Coordinated Expression of Multidrug Resistance-Associated Proteins (Mrps) in Mouse Liver during Toxicant-Induced Injury

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Following acute chemical injury, hepatocytes are generally more resistant to toxicant re-exposure. Alterations in expression of hepatobiliary transport systems may contribute to this resistance by preventing accumulation of potentially toxic chemicals. Previous data demonstrate the concomitant reduction of uptake transporters and induction of efflux transporter mRNA during chemical liver injury. The present study further characterizes the expression of multidrug resistance-associated proteins 1–4 (Mrp1–4), breast cancer resistance protein (Bcrp) and sodium-taurocholate co-transporting polypeptide (Ntcp) in mouse liver following administration of the hepatotoxicants acetaminophen (APAP) and carbon tetrachloride (CCL4). Mice received hepatotoxic doses of APAP (400 mg/kg), CCL4 (10 or 25 μL/kg), or vehicle, ip. Livers were collected at 6, 24, and 48 h for Western blot quantification and immunofluorescence analysis. Protein expression of Bcrp was unchanged with treatment. Ntcp levels were preserved in APAP-exposed livers and reduced to 30–50% of control after CCL4. Conversely, Mrp1–4 expression was differentially up-regulated. CCL4 increased Mrp1 (3.5-fold), Mrp2 (1.4-fold), and Mrp4 (26-fold) while reducing Mrp3 levels to 20% of control. Administration of APAP enhanced expression of Mrp2 (1.4-fold), Mrp3 (3.5-fold), and Mrp4 (16-fold). Immunostaining of liver sections obtained 48 h after hepatotoxicant treatment confirmed expression patterns of a subset of transporters (Bcrp, Ntcp, Mrp3, and Mrp4). Double immunofluorescence imaging demonstrated the simultaneous down-regulation of Ntcp and up-regulation of Mrp4 in hepatocytes adjacent to the central vein after CCL4. Altered expression of transporters may reduce the overall chemical burden of an injured liver during recovery and contribute to the resistance of hepatocytes to subsequent toxicant exposure.

Key Words: acetaminophen; carbon tetrachloride; hepatotoxicity; Mrp3; Mrp4; Ntcp.

Acetaminophen (APAP) is a commonly used analgesic and antipyretic agent. Although safe when taken at prescribed doses, liver damage occurs when patients ingest supratherapeutic amounts. The reproducibility of APAP-induced hepatotoxicity in rodent models makes it a widely studied compound for the identification of signaling pathways involved in hepatocyte injury and recovery which are relevant to human exposures (Park et al., 2005). Mechanistic studies often include other centrilobular hepatotoxins such as carbon tetrachloride (CCL4) that have different mechanisms of toxicity (Weber et al., 2003). Following hepatic damage elicited by either APAP or CCL4, a number of cell stress and recovery responses are coordinately up-regulated to counteract imbalances in antioxidant homeostasis and limit further hepatic disease progression (Salminen et al., 1997; Schiaffonati and Tiberio, 1997). One such cytoprotective enzyme is heme-oxygenase 1 (Ho-1). This enzyme breaks down pro-oxidant heme during liver injury (Chiu et al., 2002; Tenhunen et al., 1968). After exposure to APAP, dramatic increases in rat Ho-1 protein are prominent in centrilobular hepatocytes and macrophages (Chiu et al., 2002).

Transmembrane carrier proteins in hepatocyte cell membranes, such as sodium-taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting proteins (Oatps), are responsible for extraction of bile acids and a vast array of organic anions from sinusoidal blood. Once inside hepatocytes, bile acids and organic anions are often metabolized and/or conjugated by biotransformation enzymes. Subsequent removal of these compounds as well as xenobiotic conjugates and oxidative stress products is mediated by ATP-dependent transport proteins in the sinusoidal (e.g., multidrug resistance-associated proteins, Mrp1, 3, and 4) and canalicular (e.g., Mrp2 and breast cancer resistance protein, Bcrp) membranes.

