Biotransformation of Lithocholic Acid by Rat Hepatic Microsomes: Metabolite Analysis by Liquid Chromatography/Mass Spectrometry

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ABSTRACT:
Lithocholic acid is a lipid-soluble hepatotoxic bile acid that accumulates in the liver during cholestasis. A potential detoxification pathway for lithocholic acid involves hydroxylation by hepatic cytochrome P450 (P450) enzymes. The purpose of the present study was to identify the hepatic microsomal metabolites of lithocholic acid by liquid chromatography/mass spectrometry and to determine the P450 enzymes involved. Incubation of lithocholic acid with rat hepatic microsomes and NADPH produced murideoxycholic acid (MDCA), isolithocholic acid (ILCA), and 3-keto-5β-cholanic acid (3KCA) as major metabolites and 6-ketolithocholic acid and ursodeoxycholic acid as minor metabolites. Experiments with hepatic microsomes prepared from rats pretreated with P450 inducers and with inhibitory antibodies indicated that CYP2C and CYP3A enzymes contribute to microsomal MDCA formation. Results obtained with a panel of recombinant P450 enzymes and CYP2D6 antiserum showed that CYP2D1 can also catalyze MDCA formation. Similar experimental evidence revealed that formation of 3KCA was mediated primarily by CYP3A enzymes. ILCA formation appeared to be catalyzed by a distinct pathway mediated largely by microsomal non-P450 enzymes. Based on the results obtained using lithocholic acid and 3KCA as substrates, a mechanism for the formation of ILCA involving a geminal diol intermediate is outlined. In conclusion, lithocholic acid was extensively metabolized by multiple P450 enzymes with the predominant biotransformation pathway being hydroxylation at the 6β-position. This study provides an insight into possible routes of detoxification of lithocholic acid.

Lithocholic acid (3α-hydroxy-5β-cholanic acid) is a hydrophobic bile acid that is formed by the dehydroxylation of chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanic acid, CDCA). Removal of the 7α-hydroxy group of CDCA is catalyzed by hydratase enzymes associated with anaerobic bacteria that reside in the colon (Hofmann, 1999). Lithocholic acid is absorbed from the colon and transported to the liver, where the acidic moiety is conjugated with taurine or glycine before being excreted as a component of bile (Hofmann, 2002, 2004). Lithocholic acid, together with other biliary bile acids, facilitates the absorption, transport, and distribution of lipid-soluble nutrients from the diet and aids in the elimination of cholesterol from the body (Hofmann, 1999). Figure 1 shows the chemical structures of lithocholic acid and other bile acids (Hofmann et al., 1992).

Lithocholic acid is present in human and rat liver at a concentration of approximately 1 to 5 nmol/g tissue, which represents approximately 4 to 5% of total bile acids in human and rat liver (Setchell et al., 1997). In comparison, the major bile acids in human liver, which are cholic acid and CDCA, and the major bile acids in rat liver, which are cholic acid and muricholic acid, are present at concentrations that are 5 to 10 times greater (Setchell et al., 1997). Hepatic lithocholic acid concentrations are elevated in patients with cholestatic liver disease (Jezquel et al., 1994; Fischer et al., 1996; Erlinger, 1997; Hofmann et al., 2002; Berta et al., 2003; De Gottardi et al., 2004) and in rat models of biliary cholestasis (Setchell et al., 1997; Rost et al., 2003). The accumulation of lithocholic acid and other hydrophobic bile acids in liver has been implicated as a major factor contributing to liver injury in cholestasis because of the inherent cytotoxicity of this hydrophobic bile acid. Hepatic toxicity following chronic and acute administration of exogenous lithocholic acid or its conjugates is well documented in experimental animals (Javitt, 1966; Zaki et al., 1967; Miyai et al., 1971; Fischer et al., 1974).

The hepatotoxicity associated with lithocholic acid can be attenuated by hepatic biotransformation pathways including hydroxylation reactions catalyzed by the cytochrome P450 (P450) enzymes and Phase II reactions involving conjugation of the 3α-hydroxy group with sulfate (Kitada et al., 2003; Hofmann, 2004). The resulting metabolites are more water-soluble and more easily excreted. P450-mediated hydroxylation has been proposed to be an effective detoxification mechanism in rodents and monkeys, whereas sulfate conjugation is considered to be a more important pathway in humans (Hofmann, 2004). There is evidence indicating that P450-mediated hydroxylation of lithocholic acid is also prominent in humans. Lithocholic acid has been reported to be hydroxylated by human hepatic

ABBREVIATIONS: CDCA, chenodeoxycholic acid; P450, cytochrome P450; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; 3KCA, 3-keto-5β-cholanic acid; LC/MS, liquid chromatography/mass spectrometry; PB, phenobarbital; DEX, dexamethasone; MC, 3-methylcholanthrene; α-MCA, α-muricholic acid; β-MCA, β-muricholic acid; γ-MCA, γ-muricholic acid; UDCA, ursodeoxycholic acid; 6KLCA, 6-ketolithocholic acid; ILCA, isolithocholic acid; PXR, pregnane X receptor.
Microsomes to hydroxycylocholic acid (HDCA), murideoxycholic acid (MDCA), and CDCA (Xie et al., 2001). Formation of HDCA was shown to be catalyzed primarily by human recombinant CYP3A4 (Araya and Wikvall, 1999; Xie et al., 2001). More recently, a different metabolite, 3-keto-5β-cholanic acid (3KCA), was identified as the major metabolite of lithocholic acid with human recombinant CYP3A4 (Bodin et al., 2005). In comparison, studies with rat liver microsomes showed that 6β-hydroxylation of lithocholic acid leading to MDCA is the major pathway in the rat (Zimniak et al., 1989; Dionne et al., 1994). However, the P450 enzymes involved in the formation of MDCA in the rat were not identified.

