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Transport deficient (TR$^-$) hyperbilirubinemic rats are resistant to acetaminophen hepatotoxicity

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Abstract

The biliary excretion of acetaminophen (APAP) is reduced in transport deficient (TR$^-$) hyperbilirubinemic rats lacking the multidrug resistance-associated protein 2 (Mrp2). This mutant strain of Wistar rats has impaired biliary excretion of organic anions and increased hepatic glutathione. The rational for this study was to determine if there is an altered risk for liver damage by APAP in the absence of Mrp2. Therefore, the susceptibility of TR$^-$ rats to APAP hepatotoxicity was investigated. Male Wistar and TR$^-$ rats were fasted overnight before APAP treatment (1 g/kg). Hepatotoxicity was assessed 24 h later by plasma sorbitol dehydrogenase activity and histopathology. In other studies, TR$^-$ rats received buthionine sulfoximine before APAP to reduce hepatic glutathione to values similar to those in Wistar rats. mRNA expression of APAP metabolizing enzymes was also measured in naïve animals. Wistar rats treated with APAP showed significant elevations in plasma sorbitol dehydrogenase activity, while no increases in enzyme activity were observed in TR$^-$ rats. Histopathology was in agreement. Hepatic non-protein sulfhydryls were significantly lower in Wistar rats receiving APAP than in TR$^-$ rats. TR$^-$ rats treated with buthionine sulfoximine and APAP showed dramatic increases in hepatotoxicity. TR$^-$ rats had increased mRNA expression of several APAP metabolizing enzymes. Mrp2 expression not only is important in biliary excretion, but also influences the toxic potential of reactive intermediates by controlling intrahepatic GSH and possibly drug metabolism.

Keywords: Acetaminophen; Mrp2; TR$^-$ rat; Liver; Biliary; Hepatotoxicity

1. Introduction

Acetaminophen (APAP) and its conjugated metabolites can be found in urine and bile of mice and rats. A considerable amount of APAP is excreted in bile, mainly as the glutathione (APAP-GSH) and glucuronide (APAP-GLUC) conjugates [1,2]. Transport mechanisms for the hepatobiliary excretion of APAP and its metabolites are not completely understood. Recent studies indicate that the multidrug resistance-associated proteins 2 and 3 (Mrp2 and 3, ABCC2 and ABCC3, respectively) are involved in this process. The biliary excretion of APAP-GLUC and APAP-sulfate was reduced in isolated perfused livers from transport deficient (TR$^-$) rats [3]. This mutant strain of Wistar rats lacks expression of functional Mrp2 [4,5].

Mrp2 is involved in the biliary excretion of amphiphilic organic anions including non-bile acid organic anions, glucuronide and glutathione conjugates [6–9]. Excretion of these compounds into bile is impaired in TR$^-$ rats [10,11]. Our laboratory demonstrated that TR$^-$ rats receiving APAP have decreased biliary excretion of APAP-GSH, APAP-GLUC and APAP-*N*-acetylcysteine [1]. Our studies

Abbreviations: TR$^-$, transport deficient hyperbilirubinemic rats; Mrp, multidrug resistance-associated protein; APAP, acetaminophen; APAP-GSH, acetaminophen glutathione; APAP-GLUC, acetaminophen glucuronide; ICG, indocyanine green; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; SDH, sorbitol dehydrogenase; NPSH, non-protein sulfhydryls; BSO, buthionine sulfoximine; GSH, glutathione; PBS, phosphate buffered saline; γ-GCS, gamma-glutamylcysteine synthetase; UGT, UDP-glucuronosyltransferase; CYP450, cytochrome P450

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also showed that Mrp2 deficiency results in increased urinary excretion of APAP-GLUC in TR$^-$ rats. This is most likely due to compensatory up-regulation of the basolateral efflux transporter Mrp3 in TR$^-$ rats [12]. Studies in Mrp3$^{-/-}$ mice conclusively demonstrated that the sinusoidal efflux transport of APAP-GLUC is highly dependent on Mrp3 function [13].

A key question not previously addressed is whether there is an altered risk for hepatic damage by APAP in the absence of Mrp2. Since the organic anion indocyanine green (ICG) not only produces changes in the biliary disposition of APAP similar to those seen in TR$^-$ rats [14], but also has a protective effect against its hepatotoxicity [15], we decided to investigate the susceptibility of TR$^-$ rats to APAP toxicity.

Hepatic GSH is important in the detoxification of the reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). GSH is transported into bile by a common transport-mediated process with organic anions [16]. TR$^-$ rats have increased hepatic GSH levels, demonstrating that Mrp2 is important for GSH transport into bile [17]. Although the higher GSH content in liver should make these mutant rats more resistant to APAP toxicity, the dramatic changes in hepatobiliary disposition of APAP in these rats makes it difficult to anticipate their ultimate response to a toxic dose of APAP.

