, n-acetyl-benzoquinone imine; P450, cytochrome P450; o.p.u., optical particle unit.

* Corresponding author. Tel.: +1 214 648 3402; fax: +1 214 648 0274.
E-mail address: bonnie.miller@utsouthwestern.edu (B.C. Miller).

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.12.019

In addition to the seeming likelihood for worse outcomes to occur in patients subjected to two independent causes of liver injury such as viral hepatitis and toxic APAP metabolites, disease mechanisms operative during viral hepatitis may alter susceptibility specifically to APAP induced liver injury. In acute or chronic hepatic viral infection, host immune responses are the major cause of liver injury [10–12]. Innate cytotoxic effector mechanisms mediated by natural killer (NK) cells also play a role in drug induced liver injury as these mechanisms have been found to amplify the degree of injury mediated by hepatotoxins such as APAP and have been postulated to trigger an ongoing process of immunoallergic liver injury to APAP and other selected drugs [13–17]. Pro-inflammatory cytokines released by Kupffer cells and T cells in response to APAP also have been implicated in the progression of APAP hepatotoxicity [18–20]. Thus, it has been suggested that prior or independent immune activation may worsen the clinical outcome [21].

In the present studies, the hypothesis that acute viral hepatitis alters the susceptibility of mice to sub-lethal and lethal APAP doses was tested An adenoviral hepatitis model was used in which mice are infected by intravenous, tail vein, administration of replication-deficient adenovirus resulting in liver restricted infection. Like the major human hepatitis viruses, adenovirus is not directly cytopathic but triggers the full spectrum of innate and adaptive immune responses commonly observed in other models of viral hepatitis [22–25]. These responses in turn induce acute hepatocellular injury as adenovirally infected hepatocytes are cleared by host cytopathic immune-mediated mechanisms [11,26–30].

2. Materials and methods

2.1. Mice

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used between 6 and 14 wks of age. Mice were acclimated to the vivarium for a minimum of 1 wk prior to inclusion in experiments. Mice were injected via the tail vein with 2.5×10^{11} optical particle units (o.p.u.) per 25 g body weight of recombinant, replication-deficient adenovirus diluted in Dulbecco's phosphate buffered saline (DPBS, GIBCO®/Invitrogen™Life Technologies, Carlsbad, CA) at a concentration of $\sim 1.25 \times 10^{12}$ o.p.u./ml, as indicated, or an equal volume of DPBS. On days 3 or 7 following viral infection, mice were injected i.p. with varying doses of acetaminophen (APAP) from 75 to 500 mg/kg body weight, as indicated, or equal volume of DPBS, after an overnight fast. APAP and all other chemicals used in these studies were from Sigma–Aldrich (St. Louis, MO) unless specified otherwise. APAP was dissolved in DPBS at 55 °C, sterilized by filtration through a 0.22 μm syringe filter (Millex GP, Millipore, Billerica, MA) and maintained at 37 °C prior to injection. To provide similar injection volumes, APAP was dissolved at a concentration of 12.5 mg/ml for up to 150 mg/kg dosing, 25 mg/ml for 300 mg/kg dosing, and at 40 mg/ml for 450 and 500 mg/kg dosing. Following APAP administration, the mice were maintained for up to 48 h in cages placed partially onto heating pads to prevent hypothermia [31] and with ad lib access to food and water. Two different recombinant adenoviruses were employed in these studies, an E1 deficient, β-galactosidase expressing construct, AdV-LacZ [26] and an E1, E3 deficient adenoviral construct expressing green fluorescent protein, AdV-GFP [32]. Viruses were propagated in the AAV-293 subline (Stratagene, La Jolla, CA) and purified by centrifugation on discontinuous CsCl gradients and desalting on PD-10 chromatography columns (GE Healthcare BioSciences, Uppsala, SWE). No differences between the viruses in induction of ALT responses or protection from APAP hepatotoxicity were noted. All animal studies were carried out in compliance with accepted standards of humane animal care as described in the Guide for the Care and Use of Laboratory Animals [33] and were approved by the UT Southwestern Institutional Animal Care and Use Committee. Mice were euthanized by CO_2 inhalation consistent with the AVMA Guidelines on Euthanasia.

2.2. Serum alanine aminotransferase (ALT) assay

Blood samples (~50 μl) were taken just prior to APAP administration and at timed intervals thereafter and allowed to clot at 4 °C. Serum ALT activities were determined using Thermo Electron Infinity ALT reagent (Victoria, Australia).

2.3. APAP-adduct measurement

Following euthanasia, blood samples were drawn into EDTA-containing syringes to obtain plasma for adduct measurements; livers were harvested, snap frozen using liquid nitrogen cooled aluminum tongs, and powdered under liquid nitrogen. APAP-Cys adducts in a portion of the liver powder and in plasma were quantified by high pressure liquid chromatography and electrochemical detection (HPLC-ECD) essentially as described [34].

2.4. Liver glutathione content

Glutathione levels per mg liver protein were quantified using the Total Glutathione Quantification kit from Dojindo Molecular Technologies, Inc. (Rockville, MD). A portion of the liver powder was homogenized in ice cold PBS and an aliquot immediately extracted with trichloroacetic acid. Glutathione in the acid extract determined per the manufacturer's protocol while he remaining homogenate was used to determine liver protein. Protein concentrations were determined using the bicinchoninic acid method with reagents purchased from Sigma–Aldrich using bovine serum albumin as a standard.

2.5. Immunoblotting of liver Cyp2e1 and Cyp1a2

Frozen liver powder was homogenized in ice cold lysis buffer (10 mM Tris, pH 8.0, 100 mM KCl, 5 mM NaCl, 3.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100 and 0.05% sodium deoxycholate) and insoluble material removed by centrifugation for 10 min at 10,000 × *g*. Equal amounts of total protein in the cleared lysates (10 μg per lane) were separated on 12.5% bis-acrylamide gels by SDS-PAGE and electrophoretically transferred to nitrocellulose in pH 9.9 carbonate buffer [35]. Immunodetection was performed using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-CYP1A2 mouse monoclonal and anti-CYP 2E1 polyclonal rabbit primary antibodies were from Abcam, Inc. (Cambridge, MA). HRP-conjugated anti-mouse Ig and HRP-conjugated anti-rabbit Ig secondary antibodies were from Amersham and BioRad (Hercules, CA), respectively.

2.6. Quantitative RT-PCR of select liver mRNA

RNA was isolated by acid-guanidinium-phenol extraction and reverse transcribed using Superscript II (Invitrogen™Life Technologies, Carlsbad, CA) according to the manufacturer's instructions using random hexamers for priming. PCR was conducted in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA). PCR were performed in a final volume of 10 μl containing cDNA from 10 ng of reverse transcribed total RNA, 150 nM each forward and reverse primers and SybrGreen Universal PCR Master Mix (Applied

Biosystems). All reactions were performed in triplicate. Primer sequences for qRT-PCR for the CYP proteins were those reported by Richardson and Morgan [36]. The primer pairs for cyclophilin were those reported previously [37]. Relative mRNA levels were quantified using the delta delta Cτ method [37].

