Alternate pathways of bile acid synthesis in the cholesterol 7α-hydroxylase knockout mouse are not upregulated by either cholesterol or cholestyramine feeding

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Abstract  Bile acids are synthesized via the classic pathway initiated by cholesterol 7α-hydroxylase (CYP7A1), and via alternate pathways, one of which is initiated by sterol 27-hydroxylase (CYP27). These studies used mice lacking cholesterol 7α-hydroxylase (Cyp7a1−/−) to establish whether the loss of the classic pathway affected cholesterol homeostasis differently in males and females, and to determine if the rate of bile acid synthesis via alternate pathways was responsive to changes in the enterohepatic flux of cholesterol and bile acids. In both the Cyp7a1−/− males and females, the basal rate of bile acid synthesis was only half of that in matching Cyp7a1+/+ animals. Although bile acid pool size contracted markedly in all the Cyp7a1−/− mice, the female Cyp7a1−/− mice maintained a larger, more cholic acid-rich pool than their male counterparts. Intestinal cholesterol absorption in the Cyp7a1−/− males fell from 46% to 3%, and in the matching females from 58% to 17%. Bile acid synthesis in Cyp7a1+/+ males and females was increased 2-fold by cholesterol feeding, and 4-fold by cholestyramine treatment, but was not changed in matching Cyp7a1−/− mice by either of these manipulations. In the Cyp7a1−/− mice fed cholesterol, hepatic cholesterol concentrations increased only marginally in the males, but rose almost 3-fold in the females. CYP7A1 activity and mRNA levels were greater in females than in males, and were increased by cholesterol feeding in both sexes. CYP27 activity and mRNA levels did not vary as a function of CYP7A1 genotype, gender, or dietary cholesterol intake. We conclude that in the mouse the rate of bile acid synthesis via alternative pathways is unresponsive to changes in the enterohepatic flux of cholesterol and bile acid, and that factors governing gender-related differences in bile acid synthesis, pool size, and pool composition play an important role in determining the impact of CYP7A1 deficiency on cholesterol homeostasis in this species.—Schwarz, M., D. W. Russell, J. M. Dietschy, and S. D. Turley. Alternate pathways of bile acid synthesis in the cholesterol 7α-hydroxylase knockout mouse are not upregulated by either cholesterol or cholestyramine feeding. J. Lipid Res. 2001. 42: 1594–1603.

Supplementary key words bile acid pool size • biliary bile acid • biliary cholesterol • cholesterol absorption • cholesterol synthesis • fecal sterol excretion • liver • small intestine • sterol 27-hydroxylase

The conversion of cholesterol to bile acids in the liver and their subsequent fecal excretion represents a major route for the elimination of cholesterol from the body (1). There have been major advances in our understanding of the molecular mechanisms that regulate the rate of bile acid synthesis in accordance with the amount of cholesterol and bile acid in the enterohepatic circulation (2). Our knowledge of the pathways by which cholesterol can be converted to primary bile acids also continues to expand. These various pathways have been particularly well delineated in the mouse, as illustrated in Fig. 1. The classic or neutral pathway, common to all mammals, is initiated by cholesterol 7α-hydroxylase (CYP7A1). This enzyme is located in the endoplasmic reticulum and catalyzes the rate-limiting step in the neutral pathway (3).

Bile acids can also be synthesized via at least two alternate pathways that utilize a slightly different sequence of initial steps. Cholesterol is first converted to oxysterols by hydroxylation of the side chain, a step that can be catalyzed by three distinct enzymes: sterol 27-hydroxylase (CYP27) in mitochondria, cholesterol 25-hydroxylation in the endoplasmic reticulum, and cholesterol 24-hydroxylase (CYP46), also in the endoplasmic reticulum. The 25- and 27-hydroxycholesterol intermediates are substrates for oxysterol 7α-hydroxylase (CYP7B1) (4), whereas 24-hydroxycholesterol is the preferred substrate for another oxysterol 7α-hydroxylase, CYP39A1 (5). Irrespective of their metabolic origin, all 7α-hydroxylated oxysterols are ultimately converted to primary bile acids via several more enzymatic steps that are shared between the classic and alternate pathways. Studies using mice in which either CYP7A1, CYP27, or CYP7B1 was rendered nonfunctional by gene-targeting methods have yielded new insights into the quantitative contributions of the classic and alternate path-

Abbreviations: CYP, cytochrome P-450.  
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ways in this species (6–9). These and other studies performed with rats suggest that the proportion of total daily bile acid production via the alternate pathways is significantly greater in these animal models than appears to be the case in humans (10, 11). The regulation of the classic pathway has been studied extensively and there is now a considerable body of literature about transcriptional control of CYP7A1 in multiple species (12, 13). In contrast, comparatively little is known about the regulation of the alternate pathways. Although studies of several species have shown that CYP27 activity is responsive to changes in the enterohepatic flux of cholesterol and/or bile acids, other studies of rats suggest that bile acid synthesis via the CYP27-mediated pathway is not regulable (12, 14–18).

