HEPATIC SYNTHESIS AND URINARY ELIMINATION OF ACETAMINOPHEN GLUCURONIDE ARE EXACERBATED IN BILE DUCT-LIGATED RATS

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ABSTRACT

Renal and intestinal disposition of acetaminophen glucuronide (APAP-GLU), a common substrate for multidrug resistance-associated proteins 2 and 3 (Mrp2, Mrp3), was assessed in bile duct ligated rats (BDL) 7 days after surgery, using an in vivo perfused jejunum model with simultaneous urine collection. Doses of 150 mg/Kg b.w. (i.v.) or 1 g/Kg b.w. (i.p.) of APAP were administered, and its glucuronide was determined in bile (only Shams), urine and intestinal perfusate throughout a 150 min period. Intestinal excretion of APAP-GLU was unchanged or decreased (-58%) by BDL for the 150 mg and 1 g/Kg b.w. doses of APAP, respectively. In contrast, renal excretion was increased by 200% and 320%, respectively. Western studies revealed decreased levels of apical Mrp2 in liver and jejunum but increased levels in renal cortex from BDL animals, whereas Mrp3 was substantially increased in liver and not affected in kidney or intestine. The global synthesis of APAP-GLU, determined as the sum of cumulative excretions, was higher in BDL rats (+51 and +110%) for these same doses of APAP, as a consequence of a significant increase in functional liver mass, with no changes in specific glucuronidating activity. Expression of apical breast cancer resistance protein (Bcrp), which also transports non-toxic metabolites of APAP, was decreased by BDL in liver and renal cortex, suggesting a minor participation of this route. We demonstrate a more efficient hepatic synthesis and basolateral excretion of APAP-GLU, followed by its urinary elimination in BDL group, the latter two processes consistent with upregulation of liver Mrp3 and renal Mrp2.
INTRODUCTION

Biliary elimination of drugs is mainly mediated by members of the ABC (ATP binding cassette) family of transporters such as multidrug resistance protein 1 (Mdr1, Abcb1), multidrug resistance-associated protein 2 (Mrp2, Abcc2) and breast cancer resistance protein (Bcrp, Abcg2). Together with hepatic phase I and phase II biotransformation reactions, they constitute a coordinated system to metabolize and excrete into bile a wide variety of xenobiotics, including drugs of therapeutic application (Suzuki et al., 2003; Catania et al., 2004; Aleksunes et al., 2005). Cholestasis, defined as decreased or ceased bile flow, is the most frequent event related to liver disorders. Bile duct ligation (BDL) is an experimental model of extrahepatic cholestasis widely used to resemble bile duct obstruction. Expression and function of major canalicular transporters involved in bile flow formation, the bile salt export pump (Bsep) and Mrp2, are impaired in BDL in rats (Trauner et al., 1997; Lee et al., 2000; Paulusma et al., 2000). We have demonstrated recently that BDL rats, at 7 and 14 days post-surgery, present an impairment in the glutathione-S-transferase (GST)-mediated conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) in vivo, thus leading to decreased synthesis of the model Mrp2 substrate, dinitrophenyl-S-glutathione (DNP-SG) (Villanueva et al., 2006). It is not known, however, whether conjugation reactions mediated by another relevant phase II biotransformation pathway, UDP-glucuronosyltransferase (UGT), as well as subsequent elimination of the corresponding derivatives via Mrp2 are similarly affected in hepatic vs. extrahepatic tissues under BDL conditions.