The results of the present studies show that TR$^-$ rats are highly resistant to APAP toxicity. To investigate the role of higher hepatic GSH in this resistance, hepatic GSH content in TR$^-$ rats was normalized to values in naïve Wistar rats with the use of the GSH-depleting agent buthionine sulfoximine (BSO) prior to APAP administration. Changes in gene expression of several APAP metabolizing enzymes between strains of rats were investigated also.

2. Materials and methods

2.1. Reagents

4-Acetamidophenol (APAP), buthionine sulfoximine, glutathione, trichloroacetic acid, EDTA, 5,5′-dithiobis(2-nitrobenzoic acid), trizma hydrochloride, trizma base and β-nicotinamine adenine dinucleotide (reduced form) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of reagent grade or better.

2.2. Animals

Male Wistar rats were obtained from Charles River Laboratories (Wilmington, MA) and TR$^-$ rats were bred in our animal facilities. The mutational status of our TR$^-$ rats was confirmed in previous studies [18]. Weight-matched adult Wistar and TR$^-$ rats were used. Animals had free access to tap water and standard laboratory rodent diet. Lights were maintained on a 12:12 h light/dark cycle, and the room temperature was maintained at 22 °C. Animal studies were approved by the University of Connecticut Institutional Animal and Care Use Committee (Protocol No. A05-010).

2.3. Treatment of animals

Animals were fasted overnight prior to treatment. To elicit APAP-induced liver injury, Wistar and TR$^-$ rats received 1 g/kg APAP in 0.2% Gum Arabic, intraperitoneally (i.p.). Controls received vehicle only. Liver injury was determined 24 h later by plasma sorbitol dehydrogenase (SDH) activity and histopathology. Hepatic GSH levels were determined by the non-protein sulfhydryls (NPSH) assay. This dose of APAP was selected from pilot studies where fasted Wistar rats received doses of 750, 1000 or 1250 mg/kg. The dose of 1 g/kg was selected because it produced significant, but no overt liver toxicity or lethality.

To investigate the role of GSH in the susceptibility of TR$^-$ rats to APAP liver injury, BSO was used to modulate hepatic GSH content. Wistar and TR$^-$ rats were dosed with 0.89 g/kg of buthionine sulfoximine (BSO) in phosphate buffered saline (PBS), i.p. [19,20]. NPSH content was determined before and 3 h after BSO treatment. The goal was to decrease hepatic GSH levels in TR$^-$ rats by BSO treatment to levels in untreated Wistar rats.

To determine whether a reduction in hepatic GSH concentration by BSO treatment would increase the susceptibility of TR$^-$ rats to APAP toxicity, mutant rats were fasted overnight and dosed with 0.89 g BSO/kg or PBS vehicle 3 h prior to challenge with APAP (1 g/kg). Animals were sacrificed at 24 h for assessment of hepatotoxicity and hepatic NPSH analysis.

2.4. Biochemical assays

Plasma sorbitol dehydrogenase (SDH) activity was used as an indicator of hepatic injury. Briefly, rats were anesthetized with a combination of 100 mg ketamine/kg and 10 mg xylazine/kg, i.p., and blood was withdrawn from the abdominal aorta. Blood was collected in heparinized tubes and plasma SDH activity was measured following the procedure of Gerlach and Hiby [21].

Hepatic NPSH content was measured as an indicator of reduced glutathione (GSH). Liver samples were processed as previously described for NPSH quantification by the colorimetric procedure of Ellman [22,23]. NPSH content was determined by comparison to a GSH standard curve.

2.5. Histopathology

A piece of the left lateral lobe of the liver was fixed in 10% formalin and processed for histopathological examination as previously described [24,25]. Liver sections were scored using a scale from 0 to 5 depending on the severity of centrilobular necrosis and degeneration. Histopathology scoring was as follows: no injury = grade 0;

single to few hepatocytes = grade 1; 10–25% of hepatocytes = grade 2; 25–40% of hepatocytes = grade 3; 40–50% of hepatocytes = grade 4; or more than 50% of hepatocytes = grade 5.

2.6. mRNA analysis of phases I and II enzymes

Hepatic mRNA levels of several phases I and II enzymes known to metabolize APAP (i.e. CYP1A2, CYP2E1 and CYP3A1/23, UGT1A1, UGT1A6 and UGT1A7) were measured in TR$^-$ and Wistar rats using the QuantiGene® Branched DNA (bDNA) Signal Amplification Assay [26]. Rat gene sequences were obtained from GenBank. RNA extraction and the bDNA Signal Amplification Assay have been described in detail [27,28].