In the present study, the hepatic metabolism of lithocholic acid was investigated in rat hepatic microsomes using a liquid chromatography/mass spectrometry (LC/MS)-based assay, and the various P450 enzymes involved in metabolite formation were identified. Currently, mass spectrometry is one of the most sensitive and specific methods to quantify bile acids in tissue and subcellular fractions (Stedman et al., 2004). LC/MS was used to measure formation of the major and minor metabolites of lithocholic acid. Kinetic parameters associated with rates of metabolite formation in hepatic microsomes were also calculated. The hepatic P450 enzymes responsible for metabolite formation were determined by a combination of approaches involving P450 inducer treatments, antibody inhibition experiments, and rat recombinant P450 enzymes.

**Materials and Methods**

**Chemicals and Reagents.** Authentic bile acid standards were purchased from Steraloids Inc. (Newport, RI). Bile acid standards were dissolved in methanol as 1-mg/ml stock solutions. Additional dilutions were made in methanol for the biotransformation assay. Deuterated cholic-2,2,4,4-d4 acid, which served as an internal standard, was a gift from Dr. Jan Palaty (Children’s and Women’s Health Center, Vancouver, BC, Canada). Sodium phenobarbital (PB) was obtained from BDH Chemicals (Toronto, ON, Canada). Dexamethasone (DEX) and 3-methylcholanthrene (MC) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Baculovirus-insect cell control microsomes containing expressed rat P450-oxidoreductase and baculovirus-insect cell microsomes containing expressed rat P450 enzymes coexpressed with rat P450-oxidoreductase or with rat P450-oxidoreductase and rat cytochrome b5 (BD SUPERSONES Enzymes) were purchased from BD Biosciences (Oakville, ON, Canada). High-performance liquid chromatography-grade chemicals and solvents were purchased from Fisher Scientific (Ottawa, ON, Canada).

Rabbit CYP2D6 polyclonal antiserum (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) was purchased from BD Biosciences. The reaction of CYP2D6 antisera with rat CYP2D1 was assayed in our laboratory by immunoblot analysis using recombinant rat P450 enzymes. Rabbit anti-rat CYP2C polyclonal IgG and rabbit anti-rat CYP3A polyclonal IgG were prepared as described previously (Panesar et al., 1996; Wong and Bandiera, 1996). The inhibitory activities of the anti-CYP2C and anti-CYP3A IgG preparations toward rat hepatic microsomal testosterone 2bα- and 16a-hydroxylation and testosterone 6β-hydroxylation, respectively, had been determined previously (Law, 1995; A. Wong and S. M. Bandiera, unpublished results).

**Animal Treatment and Preparation of Hepatic Microsomes.** Male and female Long-Evans, male Wistar, and male Sprague-Dawley rats (7–8 weeks of age) were purchased from Charles River Canada Inc. (Saint-Constant, QC, Canada). On arrival, rats were housed in pairs in polycarbonate cages on corncob bedding (The Andersons Inc., Maumee, OH) with free access to water and food (Laboratory Rodent Diet, PMI Feeds Inc., Richmond, IN). Animal quarters were maintained at a constant temperature (23°C) with controlled light (14 h) and dark (10 h) cycles. Rats were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Male or female Long-Evans rats (n ≥ 5) were treated with xenobiotics as follows: PB (dissolved in water, 75 mg/kg/day), MC (dissolved in corn oil, 25 mg/kg/day), DEX (dissolved in corn oil, 100 mg/kg/day), or vehicle (0.8 ml/kg/day). Compounds were administered i.p. injection for 4 days, and rats were killed by decapitation 24 h after the last treatment. Microsomes were prepared from pooled livers as described previously (Thomas et al., 1983). Microsomes were also prepared from pooled livers from untreated Wistar rats (n = 16) or Sprague-Dawley rats (n = 4). Micromolar pellets were suspended in 0.25 M sucrose, and aliquots were stored at −75°C until needed. Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.
Lithocholic Acid Biotransformation Assay. Reaction mixtures contained lithocholic acid (0.5–300 μM), 0.5 mg of hepatic microsomal protein, 50 mM potassium phosphate buffer, pH 7.4, 3 mM magnesium chloride, and 1 mM NADPH in a final volume of 1 ml. After preincubation for 10 min at room temperature, reactions were initiated with NADPH and allowed to proceed for 30 min at 37°C. Reactions were terminated with 4 ml of dichloromethane/isopropanol (80:20). A fixed amount (0.4 μg) of internal standard (cholic-2,2,4,4-d₄ acid) was then added to each sample. Tubes were vortex-mixed for 1 min, shaken manually for 1 min, and spun at 2000g for 5 min. The top aqueous layer was carefully removed, placed into a clean tube, and re-extracted with a second 4-ml aliquot of dichloromethane/isopropanol (80:20). The final aqueous phase was discarded, and the organic phases from the second and first extraction were combined and evaporated to dryness with nitrogen. The residue was reconstituted in 0.2 ml of mobile phase (methanol/water/10 mM ammonium acetate, pH 4.6, 67:23:10) and filtered through a 3-mm, 0.45-μm syringe polytetrafluoroethylene filter. A 10-μl aliquot of each sample was analyzed by LC/MS as described by Stedman et al. (2004) with modifications as outlined below.

