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Retinoid X Receptor α Regulates the Expression of Glutathione *S*-transferase Genes and Modulates Acetaminophen-Glutathione Conjugation in Mouse Liver

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ABSTRACT

Nuclear receptors, including constitutive androstane receptor, pregnane X receptor, and retinoid X receptor (RXR), modulate acetaminophen (APAP)-induced hepatotoxicity by regulating the expression of phase I cytochrome P450 (P450) genes. It has not been fully resolved, however, whether they regulate APAP detoxification at the phase II level. The aim of the current study was to evaluate the role of RXRα in phase II enzyme-mediated detoxification of APAP. Wild-type and hepatocyte-specific RXRα knockout mice were treated with a toxic dose of APAP (500 mg/kg i.p.). Mutant mice were protected from APAP-induced hepatotoxicity, even though basal liver glutathione (GSH) levels were significantly lower in mutant mice compared with those of wild-type mice. High-performance liquid chromatography analysis of APAP metabolites revealed significantly greater levels of APAP-GSH conjugates in livers and bile of mutant mice compared with those of wild-type mice. Furthermore, hepatocyte RXRα deficiency altered the gene expression profile of the glutathione *S*-transferase (Gst) family. Basal expression of 13 of 15 *Gst* genes studied was altered in hepatocyte-specific RXRα-deficient mice. This probably led to enhanced APAP-GSH conjugation and reduced accumulation of *N*-acetyl-*p*-benzoquinone imine, a toxic electrophile that is produced by biotransformation of APAP by phase I P450 enzymes. In conclusion, the data presented in this study define an RXRα-Gst regulatory network that controls APAP-GSH conjugation. This report reveals a potential novel strategy to enhance the detoxification of APAP or other xenobiotics by manipulating Gst activity through RXRα-mediated pathways.

In the adult liver, the nuclear receptor RXRα is the most abundant among the three RXR isoforms: RXRα, -β, and -γ (Mangelsdorf et al., 1992). It dimerizes with many nuclear receptors and regulates several important biological processes, including xenobiotic detoxification. Using hepatocyte-specific RXRα knockout mice, we demonstrated previously that the level of RXRα is crucial for maintaining homeostasis of cholesterol, fatty acids, and carbohydrates as well as for the metabolism of xenobiotics (Wan et al., 2000a,b, 2003; Dai et al., 2003; Wu et al., 2004a). Absence of RXRα in hepatocytes alters the hepatic expression profile of genes, including phase I and II enzymes critical for xenobiotic metabolism (Wu et al., 2004b).

APAP is a widely used analgesic. Studies of its metabolism and toxicity have been reviewed in several publications (Bessems and Vermeulen, 2001; James et al., 2003; Sumioka et al., 2004). When taken in overdose, a fraction of APAP is converted by cytochrome P450s (P450s) to a highly reactive and electrophilic metabolite, *N*-acetyl-*p*-quinoneimine (NAPQI). NAPQI is detoxified by preferential conjugation with GSH in hepatocytes, a process catalyzed by glutathione *S*-transferases (Gsts). When cytosolic and mitochondrial GSH is depleted, NAPQI accumulates and covalently binds to cellular macromolecules (Jollow et al., 1973) and induces oxidative stress (Lores Arnaiz et al., 1995), which eventually causes liver injury. APAP overdose is a leading cause of clinical acute liver failure in the United States (Lee, 2004).

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ABBREVIATIONS: RXR, retinoid X receptor; P450, cytochrome P450; NAPQI, *N*-acetyl-*p*-benzoquinone imine; GSH, glutathione; Gst, glutathione *S*-transferase; APAP, acetaminophen; CAR, constitutive androstane receptor; PXR, pregnane X receptor; APAP-GSH, acetaminophen-glutathione; APAP-CG, acetaminophen-cysteinylglycine; APAP-CYS, acetaminophen-cysteine; APAP-GLU, acetaminophen-glucuronide; APAP-NAC, acetaminophen-mercapturate; APAP-SUL, acetaminophen-sulfate; HPLC, high-performance liquid chromatography; ALT, alanine aminotransferase; bDNA, branched DNA; Mrp2, multidrug resistance-associated protein 2; mGst, microsomal glutathione *S*-transferase; PPAR, peroxisome proliferator-activated receptor.

Even therapeutic doses of APAP may induce hepatotoxicity in the presence of risk factors, such as alcohol consumption, fasting, drug interactions, or preexisting liver diseases, which increase susceptibility to the hepatotoxic effects of APAP (Sumioka et al., 2004).

In recent years, it has been demonstrated that xenobiotic receptors play a central role in modulating APAP hepatotoxicity. Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) function as critical regulators of APAP metabolism and hepatotoxicity by regulating expression of phase I P450 genes involved in APAP bioactivation (Zhang et al., 2002; Guo et al., 2004). We reported previously that RXRα modulates APAP-induced liver injury by regulating mRNA levels of P450 genes, which participate in APAP bioactivation (Wu et al., 2004b). In the present report, we demonstrate that hepatocyte RXRα also modulates APAP detoxification by altering phase II enzyme-mediated conjugation of APAP. Our results reveal an RXRα-Gst regulatory network that controls APAP-GSH conjugation.

