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Effect of repeated administration with subtoxic doses of acetaminophen to rats on enterohepatic recirculation of a subsequent toxic dose

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ABSTRACT

Development of resistance to toxic effects of acetaminophen (APAP) was reported in rodents and humans, though the mechanism is only partially understood. We examined in rats the effect of administration with subtoxic daily doses (0.2, 0.3, and 0.6 g/kg, i.p.) of APAP on enterohepatic recirculation and liver toxicity of a subsequent i.p. toxic dose of 1 g/kg, given 24 h after APAP pre-treatment. APAP and its major metabolite APAP-glucuronide (APAP-Glu) were determined in bile, urine, serum and liver homogenate. APAP pre-treatment was not toxic, as determined by serum markers of liver damage and neither induced oxidative stress as demonstrated by assessment of ROS generation in liver or glutathione species in liver and bile. APAP pre-treatment induced a partial shift from biliary to urinary elimination of APAP-Glu after administration with the toxic dose, and decreased hepatic content and increased serum content of this conjugate, consistent with a marked up-regulation of its basolateral transporter Mrp3 relative to apical Mrp2. Preferential secretion of APAP-glu into blood decreased enterohepatic recirculation of APAP, thus attenuating liver exposition to the intact drug, as demonstrated 6 h after administration with the toxic dose. The beneficial effect of interfering the enterohepatic recirculation was alternatively tested in animals receiving activated charcoal by gavage to adsorb APAP of biliary origin. The data indicated decreased liver APAP content and glutathione consumption. We conclude that selective up-regulation of Mrp3 expression by APAP pre-treatment may contribute to development of resistance to APAP hepatotoxicity, at least in part by decreasing its enterohepatic recirculation.

1. Introduction

Acetaminophen (N-acetyl-p-aminophenol, APAP) is one of the most sold over the counter and prescribed analgesic and antipyretic drugs. Administered at therapeutics doses (FDA recommends a limit of 4 g/day), it is considered a safe drug, however accidental or intentional ingestion of larger amounts produces severe hepatotoxicity [1]. In the last decades, APAP poisoning was described as the most frequent cause of acute liver failure in USA [2]. The liver pathology is characterized by massive centrilobular necrosis that could be lethal [3]. Within the therapeutic range APAP is metabolized in the liver by glucuronidation and sulphation, generating the non-toxic metabolites, APAP-glucuronide (APAP-Glu) and APAP-sulfate, respectively [3,4]. Larger doses of the drug saturate these metabolic pathways and the excess of APAP is diverted to the CYP450 system, which generates the toxic derivative N-acetyl-p-benzoquinone imine (NAPQI). This reactive metabolite binds reduced glutathione (GSH) to form a stable conjugate and produces intrahepatic GSH depletion. NAPQI exerts its toxicity by reacting with macromolecules such as lipids, proteins and DNA [5]. Glucuronidation is a major pathway in APAP biotransformation. It represents a high-capacity, high-dose saturable pathway [6], and together with basolateral or apical transport of this conjugated derivative at the liver cell, likely affect APAP pharmacokinetics and toxicity [7]. Biliary excretion of APAP-Glu is mediated by the canalicular multidrug resistance associated protein 2 (Mrp2) [8], whereas basolateral transport may also occur and has been linked to expression levels of Mrp3 [9].

Resistance to the toxic effects of APAP has been described in response to repetitive administration of the drug in humans and experimental animals, though the mechanism is only partially

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understood [10,11]. Studies in mice demonstrated that this autoprotection was likely due to increased proliferation of liver cells and to down-regulation of the isoforms of CYP450 involved in NAPQI formation [10]. More recently, Aleksunes et al. [12] demonstrated induction of basolateral Mrp4 by APAP, which was postulated to act as a protective mechanism for the efficient elimination of cytotoxic intermediates or alternative for paracrine signaling to adjacent hepatocytes and other cell types involved in progression of and/or recovery from APAP toxicity. Increased tolerance to APAP intoxication was also observed in rats and was linked to an increased GSH availability from regenerating hepatocytes, likely as a consequence of induction of the enzymes involved in GSH synthesis [11]. We have developed a model of resistance to the toxic effects of APAP in rats based on administration of increasing doses of the drug (0.2, 0.3, 0.6 and 1.0 g/kg) for 4 consecutive days [7]. The mortality evaluated 24 h after administration of the last dose, was markedly reduced when compared to rats treated only with the toxic dose of the drug (1 g/kg). Either administration of repeated or single doses of APAP showed a preferential induction of expression of Mrp3 vs. Mrp2 in liver, with consequences on APAP-Glu pharmacokinetics. Indeed, selective induction of Mrp3 decreased biliary excretion of this metabolite and in contrast, increased its urinary elimination to a similar extent [7].