Regardless of the pathway by which bile acids are synthesized, they are rapidly secreted via the bile into the lumen of the small bowel, where they become part of a resident pool, the size of which remains essentially constant throughout life (19–21). Several different bile acid transporters located on and within both ileal enterocytes and hepatocytes function to ensure the preservation of this pool during its enterohepatic recycling (22–24). Each time the pool recycles, only a small portion of it is lost into the colon, and under steady state conditions, this is replaced by an equal amount of newly synthesized bile acid from the liver (20, 21). The preservation of the intestinal bile acid pool is critical for the absorption of essential lipids and fat-soluble vitamins. The size and composition of this pool also play a key role in regulating cholesterol balance across the whole body because together they are major determinants of the efficiency with which dietary and biliary cholesterol are absorbed (7, 25–30).

Several parameters of bile acid metabolism in the normal mouse exhibit significant sexual dimorphism, including the activities of the two oxysterol 7α-hydroxylases, CYP39A1 and CYP7B1 (5, 31, 32). The rate of bile acid synthesis and bile acid pool size have consistently been found to be higher in females than in males (32). It has not been determined, however, whether these gender-related differences prevail in mice in which the classic pathway is missing. The present studies therefore were designed to address this question by measuring various parameters of bile acid metabolism in CYP7A1-deficient male and female mice, and to use this animal model to investigate the quantitative importance and regulability of the alternate pathways of bile acid synthesis. The studies show that the rate of bile acid synthesis via the alternate pathways in the mouse is unresponsive to marked changes in the enterohepatic flux of cholesterol and bile acid, and that gender-related differences in pool size and composition are preserved in the absence of the classic pathway.

MATERIALS AND METHODS

Animals and diets

Cholesterol 7α-hydroxylase-deficient mice (Cyp7a1−/−) were generated by crossing homozygous carriers (33). The colony was maintained on a mixed strain background (C57BL/6J;129Sv), and C57BL/6J;129Sv hybrids (Cyp7a11+/+) served as control animals. To increase the survival rate of homozygous pups, the diet of nursing females was routinely supplemented with cholic acid (0.25%, w/w) until the pups were weaned at 30 days of age (34). All experiments, except one, were conducted in mice raised in our own colony, which has remained closed for the past 6 years. For comparative purposes, one experiment was carried out with male and female Cyp7a1−/− mice that were purchased as young adults directly from the Jackson Laboratory (Bar Harbor, ME). All studies were carried out with 3- to 4-month-old male and female mice fed the meal form of a cereal-based rodent diet (Wayne Lab Blox, No. 8604; Harlan Teklad, Madison, WI), which contained 0.02% (w/w) cholesterol and 5% (w/w) total lipid. This is referred to as the basal diet. In several experiments, mice were fed this diet containing added cholesterol (Byron

Fig. 1. Schematic of the pathways through which cholesterol can be converted to primary bile acids in the mouse. This schema is based on one published elsewhere (55).
Chemical, Long Island City, NY) at a final level of 1% (w/w) for 21 days, or cholestyramine at a final level of 2% (w/w) for 10 days. Mice were housed in plastic colony cages containing wood shavings in a temperature-controlled room (22°C) with light cycling. All experiments were performed in the last 3 h of the 12-h dark phase of the light cycle, and all mice were in the fed state at the time of study except those that were fasted for 6 h before harvesting of gallbladder bile. Experiments were approved by the Institutional Animal Care and Research Advisory Committee.

Plasma and tissue cholesterol levels and biliary lipid composition

Plasma and tissue total cholesterol concentrations, and the concentrations of bile acid, phospholipid, and cholesterol in gallbladder bile were measured by methods described previously (7).

Bile acid pool size and composition

Pool size was determined as the total bile acid content of the small intestine, gallbladder, and liver, which were extracted in ethanol in the presence of an internal standard ([24,2H]taurocholic acid, NEN Life Science Products, Boston, MA) and analyzed by HPLC (7). Bile acids were detected by measurement of the refractive index and identified by comparison with authentic standards. Pool size was expressed as micromoles per 100 g body weight.

Fecal bile acid and neutral sterol excretion

Stools collected from individually housed mice over 3 days were dried, weighed, and ground to a fine powder. A 1-g aliquot of this material was used to determine total bile acid content by an enzymatic method previously described (7). A second 1-g aliquot was used to quantitate the amounts of cholesterol, coprosterol, epicoprostanol, and cholestanone by gas chromatography (GC) as described in detail elsewhere (7). The excretion rates of both bile acids and neutral sterols were expressed as micromoles per day per 100 g body weight.

