Regulation of Oxysterol 7α-Hydroxylase (CYP7B1) in the Rat

Shunlin Ren, Dalila Marques, Kaye Redford, Phillip B. Hylemon, Gregorio Gil, Z. Reno Vlahcevic,† and William M. Pandak

Cholesterol metabolized to 7α-hydroxylated bile acids is a principle pathway of cholesterol degradation. Cholesterol 7α-hydroxylase (CYP7A1) is the initial and rate-determining enzyme in the "classic pathway" of bile acid synthesis. An "alternative" pathway of bile acid synthesis begins with 27-hydroxylation of cholesterol by 27-hydroxylase (CYP27), followed by 7α-hydroxylation by oxysterol 7α-hydroxylase (CYP7B1). The aim of the current study was to investigate the regulation of CYP7B1 by bile acids, cholesterol, and thyroid hormone in a previously well-studied in vivo model of bile acid synthesis, and to compare its regulation to that of CYP7A1. Three study groups were examined. In the first, male Sprague-Dawley rats with intact enterohepatic circulations were fed normal chow (controls), cholestyramine (CT), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), or cholesterol (Chol). In the second group, taurocholate (TCA) was continuously intraduodenally infused for 48 hours to chronic biliary diverted rats. In a third set of studies, squalestatin, an inhibitor of cholesterol synthesis, was intravenously infused for 48 hours. In a fourth set of studies, the diurnal variation in CYP7B1 was compared to that of CYP7A1. At the end of each study livers were harvested, and CYP7B1 and CYP7A1 activities and mRNA levels were determined. Complete biliary diversion significantly increased the specific activity (SA) of both CYP7B1 (↑212%; $P < .002$) and CYP7A1 (↑212%; $P < .007$). Intraduodenal infusion of TCA to rats with biliary diversion decreased SA of both CYP7B1 (↓29%; $P < .001$) and CYP7A1 (↓46%; $P < .01$). The addition of CA, CDCA, or DCA to rat chow led to downregulation of CYP7B1 SAs by 42% ($P < .003$), 51% ($P < .009$), and 47% ($P < .003$), and CYP7A1 SAs by 32% ± 6% ($P < .003$), 73% ± 9% ($P < .002$), and 60% ± 13% ($P < .004$), respectively. CT feeding upregulated both CYP7B1 (↑136%; $P < .004$) and CYP7A1 (↑216%; $P < .0001$) SAs. While Chol feeding significantly upregulated CYP7A1 SA, no significant increase in CYP7B1 SA was found. Conversely, as previously shown in vitro, inhibition of cholesterol synthesis significantly suppressed both CYP7A1 and CYP7B1 activity and mRNA levels. Both CYP7B1 and CYP7A1 underwent diurnal variation, with peak and trough values for CYP7B1 lagging approximately 6 hours behind CYP7A1. We conclude that, in the rat, like CYP7A1, CYP7B1 demonstrates diurnal rhythm and is regulated by bile acids and cholesterol.

CHOLESTEROL CONVERSION into 7α-hydroxylated bile acids is a principle pathway of cholesterol catabolism and secretion from the body. Cholesterol 7α-hydroxylase (CYP7A1) initiates and is the rate-determining enzyme of the "classic pathway" of bile acid synthesis.[1,2] An important "alternative" pathway of bile acid synthesis is initiated by mitochondrial sterol 27-hydroxylase (CYP27), with the subsequent 7α-hydroxylation by oxysterol 7α-hydroxylase (CYP7B1), a pathway that has been shown to contribute up to 50% of bile acid synthesis.[1-5] CYP7A1 has been demonstrated to be highly regulated by bile acids, hormones, cholesterol (oxysterols), cytokines, and second messengers.[1,2] CYP27 has been shown to be regulated by bile acids and hormones.[2] In contrast to CYP7A1, CYP27 is found not only in the liver, but in abundance in extrahepatic tissues, including the vascular endothelium. The ability of peripheral tissues to 27-hydroxylate cholesterol may represent a mechanism important for the prevention of cholesterol accumulation, not only through its participation in cholesterol degradation, but in the generation of oxysterols.[6-10] These oxysterols represent important degradation products of cholesterol. They are intermediates in the synthesis of bile acids, which appear to have regulatory effects on cholesterol homeostasis, including suppression of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase and the low-density lipoprotein (LDL) receptor, and transcriptional stimulation of CYP7A1 and ABC1 through ligand binding to LXR (liver X receptor).[6,11-13]

Recently, the cDNA encoding oxysterol 7α-hydroxylase (CYP7B1), which is capable of mediating 7α-hydroxylation of 27-hydroxycholesterol, 25-hydroxycholesterol, dehydroepiandrosterone, and pregnenolone has been isolated,[14,15] In direct contrast to CYP7A1, found only in the liver, CYP7B1 is present in many extrahepatic tissues, with high levels in the vascular endothelium.[15,16] It has been suggested that 7α-hydroxylation of 27-hydroxycholesterol is responsible for the metabolism of oxysterols to dihydroxy metabolites with less regulatory, less cytotoxic effects.[17] These more soluble dihydroxy metabolites can then be more readily released from the cell, transported to the liver, and converted to bile acids. However, the relative importance of this widely distributed enzyme needs to be more clearly defined, with its relative importance possibly being species-specific. In humans, a defect in CYP7B1 in neonates appeared lethal secondary to toxic accumulation of oxysterols.[17] In contrast, *Cyp7b1*$^{-/-}$ mice developed elevated 25- and 27-hydroxycholesterol levels, but did not present with an obvious phenotype.[18]