Control samples devoid of substrate, NADPH, or microsomes, and defined mixtures of authentic bile acid standards were routinely included in each assay. Incubations with rat recombinant P450 enzymes, instead of rat hepatic microsomes, were also carried out. Reaction mixtures contained 30 pmol of each recombinant P450 enzyme (CYP1A2, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP2D1, CYP3A1, or CYP3A2) or, in the case of insect cell control microsomes, an equivalent amount of protein (0.15 mg). To determine whether metabolic formation was P450-mediated, preliminary experiments were conducted with carbon monoxide-treated hepatic microsomes or heat-denatured microsomes. Microsome samples were boiled for 5 min in assay buffer. Carbon monoxide was bubbled into an incubation mixture containing assay buffer and microsomes for 2 min.

Assay conditions were tested using microsomes from untreated male Wistar rats to ensure that substrate and cofactor concentrations were saturating and that product formation was linear with respect to incubation time (1–60 min) and protein concentration (0.25–2 mg/ml of reaction mixture).

Antibody Inhibition. The lithocholic acid biotransformation assay was performed as described above except that microsomes were preincubated with rabbit anti-rat CYP2C IgG, anti-rat CYP3A IgG, or control IgG for 15 min at room temperature. Four concentrations of each IgG (0, 1, 2.5, and 5.0 mg of IgG/mg of microsomal protein) were tested. Formation of MDCA by rat hepatic microsomes or recombinant CYP2D1 was measured in the presence of rabbit CYP2D6 antisera or control serum. Hepatic microsomes or recombinant CYP2D1 was preincubated with various amounts of either rabbit CYP2D6 antisera or control serum (0, 10, 25, 50, and 100 μl/ml reaction mixture) for 15 min at room temperature before addition of lithocholic acid. Reactions were initiated with NADPH.

Analytical Methods. Formation of lithocholic acid metabolites was analyzed by LC/MS. Lithocholic acid and its metabolites were resolved on an XTerra MS C18 (2.1 × 150 mm, 3.5 μm) column (Waters, Milford, MA) at 40°C using a Hewlett-Packard model 1090 II liquid chromatograph (Avondale, PA). The mobile phase consisted of solvent A (methanol/water/10 mM ammonium acetate, pH 4.6, 50:40:10) and solvent B (methanol/water/10 mM ammonium acetate, pH 4.6, 85:5:10). A linear gradient was used starting from 100% solvent A to 100% solvent B from 0 to 20 min, 100% solvent B from 20 to 25 min, followed by an abrupt return to 100% solvent A at 25 min, and re-equilibration with 100% solvent A for 10 min. The flow rate was maintained at 0.15 ml/min, and total run time was 35 min/sample. The LC was interfaced to a Fisons VG Quattro mass detector (Fisons Instruments, VG-Analytical, Manchester, UK). The MS was operated in atmospheric pressure electrospray negative ionization mode, with bath gas flow and nebulizer gas flow rates of 250 and 20 l/h, respectively, a source temperature of 160°C, and capillary and cone voltages of 3 kV and 40 V, respectively. MASSLYNX version 3.1 software (Micromass, Altrincham, UK) was used for data acquisition.

Metabolites were identified by comparison of their retention times and mass to charge ratios (m/z) with those of authentic standards. Quantitative determination of bile acids was performed by selected negative ion monitoring at m/z of 411, 407, 391, 389, 375, and 373. Under these conditions, the internal standard, cholic-2,2,4,4-d₄ acid (MW 412.57), typically eluted at 17 min and was monitored at m/z 411. α-Muricholic acid (α-MCA, MW 408.57), β-muricholic acid (β-MCA, MW 408.57), γ-muricholic acid (γ-MCA, MW 408.57), and cholic acid (MW 408.57) eluted at 12, 13, 15, and 17 min, respectively, and were monitored at m/z 407. MDCA (MW 392.57), ursodeoxycholic acid (UDCA, MW 392.57), HDCA (MW 392.57), CDCA (MW 392.57), and deoxycholic acid (MW 392.57) eluted at 11, 15, 17, 20, and 20.5 min, respectively, and were monitored at m/z 391. 6-Ketolithocholic acid (6KLCA, MW 390.56) eluted at 15 min and was monitored at m/z 389. Isolithocholic acid (ILCA, MW 376.57) and lithocholic acid (MW 376.57) eluted at 23 and 26 min, respectively, and were monitored at m/z 375. 3KCA (MW 374.56) eluted at 25 min and was monitored at m/z 373. Metabolites were quantified from calibration plots of the peak area ratio of the authentic standard and internal standard plotted against the concentration of the authentic standard.