Materials and Methods

Reagents. Standards of APAP metabolites [i.e., APAP-glutathione (APAP-GSH), APAP-cysteinylglycine (APAP-CG), APAP-cysteine (APAP-CYS), APAP-mercapturate (APAP-NAC), APAP-glucuronide (APAP-GLU), and APAP-sulfate (APAP-SUL)] were generous gifts from Dr. Jose Manautou (University of Connecticut, Storrs, CT). APAP, GSH, and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade methanol and acetic acid were obtained from Fisher Scientific Co. (Springfield, NJ).

Mice. Age-matched wild-type and hepatocyte RXRα-deficient mice were used. Hepatocyte RXRα-deficient mice were generated by specifically mutating the RXRα gene in hepatocytes using lox-cre recombination as described in detail previously (Wan et al., 2000a). Mice were kept (three mice/cage) in steel microisolator cages at 22°C with a 12-h/12-h light/dark cycle. Food and water were provided ad libitum throughout the entire feeding period. To avoid gender differences in xenobiotic biotransformation, only male mice were used in the current study. Male mice (10–12 weeks old) were fasted overnight and then treated with APAP (500 mg/kg i.p.) or vehicle (phosphate-buffered saline) for 1, 2, or 18 h. Mice were fasted because unfed mice exhibit more consistent responses to APAP than do fed mice (Placke et al., 1987; Lucas et al., 2000). Mice were sacrificed at the indicated time points. Serum samples were prepared for alanine aminotransferase (ALT) analysis. Bile and urine were collected from gallbladders and bladders. Livers were immediately excised, frozen in liquid nitrogen, and kept at −80°C until use. Liver samples were also fixed in 10% formalin and stained with hematoxylin and eosin (H&E) for histological analysis. All the animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

ALT Assay. Serum ALT activity was determined spectrophotometrically using assay reagents from Catachem Inc. (Bridgeport, CT) according to the manufacturer's instructions. In brief, 20 μl of serum was added to 0.5 ml of reagents. The samples were incubated at 37°C for 5 min and then assayed for the decrease in absorbance at 340 nm. The activity of ALT was expressed as the number of international units per liter based on the following equation: ALT units/liter = [absorbance change (A)/min × assay volume (milliliters) × 1000]/[6.22 × light path (centimeters) × sample volume (milliliters)] = A/min × 2572.

Measurement of GSH Levels. GSH level was determined by the recycling method (Tietze, 1969).

HPLC Analysis. APAP and its metabolites in liver, blood, bile, and urine were analyzed using an HPLC method described by Chen et al. (2003) and Lucas et al. (2000) with minor modification. In brief, livers (0.1 g) were homogenized in 9 volumes of ice-cold methanol. Perchloric acid (3 N) was added to 2 volumes of serum and liver homogenates to precipitate proteins. Bile and urine samples were diluted with 2 volumes of ice-cold HPLC-grade methanol. After centrifugation, supernatants were filtered through 0.2-μm nylon filter and used for analysis. Aliquots (20 μl) of the processed samples were injected into a Vydac 208TP54 5-μm C8 column (4.6 × 250 mm) (P. J. Cobert Associates, St. Louis, MO). APAP and its metabolites were separated with a linear gradient and a constant flow rate of 1 ml/min. Solvent A consisted of 1% aqueous acetic acid; solvent B was 100% methanol. The mobile phase was initially kept at 5% B for 4 min followed by a 1-min linear gradient that finished at 12.5% B and then 20 min at 12.5% B. The elution of metabolites was monitored at 254 nm. Retention times of APAP and its metabolites were determined using authentic standards. Because the retention times of APAP-CG and APAP-CYS were too close to separate, they were quantified together as APAP-CG/CYS. Samples from vehicle-treated mice showed no interfering peaks. Quantification was based on integrated peak areas. The concentrations of APAP and its metabolites were calculated using an APAP standard curve.

Northern Blot Analysis. Total liver RNA was extracted by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA concentration was determined spectrophotometrically. Twenty micrograms of total RNA was separated by electrophoresis in a denaturing 1.2% (w/v) agarose gel containing 2.2 M formaldehyde. Equal loading per lane was assessed by ethidium bromide staining and hybridization with a β-actin cDNA probe. RNA was transferred to a nylon membrane by capillary blotting in 10× standard saline citrate and cross-linked by UV irradiation. Probe labeling and hybridization were performed as described previously (Wan et al., 2003). The differences in mRNA levels were determined by densitometry, normalized to β-actin mRNA levels, and expressed as fold difference from control.

Branched DNA Signal Amplification Assay. The hepatic mRNA levels of multidrug resistance-associated proteins 2 (Mrp2) were measured using a branched DNA (bDNA) assay described previously (Slitt et al., 2003). In brief, a specific Mrp2 oligonucleotide probe set was synthesized and diluted in Tris-EDTA buffer according to instructions provided by the QuantiGene bDNA signal amplification kit (Bayer Corp. Diagnostics Div., Tarrytown, NY). Total RNA (10 μg) was added to each well of a 96-well plate containing 50 μl of capture hybridization buffer and 50 μl of the diluted probe set. Subsequent hybridization and washing steps were carried out according to the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex data management software version 5.02 for analysis of luminescence from 96-well plates. The luminescence for each well is reported as relative light units per 10 μg of total RNA.