The current study examines the in vivo regulation of liver CYP7B1. The findings presented suggest that CYP7B1 in the

From the Departments of Medicine, Microbiology/Immunology, and Biochemistry/Molecular Biophysics, Veterans Affairs Medical Center and Virginia Commonwealth University, Richmond, VA.

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†Deceased.

Address reprint requests to William M. Pandak, MD, Veterans Affairs Medical Center, Division of Gastroenterology 111-N, 1201 Broad Rock Blvd, Richmond, VA 23249.s.50106

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rat is regulated by bile acids, cholesterol, and hormones, and demonstrates a diurnal rhythm.

MATERIALS AND METHODS

Materials

All chemicals purchased were of the highest available grade. Squalestatin, a potent inhibitor squalene synthetase and cholesterol biosynthesis, was kindly provided by Glaxo Research Group. Sodium salts of cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), hyocholic acid (HCA), ursodeoxycholic (UDCA), and taurocholic acid (TCA) were purchased from Calbiochem (LaJolla, CA). ^{3}H-25-hydroxycholesterol was purchased from New England Nuclear (Boston, MA). 7α- and 7β-hydroxycholesterol, and 25-hydroxycholesterol were purchased from Steraloids (Newport, RI). Poly A mRNA isolation system II was obtained from Promega (Madison, WI). Cyclodextran was purchased from Cyclodextrin Technologies Development (Gainesville, FL). Polyethylene tubing (Intramedic PE-50) and Dow Corning silastic tubing were obtained from Allegiance Health Care Corp (McGaw Park, IL). Silica gel thin-layer chromatography plates (LK6 D) were from Whatman (Clifton, NJ). Solvents were purchased from Fisher Scientific (New Lawn, NJ), and all other reagents were from Sigma Chemical Co (St Louis, MO), unless otherwise indicated. The ribonuclease protection assay (RPA) kit was purchased from Ambion (Austin, TX).

Experimental Design

Feeding experiments. Adult male Sprague-Dawley rats were procured from Charles River Laboratories (Cambridge, MA) and kept in metabolic cages in light-controlled rooms on a natural dark-light cycle. Age-matched animals weighing between 150 and 200 g were used in all feeding experiments. All rats were fed 20 g of rodent powder chow (Ralston Purina, St Louis, MO) per day, for 14 days. Rats were pair fed either plain chow (controls), or one of the following individual bile acids was mixed with their regular chow (wt/wt): 1% CA, 0.25% DCA, 1% HCA, 1% CDCA, or 1% UDCA. Additional animals were pair-fed either 2% or 4% cholesterol (Chol), or 5% cholestyramine (CT) added to the regular chow. DCA was added as 0.25% of the diet instead of 1% to avoid any possible side effects from bile acid–induced hepatotoxicity.

Bile acid pool analysis in rats fed conventional bile acids in the percentage of diet used has been reported in previous studies.[19,20] In all instances, the fed bile acid became the predominant bile acid in bile, regardless of the degree of hydrophobicity/hydrophilicity.

Intravnous-intraduodenal infusion studies. Adult male Sprague-Dawley rats weighing between 250 and 300 g were housed under controlled lighting conditions on a natural light-dark cycle. Groups of age- and weight-matched animals were used in all experiments. Under brief methoxyflurane anesthesia, biliary fistulas and intraduodenal cannulas were placed as previously described.[21] Following surgery, the rats were housed in individual metabolic cages with free access to water and chow. Diverted bile was collected every 4 hours throughout the course of the experiment. All animals received a continuous infusion of glucose-electrolyte replacement solution at 1.07 mL/h. Dietary intake, activity, and bile flow were monitored. After 72 hours of biliary diversion, experimental rats received infusion of TCA (36 μmol/h · 100 g rat^{-1}) intraduodenally, at a constant rate, for the next 24 or 48 hours. In a separate experiment, after 72 hours of biliary diversion, rats were infused intravenously with squalestatin (15 μg/h · 100 g rat^{-1}).[5]

Throughout the experiments the animals were monitored for dietary intake and activity. At the end of the experiments, animals were briefly anaesthetized, decapitated, and blood was collected in order to measure serum ALT and alkaline phosphatase levels as previously described.[20] With the exception of diurnal variation studies (see Fig 7), where animals were killed at differing periods of the light-dark cycle, animals in all groups were decapitated at 9 to 10 AM. The animal protocol used was reviewed and approved annually by the Institutional Animal Care and Use Committee of the Medical College of Virginia, Richmond, VA, and complies with the "Guide for Care and Use of Laboratory Animals" published by the National Institute of Health.