2.7. Lactate dehydrogenase (LDH) release from freshly isolated hepatocytes

Livers of control or adenovirally infected mice were perfused with calcium and magnesium free Hank's buffered salt solution (HBSS, GIBCO®) followed by 0.05% collagenase (Worthington Biochemical Corp., Lakewood, NJ) in Williams E medium (GIBCO®). Dissociated hepatocytes were filtered through 100 μM cell strainers (BD Falcon®, BD Biosciences, San Jose, CA) centrifuged (40 × *g* for 15 min at room temperature) in 50% Percoll in Williams E medium supplemented with 1% 100× penicillin–streptomycin–glutamine solution (GIBCO®) to eliminate damaged hepatocytes, then washed 3 times with the supplemented Williams E medium to remove residual Percoll. The hepatocytes were resuspended to 0.25×10^6 cells/ml in Williams E medium supplemented with 1% 100× penicillin–streptomycin–glutamine, 1% 100× insulin–transferrin–selenium (GIBCO®) and 10% fetal bovine serum and 0.1 ml aliquots distributed in 24-well culture plates. APAP (5 mM) or NAPQI (250 μM) were added to wells in triplicate; 4 h later, culture supernatants were collected for determination of released LDH activity while hepatocytes and remaining supernatant were used for determination of total LDH activity per well following cell lysis by freeze-thawing. LDH activity was determined by kinetic assay using the BioTek Synergy HT Reader (Winooski, VT) in mixtures containing 0.2 mM NADH, 5 mM pyruvate and 2.5% culture supernatant or cell lysate.

2.8. Statistical evaluation

Statistical significance was evaluated by *t*-test; differences at the level of $p < 0.01$, $p < 0.02$ and $p < 0.05$ are noted separately.

3. Results

3.1. Acetaminophen hepatotoxicity is not augmented during viral hepatitis

Administration of replication deficient, recombinant adenovirus to mice at a dose of 2.5×10^{11} o.p.u. per 25 g body weight resulted in a moderately severe hepatitis with significant elevations in serum ALT by day 3 and peak ALT levels occurring by 7 days post-infection (Fig. 1A). ALT levels plateaued from days 7–9 post-infection as was noted in multiple experiments (e.g. Fig. 2A and C). Despite marked liver injury in the infected mice, there was no significant difference in daily food intake between control and infected groups (data not shown). In mice not exposed to virus, APAP administration resulted in dose dependent increases in serum ALT levels (Fig. 1B). No hepatotoxicity was observed at a dose of 75 mg/kg. However, 150 and 300 mg/kg APAP doses resulted in ALT elevations that peaked at 24 h post-administration while 450 mg/kg was uniformly fatal prior to 24 h in the experiment shown.

To investigate whether susceptibility to APAP-induced liver injury is amplified at the peak of virally induced liver injury, a sub-lethal dose of APAP was administered to mice either infected with adenovirus or administered saline 7 days previously. As detailed in Fig. 2A, APAP administration to control mice at a dose of 300 mg/kg produced severe hepatotoxicity with serum ALT levels elevated to approximately 200 times baseline 24 h post-APAP administration. In marked contrast, mice with acute viral

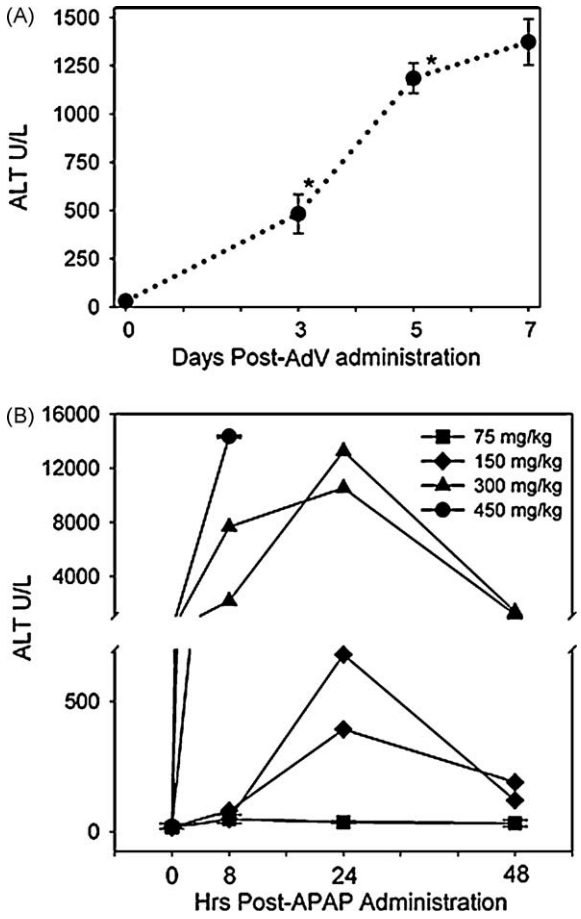

Fig. 1. Time course of serum ALT elevations following administration of (A) replication-deficient adenovirus or (B) varying APAP doses. The values presented in (A) are the mean ± SE from groups of 3–9 mice. Asterisks denote values significantly elevated ($p < 0.01$) compared to the preceding time point. The values presented in (B) are mean ± SE of groups of mice ($n = 3$ for 75 mg/kg and $n = 4$ for 450 mg/kg doses) or individual values where $n = 2$ (150 and 300 mg/kg doses). ALT values following vehicle injection alone did not differ significantly from the 0 time baseline.

hepatitis were protected from APAP hepatotoxicity. Serum ALT levels were significantly higher in infected than uninfected mice prior to APAP administration. However, APAP did not induce any further increase in serum ALT levels in the infected mice and there was no difference in serum ALT levels between infected mice receiving APAP and those given saline only (Fig. 2A). To assure that protection from APAP induced injury was not secondary to any potential contaminants in the virus preparation, mice were administered virus that had been inactivated by repeated freeze-thawing cycles and administered APAP 7 days later (Fig. 2B). Prior to APAP administration, serum ALT levels were not elevated in mice treated with inactive virus in comparison to controls and there was no difference in maximum ALT levels between the groups 24 h post-APAP administration. ALT elevation however occurred more rapidly in mice that had received inactive virus (Fig. 2B).

To determine whether the unexpected protective effect of acute viral hepatitis was observed with higher APAP doses, in a separate experiment, uninfected mice and infected mice 7 days post-infection were challenged with 500 mg/kg APAP. The uninfected mice exhibited high serum ALT levels by 8 h (Fig. 2C) and all succumbed within 48 h (Fig. 2D). In mice with acute viral hepatitis, however, even this higher APAP dose did not provoke any additional increase in serum ALT levels nor were any deaths observed (Fig. 2C and D).