Acetaminophen (APAP) is one of the analgesics most frequently used. At therapeutic doses, the drug undergoes hepatic glucuronidation and to a lesser extent, sulphation (Thomas et al., 1993). Only a small fraction of APAP results in the formation of the cytotoxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which reacts with cellular glutathione (GSH). Efficient glucuronic acid conjugation of APAP is in consequence a crucial step in prevention of APAP toxicity. A previous report suggests that biliary excretion of APAP glucuronide (APAP-GLU) is mediated by Mrp2 (Xiong et al., 2000). Additionally, this metabolite can be excreted into blood through multidrug resistance-associated protein 3 (Mrp3, Abcc3), a basolateral Mrp family member, suggested to present a higher affinity for APAP-GLU than Mrp2 (Xiong et al., 2000; Manautou et al., 2005). Mrp3 expression is low in human and rat liver under normal conditions (König et al., 1999; Soroka et al., 2001). However, the expression of apical Mrp2 is down-regulated and, in contrast, that of sinusoidal Mrp3 is up-regulated in BDL rats (Trauner et al., 1997; Paulusma et al., 2000; Donner et al., 2001; Soroka et al., 2001; Dietrich et al., 2004). More importantly, biliary secretory function is severely affected in this model of obstructive cholestasis. Thus, a vectorial change in the excretion of APAP-GLU from apical to basolateral transport is expected, causing this metabolite to be excreted into blood instead of into bile. Interestingly, APAP liver toxicity was attenuated in BDL rats, as judged by assessment of serum markers of liver damage and histology.
(Acevedo et al., 1995), thought the mechanism remains unknown. In view of the impossibility of biliary excretion of APAP and its metabolites in this model of cholestasis, extrahepatic tissues could compensate for impaired liver function. Whether derivation of non-toxic metabolites of APAP or APAP itself to extrahepatic tissues, additionally contributes to explain higher tolerance to APAP toxicity at the liver, was not explored. We here evaluated liver, renal and intestinal formation and intestinal and urinary elimination of APAP-GLU under conditions of BDL in rats, either in response to i.v. administration of a subtoxic test dose (150 mg/Kg b.w) or after i.p. administration of a toxic dose (1 g/Kg b.w.) of APAP. The results indicate that irrespective of the dose of APAP, BDL rats produced more APAP-GLU than controls, with a significant elimination through urine and a minor contribution of the intestine.

MATERIALS AND METHODS

Chemicals. Leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, sucrose, APAP, APAP-GLU, UDP-glucuronic acid (UDPGA), D-saccharic acid 1,4-lactone, Triton X-100, UDP-N-acetylglucosamine (UDP-N-AG) and palmitoyl-lysophosphatidylcholine (PLPC) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were commercial products of analytical grade purity.

Animals and surgical procedures. Adult male Wistar rats weighing 300 to 350 g (National University of Rosario, Argentina) were used. Animals had free access to food and water and received human care as outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. BDL was performed as described previously (Gartung et al., 1996) under ether anesthesia. Experiments were performed 7 days after surgery. Controls underwent a sham operation that consisted of exposure, but no ligation, of the common bile duct, and were studied 7 days later.

In vivo synthesis and excretion of APAP-GLU. Studies were performed using the in situ single-pass intestinal perfusion technique (Gotoh et al., 2000) with simultaneous urine collection (Villanueva et al., 2005). To evaluate the synthesis and disposition of APAP-GLU in response to administration with a subtoxic dose of APAP, the bile duct (only in Shams), small intestine and urinary bladder were cannulated under urethane anesthesia, as described (Villanueva et al., 2005). After a 30-min stabilization period, a single dose of 150 mg/Kg b.w. of APAP was administered i.v. as described (Ghanem et al., 2005). Bile, intestinal perfusate and urine were collected for 150 min at 10-, 15- and 30-min intervals, respectively. Biliary, intestinal and urinary flows were determined gravimetrically. Additionally, a blood sample was taken from the tail vein 5 min after administration of APAP. APAP-GLU content was assessed in bile, intestinal perfusate, urine and serum by high-performance liquid chromatography (HPLC) as described previously (Howie et al., 1997), with minor modifications (Ghanem et al., 2005).
To evaluate the synthesis and disposition of APAP-GLU in response to administration with a toxic dose of APAP, the animals were injected i.p. with 1 g/Kg b.w. of the drug. After 45 min, they were anesthetized with urethane and the bile duct (only in Shams), small intestine and urinary bladder were cannulated. One h after APAP was given; bile, intestinal perfusate and urine were collected for 150 min. At the end of this period, the animals were sacrificed by exsanguination. APAP-GLU content was assessed in all the samples by HPLC. Whereas the experiments on i.v. administration of the test dose of APAP were performed to study in detail its glucuronidation and subsequent elimination of APAP-GLU under non-saturating conditions (Ghanem et al., 2005), administration of the 1g/Kg dose, in the way of an i.p. single injection, was used to study APAP-GLU synthesis and disposition under toxic conditions.

Because of the potential role of the kidney in elimination of APAP and its major metabolite APAP-GLU in experimental obstructive cholestasis, it was also of interest to evaluate urinary excretion of intact APAP as well as its tissue content and that of APAP-GLU in renal cortex. The tissue content was evaluated at the end of the 150 min urinary collection period in rats receiving 150 mg/Kg b.w. of APAP. Homogenate preparation and subsequent HPLC analysis were performed as described (Ruiz et al., 2006).