Methods

Preparation of microsomes and mitochondria. Microsomes and mitochondria were prepared as previously described.[20] Protein concentrations were determined by the method of Bradford, using bovine serum albumin (BSA) as the standard (BioRad protein assay, BioRad Laboratories, Hercules, CA). To check the purity of organelle preparations, microsomal and mitochondrial marker assays were run. Using NADP-linked isocitrate dehydrogenase as a mitochondrial marker,[22] no mitochondrial contamination of the microsomal preparation was found. By assaying for glucose-6-phosphatase, a microsomal marker,[23] it was determined that the mitochondrial preparation could contain up to 14% microsomal contamination. Microsomal free cholesterol was quantified as previously described.[24,25]

Enzymatic assay. CYP7B1 activity was determined according to the method of Schwarz et al with modifications as described.[26] Briefly, 250 μg of microsomal protein were incubated at 37°C for 15 minutes in a shaking water bath, with 50 mmol/L of Tris acetate, pH 7.4, 1 mmol/L EDTA, 2 mmol/L DTT, 0.03% Triton X 100, 1.2 mmol/L NADPH, and 0.06 nmol of ^{3}H 25-hydroxycholesterol, in a final volume of 500 μL. In order to insure assay substrate saturation, the assay was modified from that originally described, and 1.25 nmol/L of cold 25-hydroxycholesterol was added. The reaction was stopped by addition of 6 mL of methylene chloride. The organic phase was then evaporated under nitrogen, dissolved in acetone, and assayed by thin-layer chromatography in a solvent system containing toluene/ethyl acetate (2:3). The plate was exposed to a tritium screen and the bands were quantified and analyzed with a Phosphor Imager (Molecular Dynamics, San Francisco, CA) and the appropriate software. CYP7B1 activity was further analyzed in rat microsomes as follows: (1) assay was performed using ^{14}C-cholesterol as substrate, a substrate of CYP7A1, but not CYP7B1[27]; (2) a selective inhibitor of CYP7A1, 7-oxocholesterol, was added to the assay in concentrations (10 μmol/L) that have been shown to suppress CYP7A1 by greater than 90% in an attempt to suppress activity contributed by CYP7A1[27]; (3) CYP7B1 assay was performed using labeled DHEA under saturating conditions (ie, cold DHEA added in a 25-μmol/L concentration), a substrate of CYP7B1, but not CYP7A1[27]; and (4) CYP7B1 specific activity (SA) changes were determined in mitochondria, an organelle with no detectable CYP7A1 activity. Mitochondria were tested as described earlier with microsomal markers to insure mitochondria prep purity. Furthermore, mitochondria were assayed with our existing high-performance liquid chromatography (HPLC)-based assay for CYP7A1 and found to have no detectable CYP7A1 activity (sensitive to ≥20 pmol).[28] All experimental conditions were performed employing the CYP7B1 assay described above.

CYP7A1 activity was determined in microsomes by reversed-phase HPLC as previously described.[28] In short, 1 mg of microsomal protein was incubated for 5 minutes at 37°C with 0.1 mol/L potassium phosphate buffer, pH 7.4, 50 mmol/L NaF, 5 mmol/L DTT, 1 mmol/L EDTA, 20% glycerol, and 0.015% Chaps. The reaction was initiated by adding the NADPH regeneration system, containing 5 mmol/L sodium isocitrate, 5 mmol/L $MgCl_2$, 0.5 mmol/L NADPH, and 0.075 U of isocitrate dehydrogenase, in a final volume of 1 mL and incubated for 20 minutes at 37°C in a shaking water-bath. The reaction was terminated by adding 30 μL of 20% sodium cholate. One microgram of 7β-hydroxycholesterol was added as an internal recovery standard and the final reaction was initiated by the addition of 44 μL of 0.1% of

cholesterol oxidase (Calbiochem, San Francisco, CA) in 10 mmol/L phosphate buffer, pH 7.4, 1 mmol/L DTT, and 20% glycerol, incubated for 10 minutes at 37°C. The reaction was terminated by adding 2 mL of 95% ethanol. Cholesterol metabolites were extracted by adding 6 mL of petroleum ether, vortexing, incubating for 3 minutes at 37°C, and centrifuging for 3 minutes at 1,200 × *g*. The upper layer was collected and dried under nitrogen, at 40°C. The extraction was repeated twice, and the tubes capped and stored at 4°C until ready to be analyzed by HPLC. For HPLC analysis a C-18 reverse-phase Altex Ultrasil-ODS (4.6 mm × 25 cm) column equilibrated with 70% acetonitrile and 30% ethanol vol/vol was used. The extracted products were resuspended in 100 μL of the same solvent mixture and 20 μL was injected. The initial flow rate was 0.8 mL/min for the first 18 minutes and then increased to 2.0 mL/min for another 14 minutes. The run was monitored at 240 nm.

