Disruption of the Oxysterol 7α-Hydroxylase Gene in Mice*

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Mice without oxysterol 7α-hydroxylase, an enzyme of the alternate bile acid synthesis pathway with a sexually dimorphic expression pattern, were constructed by the introduction of a null mutation at the Cyp7b1 locus. Animals heterozygous (Cyp7b1+/−) and homozygous (Cyp7b1−/−) for this mutation were grossly indistinguishable from wild-type mice. Plasma and tissue levels of 25- and 27-hydroxycholesterol, two oxysterol substrates of this enzyme with potent regulatory actions in cultured cells, were markedly elevated in Cyp7b1−/− knockout animals. Parameters of bile acid metabolism as well as plasma cholesterol and triglyceride levels in male and female Cyp7b1−/− mice were normal. The cholesterol contents of major tissues were not altered. In vivo sterol biosynthetic rates were unaffected in multiple tissues with the exception of the male kidney, which showed a ~40% decrease in de novo synthesis versus controls. We conclude that the major physiological role of the CYP7B1 oxysterol 7α-hydroxylase is to metabolize 25- and 27-hydroxycholesterol and that loss of this enzyme in the liver is compensated for by increases in the synthesis of bile acids by other pathways. A failure to catabolize oxysterols in the male kidney may lead to a decrease in de novo sterol synthesis.

7α-Hydroxylated bile acids are the end products of cholesterol (5-cholesten-3-ol) catabolism whose synthesis is required to maintain cholesterol homeostasis. Bile acids are synthesized in the liver by two metabolic pathways that differ in their initial steps (1). The classical pathway is initiated with the conversion of cholesterol to 7α-hydroxycholesterol (cholest-5-ene-3β,7α-diol) by the enzyme cholesterol 7α-hydroxylase (encoded by the Cyp7a1 locus). The alternate pathway involves an initial hydroxylation of the side chain of cholesterol to produce an oxysterol, which is then hydroxylated at the 7-position by an oxysterol 7α-hydroxylase (encoded by the Cyp7b1 locus). Once these enzymes form 7α-hydroxylated intermediates, subsequent steps leading to the synthesis of a primary bile acid are shared between the two pathways (2).

The regulation of these pathways and their individual contributions to bile acid metabolism in the mouse are currently being explored using molecular methods. Studies to date show that modulating transcription of the cholesterol 7α-hydroxy-

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lase gene regulates output from the classical pathway. Excess bile acids suppress transcription from this gene by acting as ligands for the nuclear hormone receptor FXR (3–5), whereas oxysterols increase transcription of the gene by activating the nuclear hormone receptor LXRα (6–8). Perturbations in these regulatory inputs have profound consequences for bile acid and cholesterol metabolism in the mouse. Thus, feeding bile acids leads to an approximate doubling of the bile acid pool, suppression of cholesterol 7α-hydroxylase, and a doubling of the hepatic cholesterol level (9, 10). Conversely, deletion of the LXRα gene prevents the activation of cholesterol 7α-hydroxylase and causes a pathological accumulation of hepatic cholesterol (11).

The regulation of the alternate pathway of bile acid synthesis is currently less well understood, although several studies suggest that the expression of early enzymes in the pathway may be modulated (12–15).

Deletion of the cholesterol 7α-hydroxylase gene in mice eliminates the classical pathway of bile acid synthesis and causes an 80% reduction in the bile acid pool size (9, 16). This loss also leads to an accumulation of bile acids with different chemical structures from those of animals with two functioning pathways, and it alters several parameters of cholesterol metabolism in the male (9, 16). These data indicate that the classical pathway is the major synthetic route to bile acids in mice and that the alternate pathway synthesizes ~20% of the normal bile acid pool. Mice deficient in oxysterol 7α-hydroxylase are not yet available; however, analysis of a human child lacking this enzyme illustrates the overall importance of the alternate pathway. The phenotype of this subject includes a dysfunctional liver (cholestaticis), massively elevated serum levels of oxysterols (hyperoxyroterolemia), and the absence of 7α-hydroxylated sterols and bile acids (17). The latter symptom is puzzling as the cholesterol 7α-hydroxylase gene is intact in this patient. Two explanations are postulated to underlie the absence of bile acids in this individual. First, the outcome may be due to a difference in the ontogeny of the two pathways of bile acid synthesis; the alternate pathway may be activated in humans prior to the classical pathway, leading to a dependence on the alternate pathway in neonates (17). Second, the accumulation of oxysterols may cause liver failure and a consequent loss of the classical pathway.

