Intestinal Detoxification Limits the Activation of Hepatic Pregnane X Receptor by Lithocholic Acid^S

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ABSTRACT:

The intestinal-derived secondary bile acid (BA) lithocholic acid (LCA) is hepatotoxic and is implicated in the pathogenesis of cholestatic diseases. LCA is an endogenous ligand of the xenobiotic nuclear receptor pregnane X receptor (PXR), but there is currently no consensus on the respective roles of hepatic and intestinal PXR in mediating protection against LCA in vivo. Under the conditions reported here, we show that mice lacking Pxr are resistant to LCA-mediated hepatotoxicity. This unexpected phenotype is found in association with enhanced urinary BA excretion and elevated basal expression of drug metabolism enzymes and the hepatic sulfate donor synthesis enzyme *Papss2* in *Pxr*($-/-$) mice. By subsequently comparing molecular responses to dietary and intraperi-

toneal administration of LCA, we made two other significant observations: 1) LCA feeding induces intestinal, but not hepatic, drug-metabolizing enzymes in a largely Pxr-independent manner; and 2) in contrast to LCA feeding, bypassing first-pass gut transit by intraperitoneal administration of LCA did induce hepatic detoxification machinery and in a Pxr-dependent manner. These data reconcile important discrepancies in the reported molecular responses to this BA and suggest that Pxr plays only a limited role in mediating responses to gut-derived LCA. Furthermore, the route of administration must be considered in the future planning and interpretation of experiments designed to assess hepatic responses to BAs, orally administered pharmaceuticals, and dietary toxins.

Bile acids (BAs) are crucial for the absorption of intestinal fat and fat-soluble vitamins but are also implicated in the pathology of gastrointestinal and hepatic diseases (Trauner et al., 2008). BAs are formed from cholesterol by a multienzymatic process, the rate-limiting step of which is catalyzed by the cholesterol 7 α -hydroxylase (CYP7A1) (Norlin and Wikvall, 2007). After their synthesis, BAs are actively transported from the hepatocytes, stored in the gallbladder, and released into the intestine after feeding (Portincasa et al., 2008). Primary BAs undergo bacterial modification in the intestine, resulting in the formation of hydrophobic and hepatotoxic secondary BAs, lithocholic acid (LCA), and deoxycholic acid (Ridlon et al., 2006). Approximately 95% of BAs are actively reabsorbed from the terminal ileum and transported back to the liver via the portal vein where they are detoxified and specifically targeted either for excretion or re-entry into the enterohepatic circulation.

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Gut-formed LCA is hepatotoxic and is implicated in the pathogenesis of cholestatic diseases (Pusl and Beuers, 2007; Zimmer and Gerspach, 2008; Lucangiolli et al., 2009). Unlike other BAs, LCA is thought to be actively cholestatic because it represses expression of BA transporters in the liver (Yu et al., 2002). Defense against LCA is mediated by the endobiotic and xenobiotic receptors pregnane X receptor (PXR) (Staudinger et al., 2001), constitutive androstane receptor (CAR) (Zhang et al., 2004), and vitamin D receptor (VDR) (Makishima et al., 2002). These receptors increase the expression of LCA-protective hydroxylation and sulfation enzymes. Hydroxylation of LCA by members of the cytochrome P450 (P450) family of enzymes occurs in the liver (Staudinger et al., 2001) and intestine (Makishima et al., 2002). Sulfation of LCA occurs in the liver and is mediated by sulfotransferases that require the action of the sulfate donor synthesis enzyme PAPSS2 (Sonoda et al., 2002). Sulfated LCA is exported from the hepatocyte and excreted in the urine or feces, thus reducing its concentration in the serum and the liver.