RNA isolation. RNA was isolated as previously described.[21] Briefly, 1 g of liver was homogenized in a pH 7.4 buffer, containing 4 mol/L guanidine thiocynate, 10 nmol/L Tris, 7% β-mercaptoethanol, and 2% sarcosyl. The homogenate was passed through a 23-gauge needle, heated to 65°C and centrifuged at 3,000 rpm for 10 minutes. The supernatant was then layered on top of 5.7 mol/L cesium chloride and 10 nmol/L EDTA, pH 7.4 and centrifuged at 100,000 × *g* for 16 hours. The pellet was washed with 95% ethanol, and stored in water at −20°C.

Northern blot. Northern-blot hybridization was performed as previously described.[21] Briefly, RNA was size-fractionated by electrophoresis in 1% agarose gel containing 7% formaldehyde and transferred overnight to nylon membrane. The total mRNA on the membrane was UV cross-linked and hybridized with a [^{32}P]-labeled fragment containing CYP7A1 cDNA probe. The membrane was then exposed.

Ribonuclease protection assay. A RPA probe for rat CYP7B1 mRNA was constructed. First-strand DNA synthesis was performed using the primer 5′-CGTGAATTCGAGCACATCATCTTGGCTTGC-3′ (*Eco*RI site in 5′ extension) in a reaction with reverse transcriptase and mRNA from the liver of a CT-fed rat. An aliquot from the reverse transcriptase reaction was used in a polymerase chain reaction (PCR) with the first-strand primer and the primer 5′-TCGAGATCTGGTCATTGTGTATCATTGGAGG-3′ (*Bgl*II site in 5′ extension) to produce a PCR product that was cloned into the *Eco*RI/*Bgl*II sites of pSP72 (Promega, Madison, WI). The resulting plasmid was used for production of an [α-^{32}P]-UTP-labeled RNA probe using the Maxiscript T7 in vitro transcription kit from Ambion. The RNA probe provided a 363-base protected fragment from rat CYP7B1 mRNA in the RPA assay, which was performed using the RPA III kit from Ambion.

Quantitation of both Northern blot and RPA analysis. Using rat cyclophilin as the internal loading control, the absorbencies for hybridization for CYP7A1, CYP7B1, and cyclophilin were determined by laser densitometry for each condition. The resulting indexes were compared with paired controls. Data are expressed as percent of control values (means ± SE).

Statistical Analysis

Significance of differences between groups was determined using Student's *t* test.

RESULTS

It has recently been reported that CYP7A1 is capable of 7α-hydroxylating not only cholesterol as originally proposed, but other oxysterols as well, including 25- and 27-hydroxycholesterol.[27] To then demonstrate the validity of the enzyme assay for CYP7B1 under the conditions used , the experiments outlined in the Methods were performed. The results are summarized as follows: (1) use of ^{14}C-cholesterol as substrate elicited no products, ie, no significant CYP7A1 activity under conditions employed; (2) 7-oxocholesterol, an inhibitor of CYP7A1, but CYP7B1 did not alter the CYP7B1 activity changes found with the following selected conditions: CT feeding, chronic biliary diversion, and squalestatin infusion (data not shown); (3) use of DHEA under saturating conditions (saturation studies demonstrated the need for addition of cold DHEA in at least a 25-μmol/L concentration to elicit upregulatory changes in CYP7B1 activity) elicited CYP7B1 activity changes with CT feeding (↑ 16% ± 3%; $P < .05$), which paralleled changes observed using 25-hydroxycholesterol as substrate; and (4) changes in CYP7B1 activity observed in mitochondria paralleled changes observed in microsomes. Mitochondrial preparations lacked detectable CYP7A1 activity. Of note is that although regulatory changes in rat mitochondrial CYP7B1 activity paralleled changes in microsomal CYP7B1, the basal CYP7B1 activity level in mitochondria was 55% ± 5% of that found in paired microsomes (n = 4). Finally, a distinctly different diurnal pattern of CYP7A1 and CYP7B1 activities was observed with the described enzyme assays. The above findings strongly suggest that CYP7B1 activity assay used in this study represented a reliable indicator of CYP7B1 activity.

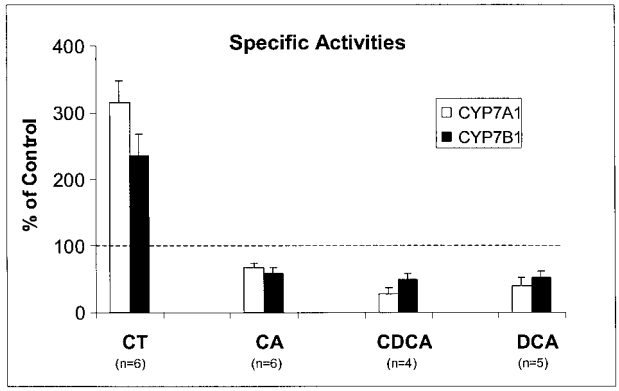

Fig 1. Effect of CT and hydrophobic bile acid feeding on the SAs of CYP7B1 and CYP7A1. Rats were fed CT (5% of diet), CA (1%), CDCA (1%), or DCA (0.25%) for 14 days. Data are expressed as a percent of paired-fed control values (means ± SE). Actual SAs (mean ± SE) for CYP7A1 and CYP7B1 of entire feeding control group were 43 ± 12 and 4.1 ± 0.23 pmol/mg of microsomal protein · min^{-1}, respectively.