We recently reported that administration of hepatotoxic doses of APAP and CCL4 results in reduced expression of mouse Ntcp and Oatp genes and increased mRNA levels of Mrp1–4 in coordination with up-regulated detoxification genes (e.g., Ho-1) (Aleksunes et al., 2005). Similar modulation of some transport genes and proteins occurs in rat liver after APAP and CCL4 treatment (Geier et al., 2002; Nakatsukasa et al., 1993; Song et al., 2003). Increased levels of MRP1 and
MMP-3 as well as the multidrug resistance 1 gene are also reported in human liver specimens following toxin-induced liver necrosis (Ros et al., 2003). The potential implications of altered expression of transporters during hepatotoxicity are two-fold. First, induction of Mrps may limit the intracellular accumulation of endogenous and exogenous chemicals during periods of stress and/or recovery from hepatotoxicity. Second, alterations in expression of these transporters likely contribute to the different pharmacokinetic profiles of numerous pharmaceuticals in individuals with altered hepatic function.

Although our recent data demonstrate the simultaneous up-regulation of efflux transporter and reduction of uptake transporter mRNA expression in mouse liver during chemical-induced injury, it is not known whether transcriptional regulation of these genes leads to corresponding changes in protein expression or if protein expression is altered through transcription-independent mechanisms (Aleksunes et al., 2005). The aim of this study was to quantitatively determine the effect of APAP and CCl4 on hepatic expression of transport proteins over time, and whether these changes were localized to specific zones of the liver. Protein analysis includes numerous transporters not previously investigated during chemical-induced liver injury in rats. In the present study, groups of male mice were injected with APAP (400 mg/kg) or CCl4 (10 or 25 \mu l/kg), which resulted in mild to moderate centrilobular hepatocellular injury as previously described (Aleksunes et al., 2005). Antibodies directed against the stress protein Ho-1, uptake transporter Ntcp, and efflux transporters Mrp1–4 and Bcrp were used for determining changes in total hepatic expression by Western blot analysis at 6, 24, and 48 h after toxicant treatment. Immunofluorescent staining of mouse liver sections for Mrp3, 4, Bcrp, and Ntcp confirmed their cellular localization to the canalicular and sinusoidal membranes and identified zonal expression patterns of transport proteins after APAP and CCl4 exposure. From these experiments, hepatotoxicant treatment resulted in the differential up-regulation of Mrp1–4 and Ho-1 proteins over the 48-h interval. These findings contrast with the reduced expression of Ntcp predominantly in centrilobular hepatocytes in response to CCl4. Since most transport protein expression patterns correlated well with changes previously reported for mRNA levels, these results indicate that altered transport protein expression in response to toxicant treatment was regulated at the transcriptional level. Coordinated expression of efflux and uptake transport systems may confer resistance to hepatocytes after subsequent toxicant exposure by limiting intracellular retention of xenobiotics and mediators of inflammation and oxidative stress.

MATERIALS AND METHODS

**Animal care and treatment.** Male C57BL/6J mice, aged 10–12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were acclimated one week upon arrival and maintained in a 12-h dark/light cycle, temperature- and humidity-controlled environment. Mice were fed laboratory rodent diet (No. 5001, PMI Feeds, St. Louis, MO) ad libitum. APAP was dissolved in 50% propylene glycol/water. CCl4 was diluted in corn oil. Groups of posted mice (n = 3–7) were administered APAP (400 mg/kg, 10 ml/kg, ip), CCl4 (10 and 25 \mu l/kg, 5 ml/kg, ip), or the respective vehicle control. The doses of APAP and CCl4 were selected in order to achieve mild to moderate, but not overt toxicity, as previously reported (Aleksunes et al., 2005). Livers were collected 6, 24, or 48 h after APAP or CCl4 administration and snap-frozen in liquid nitrogen. Frozen tissues were stored at −80°C until assayed. All animal studies were conducted in accordance with National Institutes of Health standards and the Guide for the Care and Use of Laboratory Animals.