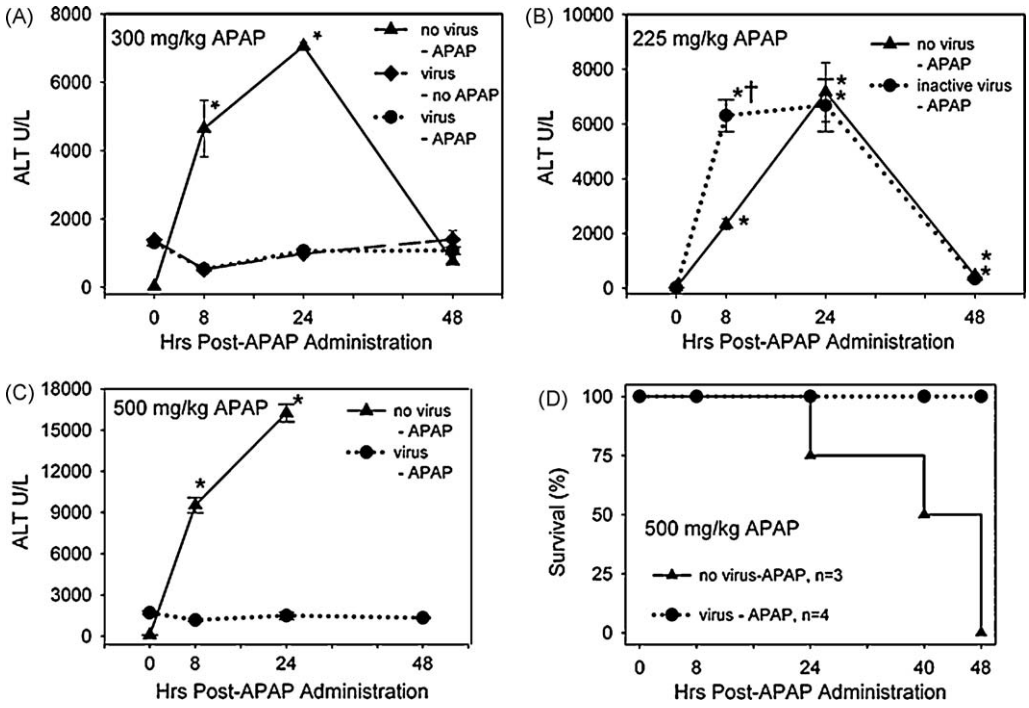

Fig. 2. Time course of ALT elevation (A–C) or mortality (D) following APAP administration to mice on day 7 of infection. Mice were injected with (A, C and D) viable adenovirus, (B) equivalent amount of inactivated virus, or (A–D) saline for no virus controls. APAP, or saline for no APAP controls, was administered 7 days later after an overnight fast and ALT levels determined at the indicated times. Values presented in panels (A–C) are the mean ± SE from 3–4 mice. Asterisks denote serum ALT levels within treatment groups significantly elevated ($p < 0.01$) compared to the corresponding pre-APAP values. In panel (B), significant differences ($p < 0.01$) between the inactive virus and uninfected mouse groups at the 8 h time point are additionally noted with a dagger.

3.2. Hepatic acetaminophen adduct formation is decreased during viral hepatitis

To investigate whether the failure of otherwise toxic APAP doses to elicit additional liver injury during viral hepatitis results from decreased metabolic activation of APAP, APAP-protein adducts were measured 4 h post-APAP administration to uninfected mice and mice infected 7 days previously (Fig. 3A). The concentration of liver APAP-protein adducts in the infected mice was less than 1/10th that in the uninfected mice; in plasma the concentration was reduced nearly 100-fold (Fig. 3A). Plasma ALT levels in the uninfected mice had increased more than 100-fold by 4 h APAP administration while ALT values in the infected mice did not change significantly (Fig. 3B).

3.3. Hepatic expression of key APAP metabolizing cytochrome P450s is decreased during acute viral hepatitis while glutathione levels are unchanged

Conjugation with glutathione is an important detoxification pathway for reactive APAP metabolites and decreased hepatic glutathione levels can lower the threshold dose resulting in adduct formation and APAP hepatotoxicity. However, as detailed in Fig. 4, after overnight fasting to mimic APAP administration conditions, hepatic glutathione levels were comparable in control and infected mice 7 days post-virus administration.

To investigate whether decreased cytochrome P450 metabolism of APAP might be responsible for decreased adduct formation in the infected mice, mRNA and protein expression of the major P450 isoforms responsible for APAP metabolism in liver was examined. Messenger RNA levels for Cyp2e1, the isoform primarily responsible for APAP metabolism [9] were reduced over 80% in the infected mice while mRNA levels for Cyp1a2, a minor APAP metabolizing isoform, were not significantly different (Fig. 5A). Messenger RNA levels for Cyp3a11, an isoform which may play a

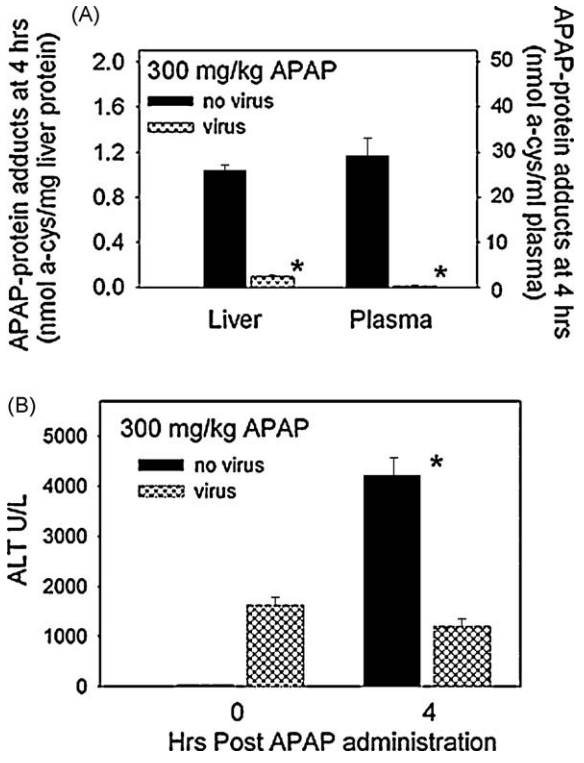

Fig. 3. Effect of concurrent adenoviral hepatitis on (A) APAP-protein adduct levels and (B) ALT levels 4 h post-APAP administration on day 7 of infection After an overnight fast, APAP was administered to mice that had been injected with virus or saline 7 days previously. Livers and plasma were harvested for adduct measurements ~4 h later. The values presented are the mean ± SE of 4 mice in ea group. In panel (A), asterisks denote values significantly different ($p < 0.01$) between virus and control groups. In panel (B), asterisks denote values within groups that differ significantly between time points.