Intestinal cholesterol absorption and whole animal sterol synthesis

Cholesterol absorption was measured by a fecal dual-isotope ratio method (7). A mixture of 2 µCi of [5,6-3H]sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and 1 µCi of [4,4-2H]cholesterol (New England Nuclear, Boston, MA) was administered intragastrically by gavage. Mice were then housed individually in fresh cages and stools were collected over the next 3 days. Aliquots of stool and of the original dosing mixture were extracted, and the percent cholesterol absorption was calculated from the ratio of 14C to 3H as described (7). Whole animal sterol synthesis rates were measured in vivo with 3H2O as described earlier (7, 35). These rates were expressed as the micromoles of 3H2O incorporated into sterols per h per 100 g body weight.

Enzyme activities

Mitochondrial and microsomal membrane fractions were prepared from freshly excised individual liver samples by sequential centrifugation as described (8). Total protein content was determined using bichoninic acid (Pierce, Rockford, IL). All assays were performed immediately after preparation of the membrane fractions. Microsomal fractions were used for measurement of the activity of CYP7A1 by an HPLC method described by Chiang (36). Briefly, 7α-hydroxycholesterol is formed from endogenous cholesterol and is quantitated after conversion to 7α-hydroxy-4-cholesten-3-one, using cholesterol oxidase. CYP27 activity was determined by a modification of the protocol by Petrack and Lattaro (37). Mitochondria (0.5 mg) were incubated at 37°C for 20 min in 100 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM EDTA and 1 mM dithiothreitol in the presence of an NADPH-regenerating system (12 mM NADPH, 5 mM dl-sodium isocitrate, 0.2 U of isocitric dehydrogenase) and exogenous [3H]cholesterol (250 nmol, 2 mCi/mmol), which was delivered in 2-hydroxypropyl-β-cyclodextrin (0.8%, w/v). Incubations were terminated by the addition of 6 ml of methylene chloride. Steroid products were extracted, dried under nitrogen, redissolved in 50 µl of acetone, and analyzed by thin-layer chromatography on adsorption silica gel plates (LK5D) (Whatman, Ann Arbor, MI). Plates were developed in toluene–ethylacetate 2:3 (v/v), radiolabeled products were quantitated with a system 200 imaging scanner (Bioscan, Washington, DC), and 27-hydroxycholesterol was identified by comparing its Rf value with that of an authentic standard (Research Plus, Bayonne, NJ).

The downstream metabolism of 27-hydroxycholesterol in the in vitro system described here warrants several comments because it plays a critical role in product quantitation (38). 27-Hydroxycholesterol is further metabolized to 7α,27-dihydroxycholesterol by the action of oxysterol 7α-hydroxylase (CYP7B1). This enzyme is microsomal, but because of cross-contamination of subcellular fractions during sequential centrifugation its activity is consistently detected in mitochondrial fractions as well. As the activity of CYP7B1 in mouse liver is about 3-fold higher in males than it is in females (32), the newly formed 27-hydroxycholesterol is metabolized at a much lower rate in females than it is in males. This gives rise to an apparent gender difference in the amount of detectable 27-hydroxycholesterol after incubation of cholesterol in the above-described system. To demonstrate that this difference is entirely due to the sexual dimorphism of CYP7B1, we measured CYP27 activity in mitochondrial fractions prepared from the livers of males and female Cyp7b1−/− mice as well as from matching Cyp7b1+/− mice (data not shown). In CYP7B1-deficient mice, equal amounts of 27-hydroxycholesterol were formed from cholesterol irrespective of gender, and there was no downstream conversion of this metabolite. Clearly, then, the most accurate measure of CYP27 activity is one that takes into account the marked gender-related difference in CYP7B1 activity. Hence in the present studies, the activity of CYP27 in each group reflects the inclusion of CYP7B1 activity in those same animals.

RNA analysis

Total RNA was prepared from individual liver samples, using an RNA isolation kit (Tel-Test B, Friendswood, TX). Equal amounts of total RNA from individual animals were pooled and used for mRNA isolation, using an RNA purification kit (Amerham Pharmacia Biotech, Piscataway, NJ). For Northern blotting, 5 µg of poly(A) RNA was fractionated on a formaldehyde–agarose gel. Filters hybridized with full-length radiolabeled cDNA probes encoding murine CYP7A1 and CYP27, respectively, were washed stringently and were exposed to Kodak (Rochester, NY) X-OMAT AR film at −80°C, using an intensifying screen.

Analysis of data

All data are reported as the mean ± 1 SEM in the specified number of individual animals. Differences between mean values were tested for statistical significance (P < 0.05) by the two-tailed Student’s t test.