Data Analysis and Calculation of Enzyme Kinetic Parameters. Data were analyzed using the SigmaPlot Enzyme Kinetics Module (version 1.1, Systat Software Inc., Richmond, CA). Metabolite formation as a function of substrate concentration was analyzed by nonlinear regression analysis, and apparent Km, K', and Vmax values were generated using the Hill equation (eq. 1) or the substrate inhibition kinetic equation (eq. 2) for the major metabolites or the Michaelis-Menten equation (eq. 3) for the minor metabolites of lithocholic acid:

\[ v = \frac{V_{\text{max}} \times [S]^{n}}{K' + [S]^{n}} \]  

\[ v = \frac{V_{\text{max}}}{1 + K_{s}[S] + [S]^{n}K'_{s}} \]  

\[ v = \frac{V_{\text{max}} \times [S]}{K_{m} + [S]} \]

where \( v \) is initial velocity of the reaction, \( V_{\text{max}} \) is the maximal velocity, \([S]\) is the substrate concentration, \( K' \) is the Hill dissociation constant, \( n \) is the Hill coefficient representing cooperativity of the reaction. \( K_{s} \) is the Michaelis-Menten constant, and \( K_{m} \) is the dissociation constant of substrate binding to the inhibitory site.

Statistical Analysis. Comparisons of rates of metabolism formation were made using the unpaired t test with Welch’s correction. A p value of <0.05 was considered statistically significant.

Results

Biotransformation of Lithocholic Acid and Metabolite Identification. A mixture of 13 bile acid standards (α-MCA, β-MCA, γ-MCA, cholic acid, MDCA, UDCA, HDCA, CDCA, deoxycholic acid, 6KLCA, ILCA, 3KCA, and lithocholic acid) was initially analyzed to ensure that potential metabolites were resolved with the LC conditions selected. Baseline separation of the bile acid standards was achieved, with the exception of α-MCA and β-MCA, which were distinguished by spiking with authentic standards.

Incubation of lithocholic acid with liver microsomes from male Wistar rats yielded seven metabolites that were identified as β-MCA, MDCA, ILCA, UDCA, HDCA, CDCA, and 3KCA (Fig. 2) by comparison with authentic standards. The major metabolites were MDCA, ILCA, and 3KCA. 6KLCA and UDCA were minor metabolites, whereas β-MCA and HDCA were produced at levels close to or below the limit of quantification. Four other peaks (M-1, m/z 407; M-2, m/z 407; M-3, m/z 391; and M-4, m/z 389) corresponding to trace metabolites were detected but were not identified because the retention times of these metabolites did not match those of the authentic standards. To determine whether lithocholic acid biotransformation differed among rat strains, incubations were performed with hepatic microsomes from male Wistar, Long-Evans, and Sprague-Dawley rats. The same metabolite profile was obtained for all three rat strains (results not shown). Metabolite formation was not observed when lithocholic acid was incubated with carbon monoxide-treated or
boiled microsomal preparations or when NADPH was omitted from the reaction mixture.

Although CDCA was previously reported to be a metabolite of lithocholic acid in the rat (Zimniak., 1989), our results indicate that CDCA is not a metabolite. A peak at \( m/z \) 391 with the same retention time as CDCA was observed but was determined to be a contaminant of lithocholic acid as it was detected with reaction mixtures that contained substrate and NADPH but not hepatic microsomes and the area of the CDCA peak did not change following incubation with increasing concentrations of hepatic microsomes.

Kinetic Analysis of Hepatic Microsomal Metabolite Formation.

Formation of the major and minor metabolites of lithocholic acid was evaluated over a range of substrate concentrations (0.5–300 \( \mu \)M). An incubation time of 30 min and a microsomal protein concentration of 0.5 mg/ml were found to be optimal and were used in subsequent experiments. A lithocholic acid concentration of 100 or 250 \( \mu \)M, depending on the metabolite, was found to be saturating. Hepatic microsomal MDCA formation exhibited typical Michaelis-Menten kinetics up to a substrate concentration of 100 \( \mu \)M. At higher concentrations of lithocholic acid, the rate of MDCA formation decreased, possibly as a result of substrate inhibition (Fig. 3A). Formation of ILCA and 3KCA exhibited sigmoidal kinetic profiles. Decreased ILCA and 3KCA formation was observed at substrate concentrations greater than 100 \( \mu \)M for ILCA and 250 \( \mu \)M for 3KCA (Fig. 3, B and C). Hence, saturating substrate concentrations of 100 \( \mu \)M for MDCA and ILCA formation and 250 \( \mu \)M for 3KCA formation were selected for further experiments. Nonlinear Eadie-Hofstee plots of MDCA, ILCA, and 3KCA formation, signifying atypical enzyme kinetics, were obtained (Fig. 3, A–C). Of the minor metabolites that could be quantified, formation of 6KLCA and UDCA followed typical Michaelis-Menten kinetics up to a substrate concentration of 100 \( \mu \)M (results not shown).