LCA activates both mouse and human PXR (Staudinger et al., 2001) and is one of its few endogenous ligands. Reported mouse Pxr target genes include LCA-protective P450 enzymes *Cyp3a11* (Staudinger et al., 2001) and *Cyp2c55* (van Waterschoot et al., 2009), the sulfotransferase *Sult2a1* (Staudinger et al., 2001), and transporters *Oatp2* (Staudinger et al., 2001) and *Mrp2* (Guo et al., 2003). However, LCA induces intestinal expression of *Cyp3a11* by Pxr-indepen-

ABBREVIATIONS: BA, bile acid; LCA, lithocholic acid; PXR, pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor; P450, cytochrome P450; ALT, alanine aminotransferase; PCR, polymerase chain reaction.

dent mechanisms involving Vdr (Makishima et al., 2002), and in the liver the role of Pxr in protection against LCA is complemented by Car, which can induce several Pxr target genes and, in addition, *Cyp2b10* (Zhang et al., 2004; Ding and Staudinger, 2005; Stedman et al., 2005; Uppal et al., 2005; Scheer et al., 2008). These findings, as well as the fact that previous studies have not specifically considered the separate roles of the intestine and the liver, have contributed to conflicting reports on the regulation of Pxr target genes by LCA. As such, there is currently no consensus on the relative toxicity of LCA in wild-type and *Pxr*($-/-$) mice, on the basal level of hepatic P450 expression in *Pxr*($-/-$) animals, or on the induction of hepatic *Cyp3a11* by LCA (Xie et al., 2000, 2001; Staudinger et al., 2001; Kitada et al., 2003; Stedman et al., 2004; Zhang et al., 2004; Uppal et al., 2005; Fickert et al., 2006; Teng and Piquette-Miller, 2007; Scheer et al., 2008; Beilke et al., 2009). Using wild-type and *Pxr*($-/-$) animals, we aimed to address these discrepancies by conducting LCA feeding experiments and intraperitoneal injections of LCA that bypass intestinal metabolism.

Materials and Methods

LCA and corn oil were purchased from Sigma-Aldrich (St. Louis, MO). Diets were produced by Special Diet Service (Witham, Essex, UK). For production of the 0.5% LCA diet, standard chow was mixed with LCA powder (Sigma-Aldrich) before pelleting. *Pxr*($-/-$) mice have been generated by two independent laboratories (Xie et al., 2000; Staudinger et al., 2001). The *Pxr*($-/-$) mice used for this study (Staudinger et al., 2001) were provided by GlaxoSmithKline (Harlow, Essex, UK), and matched wild-type C57BL/6 mice were used as controls.

Animals and Treatments. Studies were conducted in accordance with the Animals (Scientific Procedures) Act 1986. Mice were housed under standard conditions and were age- and weight-matched for experimental procedures.

Oral administration of LCA was by dietary modification. Therefore, LCA was delivered to the intestinal lumen without introducing potential vehicle effects associated with oral gavage. The 0.5% LCA diet was administered to both male and female mice. Animal body weight and food consumption were measured daily to determine LCA intake.

Injection experiments were carried out on male mice. Animal welfare considerations limited the gauge of needle and the daily volume of vehicle that could be administered. Because LCA forms a thick suspension in corn oil, mice received the maximum daily dose that could be administered: twice-daily injections of 0.125 mg/g LCA for 4 days. Control animals received an equal volume of vehicle, 100 μ L.

In all cases, food was removed for 4 h before sacrifice, which took place at 1 to 2 PM. Tissues were snap-frozen and stored at -80°C . A piece of the median lobe of the liver was fixed in 10% formalin for histological analysis.

Histology. Hematoxylin and eosin-stained 5- μ m sections were assessed by a single pathologist who was blinded to the treatment/duration and genotype of the sample. Histopathologic changes were assessed according to the following morphologic criteria.

Lesion score. The proportion of the liver affected by the individual changes described below, relative to the total area of liver section, was presented on an ordinal scale of 1 to 10.

Multifocal hepatocellular necrosis. Coagulative necrosis of hepatocytes, which was multifocal to coalescing at moderate grade or above, originating adjacent to the portal tract and extending to midzonal and centrilobular regions was associated with variable congestion/hemorrhage. Biliary proliferation is the increased numbers of variably basophilic bile ducts and biliary epithelial cells. Neutrophilic inflammation is the infiltration of inflammatory cells rich in neutrophil polymorphonuclear cells, associated with foci of hepatocellular necrosis and/or adjacent portal tracts. An assessment of mitotic increase was also made.