**Preparation of crude membrane and microsomal fractions.** Livers were homogenized in sucrose-Tris (ST) buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4) containing 50 μg/ml aprotenin and centrifuged at 100,000 × g for 60 min at 4°C. The resulting pellet constituted the crude membrane fraction and was resuspended in ST buffer. For isolation of microsomes, homogenates were first centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was then centrifuged at 100,000 × g for 60 min. ST buffer was used to resuspend the microsomal pellet. Protein concentration was determined by the method of Lowry using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

**Western blot analysis of Ho-1 protein.** Microsomal proteins (40 μg protein/lane) were boiled for 3 min and electrophoretically resolved using polyacrylamide gels (12% resolving, 4% stacking) and transblotted overnight at 4°C onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Immunoochemical detection of Ho-1 (32 kDa) was performed using anti-Ho-1 antibody (SPA895, Stressgen, San Diego, CA). Membranes were blocked in 10% nonfat dry milk (NFDM) in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw) for 2 h and incubated with primary antibody diluted 1:2000 in NFDM in PBS-Tw for 2 h. A peroxidase-labeled secondary antibody (Amersham Life Science, Arlington Heights, IL, na934) diluted 1:2000 in 5% NFDM in PBS-Tw for 1 h was then applied to PVDF membranes.

**Western blot analysis of transport proteins.** Membranes (50 μg protein/lane) were electrophoretically resolved without boiling using polyacrylamide gels (8% resolving, 4% stacking) and transblotted overnight at 4°C onto PVDF-Plus membrane (Micron Separations). Immunoochemical detection of Mrp1 (~190 kDa), Mrp2 (~190–200 kDa), Mrp3 (~180–190 kDa), Mrp4 (~160–170 kDa), and Bcrp (~175 kDa) protein was performed using MRPr1, M2III-5, M3II-2, M4I-10, M10, BXP-53 antibodies, respectively. Anti-Mrp and Bcrp antibodies were provided by George Scheffer, VU Medical Center, Amsterdam, the Netherlands. Membranes were blocked with 1% NFDM in PBS-Tw for 1 h and incubated for 1 h with the primary antibody diluted in blocking buffer (1:2000 for MRPr1, M3II-2, M4I-10, 1:600 for M3II-3, 1:5000 for BXP-53). A species-appropriate peroxidase-labeled secondary antibody (Sigma Chemical Co., St. Louis, MO) was diluted (1:2000) in blocking buffer and incubated with blots for 1 h. Immunoochemical detection of Ntcp protein (~50–52 kDa) was performed using anti-rat Ntcp K polyclonal antibody (provided by Bruno Steiger, University Hospital, Zurich, Switzerland). PVDF membranes were blocked with 5% NFDM in 0.1% Tris-buffered saline containing 0.05% Tween 20 (TBS-Tw) for 1 h and incubated for 1 h with the primary antibody diluted in blocking buffer (1:5000). Donkey anti-rabbit peroxidase-labeled secondary antibody (Amersham Life Science) was diluted (1:1000) in TBS-Tw and applied to blots for 1 h. Equal protein loading was confirmed by coomassie blue staining of blots (data not shown). Protein-antibody complexes were detected using an ECL chemiluminescent kit (Amersham Life Science) and exposed to Fuji Medical X-ray film (Fisher Scientific, Pittsburgh, PA). The intensity of protein bands was quantified using a PDI Image Analyzer (Protein and DNA ImageWare System, PDI, Inc., Huntington Station, NY). Intensity values were normalized to pooled controls (oh) and expressed as relative protein expression.

