Shift from Biliary to Urinary Elimination of Acetaminophen-Glucuronide in Acetaminophen-Pretreated Rats

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ABSTRACT

Despite its toxicity, acetaminophen (APAP) is used increasingly as an analgesic, antipyretic, and anti-inflammatory agent. We examined the effect of prior exposure to APAP on its biliary and urinary elimination. The biliary and urinary elimination of a test dose of APAP (150 mg/kg i.v.) was determined in male Wistar rats 24 h after pretreatment with vehicle, a single dose (1.0 g/kg i.p.), or increasing daily doses (0.2, 0.3, 0.6, and 1.0 g/kg/day i.p.) of APAP. Although elimination of the parent APAP was minimally affected, biliary excretion of APAP glucuronide was significantly decreased 70 and 80%, whereas urinary excretion was significantly increased 90 and 100% in the groups pretreated with APAP, relative to vehicle controls. Western analysis and confocal immunofluorescent microscopy indicated a marked increase in hepatic expression of multidrug resistance-associated protein 3 (Mrp3) in both groups pretreated with APAP, relative to expression of Mrp2. ATP-dependent transport of [3H]taurocholate, an Mrp3 substrate, was significantly increased in basolateral liver plasma membrane vesicles from rats pretreated with repeated doses of APAP relative to controls. Enterohepatic recirculation of APAP glucuronide after administration of the same test dose of the drug was significantly decreased in rats pretreated with repeated doses of APAP. These data indicate that APAP pretreatment induced a shift from biliary to urinary elimination of APAP glucuronide, consistent with the increased expression of Mrp3 in the basolateral domain of the hepatocyte. We postulate that decreased enterohepatic recirculation contributes to decreased APAP hepatotoxicity by reducing liver exposure.

The increasing popularity of acetaminophen (N-acetyl-p-aminophenol, APAP) as a nonprescription analgesic and antipyretic has resulted in a significant increment in the incidence of accidental and intentional poisonings with this drug (Kaplowitz, 2004). It is generally accepted as a safe drug when administered within the therapeutic range (Rumack, 2004). However, in large single-dose ingestion, it causes massive centrilobular necrosis, inducing severe hepatotoxicity in human and experimental animals that may be lethal (Thomas, 1993; Bessems and Vermeulen, 2001).

APAP is metabolized in the liver mainly by glucuronidation and sulfation, thus generating the nontoxic metabolites, APAP-glucuronide (APAP-glu) and APAP sulfate (Thomas, 1993). At high doses of APAP, these metabolic paths are saturated and a substantial portion of the drug is metabolized by the CYP450 system, generating a reactive metabolite, NAPQI. This compound reacts with reduced GSH, leading to GSH depletion, or it covalently binds to cellular proteins, as likely explanations for APAP hepatotoxicity (Mitchell et al., 1973; Linscheer et al., 1980). APAP-glu excretion in bile is mediated by the canalicular multidrug resistance-associated protein 2 (Mrp2; Abcc2) (Xiong et al., 1994) and represents approximately 7% of a single dose (100 mg/kg) of APAP administered systemically to rats (Brouwer and Jones, 1990). Basolateral efflux of APAP-glu in liver may also occur and has been linked to the expression of Mrp3.

ABBREVIATIONS: APAP, acetaminophen; APAP-glu, acetaminophen-glucuronide; Mrp1, Mrp2, and Mrp3, multidrug resistance-associated proteins 1, 2, and 3; NAPQI, N-acetyl-p-benzoquinone imine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GST, glutathione S-transferase; GSH, glutathione; GSSG, oxidized glutathione; TC, taurocholate; UGT, UDP-glucuronosyltransferase.
free access to food and water and were maintained on a 12-h auto-

Chemicals

pretreated with phenobarbital, *trans*-stilbene oxide, diallyl sulfide, or oltipraz correlated well with increased basolateral and decreased biliary clearance of APAP-glu (Xiong et al., 2002; Slitt et al., 2003). Thus, increased expression of the basolateral Mrp3 transporter relative to the canicular Mrp2 transporter is associated with a shift from biliary to basolateral and thereby urinary excretion of APAP-glu.