BA Measurements. Total BAs were determined enzymatically and according to the manufacturer's instructions (Sentinel Diagnostics, Milan, Italy). In the initial step of the reaction, the enzyme 3- α hydroxysteroid dehydrogenase converts BAs to 3-keto steroids and NADH. The NADH then reacts with

nitrotriazolium blue to form a formazan dye, which is proportional in intensity to the BA concentration in the sample.

Serum bile acids were measured directly. Fecal BAs were extracted by homogenization in a dry weight-corrected volume of ethanol, followed by 4 h of shaking at 55°C . Urinary BAs were determined after an on-column (Varian, Inc., Palo Alto, CA) extraction.

Alanine Aminotransferase Activity. Serum alanine aminotransferase (ALT) was measured by using reagents from Thermo Fisher Scientific (Waltham, MA).

Gene Expression Analysis. To avoid gene expression alterations resulting from the severe liver damage induced by 7 days of LCA feeding, gene and protein expression was investigated after 3 days of LCA feeding. Tissues were homogenized in TRIzol (Invitrogen, Carlsbad, CA), and the RNA was extracted by standard methods. cDNA was synthesized from 1 μ g of RNA using the First-Strand cDNA Synthesis System for reverse transcription-polymerase chain reaction (PCR) (Invitrogen). Gene expression was determined by real-time quantitative PCR using the Opticon2 thermal cycler (MJ Research, Watertown, MA) with SYBR Green (Sigma Chemical, Poole, Dorset, UK) detection. PCR primer sequences were as published (Kitada et al., 2003; Teng and Piquette-Miller, 2005; Zollner et al., 2006) or, for *Oatp2*, *Cyp2b10*, and *Cyp2c55*, were proprietary (QIAGEN, Crawley, West Sussex, UK). Expression levels were normalized to *Cyclophilin*.

Protein Expression. Total protein, pooled from five or six mice, was separated by polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-Cyp3a (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibody. Anti-goat (Dako Denmark A/S, Glostrup, Denmark) secondary antibody was used, and immune complexes were detected using the enhanced chemiluminescence system (Pierce Chemical, Rockford, IL). GAPDH was used to assess protein loading.

Statistical Analysis. Error bars represent S.E.M. Significance was determined by one- or two-way analysis of variance, as appropriate. In all cases, $p < 0.05$ was considered statistically significant.

Results

Enhanced Urinary BA Excretion Protects *Pxr*($-/-$) Mice against LCA-Mediated Hepatotoxicity. *Pxr*($-/-$) mice are resistant to hepatotoxicity caused by cholic acid (Teng and Piquette-Miller, 2007) and compared with wild-type mice are reported to be both equally sensitive (Staudinger et al., 2001) and more sensitive (Xie et al., 2001) to hepatotoxicity caused by LCA. As such, there are conflicting reports on the sensitivity of *Pxr*($-/-$) mice to BA-mediated hepatotoxicity. We aimed to address these discrepancies by administering LCA to wild-type and *Pxr*($-/-$) mice by dietary modification and by intraperitoneal injection, which bypasses the metabolic actions of the ileum.

Male and female wild-type and *Pxr*($-/-$) mice were fed a 0.5% LCA diet for 7 days. Food consumption per gram of mouse body weight was not significantly affected by the diet or the genotype. The estimated dietary dose of LCA was calculated as 1.15 mg/g/day. Unexpectedly, and independent of gender, the LCA diet caused significant body weight loss in wild-type but not *Pxr*($-/-$) mice (Fig. 1A). Histological liver sections were quantitatively examined (see under *Materials and Methods*) in male mice after LCA feeding. Although all the mice showed some degree of liver damage, there was a significant reduction in the incidence and severity of multifocal hepatocellular necrosis, biliary proliferation, and overall lesion score in *Pxr*($-/-$) mice compared with wild-type animals (Fig. 1, B and C). Consistent with these histological findings, serum ALT levels were already 6-fold higher in wild-type mice than in *Pxr*($-/-$) animals on the 3rd day of LCA feeding (Fig. 1D). The alterations in histological and biochemical parameters after dietary LCA administration in wild-type and *Pxr*($-/-$) mice were similar to those after intraperitoneal administration of the BA (Supplemental Fig. 1). As