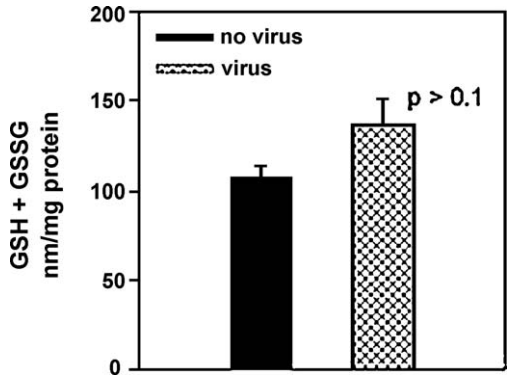

Fig. 4. Hepatic glutathione levels in mice with or without acute viral hepatitis on day 7 of infection. Total glutathione (GSH + GSSG) levels in liver were determined after overnight fasting. The values presented are the mean ± SE of 4 mice in ea group and are not significantly different ($p > 0.1$).

minor role in APAP metabolism [38], were modestly decreased in the infected mice. Immunoblot analysis revealed that, consistent with the mRNA results, Cyp2e1 but not Cyp1a2 protein levels were significantly decreased on day 7 post-infection (Fig. 5B).

3.3.1. Hepatocytes isolated from adenovirally infected mice are resistant to APAP but not to NAPQI induced injury

To investigate whether mechanisms in addition to decreased metabolic activation of APAP contributed to the marked decrease in APAP hepatotoxicity in infected mice at the peak of virally induced liver injury, freshly isolated hepatocytes from uninfected and infected mice were treated with APAP (5 mM) or NAPQI (250 μM) in vitro and lactate dehydrogenase release measured 4 h later. As detailed in Fig. 6, NAPQI treatment induced similar levels of LDH from both infected and uninfected hepatocytes. In the uninfected hepatocytes, LDH release also was significantly elevated by APAP treatment at even this early time point but no increase was seen in the infected hepatocyte cultures (Fig. 6).

3.4. APAP hepatotoxicity is reduced in mice with viral hepatitis 3 days post-infection

Hepatotoxicity of 300 mg/kg APAP also was evaluated on day 3 following infection, as detailed in Fig. 7, to assess whether protection, or possibly potentiation, occurred prior to the peak of virally induced liver injury. Consistent with the time course of viral hepatitis in this model (see Fig. 1), serum ALT levels in the infected mice continued to increase during the 48 h period following APAP administration but did not differ from those in infected mice receiving saline only. Moreover, the increase in serum ALT levels due to the ongoing hepatitis was more than an order of magnitude lower than ALT increases elicited by APAP administration to non-infected mice (Fig. 7). To examine expression of APAP metabolizing cytochrome P450s on day 3 following infection, Cyp1a2 and Cyp2e1 liver mRNA and protein levels were examined in a separate set of mice (Fig. 8). As detailed in Fig. 7A, both Cyp1a2 and 2e1 hepatic mRNA levels were decreased on day 3 following infection, to approximately 50 and 35% of control levels, respectively, while Cyp3a11 mRNA levels in the infected mice were not different from controls. Immunoblot analysis revealed reduction of both Cyp1a2 and Cyp2e1 protein levels (Fig. 8B), although the relative decrease in Cyp2e1 protein was not as extensive as on day 7 following infection (Fig. 5). APAP-protein adducts levels in liver and plasma 4 h following APAP administration to mice infected 3 days previously also were significantly less than in matched controls (Fig. 9A). As on day 7, there was a dramatic reduction in plasma APAP-protein adduct levels in the infected mice, approximately 90% compared to

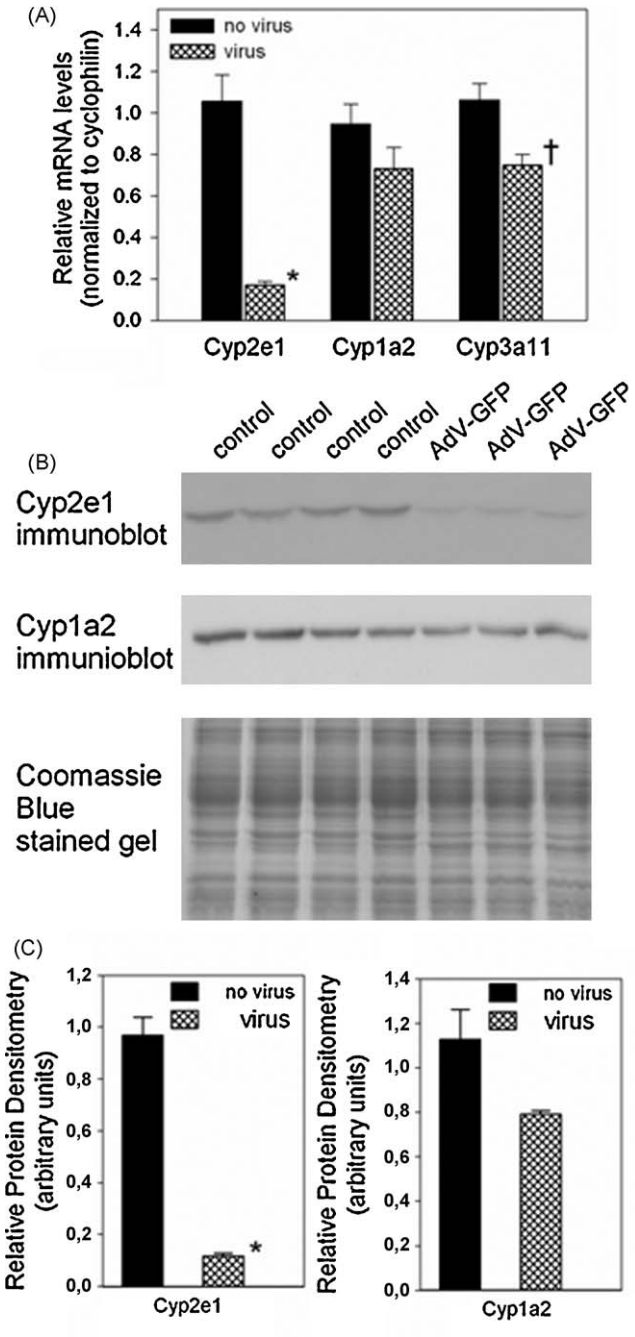

Fig. 5. Effect of acute adenoviral hepatitis on messenger RNA (A) and protein (B and C) expression of APAP metabolizing cytochrome P450s on day 7 of infection. (A) Cyp2e1, Cyp1a2 and Cyp3a11 mRNA levels were determined by qRT-PCR and normalized to cyclophilin mRNA measured in the same sample. The values presented are the mean ± SE of the average normalized value from 4 mice in each group. Significant differences between groups with p value <0.01 are indicated with an asterisk, p value <0.05 is indicated with a dagger. (B) Equal amounts of total liver protein (10 μg per lane) were subjected to SDS-PAGE on replicate gels and analyzed by immunoblotting or stained for total protein with Coomassie Blue as a loading control. Quantitative densitometry of the immunoblots is shown in (C) with significant differences between groups ($p < 0.01$) noted with an asterisk.

controls. In addition, a 55% reduction in liver APAP-protein adduct levels was observed in APAP treated infected mice.