2.7. Statistical analysis

Results are expressed as means ± S.E.M. of at least three animals per treatment group. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Duncan's new multiple range test. Histopathology scores were ranked and then subjected to ANOVA, followed by Duncan's new multiple range tests. The level of significance was set at $p < 0.05$.

3. Results

3.1. Studies on the susceptibility of TR$^-$ rats to APAP hepatotoxicity

Plasma SDH activity in Wistar and mutant rats receiving Gum Arabic was negligible (Fig. 1). However, Wistar rats receiving APAP showed SDH values of 646.3 ± 164 U/ml. By contrast, plasma SDH values in TR$^-$ rats were drastically lower (approximately 50 U/ml), indicating that the mutant rats are resistant to APAP hepatotoxicity. These results were in agreement with the histopathology. As depicted in Table 1, 100% of the animals treated with vehicle received a score of zero. Similarly, TR$^-$ rats receiving APAP did not exhibit significant liver injury (all animals had scores lower than 2). On the other hand, all Wistar rats treated with APAP had scores higher than two. Fifty percent of the animals in this group received the highest possible score of 5.

Table 1
APAP-induced hepatic centrilobular necrosis in Wistar and TR$^-$ rats

Treatments	Histopathology scores						
	0	1	2	3	4	5	>2 (%)
Wistar rats							
Gum Arabic *a*	4	0	0	0	0	0	0
APAP (1 g/kg) *b*	0	0	0	1	1	2	100
TR$^-$ rats							
Gum Arabic *a*	4	0	0	0	0	0	0
APAP (1 g/kg) *a*	4	2	0	0	0	0	0

Note: After overnight fasting, rats were challenged with 1 g APAP/kg, i.p. Rats were killed at 24 h; livers were removed, examined histologically and graded for severity of hepatocellular necrosis. Scores greater than 2 are indicative of significant necrosis. Treatment groups with different letters are significantly different from one another ($p \leq 0.05$).

3.2. Hepatic NPSH content in TR$^-$ in response to toxic APAP dosing

Fig. 2 shows that hepatic NPSH content at 24 h after APAP administration in Wistar rats was 47% of their Gum Arabic controls (Fig. 2). NPSH data at 24 h offers some indication of the severity of toxicity and capacity to replenish GSH in response to APAP. Control TR$^-$ rats had approximately 1.6 times more hepatic NPSH than vehicle treated Wistar rats. Mutant rats treated with either

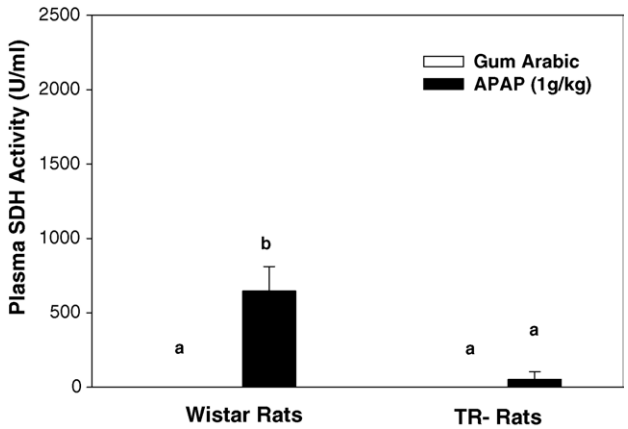

Fig. 1. Effect of APAP on plasma SDH activity in Wistar and TR$^-$ rats. Wistar and TR$^-$ rats were fasted overnight and then challenged with 1 g APAP/kg, i.p. Controls received Gum Arabic vehicle. Rats were killed 24 h later and blood was collected for determination of plasma SDH activity. Values are means ± S.E.M., $n = 4$–6 (letters a and b). Treatment groups with different letters are significantly different from one another ($p < 0.05$).

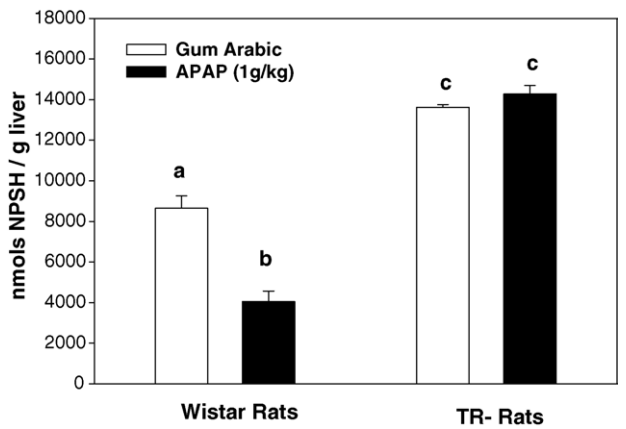

Fig. 2. Effect of APAP on hepatic NPSH content in Wistar and TR$^-$ rats. After overnight fasting, animals received 1 g APAP/kg or vehicle (Gum Arabic), i.p. Rats were killed at 24 h later for determination of hepatic NPSH content. Values are means ± S.E.M., $n = 4$–6 (letters a–c). Treatment groups with different letters are significantly different from one another ($p < 0.05$).