It has been demonstrated that once APAP-Glu is excreted into bile, the intestinal microflora hydrolyzes this conjugate and the liberated APAP is reabsorbed at a considerable rate, leading to drug enterohepatic recirculation [13]. This was confirmed by using activated charcoal (AC), which is effective in adsorbing APAP onto its surface [14]. Indeed, after i.v. administration of APAP to rats, urinary elimination of APAP metabolites decreased substantially in animals given AC orally, as a consequence of drug sequestration at the intestinal lumen [15]. These findings together with our previous results on selective regulation of Mrp3 vs. Mrp2 by toxic protocols of APAP, and consequent re-direction of APAP-Glu from bile to blood, led us to postulate a link between decreased recirculation of the intact drug and development of resistance to its toxic effects [7].

In this study we explored if treatment with subtoxic doses of APAP was key in development of drug resistance. We first evaluated if administration with 0.2, 0.3 and 0.6 g/kg of APAP, daily, induces liver damage or oxidative stress and if it produces the same effect on Mrps expression as reported for the protocol including the 1 g/kg dose [7]. Then, and more importantly, we evaluated if this particular protocol influences APAP and APAP-Glu pharmacokinetics in response to a subsequent toxic dose. We particularly explored the impact on enterohepatic recirculation and intrahepatic concentration of intact APAP. The results indicate that the current protocol was not toxic and increased expression of Mrp3 to a higher extent than that of Mrp2. This selective regulation influenced APAP and APAP-Glu pharmacokinetics of a subsequent toxic dose, significantly reducing its enterohepatic recirculation and liver content.

2. Methods and materials

2.1. Chemicals

APAP, APAP-Glu, AC, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, NADPH, GSH, and glutathione reductase were from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade purity.

2.2. 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 automatically timed light and dark cycle. All procedures involving animals were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

The following experimental groups were used:

(i) *APAP group*. APAP was administered i.p. at increasing daily doses of 0.2, 0.3, and 0.6 g/kg b.w., as a 100 mg/ml in 1:4 polyethylene glycol 400:saline solution.
(ii) *Control group*. The animals received the same increasing volumes of vehicle administered i.p. for 3 days.

Unless otherwise stated, all studies were performed 24 h after the last dose of APAP or vehicle.

2.3. Sample collection

The rats were anesthetized with sodium pentobarbital (50 mg/kg b.w., i.p.), and sacrificed by exsanguination. The livers were immediately perfused for 30 s with ice-cold saline and removed for Western blot and confocal microscopy studies. Aliquots were gently frozen in liquid nitrogen and preserved at −80 °C until used for plasma membrane preparation, or frozen in liquid nitrogen-precooled isopentane for immunofluorescence studies. Sera were separated from blood samples to assess markers of liver damage. Two additional sets of animals were used to study oxidative stress status. In one study, rats were anesthetized 15 min after the last administration with APAP or solvent and the liver surface was exposed by laparotomy to study *in situ* liver chemiluminescence at 30, 40 or 50 min post-administration. At 60 min the animals were sacrificed and the livers removed for glutathione determinations. In a second study, animals were anesthetized 5 h after the last administration with APAP or solvent and bile duct was cannulated using a polyethylene tubing (PE10). Bile was collected for 10 min after a 30 min stabilization period. The animals were immediately sacrificed by exsanguination and the livers removed and homogenized for glutathione determinations [16].

2.4. Pharmacokinetics of a toxic dose of APAP

The pharmacokinetics of the intact drug and its major metabolite, APAP-Glu, were specifically evaluated. The animals were injected with a toxic dose of APAP (1 g/kg b.w.) using the same solution described above. After 45 min, they were anesthetized with sodium pentobarbital (50 mg/kg b.w., i.p.) and thus maintained throughout. Body temperature was measured with a rectal probe and maintained at 37 °C with a heating lamp. The bile duct and urinary bladder were cannulated using polyethylene tubing (PE10 and PE75 respectively). One hour after APAP was given, bile and urine were collected for 60 min. At the end of this period, the animals were sacrificed by exsanguination and the livers removed. A portion of the organ was homogenized [7] and sera were separated from blood samples. Bile and urinary flow was determined gravimetrically. Appropriate aliquots of bile, urine, sera and liver homogenate were used for assessment of the content of APAP and its glucuronide by HPLC. Total blood content of APAP and APAP-Glu was estimated considering an average blood volume of 7% of total b.w.

2.5. Liver concentration of APAP, APAP-Glu, and glutathione species in animals with preserved enterohepatic circuit

Control or APAP pre-treated animals were injected with a toxic dose of APAP (1 g/kg b.w.) as described above, and either 2 or 6 h later, they were anesthetized and sacrificed by exsanguination. The liver were removed, homogenized and used for glutathione species determination and for assessment of the content of APAP and its glucuronide by HPLC.