FIG. 1. *Pxr*($-/-$) mice are protected against hepatotoxicity caused by LCA feeding. A, body weight in male and female mice after 7 days of 0.5% LCA feeding. B, liver histology in male mice after 7 days of feeding. Arrows show biliary proliferation and neutrophilic inflammatory cell infiltration extending from peribiliary regions into the hepatic parenchyma. Arrowheads show multifocal hepatocellular coagulative necrosis with variable neutrophilic inflammatory cell infiltrate. C, scoring of histological liver damage. D, serum ALT after 3 days of 0.5% LCA feeding. Urinary BAs (E) and serum BAs (F) after 7 days of LCA feeding. $n = 6$ per group. *, $p < 0.05$ compared with chow-fed; #, $p < 0.05$ compared with wild-type.

such, *Pxr*($-/-$) mice are resistant to hepatotoxicity caused by intraperitoneal administration of LCA and LCA feeding.

Neither fecal BA excretion nor urinary flow rate was altered in LCA-fed *Pxr*($-/-$) compared with wild-type animals. However, it is interesting to note that the concentration of BAs in the urine of LCA-fed *Pxr*($-/-$) mice was 2.5-fold higher than in LCA-fed wild-type animals (Fig. 1E). Associated with enhanced urinary BA excretion, LCA-fed *Pxr*($-/-$) mice had 7-fold lower serum BAs than LCA-fed wild-type mice (Fig. 1F). Therefore, we propose that *Pxr*($-/-$) mice are resistant to LCA-mediated hepatotoxicity as a result of enhanced urinary BA excretion.

Hepatic Transporters Are Similarly Affected by LCA Feeding and LCA Injection in Wild-Type and *Pxr*($-/-$) Mice. We hypothesized that hepatoprotection against LCA in mice lacking *Pxr* is caused by increased basal or LCA-responsive levels of BA transporters or P450 enzymes. To test this possibility, we initially monitored the mRNA expression of a number of BA-responsive transporters and

regulators of BA biosynthesis in the liver and intestine of LCA-treated wild-type and *Pxr*($-/-$) mice. Intestinal *Fgf15*, *Slc10a2*, *Fabp6*, *Osta*, and *Ostb* and hepatic *Cyp7a1*, *Slc10a1*, *Slc10a1*, *Slc10b2*, *Abcb11*, *Abcc2*, *Abcc3*, *Abcc4*, and *Abcb1a* responded to LCA feeding similarly in wild-type and *Pxr*($-/-$) mice (Table 1). We note that the magnitude of gene expression effect was, on the whole, greater in the wild-type than *Pxr*($-/-$) mice; this reaction is possibly a result of the reduced serum BAs in the knockout animals. In addition, hepatic genes were similarly affected by both LCA feeding and LCA injection (Table 1). Finally, the basal level of the hepatic transporters assessed here was not significantly altered in the *Pxr*($-/-$) mice (Supplemental Fig. 2). Therefore, these genes respond in a similar manner to LCA feeding and LCA injection, and their expression pattern is not sufficient to explain the hepatoprotection of *Pxr*($-/-$) mice against LCA.

Pxr-Mediated Induction of P450 Enzymes Fails to Protect Wild-Type Mice against LCA Toxicity. We next assessed the potential contribution of P450 enzymes in protecting *Pxr*($-/-$) and

TABLE 1

Effect of LCA feeding (0.5% for 3 days) and LCA injection (0.125 mg/g for 4 days) on hepatic and intestinal transporters and regulators of bile acid biosynthesis

Male mice; $n = 6$ for feeding experiments and $n = 5$ for injections.