To resolve this issue and to gain further insight into the role of the alternate pathway in bile acid and cholesterol metabolism, we have produced a line of mice with a disruption in the Cyp7b1 gene encoding oxysterol 7α-hydroxylase. Bile acid metabolism is normal in these animals, suggesting that either the classical pathway or another synthetic route can fully compensate for the absence of the alternate pathway. Serum and tissue levels of two oxysterols accumulate in micromolar amounts and may adversely affect de novo cholesterol synthesis in the male kidney. The data indicate that the major physiological role of the CYP7B1 oxysterol 7α-hydroxylase is to inactivate oxysterols.
EXPERIMENTAL PROCEDURES

Construction of Targeting Vector—Two overlapping DNA fragments spanning the 3′-end of the Cyp7b1 gene were isolated by standard procedures (18) from a bacteriophage λ library prepared from 129SvEv genomic DNA. This library was a kind gift from Martin Matzuk (Baylor College of Medicine, Houston, TX). A short arm consisting of a 1.6-kilobase pair SacI fragment from the last exon of the gene was inserted on the 3′-side of a Neo gene in the psiBluescript-NeoIHSV vector (19). A long arm consisting of a 12-kilobase pair SacI fragment spanning introns 4 and 5 of the Cyp7b1 gene was inserted on the 5′-side of the Neo gene to produce the final targeting vector (see Fig. 1A). Homologous recombination should result in deletion of most of exon 6 from the Cyp7b1 gene, which normally encodes amino acids 410–507 of the protein.

Embryonic Stem Cell Culture—Mouse 11C embryonic stem (ES)1 cells derived from the 129SvEv mouse strain were cultured on mitotically inactivated, leukemia inhibitory factor-producing STO feeder cells in Dulbecco’s minimal essential medium supplemented with 15% (v/v) fetal calf serum, 0.1 mM nonessential amino acids, 2 mM l-glutamine, and 1 mM β-mercaptoethanol. Growth was at 37 °C in an atmosphere of 5% CO2. Approximately 2 × 106 ES cells were electroporated with 100 μg of SalI-linearized targeting vector to initiate homologous recombination. Electroporated cells were cultured in the presence of G418 (190 μg/ml) and ganciclovir (2 μM) for 12 days prior to subcloning of single ES cell colonies. Those that had undergone homologous recombination were identified by Southern blot analyses of Psfl-digested genomic DNA using the 3′-end probe described above. The arm fragment in the targeting vector. The frequency of clones with homologous recombination events was 5.7%. Positive clones were expanded and injected into C57BL/6J blastocysts. High percentage male chimeras from two different ES cell clones were crossed with female C57BL/6J mice to generate two independent lines of animals carrying the disrupted Cyp7b1 allele. The experiments reported here were performed in mice of mixed genetic background (C57BL/6J-129SvEv). No phenotypic differences were observed between knockout mice derived from the two independent ES cell lines.

RNA and Protein Blotting—Total RNA was extracted from previously frozen liver tissue using RNA Stat-60 kits (Tel-Test “B,” Friendswood, TX). Polyadenylated mRNA was prepared from pooled total RNA using an mRNA purification kit (Amersham Pharmacia Biotech).

Total liver extracts were prepared by homogenizing previously frozen liver tissue in 0.25 M sucrose buffer (0.25 M sucrose, 20 mM Tris acetate (pH 7.4) 1 mM EDTA, 50 μg/ml N-acetyl-Leu-Leu-norleucinal, 0.1 mM Pefabloc, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotonin) using a Polytron, followed by centrifugation at 600 × g for 10 min. Homogenates from three to six mice of the same sex and genotype were pooled for immunoblot analysis. Mitochondrial membranes were prepared from pooled homogenates by centrifugation at 10,000 × g for 20 min. The resulting pellets, which contained the mitochondria, were washed once with potassium phosphate buffer (10 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, and 5 mM dithiothreitol) and resuspended in the same buffer containing 20% (v/v) glycerol prior to analysis. To prepare microsomal membranes, supernatants from the 10,000 × g centrifugation were subjected to a second centrifugation step at 100,000 × g for 30 min at 4 °C. The resulting pellets were resuspended in 50 mM Tris acetate (pH 7.4), 1 mM EDTA, and 20% (v/v) glycerol prior to immunoblot and enzymatic analyses. For immunoblotting, aliquots of protein (55–150 μg) were separated by electrophoresis on 10% acrylamide gels containing SDS and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). A rabbit-generated polyclonal antibody that recognizes amino acids 266–281 of the mouse Cyp7B1 oxysterol 7α-hydroxylase was used to detect the protein (13). A rabbit-generated polyclonal antibody that recognizes amino acids 476–490 of the murine cholesterol 7α-hydroxylase was used to detect Cyp7a1. A polyclonal antibody against the immunoglobulin heavy chain-binding protein was purchased from Stressgen Biotech Corp. (Victoria, Canada), and an antibody that recognizes the mitochondrial carnitine palmitoyltransferase-2 protein was from Vickie Esser and J. Dennis McGarry (University of Texas Southwestern Medical Center).