4. Discussion

The relatively few clinical studies available on APAP use in patients with active liver disease offer conflicting conclusions.

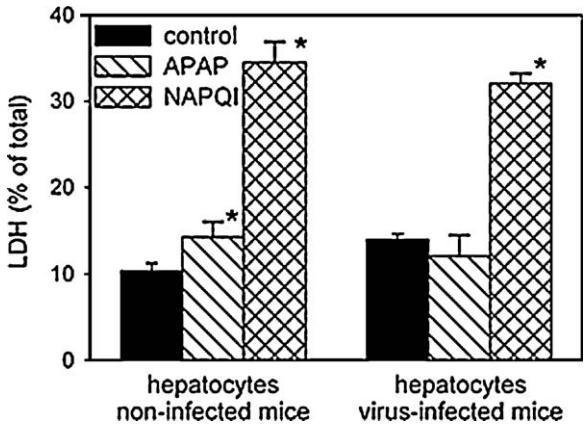

Fig. 6. APAP and NAPQI mediated LDH release in hepatocytes from control and virus infected mice. Hepatocytes isolated from a non-infected or adenovirally infected mouse were cultured for 4 h in the presence or absence of 5 mM APAP or 250 μM NAPQI, LDH release into the culture medium was determined and expressed as a percent of total LDH activity (cellular plus released) in the cultures. The values presented are the mean ± SD of triplicate wells; asterisks denote values significantly different ($p < 0.01$) between NAPQI treated and control cultures.

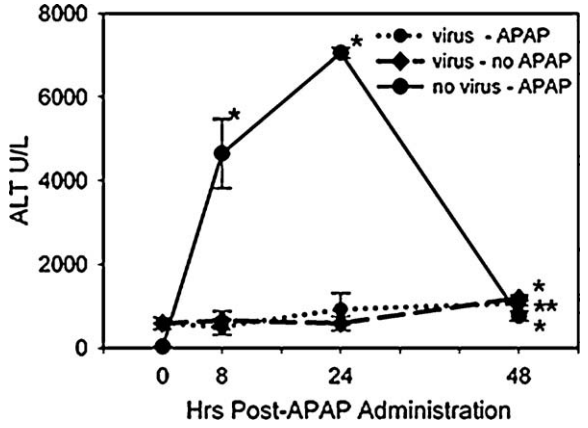

Fig. 7. Time course of ALT elevation following APAP administration to mice on day 3 of adenoviral infection. After an overnight fast, groups of mice that had been injected 3 days previously with adenovirus were administered APAP (virus APAP) or equal volume of saline (virus-no APAP) and ALT levels determined at the indicated times. The values presented are the mean ± SE from 4 mice in ea group. Single asterisks denote serum ALT levels within treatment groups significantly elevated ($p < 0.01$) compared to the corresponding pre-APAP values while the double asterisk indicates $p < 0.02$ for the virus—no APAP group at 48 h. Data from the no virus APAP treated control group previously displayed in Fig. 2a is repeated here to facilitate comparison of the magnitude of hepatoprotection.

Some studies have suggested the relative safety of APAP use in the setting of chronic liver disease [39]. In contrast, Myers et al. found that APAP hepatotoxicity was more common in patients with pre-existing liver disease [40] and Nguyen et al. found that HCV infection is an independent predictor for APAP injury and its subsequent complications [41]. In patients with acute viral hepatitis, Yaghi et al. found that APAP use at therapeutic doses was associated with greater alterations of surrogate markers of the severity of acute viral hepatitis [5] and anecdotal associations between APAP use during acute viral hepatitis and worse clinical outcomes also have been reported (e.g. [42]). However, other studies seeking to detect evidence for additive effects of APAP use on liver injury during acute viral hepatitis or other causes of liver injury have instead found that some bioanalytical methods for the detection of APAP yield high rates of false positives in the presence of high bilirubin levels and thus may have resulted in overestimation of APAP use during acute liver disease in some studies [43].

Using a mouse model of adenoviral hepatitis, results of the studies presented here provide evidence that not only is there no potentiation of APAP induced liver injury during acute viral hepatitis but instead there is a remarkable degree of protection from the hepatoxic effects of APAP overdose. In non-infected mice, the magnitude of APAP induced ALT elevation increased sharply with incremental increases above the threshold hepatotoxic dose. However even 500 mg/kg APAP, a supralethal dose in non-infected mice, produced no additional rise in ALT above levels resulting from the viral hepatitis when administered to mice at the peak of virally induced liver injury.

Protection from APAP hepatotoxicity was associated with down-regulated expression of Cyp2e1 and corresponding reduction in APAP-protein adduct formation reflecting decreased APAP metabolism to reactive species. In wild-type mice, liver APAP-protein adducts reach peak levels approximately 2 h following APAP ingestion, then decline rapidly over the next 4–6 h concomitant with increasing levels of plasma ALT and APAP-protein adducts released from damaged hepatocytes [44]. In the studies reported here, the significantly lower levels of APAP-protein adducts in livers of infected mice after 3 or 7 days of infection compared to controls were coupled with proportionately even lower levels of plasma APAP adducts at 4 h post-APAP administration.

The lower levels of hepatic APAP-protein adducts in mice given 300 mg/kg APAP after 7 days of infection compared to 3 days is consistent with the continued decline in Cyp2e1 expression during this period despite the substantial recovery in Cyp1a2 expression. Due to in vivo differences in K_m and V_{max}, at doses below ~450 mg/kg, P450 metabolism of APAP is mediated almost exclusively by CYP2E1 while at higher doses, CYP1A2 contributes [45]. The in vivo significance of the differing affinities of CYP2E1 and CYP1A2 for APAP is underscored by the observation that Cyp2e1 knockout mice exhibit almost complete resistance to APAP at doses below 600 mg/kg but with higher doses complete protection is seen only in mice deficient in both Cyp2e1 and Cyp1a2 [46,47].