RESULTS

As shown in Table 1, there were no consistent differences in body weight, liver weight, hepatic cholesterol concentrations, or plasma cholesterol concentrations between Cyp7a1−/− and Cyp7a1+/+ mice of either gender.
when fed the basal diet. Hepatic cholesterol concentrations were about the same in all groups except the Cyp7a1−/− females, in which a small elevation was evident. Plasma cholesterol concentrations in females of both genotypes were marginally lower than those in matching males. However, there was no evidence of hypercholesterolemia in any of the 41 male and female Cyp7a1−/− mice that were studied. This was also true of the Cyp7a1+/− mice that were obtained directly from Jackson Laboratory. The values for the Cyp7a1−/− males and females from that source were 117.6 ± 16.8 mg/dl (n = 6) and 93.2 ± 8.5 mg/dl (n = 6), respectively. Although the absolute concentration of cholesterol in gallbladder bile did not vary as a function of either gender or genotype, the relative cholesterol content of the bile was marginally elevated in the Cyp7a1−/− mice because of the lower bile acid concentrations in these animals.

Previous studies using different strains of normal wild-type mice documented significant gender-related differences in bile acid pool size and composition, and in the efficiency of intestinal cholesterol absorption (32). Therefore, in the first group of experiments we investigated whether such differences prevailed in mice lacking CYP7A1. The data in Fig. 2 show intestinal cholesterol absorption as a function of bile acid pool size in Cyp7a1−/− male and female mice and their respective matching Cyp7a1+/+ controls fed the basal diet. In the Cyp7a1−/− males and females pool size fell from 63 to 21 μmol/100 g body weight and from 85 to 33 μmol/100 g body weight, respectively. In the case of the males this led to a dramatic reduction in the efficiency of intestinal cholesterol absorption (from 46% to 3%). The reduction in absorption was less pronounced in the Cyp7a1−/− females (from 58% to 17%). This correlated directly with the preservation of a larger, more cholic acid-rich pool in the Cyp7a1−/− females than was the case in their male counterparts. As shown by the data in the inset in Fig. 2, the ratio of cholic to muricholic acid in the pool of the females, irrespective of genotype, was consistently higher than in the corresponding males. This was particularly the case in the Cyp7a1−/− animals, in which the ratio for females (1.8 ± 0.2) was 2.6-fold higher than in matching males (0.7 ± 0.1). Together, then, the data in Fig. 2 demonstrate the major regulatory role that bile acid pool size and composition play in dictating the level of intestinal cholesterol absorption, and that the gender difference in pool size and composition seen in Cyp7a1+/+ mice is preserved in mice lacking CYP7A1. A comparison of the data in Fig. 2 and Table 1 shows that there was no correlation between the level of cholesterol absorption and biliary cholesterol concentration.

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**Fig. 2.** Intestinal cholesterol absorption as a function of bile acid pool size in Cyp7a1+/+ and Cyp7a1−/− mice. Male and female mice of each genotype, 3 to 4 months of age, were housed individually and fed the basal diet without added cholesterol. Cholesterol absorption was measured by a fecal isotope ratio method (7). Some of these animals from each group were subsequently used for the determination of bile acid pool size and composition (7). Pool size represents the total bile acid content of the small intestine, gallbladder, and liver. The ratio of cholic acid (CA) to muricholic acid (MCA) in the pool is shown in the inset. For the cholesterol absorption measurements, values represent the mean ± 1 SEM of data from 10 and 14 Cyp7a1+/+ males and females, respectively, and from 40 and 31 Cyp7a1−/− males and females, respectively. For the bile acid pool size and composition measurements, values represent the mean ± 1 SEM of data from 8 and 7 Cyp7a1+/+ males and females, respectively, and from 13 and 16 Cyp7a1−/− males and females, respectively. For the data in the inset, an asterisk denotes that the value for the Cyp7a1−/− animals is significantly different from that for the Cyp7a1+/+ mice of the same gender (P < 0.05). The double dagger denotes that the value for the females is significantly different from that for males of the same genotype (P < 0.05).

