Retinoids Modulate Thioacetamide-Induced Acute Hepatotoxicity

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The literature indicates that retinoids can influence the metabolism and actions of xenobiotics and conversely that xenobiotics can influence the metabolism and actions of retinoids. We were interested in understanding the degree to which hepatic retinoid stores, accumulated over a lifetime, affect xenobiotic metabolism, and actions. To investigate this, we induced liver injury through administration of the hepatotoxin thioacetamide (TAA) to Chow fed wild type (WT) mice and lecithin:retinol acyltransferase-deficient (Lrat−/−) mice that are genetically unable to accumulate hepatic retinoid stores. Within 48 h of TAA treatment, WT mice develop liver injury as evidenced by focal necrotic areas and increases in serum ALT activity and myeloperoxidase activity in hepatic parenchyma. Simultaneously, features of hepatic encephalopathy develop, as evidenced by a 25% increase in blood ammonia and a threefold reduction of blood glucose levels. This is accompanied by reduced hepatic glutathione, and in-}

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considerable evidence in the literature for significant retinoid-xenobiotic interactions within the body.

Recently, we reported that $\text{Lrat}^{-/}$ mice, a strain of mice lacking the enzyme responsible for retinyl ester formation and hence retinoid stores in HSCs (O’Byrne et al., 2005), are less susceptible to diethylnitrosamine (DEN) induced hepatic tumorigenesis than matched wild type (WT) mice (Shirakami et al., 2012). This observation was surprising to us because both dietary retinoid intake in humans and retinoid acid administration to cancer cells in culture or experimentally induced cancers in animals have often been found to convey chemoprotection against cancer development (Tang and Gudas, 2011). To assess whether this hepatic response in the total absence of retinoid stores is specific to DEN or whether it can be more generalized to other hepatic toxins, we undertook studies of thioacetamide (TAA)-induced hepatic toxicity in age-, diet-, gender-, and genetic background-matched $\text{Lrat}^{-/}$ and WT mice. We chose to study specifically TAA because of its efficacy in inducing hepatic failure in rodents (Butterworth et al., 2009; Rahman and Hodgson, 2000), its high specificity for the liver, its regiospecificity for the perivenous area, and the short window of time between its necrogenic effects and liver failure (Chilakapati et al., 2007; Mehendale, 2005).

**MATERIALS AND METHODS**

Animal husbandry and dietary regimens. All mice employed in our studies (males weighing 20–25 g, 10–12 weeks of age) were treated and maintained according to the NIH Guide for the Care and Use of Laboratory Animals (2011). The $\text{Lrat}^{-/}$ mice were derived from ones originally described on a mixed genetic background. During the breeding and lactation periods, all mice were maintained on breeder chow congenic in this genetic background. During the breeding and lactation periods, all mice were maintained on breeder chow that contained 15 IU retinol g diet. After weaning, mice were maintained on a standard chow diet that also contained 15 IU retinol g diet.

Acute hepatic failure induction. Acute hepatic injury was induced by a single intraperitoneal (ip) injection of a dose of 500 mg/kg TAA (Sigma-Aldrich Co.) dissolved in saline (0.9% wt/vol NaCl). Control-treated mice received the same volume of saline via ip injection. Routinely, six mice per group were studied. For retinoid-supplementation studies, separate groups of mice (six for each genotype) received 3000 IU of retinyl acetate in vegetable oil by gavage at 12 h intervals after either TAA or saline administration. At the time of sacrifice, 48 h after TAA injection, mice were weighed, blood was taken from the inferior vena cava, and the liver was immediately removed. The dissected livers were rapidly weighed and either used immediately for microsomal fraction isolation or frozen in liquid N$_2$ and stored at −80°C until analysis. Sections from the dissected livers were also fixed in 10% neutral buffered formalin for histological analysis.

**Histology.** For paraffin sections, livers were first fixed in neutral buffered formalin and then processed into paraffin blocks according to standard protocols (Fischer et al., 2008). The embedded tissues were cut into 6 μm slices, mounted on charged adhesive slides, and dried overnight at 50°C. Slides were deparaffinized in xylene and rehydrated in graded alcohol and distilled water. Representative sections were stained with hematoxylin and eosin (H&E) according to standard protocols (Fischer et al., 2008).

Liver function tests. Alanine aminotransferase (ALT) enzymatic activity was determined in mouse serum using a kit from Felicit Diagnostics (Felicit Diagnostics, Ukraine), according to the manufacturer’s instructions.