**Western blotting studies.** Because of the different localizations of Mrp2 and Bcrp with respect to Mrp3 (apical vs. basolateral), and to perform a comparative analysis of their expression in response to BDL, we prepared homogenates from liver, jejunum and renal cortex, which were solubilized with Triton X-100 as described (Cao et al., 2002). The final preparations were used immediately in western blot studies using a monoclonal antibody to human MRP2 (M2 III-6, Alexis Biochemicals, Carlsbad, CA), a rabbit polyclonal antibody to mouse Bcrp (M-70, Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit polyclonal antibody to rat Mrp3 (Ogawa et al., 2000), respectively. Expression of UGT1A6, demonstrated to be a major isoform involved in APAP glucuronidation (Kessler et al., 2002), was evaluated by western blotting of liver, jejunum and renal cortex microsomal preparations (Catania et al., 1998) using a specific rabbit polyclonal anti-rat antibody (Ikushiro et al., 1995). The immunoreactive bands were quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

**UGT activity.** Microsomal APAP glucuronidating activity was measured as described previously (Kessler et al., 2002), except that PLPC (0.15 mg/mg protein) was used to fully activate the microsomal suspension and D-saccharic acid 1,4-lactone (2 mM) was routinely included in the incubation media to inhibit enzymatic hydrolysis of APAP-GLU. Hepatic UGT activity was alternatively assessed in the presence of UDP-N-AG, the physiologic activator of UGTs, instead of PLPC. UDP-N-AG was incorporated to the incubation mixtures at a 2 mM final concentration. The APAP-GLU formed was detected by HPLC.
Statistical Analysis. Data are presented as mean ± SD. Statistical analysis was performed using the Student’s t test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

BDL induced a significant increase in the liver weight to body weight ratio by 68% over Shams (0.052 ± 0.004 vs. 0.031 ± 0.003 for BDL and Sham rats, respectively, $p < 0.05$, $N = 3$) and in the mass of both kidneys relative to body mass (+33%, 0.008 ± 0.001 vs. 0.006 ± 0.001, $p < 0.05$, $N = 3$). In contrast, the relative mass of the fragment of small intestine perfused in vivo for transport studies was not affected by BDL (0.013 ± 0.003 vs. 0.015 ± 0.002, $N = 3$). This portion of the small intestine (~50 cm long) corresponds mainly to jejunum, where the highest expression and activity of Mrp2 were reported (Gotoh et al., 2000; Mottino et al., 2000), whereas expression of Mrp3 and Bcrp were the lowest (Rost et al., 2002; Tanaka et al., 2005). Excretion rate of APAP-GLU by these three organs is shown in Fig 1. Biliary elimination of APAP-GLU after administration of a subtoxic, test dose, of APAP (150 mg/Kg b.w.) to Sham rats showed its maximum value at 40 min, and thereafter decreased with time (Fig 1A). Although statistically significant differences in intestinal excretion of APAP-GLU were observed in some periods in response to BDL (Fig 1B), these differences had little impact on the cumulative measure (inset), which did not differ between groups. In contrast, urinary excretion of APAP-GLU was increased significantly by BDL from 60 min onwards (Fig 1C). Cumulative renal excretion of the glucuronide was increased by 200% in BDL rats respect to Shams (inset in Fig 1C). Total excretion of APAP-GLU was also calculated for each organ and expressed as percentage of the dose of APAP. The data are shown in Fig 1D (left). Under normal conditions, biliary and urinary excretion of APAP-GLU accounted for elimination of 13 and 9% of the administered APAP, respectively, with a minor contribution for the intestinal excretion (~1%). The data also indicate that normal contribution of biliary excretion of APAP-GLU, absent in BDL rats, was overrode by its renal excretion, which exhibited an increase of 264% with respect to Shams. Intestinal elimination was not affected in BDL group. The total amount of APAP-GLU eliminated by hepatic and extrahepatic tissues after administration of the test dose was significantly increased (+51%) in BDL group, as seen in the same figure, clearly indicating exacerbated production of the glucuronide derivative in this group when compared to Shams. We additionally evaluated serum APAP-GLU levels 5 min after administration with this same dose of APAP. The data indicate higher values for BDL rats (1194 ± 247 µM) than for Shams (496 ± 64 µM), $p < 0.05$, $N = 3$, suggesting more efficient basolateral secretion into blood. Urinary excretion of intact APAP was also increased in BDL rats (18.1 ± 0.3% of the dose) when compared to Shams (11.9 ± 2.4% of the dose), $p < 0.05$, $N = 3$, likely reflecting impossibility of excretion through bile. Renal tissue content of APAP-GLU by the end of the experiment was higher in BDL (3.4 ± 0.2% of the dose) vs. Sham (2.3 ± 0.2% of the dose) rats, $p < 0.01$, $N = 3$, as it
is the content of intact APAP (1.3 ± 0.1\% and 0.9 ± 0.1\% of the dose for BDL and Sham rats, respectively, \( p < 0.05, N = 3 \)). These data are consistent with increased exposition of the kidney to APAP and its glucuronide, as a consequence of a failure in their biliary elimination.