Apparent \( K_m \) and \( V_{max} \) values for hepatic microsomal MDCA formation were calculated using eq. 2. Apparent \( K' \) and \( V_{max} \) values for hepatic microsomal ILCA and 3KCA formation were calculated using eq. 1 (Table 1). Positive cooperativity (\( n > 2 \)) was indicated for ILCA and 3KCA formation. The apparent \( V_{max} \) value for MDCA formation was approximately 7 and 14 times higher than the values for ILCA and 3KCA formation, respectively, showing that MDCA was the predominant microsomal metabolite of lithocholic acid in rats. On the other hand, the lower apparent \( K' \) value associated with ILCA formation (29.3 ± 5.9 \( \mu \)M) suggests that this metabolite would be preferentially formed at low lithocholic acid concentrations. The apparent \( V_{max} \) values calculated for UDCA and 6KLCA formation...
To identify the P450 enzymes involved in lithocholic acid biotransformation, experiments were performed with hepatic microsomes prepared from male and female Long-Evans rats that had been pretreated with MC, PB, or DEX. Comparison of rates of formation of the metabolites revealed a distinct sex difference for some of the metabolites (Table 2). Rates of MDCA and ILCA formation for control male rats were approximately 2 and 30 times greater, respectively, than for control female rats, whereas the rate of formation of 3KCA was similar for control male and female rats. Treatment with PB or MC decreased formation rates of MDCA and ILCA for male rats. The rate of formation of 3KCA was slightly, but not significantly, greater for both sexes following DEX treatment, whereas formation of MDCA and ILCA was not affected. With respect to the minor metabolites, rates of formation of 6KLCA and UDCA were greater for male than female rats, and formation of both metabolites was increased following treatment with DEX but not PB or MC (data not shown).

Antibody Inhibition Studies. The role of CYP2C and CYP3A enzymes in lithocholic acid biotransformation was investigated using antibodies against CYP2C and CYP3A enzymes. Rates of formation of MDCA and 3KCA were each inhibited by approximately 50% by anti-CYP2C IgG (at 5 mg of IgG/mg of protein), whereas ILCA formation was not affected (Fig. 4). Anti-CYP3A IgG (at 5 mg of IgG/mg of protein) inhibited the rates of formation of MDCA, ILCA, and 3KCA by 40 to 60% (Fig. 4). Formation of 6KLCA and UDCA was not affected by anti-CYP2C IgG but was inhibited by approximately 20 and 30%, respectively, following incubation of microsomes with anti-CYP3A IgG (at 5 mg of IgG/mg of protein, data not shown).

Biotransformation Studies with Recombinant P450 Enzymes. The contribution of individual P450 enzymes in lithocholic acid biotransformation was evaluated using a panel of nine rat recombinant P450 enzymes (Fig. 5). Initial experiments were conducted to determine P450 concentrations that would ensure linearity of product formation with incubation time. An incubation time of 30 min and rat recombinant P450 enzyme concentration of 30 pmol of P450/ml were found to be optimal. Under the experimental conditions used, CYP2D1 was the most active P450 enzyme catalyzing MDCA formation. In comparison, formation of ILCA was catalyzed at a relatively low rate by several recombinant P450 enzymes including CYP1A2, CYP2C6, CYP2D1, CYP3A1, and CYP3A2 but not by CYP2A2 or CYP2C13. The rate of formation of ILCA by the recombinant P450 enzymes was approximately 4 to 16 times less than the rate obtained with hepatic microsomes, when activity values were expressed per nanomole of total P450. CYP3A2, followed by CYP3A1 and CYP2C11, were the most active P450 enzymes catalyzing 3KCA formation. The rate of 3KCA formation by recombinant CYP3A2 was found to be greater, when expressed per nanomole of total P450, than that obtained with hepatic microsomes (approximately 31 versus 0.6 pmol/min/pmol P450, respectively). Formation of 6KLCA and UDCA by rat recombinant P450 enzymes was also assessed. 6KLCA formation was catalyzed mainly by CYP3A1 and CYP3A2, whereas UDCA formation was catalyzed solely by CYP2A2 (results not shown). There was no evidence of β-MCA or CDCA formation by the rat recombinant P450 enzymes.

To verify the involvement of CYP2D1 in MDCA formation, antibody inhibition experiments were conducted using CYP2D6 antiserum, which has been reported to cross-react with CYP2D1 (Umehara et al., 1997). MDCA formation by recombinant CYP2D1 was inhibited by 90% at the highest concentration of CYP2D6 antiserum tested (Fig. 6). In contrast, CYP2D6 antiserum had no effect on MDCA formation by hepatic microsomes.