2.6. Liver concentration of APAP, APAP-Glu, and glutathione species in animals with interrupted enterohepatic circuit

To evaluate in a more direct way if the interruption in APAP enterohepatic circulation modifies liver exposition, we administered APAP and AC simultaneously, to adsorb the drug from biliary origin. We evaluated if this treatment produced changes in intrahepatic concentration of APAP, APAP-Glu and glutathione species as an indirect index of NAPQI formation. Rats were administered with a suspension of AC in water by gavage (0.8 g/kg b.w.) and simultaneously with a toxic dose of APAP (1 g/kg b.w., i.p.) (AC + APAP group) or with water alone and APAP at the same dose (Water + APAP group). After 45 min, the rats were anesthetized with sodium pentobarbital and the common bile duct and urinary bladder were cannulated with polyethylene tubing (PE10 and PE75 respectively). One hour after APAP was given, bile and urine were collected for 60 min. At the end of this period, the animals were sacrificed by exsanguination and the livers were removed, homogenized and used in determination of APAP, APAP-Glu and glutathione species. APAP and APAP-Glu were also determined in serum, bile and urine samples.

It was important to evaluate if administration with AC itself affects liver content of glutathione species. For this purpose, a third group of animals received AC by gavage and APAP vehicle by i.p. injection (AC + APAP Solv group). Two hours later, the animals were sacrificed by exsanguination and the livers were removed and used in determination of glutathione species.

2.7. Western blot studies

Preparation of crude plasma membranes and subsequent immunoblotting were performed as previously described [17] using a mouse monoclonal antibody to human Mrp2 (M_2 III-6, Alexis Biochemicals, Carlsbad, CA) and a rabbit polyclonal antibody to rat Mrp3 [18].

2.8. Immunofluorescence microscopy

Liver slices (5 μm) were prepared with a Zeiss Microm HM5000 microtome cryostat (MICROM Laborgeräte, Walldorf, Germany), air dried for 1 h, and fixed for 10 min with cold acetone (−20 °C). Tissue sections were incubated overnight with the monoclonal anti-human Mrp2 M_2 III-6 (1:100) and rabbit anti-human Mrp3 (1:100, Sigma Chemical Company) antibodies, followed by incubation with the appropriate fluorescent secondary antibodies. Confocal microscope analysis was performed as described [17].

2.9. Analytical methods

The content of APAP and APAP-Glu in liver homogenate, plasma, bile and urine was assayed by HPLC [19]. Proteins from these samples were removed by precipitation with equal volumes of trichloroacetic acid solution (25%, w/w) followed by centrifugation, previous to HPLC analysis. Retention times of APAP and APAP-Glu were determined by authentic standards.

Liver damage induced by administration with repeated doses of APAP was assessed in serum samples by measuring activity of the enzymes AST and ALT with commercial kits (Human, Wasebaden, Germany).

Oxidized (GSSG) and total (GSH + GSSG) glutathione concentrations in liver homogenate or bile were determined spectrophotometrically by using the recycling method of Tietze [20] as modified by Griffith [21]. The results were expressed as equivalents of GSH. Concentration of GSH was calculated as the difference between total and oxidized glutathione.

In situ liver chemiluminescence was studied by liver surface exposition to a Johnson Foundation photoncounter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA) [22]. Chemiluminescence was expressed in counts per second per unit of liver surface (cps/cm^2).

2.10. Statistical analysis

Data are presented as the means ± SD. Statistical analysis was performed using Student *t*-test unless otherwise stated. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of repeated doses of APAP on liver toxicity and oxidative stress

Table 1 shows the effect of APAP, administered at daily doses of 0.2, 0.3 and 0.6 g/kg b.w. on serum markers of liver damage. This treatment did not produce any significant increment in the markers of liver necrosis AST and ALT. Similarly, the cholestatic marker ALP was neither affected. These results strongly suggest that this protocol was subtoxic.

ROS production estimated through detection of chemiluminescence at the surface of liver was previously found to increase early after administration of a toxic dose of APAP to mice [23]. We found no changes in response to our current non-toxic protocol, either at 30, 40 or 50 min post-administration of the last APAP dose (23 ± 2 and 26 ± 5 cps/cm^2 of liver for controls and APAP respectively, at 50 min, $N = 10$). Additionally, no changes in GSH or GSSG content

Table 1
Liver and biliary content of glutathione species and serum markers of liver damage.

		Determination	APAP/solvent post-administration time	APAP	Control
Serum markers of liver damage		ASAT (UI/L)	24 h	100±40	85±18
		ALAT (UI/L)		42±7	44±10
		ALP (UI/L)		499±75	443±53
Glutathione content	Liver	GSH (ηmol/g liver)	1 h	8050±1103	8188±998
		GSSG (ηmol/g liver)		590±110	712±98
		Total glutathione (ηmol/g liver)		8647±1476	8901±1477
	Liver	GSH (ηmol/g liver)	6 h	7205±1063	7600±1360
		GSSG (ηmol/g liver)		572±91	490±85
		Total glutathione (ηmol/g liver)		7780±1010	8093±1310
	Bile	Bile Flow (ul/min/g liver)	6 h	2.88±0.33 *	1.67±0.21
		GSH (ηmol/min/g liver)		7.50±2.25	7.21±1.94
		GSSG (ηmol/min/g liver)		1.27±0.21	1.88±0.62
		Total glutathione (ηmol/min/g liver)		8.77±2.13	9.10±2.40