In freshly isolated hepatocytes in vitro, as in vivo, APAP toxicity occurs in two phases [48]; the first, metabolic, phase occurs over a period of about 2 h and includes cytochrome P450 mediated APAP activation to NAPQI. Cell death, which increases over the subsequent 2–12 h, occurs in the second phase when removal of APAP from the cells no longer reduces toxicity [44,49]. Cytotoxicity caused by direct NAPQI addition to isolated hepatocytes also is time dependent, evolving over 10 min to 2 h in a concentration dependent manner, suggesting a requirement for additional cellular mechanisms subsequent to NAPQI initiated damage [50]. In the studies reported here, the comparable sensitivity of hepatocytes from adenovirally infected and control mice to NAPQI mediated cytotoxicity provides additional evidence that decreased Cyp2e1 metabolism of APAP to NAPQI is the primary mechanism contributing to the in vivo resistance of mice with acute viral hepatitis to APAP induced injury.

Expression of the various hepatic cytochrome P450s can be regulated by a wide range of intrinsic and extrinsic factors and at multiple levels from gene transcription to protein turnover. Altered hepatic CYP mRNA and protein expression is well known to occur in extra-hepatic or systemic inflammatory models (recent reviews include [51,52]). Consistent with the complex and differential regulation of different P450s however, the specific patterns of altered cytochrome P450 expression are distinct in different models of inflammation. For instance, on day 6 following infection with the enteropathogenic bacteria *citrobacter Rodentium*, hepatic Cyp3a11 mRNA levels in wild-type mice were decreased to 16% of control levels while Cyp1a2 and Cyp2e1 mRNA levels were not affected [53]. Administration of LPS to wild-type mice however rapidly and uniformly reduced mRNA expression of

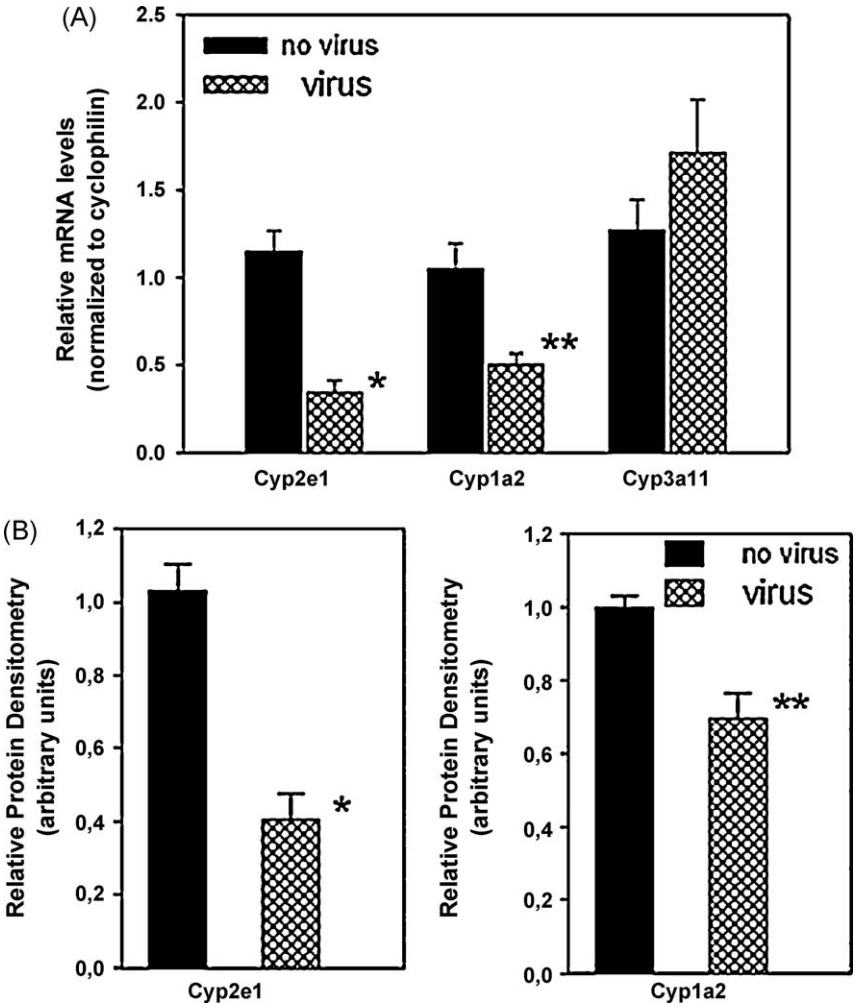

Fig. 8. Effect of acute adenoviral hepatitis on messenger RNA (A) and protein (B) expression of APAP metabolizing cytochrome P450s on day 3 of infection. (A) Cyp2e1, Cyp1a2 and Cyp3a11 mRNA levels were determined by qRT-PCR and normalized to cyclophilin mRNA measured in the same sample. The values presented are the mean ± SE of the average normalized value from 4 mice in each group. (B) Equal amounts of total liver protein (10 μg per lane) were subjected to SDS-PAGE on replicate gels and analyzed by immunoblotting. Quantitative densitometry of the immunoblots is shown, the values represent the mean ± SE from 4 mice in ea group. In both panels, significant differences between groups are noted with a single asterisk indicating p values <0.01 and double asterisks indicating a p value <0.02.

almost all hepatic P450 s examined, which included Cyp1a2, 2e1 and 3a11 [53]. Consistent with down-regulation of the primary APAP metabolizing P450s, LPS pre-treatment induces significant protection from APAP toxicity [54]. Co-administration of non-toxic LPS doses in rodents, however, potentiates liver injury of multiple other hepatotoxic drugs (e.g. [55–58]). It is therefore of particular importance to note in the studies presented here that the protective effect of adenoviral hepatitis was not elicited by any potential contaminants in the adenovirus preparation or from a response to denatured viral proteins. The more rapid induction of APAP induced liver injury in mice administered inactivated virus (Fig. 2B) in fact suggests that in the absence of infection, the immune response to denatured virus may prime the liver for APAP induced injury.

While the present studies focused on APAP hepatotoxicity during acute viral hepatitis, it is interesting to note that there is evidence for down-regulated CYP2E1 expression in patients with chronic hepatitis C infection. In a molecular profiling study, CYP2E1 mRNA levels in liver biopsies from 21 HCV patients with stage 1 fibrosis were significantly reduced compared to 16 normal controls [59]. Normal controls consisted of patients with moderately elevated ALT activity in whom all causes of liver disease had been ruled out and whose biopsy specimens were histologically normal. In an independent study, CYP1A2, CYP2E1 and CYP3A4 mRNA levels in patients with chronic hepatitis C infection were found to decrease with increasing stage of fibrosis [60]. However, the HCV patients with stage 1 fibrosis had higher levels of these mRNAs than histologically normal specimens from patients with metastatic liver cancer originating from colon cancer. Thus, as discussed by the authors, it is unclear from the study results whether the specific P450 mRNA levels are reduced in liver tissue adjacent to cancerous lesions even more extensively than in patients with chronic HCV, or whether there is an initial increase in expression of these P450s during chronic HCV infection which then declines with disease progression.