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**Table 1.** Hepatic and plasma cholesterol and biliary lipid concentrations in Cyp7a1+/+ and Cyp7a1−/− mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male Cyp7a1+/+</th>
<th>Male Cyp7a1−/−</th>
<th>Female Cyp7a1+/+</th>
<th>Female Cyp7a1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27 ± 1 (19)</td>
<td>26 ± 1 (26)</td>
<td>25 ± 1 (18)</td>
<td>22 ± 1 (22)</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.34 ± 0.04 (17)</td>
<td>1.51 ± 0.07 (22)</td>
<td>1.28 ± 0.05 (11)</td>
<td>1.26 ± 0.04 (19)</td>
</tr>
<tr>
<td>Hepatic total cholesterol concen-</td>
<td>40.4 ± 0.05 (17)</td>
<td>23.9 ± 0.06 (22)</td>
<td>25.2 ± 0.09 (11)</td>
<td>29.7 ± 0.17 (19)</td>
</tr>
<tr>
<td>Plasma total cholesterol concen-</td>
<td>87.2 ± 4.4 (17)</td>
<td>83.2 ± 4.3 (22)</td>
<td>74.6 ± 5.7 (11)</td>
<td>70.6 ± 3.7 (19)</td>
</tr>
<tr>
<td>Biliary cholesterol concen. (μmol/ml)</td>
<td>1.9 ± 0.1 (8)</td>
<td>2.0 ± 0.2 (6)</td>
<td>2.4 ± 0.3 (6)</td>
<td>2.1 ± 0.1 (5)</td>
</tr>
<tr>
<td>Biliary phospholipid concen. (μmol/ml)</td>
<td>17.4 ± 1.8 (8)</td>
<td>15.0 ± 1.8 (6)</td>
<td>28.3 ± 3.1 (6)</td>
<td>25.2 ± 4.7 (5)</td>
</tr>
<tr>
<td>Biliary bile acid concen. (μmol/ml)</td>
<td>160.1 ± 11.1 (8)</td>
<td>123.7 ± 12.4 (6)</td>
<td>208.5 ± 7.7 (6)</td>
<td>110.4 ± 13.2 (5)</td>
</tr>
<tr>
<td>Molar ratio of cholesterol in bile (%)</td>
<td>1.1 ± 0.1 (8)</td>
<td>1.5 ± 0.2 (6)</td>
<td>1.0 ± 0.1 (6)</td>
<td>1.6 ± 0.2 (5)</td>
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All mice were fed ad libitum a basal rodent diet without added cholesterol. They were 3 to 4 months old when studied. The data represent the mean ± 1 SEM of values for the number of animals indicated in parentheses.

* The value for females is significantly different from that for males of the same genotype (P < 0.05).

* The value for Cyp7a1−/− animals is significantly different from that for the Cyp7a1+/+ mice of the same gender (P < 0.05).
In these same four groups of mice, there was an inverse correlation between the rate of fecal neutral sterol excretion and the level of cholesterol absorption (Fig. 3). Clearly, the diminished level of absorption in the Cyp7a1\(^{-/-}\) animals was reflected in a greater loss of sterol in the feces. This resulted not just from the excretion of greater amounts of unabsorbed dietary cholesterol, but of additional endogenously derived cholesterol arising from the compensatory increase in cholesterol synthesis (Fig. 3, inset). These data again revealed a marked gender difference among the Cyp7a1\(^{-/-}\) mice. Thus, in the Cyp7a1\(^{-/-}\) males, in which cholesterol absorption was reduced almost to zero, there was a greater compensatory increase in cholesterol synthesis, and a marginally higher loss of sterol in the feces compared with matching Cyp7a1\(^{+/+}\) females. Together, the data in Figs. 2 and 3 demonstrate that CYP7A1 deficiency had a greater impact on cholesterol balance in males than in females. This difference correlated directly with the ability of the females to maintain a larger, more cholic acid-rich pool than the males under circumstances in which only the alternate pathways of bile acid synthesis were operational.

The next series of experiments was designed to test the regulability of the alternate pathways of bile acid synthesis by either interrupting the enterohepatic circulation of bile acids by feeding cholestyramine, or by increasing the delivery of chylomicron cholesterol to the liver by feeding a high cholesterol diet. In these studies the rate of bile acid synthesis was measured as the daily rate of fecal bile acid excretion per 100 g body weight. In the cholestyramine feeding experiment, groups of matching male and female Cyp7a1\(^{+/+}\) and Cyp7a1\(^{-/-}\) mice were initially fed only the basal diet before being switched to the diet containing 2% (w/w) of the resin. Stool collections were made from each animal before and during cholestyramine feeding. As shown in Fig. 4, there was a greater than 4-fold increase in bile acid synthesis in both the Cyp7a1\(^{+/+}\) males (Fig. 4A) and Cyp7a1\(^{-/-}\) females (Fig. 4B) fed the resin. In marked contrast, no such upregulation was seen in the matching Cyp7a1\(^{-/-}\) males or females.

In the cholesterol feeding study, the basal and cholesterol-rich diets were fed simultaneously to separate groups of matching male and female mice of each genotype. The data in Fig. 5 show that in both the males (Fig. 5A) and females (Fig. 5B) there was clearly an upregulation of bile acid synthesis in the Cyp7a1\(^{-/-}\) mice, but not in the matching Cyp7a1\(^{+/+}\) animals. Similar results were found for bile acid pool size in these same groups of animals