Measurement of myeloperoxidase activity in liver tissue was performed exactly as described earlier by others (Schierwagen et al., 1990). Briefly, 50 mg of liver tissue was homogenized on ice in 500 μl of 50mM potassium phosphate (KPO$_4$) buffer, pH 7.4, using a Potter homogenizer, followed by centrifugation at 15,000 x g for 15 min at 4°C. The pellet was washed in the same buffer and centrifuged for 5 min at 15,000 x g, followed by resuspension in 10 volumes of 50mM KPO$_4$ buffer, pH 6.0, containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide (Fluka Chemie Buchs, Switzerland) and incubation at 60°C for 2 h. The resuspended pellet was then sonicated for 10 s. After sonication, samples were subjected to three freeze-thaw cycles and again sonicated for 10 s, followed by centrifugation at 15,000 x g for 15 min. An aliquot of supernatant (100 μl) was mixed with 100 μl of o-dianisidine dihydrochloride (10 mg/ml in KPO$_4$ buffer; Sigma-Aldrich Co.), 0.3% hydrogen peroxide (H$_2$O$_2$) was added and the absorbance was measured at 405 nm for 1 min to determine myeloperoxidase specific activity.

Blood ammonia levels were measured using a standard method (Huizenga et al., 1994). Blood glucose levels were measured employing the glucose oxidase method using a kit from Felicí Diagnostics (Felicit Diagnostics), according to the manufacturer’s instructions.

Oxidative damage measurement. The degree of oxidative modification of hepatic proteins was determined through assessments of the levels of protein carbonylation (Levine et al., 1990) and of protein thiol groups (Murphy and Kehrer, 1989). Lipid peroxidation in liver was determined by assessing the level of thiobarbituric acid-reactive substances (TBRAS) (Ohkawa et al., 1979). Hepatic reduced glutathione level was determined as described by Ellman (1959).

Preparation of liver microsomes. Liver microsomes were prepared according to the method of Schenman and Cinti.
(1978), with modifications. Briefly, livers were removed and homogenized using a Potter homogenizer in 10 volumes of ice-cold 0.25M sucrose. The liver homogenate was filtered through a 100 μm nylon mesh to remove debris and then centrifuged at 12,000 × g for 15 min at 4°C. Tris-HCl buffer (10mM, pH 7.4) containing 80mM CaCl2 and 160mM MgCl2 was added to the resulting supernatant (9 volumes of supernatant + 1 volume of buffer). The samples were gently mixed for 10 min at 4°C followed by centrifugation for 15 min at 9000 × g. The resulting pellet consisting of liver microsomes was washed twice with ice-cold 0.25M sucrose. The purity of each microsomal fraction was assessed for marker enzymes for other subcellular organelles, as described in Archakov et al. (1973). Specifically, we assessed succinate dehydrogenase (a marker enzyme for the membrane of endoplasmic reticulum, and Na+/K+-ATPase (a marker enzyme for the plasma membrane) activities. For all of our studies, we employed only microsome preparations for which contaminating marker enzyme assays did not exceed 10% of the activity measured in the crude liver homogenate used for microsome isolation. Aliquots of liver microsomes were stored at –80°C until use.

The content of microsomal protein was determined according to the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

**Microsomal monooxygenase activities.** The aniline p-hydroxylase activity of hepatic cytochrome P450s was determined by the method of Archakov et al. (1974). The reaction mixture consisted of 40mM Tris-HCl buffer, pH 7.3, containing 16mM MgCl2, 3mM NADPH, and 2 mg of microsomal protein. The reaction was initiated by adding aniline to a final concentration 3mM. In control samples, NADPH was added after the termination of the reaction. Test and control samples were incubated at 37°C for 20 min with constant shaking. The reaction was terminated through addition of 15% trichloroacetic acid, followed by centrifugation at 3500 × g for 10 min. Following centrifugation, 10% (wt/vol) Na2CO3 and 2% (wt/vol) phenol in 0.2M NaOH were added to the supernatant. The samples were incubated in a water bath at 37°C for 30 min. To assess enzymatic activity, the absorbance was determined spectrophotometrically at 630 nm using a molar extinction coefficient for p-aminophenol of 13.3/mM/cm. Aniline p-hydroxylase activity was expressed as nmol/min/mg microsomal protein.

To assess the N-demethylase activity of hepatic cytochrome P450s, a reaction mixture consisting of 40mM Tris-HCl buffer, pH 7.6, containing 3mM NADPH, 16mM MgCl2, and 1.5 mg microsomal protein was employed. The reaction was initiated by addition of N,N-dimethylaniline to a final concentration 6mM. Test and control (no NADPH added) samples were incubated at 37°C for 30 min with vigorous shaking. The reaction was terminated by addition of an equal volume of 25% (wt/vol) Zn2SO4 in a saturated BaOH, followed by centrifugation at 3500 × g for 10 min. The formaldehyde content in the supernatant was determined employing the Nash color reaction (Nash, 1953). Color intensity was determined spectrophotometrically at 412 nm. The activity was calculated using a formaldehyde molar extinction coefficient of 1.5/mM/cm and expressed as nmol/min/mg microsomal protein.