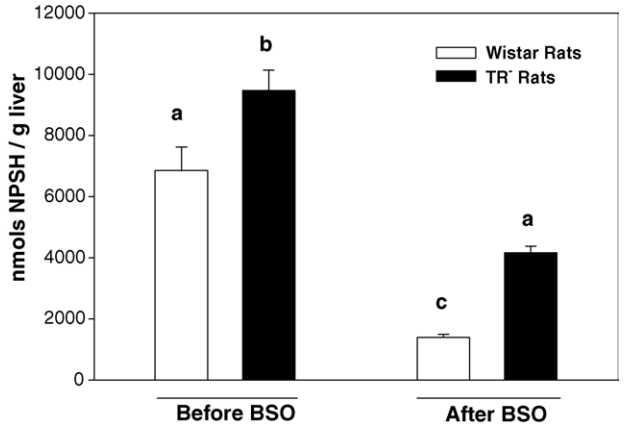

Fig. 3. Effect of buthionine sulfoximine (BSO) on hepatic NPSH levels in Wistar and TR$^-$ rats before and at 3 h after treatment. Animals were fasted overnight and dosed the next morning with 0.89 g BSO/kg. NPSH content was determined before and at 3 h after treatment. Values are means ± S.E.M., $n = 3$–4 (letters a–c). Treatment groups with different letters are significantly different from one another ($p < 0.05$).

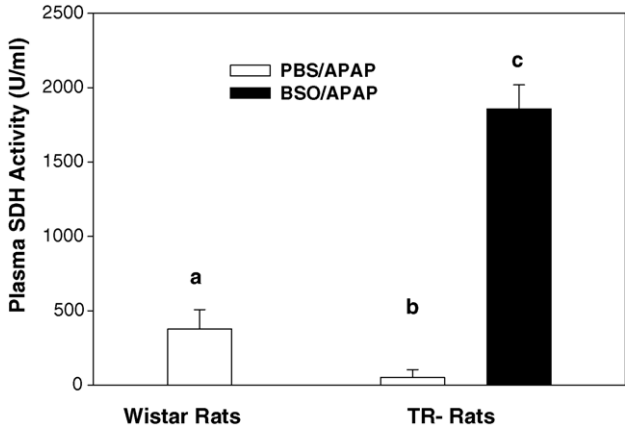

Fig. 4. Effect of BSO on plasma SDH activity 24 h after APAP challenge in TR$^-$ rats. Wistar and TR$^-$ rats were fasted overnight. The following morning, TR$^-$ rats were treated with 0.98 g BSO/kg, i.p., and then challenged with APAP (1 g/kg, i.p.) 3 h later. Wistar rats without BSO treatment were also challenged with APAP. Rats were killed at 24 h after APAP administration and blood was collected for determination of plasma SDH activity. Treatment groups with different letters are significantly different from one another ($p < 0.05$).

vehicle or APAP (1 g/kg) had similar hepatic NPSH levels (13 621 ± 134 nmol/g versus 14 282 ± 411 nmol/g liver). This suggests that the higher basal GSH concentration in the liver of mutant rats prevents these values from changing as significantly as in Wistar rats in response to APAP.

3.3. Modulation of hepatic NPSH content in TR$^-$ rats following BSO treatment

Hepatic NPSH values before and 3 h after BSO treatment are depicted in Fig. 3. Fasted, Wistar rats had NPSH values of 6863 ± 758 nmol/g liver before BSO treatment, while mutant rats had significantly higher NPSH (9474 ± 660 nmol/g liver). At 3 h after BSO treatment, NPSH levels were decreased by 80% and 56% in Wistar and TR$^-$ rats, respectively. Levels of NPSH in mutant rats at 3 h after BSO treatment were not significantly different from those in Wistar rats before treatment.