Statistical Analysis. Data are given as mean ± S.D. Statistical analysis was performed using Student's t test or one-way analysis of variance. Significance was defined by $P < 0.05$.

Results

Hepatocyte RXRα-Deficient Mice Are Resistant to APAP-Induced Hepatotoxicity. To evaluate the effect of RXRα on hepatocyte susceptibility to APAP toxicity, both wild-type and hepatocyte RXRα-deficient mice were treated with APAP (500 mg/kg i.p.) or vehicle and sacrificed 2 and 18 h after treatment. Liver injury was evaluated histologically and by measuring serum ALT levels. Two hours after APAP administration, serum ALT levels were not significantly changed (Fig. 1A). Histopathological analysis revealed that modest cellular damage occurred in the livers of wild-type mice, but not hepatocyte RXRα-deficient mice, by 2 h after treatment (Fig. 1B). Eighteen hours after APAP injec-

tion, serum ALT levels were 5-fold higher in wild-type mice than that in hepatocyte RXRα-deficient mice (Fig. 1A). Consistent with this observation, massive centrilobular necrosis was present in the livers of wild-type mice, but not hepatocyte RXRα-deficient mice (Fig. 1B). Vehicle-treated wild-type and hepatocyte RXRα-deficient animals displayed normal liver histology (data not shown). These data show that hepatocyte RXRα-deficient mice are protected from APAP toxicity. Furthermore, because these mice are protected as early as 2 h after APAP treatment, this may suggest that the mechanism underlying protection is an early event.

Hepatic GSH Is Rapidly Depleted in Wild-Type and Hepatocyte RXRα-Deficient Mice after APAP Treatment. GSH is critical for detoxification of NAPQI. Total GSH content was assayed using livers harvested from mice 2 h after vehicle or APAP administration. The results showed that basal hepatic GSH levels in hepatocyte RXRα-deficient mice were significantly lower than that in wild-type mice (Fig. 2). After APAP treatment, hepatic GSH levels were depleted in both genotypes of mice (Fig. 2). These data suggest that the GSH levels may not have a significant impact on the extent of APAP-induced liver injury in wild-type versus hepatocyte RXRα-deficient mice.

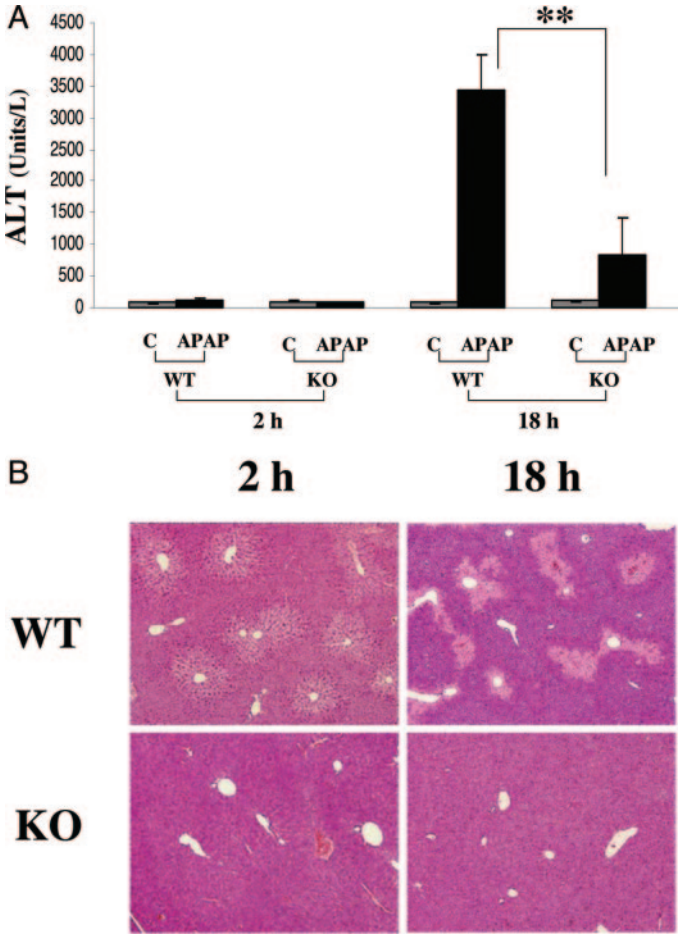

Fig. 1. Hepatocyte RXRα-deficient mice are resistant to APAP hepatotoxicity. Wild-type (WT) and hepatocyte RXRα-deficient (KO) mice were given vehicle (C) or APAP (500 mg/kg i.p.). There were five mice in each group. A, serum ALT levels of wild-type and hepatocyte RXRα-deficient mice 2 and 18 h after APAP treatment. Blood samples were collected at 2 and 18 h after APAP treatment for determination of serum ALT activity. Note that ALT activity was significantly higher in wild-type than that in hepatocyte RXRα-deficient mice 18 h after APAP treatment ($P < 0.01$). B, H&E staining of liver tissue sections from wild-type and hepatocyte RXRα-deficient mice 2 and 18 h after APAP treatment. Liver samples from all animals were analyzed, but only representative histology is presented. Note that extensive hepatic centrilobular necrosis occurred in wild-type, but not in hepatocyte RXRα-deficient, mice 18 h after APAP administration.