**Immunofluorescence staining and confocal laser scanning microscopy.** Livers were embedded in Optimal Cutting Temperature (OCT) compound and brought to −10°C. Cryosections (5–7 μm) were thaw-mounted.
onto Superfrost glass slides (Fisher Scientific) and stored at −80°C with desiccant until use. Tissue sections were fixed with 4% paraformaldehyde for 5 min. All antibody solutions were filtered through 0.22 μm membrane syringe-driven filter units (Osmonics Inc., Minnetonka, MN) prior to use. Immunofluorescence analysis was limited to Mrp3, Mrp4, Bcrp, and Ntcp. Antibodies used for Western blot immunostaining of Mrp1, Mrp2, and Ho-1 were not suitable for immunofluorescent detection in this study. Immunostaining of liver sections was performed at the 48 h time point (except for Mrp4 which was stained at 24 and 48 h) since alterations in Mrp3 and Ntcp protein expression as seen on Western blot were greatest at 48 h. For Mrp3, Mrp4, and Bcrp staining, sections were blocked with 5% goat serum/phosphate-buffered saline with 0.1% Triton X (PBS-Tx) for 1 h and then incubated with M2II-2, M1-J-10, or BXP-53 primary antibody diluted 1:100 in 5% goat serum/PBS-Tx for 2 h at room temperature. After incubation with primary antibody, the sections were washed three times in PBS-Tx and incubated for 1 h with goat anti-rat IgG Alexa 488 IgG (Invitrogen Corporation, Carlsbad, CA) diluted 1:200 in 5% goat serum/PBS-Tx. Ntcp staining was performed as previous described (Stieger et al., 1994). Briefly, sections were blocked with 0.1% saponin and 2% gelatin for 30 min and incubated with a primary antibody against Ntcp (rabbit anti-rat K4 antibody) for 2 h at a dilution of 1:100 in 0.1% saponin (w/v) and 1% (w/v) bovine serum albumin in PBS. Sections were incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibody to goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:200 in PBS, 0.1% (w/v) saponin and 1% (w/v) bovine serum albumin. Slides were washed in PBS and then rinsed twice in distilled deionized water. The sections were air dried and mounted in Prolong Gold with 4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen Corp.). Images were acquired on a Leica TCS-SP laser scanning confocal microscope (Leica Microsystems Inc., Exton, PA) equipped with Argon, NeHe, and UV lasers, which allowed excitation at 488, 633, and 358 nm wavelengths for detection of Alexa 488 and FITC, Alexa 633, and DAPI, respectively. Each fluorescent channel was acquired sequentially and then merged to create the final image. Negative control staining was performed by incubating sections without primary antibody.

Appropriate secondary antibodies were used for simultaneous imaging of Ntcp and Mrp4 or Bcrp. Tissue sections were incubated with goat anti-rat Alexa 488 (Invitrogen Corp.) and goat anti-rabbit Vectastain biotinylated IgG (Vector Laboratories, Burlingame, CA) at dilutions of 1:100 for 1 h. Streptavidin Alexa 633 (Invitrogen Corp.) was applied for 20 min.

Statistical analysis. Quantitative results were expressed as mean relative protein expression ± standard error. Immunoblot data were normalized to control values (0 h) and analyzed using a one-way ANOVA followed by Neuman-Keuls multiple range test (p < 0.05).

RESULTS

Western Blot of Ho-1 Protein after Hepatotoxicant Treatment

The hepatotoxicity of APAP (400 mg/kg) and CCl4 (25 μl/kg) as assessed by plasma alanine aminotransferase (ALT) and histopathological analysis has been previously reported (Aleksunes et al., 2005). Briefly, APAP increased mean plasma ALT activity at 6 h to 230 U/L and remained elevated over the 48-h period although histopathological damage had resolved by 48 h. CCl4 increased ALT at 6 h (mean ALT 145 U/L) which continued to increase through 48 h (mean ALT 4558 U/L). Using these doses, mild to moderate hepatocellular degeneration and necrosis is observed after APAP while more extensive centrilobular injury is seen with exposure to CCl4. Temporal expression of the prototypical stress protein Ho-1 after APAP and CCl4 was analyzed. This study demonstrates up-regulation of mouse Ho-1 protein levels at 6, 24, and 48 h after chemical treatment (Figs. 1A and 1B). Maximal Ho-1 expression (9 to 18-fold) was seen 24 h after APAP and CCl4. Up-regulation of Ho-1 in mouse liver persisted through the 48 h time point with 6 to 7-fold induction over controls.

Western Blot of Canalicular Transport Proteins after Hepatotoxicant Treatment

No change in levels of the canalicular efflux protein Bcrp after APAP and CCl4 were observed during the 48-h study period (Fig. 2A). CCl4 increased Mrp2 protein 1.4-fold at both 6 and 48 h. The expression of Mrp2 protein in mouse liver was unaltered at 6 and 24 h after APAP but increased 1.6-fold by 48 h. Representative 48 h blots are shown in Figure 2B.