3.4. Role of higher hepatic GSH content in the resistance of TR$^-$ rats to APAP toxicity

In Fig. 4, plasma SDH activity in Wistar rats pretreated with PBS vehicle and then challenged with APAP was elevated, while TR$^-$ rats receiving the same treatment were less susceptible as previously seen in Fig. 1. Mutant rats pretreated with BSO prior to APAP had SDH activity elevated to 1984 ± 175 U/ml. This represents a three-fold increase in SDH activity from APAP alone in Wistar rats (Fig. 1). These results indicate that BSO treatment dramatically increased APAP liver toxicity in mutant rats, far surpassing the response of Wistar rats to the same dose of APAP. Wistar rats treated with BSO and APAP were not included in this analysis since this treatment was shown to produce 100% mortality by 24 h in pilot studies (data not shown). Histopathology scores from the BSO and APAP study are shown in Table 2. Three out of six Wistar rats challenged with APAP had significant hepatic injury (histopathology scores higher than two). However, none of the mutant rats in the APAP only group showed significant necrosis. By contrast, all mutant rats treated with BSO before APAP challenge had scores higher than 2.

The 24 h hepatic NPSH values in mutant rats pretreated with BSO and challenged with APAP are presented in Fig. 5. Liver NPSH content was 7014 ± 497 nmol/g liver in Wistar rats treated with PBS vehicle and APAP. PBS/APAP treated mutant rats had much higher NPSH levels (13 807 ± 3120 nmol/g liver). TR$^-$ rats treated with BSO and APAP had approximately 3% of NPSH values in mutant rats receiving PBS and APAP only. This greatly compromised GSH recovery correlates well with increased susceptibility to APAP toxicity.

Representative photomicrographs of liver sections stained with H&E are presented in Fig. 6. Panel A is

Table 2
Effect of BSO pretreatment on APAP-induced centrilobular necrosis in Wistar and TR$^-$ rats

Treatments	Histopathology scores						
	0	1	2	3	4	5	>2 (%)
Wistar rats							
APAP (1 g/kg) *a*	1	1	1	2	1	0	50
TR$^-$ rats							
BSO/APAP *b*	0	0	0	0	3	0	100
APAP (1 g/kg) *c*	0	2	1	0	0	0	0

Note: After overnight fasting, rats were challenged with 1 g APAP/kg, i.p with or without prior treatment with BSO (0.89 g/kg, i.p.). Rats were killed at 24 h, livers were removed, examined histologically and graded for severity of hepatocellular necrosis. Scores greater than 2 are indicative of significant necrosis. Treatment groups with different letters are significantly different from one another ($p \leq 0.05$).

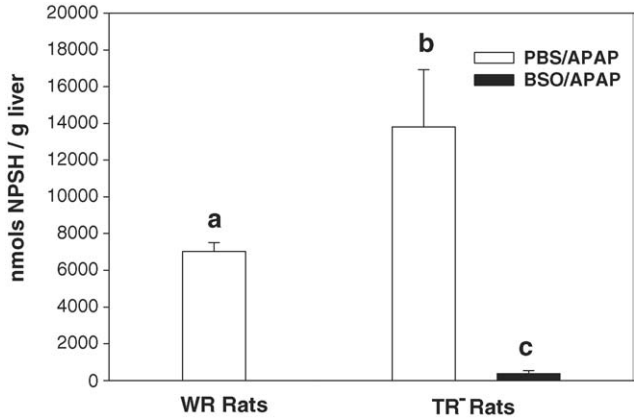

Fig. 5. Effect of BSO on hepatic NPSH levels 24 h after APAP challenge in TR$^-$ rats. Wistar and TR$^-$ rats were fasted overnight. The following morning, TR$^-$ rats were treated with 0.98 g BSO/kg, i.p., and then challenged with APAP (1 g/kg, i.p.) 3 h later. Wistar rats without BSO treatment were also challenged with APAP. Rats were killed at 24 h after APAP administration and blood was collected for determination of hepatic NPSH content. Treatment groups with different letters are significantly different from one another ($p < 0.05$).

representative of liver sections from vehicle treated Wistar rats. Liver from vehicle treated TR$^-$ rats were histologically normal and had no apparent morphologic differences with the untreated Wistar rats (data not shown). Panel B is representative of the severity of hepatic centrilobular necrosis produced by APAP in Wistar rats. In panel C, minimal or no evidence of hepatocellular necrosis is seen in TR$^-$ rats receiving APAP. However, treatment of TR$^-$ rats with BSO and APAP resulted in hepatic centrilobular necrosis that was greater in severity than that seen in Wistar rats receiving APAP only (panel D).

3.5. mRNA levels for several APAP metabolizing enzymes in TR$^-$ rat liver

The steady-state levels of CYP3A1/23 and UGT1A6 mRNA in naïve TR$^-$ rats were approximately 300% and 200% of those in Wistar rats, respectively (Fig. 7). CYP2E1 and UGT1A1 mRNA expression was also significantly higher in TR$^-$ rats. No significant differences in mRNA levels of CYP1A2 and UGT1A7 were detected between genotypes. Since CYP3A, CYP2E1 and UGT1A6 are known to metabolize APAP, these results suggest that both APAP bioactivation and glucuronidation pathways are induced in TR$^-$ rats.