APAP-GSH Conjugation Is Enhanced in Hepatocyte RXRα-Deficient Mice. APAP is metabolized through multiple pathways. To elucidate the mechanism by which hepatocyte RXRα-deficient are protected from APAP hepatotoxicity, HPLC assays were performed to measure APAP and its metabolites. Liver, serum, urine, and bile samples were collected from wild-type and hepatocyte RXRα-deficient mice 1 h after APAP administration. The results are summarized in Table 1.

Free APAP concentrations were not significantly different between wild-type and hepatocyte RXRα-deficient mice for all four sample groups. This suggested that RXRα deficiency in hepatocytes did not influence the rate of APAP disappearance in the liver, bile, urine, and blood. With overdose, oxidation of a small portion of APAP by P450 enzymes gives rise to NAPQI, which is inactivated by conjugation with GSH. APAP-GSH is mainly excreted into bile. It is noteworthy that APAP-GSH concentrations in hepatocyte RXRα-null mice were 7-fold higher in liver and 2-fold higher in bile compared with those of wild-type mice. APAP-GSH can be sequentially metabolized to APAP-CG, APAP-CYS, and APAP-NAC. As shown in Table 1, all three APAP metabolites were detected in bile, urine, and serum but not in liver. However, no significant differences were observed between the two genotypes of mice, indicating that RXRα deficiency did not alter the sequential metabolism of APAP-GSH to APAP-CG, APAP-CYS, and then APAP-NAC. APAP-SUL and APAP-GLU are two major conjugates formed during APAP detoxification. In both genotypes, APAP-SUL was mainly excreted into the urine, whereas APAP-GLU, the most abundant APAP metabolite, was excreted into the urine and bile. In RXRα-deficient mice, disposition and concentration of APAP-SUL or APAP-GLU were similar to that in wild-type mice. This result suggested that hepatocyte RXRα deficiency did not significantly alter APAP-SUL and APAP-GLU conjugation and disposition. These findings strongly imply that con-

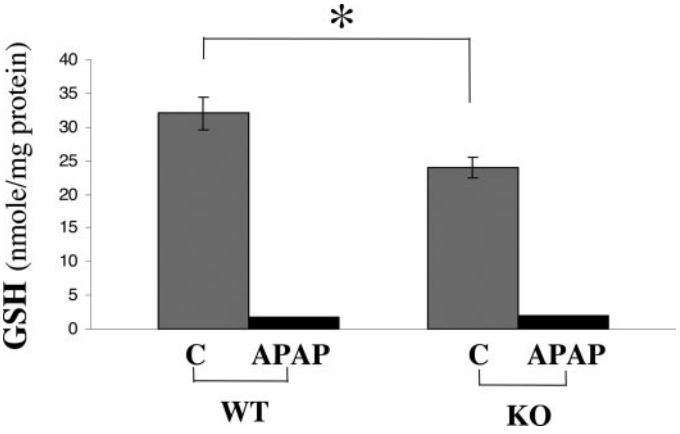

Fig. 2. Hepatic GSH levels in APAP- or vehicle (C)-treated animals. Liver samples were collected 2 h after APAP or vehicle treatment, and GSH content was measured. Note that the basal level of GSH was lower in hepatocyte RXRα-deficient (KO) mice than that in wild-type (WT) mice ($P < 0.05$; $n = 5$). GSH was rapidly depleted in both genotypes of mice after APAP administration.

jugation of NAPQI with GSH was enhanced in hepatocyte RXRα-null mice.

To confirm these findings, we measured APAP metabolites in liver and serum collected 2 h after APAP administration (Table 2). Free APAP concentrations in liver and serum were comparable between wild-type and knockout animals. Hepatic APAP-GSH concentrations were approximately 15-fold higher in hepatocyte RXRα-null mice than that in wild-type mice, which is consistent with the observation from the livers collected 1 h after APAP treatment. Trace amounts of APAP-GSH and APAP-CG/CYS were detected in serum and liver with no significant differences between wild-type and hepatocyte RXRα-deficient mice. The other metabolites were not detectable in the liver and serum.

Hepatobiliary excretion of APAP-GSH is mediated by Mrp2 (Chen et al., 2003). To evaluate whether RXRα is involved in the regulation of Mrp2 gene, mRNA levels of Mrp2 were measured using a bDNA assay in the livers of hepatocyte RXRα-deficient and wild-type mice. The result indicated that absence of RXRα does not alter the expression of Mrp2 gene (Fig. 3).