SAs in control rats demonstrated a sexual dimorphism for CYP7B1 but not for CYP7A1. The SAs for CYP7A1 were 35.6 ± 3.3 and 35.3 ± 5 pmol/mg microsomal protein-min^{-1} for male (n = 15) and female (n = 11) chow-fed controls, respectively. Female CYP7A1 activity was 99% ± 14% (P = not significant [NS]) of males. The SA activities for CYP7B1 were 4.8 ± 0.46 and 3.6 ± 0.27 pmol/mg of microsomal protein · min^{-1} for male (n = 18) and female (n = 11) chow-fed controls, respectively. Female CYP7B1 activity was 83% ± 3% ($P < .06$) of male.

Figure 1 shows the SAs of CYP7B1, and for comparative purposes, CYP7A1, after feeding CT, CA, CDCA, or DCA for 14 days, respectively. CT or bile acids were added to the

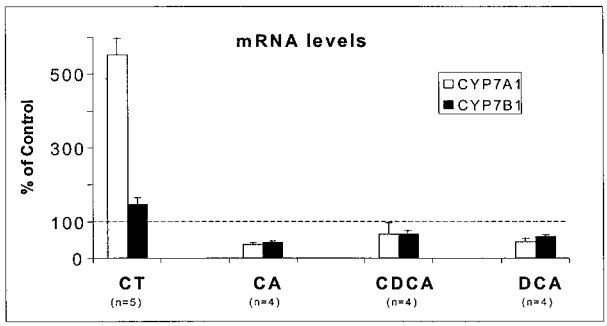

Fig 2. Effect of CT and hydrophobic bile acid feeding on the steady-state mRNA levels of CYP7B1 and CYP7A1. Rats were fed CT (5% of diet), CA (1%), CDCA (1%), or DCA (0.25%) for 14 days. Using rat cyclophilin as the internal loading control, the absorbencies for hybridization for CYP7A1, CYP7B1, and cyclophilin were determined by laser densitometry for each condition. The resulting indexes were compared with paired controls. Data are expressed as percent of control values (means ± SE).

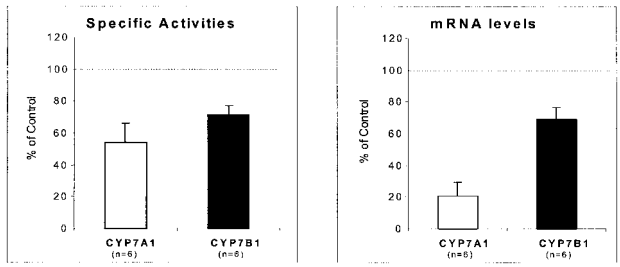

Fig 4. Effect of intraduodenal infusion of TCA on the SAs and steady-state mRNA levels of CYP7B1 and CYP7A1. After 72 hours of CBD, the sodium salt of TCA (36 μmol/h · 100 g rat^{-1}) was continuously infused intraduodenally for 48 hours. Shown are SAs and mRNA levels after 48 hours of TCA infusion. Data are expressed as percent of chronic biliary diverted paired controls (means ± SE; see Methods). Actual SAs (mean ± SE) for CYP7A1 and CYP7B1 of control group were 89.8 ± 11.7 and 9.7 ± 1.5 pmols/mg of microsomal protein · min^{-1}, respectively.

chow-fed to rats with an intact enterohepatic circulation. CT feeding increased CYP7B1 and CYP7A1 activities by 2.4-fold ($P < .004$) and 3.2-fold ($P < .00001$) over paired controls, respectively. CA, CDCA, and DCA feeding decreased CYP7B1 activity 42% ± 9% ($P < .003$), 51% ± 10% ($P < .009$), and 47% ± 9% ($P < .003$), respectively. CYP7A1 activity was decreased 32% ± 6% ($P < .003$), 73% ± 9% ($P < .0002$), and 60% ± 13% ($P < .004$), respectively. No significant differences in hepatic microsomal free cholesterol or serum cholesterol levels have been observed after feeding CT or the respective bile acids in our previous studies.[25]

The effects of CT, CA, CDCA, and DCA on CYP7B1 and CYP7A1 mRNA levels are shown in Fig 2. The alterations in CYP7B1 mRNA levels were similar to those in specific activities. CT increased both CYP7B1 and CYP7A1 mRNA levels 1.5-fold ($P < .05$) and 5.5-fold ($P < .0001$), respectively, over paired controls. The addition of CA, CDCA, and DCA to rat chow led to a decrease in CYP7B1 mRNA levels by 57% ± 3% ($P < .0001$), 43% ± 8% ($P < .007$), and 41% ± 5% ($P < .0002$), respectively. Decreases in CYP7A1 mRNA levels were similar, being decreased 63% ± 6% ($P < .0001$), 43% ± 3% ($P < .02$), and 54% ± 8% ($P < .0001$) of pair-fed controls, respectively.