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**Fig. 3.** Fecal neutral sterol excretion as a function of intestinal cholesterol absorption in Cyp7a1\(^{+/+}\) and Cyp7a1\(^{-/-}\) mice. Male and female mice of each genotype were housed individually and fed the basal diet without added cholesterol. Cholesterol absorption was measured by a fecal isotope ratio method. In most of these same mice, the rate of fecal neutral sterol (cholesterol and its derivatives coprostanol, epicoprostanol, and cholestanone) excretion was also determined (7). In separate, smaller groups of matching mice, the rate of whole animal sterol synthesis was determined (7). These rates represent the micromoles of \(^3\)H\(_2\)O incorporated into sterols per hour per 100 g body weight. The numbers of animals in each group in which cholesterol absorption measurements were made are the same as those given in the legend to Fig. 2. For the fecal neutral sterol excretion measurements, values represent the mean ± 1 SEM of data from 10 and 14 Cyp7a1\(^{+/+}\) males and females, respectively, and from 28 and 29 Cyp7a1\(^{-/-}\) males and females, respectively. For the whole animal sterol synthesis measurements (inset), values represent the mean ± 1 SEM of data from six and eight Cyp7a1\(^{+/+}\) males and females, respectively, and from five and seven Cyp7a1\(^{-/-}\) males and females, respectively. An asterisk denotes that the value for the Cyp7a1\(^{-/-}\) animals is significantly different from that for the Cyp7a1\(^{+/+}\) mice of the same gender \((P<0.05)\). The double dagger denotes that the value for the females is significantly different from that for males of the same genotype \((P<0.05)\).

**Fig. 4.** Rate of fecal bile acid excretion in Cyp7a1\(^{+/+}\) and Cyp7a1\(^{-/-}\) mice fed cholestyramine. Male and female mice of each genotype were housed individually and fed the basal diet without any additions. Stools were collected from each animal over a 3-day period. All mice were then switched to the same basal diet containing cholestyramine for the next 10 days. Stools were collected over the last 3 days of this feeding period. The rate of fecal bile acid excretion from each animal before and during cholestyramine feeding was then determined. Values represent the mean ± 1 SEM of data from five and five Cyp7a1\(^{+/+}\) males and females, respectively, and from five and five Cyp7a1\(^{-/-}\) males and females, respectively. An asterisk denotes that the value for the period during cholestyramine feeding is significantly different from that found when the mice received the basal diet alone \((P<0.05)\). The double dagger denotes that the value for the females is significantly different from that for the males of the same genotype that were fed the same diet \((P<0.05)\).
more than it was in the males (Fig. 7A). Third, in contrast cholesterol levels in the females (Fig. 7B) was appreciably elevated (Fig. 7A and B). Second, in the case of the Cyp7a1-/- males and females, respectively, and from 35 and 25 Cyp7a1-/- males and females, respectively. Values for the mice fed the basal diet alone represent the mean ± 1 SEM of data from 8 and 9 Cyp7a1+/- males and females, respectively, and from 35 and 25 Cyp7a1-/- males and females, respectively. An asterisk denotes that the value for the animals fed the high cholesterol diet is significantly different from that for animals of the same genotype and gender that were fed the basal diet alone (P < 0.05). The double dagger denotes that the value for the females is significantly different from that for males of the same genotype that were fed the same diet (P < 0.05). (Fig. 6). Thus, while there was a marginal expansion of pool size in the cholesterol Cyp7a1+/- males (Fig. 6A) and Cyp7a1+/- females (Fig. 6B), this did not occur in the matching Cyp7a1-/- mice. In all groups of mice the two predominant bile acids in the pool were cholic and muricholic acid. In the Cyp7a1+/+ females, and particularly in the Cyp7a1-/- females, the ratio of cholic to muricholic acid was significantly greater than it was in males of the same genotype given the same diet (Fig. 6C and D). Cholesterol feeding lowered the ratio of cholic to muricholic acid in both male and female Cyp7a1+/- mice, but had no such effect in the Cyp7a1-/- animals of either gender.

The hepatic cholesterol concentrations in the eight groups of mice used in the cholesterol-feeding experiment are given in Fig. 7. Several gender- and genotype-related effects on hepatic cholesterol accumulation were evident. First, when the basal diet alone was fed, hepatic cholesterol levels were about the same in all groups, except in the Cyp7a1-/- females, in which they were marginally elevated (Fig. 7A and B). Second, in the case of the Cyp7a1+/- animals fed cholesterol, the increase in hepatic cholesterol levels in the females (Fig. 7B) was appreciably more than it was in the males (Fig. 7A). Third, in contrast to their Cyp7a1+/- controls, the Cyp7a1-/- males showed almost no change in hepatic cholesterol content when fed the high cholesterol diet (Fig. 7A). A strikingly different result was seen for the cholesterol-fed Cyp7a1-/- females, which showed about the same 3-fold increase in liver cholesterol levels as did their matching Cyp7a1+/- controls (Fig. 7B). However, among the 23 Cyp7a1-/- females fed cholesterol, there was wide individual variability in their responsiveness, with hepatic cholesterol concentrations ranging from 2.5 to 35 mg/g. Cholesterol feeding did not significantly raise plasma total cholesterol concentrations in males or females of either genotype (data not shown).

The final sets of data describe the activity (Fig. 8) and mRNA (Fig. 9) levels of CYP7A1 and CYP27 in groups of mice that matched those used in the cholesterol-feeding studies described in Figs. 5–7. CYP7A1 activity (Fig. 8A and B) and mRNA (Fig. 9) levels were increased in the cholesterol-fed mice, irrespective of gender, and were marginally higher in female mice than in male mice, irrespective of diet. In contrast, CYP27 activity (Fig. 8C and D) and mRNA...
levels (Fig. 9) did not consistently vary as a function of CYP7A1 genotype, gender, or dietary cholesterol intake.