Western Blot of Sinusoidal Transport Proteins after Hepatotoxicant Treatment

Transport proteins responsible for sinusoidal uptake (Ntcp) and efflux (Mrp1, 3, 4) were differentially expressed following APAP and CCl4-induced hepatic injury (Figs. 3A and 3B). Of note, the scales depicting relative protein expression are different for each of the transporters (Fig. 3A). Representative 48 h blots are shown in Fig. 3B. Compared with control mouse livers, Ntcp expression was reduced to 47% and 30% at 24 and 48 h after CCl4, respectively, while no significant changes were observed in mice with APAP treatment. A slight and statistically

FIG. 1. Western blot analysis of heat shock protein Ho-1 expression in livers from APAP- and CCl4-treated mice. Western immunoblots were performed using liver microsomal protein fractions from C57BL/6J mice at 6, 24, and 48 h after treatment with APAP (400 mg/kg), CCl4 (25 μl/kg), or appropriate vehicle control (Con). The data are presented as mean relative Ho-1 protein expression ± SE (A) and as individual blots at 6, 24, and 48 h (B). Asterisks (*) represent a statistical difference (p < 0.05) from pooled 0-h control mice (n = 4–7 animals/time point).
FIG. 2. Western blot analysis of canalicular transport proteins Bcrp and Mrp2 expression in livers from APAP- and CCl4-treated mice. Western immunoblots were performed using liver membrane protein fractions from C57BL/6J mice at 6, 24, and 48 h after treatment with APAP (400 mg/kg), CCl4 (25 µl/kg), or appropriate vehicle control (Con). The data are presented as mean relative Mrp2 or Bcrp protein expression ± SE (A) and as representative blots at 48 h (B). Asterisks (*) represent a statistical difference (p < 0.05) from pooled 0-h control mice (n = 4–7 animals/time point).

FIG. 3. Western blot analysis of sinusoidal transport proteins Mrp1, Mrp3, Mrp4, and Ntcp expression in livers from APAP- and CCl4-treated mice. Western immunoblots were performed using liver membrane protein fractions from C57BL/6J mice at 6, 24, and 48 h after treatment with APAP (400 mg/kg), CCl4 (25 µl/kg), or appropriate vehicle control (Con). The data are presented as mean relative Mrp1, Mrp3, Mrp4, or Ntcp protein expression ± SE (A) and as representative blots at 48 h (B). Notably, the scales for mean relative protein expression differ for each transport protein. Asterisks (*) represent a statistical difference (p < 0.05) from pooled 0-h control mice (n = 4–7 animals/time point).
significant (1.3-fold) increase in liver Ntcp at 6 h after CCl₄ was seen before the decline in protein expression at 24 h.

Notably, enhanced expression of Mrp1 protein was detected in response to CCl₄ at 24 (2-fold) and 48 h (3.5-fold) (Figs. 3A and 3B). These changes were not observed with APAP treatment. Conversely, APAP, but not CCl₄, increased Mrp3 expression in mouse liver 3.5-fold at 48 h. Instead, CCl₄ administration decreased hepatic Mrp3 to 26 and 21% of control livers at 24 and 48 h, respectively (Figs. 3A and 3B).

Using the M₄I-10 antibody, expression of Mrp4 is almost undetectable in normal mouse liver. The specificity of this antibody has been previously confirmed using tissue homogenate from Mrp4-/- mice (Leggas et al., 2004). These data are consistent with previous reports demonstrating very low levels of Mrp4 mRNA in mouse liver (Aleksunes et al., 2005; Maher et al., 2005). Although difficult to discern from the representative 48-h blots shown in Figure 3B, quantifiable bands were detected in membrane preparations from control mice using image analysis software. Exposure to APAP and CCl₄ resulted in 5- and 7-fold increase of Mrp4 at 24 h, respectively (Fig. 3A). A more profound induction of hepatic Mrp4 protein was seen at 48 h after APAP (16-fold) and CCl₄ (26-fold) (Figs. 3A and 3B).

**Immunofluorescent Localization of Transport Proteins after Hepatotoxicant Treatment**

Immunofluorescent staining of Mrp3, Mrp4, Bcrp, and Ntcp was performed on liver sections obtained from vehicle and hepatotoxicant-treated mice in order to determine patterns of cellular and zonal transporter expression. In Figure 4, Ntcp (red) is uniformly expressed at high levels in normal mouse liver and exhibits a “honeycomb” appearance which is characteristic of its sinusoidal localization. Conversely, Bcrp (green) staining is consistent with localization to canalicular junctions between adjacent hepatocytes. No overlap of Ntcp and Bcrp staining is observed. Low magnification images demonstrated no zonal differences in Bcrp staining in control mouse liver (data not shown). Similar to Western blot staining, no change in expression of canalicular Bcrp was seen in mouse liver after exposure to APAP or CCl₄ (Figs. 5B and 5C) compared to control (Fig. 5A).