		LCA Feeding Compared with Chow		LCA Injection Compared with Vehicle	
		Wild Type	<i>Pxr</i> (-/-)	Wild Type	<i>Pxr</i> (-/-)
Hepatic expression	<i>Cyp7a1</i>	0.08*	0.30*	0.20*	0.59*
	<i>Slc10a1</i>	0.76	0.84	0.26*	0.80
	<i>Slco1a1</i>	0.71	0.66	0.11*	0.67
	<i>Slco1b2</i>	0.61*	0.74	0.12*	0.64*
	<i>Abcb11</i>	0.69*	0.89	0.42*	0.77
	<i>Abcc2</i>	0.63*	0.91	0.41*	0.49*
	<i>Abcc3</i>	0.75	0.73	1.34	0.46
	<i>Abcc4</i>	0.95	0.79	0.96	1.43
	<i>Abcb1a</i>	0.80	0.80	1.20	1.08
	<i>Fgf15</i>	6.30*	6.27*		
Intestinal expression	<i>Fabp6</i>	1.32	1.98*		
	<i>Osta</i>	0.78*	0.98		
	<i>Ostb</i>	0.75	0.97		

* $p < 0.05$.

wild-type mice against LCA-mediated hepatotoxicity. There are conflicting reports on the effect of LCA on hepatic P450 gene expression in wild-type mice (Staudinger et al., 2001; Xie et al., 2001; Kitada et al., 2003; Zhang et al., 2004; Uppal et al., 2005; Fickert et al., 2006). We found that in wild-type mice, the expression of *Cyp3a11*, *Cyp2c55*, and *Cyp2b10* was enhanced in the intestine by LCA feeding (+4-, +1.5-, and +11-fold, respectively) but repressed in the liver by LCA feeding (-1.6-, -3.5-, and -7-fold, respectively) (Fig. 2, A and B). Supporting an active role for the intestine in LCA hydroxylation, we then found that intraperitoneal administration of LCA bypassed intestinal drug-metabolizing enzymes and induced hepatic expression of *Cyp3a11* (+1.5-fold), *Cyp2c55* (+1.7-fold; $p = 0.070$), and *Cyp2b10* (+47-fold) (Fig. 2C). In support of these gene expression data, total hepatic Cyp3a protein was also induced by injecting LCA but not by feeding LCA to wild-type mice (Fig. 3). Therefore, LCA feeding induces intestinal but not hepatic P450 enzymes, whereas injecting LCA bypasses the metabolic actions of the gut and activates hepatic detoxification machinery. These data provide evidence of a reciprocal relationship between intestinal and hepatic drug-metabolizing enzymes that may explain discrepancies in the reported hepatic P450 gene responses to LCA.

FIG. 2. The enterohepatic response of P450 enzymes to LCA. A, intestinal gene expression after 3 days of LCA feeding. B, hepatic gene expression after 3 days of LCA feeding. C, hepatic gene expression after intraperitoneal injection of LCA. $n = 5$ or more per group. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with wild-type.

FIG. 3. Hepatic Cyp3a protein expression after 3 days of LCA feeding and LCA injection in wild-type and *Pxr*(-/-) mice. $n = 5$ or more per group.

Several studies have reported enhanced basal expression of hepatic P450 enzymes in mice lacking *Pxr* (Staudinger et al., 2001; van Waterschoot et al., 2009), but this effect is not a consistent finding (Xie et al., 2000). We found that the basal expression of *Cyp3a11* and *Cyp2c55* was elevated in the liver (+1.6- and +3.7-fold, respectively) but reduced in the intestine (-3.6- and -3.3-fold, respectively) of *Pxr*(-/-) mice (Fig. 2, A and B). Adaptive activation of Car could drive the hepatic up-regulation of these genes in *Pxr*(-/-) mice (Zhang et al., 2004). This does not seem to be the case here because hepatic expression of the principal Car target gene, *Cyp2b10*, was similar in wild-type and *Pxr*(-/-) mice (Fig. 2B). By contrast, the intestinal expression of *Cyp2b10* was 4-fold higher in mice lacking *Pxr* than in controls (Fig. 2A).