Cumulative elimination of APAP-GLU in response to administration of the 1 g/Kg b.w. dose of APAP is shown in Fig 1D (right) as percentage of the total dose of APAP. Intestinal contribution in Shams was minor, as already seen for the test dose, and was further decreased by BDL surgery (-55\%). In contrast, a substantial increase in APAP-GLU urinary excretion was observed in BDL rats when compared to Shams (+397\%), which again, overrode normal contribution of biliary elimination. Fig 1D shows that the amount of APAP-GLU excreted by all tissues, was exacerbated in BDL rats when compared to Shams (+110\%), clearly indicating overproduction of the glucuronide derivative, as reported above for the test dose.

APAP-GLU is a model substrate for both Mrp2 and Mrp3 (Xiong et al., 2000; Manautou et al., 2005). Though Mrp2 has been considered a crucial step in liver elimination of endogenous and exogenous glucuronides, more recently, Mrp3 was found to play also a significant role, as clearly demonstrated using the Mrp3 null mice model (Belinsky et al., 2005; Manautou et al., 2005; Zelcer et al., 2006). To establish a potential association between disposition of this metabolite and its plasma membrane transport in the three tissues, we estimated the expression of Mrp2 and Mrp3 by western blotting. Fig 2A shows a significant decrease in the content of hepatic and intestinal Mrp2 protein in BDL rats by 42\% and 47\%, respectively, with respect to controls, whereas it was increased by 57\% in renal cortex. These data on tissue-dependent regulation of this transporter under conditions of obstructive cholestasis agree well with previous reports (Trauner et al., 1997; Paulusma et al., 2000; Lee et al., 2001; Tanaka et al., 2002; Dietrich et al., 2004; Villanueva et al., 2006). BDL treatment also resulted in a marked induction of hepatic Mrp3 (+202\%), in agreement with previous reports (Donner et al., 2001; Soroka et al., 2001). The current western blot studies further demonstrate that expression of Mrp3 in jejunum and renal cortex was not affected by BDL surgery (Fig 2A). Because of its high affinity for APAP-GLU, the remarkable induction of hepatic Mrp3 could have explained an increase in blood accumulation of this metabolite under BDL conditions, even in absence of increased glucuronidation capacity. Up-regulation of renal Mrp2 may account for subsequent tubular secretion and thereby urinary elimination of this metabolite, which was found to be significantly increased in response to administration of either a test or a toxic dose of APAP. It should be considered, however, the possibility of a proportion of this metabolite to be eliminated in urine by glomerular filtration. This needs further examination. In contrast to renal findings, contribution of jejunum to elimination of APAP-GLU was of much less relevance. Moreover, decreased expression of Mrp2 detected in intestine from BDL animals correlates well with impaired excretion of
APAP-GLU, as detected \textit{in vivo} under saturating conditions resulting from administration with the 1 g/Kg b.w. dose of APAP.

Bcrp is involved in apical transport of sulfate conjugate of several drugs, whereas glucurononides are transported to a lesser extent (Suzuki et al., 2003; Zamek-Gliszczynski et al., 2005; Zamek-Gliszczynski et al., 2006). Because some overlap exists in substrate specificity between Bcrp and Mrps, it was also of interest to explore the effect of BDL on expression of Bcrp in homogenate from the different tissues. The data in panel A from Fig 2 clearly show downregulation in liver and renal cortex. Intestinal content was below the limits of detection with the current methodology. Decreased levels of Bcrp in kidney strongly suggests a minor role for this pathway as a compensatory mechanism to eliminate the non-toxic metabolites of APAP, APAP-GLU and APAP sulfate, under conditions of experimental obstructive cholestasis.