Increased resistance to APAP hepatotoxicity in response to repetitive administration of the drug has been observed in humans and experimental animals, although the mechanisms are only partially understood. Studies in mice demonstrated that autoprotection was probably because of down-

Regulation of the CYP450s involved in the formation of NAPQI and because of a potentiated proliferative response (Shayiq et al., 1999). Increased tolerance to APAP intoxication was also observed in rats and was linked to increased GSH availability from regenerating hepatocytes because of induction of the enzymes involved in GSH synthesis (Dalhoff et al., 2001). The evidence thus indicates that APAP pretreat-

ment may influence its toxic effects by modifying key meta-

bolic pathways. The possibility that APAP hepatotoxicity is also affected by changes in expression or activity of trans-

porters involved in APAP metabolite disposition has never been explored. Aleksunes et al. (2005) demonstrated that APAP induced an increase in Mrp2 and Mrp3 mRNA expression in mice, although the effect on protein levels or activities was not evaluated. In a recent study, we found an increase in Mrp2 protein level in liver from rats treated with a single 1.0 g/kg dose of APAP, together with increased biliary excretion of the Mrp2 substrate oxidized glutathione (GSSG) (Ghanem et al., 2004). We proposed that Mrp2 induction by APAP may represent an adaptive mechanism to accelerate liver dispo-

sition of the nontoxic metabolite APAP-glu and to increase elimination of GSSG to maintain the redox equilibrium in the hepatocyte. To test the former possibility, we evaluated the biliary and urinary disposition of APAP-glu in rats pre-

treated with a single dose of APAP (1.0 g/kg) or with repeated and increasing doses of the drug (0.2–1.0 g/kg). The influence on expression of Mrp2 was also explored. Because of the potential impact of basolateral Mrps on liver to blood trans-

port and subsequent urinary elimination of APAP-glu, we also evaluated the expression of Mrp1 and Mrp3 in the same animals. The data indicate that both single and repeated doses of APAP led to a shift from biliary to urinary excretion of APAP-glu, correlating well with a marked induction of Mrp3 relative to Mrp2.

Materials and Methods

Chemicals

APAP, APAP-glu, sucrose, sodium taurocholate (TC), creatine phosphate, creatine phosphokinase, NADPH, glutathione reductase, UDP-glucuronosyl, and bovine serum albumin were from Sigma-Aldrich (St. Louis, MO). [3H]TC (2.1 Ci/mmol; 98% purity) was ob-

tained from PerkinElmer Life and Analytical Sciences (Boston, MA). All other chemicals were of analytical grade and used as supplied.

Animals and Experimental Protocols

Male Wistar rats (250–290 g) were used throughout. The rats had free access to food and water and were maintained on a 12-h auto-
matically timed light and dark cycle. All of the procedures involving animals were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The following experimental groups were used.

Single Protocol. Animals were injected with a single dose of APAP, 1.0 g/kg b.wt. i.p. APAP was administered as a solution of 100 mg/ml in 1:4 polyethylene glycol 400:saline. Control animals received the vehicle. Studies were performed 24 h later. This group is termed Single APAP.

Repeated Protocol. APAP was administered i.p. at increasing daily doses of 0.2 g/kg on day 1, 0.3 g/kg on day 2; 0.6 g/kg on day 3, and 1.0 g/kg on day 4. Administration of increasing nontoxic doses of APAP has been demonstrated to confer resistance to a subsequent lethal dose either in rats or mice (Shayiq et al., 1999; Dalhoff et al., 2001). Control animals received the same increasing volume of ve-

hicle for 4 days. Studies were performed 24 h after the last dose of APAP or vehicle. This group is termed Repeated APAP.

Different sets of animals were used in the different experimental designs. 1) Single APAP (*n* = 4), Repeated APAP (*n* = 4), and their respective controls (*n* = 3 each) were used for assessment of biliary and urinary excretion of APAP and APAP-glu. 2) Repeated APAP (*n* = 4) and their controls (*n* = 3) with external biliary drainage versus Repeated APAP (*n* = 3) and their controls (*n* = 3) with preserved enterohepatic recirculation were used for assessment of enterohepatic recirculation of APAP. 3) Single APAP (*n* = 6), Re-

peated APAP (*n* = 4), and their respective controls (*n* = 2 each) were used for assessment of renal function. 4) A maximum number of four Single APAP animals, four Repeated APAP animals, and three of their respective controls were used in assessment of serum marker activities, intrahepatic GSH levels, UDP-glucuronosyltransferase (UGT) activity, and Western blot and confocal microscopy studies. 5) Repeated APAP (*n* = 3) and their controls (*n* = 3) were used for assessment of Mrp3 activity. 6) All of these animals, together with those that did not survive the APAP treatment, were grouped for calculation of survival.