In summary, in the acute viral hepatitis model employed in these studies, decreased Cyp2e1 mediated APAP metabolism evidenced by decreased APAP-adduct formation affords significant protection from APAP hepatotoxicity. These findings provide an example of the dramatic and unexpected effects on drug metabolism that may result from the changes in hepatic cytochrome P450 expression that occur during the course of even acute, self-limited liver disease.

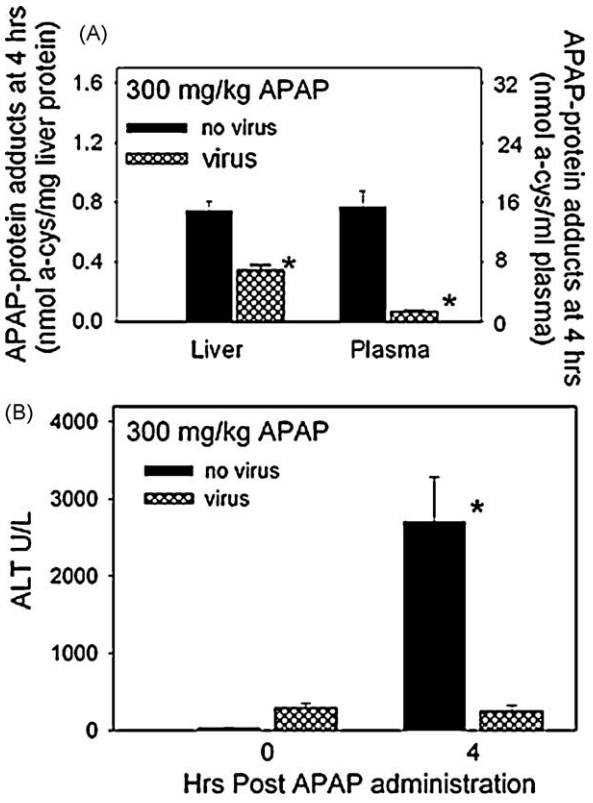

Fig. 9. Effect of concurrent adenoviral hepatitis on (A) APAP-protein adduct levels and (B) ALT levels 4 h post-APAP administration on day 3 of infection. After an overnight fast, APAP was administered to mice that had been injected with virus or saline 3 days previously. Livers and plasma were harvested for adduct measurements ~4 h later. The values presented are the mean ± SE of 4 mice in ea group. In panel (A), asterisks denote values significantly different ($p < 0.01$) between virus and control groups. In panel (B), asterisks denote values within groups that differ significantly between time points.

Acknowledgements

This research was supported by National Institutes of Health Grant 5R01 DK53933.

References

[1] Arundel C, Lewis JH. Drug-induced liver disease in 2006. Curr Opin Gastroenterol 2007;23:244–54.
[2] Lee WM, Seremba E. Etiologies of acute liver failure. Curr Opin Crit Care 2008;14:198–201.
[3] Schmidt LE, Larsen FS. MELD score as a predictor of liver failure and death in patients with acetaminophen-induced liver injury. Hepatology 2007;45:789–96.
[4] Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 2005;42:1364–72.
[5] Yaghi C, Honein K, Boujaoude J, Slim R, Moucari R, Sayegh R. Influence of acetaminophen at therapeutic doses on surrogate markers of severity of acute viral hepatitis. Gastroenterol Clin Biol 2006;30:763–8.
[6] Fontana RJ. "Unintentional" acetaminophen overdose on the rise: who is responsible? Dr Robert J Fontana is interviewed by Paul C Adams. Can J Gastroenterol 2006;20:319–24.
[7] Kuehn BM. FDA focuses on drugs and liver damage: labeling and other changes for acetaminophen. J Am Med Assoc 2009;302:369–71.
[8] Mehta G, Rothstein KD. Health maintenance issues in cirrhosis. Med Clin North Am 2009;93:901–15. viii–ix.
[9] Nelson SD, Bruschi SA. Mechanisms of acetaminophen-induced liver disease. In: Kaplowitz N, DeLeve LD, editors. Drug-induced Liver Disease. New York: Informa Healthcare USA, Inc.; 2007. p. 353–88.
[10] Bertoletti A, Ferrari C. Kinetics of the immune response during HBV and HCV infection. Hepatology 2003;38:4–13.
[11] Abougergi MS, Gidner SJ, Spady DK, Miller BC, Thiele DL. Fas and TNFR1, but not cytolytic granule-dependent mechanisms, mediate clearance of murine liver adenoviral infection. Hepatology 2005;41:97–105.
[12] Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 2003;77:68–76.
[13] Seguin B, Uetrecht J. The danger hypothesis applied to idiosyncratic drug reactions. Curr Opin Allergy Clin Immunol 2003;3:235–42.
[14] Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. Gastroenterology 2004;127:1760–74.
[15] Liu ZX, Han D, Gunawan B, Kaplowitz N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology 2006;43:1220–30.
[16] Liu ZX, Kaplowitz N. Immune-mediated drug-induced liver disease. Clin Liver Dis 2002;6:755–74.
[17] Angelini G, Gardella S, Ardy M, Ciriolo MR, Filomeni G, Di Trapani G, et al. Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. Proc Natl Acad Sci USA 2002;99:1491–6.
[18] Blazka ME, Wilmer JL, Holladay SD, Wilson RE, Luster MI. Role of proinflammatory cytokines in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 1995;133:43–52.
[19] Ishida Y, Kondo T, Ohshima T, Fujiwara H, Iwakura Y, Mukaida N. A pivotal involvement of IFN-gamma in the pathogenesis of acetaminophen-induced acute liver injury. FASEB J 2002;16:1227–36.
[20] Numata K, Kubo M, Watanabe H, Takagi K, Mizuta H, Okada S, et al. Overexpression of suppressor of cytokine signaling-3 in T cells exacerbates acetaminophen-induced hepatotoxicity. J Immunol 2007;178:3777–85.
[21] Jaeschke H. Innate immunity and acetaminophen-induced liver injury: why so many controversies? Hepatology 2008;48:699–701.
[22] Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, et al. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther 2001;3:697–707.
[23] Lieber A, He CY, Meuse L, Schowalter D, Kirillova I, Winther B, et al. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J Virol 1997;71:8798–807.
[24] Yang Y, Wilson JM. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. J Immunol 1995;155:2564–70.
[25] Yang Y, Xiang Z, Ertl HC, Wilson JM. Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. Proc Natl Acad Sci USA 1995;92:7257–61.
[26] Kafrouni MI, Brown GR, Thiele DL. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. J Immunol 2001;167:1566–74.
[27] Kafrouni MI, Brown GR, Thiele DL. The role of TNF-TNFR2 interactions in generation of CTL responses and clearance of hepatic adenovirus infection. J Leukoc Biol 2003;74:564–71.
[28] Stout-Delgado HW, Getachew Y, Rogers TE, Miller BC, Thiele DL. The role of serpinb9/serine protease inhibitor 6 in preventing granzyme B-dependent hepatotoxicity. Hepatology 2007;46:1530–40.
[29] Chen J, Zajac AJ, McPherson SA, Hsu HC, Yang P, Wu Q, et al. Primary adenovirus-specific cytotoxic T lymphocyte response occurs after viral clearance and liver enzyme elevation. Gene Ther 2005;12:1079–88.
[30] Xu X, Zhang HG, Liu ZY, Wu Q, Yang PA, Sun SH, et al. Defective clearance of adenovirus in IRF-1 mice associated with defects in NK and T cells but not macrophages. Scand J Immunol 2004;60:89–99.
[31] Vaquero J, Belanger M, James L, Herrero R, Desjardins P, Cote J, et al. Mild hypothermia attenuates liver injury and improves survival in mice with acetaminophen toxicity. Gastroenterology 2007;132:372–83.
[32] He T-C, Zhou S, Costa Ld, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 1998;95:2509–14.
[33] National Research Council. Guide for the care and use of laboratory animals, 7th ed., Washington: National Academy Press; 1996.
[34] Muldrew KL, James LP, Coop L, McCullough SS, Hendrickson HP, Hinson JA, et al. Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of acetaminophen using high-performance liquid chromatography with electrochemical detection. Drug Metab Dispos 2002;30:446–51.
[35] Dunn SD. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. Anal Biochem 1986;157:144–53.
[36] Richardson TA, Morgan ET. Hepatic cytochrome P450 gene regulation during endotoxin-induced inflammation in nuclear receptor knockout mice. J Pharmacol Exp Ther 2005;314:703–9.
[37] Stout-Delgado HW, Getachew Y, Miller BC, Thiele DL. Intrahepatic lymphocyte expression of dipeptidyl peptidase I-processed granzyme B and perforin induces hepatocyte expression of serine proteinase inhibitor 6 (Serpinb9/SPI-6). J Immunol 2007;179:6561–7.
[38] Guo GL, Moffit JS, Nicol CJ, Ward JM, Aleksunes LA, Slitt AL, et al. Enhanced acetaminophen toxicity by activation of the pregnane X receptor. Toxicol Sci 2004;82:374–80.
[39] Benson GD. Acetaminophen in chronic liver disease. Clin Pharmacol Ther 1983;33:95–101.
[40] Myers RP, Shaheen AA, Li B, Dean S, Quan H. Impact of liver disease, alcohol abuse, and unintentional ingestions on the outcomes of acetaminophen overdose. Clin Gastroenterol Hepatol 2008;6:918–25.
[41] Nguyen TH, Mai G, Villiger P, Oberholzer J, Salmon P, Morel P, et al. Treatment of acetaminophen-induced acute liver failure in the mouse with conditionally immortalized human hepatocytes. J Hepatol 2005;43:1031–7.