RXRα Modulates APAP-GSH Conjugation by Regulating Gst Gene Expression. Conjugation of NAPQI with GSH is catalyzed by Gsts. The significant difference in APAP-GSH formation between wild-type and RXRα-deficient mice implies a possible difference in Gst levels. Therefore, we postulated that RXRα modulates APAP-GSH conjugation by regulating *Gst* gene expression. Total liver RNA was extracted from both genotypes of mice 2 h after vehicle or APAP injection. The cDNA fragment specific to each member of the Gst family was identified by cDNA sequence comparison and amplified by reverse transcriptase-polymerase chain reaction to generate a probe. Because of the greater than 95% sequence identity between Gst α1 and α2 or Gst μ1 and μ3, the common probes Gst α1/2, able to detect both Gst α1 and α2 mRNA signals, and Gst μ1/3, able to detect both Gst μ1 and μ3, were prepared. The mRNA levels were measured by Northern blot experiments using densitometry. Data were normalized with β-actin mRNA levels. The results (Fig. 4) showed alterations in the gene expression profiles of Gst family members because of RXRα deficiency or APAP treatment. With respect to basal mRNA levels, a 1.5- to 3.5-fold up-regulation of Gst α1/2, μ1/3, μ2, and μ4 was observed in knockout mice. In addition, a 0.5- to 5-fold down-regulation of Gst α3, μ3v (Gst μ3 variant, GenBank accession no. XM_137047), π1, θ1, θ2, θ3, ζ1, and ω1 was seen in knockouts. No significant differences in basal mRNA levels of Gst α4, κ1, and microsomal Gst (mGst) 1 were observed in hepatocyte RXRα-null mice compared with wild-type mice. These findings suggest that RXRα is an important regulator of *Gst* gene expression. In wild-type mice, mRNA expression of all the Gst family members examined was inhibited by APAP treatment. In particular, Gst α3, α4, μ3v, π1, θ1, θ2, ζ1, ω1, and κ1 were decreased 0.5- to 5-fold. In hepatocyte RXRα-deficient mice, APAP treatment had a differential influence on Gst mRNA expression. The mRNA expression of Gst μ1/3 and μ2 was increased, whereas expression of Gst α1/2, α4, and θ2 was decreased. Others did not appreciably change. After APAP treatment, expression of Gst μ2, μ4, α1/2, and μ1/3 was 5-, 3-, 3-, and 2-fold higher, respectively, in hepatocyte RXRα-deficient mice compared with wild-type mice. These Gsts were up-regulated to the greatest extent as a result of hepatocyte RXRα deficiency without APAP administration. Therefore, up-regulation of Gst μ2, μ4, α1/2, and μ1/3 may contribute to the enhancement of APAP-GSH conjugation in hepatocyte RXRα-deficient mice.

Discussion

RXRα is an obligatory partner of two major xenobiotic receptors, CAR and PXR. Modulation of APAP-induced hepatotoxicity by RXRα, CAR, and PXR has been evaluated using hepatocyte RXRα-deficient mice, CAR knockouts, PXR knockouts, CAR/PXR double knockouts, and humanized CAR mouse models. Our previous studies demonstrated that hepatocyte RXRα-deficient mice are resistant to APAP-induced hepatotoxicity by expressing lower levels of Cyp1a2 and Cyp3a11 mRNAs (Wu et al., 2004). CAR knockout mice are protected from APAP hepatotoxicity as a result of lack of induction of Cyp1a2, Cyp3a11, and Gst π expression by

TABLE 1
HPLC analysis of APAP and its metabolites 1 h after APAP treatment
Data are mean ± S.D. (n = 5).

	Liver	Bile	Urine	Serum
	nmol/g	*nmol/ml*	*nmol/ml*	*nmol/ml*
APAP				
WT	2544 ± 606	3954 ± 291	19,772 ± 4409	1628 ± 319
KO	3196 ± 541	3691 ± 1529	17,222 ± 6744	1071 ± 113
APAP-GSH				
WT	518 ± 138	9480 ± 1841	N.D.	N.D.
KO	3594 ± 273**	18,706 ± 6388*	N.D.	N.D.
APAP-CG/CYS				
WT	N.D.	520 ± 89	10,430 ± 1674	102 ± 5
KO	N.D.	724 ± 210	9284 ± 2581	87 ± 2
APAP-NAC				
WT	N.D.	633 ± 186	11,708 ± 1099	665 ± 86
KO	N.D.	1158 ± 342	12,462 ± 2903	454 ± 46
APAP-SUL				
WT	N.D.	N.D.	4798 ± 880	185 ± 50
KO	N.D.	N.D.	7139 ± 1981	81 ± 24
APAP-GLU				
WT	N.D.	1016 ± 494	61,354 ± 9093	137 ± 20
KO	N.D.	2611 ± 1215	45,753 ± 6973	180 ± 24

WT, wild type; KO, knockout; N.D., not detectable.
* $P < 0.05$; ** $P < 0.01$.