Rats were treated with daily increasing subtoxic doses of APAP for 3 days (APAP group) or with solvent (control group). The second column indicates the time post-administration of the last dose of APAP or solvent. ASAT: Aspartate amino transferase. ALAT: Alanine amino transferase; ALP: alkaline phosphatase; GSH: reduced glutathione; GSSG: oxidized glutathione. Data are means ± SD of 4 animals per group. (*) Significantly different from control group ($P < 0.05$).

were detected in liver 1 or 6 h after APAP administration (Table 1). Bile flow determined at 6 h was significantly increased in APAP group likely as a consequence of APAP metabolites excretion [16]. In spite of this, neither GSH nor GSSG biliary excretion rates were affected by the treatment (Table 1). Overall, these data suggest the absence of oxidative stress in rats following the current protocol of APAP administration.

3.2. Effect of repeated doses of APAP on liver expression of Mrp2 and Mrp3

Panel A in Fig. 1 shows the content of Mrp2 and Mrp3 in crude plasma membranes, as detected by Western blotting. The expression of both transporters was increased in APAP pre-treated rats, though to a different extent. While Mrp2 content was increased by 40%, Mrp3 levels were increased by 470%, when compared to controls. This marked induction of Mrp3 relative to Mrp2 was confirmed by confocal microscopy. Panel B in Fig. 1 shows a very low expression of Mrp3 restricted to the basolateral membrane of perivenous hepatocytes surrounding the central vein (CV) in control rats (lower-right panel), as previously described [24]. The expression of Mrp2 (upper-right panel) was restricted to the canaliculus, with similar extent of detection at the perivenous and periportal hepatocytes. Repeated APAP treatment produced a marked increase in immunodetection of Mrp3, which was extended to periportal hepatocytes (lower-left panel), correlating well with Western blot studies, whereas Mrp2 exhibited a same pattern of staining as controls (upper-left panel).

3.3. Effect of repeated doses of APAP on the pharmacokinetics of a toxic dose

APAP and APAP-Glu pharmacokinetics were specifically studied. Table 2 shows cumulative biliary and urinary excretion of APAP-Glu and its concentration in blood and liver at the end of the experiment. The animals pre-treated with APAP exhibited a