A low magnification view of normal mouse liver shows sinusoidal Mrp3 staining predominantly in hepatocytes of the centrilobular region with a gradual decrease in intensity near the portal vein (Supplementary Fig. 1). This observation is consistent with preferential expression of Mrp3 in the perivenous region of rat and human liver. Increased staining of Mrp3 after APAP was prominent on the membranes of hepatocytes throughout the liver, with greater staining in centrilobular cells (Fig. 5E) compared to control (Fig. 5D). Conversely, a marked reduction of Mrp3 was observed in livers from mice treated with CCl₄ as seen by western blot immunostaining (Fig. 5F).

Similar to Western blot analysis of control mouse liver, minimal immunofluorescent staining of Mrp4 was seen in the liver lobule (Fig. 5G). Strong Mrp4 staining was observed in centrilobular hepatocytes after APAP and CCl₄ (Figs. 5H and 5I). The labeling of Mrp4 consisted of a single or double layer of cells surrounding the central vein after 48 h after APAP treatment (Fig. 5H). CCl₄ exposure yielded diffuse, punctate staining of Mrp4 in liver cells, also in the perivenous region at 48 h (Fig. 5I).

Ntcp was the only uptake carrier selected for protein analysis in this study. Low magnification images demonstrated uniform Ntcp staining throughout the normal mouse liver (data not shown). CCl₄ treatment markedly reduced the expression of Ntcp at 48 h (Fig. 5L) compared to control (Fig. 5J). In contrast, no change in intensity of Ntcp staining was seen in sections from mice after APAP, consistent with the lack of mRNA and total protein changes in these livers (Fig. 5K) (Aleksunes et al., 2005).

In order to further address the irregular cellular pattern of Mrp4 staining in response to CCl₄, liver sections from mice treated with a lower dose of CCl₄ (10 µl/kg) were also stained. At this dose, mild increases in serum ALT in the absence of histopathological injury are observed. Increased expression of Mrp4 in response to CCl₄ (10 µl/kg) was detected in centrilobular hepatocytes at 24 and 48 h (Supplementary Figs. 2B and 2E, respectively). This induction occurred in the absence of histopathological damage and was entirely localized to the cell membrane. Images from CCl₄ (25 µl/kg) were also included for comparison (Supplementary Figs. 2C and 2F).
Double immunostaining directed against Ntcp and Mrp4 confirmed high basal expression of Ntcp and low Mrp4 levels in the liver lobule of normal mice (Fig. 6A). At this magnification, the selective up-regulation of Mrp4 in centrilobular hepatocytes after APAP and CCl4 (10 μl/kg) is evident (Figs. 6B and 6C, respectively). As noted above, Mrp4 staining is diffuse at the higher dose of CCl4 (25 μl/kg) and inconsistent with complete localization to the plasma membrane. In this group of mice, double immunostaining demonstrated a simultaneous decrease of Ntcp in centrilobular hepatocytes (Fig. 6D).

Protein analysis of other transporters for which mRNA expression was previously assessed in response to APAP and CCl4 (Aleksunes et al., 2005) was not included in the current studies since there are no specific antibodies to detect these proteins in mice (e.g., organic anion transporter polypeptides).

**DISCUSSION**

Recent research has highlighted the induction of stress proteins, such as Ho-1, in rodent liver in response to both APAP and CCl4 exposure (Chiu et al., 2002; Nakahira et al., 2003). Ho-1 is a highly inducible heat shock protein which is responsible for heme catabolism (Tenthunen et al., 1968).
pro-oxidant heme is liberated from drug metabolizing enzymes during hepatocellular injury. Therefore, an up-regulation of Ho-1 is thought to be a compensatory response of centrilobular hepatocytes to limit further cellular damage by eliminating this molecule during oxidative stress (Bauer and Bauer, 2002). Given these findings, we measured Ho-1 protein at 6, 24, and 48 h after toxicant treatment in order to compare temporal changes in the expression of transport proteins to a known indicator of the hepatic stress response.