We next assessed the role of *Pxr* in mediating P450 gene responses to LCA feeding. In *Pxr*(-/-) mice, dietary LCA administration induced the intestinal expression of *Cyp3a11* (+5-fold) and *Cyp2c55* (+6.7-fold) but not *Cyp2b10* (Fig. 2A). Furthermore, LCA feeding reduced the hepatic expression of *Cyp3a11*, *Cyp2c55*, and *Cyp2b10* (-1.4-, -7-, and -4.2-fold, respectively) in *Pxr*(-/-) animals (Fig. 2, A and B). With the exception of intestinal *Cyp2b10*, these findings are consistent with the effects in wild-type animals (Fig. 2, A and B). We conclude that *Pxr* is largely not required to maintain P450 responses to LCA feeding in the intestine or the liver.

In contrast to LCA feeding, injecting LCA induced hepatic P450 enzymes in wild-type mice (Figs. 2C and 3). We found that LCA

injections did not induce the expression of *Cyp3a11*, *Cyp2c55*, *Cyp2b10*, or total *Cyp3a* in the liver of *Pxr*($-/-$) mice (Figs. 2C and 3). Therefore, Pxr is required for the normal hepatic induction of P450 enzymes in response to intraperitoneal injection of LCA.

More importantly, we found that LCA-protective P450 enzymes in the liver were not expressed at a consistently higher level in LCA-treated *Pxr*($-/-$) mice compared with LCA-treated wild-type animals (Figs. 2 and 3). As such, the expression pattern of the drug-metabolizing enzymes tested here is unlikely to be sufficient to explain the phenotype of protection of *Pxr*($-/-$) mice against LCA feeding and LCA injection.

Taken together, these data suggest that a mechanism independent of P450 enzymes is responsible for protecting *Pxr*($-/-$) mice against LCA-mediated hepatotoxicity. Furthermore, LCA feeding and LCA injection differentially affect phase I gene and protein expression in the liver such that Pxr is activated by intraperitoneal but not by dietary LCA administration.

Elevated Hepatic Expression of *Papss2* Contributes to Protecting *Pxr*($-/-$) Mice against LCA-Mediated Hepatotoxicity. We have shown that enhanced urinary BA excretion is likely to be the primary mechanism protecting *Pxr*($-/-$) mice against LCA-mediated hepatotoxicity (Fig. 1). Furthermore, by comparing molecular responses to LCA feeding and LCA injection, we have found that the response of drug-metabolizing P450 enzymes to LCA is insufficient to explain the phenotype of hepatoprotection of the *Pxr*($-/-$) mice against this BA (Figs. 2 and 3). Because hepatic sulfation targets BAs for excretion in urine, we assessed the expression of the sulfotransferase *Sult2a1* and the universal sulfate donor synthesis enzyme *Papss2* in the livers of wild-type and *Pxr*($-/-$) mice in response to LCA feeding and LCA injection.

Consistent with the effects on hepatic P450 enzymes, hepatic *Sult2a1* was repressed by LCA feeding (-2.0 -fold) and induced by LCA injection in wild-type mice ($+9.4$ -fold) (Fig. 4). Furthermore, consistent with *Sult2a1* being a Pxr target gene, we found that hepatic *Sult2a1* was not induced by LCA injections in *Pxr*($-/-$) mice (Fig. 4). As a result, *Sult2a1* was significantly higher ($+7.2$ -fold) in LCA-injected wild-type mice than in LCA-injected *Pxr*($-/-$) mice (Fig. 4) and therefore does not explain the resistance of mice lacking Pxr to LCA toxicity.

Finally, we monitored the expression of *Papss2*, the enzyme that synthesizes the universal sulfate group that is transferred to BAs by the sulfotransferase enzymes. We found that both LCA feeding and LCA injection repressed the expression of *Papss2* in wild-type (-1.9 - and -1.7 -fold, respectively) and in *Pxr*($-/-$) mice (-1.3 - and -1.7 -fold, respectively) (Fig. 4). However, it is interesting to note that the

basal and LCA-responsive level of *Papss2* expression was more than 2-fold ($p < 0.05$) higher in *Pxr*($-/-$) mice than in wild-type controls (Fig. 4). Therefore, enhanced hepatic expression of *Papss2* could contribute to the increased urinary BA excretion and protection of *Pxr*($-/-$) mice against LCA-mediated hepatotoxicity.