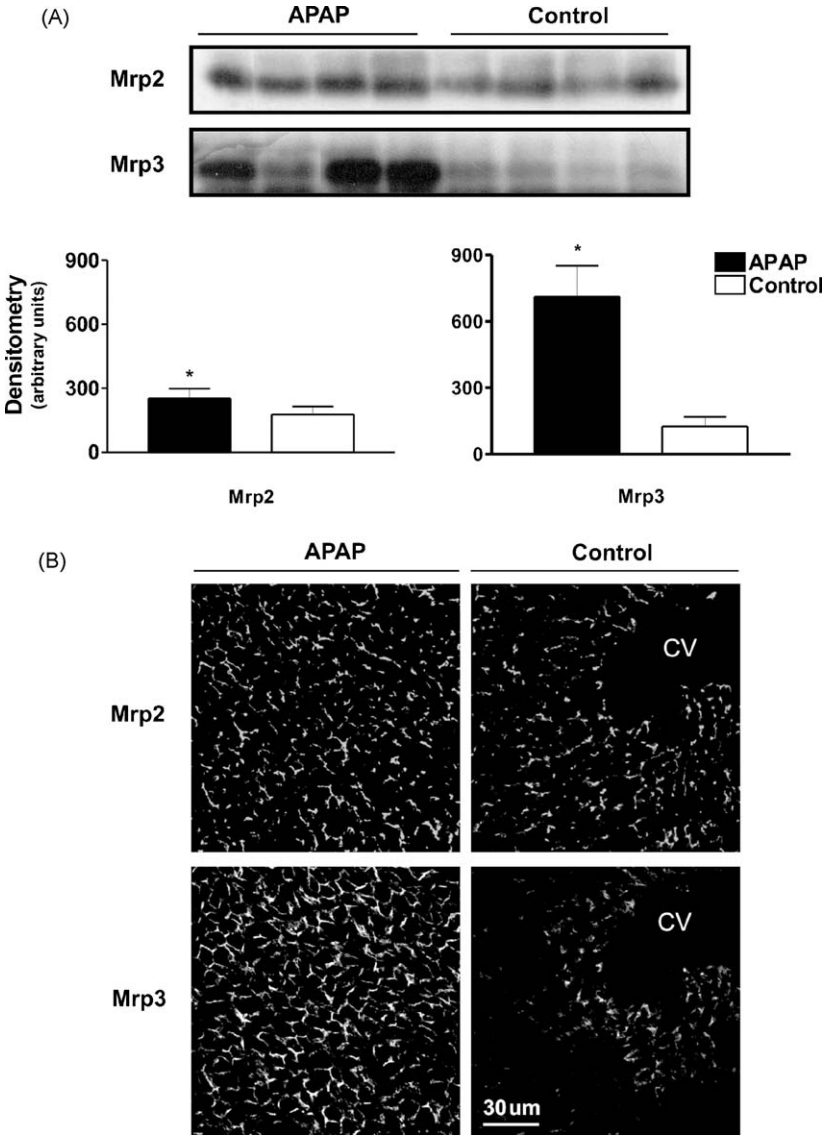

Fig. 1. Liver expression of Mrp2 and Mrp3. *Panel A*: Western blot analysis was performed using mixed plasma membranes (15 μg of protein per well) and developed with a mouse monoclonal antibody to human Mrp2 (M_2 III-6, Alexis Biochemicals) and a rabbit polyclonal antibody to rat Mrp3 [18]. Uniformity of protein loading and transference from gel to membrane was controlled with Ponceau S. Desitometry was performed in four animals per group and expressed in arbitrary units. Data are means ± SD. *Significantly different from control group ($P < 0.05$). *Panel B*: Mrp2 and Mrp3 were simultaneously detected by confocal microscopy using a monoclonal anti-human Mrp2 M_2 III-6 antibody (1:100) and a rabbit anti-human Mrp3 antibody (1:100, Sigma Chemical Company). A same field in each group is shown as stained with anti-Mrp2 (upper panels) and anti-Mrp3 (lower panels). Similar patterns of staining were observed in 3 independent preparations per group. CV: Central vein.

Table 2
APAP and APAP-Glu pharmacokinetics after a toxic dose of APAP.

	APAP-Glu (% dose)		APAP (% dose)	
	APAP	Control	APAP	Control
Biliary excretion	8.58 ± 0.98*	11.62 ± 0.98	2.11 ± 0.67	2.38 ± 0.62
Urinary excretion	13.09 ± 3.90*	6.60 ± 1.99	4.08 ± 1.09	4.41 ± 0.63
Blood content	7.03 ± 2.61*	2.96 ± 0.67	18.50 ± 1.58	17.50 ± 0.75
Liver content	5.09 ± 1.43*	8.19 ± 1.90	7.26 ± 1.08	9.13 ± 2.12
Total	33.79 ± 8.92	29.37 ± 5.54	31.95 ± 4.42	33.42 ± 4.12

Rats were treated with daily increasing subtoxic doses of APAP for 3 days (APAP group) or its solvent (control group). Pharmacokinetics of a subsequent toxic dose was examined 24 h later. Sixty minutes after administration with the toxic dose of APAP, bile and urine were collected for another 60 min. At the end of the experiment, a blood sample was taken and the liver was removed. APAP and APAP-Glu concentrations were determined in all the specimens by HPLC. Data are means ± SD of 4 animals per group.

* Significantly different from control group ($P < 0.05$).

significant decrease in biliary APAP-Glu excretion (−26%), whereas the excretion of this metabolite was significantly increased in urine (+98%), when compared to controls. While hepatic content of APAP-Glu was reduced by 38%, its blood concentration substantially increased (+137%) in APAP pre-treated rats. Overall, these data are consistent with a partial shift in the preferential route of APAP-Glu disposition as a consequence of its increased basolateral transport, associated with preferential induction of Mrp3. Table 2 also shows that the distribution of intact APAP in the different compartments was not affected by the current protocol.

3.4. Effect of repeated doses of APAP on enterohepatic recirculation of a toxic dose

Liver retention of APAP and its glucuronide was evaluated 2 or 6 h after i.p. injection with the 1 g/kg b.w. of APAP. To preserve enterohepatic recirculation, no bile duct cannulation was performed, and thus, intact APAP and APAP-Glu were assessed only in hepatic homogenates. Fig. 2 shows that APAP-Glu content was decreased in animals pre-treated with the subtoxic protocol at both times studied. These decreases were of 48% and 68% when compared to controls, for the 2 and 6 h periods, respectively. While the hepatic content of intact APAP showed only a tendency to decrease at 2 h, this measure was significantly decreased at 6 h (−26%) by APAP pre-treatment. Interestingly, intrahepatic concentration of APAP-Glu detected in both experimental groups was similar between 2 and 6 h-periods, in spite that intact APAP at 6 h

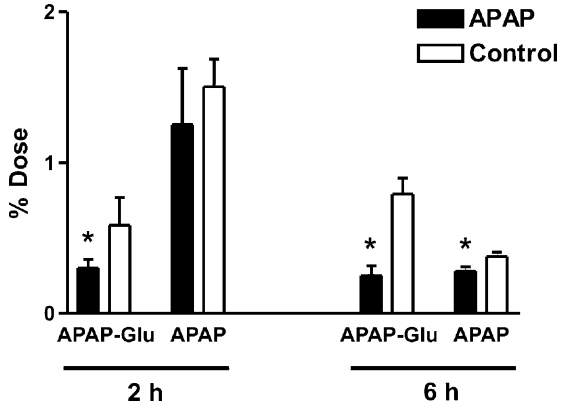

Fig. 2. Content of APAP and APAP-Glu in liver with preserved enterohepatic recirculation. Hepatic concentration of APAP and APAP-Glu was determined by HPLC at 2 or 6 h post-administration with a toxic dose of APAP, and expressed as percentage of this dose. Data are means ± SD of 4 animals per group. *Significantly different from control group ($P < 0.05$).

represented about one third the value detected at 2 h. This may reflect a maximal capability for glucuronide formation followed by efficient efflux from the liver cell at both periods currently studied.