In the present study, the effect of hepatocellular injury on expression of transport proteins is examined in male C57BL/6J mouse liver over time. In general, APAP and CCl4-mediated damage increased levels of transport proteins (in particular, the Mrps) involved in export of chemicals from the hepatocyte into blood or bile. A reciprocal reduction in the bile acid influx carrier Ntcp is seen with CCl4-induced injury. The majority of changes in transporter expression occurred between 24 and 48 h after toxicant treatment and were preceded by induction of the cellular stress protein Ho-1.

An understanding of the substrate specificity of organic anion transporters may provide some insight into the potential functional significance of altered expression during hepatotoxicity. Mrp1 is one of the most characterized Mrps and is capable of transporting substances that can influence the outcome of toxicant treatment including leukotriene C4, glutathione conjugates of 4-hydroxynonenol and prostaglandin A2 and factors involved in cell cycle regulation (Evers et al., 1997; Keppler et al., 1997; Renes et al., 2000). Induction of Mrp3 (as seen with APAP) may help to limit intracellular accumulation of glucuronide conjugates as well as bile acids in hepatocytes (Zelcer et al., 2003b). Lastly, Mrp4 is able to efflux a number of substrates associated with cell stress including prostaglandins E1 and E2, glutathione, cyclic nucleotides (cAMP and cGMP) as well as monoanionic bile salts and bile acid conjugates (Bai et al., 2004; Chen et al., 2001; Reid et al., 2003; Rius et al., 2003; Zelcer et al., 2003a). Increased basolateral excretion of these substrates to sinusoidal blood may serve not only as a protective mechanism for efficient elimination but also for paracrine signaling to adjacent hepatocytes and other cell types (including Kupffer and stellate cells) involved in progression of and/or recovery from liver disease. By contrast, reduced expression of Ntcp after CCl4 likely minimizes intracellular

**FIG. 6.** Immunofluorescent co-localization analysis of Ntcp and Mrp4 expression in livers from APAP- and CCl4-treated mice. Double labeling indirect immunofluorescence against Mrp4 (green) and Ntcp (red) was performed on liver cryosections (5–7 μm) obtained at 48 h from control (A), APAP- 400 mg/kg (B), CCl4- 10 μl/kg (C), and CCl4- 25 μl/kg (D) treated mice. Bar, 83.33 μm.
levels of toxic bile acids by limiting influx into centrilobular hepatocytes.

The expression of some hepatic genes and proteins is compartmentalized into three different zones which reflect the spatial distribution of cells near the central or portal veins. Numerous metabolism-related pathways including xenobiotic metabolism and conjugation, amino acid utilization, cholesterol synthesis, bile formation and nuclear hormone receptors exhibit zonal patterns of regulation and/or expression in normal liver (Jungermann and Katz, 1989). Similarly, rat and human Mrp3 are preferentially expressed in cells around the central vein (Ros et al., 2003). Although constitutive levels of Mrp3 are greater in mouse liver, this zonal selectivity is conserved across these three species (Fig. 6). The function of this efflux pump includes secretion of glucuronides into sinusoidal blood (Belinsky et al., 2005; Hirohashi et al., 1999; Manautou et al., in press). Fittingly, glucuronide conjugates are predominantly formed in centrilobular hepatocytes (Jungermann and Katz, 1989). Induction of mouse Mrp3 after APAP may lead to more efficient hepatic excretion of glucuronide conjugates similar to previous reports characterizing APAP disposition in rats with up-regulated hepatic Mrp3 expression (Slitt et al., 2003; Xiong et al., 2002). Mouse Mrp3 levels are also elevated during cholestatic liver injury (Bohan et al., 2003). Conversely, reduced Mrp3 staining is observed during CCl4 damage possibly reflecting differential turnover or degradation of Mrp3 protein depending on the etiology or extent of hepatic stress and/or injury.