Chronic biliary diversion (CBD) led to a 4.9-fold increase ($P < .008$) in CYP7A1 mRNA levels with only a 23% increase ($P < .006$) noted in CYP7B1 mRNA levels. CBD increased both CYP7A1 and CYP7B1 activity by approximately 3-fold ($P < .002$ and $P < .007$, respectively) (Fig 3).

Figure 4 shows the SAs and mRNA levels of CYP7B1 after continuous intraduodenal infusion of TCA (36 μmol/h · 100 g rat^{-1}) for 48 hours. CYP7B1 activity and mRNA levels were 71% ± 6% ($P < .0002$) and 70% ± 9% ($P < .0002$) of controls compared with CYP7A1 activity and mRNA levels, 54% ± 12% ($P < .012$) and 21% ± 9% ($P < .0001$) of controls.

The effects of a diet high in Chol (2% or 4%) on the SAs of CYP7B1 are shown in Fig 5. CYP7B1 activities after feeding

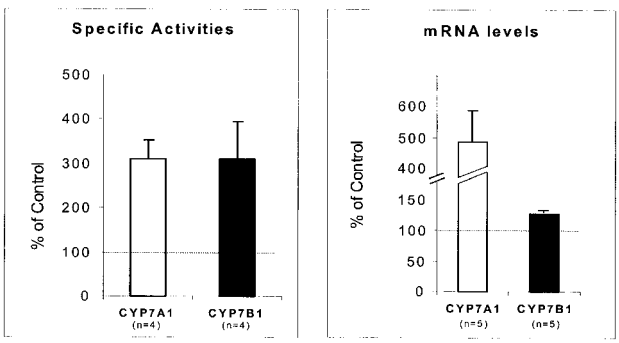

Fig 3. Effect of biliary diversion (CBD) on the SAs and steady-state mRNA levels of CYP7B1 and CYP7A1. Shown are the SAs and mRNA levels after 120 hours of CBD. Data are expressed as percent of control (sham-operated) values (means ± SE; see Methods). Actual SAs (means ± SE) for CYP7A1 and CYP7B1 of the control group were 29.8 ± 7.3 and 3.6 ± 0.5 pmol/mg of microsomal protein · min^{-1}, respectively.

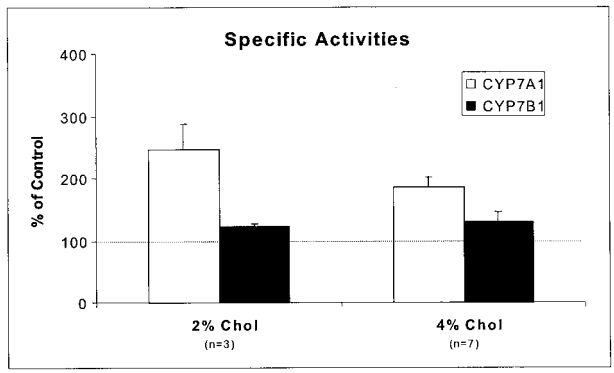

Fig 5. Effect of Chol feeding on the SAs of CYP7B1 and CYP7A1, and on rat serum cholesterol levels. Rats were pair-fed Chol (2% or 4% of diet) for 14 days. Data are expressed as percent of paired controls (means ± SE; see Methods). Actual SAs of CYP7A1 and CYP7B1 for 2% and 4% paired controls were 39.2 ± 6.8 and 4 ± 0.3 pmol/mg of microsomal protein · min^{-1}, and 37.7 ± 4.2 and 4.6 ± 0.4 pmol/mg of microsomal protein · min^{-1}, respectively.

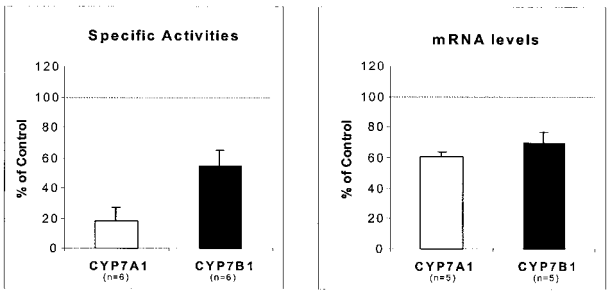

Fig 6. Effect of intravenous infusion of squalestatin on the SAs and steady-state mRNA levels of CYP7B1 and CYP7A1. After 72 hours of CBD, squalestatin (15 μg/h) was continuously infused for 48 hours. Shown are the SAs and mRNA levels after 48 hours of squalestatin infusion. Data are expressed as percent of chronic biliary diverted paired controls (means ± SE). Actual SAs (mean ± SE) for CYP7A1 and CYP7B1 of control group were 87 ± 12.7 and 9.7 ± 1.5 pmol/mg of microsomal protein · min^{-1}, respectively.

a diet enriched with 2% Chol (122% ± 5% of controls; P = NS) and 4% cholesterol (130% ± 16% of controls; P = NS) were not significantly different from controls. In separate studies in primary rat hepatocytes,[29] mevalonate (cholesterol precursor) or cholesterol addition to the hepatocyte culture medium led to a small, but significant increase in CYP7B1 activity. In contrast, CYP7A1 levels after feeding a diet enriched with 2% cholesterol (↑ 2.4-fold; $P < .01$) and 4% Chol (↑ 1.9-fold; $P > .0007$) were significantly increased over controls. Chol feeding had no significant impact on serum cholesterol levels in the rat (control rat serum cholesterol levels, 47 ± 1.3 mg/dL). Hepatic microsomal free cholesterol levels were significantly increased with 4% Chol feeding (↑ 1.5-fold; $P > .01$), but were not altered by 2% Chol feeding.