Assessment of Biliary and Urinary Excretion of APAP and APAP-glu

The elimination of APAP and APAP-glu in urine and bile was determined after administration of a test dose of APAP (150 mg/kg i.v.) to groups of animals that had been treated with APAP or vehicle according to the Single and Repeated protocols described above. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and thus maintained throughout. Animals from APAP groups and controls were administered the same total doses of pentobarbital throughout the experiment to avoid any differential impact of the anesthetic on bile flow. Body temperature was measured with a rectal probe and maintained at 37°C with a heating lamp. The jugular vein and the common bile duct were cannulated with poly-

ethylene tubing (PE50 and PE10, respectively). The urinary bladder of the animals was exteriorized through a midline incision and cannulated with polyethylene tubing (PE75). Appropriate volumes of 5% bovine serum albumin in saline were administered intravenously throughout the experiment to replenish body fluids. After a 30-min stabilization period, two initial bile samples and one urine sample were collected. APAP (150 mg/kg; 50 mg/ml in 1:3 propylene glycol: saline solution) was administered i.v., and bile (at 15-min intervals) and urine (at 30-min intervals) samples were collected for 3 h. Bile and urinary flow were determined gravimetrically. Samples of bile and urine were used for assessment of content of APAP and its glucuronide by HPLC. Analysis of APAP and APAP-glu content in basal bile and urine samples before administration of the test dose indicated no detectable levels in either Single or Repeated protocols.

Assessment of Enterohepatic Recirculation of APAP

In a different set of animals, we evaluated the effect of Repeated APAP pretreatment on enterohepatic recirculation of the drug. The
rats were anesthetized, and the jugular vein was cannulated as described above. A test dose of APAP (150 mg/kg) was administered i.v., and 150 min later, the common bile duct was cannulated and bile was collected for 30 min. Bile flow was determined gravimetrically. Samples of bile were used for determination of APAP-glu by HPLC. Thus, the data obtained were compared with those obtained after administration of the same test dose and during the equivalent period of bile collection but under conditions of permanent biliary drainage, as described above. The 150- to 180-min time period was chosen based on the findings demonstrating no differences in APAP-glu biliary excretion between controls and Repeated APAP groups under conditions of external biliary drainage (see two last periods in Fig. 1A). Higher excretion values in controls but not in the APAP group under conditions of preserved recirculation would indicate that Repeated APAP pretreatment interfered with the enterohepatic recirculation.

**Renal Function**

Functional parameters were assessed in vivo in a different set of APAP-treated animals. The rats were anesthetized, and the urinary bladder was cannulated. After a 30-min stabilization period, urine was collected for 60 min. At the end of this period, blood was collected by cardiac puncture and serum was immediately separated by centrifugation. Urinary flow was determined gravimetrically. Samples of serum and urine were used for determination of renal functional parameters.

**Collection of Liver Samples**

In a different set of animals, which were sacrificed by cardiac puncture under pentobarbital anesthesia, livers were rinsed with PBS and portions of the organ were gently frozen in liquid nitrogen and preserved at ~80°C until used for crude plasma and microsomal membrane preparation, frozen in precooled isopentane for confocal microscopy, or used in assessment of total GSH content. Serum samples were separated for determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. For preparation of basolateral plasma membranes (BLPM), livers were removed and immediately used for membrane preparation.

**Preparation of Liver Membranes**

Crude plasma membranes (Meier et al., 1984) and microsomal membranes (Luquita et al., 1994) were prepared from each experimental group and were used in Western blot studies to determine the content of Mrp1–3 and in the assessment of UGT activity toward APAP, respectively. BLPM vesicles were prepared by continuous sucrose separation (Meier et al., 1984) in two days in total from...
control or Repeated APAP rats and were used in transport studies. Protein concentration was measured as described previously (Lowry et al., 1951). \( \text{Na}^+ \cdot \text{K}^- \cdot \text{ATPase} \) (micromole Pi per hour per milligram of protein) activity in BLPM vesicles prepared from control rats was \( 24.6 \pm 5.0 \) (mean \( \pm \) S.D., \( n = 3 \)) and was not different in the Repeated APAP group (data not shown). This activity represents an enrichment of 28 \pm 9-fold with respect to homogenates. The activity of 5'-nucleotidase (micromole per hour per milligram of protein) in BLPM membranes from control rats was \( 4.7 \pm 1.5 \) (mean \( \pm \) S.D., \( n = 3 \)) and was not affected by APAP pretreatment (data not shown). This value represents a \( 3.7 \pm 0.2 \)-fold increase with respect to homogenates and indicate minimal contamination with canalicul membranes, as described previously (Meier et al., 1984). Analysis of activities of UDP-glucuronosyltransferase, acid phosphatase, and aspartate aminotransferase, markers of microsomal, lysosomal, and mitochondrial membranes, respectively, indicated no appreciable contamination with these fractions (data not shown).