APAP (Zhang et al., 2002). Humanized CAR mice pretreated with phenobarbital show significantly increased liver toxicity induced by APAP compared with mice that were not pretreated (Zhang et al., 2002). PXR knockout mice are more sensitive to APAP hepatotoxicity because of a higher basal level of Cyp3a11 mRNA compared with wild-type mice (Guo et al., 2004). Treatment with pregnenolone 16α-carbonitrile markedly enhances the susceptibility to APAP-induced liver injury mainly by inducing Cyp3a11 expression in wild-type mice but not in PXR null mice (Guo et al., 2004). Another independent study, using a different PXR-null mouse line, showed no difference in APAP toxicity between wild-type and PXR null mice (Zhang et al., 2004). The discrepancy between these two studies might be attributable to the difference in basal Cyp3a11 mRNA levels in the two PXR knockout mouse lines established independently. PXR/CAR double knockout mice exhibit decreased APAP toxicity compared with wild-type mice. CAR knockout mice show similar sensitivity to APAP hepatotoxicity as PXR/CAR double knockout mice (Zhang et al., 2004). All of the above-mentioned studies primarily focused on the role of these nuclear receptors in phase I bioactivation of APAP. Our current study demonstrates a critical role for RXRα in phase II detoxification of APAP. RXRα also modulates APAP-GSH conjugation and determines the susceptibility of APAP hepatotoxicity at the phase II level. The APAP metabolic pathways and molecular mechanisms for RXRα, PXR, and CAR modulating APAP-induced hepatotoxicity are summarized in Fig. 5.

Our results reveal the molecular mechanism underlying the protection of hepatocyte RXRα-deficient mice from APAP overdose at the phase II level. NAPQI is detoxified by conjugation with GSH. Therefore, GSH levels are very important for the elimination of this toxic intermediate. RXRα regulates GSH homeostasis by regulating glutamate-cysteine ligase catalytic subunit, which is an essential enzyme involved in GSH synthesis (Wu et al., 2004b). Despite being protected from APAP-induced hepatotoxicity, GSH levels are significantly lower in hepatocyte RXRα-null mice than that in wild-type mice. After APAP treatment, GSH was depleted in both genotypes of mice. Hence, it is unlikely that the RXRα-GSH synthesis pathway contributes to the mechanism underlying the resistance of hepatocyte RXRα knockout mice to APAP toxicity. Systematically measuring APAP and its metabolites uncovered an exciting phenomenon. The concentrations of APAP-GSH conjugates in the liver were significantly higher in hepatocyte RXRα null mice than that in wild-type mice. APAP-GSH conjugates are mainly excreted into the bile (Chen et al., 2000, 2003). Higher concentrations of APAP-GSH were also observed in the bile of hepatocyte RXRα mutant mice compared with that of wild-type mice. Consistent with this finding, the total amount of APAP-GSH in bile

TABLE 2
HPLC analysis of APAP and its metabolites 2 h after APAP treatment
Data are mean ± S.D. (n = 5).

	Liver	Serum
	nmol/g	*nmol/ml*
APAP		
WT	932 ± 263	68 ± 22
KO	1496 ± 559	130 ± 41
APAP-GSH		
WT	217 ± 53	21 ± 7
KO	3194 ± 666**	28 ± 6
APAP-CG/CYS		
WT	6 ± 3	N.D.
KO	11 ± 4	N.D.
APAP-NAC		
WT	N.D.	N.D.
KO	N.D.	N.D.
APAP-SUL		
WT	N.D.	N.D.
KO	N.D.	N.D.
APAP-GLU		
WT	N.D.	N.D.
KO	N.D.	N.D.

WT, wild type; KO, knockout; N.D., not detectable.
** $P < 0.01$.

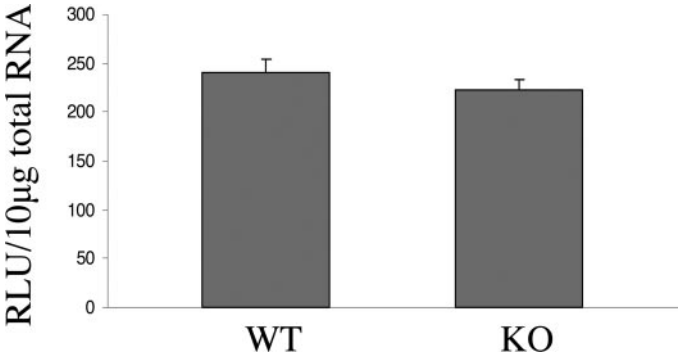

Fig. 3. bDNA assay of Mrp2 mRNA expression in the livers of wild-type (WT) and hepatocyte RXRα-deficient (KO) mice. Total liver RNA was extracted from the livers of wild-type and mutant mice and subjected to the bDNA assay for measurement of Mrp2 mRNA levels. Note that there is no significant difference in hepatic expression of Mrp2 gene between wild-type and hepatocyte RXRα-deficient mice ($P > 0.05$; n = 5).