In rats with CBD, continuous squalestatin infusion (Fig 6) for 48 hours resulted in a 54% ± 10% ($P < .0003$) decrease in SA and 31% ± 7% ($P < .0003$) decrease in mRNA levels for CYP7B1. As previously shown, CYP7A1 SA (18% ± 8% of control; $P < .0001$) and mRNA levels (61% ± 2% of control; $P < .002$) significantly decreased with squalestatin infusion.

Figure 7 shows the SAs of CYP7B1 and CYP7A1 over a 24-hour cycle (diurnal variation). Four to 11 animals were tested for each time point with greatest values at the midnight and noon time points. There were similarities and differences in diurnal variations between the 2 enzymes. Both CYP7B1 and CYP7A1 demonstrated a diurnal variation, but with a slightly different phase. As previously reported, the nadir of CYP7A1 SAs is at noon, whereas it peaks at midnight. In contrast, the peak SA for CYP7B1 presented 6 hours (ie, 6 AM) after the CYP7A1 peak.

DISCUSSION

In this in vivo study in the rat, the effects of bile acid and cholesterol, diurnal rhythm, and sexual dimorphism on the regulation of liver CYP7B1 were explored. The changes observed in CYP7B1 SAs were paralleled by changes in mRNA levels, suggesting regulation at the transcriptional level. The observed changes in CYP7B1 paralleled changes in CYP7A1. Similar regulatory findings have recently been observed in primary rat hepatocyte cultures, lending support to the findings of this study.[29]

The regulation of CYP7B1 has not been thoroughly studied. CYP7B1 has been traditionally considered part of an "alternative pathway," a bile acid synthesis pathway initiated by mitochondrial 27-hydroxylation of cholesterol.[1,2,30] Although this alternative pathway exists as a distinct pathway from the predominant "classic pathway" initiated by the highly regulated CYP7A1, the 2 pathways are interdependent as evidenced by the ability of CDCA, an end product of both pathways, to feedback repress CYP7A1. Therefore, a similarity in the response of both CYP7A1 and CYP7B1 under like conditions would not be unexpected.

Although intensely studied, the true role of the alternative pathway also has not been clearly determined. It has been suggested that the alternative pathway represents a more primitive pathway of bile acid synthesis that appears early, and, therefore, could be hypothesized to be regulated in a fashion similar to the classic pathway.[1,30-32] However, whether the synthesis of bile acids is the alternative pathway's primary function, or whether it is to generate regulatory oxysterols that are subsequently further metabolized to bile acids, is unclear.[10]

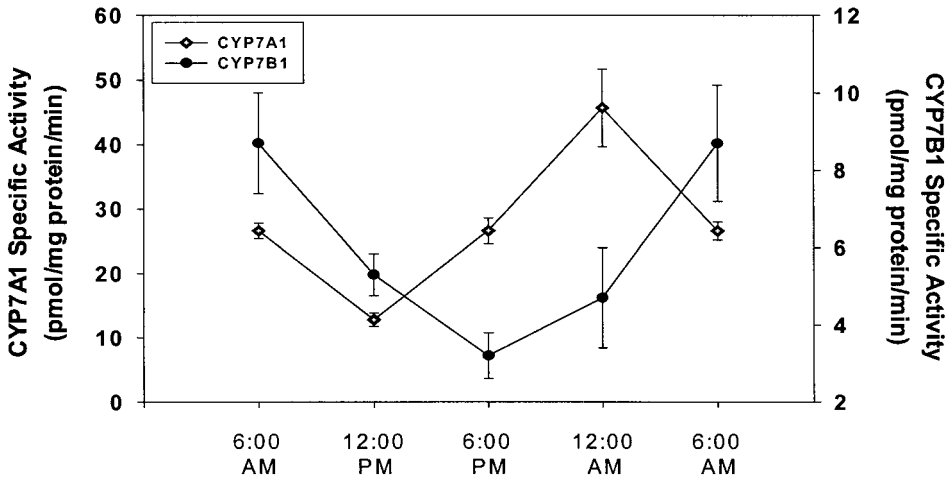

Fig 7. Diurnal variation of CYP7B1 compared to CYP7A1. SAs of CYP7B1 and CYP7A1 were measured every 6 hours over a 24-hour period. SAs are shown in pmol/mg · min^{-1} for CYP7A1 and for CYP7B1 (n ≥ 4 values for each time point).