Conversion of lithocholic acid to ILCA involves epimerization of the hydroxyl group at the 3-position. To determine whether ILCA formation could occur by a stepwise process, 3KCA was incubated with hepatic microsomes prepared from control male rats and with a
LITHOCHOLIC ACID BIOTRANSFORMATION BY HEPATIC P450 ENZYMES

TABLE 1
Kinetic parameters of lithocholic acid metabolite formation by rat hepatic microsomes

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Apparent $V_{max}$</th>
<th>Apparent $K_{m}$</th>
<th>Apparent $K'$</th>
<th>n</th>
<th>Apparent $K_i$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pmol/min/mg protein</td>
<td>µM</td>
<td>µM</td>
<td></td>
<td>µM</td>
</tr>
<tr>
<td>MDCA*</td>
<td>5920 ± 1180</td>
<td>56.0 ± 17.8</td>
<td>N.A.</td>
<td>N.A.</td>
<td>194 ± 74.6</td>
</tr>
<tr>
<td>ILCA*</td>
<td>803 ± 91.8</td>
<td>N.A.</td>
<td>29.3 ± 5.9</td>
<td>2.3 ± 0.8</td>
<td>N.A.</td>
</tr>
<tr>
<td>3KCA</td>
<td>413 ± 25.8</td>
<td>71.6 ± 6.5</td>
<td>2.5 ± 0.6</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>6KLCA</td>
<td>44.5 ± 2.1</td>
<td>1.6 ± 0.3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>UDCA*</td>
<td>22.4 ± 1.7</td>
<td>15.3 ± 3.7</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not applicable.
* Kinetic parameters for MDCA formation were calculated using the substrate inhibition kinetics model (eq. 2).
† Kinetic parameters for ILCA and 3KCA formation were calculated using the sigmoidal kinetics model, Hill equation (eq. 1).
§ Kinetic parameters for 6KLCA and UDCA formation were calculated using the Michaelis-Menten equation.

TABLE 2
Effect of sex and treatment with P450 inducers on lithocholic acid metabolite formation by rat hepatic microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of Metabolite Formation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MDCA</td>
</tr>
<tr>
<td>Male rats</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2900 ± 302</td>
</tr>
<tr>
<td>MC</td>
<td>1010 ± 171*</td>
</tr>
<tr>
<td>PB</td>
<td>633 ± 150*</td>
</tr>
<tr>
<td>DEX</td>
<td>1810</td>
</tr>
<tr>
<td>Female rats</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1220 ± 182*</td>
</tr>
<tr>
<td>PB</td>
<td>1250</td>
</tr>
<tr>
<td>DEX</td>
<td>781 ± 122</td>
</tr>
</tbody>
</table>

* p < 0.05
† Values represent the average of duplicate determinations.

Discussion

Lithocholic acid was extensively metabolized by rat hepatic microsomes to three major (MDCA, ILCA, and 3KCA) and two minor (6KLCA and UDCA) products, as determined by LC/MS. The predominant biotransformation pathway involved hydroxylation of lithocholic acid at the 6β-position and led to formation of MDCA. This result is consistent with previous studies of in vivo and in vitro studies, which described hepatic 6β-hydroxylation of bile acids as a major pathway in rat (Voigt et al., 1968; Gustafsson, 1978) and identified MDCA as a major metabolite of lithocholic acid (Zimniak et al., 1989; Dionne et al., 1994). However, the P450 enzymes catalyzing MDCA formation were not identified in previous studies. Our experiments with hepatic microsomes prepared from rats pretreated with P450 inducers and with inhibitory P450 antibodies indicated that CYP2C and CYP3A enzymes contribute to microsomal MDCA formation, whereas results obtained with a panel of rat recombinant P450 enzymes showed that CYP2D1 also catalyzes MDCA formation. The lack of effect by CYP2D6 antisera on hepatic microsomal MDCA formation suggests that there is little or no contribution by CYP2D1 to the activity of hepatic microsomes.

Panel of rat recombinant P450 enzymes. Hepatic microsomes catalyzed ILCA formation from both lithocholic acid and 3KCA. Formation of ILCA from 3KCA was not catalyzed by any of the recombinant P450 enzymes. Formation of 3KCA by recombinant CYP3A2 was further evaluated at substrate concentrations of 0.5 to 100 µM (Fig. 7). Formation of 3KCA by recombinant CYP3A2 followed typical Michaelis-Menten kinetics, which is in contrast to the atypical sigmoidal kinetic pattern observed with hepatic microsomes. These data suggest that formation of 3KCA in hepatic microsomes involved more than a single P450 enzyme. A $V_{max}$ value of 31.5 pmol/min/µmol P450 and a $K_m$ value of 18.9 µM were obtained for 3KCA formation by recombinant CYP3A2.