### Western Blot Studies

Immunoblotting was performed with crude plasma membranes as described previously (Mottino et al., 2002) using a rat monoclonal antibody to human MRP1 (Alexis Biochemicals, Carlsbad, CA), a mouse monoclonal antibody to human MRP2 (M_II_3-6; Alexis Biochemicals), and a rabbit polyclonal antibody to rat Mrp3 (Ogawa et al., 2000). Subsequent densitometry was performed using the Gel-Pro Analyzer (Media Cybernetics, Inc., Silver Spring, MD) software.

### BLPM Transport Studies

BLPM were diluted with suspension buffer (10 mM Tris-HCl, and 250 mM sucrose, pH 7.4) to 5.0 to 5.5 mg/ml protein and vesiculated by 20 passages through a 25-gauge needle. ATP-dependent uptake of the Mrp3 substrate TC into liver BLPM was determined as described previously for intestinal BLPM (Shoji et al., 2004). Some modifications were introduced. In brief, 30 \( \mu l \) of transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM MgCl_2, pH 7.4) containing radiolabeled TC (1.5 \( \mu l \)) was preincubated at 37°C for 3 min and then rapidly mixed with 15 \( \mu l \) of membrane vesicles (80 \( \mu g \) of protein). The reaction mixture contained 5 mM ATP and an ATP-regenerating system (10 mM creatine phosphate and 100 \( \mu g \) creatine phosphokinase) or 5 mM AMP. The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4), at 20, 40, 60, and 120 s. This mixture was filtered through a 0.45-\( \mu m \) membrane filter (Millipore Corporation, Billerica, MA) and washed twice with 3.5 ml of stop solution. Radioactivity retained on the filter was determined in a scintillation analyzer (RackBeta 1214; Pharmacia Wallac Oy, Turku, Finland).

### Analytical Methods

APAP and APAP-glu in bile and urine were assayed by HPLC (Howie et al., 1977). Retention times of APAP and APAP-glu were determined by authentic standards. Proteins from bile and urine samples were removed by precipitation with equal volumes of trichloroacetic acid solution (25% w/v) followed by centrifugation before HPLC analysis.

Liver toxicity was assessed by measuring AST and ALT activity in serum using commercial kits (Visible Human Explorer; Human, Wiesbaden, Germany). For assessment of total GSH (reduced + oxidized) in liver samples, the tissue was homogenized (20% w/v in saline), 2 volumes of the homogenate were mixed with 1 volume of 10% sulfosalicylic acid and centrifuged at 5000 rpm for 5 min, and the supernatant was used in the GSH assay (Tietze, 1969). Survival was assessed by calculating the ratio between the number of animals surviving the treatment and the total number of animals treated with the drug.

Incubation conditions for assessment of UGT activity were as described previously (Kessler et al., 2002), with the exception that Triton X-100 was incorporated to activate microsomes (Luqita et al., 1994). At the end of the incubation, mixtures were deproteinized as described above and the formed APAP-glu was detected in supernatants by HPLC (Howie et al., 1977).

### Statistical Analysis

Data are presented as the means \( \pm \) S.D. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni test, unless otherwise stated. Values of \( P < 0.05 \) were considered to be statistically significant.