4. Discussion

The human Dubin–Johnson syndrome is a rare autosomal recessive liver disorder characterized by chronic

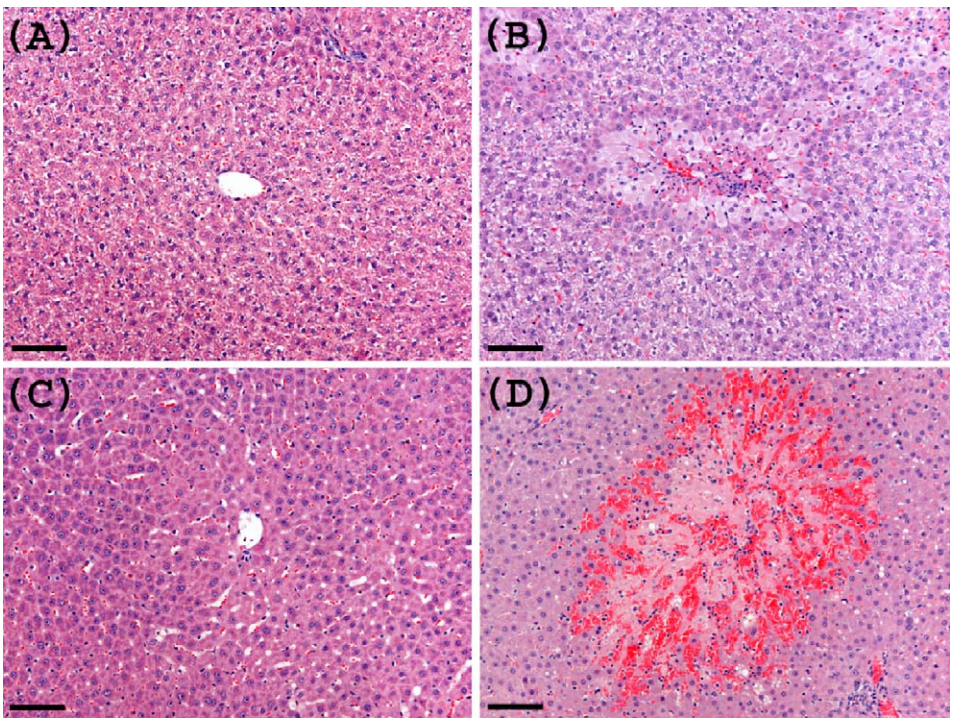

Fig. 6. Liver histopathology in Wistar and TR$^-$ rats. All animals were fasted overnight. The following morning, Wistar rats were treated with either APAP (1 g/kg, i.p) or Gum Arabic vehicle. TR$^-$ rats were treated with 0.89 g BSO/kg or PBS vehicle, i.p., and challenged with APAP or Gum Arabic 3 h later. Rats were killed 24 h later and livers were obtained and processed for histopathology as described in Section 2. For orientation purposes, the central vein is located in the center of each picture. Representative photomicrographs of (A) normal liver histology in Wistar rat treated with Gum Arabic, (B) centrilobular necrosis (grade 3) in Wistar rat treated with APAP, (C) normal liver histology in TR$^-$ rat treated with APAP only, and (D) centrilobular necrosis (grade 4) in TR$^-$ rat treated with BSO and APAP. Liver centrilobular necrosis is circumferential coagulative necrosis of hepatocytes. In comparison to Wistar rats treated with APAP only, the TR$^-$ rats treated with APAP alone lacked or had decreased centrilobular necrosis while the TR$^-$ rats treated with both BSO and APAP had a greater extent of necrosis. Bars represent 100 mm. Sections were stained with hematoxylin and eosin.