DISCUSSION

The study of the quantitative importance and regulability of the alternate pathways of bile acid synthesis is challenging because at least three separate pathways are involved. To date, most studies of these questions have focused on the pathway initiated by CYP27, which in humans apparently accounts for only about 9% of total bile acid synthesis (11). The percent contribution of this pathway to bile acid production in other species continues to be investigated. It cannot be determined by deleting the gene for CYP27 because this also obstructs the classic pathway, which requires CYP27 for oxidation of the cholesterol side chain (3). Thus, while the contribution of the CYP27-mediated pathway remains unclear, that from the pathway initiated by CYP46 is probably trivial because 24-hydroxycholesterol formation takes place predominantly in the brain, where the rate of cholesterol turnover is only a fraction of that in the other major organs (39–41). Similarly, bile acid synthesis via cholesterol 25-hydroxylase is likely to be quantitatively minor given that the tissue levels of mRNA for this enzyme are low in the mouse (42). Despite the lack of information regarding the quantitative importance of the CYP27 pathway in different animal models, the activity and/or mRNA levels of this enzyme are routinely used as markers for the output of bile acid from this pathway. Such studies in rabbits, rats, and mice imply that there might be species differences in the extent to which the CYP27-mediated pathway responds to changes in the enterohepatic flux of cholesterol and bile acid (10, 14–18). The resolution of this question is of major importance to the development of therapies aimed at lowering plasma LDL cholesterol concentrations by promoting the production and excretion of bile acids.

Three points regarding the design of the present studies warrant emphasis. First, the Cyp7a1 knockout mouse provided a well-defined model in which the regulability of the alternate pathways of bile acid synthesis could be studied without interference from concomitant changes in the CYP7A1-mediated pathway. Second, because of well-documented gender-related effects on bile acid metabolism in various species, particularly the mouse, our experimen...
ments were performed in matching groups of male and female Cyp7a1−/− animals, together with their corresponding Cyp7a1+/+ controls. Third, as all mice were studied under steady state conditions, we used fecal bile acid excretion as a measure of the rate of bile acid synthesis. In the cholesterol-feeding studies, these excretion data were supported by measurements of the activity and mRNA level of both CYP7A1 and CYP27.

Three major conclusions can be drawn from these studies. The first, which is based on the data for the mice fed only the low cholesterol basal diet, is that the gender difference in total bile acid synthesis in this species can be attributed entirely to a difference in production via the classic pathway, not the alternate pathways. Thus, in the absence of CYP7A1, the basal rate of bile acid excretion in both sexes fell to about 5 μmol/day per 100 g body weight from a value of about 9 μmol/day per 100 g body weight in the males, and of about 14 μmol/day per 100 g body weight in the females. The activity of CYP7A1, and especially the mRNA levels for this enzyme, were higher in the females than in the males. In contrast, the activity and mRNA level of CYP27 were about the same, irrespective of CYP7A1 genotype, or of gender. Clearly, in neither the males nor the females did the loss of CYP7A1 result in a compensatory upregulation of CYP27. There are few published data relating to gender differences concerning these enzymes in other species, although in rats the activity of CYP7A1 was reported to be higher in females (43). The opposite finding has been described in hamsters (38). In humans, the rate of bile acid synthesis is marginally lower in females than in males, but it is unknown whether this reflects lower CYP7A1 activity (44).

The second conclusion, also drawn from the data for the mice fed the basal diet, relates to the cause and consequences of the larger, more cholic acid-rich bile acid pool in the females. Although the higher rate of bile acid production via the classic pathway possibly contributed to these different pool features in the females, this could not have been the only factor because in the mice lacking CYP7A1, the pool remained marginally larger and decisively more hydrophobic in the females, even though their rate of bile acid synthesis via the alternate pathways was the same as in their male counterparts. The increased hydrophobicity of the bile acid pool may be due to sexually dimorphic expression patterns of the CYP7B1 and CYP39A1 7α-hydroxylases (5, 32) or of sterol 12α-hydroxylase (CYP8B1) (45). It may also reflect gender differences in the bacterial modification of bile acids entering the pool from the liver. The increased size of the pool in females may relate to gender differences in the activity of one or more of the hepatic or ileal bile acid transporters. Irrespective of why pool characteristics remain different in the females when the classic pathway is absent, it is likely that the existence of a larger, more cholic acid-rich pool in these animals fully accounts for their ability to maintain a modestly efficient level of cholesterol absorption (17%) compared with the level manifest in their male counterparts (3%). It is well documented in many species, including the mouse, that even relatively subtle shifts in pool size and composition can dramatically alter the amount of chylomicron cholesterol that reaches the liver from the small bowel (7, 25, 29, 30). This differential in the efficiency of cholesterol absorption between the male and female Cyp7a1−/− mice was associated with significant quantitative, but not qualitative, differences between the sexes in the ensuing adaptive changes in whole body sterol balance. Thus, compared with their male counterparts, the Cyp7a1−/− females manifested less of a compensatory upregulation of whole body sterol synthesis, and excreted marginally less cholesterol in their stools. As a result of their compensatory adjustments in cholesterol synthesis and excretion, both the male and female Cyp7a1−/− mice were able to maintain normal plasma total cholesterol concentrations, and circumvent overt cholesterol accumulation in their livers under circumstances in which their daily loss of cholesterol as acidic steroids was dramatically reduced. While these findings are not new for the Cyp7a1−/− males (7), it is noteworthy that their female counterparts also maintained essentially normal cholesterol homeostasis when their rate of bile acid synthesis fell to only 40% of normal values compared with 56% in the case of the Cyp7a1−/− males.