### Results

**Effect of APAP on Liver Toxicity, Hepatic GSH Content, and Survival.** Table 1 shows significantly increased values of AST and ALT in the Single APAP group, as reported previously for the same dosage of APAP (Ghanem et al., 2004), clearly indicating hepatotoxicity induced by the drug. Less pronounced changes in these parameters were obtained in the Repeated APAP group when compared with Single APAP group. GSH content in liver was not affected by the Single protocol but increased by 31% in the Repeated group. These data were consistent with previous reports indicating that GSH content returned to normal values 18 to 24 h after injection of 0.5 g/kg APAP to rats (Noriega et al., 2000) and with increased capability for GSH synthesis by

### Table 1

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<th>Control</th>
<th>Single APAP</th>
<th>Repeated APAP</th>
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<tr>
<td>AST (U/l)</td>
<td>69 ( \pm ) 27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>305 ( \pm ) 72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200 ( \pm ) 86</td>
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<tr>
<td>ALT (U/l)</td>
<td>47 ( \pm ) 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>238 ( \pm ) 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160 ( \pm ) 13</td>
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<td>Hepatic GSH content (nmol/g liver)</td>
<td>7878 ( \pm ) 699&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6548 ( \pm ) 1373&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10,341 ( \pm ) 987</td>
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<tr>
<td>Percent survival at 24 h</td>
<td>100 (25)</td>
<td>56 (25)</td>
<td>96 (23)</td>
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<sup>a</sup> Different from Single and Repeated APAP groups \((P < 0.01)\).

<sup>b</sup> Different from Repeated APAP group \((P < 0.05)\).
regenerating liver under conditions of chronic treatment (Dalhoff et al., 2001). Table 1 also shows that survival was significantly higher in the Repeated APAP treatment group, indicating development of resistance to drug toxicity in this group relative to rats in the Single APAP group.

**Effect of APAP Pretreatment on Excretion of APAP and APAP-glu.** Basal bile flow was not affected by APAP pretreatment (1.9 ± 0.4, 2.5 ± 0.7, and 2.4 ± 0.8 μl/min/g liver in control, Single APAP, and Repeated APAP groups, respectively, n = 4 – 6). The test dose of APAP increased this measure by up to 40%, coincidently with the peak of excretion of APAP-glu in all of the groups (data not shown). Both Single APAP and Repeated APAP protocols markedly decreased biliary excretion of APAP-glu with respect to controls, whereas no substantial differences were observed in the rate of excretion of APAP-glu between the two APAP pretreatment protocols (Fig. 1A). Cumulative biliary excretion of APAP-glu was decreased by 70 and 80% in response to Single APAP and Repeated APAP treatment, respectively (Fig. 1E).

In contrast, the rate of urinary excretion of APAP-glu was increased significantly by both protocols (Fig. 1B). As occurred with biliary excretion, no differences in the rate of urinary excretion of APAP-glu were detected between Single APAP and Repeated APAP groups. Cumulative renal excretion of the glucuronide was increased by 90 and 100% in Single APAP and Repeated APAP, respectively (Fig. 1F). Comparison of the data in Fig. 1, E and F, indicates that diminution of the percentage contribution of biliary APAP-glu to overall elimination of the drug was compensated by increased renal excretion. Excretion rates of parent APAP are depicted in Fig. 1, C and D. In control animals, the parent drug contributed minimally to total APAP biliary elimination and moderately to its urinary elimination. Although statistically significant differences in biliary or urinary excretion of APAP were observed in some periods in response to both protocols of administration (Fig. 1, C and D), these differences had little impact on the cumulative measures (Fig. 1, E and F). Specifically, the decrease in cumulative urinary excretion of APAP in the Single group (Fig. 1F) could be tentatively associated with the impairment in creatinine clearance (see Table 2), because intact APAP is not extensively bound to plasma proteins and it is likely to undergo considerable glomerular filtration (Forrest et al., 1982). Urinary excretion peak of both APAP-glu and intact APAP exhibited a delay from 60 to 120 to 150 min in APAP-pretreated animals (Fig. 1, B and D).

**Effect of Repeated APAP Protocol on Enterohepatic Recirculation of the Drug.** After its biliary excretion, APAP-glu undergoes intestinal hydrolysis and the released intact APAP is partially reabsorbed, thus indicating enterohepatic recirculation (Watari et al., 1983). Biliary excretion rates of APAP-glu in animals with preserved enterohepatic recirculation are depicted in Fig. 2, together with the values obtained for the same periods under nonrecirculating conditions. Under conditions of preserved recirculation, this measure was decreased by 70% in the Repeated APAP group versus controls. In contrast, there was no difference between these two groups under conditions of permanent biliary drainage. The difference in biliary excretion of the glucuronide in control animals with preserved versus nonpreserved recirculation confirmed APAP enterohepatic recirculation in our model. This difference was not observed in rats pretreated with APAP, suggesting minimal contribution of a preserved enterohepatic circuit to biliary excretion of APAP-glu at 150 to 180 min after injection of the APAP test dose.