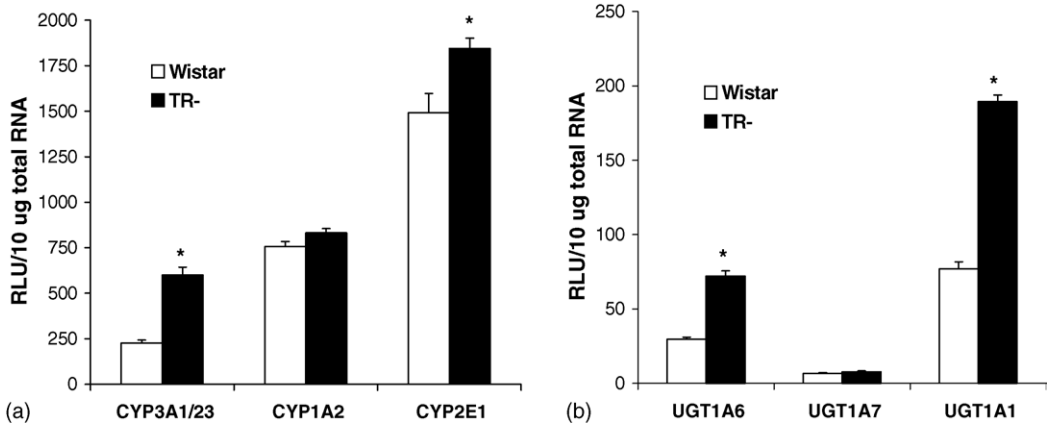

Fig. 7. Hepatic CYP450 and UGT mRNA expression in livers from Wistar and TR$^-$ rats. Total RNA was isolated from livers of untreated Wistar and TR$^-$ rats. RNA was analyzed by bDNA assay for expression of (a) CYP3A1/23, 1A2, 2E, and (b) UGT 1A6, 1A7 and 1A1. The data are presented as mean relative light units ± S.E. (n = 4–12 animals). Bars represent control levels for each genotype. Values in TR$^-$ rats with an asterisk are significantly different from their respective Wistar rat controls ($p < 0.05$).

conjugated hyperbilirubinemia [29]. Patients with this condition have a compromised capacity for excreting conjugated bilirubin and other organic anions into bile. A highly homologous phenotype has been described for the transport-deficient (TR$^-$) hyperbilirubinemic rat [30,31].

Recent experiments in our laboratory and others documented a role for Mrp2 in the biliary disposition of APAP [1,3]. However, those studies did not examine whether there is an altered risk for liver injury by APAP in the absence of Mrp2. To investigate this, Wistar and TR$^-$ rats received a toxic dose of APAP and hepatotoxicity was assessed 24 h later. Plasma SDH activity and liver histopathology revealed that mutant rats are highly resistant to APAP hepatotoxicity. It is worth noting that liver GSH content was significantly higher in TR$^-$ rats treated with APAP than in vehicle-treated Wistar rats, and nearly equal to control TR$^-$ rats.

To further study the importance of hepatic GSH in the response of TR$^-$ rats to APAP, GSH levels were modulated by the administration of buthionine sulfoximine (BSO). BSO is a specific inhibitor of gamma-glutamylcysteine synthetase (γ-GCS) that blocks GSH synthesis, thus reducing its concentration in the liver [32]. The dose of BSO used in our studies was also used by Dietrich [20] in TR$^-$ rats to study the role of Mrp2 and GSH in the intrahepatic cycling of toxins such as α-naphthylisothiocyanate.

In our experiments, BSO decreased GSH levels in Wistar and TR$^-$ rats by 80% and 56%, respectively. BSO is known to have a more pronounced inhibitory effect in tissues with high GSH turnover [33]. Normal liver has a high capacity for exporting GSH and there is constant need for replenishing it. In contrast, GSH turnover is expected to be lower in TR$^-$ rats due to its low biliary excretion and retention in hepatocytes. As a result, the γ-GCS pathway might not be affected by BSO as prominently in TR$^-$. However, both strains or rats showed decreases in NPSH of a similar absolute magnitude (approximately 5000 nmol/g liver), which indicates that BSO inhibits GSH synthesis in both genotypes equally.

Upon APAP challenge, mutant rats pretreated with BSO showed a dramatic increase in hepatotoxicity. This indicates that higher GSH content does contribute to the resistance exhibited by these mutant rats, since reduction of GSH rendered them more susceptible to liver injury than Wistar rats receiving APAP only. Similar results were obtained in a separate study using a lower dose of BSO (0.45 g/kg). NPSH values in TR$^-$ rats at 3 h after this dose of BSO were 5303 ± 623 nmol/g liver, in comparison to 4699 ± 422 nmol/g liver in Wistar rats before BSO. Animals pretreated with the lower dose of BSO receiving the same dose of APAP showed plasma SDH activity of 1489 ± 611 U/ml, which is similar to that in mutants receiving twice as much BSO.

The low susceptibility of Mrp2 mutant rats to APAP toxicity may also be due to impaired biliary excretion of other organic anions with hepatoprotective properties. As with GSH, other endogenous substances with antioxidant properties requiring biliary excretion could be retained intracellularly in the absence of Mrp2; thus, protecting from APAP hepatotoxicity. An example of such compound is bilirubin [34]. Bilirubin is a known potent antioxidant and is readily transported into the bile conjugated with glucuronic acid [35]. In TR$^-$ rats, significant retention of bilirubin metabolites occurs [36].