Discussion

Lithocholic acid was extensively metabolized by rat hepatic microsomes to three major (MDCA, ILCA, and 3KCA) and two minor (6KLCA and UDCA) products, as determined by LC/MS. The predominant biotransformation pathway involved hydroxylation of lithocholic acid at the 6β-position and led to formation of MDCA. This result is consistent with previous studies of in vivo and in vitro studies, which described hepatic 6β-hydroxylation of bile acids as a major pathway in rat (Voigt et al., 1968; Gustafsson, 1978) and identified MDCA as a major metabolite of lithocholic acid (Zimniak et al., 1989; Dionne et al., 1994). However, the P450 enzymes catalyzing MDCA formation were not identified in previous studies. Our experiments with hepatic microsomes prepared from rats pretreated with P450 inducers and with inhibitory P450 antibodies indicated that CYP2C and CYP3A enzymes contribute to microsomal MDCA formation, whereas results obtained with a panel of rat recombinant P450 enzymes showed that CYP2D1 also catalyzes MDCA formation. The lack of effect by CYP2D6 antisera on hepatic microsomal MDCA formation suggests that there is little or no contribution by CYP2D1 to the activity of hepatic microsomes. This may be explained by the relatively low level of expression of CYP2D1. Determination of CYP2D1 levels in Hepatic microsomes prepared from rats pretreated with P450 inducers and from control female rats (p < 0.07). Collectively, the results implicate that several P450 enzymes, which are expressed at higher levels than CYP2D1, are involved in MDCA formation as shown in Fig. 5A.

Lithocholic acid concentrations of 5 to 10 µM have been reported in the liver of cholestatic patients and in rat models of biliary cholestasis (Jezequel et al., 1994; Fischer et al., 1996; Erlinger, 1997; Setchell et al., 1997; Hofmann, 2002; Berta et al., 2003; Rost et al., 2003; De Gottardi et al., 2004). A lithocholic acid concentration of 100 µM was found to be saturating for hepatic microsomal MDCA formation in the present study. MDCA was the major metabolite obtained in the in vitro biotransformation assay, with a rate of formation of 250 to 500 pmol/min/mg at a lithocholic acid concentration of 5 µM, which approximates the physiological hepatic concentration. Thus, our study shows that 6β-hydroxylation is the major P450-mediated hepatic pathway of lithocholic acid biotransformation in the rat. In contrast, hydroxylation of lithocholic acid at the 6α-position has been proposed to be the predominant P450-mediated pathway in humans (Araya and Wikvall, 1999; Xie et al., 2001). Only a trace amount of HDCA was produced by rat liver microsomes in the present study.

ILCA, the 3β-isomer of lithocholic acid, was the second most abundant microsomal metabolite detected in our study. ILCA was reported to be a major metabolite in human feces (Norman and Palmer, 1964; Palmer, 1971) and was identified as an in vivo metabolite of rats fed sulfated lithocholic acid (Palmer, 1971; Zimniak et al., 1989; Dionne et al., 1994) but was not found to be a metabolite of lithocholic acid produced by rat liver microsomes (Palmer, 1971; Zimniak et al., 1989; Dionne et al., 1994). In our study, conversion of lithocholic acid to ILCA was observed with some rat recombinant P450 enzyme preparations but at a relatively low rate. Hepatic microsomal ILCA formation was not induced by pretreatment with MC, PB, or DEX, was not inhibited by anti-CYP2C IgG, and was slightly
The results suggest that microsomal enzymes other than P450 enzymes were involved in ILCA formation. Epimerization of the hydroxyl group on steroid molecules is most often catalyzed by hepatic hydroxysteroid dehydrogenase and steroid oxidoreductase enzymes and is not a common P450-mediated reaction (Penning et al., 1986). A possible mechanism for the formation of ILCA is shown in Fig. 8. We speculate that microsomal ILCA formation can proceed by two pathways. One pathway involves conversion of lithocholic acid to ILCA, possibly through a geminal diol intermediate with subsequent loss of a hydroxyl group. The geminal diol is formed by β-hydroxylation at an existing α-hydroxy position (as suggested by Bodin et al., 2005). The geminal diol intermediate can spontaneously rearrange to form either ILCA or 3KCA. A second pathway involves 3β-oxidation of lithocholic acid followed by dehydration to form 3KCA, with subsequent reduction of 3KCA to ILCA.

Fig. 4. Effect of anti-CYP2C IgG and anti-CYP3A IgG on hepatic microsomal MDCA, ILCA, and 3KCA formation. Hepatic microsomes from untreated male rats (0.5 mg) were incubated with various concentrations of rabbit anti-rat CYP2C polyspecific IgG (○), anti-CYP3A polyspecific IgG (▼), and control IgG (■) for 15 min at room temperature before addition of lithocholic acid (100 μM). Results are expressed as percentage of the activity obtained in the presence of 0 mg of IgG.