**Effect of APAP on UGT Activity.** Microsomal UGT activity toward APAP in control rats was 3.64 ± 1.50 nmol/min/mg protein (n = 6, which includes animals in experiments receiving both single and repeated doses of solvent). Pre-treatment with APAP either as Single (4.22 ± 1.32 nmol/min/mg protein, n = 4) or Repeated (5.54 ± 2.01 nmol/min/mg protein, n = 4) protocol did not significantly modify enzyme activity.

**Effect of APAP on Renal Function.** Animals receiving a single dose of APAP exhibited a significant decrease in urinary flow rate and creatinine clearance when compared with controls (Table 2). The Repeated APAP group showed a similar decrease in the urinary flow rate but without alteration in the creatinine clearance. All groups showed a fractional excretion of sodium lower than 1%, indicating preservation of concentric tubular function in response to APAP. Decreased urinary flow rate in the Repeated APAP group, in spite of preserved creatinine clearance, probably resulted from increased tubular reabsorption, as indicated by the values of fractional excretion of sodium.

| TABLE 2 |
| Renal function parameters |
| Single and Repeated vehicle rats rendered similar values and were grouped. Values are expressed as mean ± S.D. of four to six animals per group. |
| | Control | Single APAP | Repeated APAP |
| Urinary flow (μl/min/g kidney) | 4.97 ± 1.43<sup>a</sup> | 2.95 ± 0.87 | 2.70 ± 1.59 |
| Creatinine clearance (ml/min/g kidney) | 0.62 ± 0.12<sup>b</sup> | 0.23 ± 0.32 | 0.44 ± 0.17 |
| Fractional excretion of sodium (%) | 0.68 ± 0.12<sup>a</sup> | 0.28 ± 0.14 | 0.06 ± 0.02 |

<sup>a</sup> Different from Single and Repeated APAP groups (P < 0.05).

<sup>b</sup> Different from Single APAP group (P < 0.05).
Effect of APAP on Expression of Mrp1, Mrp2, and Mrp3. Expression of Mrp1, as detected by Western blotting, was not affected by either Single APAP or Repeated APAP (Fig. 3). In contrast, both protocols induced a similar increase in Mrp2 content of approximately 65%. The most striking finding was the marked increase in Mrp3 levels (greater than 400%), which was clearly higher in magnitude than that for Mrp2. Although the Mrp1 signal was of similar level as Mrp2 in spite of its usual low expression in normal rat liver (Croccenzi et al., 2004), this was probably the result of differences in sensitivity of the respective antibodies in addition to different times of exposure of the films to chemiluminescence reagents. The marked induction of Mrp3 relative to Mrp2 in response to APAP was confirmed by immunofluorescence. Figure 4 shows a very low expression of Mrp3 restricted to the basolateral membrane of perivenous hepatocytes surrounding the central vein in control rats (green fluorescence), as described previously (Soroka et al., 2001). The inset in the upper panel shows a typical pattern of immunofluorescence of Mrp2 in the canaliculus (red fluorescence) and of Mrp3 (green fluorescence) in the basolateral domain (see white arrow). The yellow fluorescence seen occasionally in canicular or pericanicular regions may reflect some degree of cross-reactivity between anti-MRP2 and -MRP3 commercial antibodies. APAP treatment markedly increased immunodetection of Mrp3, which was extended to periportal hepatocytes in both Single and Repeated APAP treatment protocols. Although no quantitative analysis was performed in these studies, simultaneous immunodetection of Mrp2 and Mrp3 in the perivenous region clearly demonstrates a significant increase in expression of basolateral Mrp3 relative to canicular Mrp2 in APAP livers (see insets).

Effect of APAP on TC Uptake by Liver BLPM Vesicles. Consistent with increased Mrp3 expression by Western analysis, ATP-dependent transport of TC was significantly increased in the Repeated APAP group versus controls (Fig. 5). Data on the purity of the BLPM in both groups do not support greater contamination of BLPM with canalicular membranes. Furthermore, we have shown that a single 1 g/kg dose of APAP does not change in vivo biliary secretion of endogenous bile salts (Ghanem et al., 2004) or bile salt export pump levels as detected by Western blotting in crude plasma membranes (C. I. Ghanem and A. D. Mottino, unpublished results). Taken together, these data support an increase in transport activity due to the increased expression of Mrp3 in the basolateral domain of the plasma membrane.