Discussion

We found that *Pxr*($-/-$) mice were resistant to hepatotoxicity caused by the endogenous PXR ligand LCA. We investigated the mechanism of protection and found enhanced urinary BA excretion in association with elevated hepatic expression of the sulfate donor synthesis enzyme *Papss2* in *Pxr*($-/-$) mice. It is noteworthy that by comparing intestinal and hepatic responses to LCA and by performing feeding and injection experiments, we have also reconciled a number of important discrepancies in the reported molecular responses to LCA that are likely to be relevant to other systems.

The data presented here are not consistent with reports that *Pxr*($-/-$) mice are more sensitive to LCA toxicity than wild-type mice (Xie et al., 2001). However, some reports show approximately equal hepatotoxicity in LCA-treated wild-type and *Pxr*($-/-$) mice (Staudinger et al., 2001; Xie et al., 2001), and one article shows that the knockout animals are protected against cholic acid-mediated toxicity (Teng and Piquette-Miller, 2007). Therefore, there are conflicting reports on the relative toxicity of BAs in wild-type and *Pxr*($-/-$) animals. These are likely to be the combined result of a number of factors including the age, strain, and sex of mouse, the housing conditions, and the duration and dose of LCA administered. For example, we administered LCA by intraperitoneal injection at the same dose used previously (Staudinger et al., 2001), and although we replicated the molecular responses, the phenotype of the *Pxr*($-/-$) animals was somewhat different. The predicted dose of LCA after the dietary modification used here (1.15 mg/g, i.e., approximately 23 mg/day/mouse) is significantly higher than that administered by Xie et al. (2001), who used oral gavage at 8 mg/day/mouse.

Mice deficient of Pxr have been generated in two laboratories, and it is noteworthy that, unlike the animals used in this study (Staudinger et al., 2001; Xie et al., 2001), the knockout mice generated by Xie and Evans (2002) do not express elevated basal levels of hepatic drug-metabolizing enzymes. This effect suggests that the altered basal gene expression in these mice may influence their phenotype. There is now indirect evidence that altered standard diet composition can influence basal gene expression in drug-metabolizing enzyme knockout mice (van Waterschoot et al., 2009). Therefore, discrepancies in the reported basal gene expression in *Pxr*($-/-$) mice (Xie et al., 2000; Staudinger et al., 2001; Teng and Piquette-Miller, 2007) are possibly

FIG. 4. mRNA expression of phase II sulfation enzymes after 3 days of LCA feeding (A) and injection (B) in wild-type and *Pxr*($-/-$) mice. $n \geq 5$ per group. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with wild-type.

mediated by interlaboratory variation in standard diet composition (van Waterschoot et al., 2009) and may also explain the differences in the reported phenotypic response of these knockout mice to BA challenge (Staudinger et al., 2001; Xie et al., 2001; Teng and Piquette-Miller, 2007). The mechanisms driving enhanced basal hepatic expression of *Cyp3a11* and *Cyp2c55* in *Pxr*($-/-$) mice are not known. However, because *Pxr*($-/-$) mice do not express elevated levels of the hepatic Car target gene *Cyp2b10*, adaptive basal activation of Car may not be responsible for the basal expression of *Cyp3a11* and *Cyp2c55* in mice lacking *Pxr*. Whereas basal induction of P450 enzymes may form a component of the mechanism protecting *Pxr*($-/-$) mice against LCA toxicity, the level of their expression in response to the LCA challenge suggests that they are not sufficient to explain the hepatoprotective phenotype.