[42] Kc S. Acute liver failure caused by hepatitis E virus and paracetamol. JNMA J Nepal Med Assoc 2007;46:74–6.
[43] Polson J, Wians Jr FH, Orsulak P, Fuller D, Murray NG, Koff JM, et al. False positive acetaminophen concentrations in patients with liver injury. Clin Chim Acta 2008;31:24–30.
[44] Pumford NR, Hinson JA, Potter DW, Rowland KL, Benson RW, Roberts DW. Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen adducts in serum and liver proteins of acetaminophen-treated mice. J Pharmacol Exp Ther 1989;248:190–6.
[45] Bessems JG, Vermeulen NP. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit Rev Toxicol 2001;31:55–138.
[46] Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ. Role of CYP2E1 in the hepatotoxicity of acetaminophen. J Biol Chem 1996;271: 12063–7.
[47] Zaher H, Buters JT, Ward JM, Bruno MK, Lucas AM, Stern ST, et al. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol 1998;152:193–9.
[48] Reid AB, Kurten RC, McCullough SS, Brock RW, Hinson JA. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther 2005;312:509–16.
[49] Bajt ML, Knight TR, Lemasters JJ, Jaeschke H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. Toxicol Sci 2004;80:343–9.
[50] Holme JA, Dahlin DC, Nelson SD, Dybing E. Cytotoxic effects of N-acetyl-p-benzoquinone imine, a common arylating intermediate of paracetamol and N-hydroxyparacetamol. Biochem Pharmacol 1984;33:401–6.
[51] Renton KW. Regulation of drug metabolism and disposition during inflammation and infection. Expert Opin Drug Metab Toxicol 2005;1:629–40.
[52] Morgan ET. Regulation of cytochrome p450 by inflammatory mediators: why and how? Drug Metab Dispos 2001;29:207–12.
[53] Richardson TA, Sherman M, Antonovic L, Kardar SS, Strobel HW, Kalman D, et al. Hepatic and renal cytochrome p450 gene regulation during citrobacter rodentium infection in wild-type and toll-like receptor 4 mutant mice. Drug Metab Dispos 2006;34:354–60.
[54] Liu J, Sendelbach LE, Parkinson A, Klaassen CD. Endotoxin pretreatment protects against the hepatotoxicity of acetaminophen and carbon tetrachloride: role of cytochrome P450 suppression. Toxicology 2000;147:167–76.
[55] Deng X, Stachlewitz RF, Liguori MJ, Blomme EA, Waring JF, Luyendyk JP, et al. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J Pharmacol Exp Ther 2006;319:1191–9.
[56] Tukov FF, Luyendyk JP, Ganey PE, Roth RA. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. Toxicol Sci 2007;100:267–80.
[57] Shaw PJ, Ditewig AC, Waring JF, Liguori MJ, Blomme EA, Ganey PE, et al. Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. Toxicol Sci 2009;107:270–80.
[58] Shaw PJ, Ganey PE, Roth RA. Tumor necrosis factor alpha is a proximal mediator of synergistic hepatotoxicity from trovafloxacin/lipopolysaccharide coexposure. J Pharmacol Exp Ther 2009;328:62–8.
[59] Bieche I, Asselah T, Laurendeau I, Vidaud D, Degot C, Paradis V, et al. Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection. Virology 2005;332:130–44.
[60] Nakai K, Tanaka H, Hanada K, Ogata H, Suzuki F, Kumada H, et al. Decreased expression of cytochromes P450 1A2, 2E1, and 3A4 and drug transporters Na+-taurocholate-cotransporting polypeptide, organic cation transporter 1, and organic anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic hepatitis C patients. Drug Metab Dispos 2008;36:1786–93.