Flavin-containing monooxygenase (FMO) activity was determined by the method of Ziegler & Pettit (Pettit et al., 1964) with some modifications. The reaction mixture consisted of 40mM Tris-HCl, pH 7.6, containing 3mM NADPH, 16mM MgCl2, and 1.5 mg microsomal protein. The reaction was initiated by addition of N,N-dimethylaniline to a final concentration 6mM. The test and control (no NADPH added) samples were incubated at 37°C for 30 min with vigorous shaking. The reaction was terminated by addition of 0.9M HClO4. Precipitated protein was pelleted by centrifugation at 3500 × g for 10 min. The clarified supernatants were then transferred to graduated test tubes and pHs were adjusted to 9.4 through addition of 1M NaOH. To eliminate unoxidized dimethylaniline, the samples were extracted three times with diethyl ether, each after vigorous shaking for 2 min. After the third extraction, the samples were left open for 20 min to allow for evaporation of the diethyl ether. After the ether had evaporated, the pH was adjusted to 2.4 through addition of several microliters of 5% trichloroacetic acid. Subsequently, 0.2 ml of 0.1M NaNO2 was added, followed by adjustment of the final volume to 3 ml through addition of citrate buffer (pH 2.4). For color development, the tubes were placed in a water bath at 60°C for 5 min. The absorbance was measured at 420 nm. To calculate enzyme specific activity expressed as nmol/min/mg microsomal protein, a molar extinction coefficient for p-nitrosodimethylaniline of 8.2/mM/cm was employed.

**Statistical analysis.** All data are presented as means ± SD. Student’s t-test was used to analyze differences between the control and knock-out strains. Statistical comparisons involving larger groups were first analyzed by a one-way ANOVA followed by multiple comparisons employing Tukey’s HSD post hoc test. p-values <0.05 were considered statistically significant.

**RESULTS**

To understand whether hepatic retinoid stores influence the metabolism and toxicity of TAA, WT, and Lrat−/− mice were treated with a single ip dose of TAA, a hepatotoxin known to cause liver failure (Butterworth et al., 2009; Rahman and Hodgson, 2000). We first assessed the effects of different doses of TAA on the survival of matched WT and Lrat−/− mice, which lack any hepatic retinoid stores (O’Byrne et al., 2005). To this end, we initially employed a range of TAA concentrations (200–1000 mg/kg body weight) that is standardly used in the literature to investigate acute TAA toxicity. As can be seen from Figure 1, a dose of 450 mg TAA/kg body weight resulted in mor-
tality in WT mice, with progressively more mortality observed with increasingly larger doses. Mortality due to TAA administration was first observed for Lrat\(^{-/-}\) mice at a larger dose of 600 mg/kg body weight. The data provided in Figure 1 establish that the Lrat\(^{-/-}\) mice are less susceptible to acute TAA toxicity than gender-, diet-, and genetic background-matched WT mice.

Because 500 mg TAA/kg body weight was the highest TAA dose that did not induce mortality in Lrat\(^{-/-}\) mice, which resulted in a >35% mortality rate in WT mice, we employed this dose in our subsequent studies. For these studies, WT and Lrat\(^{-/-}\) mice were treated with a dose of TAA, administered as a single ip injection of 500 mg TAA/kg body weight. This treatment led to the development of extensive liver injury within 48 h in WT mice, as evidenced by the appearance of focal necrotic areas (Supplementary fig. 1) and increases, by 2 orders of magnitude, in serum ALT activity and myeloperoxidase activity in hepatic parenchyma compared with control values (Figs. 2A and 2B). This pronounced hepatic toxicity was not seen for Lrat\(^{-/-}\) mice. These mutant mice exhibited normal levels of serum ALT activity and no histological evidence for hepatic parenchyma damage (Fig. 2A, Supplementary fig. 1). The inflammation-associated recruitment of neutrophils was also attenuated in the livers of Lrat\(^{-/-}\) mice (Fig. 2B).