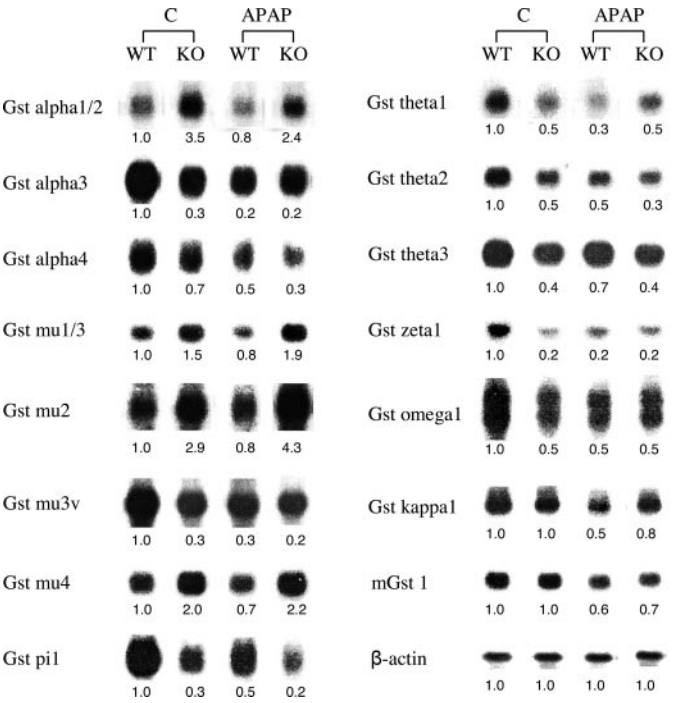

Fig. 4. Northern blot analysis of the gene expression of Gst family members. Wild-type (WT) and hepatocyte RXRα-deficient (KO) mice were sacrificed 2 h after APAP or vehicle (C) treatment. There were five mice in each group. Total liver RNA was extracted and subjected to Northern blot analysis with the indicated probes. Gst α1/2 probe detected both Gst α1 and 2 mRNAs. Gst μ1/3 probe detected both Gst μ1/3 mRNAs. The mRNA levels were determined by densitometry, normalized to β-actin mRNA levels, and expressed as fold difference. Note that gene expression of the Gst family members was altered because of RXRα deficiency or APAP treatment.

is also significantly higher in mutant than in wild-type mice. Chen et al. (2003) found that absence of a multidrug resistance protein, Mrp2, completely blocks the hepatobiliary excretion of APAP-GSH. This finding demonstrates that Mrp2 is a key transporter responsible for hepatobiliary excretion of APAP-GSH. However, hepatocyte RXRα deficiency does not change Mrp2 gene expression (Fig. 3). Therefore, we anticipate that absence of RXRα may not influence the hepatobiliary excretion of APAP-GSH. Similar concentrations of APAP-CG/CYS and APAP-NAC conjugates were observed between the two genotypes of mice, indicating that the sequential conversion of APAP-GSH to these metabolites occurs normally in hepatocyte RXRα knockout mice. Therefore, increased accumulation of APAP-GSH conjugates in the liver and bile was probably due to enhanced APAP-GSH conjugation in hepatocyte RXRα-deficient mice, which reduced NAPQI accumulation (Fig. 5). A mass balance needs to be made to confirm whether only GSH conjugation is affected.

Our data establish that RXRα is an important transcriptional regulator of the Gst family. Except for Gst α4, mGst1, and Gst κ1, all other Gst mRNAs were either up-regulated or down-regulated in the livers of hepatocyte RXRα-null mice compared with wild-type mice. Further investigation is required to elucidate whether RXRα directly or indirectly regulates these Gsts. Several transcription factors have been shown to be involved in the regulation of Gst family. Nuclear factor E2-related factor 2 (Nrf2) regulates hepatic gene expression of Gst μ1, μ2, μ3, α2, α4, θ2, and mGst 3 (Kwak et al., 2003; Fig. 5). Although the response elements have not been identified, CAR regulates expression of genes encoding Gst α1, α4, μ1, and μ2 in the liver (Maglich et al., 2002; Rosenfeld et al., 2003; Fig. 5). Gst α1, α3, μ1, θ1, and π2 are PXR target genes (Maglich et al., 2002; Rosenfeld et al., 2003; Fig. 5). RXR/peroxisome proliferator-activated receptor (PPAR) heterodimers promote Gst α2 induction by activating PPAR-responsive enhancer module in the Gst α2 gene (Park et al., 2004; Fig. 5).

Gst enzymes detoxify a broad range of endobiotic and xenobiotic chemicals by covalently linking GSH to a hydrophobic substrate, forming less reactive and more polar GSH conjugates. A systematic study of all of these enzymes, with respect to substrate specificity or preference, has not been undertaken (Hayes et al., 2005). Among the members regulated by RXRα, Gst α1/2, μ1/3, μ2, and μ4 mRNA levels were significantly elevated by a factor of 1.5- to 3-fold in hepatocytes lacking RXRα. Two hours after APAP treatment, mRNA levels of these Gsts were 2- to 5-fold higher in the liver of hepatocyte RXRα-deficient mice than that in wild-type mice. The elevation of mRNA expression of these Gsts may contribute to the enhancement of APAP-GSH conjugation in hepatocyte RXRα knockout mice. However, their efficiency in catalyzing APAP-GSH conjugation remains to be investigated. An in vitro assay showed that Gst π isoforms are particularly efficient in detoxifying NAPQI (Coles et al., 1988). Conversely, Gst π knockout mice are resistant to APAP hepatotoxicity (Henderson et al., 2000). Becausethe expression of Gst π gene is suppressed in hepatocyte RXRα-null mice, Gst π does not contribute in vivo to the formation of APAP-GSH conjugates, but it plays a novel and unexpected role