The implications of up-regulation of the basolateral transporter Mrp3 in TR$^-$ rats and the presence of the canalicular transporter breast cancer-related protein (Bcrp) in the resistance of TR$^-$ rats to APAP toxicity were considered. It is well known that hepatic expression of Mrp3 is elevated in TR$^-$ rat liver [37], which can re-direct the excretion of chemicals from bile into sinusoidal blood. Mrp3 has preference toward glucuronide conjugates of xenobiotics [38]. We recently showed that only the biliary and basolateral disposition of APAP-glucuronide is significantly altered in mice lacking Mrp3 [13]. Disposition of APAP itself or any of the other metabolites is unchanged in Mrp3$^{-/-}$ mice. Furthermore, liver perfusion experiments

also show that basolateral and biliary elimination of GSH in Mrp3$^{-/-}$ mice is unaltered. These results indicate that Mrp3 is unlikely to contribute to the lower susceptibility of TR$^-$ rats to APAP hepatotoxicity. In regards to Bcrp, its expression has also been examined in TR$^-$ rats and no differences were detected between mutant and normal Wistar rats (Curtis D. Klaassen, Yuji Tanaka, KUMC, personal communication). Therefore, Bcrp is not expected to contribute to the observed resistance of TR$^-$ rats to APAP either.

We also considered the possibility that the resistance of these mutants to toxicity could be attributed to compensatory changes in metabolic pathways involved in APAP bioactivation or detoxification. Constitutive mRNA levels for UGT1A6 and 1A1 were significantly higher in TR$^-$ rats. This suggests that enhanced glucuronidation may also contribute to the decreased susceptibility of these mutant rats to APAP.

Changes in metabolite profiles in urine and bile are often indicative of alterations in activity of biotransformation pathways. Our previous studies on the hepatobiliary disposition of APAP in TR$^-$ rats explored this [1]. However, this in vivo approach was deemed far more complex in TR$^-$ rats since both biotransformation and transport processes contribute to the disposition of APAP from the liver, with the later being considerably altered. This makes the presence of APAP metabolites in urine and bile of TR$^-$ rats a non-reliable indicator of potential changes in metabolism. We relied instead on gene expression analysis of drug metabolizing enzymes.

Liver steady-state levels of CYP3A1/23 and 2E1 mRNA were significantly higher in TR$^-$ rats in comparison to Wistar controls. These CYP450 isoforms are known to catalyze the conversion of APAP to NAPQI [39]. Increases in CYP3A and 2E1 should lead to more NAPQI formation in mutant rats, which in turn should lead to greater hepatotoxicity. However, the opposite was seen. We attribute this to the higher hepatic GSH content in these animals. Bile and urine analysis from our previous studies was not indicative of enhanced NAPQI formation and conjugation with GSH in TR$^-$ rats because in the absence of Mrp2, the disposition of thiol-derived conjugates of APAP is greatly altered [18]. However, APAP-GSH accumulated in the liver of TR$^-$ rats, which could be reflective of both altered hepatobiliary excretion and higher NAPQI generation. Given the high levels of basal gene expression of biotransformation enzymes tested in TR$^-$ rats, the magnitude of these changes should have an impact on APAP biotrasformation. A recent paper by Newton et al. [40] showed that total CYP450 content, NADPH-cytochrome P450 reductase and the activity of several CYP450-mediated reactions were all significantly higher in TR$^-$ rats than in Wistar rats. This further supports the biological significance of higher mRNA expression for APAP metabolizing enzymes in TR$^-$ rats. Furthermore, the dramatic increases in liver injury in TR$^-$ treated with BSO and APAP in comparison to Wistar rats receiving APAP only suggest that the GSH-lowering effect of BSO unmasked the higher capacity of TR$^-$ rats to bioactivate APAP via higher constitutive expression of CYP4503A1/23 and 2E1.

In summary, these experiments demonstrate that TR$^-$ rats are resistant to APAP hepatotoxicity and that the reduced susceptibility is related to higher hepatic GSH content. Changes in APAP biotransformation may also contribute to this resistance. These studies are of great clinical significance since they raise important questions about the potential susceptibility of patients with Dubin–Johnson Syndrome and related liver disorders to APAP. One important question is whether patients with Dubin–Johnson Syndrome also have higher hepatic GSH. This should make them more resistant to APAP and other hepatotoxicants working via reactive metabolites that are neutralized by conjugation with GSH. Another unknown is whether the changes in gene expression of APAP metabolizing enzymes seen in TR$^-$ rats are also present in Dubin–Johnson patients. Higher CYP450 bioactivating activity can affect drug metabolism in these individuals. Altered glucuronidation can similarly change pharmacokinetics and toxic actions of chemicals. We can conclude from our studies that Mrp2 not only plays an important role in biliary excretion, but also affects toxicity outcome for reactive intermediates by controlling intrahepatic GSH levels and possibly xenobiotic metabolizing enzymes.

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