Elevated urinary BA excretion in *Pxr*($-/-$) mice has been reported before, but not in conjunction with resistance of the animals against LCA-mediated toxicity (Staudinger et al., 2001), possibly because of masking of the phenotype by other factors, such as housing conditions or the time course of the study. Increased urinary BA excretion indicates enhanced hepatic sulfation. However, expression of *Sult2a1*, which is thought to be a key sulfotransferase, is higher in LCA-injected wild-type than *Pxr*($-/-$) animals and therefore is unlikely to contribute to the enhanced BA excretion in LCA-treated *Pxr*($-/-$) mice. *Papss2* is the universal sulfate donor synthesis enzyme that is required for BAs' sulfation and subsequent excretion in urine (Xu et al., 2003). We found that *Pxr*($-/-$) mice express consistently higher levels of *Papss2* than wild-type mice after both LCA feeding and injection. It is not known whether elevated cellular *Papss2* is sufficient to increase LCA sulfation and excretion in the absence of up-regulated expression of the sulfotransferase enzymes. However, it is plausible that increased expression of *Papss2* contributes to the enhanced urinary excretion of BAs in LCA-treated *Pxr*($-/-$) mice.

LCA is formed in the intestine; therefore, intraperitoneal injection, which bypasses the metabolic action of the gut, is not a physiologically relevant route of administration. By comparing LCA feeding and LCA injection experiments, we have provided new and important information on the enterohepatic response to this BA, as well as an explanation for why hepatic *Cyp3a11* is reported to be unaffected (Kitada et al., 2003; Beilke et al., 2009), induced (Staudinger et al., 2001; Xie et al., 2001; Stedman et al., 2004; Zhang et al., 2004; Fickert et al., 2006), and even repressed (Uppal et al., 2005) by different LCA treatments. Our LCA feeding experiments confirmed an active role for the intestine in LCA hydroxylation (Makishima et al., 2002) and provided additional evidence, with regard to LCA metabolism, that detoxifying systems in the intestine can influence those in the liver (van Waterschoot et al., 2009). LCA feeding did not increase the hepatic expression of drug-metabolizing enzymes, and the induction of intestinal *Cyp3a11* and *Cyp2c55* occurred through mechanisms independent of *Pxr*. Therefore, the intestinal induction of *Cyp3a11* and *Cyp2c55* in response to LCA feeding was likely mediated by Vdr and/or Car (Makishima et al., 2002; Zhang et al., 2004). Indeed, the only evidence we found for a *Pxr*-mediated response to LCA feeding in the intestine was the unexpected absence of intestinal up-regulation of the principal Car target gene, *Cyp2b10*, in *Pxr*($-/-$) mice.

The intestine plays a functional role in LCA metabolism because intraperitoneal injection of LCA bypasses gut metabolism and caused *Pxr*-dependent induction of hepatic detoxification machinery that did not occur in response to LCA feeding. This finding suggests that metabolism in the intestine may alter the structure of LCA such that it is no longer a ligand for hepatic *Pxr*. Therefore, *Pxr* is required to mediate induction of liver metabolism after intraperitoneal LCA ad-

ministration but not after LCA feeding. This is an important finding for the understanding of the role of the intestine, as well as hepatic *Pxr*, in protection against LCA hepatotoxicity.

LCA is implicated in the pathogenesis of cholestatic diseases (Lucangioli et al., 2009), and this work highlights the lack of knowledge of the molecular pathology of this actively cholestatic compound. Elevated basal expression of drug-metabolizing enzymes limits the value of *Pxr*($-/-$) mice as a model for studying the involvement of this receptor in response to xenobiotic stress. However, the hepatoprotective phenotype of the *Pxr*($-/-$) mouse provides useful insights into mechanisms of protection against cholestatic compounds, and this information could be used to target novel therapeutic agents, possibly to *PAPSS2*. We have provided evidence that activation of hepatic *Pxr* is not a primary mechanism of protection against hepatotoxicity caused by dietary administration of LCA. Our finding that intestinal hydroxylation influences liver metabolism should be considered in the future planning and interpretation of experiments designed to assess the molecular toxicity of BAs, dietary toxins, and orally administered pharmaceuticals.

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