The hepatic parenchymal injury observed in WT mice was accompanied by a loss of liver function. Forty-eight hours after TAA administration to WT mice, features of hepatic encephalopathy were present, as evidenced by a 25% increase in blood ammonia levels and a threefold reduction of blood glucose levels (Figs. 2C and 2D). However, these changes were not observed in Lrat\(^{-/-}\) animals whose blood ammonia levels were not statistically different from the values of the untreated group. Although a 35% reduction in blood glucose level was observed in Lrat\(^{-/-}\) mice after TAA injection, these values were still significantly larger by 30% than for WT mice subjected to TAA administration.

Because TAA hepatotoxicity requires TAA bioactivation to highly reactive sulfoxide (TASO) and dioxide (TASO\(_2\)) prooxidant species (Chilakapati et al., 2005, 2007; Hajovsky et al., 2012), we measured lipid and protein oxidative damage products in the livers of treated matched WT and Lrat\(^{-/-}\) mice. TAA treatment of WT mice resulted in a significantly lower level of reduced hepatic glutathione, and protein sulfhydryl groups, and significantly elevated levels of protein carbonyl derivatives and thiobarbituric acid reactive substances (TBARS). For the TAA-treated Lrat\(^{-/-}\) mice (Fig. 3), these parameters were not different than those of sham treated controls (either WT or Lrat\(^{-/-}\) mice injected with the saline vehicle).

We hypothesized that the absence of lipid and protein oxidative damage products in livers of TAA-treated Lrat\(^{-/-}\) mice might be as a result of defective drug metabolism and activation of the TAA, owing to the lack of hepatic retinoid stores in Lrat\(^{-/-}\) mice. We assessed hepatic drug detoxification activities for both WT and Lrat\(^{-/-}\) mice. After TAA administration, we observed both lower CYP hydroxylation activity and lower FMO oxygenase activity in microsomes prepared from Lrat\(^{-/-}\) livers compared with activities determined for WT microsomes (Fig. 4).

To confirm that TAA hepatotoxicity depends on hepatic retinoid availability, we administered orally 3000 IU retinyl acetate at each of four 12 h intervals to the mice after TAA administration. This is a relatively large dose of retinoid given that our mice consuming a chow diet would only be consuming 45–50 IU/day. When retinyl acetate was administered alone not in conjunction with TAA, we did not observe any adverse effect, including hepatic injury or oxidative damage, in Lrat\(^{-/-}\) mice for the short period (48 h) of supplementation employed in our studies. Nor did we observe an effect in WT mice after supplementation (data not shown). However, following TAA injection, retinyl acetate administration led to the development of acute hepatotoxicity in Lrat\(^{-/-}\) mice, including the appearance of necrotic areas in parenchyma (Supplementary fig. 1), an increase in serum ALT and hepatic myeloperoxidase activities, accompanied by the development of hepatic encephalopathy (Fig. 2). Upon retinyl acetate supplementation, CYP hydroxylation and FMO oxygenase activities were increased in microsomes of Lrat\(^{-/-}\) livers (Fig. 4) and protein and lipid oxidation markers were detected as well (Fig. 3), at levels which were identical to those observed for TAA-treated WT mice. Furthermore, many parameters of acute hepatotoxicity were further aggravated in TAA-injected WT mice following administration of retinyl acetate (Figs. 2–4).

**DISCUSSION**

Our data convincingly establish that the complete absence of hepatic retinoid stores in Lrat\(^{-/-}\) mice results in diminished TAA-induced hepatic toxicity compared with matched WT mice. This protection was lost upon oral administration
FIG. 2. Biochemical features of acute hepatotoxicity and hepatic encephalopathy in wild type and Lrat\textsuperscript{−/−} mice administered a single intraperitoneal injection of 500 mg/kg thioacetamide. Serum ALT activity (panel A), hepatic parenchymal myeloperoxidase activity (panel B), blood ammonia concentrations (panel C), and blood glucose concentrations (panel D) were determined 48 h after injection of saline or TAA, with or without oral retinyl acetate administration. Values marked with different letters (a, b, and c) are statistically different, \( p < 0.05 \). All values are given as the mean \( \pm 1\text{SD} \), \( n = 6 \) for each group.

of retinyl acetate to Lrat\textsuperscript{−/−} mice. The Lrat\textsuperscript{−/−} mice, when dosed with retinyl acetate, displayed a similar degree of hepatic toxicity to that observed for TAA-treated WT mice. This provides strong support for the unexpected conclusion that hepatic retinoid stores accumulated over a lifetime from the diet help to facilitate liver toxicity associated with TAA exposure. This conclusion is in agreement with our published finding that mice lacking hepatic retinoid stores are less susceptible to DEN-induced hepatocellular carcinoma (Shirakami et al., 2012). The present finding raises the possibility that hepatic retinoid stores may more broadly contribute to chemically induced liver injury.