Dramatic up-regulation of Mrp4 in mouse liver occurs after administration of both minimally and moderately hepatotoxic doses of APAP and CCl4. Interestingly, marked Mrp4 induction persists at 48 h after APAP when resolution of histopathological injury has occurred. Increased Mrp4 is predominantly confined to the last 1 to 3 rows of hepatocytes around the terminal hepatic venules. This is the first documentation of zonal changes in transport protein expression in liver after toxicant treatment. Notably, Mrp4 protein exhibits a diffuse staining pattern within liver cells after the higher dose of CCl4 used in this study. The extent to which this staining represents intracellular and/or membrane-bound Mrp4 is not presently known. Although this altered staining pattern is likely the result of reduced membrane integrity in damaged hepatocytes, the dramatic up-regulation of Mrp4 protein as seen in this study may have saturated cytoskeletal cellular trafficking, thus contributing to this diffuse staining pattern. It is also important to note that Bcrp membrane staining was minimally affected in centrilobular areas of CCl4-induced hepatocellular damage at the high dose. Therefore, it is reasonable to conclude that the Mrp4 staining pattern with CCl4 is the outcome of both abnormal trafficking of newly synthesized protein and cell injury.

Due to irregular cellular staining of Mrp4 at the high dose, it is difficult to anticipate changes in Mrp4-mediated sinusoidal efflux during CCl4-induced damage. Further characterization is necessary to mechanistically understand the unique patterns of Mrp protein localization during hepatotoxicity and their functional consequences.

Recent work demonstrates similar up-regulation of MRP proteins and decrease in NTCP expression in human liver specimens following APAP overdose (unpublished observations from our laboratory). Drug-induced liver injury accounted for more than 50% of all cases of acute liver failure in the United States from 1997–2002, with 40% of these attributed to APAP ingestion (Lee, 2003). The popularity of this analgesic and the potential for drug-related morbidity and mortality makes it a significant human health problem. Although CCl4 exposure is not of great human risk, from a mechanistic standpoint it is important to study the effect of two toxicants with not only distinct modes of action, but also differing means of hepatic disposition. Notably, APAP-induced hepatotoxicity has been associated with covalent adduct formation, depletion of cellular antioxidants such as glutathione as well as generation of reactive oxygen and nitrogen species. CCl4-mediated liver injury is generally characterized by lipid peroxide formation and altered redox status (Brattin et al., 1985). Moreover, while APAP conjugates are eliminated predominantly via transporters, studies by Page and Carlson found negligible biliary excretion of CCl4 in rats suggesting transport-independent clearance (Chen et al., 2003; Page and Carlson, 1994; Xiong et al., 2000).

Importantly, drug-induced liver injury impairs hepatobiliary function and results in altered pharmacokinetics for a number of pharmaceuticals. In turn, clinicians are required to adjust dosages and dosing intervals to compensate for reduced hepatic function in patients. These data demonstrate altered expression of multiple transport proteins during drug-induced liver injury and may serve to explain corresponding changes in xenobiotic handling.

Autoprotection or acquired resistance to supratherapeutic doses of APAP has been reported in both rodents and humans. Presently, only case reports observing human autoprotection in a select number of patients are available. However, among clinicians it is generally thought that patients develop tolerance to the hepatotoxic effects of supratherapeutic APAP doses with repeated exposure. One such clinical case was the basis for studies documenting the same phenomenon in mice (Shayiq et al., 1999). It is well known that mouse models recapitulate the hepatotoxicity observed in humans following APAP exposure (e.g., toxic doses, bioactivation of APAP, biological sequence of injury). Use of animal models to study the underlying mechanisms of autoprotection has demonstrated minimal involvement of Phase I and II metabolic pathways in this resistance (Chanda et al., 1995; Roberts et al., 1983). Studies are necessary to address if altered transporter expression after sublethal hepatotoxicant administration confers hepatocyte protection to subsequent doses.

This study comprehensively characterizes for the first time the temporal and zonal changes in hepatobiliary transporter expression during chemical-induced hepatotoxicity. In
particular, coordinated induction of efflux and stress proteins (e.g., Mrps and Ho-1) with a corresponding reduction of uptake carriers (e.g., Ntcp in the CCl₄ model) strongly suggest an adaptive defense mechanism for elimination of xenobiotics and oxidative stress products generated during chemical liver damage. A better understanding of the altered expression of transporters in both mouse and human liver is necessary to address the functional contribution of transport mechanisms to the impaired hepatic clearance of xenobiotics during injury as well as conferring resistance to subsequent toxicant exposure.

SUPPLEMENTARY DATA

Supplementary data are available online at www.toxsci.oxfordjournals.org.

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