Because of the findings on increased production of APAP-GLU \textit{in vivo}, it was of interest to explore the origin of overproduction of this metabolite. UGT activity towards APAP was first examined \textit{in vitro} in fully activated microsomes. The data (nmol/min/mg protein) indicate that neither liver (4.1 ± 0.4 vs. 4.9 ± 0.9, N = 3), nor jejunum (1.1 ± 0.3 vs. 1.4 ± 0.3, N = 3) or renal cortex (2.5 ± 0.2 vs. 2.1 ± 0.1, N = 3) exhibited any change in UGT activity in BDL when compared to Sham rats. These data correlate well with the data on detection of a major UGT isoform involved in APAP conjugation by western blotting. Fig 2B shows that only intestinal UGT1A6 was affected by BDL, with an increase of 27% over Shams. This likely had no consequences on APAP glucuronidating activity as reported above. Because UGT activity detected in fully activated microsomes likely correlates with the number of catalytic units or enzyme molecules (and thus with western blot studies) but not necessarily with the \textit{in situ} activity as conditioned by the lipid environment, we performed an additional assay using liver microsomes in the presence of the physiologic activator UDP-N-AG, which does not produce membrane perturbation (Zakim et al., 1977; Hauser et al., 1988). The study indicates that UGT activity did not differ between BDL (1.5 ± 0.1 nmol/min/mg protein) and Sham (1.7 ± 0.8 nmol/min/mg protein) rats (N = 3). Microsomal protein yield per liver mass unit was neither affected (36 ± 5 and 41 ± 6 mg per g of liver weight in DBL vs. Sham rats, respectively). In consequence, UGT activity per liver mass unit is also expected to be preserved in BDL rats. Because a net increase of the liver weight was observed in BDL vs. Sham rats (16.5 ± 1.3 vs. 10.4 ± 0.3 g, \(p < 0.01\), N = 3), it is likely that the higher amount of APAP-GLU produced in cholestatic rats and expressed as percentage of the dose of administered APAP, resulted from a net increase in functional liver mass. The possibility that the renal cortex or the intestine also contributed to production of the excess of APAP-GLU in BDL group is less likely, since their UGT activities were lower than that of the liver, and the increase in kidney mass in response to BDL was less relevant when compared with the liver one.
Liver glutathione conjugation of CDNB was severely impaired in BDL rats, whereas renal cortex constituted the main place for its metabolism and subsequent elimination through urine (Villanueva et al., 2006). It is known that Mrp3 has preference for glucuronides rather than for glutathione conjugates as substrates (Hirohashi et al., 1999; Zelcer et al., 2001). Taken together, the evidence clearly demonstrates tissue-specific differences in conjugation of different phase II substrates, as well as in re-distribution and disposition of the corresponding conjugates, in this model of obstructive cholestasis.

In summary, we demonstrate that cholestasis by BDL led to a marked increase in liver synthesis of APAP-GLU followed by its efficient urinary excretion, whereas intestinal elimination played a minor role. This was consistent with upregulation of basolateral Mrp3 in liver and apical Mrp2 in renal cortex. This may contribute to explain reduced sensitivity of BDL rats to APAP toxicity, as previously reported (Acevedo et al., 1995). In contrast, downregulation of renal Bcrp would indicate a minor role for this protein in exacerbated urinary elimination of non-toxic metabolites of APAP.

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Footnotes:

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FIGURE LEGENDS

**Fig 1. Biliary, intestinal and urinary excretion of APAP-GLU.**

Excretion of APAP-GLU, a common substrate for both Mrp2 and Mrp3, in bile (A), intestinal perfusate (B), and urine (C) was evaluated at 10, 15 or 30 min periods, respectively, after administration of a 150 mg/Kg b.w. dose of APAP. Insets depict cumulative excretion of APAP-GLU by 150 min. The total amount of APAP-GLU excreted by the whole organs, expressed as percentage of the amount of APAP administered, is depicted in Fig 1D for the dose of 150 mg/Kg b.w. (left) or for the dose of 1 g/Kg b.w. (right). Data are means ± SD of 3 animals per group.
* significantly different from Sham group (p < 0.05).
** significantly different from Sham group (p < 0.01).

**Fig 2. Expression of Mrp2, Mrp3, Bcrp (panel A) and UGT1A6 (panel B) in liver, intestine and kidney.**

Twenty µg of protein from hepatic homogenates and 40 µg from jejunum and renal cortex homogenates were loaded in the gels for simultaneous detection of Mrp2 and Mrp3. 60 µg of protein from liver and renal cortex homogenates were loaded in the gels for detection of Bcrp. Fifteen µg of protein from hepatic and renal cortex microsomes and 30 µg from jejunum microsomes were loaded in the gels for detection of UGT1A6. These amounts of protein gave a densitometric signal in the linear range of the response curve for the different antibodies. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S. Data on densitometry are means ± SD of 3 animals per group. Bcrp was not detected in jejunum in spite that 100 µg of protein were loaded in the gel.
* significantly different from Sham group (p < 0.05).