3.5. Effect of interruption of APAP enterohepatic recirculation on APAP, APAP-Glu, and glutathione liver contents

It has been well established that AC has the ability to adsorb APAP onto its surface [14]. Thus, if AC is given simultaneously with APAP, it is expected to decrease intestinal intraluminal concentration of APAP of biliary origin. While AC was given by gavage, the toxic dose of APAP was administered i.p. We assessed not only APAP and APAP-Glu concentration in the usual compartments currently studied, but also evaluated the potential impact of enterohepatic circuit interruption on APAP-induced glutathione consumption. Panel A in Fig. 3 shows that APAP cumulative biliary and urinary excretion were significantly decreased in animals administered with AC (−44% and −22%, respectively) when compared to animals receiving water. Blood and liver concentrations were also decreased by this same treatment (−44% and −33%, respectively). As a consequence, the total amount of intact APAP, as detected in the different compartments, decreased significantly (−36%) in response to AC administration. In contrast, no changes in total APAP-Glu content were observed in any compartment. This may be due to the fact that a maximal capability for glucuronide formation was achieved at the dose of APAP administered.

As it is known, NAPQI generated by CYP450 reacts with GSH to form a stable conjugated [4]. Thus, assessment of liver content of glutathione species is considered an indirect estimation of NAPQI formation. Since GSH is a major protection against oxidative stress by conversion to GSSG, assessment of both species may be also indicative of oxidative stress induced by APAP. We first evaluated if the treatment with AC itself may affect these measures. We found that neither hepatic content of GSH nor that of GSSG is affected 2 h after administration of normal rats with AC when compared to rats only receiving water (data not shown). Panel B in Fig. 3 shows that a toxic dose of APAP, either when administered with AC or with water (solvent of AC), induced a decrease in total glutathione and GSH when compared to the group that was only administered with AC by gavage. However, this decrease was of higher extent for the animals receiving APAP and water than for those receiving APAP and AC (−65% vs. −44% in average), which indicates that AC produced a partial protection on GSH consumption induced by APAP. Surprisingly, liver GSSG content was not affected by APAP administration in any group. This could be linked to the efficient capability of liver to excrete the excess of GSSG produced in conditions of oxidative stress, thus leading to steady intracellular levels [25]. There was no difference in GSSG content between AC + APAP and water + APAP groups either, in spite that this latter group presented increased consumption of GSH, which, again, would indicate an efficient regulation based on elimination from the cell. In support to this possibility we found that GSSG biliary excretion was significantly increased in the group water + APAP (4.6 ± 0.5 nmol/min/g of liver) when compared to the AC + APAP group (2.9 ± 0.9 nmol/min/g of liver) ($N = 4$, $P < 0.05$).

4. Discussion

APAP overdose is one of the main causes of acute liver failure, produced by accidental or intentional overdose ingestion [1,2]. The evidence indicates that chronic ingestion of the drug (several tablets per day, for weeks) leads to development of tolerance to the toxic effects of APAP, usually delaying the onset of liver injury. Tolerance to APAP toxicity also occurs in experimental animals such as mice and rats and the mechanism is likely multifactorial [10–12]. We here provide evidence that administration of repeated subtoxic doses of