Fig. 5. Comparison of MDCA, ILCA, and 3KCA formation by a panel of rat recombinant P450 enzymes. Metabolite formation (activity) was measured following a 30-min incubation of lithocholic acid (100 μM) with baculovirus-insect cell microsomes containing expressed rat P450 enzymes (30 pmol). Insect cell microsomes containing expressed P450 enzymes, except for CYP1A2 and CYP2D1, also contained coexpressed P450 oxidoreductase and cytochrome b₅. Microsomes containing expressed CYP1A2 and CYP2D1 contained coexpressed P450 oxidoreductase but not cytochrome b₅. There was no obvious correlation between rates of metabolite formation and P450 oxidoreductase or cytochrome b₅ levels in the recombinant P450 preparations. Plots show the mean values ± S.E.M. of triplicate determinations.
Our experiments conducted using 3KCA as substrate showed that although ILCA is a major hepatic microsomal metabolite of 3KCA, none of the recombinant P450 enzymes contributed significantly to that conversion. Accordingly, we believe that both pathways are mediated largely by non-P450 enzymes. An example of a microsomal enzyme that can catalyze this reaction is 3β-hydroxy-Δ5-C27-steroid oxidoreductase enzyme, which was purified from rabbit liver microsomes and was shown to catalyze the reversible oxidation of the 3β-hydroxy group of C27-steroids (Wikvall, 1981).

3KCA was the third most abundant hepatic microsomal metabolite of lithocholic acid in our study. A recent report identified 3KCA as the major lithocholic acid metabolite (74% of total) formed by human recombinant CYP3A4 (Bodin et al., 2005), and two earlier studies found 3KCA as a major in vivo product of rats fed a lithocholic acid-enriched diet (Thomas et al., 1964; Sakai et al., 1980). Formation of 3KCA from lithocholic acid entails oxidation of a hydroxyl group and can be catalyzed by P450 enzymes by the mechanism described above. In the present study, treatment of male and female rats with DEX led to increased 3KCA formation, suggesting that CYP3A enzymes contribute to formation of this metabolite. Results of the antibody inhibition experiments substantiate the involvement of CYP3A and CYP2C in 3KCA formation. Of the recombinant enzymes examined, the highest catalytic activity was obtained with CYP3A2. Taken together, the data provide convincing evidence that 3KCA formation in rat hepatic microsomes was catalyzed primarily by CYP3A. A proposed mechanism for 3KCA formation was outlined above (see Fig. 8).

Minor metabolites, namely, 6KLCA and UDCA, as well as metabolites that were detected but could not be quantified, namely, HDCA and β-MCA, were also reported by Zimniak et al. (1989). Our results suggest that 6KLCA formation was catalyzed mainly by CYP3A enzymes, whereas UDCA formation was catalyzed by CYP2A enzymes. Appreciable formation of β-MCA was apparent only at low substrate concentrations, suggesting that β-MCA was converted to other metabolites. CDCA, which was reported to be a metabolite in previous studies (Thomas et al., 1964; Zimniak et al., 1989), was identified herein as a contaminant of lithocholic acid. We found no evidence of CDCA formation by either rat hepatic microsomes or rat recombinant P450 enzymes.

Our study focused primarily on the contribution of P450 enzymes to the biotransformation of lithocholic acid in hepatic microsomes. We are aware that Phase II enzymes such as sulfotransferases and glucuronosyl transferases also play an important role in lithocholic acid biotransformation. Glucuronosyl transferases are microsomal enzymes that facilitate conversion of lithocholic acid and its metabolites, such as MDCA, to their ester glucuronide conjugates, thereby affecting MDCA formation. Moreover, nuclear hormone receptors, such as the pregnane X receptor (PXR), constitutive androstane receptor, liver X receptor, and farnesoid X receptor, can be activated by bile acids and help regulate bile acid homeostasis (Staudinger et al., 2001; Makishima, 2005). Some of these receptors, such as PXR, are involved in the inducible expression of P450 enzymes and sulfotransferases and glucuronosyl transferases (Tien and Negishi, 2006). Thus, treatment with DEX and other inducers used in the present study could alter formation of metabolites such as MDCA through an effect on Phase II enzymes, apart from the effect on P450 enzymes. This may partly explain the decreased MDCA formation observed after pretreatment with DEX. The concentration of DEX used in our study (100 mg/kg) is sufficient to activate PXR in rodents (Hartley et al., 2004).

In summary, LC/MS proved to be an effective method for resolving and identifying the biotransformation products of lithocholic acid. Lithocholic acid, like many P450 substrates, is metabolized by multiple P450 enzymes, which catalyze overlapping pathways. Our study suggests a major role for hepatic CYP2C and CYP3A enzymes in lithocholic acid biotransformation. In rats and humans, the contribution of individual P450 enzymes to the various metabolic pathways is determined by their level of expression and the tissue concentration of lithocholic acid. CYP2C enzymes are the predominant P450 enzyme subfamily expressed in untreated rat liver and together with CYP3A enzymes, which predominate in human liver, are expected to be the main catalysts of lithocholic acid biotransformation in rats and possibly in humans. Because CYP3A is involved in the formation of most of the major metabolites of lithocholic acid, induction of CYP3A enzymes offers a potential mechanism to lessen the hepatotoxicity associated with high tissue levels of lithocholic acid. Based on results...
obtained, a scheme for P450-mediated formation of major lithocholic acid metabolites in rat hepatic microsomes is proposed in Fig. 9.

The LC/MS analytical method used herein provides a sound foundation for future bile acid biotransformation studies using human hepatic microsomes and purified or recombinant P450 enzymes, studies that are currently underway in our laboratory.

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