The third conclusion concerns the unresponsiveness of the alternate pathways of bile acid synthesis to marked
changes in the enterohepatic flux of cholesterol or bile acid, and the impact that this had on whole animal cholesterol homeostasis when dietary cholesterol intake was overly increased. The experiment with cholestyramine feeding clearly showed that while this agent produced a major derepression of bile acid synthesis in the Cyp7a1+/+ males and females, it was completely ineffective in their Cyp7a1−/− counterparts. The activity and mRNA levels for CYP7A1 and CYP27 were not determined in the cholestyramine-fed mice. However, it is well documented in several species, including the mouse, that not only does this treatment markedly increase CYP7A1 activity, but that it also induces a parallel increase in total bile acid synthesis (46–48). Other studies have shown that the interruption of the enterohepatic circulation of bile acids by either cholestyramine treatment or biliary drainage also raises the activity and mRNA level of CYP27 in some, but not all, species (12, 15, 17). It is unclear, however, whether such derepression of CYP27 significantly alters the amount of bile acid generated via this pathway in these models. The present studies suggest that, at least in the mouse, bile acid output from the combined alternative pathways remains independent of the amount of bile acid undergoing enterohepatic recirculation.

The cholesterol-feeding experiment provided a clearer insight into the relative importance of the classic and alternate pathways in the handling of chylomicron cholesterol by the liver. Thus, in both the male and female Cyp7a1+/+ mice fed cholesterol there was a significant increase in bile acid synthesis, and a generally parallel increase in the activity and mRNA level of CYP7A1. These animals also showed a modest expansion of their bile acid pool. The activity and mRNA level of CYP27 were not changed by cholesterol feeding in either the male or female Cyp7a1+/+ or Cyp7a1−/− mice. Bile acid synthesis and pool size also were unchanged in the Cyp7a1−/− mice fed cholesterol. In the case of the Cyp7a1−/− males, this result was predictable because their efficiency of cholesterol absorption was so low that the small additional amount of chylomicron cholesterol delivered to the liver was likely fully compensated for by an adjustment in de novo cholesterol synthesis. Hence, it was not surprising that hepatic cholesterol concentrations in the majority of the cholesterol-fed Cyp7a1−/− males were the same as those in the Cyp7a1+/+ males fed only the basal diet. This was clearly not the case in the cholesterol-fed Cyp7a1−/− females, in which the more efficient absorption of cholesterol, combined with the inability of the liver to channel this sterol into bile acid synthesis via the alternate pathways, led to the same degree of hepatic cholesterol accumulation as was seen in the Cyp7a1+/+ females fed cholesterol. Despite their elevated hepatic cholesterol levels, neither the Cyp7a1+/+ nor the Cyp7a1−/− females fed cholesterol became hypercholesterolemic, presumably because of the remarkably high rate of hepatic LDL clearance found in this species (49). Together, these data from the cholestyramine-feeding study demonstrate that, in the mouse, the alternate pathways of bile acid synthesis are not subject to the liver X receptor- and farnesoid X receptor-mediated control mechanisms that regulate CYP7A1 and the classic pathway (2).

Finally, two points should be made concerning the relevance of these studies to the development of new treatments for lowering plasma LDL cholesterol concentrations in the general population (50, 51). One is that, although significant differences in bile acid metabolism exist between humans and mice, the current data imply that cholesterol-lowering treatments that are designed to drive bile acid synthesis and excretion should focus on the classic pathway, not the alternate pathways, of bile acid synthesis. The other point concerns the fundamentally important role that the bile acid pool plays in dictating the efficiency of cholesterol absorption and the balance of cholesterol across the whole body. This fact is particularly relevant to current attempts to determine whether the uptake of cholesterol by the enterocyte is protein mediated (52–54). If such a transporter(s) is identified, then its suitability as a target for pharmacological control will depend partly on the changes in bile acid pool size and composition that ultimately result from blocking the enterohepatic flux of cholesterol.11

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