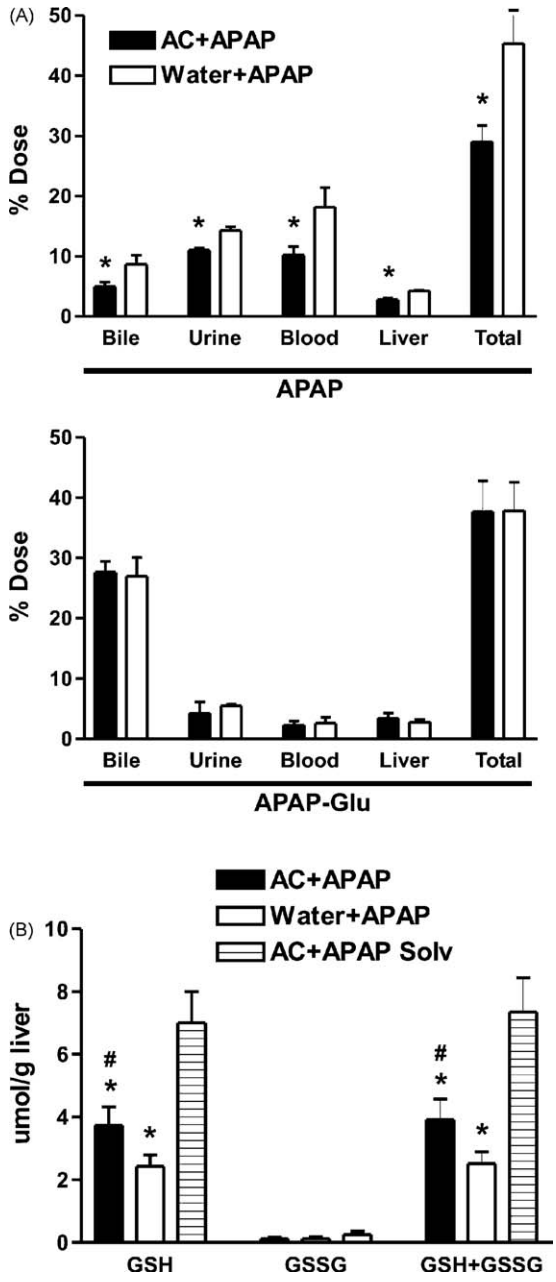

Fig. 3. Content of APAP, APAP-Glu, and glutathione species in liver with interruption of enterohepatic recirculation. *Panel A*: Sixty min after simultaneous administration of activated charcoal (AC) or water by gavage and an i.p. toxic dose of APAP, bile and urine were collected for another 60 min. At the end of the experiment a blood sample was taken and the liver was removed. APAP and APAP-Glu concentrations were determined by HPLC, and expressed as percentage of the dose of APAP administered. Data are means ± SD of 4 animals per group. *Significantly different from control group ($P < 0.05$). *Panel B*: Total glutathione and GSSG were determined in liver samples taken as described in A. GSH concentration was calculated as a difference between both measures. To test the effect of AC itself, extra control animals were administered simultaneously with AC by gavage and with APAP vehicle i.p., and compared with animals receiving both solvents. Liver content of glutathione species was not different between these groups (data not shown). Data are means ± SD of 4 animals per group. Significance was evaluated by one-way ANOVA, followed by the Bonferroni's test. *Significantly different from AC + APAP Solv group ($P < 0.05$). #Significantly different from water + APAP group ($P < 0.05$).

APAP, leading to a preferential induction of Mrp3 vs. Mrp2, substantially affects the enterohepatic recirculation, and in consequence, liver exposition to a subsequent toxic dose. These alterations may contribute to explain the resistance to liver toxicity of the drug, in addition to other well characterized mechanisms [10–12].

The altered vectorial excretion of APAP-Glu from bile to urine agrees well with previous studies reported by Xiong et al. and Slitt et al. using different Mrp3 inducers [26,27]. Furthermore, it was demonstrated in rodents that APAP-Glu is a substrate for both canalicular Mrp2 and basolateral Mrp3 [9,26,27], the affinity for Mrp3 being several times higher than for Mrp2 [9,26,27]. Clearly, induction of basolateral transporters such as Mrp3 may explain increased detection of APAP-Glu in urine. Allegaert et al. have studied the urinary excretion of APAP-Glu, APAP-sulfate and intact APAP in human newborns treated with propacetamol, the diethylglycidyl-ester of paracetamol [28]. Following intravenous administration, this pro-drug is rapidly hydrolyzed by plasma esterases releasing APAP. The authors observed that the proportion of APAP-Glu relative to total species of APAP was increased in urine when the pro-drug was administered repeatedly vs. singly, whereas the proportion of intact APAP was decreased. More recently, Gelotte et al. reported that healthy volunteers which received therapeutic or supratherapeutic doses of APAP (4, 6 or 8 g/day) for 3 consecutive days, presented at the third day lower values of APAP accumulation in plasma than those expected from the half-life of a single administration. The authors also observed an increase in the clearance of APAP-Glu after administration of the third dose [29]. Both groups attributed their observations to an increment in UDP-glucuronosyltransferase (UGT) activity in response to repeated administration with APAP. The possibility arises that the induction of basolateral transporters (e.g. Mrp3) produce a more efficient basolateral secretion of the glucuronide and subsequent urinary elimination, as described by Allegaert et al. and Gelotte et al. It was reported that explants liver specimens from patients following APAP acute intoxication exhibited induction of basolateral Mrp4 and Mrp5 but not of Mrp3 [30]. Mrp4 and Mrp5 differ structurally and functionally from Mrp1–3 in the sense that they preferentially transport nucleotides, whereas other typical Mrp substrates are transported with lower affinities [31]. Unfortunately, it is currently unknown if Mrp4 or Mrp5 of human origin participates in APAP-Glu transport. Whether Mrp3 is induced in humans developing resistance to APAP toxicity in response to chronic treatment with the drug as we described in rats [7] was neither explored.