Discussion

APAP overdose represents the leading cause for calls to poison control centers in many countries and produces a considerable number of deaths due to acute liver failure each year (Lee, 2004). It was reported that chronic ingestion of an excess of APAP (several tablets per day for weeks) leads to development of tolerance to the drug, usually delaying the onset of liver injury. Tolerance to the toxic effects of APAP in experimental animals has been linked to changes in activity of specific metabolic pathways involved in APAP bioactivation or GSH synthesis (Shayiq et al., 1999; Dalhoff et al., 2001). Herein, we provide evidence that APAP pretreatment markedly induces liver basolateral Mrp3 versus canalicular Mrp2, leading to a shift from biliary to urinary excretion of its major metabolite APAP-glu and decreased enterohepatic recirculation of APAP. These findings probably contribute to the decreased liver damage, as indicated by the decreased serum AST and ALT levels and decreased mortality after pretreatment with APAP in Repeated APAP protocol (Table 1).

We recently demonstrated that an acute toxic dose (1.0 g/kg) of APAP increased the hepatic expression of Mrp2 and increased the biliary excretion of its model substrates dinitrophenyl-S-glutathione and GSSG (Ghanem et al., 2004).
The current data indicate that pretreatment with APAP in the Repeated APAP protocol maintained but did not further increase Mrp2 expression. Surprisingly, in spite of this increase in Mrp2 expression, the biliary excretion of APAP-glu was markedly decreased in APAP-pretreated groups, whereas its urinary excretion was increased. Although APAP-glu is a substrate for both canalicular Mrp2 and basolateral Mrp3 in rodents, constitutive expression of Mrp3 in normal rat liver is very low relative to canalicular Mrp2. Because the affinity of APAP-glu for Mrp3 is estimated to be several times higher than that for Mrp2 (Xiong et al., 2002; Manautou et al., 2004), induction of Mrp3 leads to preferential efflux of APAP-glu across the basolateral membrane of liver into blood versus biliary excretion (Xiong et al., 2002; Slitt et al., 2003). Moreover, Mrp3-mediated excretion of APAP-glu critically modulates its intrahepatic levels (Manautou et al., 2004). In our study, both APAP pretreatment protocols induced Mrp3 expression to a much greater extent than Mrp2; this selective regulation almost certainly accounts for the vectorial change in disposition of APAP-glu. These findings agree well with studies by Xiong et al. (2002) and Slitt et al. (2003) that used different Mrp3 inducers and demonstrated increased urinary excretion of the glucuronide derivative.

Renal elimination of APAP-glu was markedly increased in APAP-pretreated groups, irrespective of the protocol. A single 1-g/kg dose of APAP has been shown to induce impairment of the glomerular filtration rate, as estimated by creatinine clearance (Trumper et al., 1998). Our study also showed decreased creatinine clearance in rats pretreated with a single dose of APAP, whereas pretreatment in the Repeated APAP protocol did not affect this parameter. Despite this impairment in renal function, it is clear that the kidneys were able to increase the urinary excretion of APAP-glu. Although APAP-glu urinary excretion has been postulated to result from both glomerular filtration and active tubular secretion under normal conditions (Forrest et al., 1982), it is not known whether APAP-glu is preferentially eliminated in urine by filtration or tubular secretion after APAP pretreatment.

After intraduodenal administration of APAP-glu in rats in vivo, the glucuronide is hydrolyzed by the microflora and the liberated APAP is partially reabsorbed (Watari et al., 1983). Our data in Fig. 1 showing decreased biliary excretion of APAP-glu thus constitute a major finding, with potential consequences for the retention of the parent compound in the enterohepatic circulation and consequent liver toxicity. To test this possibility, we performed experiments in animals with intact enterohepatic recirculation, which was preserved until 150 min after injection with a test dose of APAP. The
data clearly demonstrate that the Repeated APAP protocol decreased enterohepatic recirculation of the drug. The shift from biliary to urinary excretion of only 10% of administered APAP as APAP-glutathione is of minor relevance because of its low magnitude. However, such 10% represented a two-thirds decrease with respect to the excretion in control rats (see Fig. 1E). Because APAP-glutathione is the main metabolite of APAP in rats and its biliary excretion constitutes the major determinant of APAP recirculation (Watari et al., 1983), our finding indicates that pretreatment with APAP may have a significant impact on liver exposure to the drug. We hypothesize that preferential induction of Mrp3 versus Mrp2, thus minimizing exposure of the liver to APAP and subsequent bioactivation of the drug, may contribute to the development of resistance under conditions of chronic treatment. In the Single protocol, survival after a large acute single dose of APAP could be associated with the ability of some animals to quickly induce basolateral versus canalicular Mrps. Interestingly, we observed more variation in the extent of Mrp3 induction in animals from the Single protocol than those from the Repeated protocol as detected by Western blotting (see Fig. 3). Unfortunately, because of technical limitations, it is not possible to evaluate whether animals that did not survive the Single protocol were unable to induce Mrp3.