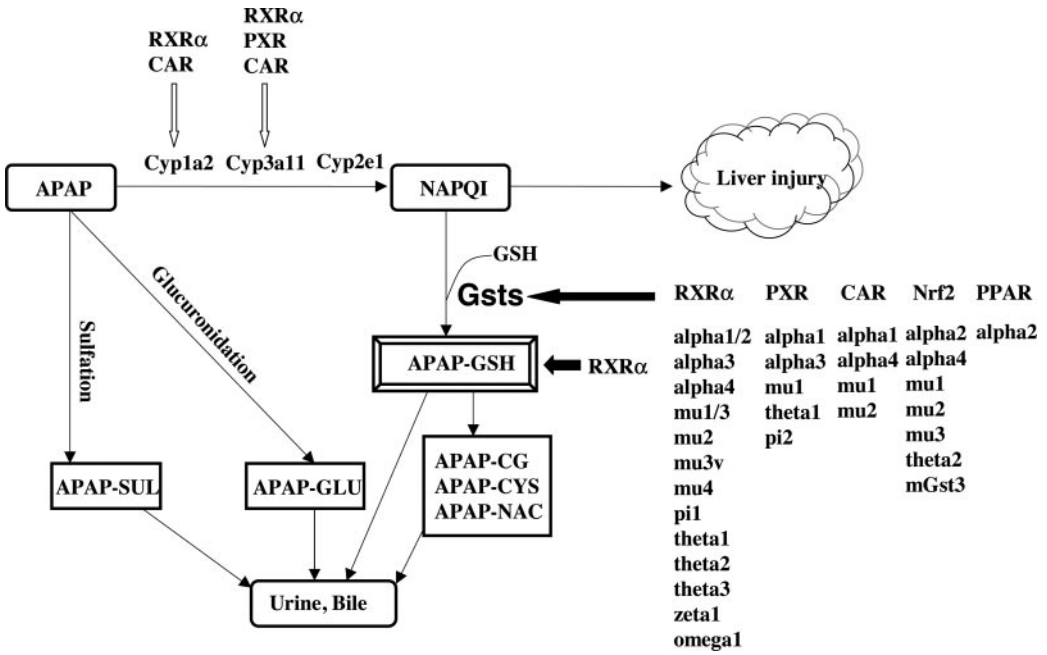

Fig. 5. Metabolic pathways of APAP and the role of RXRα, CAR, and PXR in modulating APAP-induced hepatotoxicity. At therapeutic doses, the majority of APAP goes through the glucuronidation and sulfation pathways, and then it is excreted into the bile and urine. A small portion of APAP is oxidized by P450s such as Cyp1a2, Cyp3a11, and Cyp2e1 to the toxic electrophile NAPQI, which is detoxified by conjugation with reduced GSH to form APAP-GSH, a process catalyzed by Gsts. APAP-GSH is sequentially converted to several intermediate metabolites (APAP-CG, APAP-CYS, and APAP-NAC), which are excreted into the urine and bile. At toxic doses, the sulfation and glucuronidation pathways become saturated, causing more NAPQI to be formed along with the rapid depletion of hepatic GSH. Accumulated NAPQI causes severe liver injury. At the phase I level, RXRα, PXR, and CAR modulate APAP bioactivation by regulating the gene expression of Cyp1a2 and/or Cyp3a11. At the phase II level, all three nuclear receptors regulate the expression of *Gst* genes. The target *Gst* genes of RXRα, PXR, CAR, PPAR, and nuclear factor E2-related factor 2 are listed below each regulator. However, it is unknown which Gst participates in catalyzing APAP-GSH conjugation. APAP-GSH conjugation is regulated by RXRα as demonstrated in the current study.

in APAP hepatic toxicity (Henderson et al., 2000; Elsby et al., 2003).

New therapeutic strategies are emerging as there are advances in APAP toxicology research, although the precise mechanism by which APAP or its metabolites cause cell injury is still unknown. *N*-Acetylcysteine, one of the cysteine prodrugs that stimulates hepatic GSH synthesis, is the most widely used drug for treating APAP-induced liver injury in clinical practice (Sumioka et al., 2004). Because Cyp2e1 is a very important phase I enzyme for bioactivation of APAP into NAPQI (Lee et al., 1996; Zaher et al., 1998), Cyp2e1 inhibitors could serve as an antidote for APAP overdose (Hu et al., 1996; Sumioka et al., 2001). Oxidative stress contributes to APAP hepatotoxicity (Wendel et al., 1979; Albano et al., 1983, 1985), and antioxidants have been confirmed to be effective in treating APAP-induced liver injury (Amimoto et al., 1995). Because the nuclear receptor CAR modulates APAP hepatotoxicity, CAR inhibitors also have therapeutic potential (Zhang et al., 2002). Our research defined a pathway of RXRα, Gsts, and APAP-GSH conjugation that modulates APAP detoxification. Thus, elevation of *Gst* gene expression and enzyme activity may enhance APAP detoxification, which would provides a possible new therapeutic strategy for treating APAP overdose.

In summary, our current study demonstrates that RXRα regulates gene expression of the Gst family, modulates APAP-GSH conjugation, controls NAPQI detoxification, and determines susceptibility to APAP hepatotoxicity at the phase II level. The data presented in this report reveal a new mechanism of modulation of APAP hepatotoxicity and a novel potential strategy to treat APAP or other drug-induced hepatotoxicity through nuclear receptor-mediated regulation of Gst activity.

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