We have previously demonstrated a similar differential pattern of induction of Mrp3 vs. Mrp2 as currently reported, by either acute or chronic protocols including a 1 g/kg toxic dose of APAP [7]. Though to a different extent, induction of liver toxicity and oxidative stress was confirmed for both protocols. We thus postulated that induction of Mrps was secondary to oxidative stress, which is known to modulate regulation of several different genes. The current results suggest that induction of both transporters may also occur in the absence of any apparent manifestation of liver toxicity or oxidative stress induced by APAP. Several drugs, such as phenobarbital, ethoxyquin and oltipraz [32,33], have the ability to bind specific receptors, such as nuclear Constitutive Androstane Receptor (CAR) and transcription factor E2-related factor 2 (Nrf2), which in turn are demonstrated to induce liver expression of Mrp2 [27,33–36] and Mrp3 [27,32,37,38]. Interestingly, it was recently found that Nrf2 mediates the induction of Mrp3 and Mrp4 after APAP treatment to mice [39], and that NAPQI may directly activate Nrf2 [40] explaining, at least in part, translocation of Nrf2 to the nucleus following administration of either hepatotoxic or non-hepatotoxic doses of APAP to mice [41]. We hypothesize a direct interaction of APAP or its metabolites (e.g. NAPQI) to either of the receptors involved in regulation of Mrp gene expression under the current subtoxic protocol of APAP administration.

Development of tolerance to the toxic effects of APAP was mainly explored in rodents and found to be associated with down-regulation of CYP450 isoforms involved in conversion of APAP into

NAPQI, such as CYP2E1 and CYP1A2 [10,11]. Alternatively, development of resistance was attributed to overproduction of GSH as a result of exacerbated cellular proliferation [11]. Additional mechanisms with participation of modulation of Mrps by APAP may be also plausible. Our current results suggest that significant re-direction of APAP-Glu from bile to urine after administration of a 1 g/kg dose of the parent drug, and concomitant decrease in enterohepatic recirculation and liver exposition to intact APAP represent also a contributing factor to explain development of tolerance to drug toxicity. To test this possibility in a more direct way we interfered APAP enterohepatic recirculation using a strategy different from that of pre-treatment with repeated subtoxic doses of the drug. APAP-Glu is by far the major conjugated metabolite excreted into bile in rats. Watari et al. observed that following intraduodenal injection of APAP-Glu in rats *in vivo*, this compound is hydrolyzed by the microflora and the liberated APAP is reabsorbed at a considerable rate, strongly indicating enterohepatic recirculation [14]. It is known that AC is effective in adsorbing APAP [14], and that after its oral administration, urinary elimination of total APAP, expressed as % of the dose, decreases markedly when the drug was given intravenously at a subtoxic, 100 mg/kg dose, this decrease mainly occurring at the expense of APAP conjugates [15]. We thus performed experiments in animals receiving AC by gavage and simultaneously APAP, as an i.p. toxic, 1 g/kg dose. The results confirmed the decrease in the amount of total APAP in all the samples examined, including the urine, though at the expense of intact APAP. Our data also indicate a decrease in hepatic content of the intact drug as well as attenuation of GSH consumption, strongly suggesting that AC treatment was able to decrease the enterohepatic recirculation of APAP and in consequence, liver injury. Preferential induction of Mrp3 vs. Mrp2, leading to preferential basolateral vs. biliary elimination of APAP-Glu and concomitant remotion of a major component in the enterohepatic reservoir, clearly differs from the action of AC, which likely retains intact APAP (and eventually its metabolites) into the intestinal lumen. In spite of this, both strategies seem to be effective in reducing enterohepatic recirculation and deleterious effects of APAP.

It is known that 70% of a therapeutic dose of APAP is absorbed during the first 30 min following its ingestion by humans [42]. Our results on interference of APAP recirculation by AC could explain the reported efficacy of this agent to protect from liver toxicity, even when administered to patients several hours after APAP intoxication [43,44]. Spiller et al. reported that patients intoxicated who received N-acetylcysteine together with AC up to 3 or 4 h after APAP ingestion, showed lesser incidence of liver injury than those only receiving N-acetylcysteine [43]. Sato et al. demonstrated in a randomized trial, that healthy volunteers who received AC 3 h after administration with a 3 g dose of APAP, showed lower plasma concentrations of the drug than those only receiving its solvent [44]. These observations can only be explained assuming a decrease in enterohepatic recirculation of APAP, thus shortening the permanence of the drug in the enterohepatic circuit. Our data demonstrating that APAP content in liver was decreased by the subtoxic protocol at 6, but not at 2 h post-administration with the toxic dose, is also consistent with a protective role for interruption of APAP recirculation, preferentially at a post-absorptive period (e.g. from 2 h onwards). Different mechanism/s, such as those previously proposed [10–12], may more appropriately explain attenuation of APAP liver toxicity shortly after APAP administration.

In conclusion, we demonstrated a shift from apical to basolateral excretion of APAP-Glu in response to repeated administration with subtoxic doses of APAP, as a consequence of a marked induction of Mrp3 relative to Mrp2. This led to a decrease in hepatic exposition to the intact drug after administration with a toxic dose, likely contributing to the development of resistance to APAP hepatotoxicity, as largely described for chronic protocols.

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