It is known that saturation of UGT-mediated conjugation of APAP under conditions of drug overdose leads to increased production of NAPQI (Rumack, 2004). The relevance of the glucuronidation pathway was recently confirmed by Kostrubsky et al. (2005), who demonstrated that inhibition of APAP glucuronidation in cultured human hepatocytes leads to increased hepatotoxicity. In the current study, neither the Single nor the Repeated protocol affected UGT activity toward APAP. This was in agreement with preserved cumulative total (biliary + urinary) excretion of APAP-glutathione in both groups of APAP-pretreated rats (see Fig. 1E) and would indicate that decreased toxicity to APAP did not result from increased hepatic glucuronidation. We postulate that decreased CYP450-mediated production of NAPQI (Shayiq et al., 1999) resulted in increased synthesis of GSH (Dalhoff et al., 2001), together with induction of basolateral Mrp3, may act concurrently in the development of tolerance to APAP given chronically.

Tredger et al. (1995) reported a case of a 58-year-old woman who consumed 15 to 20 g of APAP daily without developing liver damage. These authors found a delayed but a 2-fold increase in plasma levels and urinary excretion of APAP-glutathione and APAP sulfmate in this patient relative to a group of normal volunteers. These authors concluded that resistance to APAP toxicity may have derived from a combination of factors, including delayed absorption of the drug, increased metabolism by conjugation, and reduced CYP450-mediated formation of cytotoxic metabolites. Our data demonstrating increased urinary excretion of the nontoxic glucuronide metabolite of APAP agrees well with those of Tredger et al. (1995) and further suggest a role for induction of Mrp3 in preferential secretion of APAP-glutathione from liver to blood in response to APAP pretreatment. Tredger’s results also suggest a role for increased urinary excretion of APAP sulfmate in the development of tolerance to APAP. Further studies would be necessary to confirm this possibility in experimental animals.

The mechanism by which APAP increases Mrp3 expression is not known. Our current and previous (Ghanem et al., 2004) data agree well with those from Aleksunes et al. (2005) showing increased expression of Mrp2 and Mrp3 mRNA 6 and 24 h after a single dose (0.4 g/kg) of APAP in mice, which would suggest transcriptional regulation of Mrp2 and Mrp3 genes by APAP. Using Mrp3-null mice, Belinsky et al. (2005) demonstrated that this pump functions as an alternative route for secretion of bile acids and endogenous glucuronides in cholestatic hepatocytes. Increased Mrp3 expression is seen in animals hereditarily deficient in Mrp2 or with experimental obstructive cholestasis, as well as in some forms of human cholestatic disease (Hirohashi et al., 1998; König et al., 1999; Shoda et al., 2001; Soroka et al., 2001; Scheffer et al., 2002).

Because some bile salts are Mrp3 substrates, up-regulation of the transporter is thought to limit hepatocellular injury resulting from accumulation of these compounds during cholestasis (Bohan et al., 2003). Interestingly, our study demonstrated an unusual coexistence of increased expression of both Mrp2 and Mrp3. Increased expression of Mrp3 in response to APAP does not seem to play a role in protection against bile salt accumulation, because biliary secretion of bile salts is not affected by APAP (Ghanem et al., 2004). Because neither Mrp2 expression nor its activity was decreased but rather increased by APAP, we also ruled out the occurrence of harmful effects resulting from accumulation of Mrp2 endogenous substrates such as bilirubin.

In conclusion, we have demonstrated that pretreatment with APAP led to a marked increase in the hepatic expression and activity of Mrp3 that was correlated with a significant shift from canalicular to basolateral efflux of APAP-glutathione and a decrease in its enterohepatic recirculation. This decreased enterohepatic recirculation is postulated to contribute to APAP-decreased hepatotoxicity by minimizing exposure to the liver and APAP activation to the toxic reactive metabolite.

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References


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