Furthermore, CYP7B1 serves as a 7α-hydroxylating enzyme found in both the mitochondria and microsomes whose original function may have been to detoxify accumulating potentially cytotoxic oxysterols such as 27-hydroxycholesterol and its metabolites.[33] Based on these premises, the alternative pathway, although able to synthesize bile acids, may exist primarily for other purposes; a hypothesis supported by the ubiquitous existence of CYP27 and CYP7B1 in the body.[15,16,30]

As mentioned, oxysterols are considered regulators of cholesterol homeostasis, with in vitro effects such as repression of sterol responsive genes (ie, HMG-CoA reductase, LDL receptor, fatty acid synthetase).[6,11] In direct support of this statement, increasing 7α-hydroxylation of 27-hydroxycholesterol via overexpression of CYP7B1 activity in CHO cells has been shown to prevent the regulatory effects of this oxysterol on HMG-CoA reductase.[10] However, in *Cyp7b1*$^{-/-}$ mice, where 25- and 27-hydroxycholesterol plasma levels are increased, no major differences in cholesterol homeostasis have been observed.[18] The absence of generalized suppression of sterol synthesis in this and other in vivo models in the presence of the increased oxysterols, 25- and 27-hydroxycholesterol, is unclear, but will need to be explored if the physiologic role of oxysterols as regulators of cholesterol metabolism is to be understood.[18,34]

The ability of cholesterol to regulate CYP7B1 is manifested through the repression of CYP7B1 activity by the cholesterol synthesis inhibitor, squalestatin (Fig 6). In primary rat hepatocytes this repression was also observed, and could be prevented with simultaneous cholesterol addition. In support of this type of regulation is the identification of SRE-like site in the human CYP7B1 promoter.[35] Whether this represents a true sterol regulatory site mediated through previously defined pathways is uncertain. Of interest is that although the depletion of cholesterol led to a significant downregulation of CYP7B1, surplus Chol feeding, resulting in a significant increase in microsomal cholesterol levels, did not lead to any significant change in CYP7B1 activity (Fig 5). The reason for CYP7B1 responsiveness to cholesterol depletion but not to surplus in this model is currently unclear. This lack of CYP7B1 responsiveness to cholesterol surplus, however, has been observed previously both in rats and in mice.[16,36,37]

The concept that feedback repression of bile acid biosynthesis was mediated solely through the regulation of CYP7A1 is being modified. It has now been shown *Cyp7a1*$^{-/-}$ mice can continue to synthesize bile acids (albeit having a smaller bile acid pool), demonstrating their ability to 7α-hydroxylate cholesterol and synthesize bile acids through an alternative pathway or pathways.[18,38] Furthermore, CYP7B1 enzyme activity, protein, and mRNA levels in *Cyp7a1*$^{-/-}$ mice were increased over control levels, an observation that would lend support to the findings of this study.[26] However, Toll et al, in rat liver, failed to see any stimulation of CYP7B1-mediated 7α-hydroxylation activity by the bile acid binding resin, cholestyramine.[39] In the mouse, Schwartz et al also reported no effects of CT feeding on CYP7B1 mRNA expression, but interestingly found a significant decrease in CYP7B1 mRNA levels with cholate feeding.[15] In vitro, using CYP7B1 promoter/luc constructs, Chiang et al have also demonstrated CDCA- mediated suppression of CYP7B1 transcriptional activity (personal communication). The results of this study in the rat not only demonstrate a suppression of CYP7B1 mRNA levels with bile acids, but suppression of CYP7B1 activity as well. These findings have been corroborated in primary rat hepatocytes, where bile acids repressed CYP7B1 mRNA and SA levels.[29] Conversely, CYPB1 mRNA levels and SA levels were increased with chronic biliary diversion and CT feeding. The discrepancy in previous studies findings with CT versus bile acid feeding is not easily reconciled. It is unclear, however, whether CYP7B1 activity in those studies, using CT- treated microsomes, was assayed under substrate saturating conditions (see Methods and Results). Therefore, at least in the rat, the preponderance of data supports the ability of bile acids to elicit negative feedback regulation on CYP7B1—regulation that appears to be mediated predominantly at the transcriptional level. However, the relative importance of these alternative pathways may be species- and/or disease state–dependent,[2,40-43] as well as of more moderate response than observed in these studies if more moderate manipulations are tested.

Although a clear sexual dimorphism was demonstrated for CYP7B1 in the mouse model (male ~3-fold greater than female),[37] only a smaller trend was apparent in the rat. Furthermore, the sexual dimorphism previously observed for CYP7A1 in the rat (female greater than male) was not apparent in these studies. These findings, however, were consistent with the lack of sexual dimorphism demonstrated in the mouse.[37]

Surprisingly, the diurnal rhythm of CYP7B1 is similar in its pattern to CYP7A1, but different in the appearance of a 6-hour time shift. There is no clear explanation for this relationship. It is possible that accumulation of oxysterols is greatest at this time, and that feedback regulation is mediated through the cellular levels of these oxysterols.

In summary, in the rat CYP7B1 activity, like CYP7A1, is regulated by bile acids and cholesterol, and exhibits a diurnal rhythm. As with CYP7A1, these regulatory mediators appear to regulate CYP7B1 at the level of gene transcription.

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