What are the molecular processes that underlie the association between hepatic retinoids and chemically induced hepatotoxicity? Our data establish that TAA treatment of WT mice results in a significantly decreased level of reduced hepatic glutathione and increased levels of oxidized proteins and TBARS compared with Lrat\textsuperscript{−/−} mice. This was accompanied by lower levels of CYP hydroxylation activity and FMO oxygenase activity in livers of Lrat\textsuperscript{−/−} mice. When mice are treated with retinyl acetate, these differences are completely abolished. Thus, the presence of hepatic retinoids must be contributing to increased levels of oxidative stress within the liver as well as increases in the activity levels of the CYP and FMO systems. Because TAA is bioactivated by the hepatic CYP and/or FMO systems and the products formed upon bioactivation account for the hepatic injury associated with TAA (Kang et al., 2008; Kim et al., 2000; Wang et al., 2000), our findings are in agreement with the literature. Moreover, this literature suggests that CYP2E1 is centrally
involved in mediating TAA induced oxidative stress and liver injury (Kang et al., 2008; Wang et al., 2000). Although we did not specifically assess CYP2E1 mRNA or protein levels in this study, we earlier showed that both CYP2E1 mRNA and protein levels were lower 4, 24, and 48 h after administration of DEN to Lrat−/− mice compared with treated WT mice (Shirakami et al., 2012). Thus, we hypothesize that hepatic retinoids may be needed to maintain high levels of CYP2E1 and other CYPs and FMOs involved in xenobiotic metabolism in the liver. Although retinoic acid is known to regulate the rate of transcription of a number of CYP enzymes (Kedishvili, 2013), as far as we are aware, there is no information regarding whether retinoic acid contributes to the transcriptional regulation of CYP2E1.

The literature indicates that repeated administration of relatively high doses of retinol (≈5000 IU) to mice for 4 days prior to administration of acetaminophen potentiates the acute hepatotoxicity of this compound (Bray et al., 2001; Bray and Rosengren 2001; Pumford et al., 1990). Because the mice employed in these published studies were WT mice that were also maintained on a chow diet throughout life, our finding that oral retinyl acetate administration to WT mice enhances a number of features of the hepatoxicity associated with TAA administration is consistent with the earlier work. Hence, we should not view the findings from our work as being too surprising. (We remind the reader that oral retinyl acetate will be hydrolyzed in the gut and absorbed as retinol.) Strikingly though, our studies
FIG. 4. Hepatic monooxygenase system activities in wild type and Lrat<sup>−/−</sup> mice administered a single intraperitoneal injection of 500 mg/kg thioacetamide. 3-Hydroxylase (panel A), N-demethylase (panel B), and N-oxygenase (panel C) activities were determined for microsomes isolated from livers of mice at 48 h after injection of saline or TAA, either with or without oral retinyl acetate administration. Values marked with different letters (a, b, and c) are statistically different, <i>p</i> < 0.05. All values are given as the mean ± 1SD, <i>n</i> = 6 for each group.

establish that the absence of hepatic retinoid stores arising from the ablation of the Lrat gene completely abolishes the hepatic toxicity associated with TAA administration. Within the liver, the great majority of hepatic retinoid is found within the lipid droplets of HSCs (Blaner et al., 2009). This suggests a dynamic role for HSC retinoids in the TAA-induced disease process that also involves the other cell types present in the liver.

What is the practical significance of our findings for understanding TAA-induced injury? Our data indicate that the severity of the effects of chemicals used to induce hepatic injury, ones like DEN and TAA, may be different depending on the retinoid status of the liver. Greater toxicity would likely be observed when hepatic retinoid stores are large, or dietary retinoid intake is high. Hepatic retinoid stores reflect dietary retinoid intake over the entire lifetime. This point may need to be considered in the design of experiments and/or in the interpretation of data obtained from studies of chemically induced liver injury. Our observations may also hold some therapeutic values to patients who have been acutely exposed to hepatotoxins like TAA. Based on our data, it would seem reasonable that such patients might be advised to immediately limit their dietary intake of retinoid.

In summary, hepatic retinoid stores and/or newly administered dietary retinoid potentiates TAA-induced liver injury.
Hepatotoxicity was associated with higher levels of hepatic CYP and FMO activities and greater bioactivation of TAA. The bioactivated metabolites being formed increase the levels of oxidative stress experienced by the liver, facilitating disease development. These observations are however counterintuitive because retinoids are normally seen as agents promoting optimal health, and the presence of substantial hepatic stores as being beneficial. This raises an issue as to the extent to which retinoid stores and dietary intake need to be considered in the design and interpretation of studies involving TAA and possibly related compounds.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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**REFERENCES**