Enzyme Assays—Total protein homogenates were prepared from liver tissue of Cyp7b1 knockout mice. Cyp7a1 and cholesterol 7α-hydroxylase activities were assayed in a 0.5-ml reaction containing 250 μg of protein, 1 μM 25-[3H]hydroxycholesterol (delivered in ethanol), 50 mM Tris acetate (pH 7.4), and 1 mM EDTA. The reaction was prewarmed in a 37 °C water bath for 5 min prior to the addition of 1.5 mM NADPH. After 15 min at 37 °C, 6 ml of methylene chloride was added to terminate the reaction and to extract sterols. The resulting organic phase was evaporated to dryness under nitrogen, and the pellets were dissolved in 40 μl of methanol (2:1, IPA-water). The solutions were analyzed by thin-layer chromatography on Silica Gel LK6D 150-A plates (Whatman) in a solvent system of toluene/ethyl acetate (2:3, v/v).

Measurement of Oxysterols—Concentrations of 24-hydroxycholesterol (cholest-5-ene-3β,24-diol), 25-hydroxycholesterol (cholest-5-ene-3β,25-diol), and 27-hydroxycholesterol (cholest-5-ene-3β,27-diol) in plasma were determined by isotope dilution-mass spectrometry (20). Decreased cholesterol standards (from I. Björkhem, Karolinska Institute, Huddinge, Sweden) were added to a defined volume of mouse plasma (0.3 ml) or amount of homogenized tissue (50 mg). Samples were saponified with KOH in ethanol/water, extracted with chloroform, and purified on Isolute Silica cartridge columns. Two ml (rather than 5 ml) of 30% isopropyl alcohol in hexane was used to elute oxysterols from the column since the former volume was found sufficient for the elution of 24-, 25-, and 27-hydroxycholesterol. These oxysterols are either minor or unrecognized autoxidation products of cholesterol (21), and rigorous precautions to exclude air during sample workup were not taken. After derivatization to trimethylsilyl ethers, samples were analyzed by gas chromatography-mass spectrometry as described (20).

Animal Diets—Mice were housed in plastic cages in a temperature-controlled room (22 °C) with 12-h light cycling. Animals were fed ad libitum a commercial diet containing 7% (w/w) protein (mouse diet 7001, Harlan Teklad, Madison, WI) containing 0.2 mg of cholesterol and 40 mg of total lipid per g of diet. Cholesterol absorption, sterol synthesis rates, bile acid pool sizes and compositions, and plasma and tissue cholesterol levels were measured in 3–4-month-old mice.

Sexual dimorphism in Cyp7B1 expression was examined in 10-week-old male and female wild-type C57BL/6J mice (stock 006664, Jackson Laboratory, Bar Harbor, ME). Male mice bearing a mutation in the androgen receptor were purchased from the same vendor (stock 001809). Orchiectomies were performed using standard methods of animal surgery. Where indicated, castrated mice were treated with dihydrotestosterone (1.5 mg in a 21-day release pellet; Innovative Research of America, Toledo, OH) or estradiol (0.5 mg in a 21-day release pellet) as described previously (22).

Cholesterol Balance Studies—The in vivo rate of sterol synthesis in the liver and major extrahepatic organs was measured as described (9). Wild-type and Cyp7b1−/− mice (n = six per genotype and sex) were used in these measurements. Intestinal cholesterol absorption was measured by a fecal dual-isotope ratio method in groups of 10 animals per genotype and sex (9, 23). Bile acid pool size and composition were determined using a high pressure liquid chromatography method (9). Plasma total cholesterol and triglyceride concentrations were measured using a reagent mixture (catalog no. 1127771, Roche Molecular Biochemicals). For tissue cholesterol measurements, organs from six mice of each genotype and sex were collected, saponified in ethanol/KOH, and extracted with petroleum ether in the presence of stigmastanol as an internal recovery standard. The amount of extracted cholesterol was measured by gas chromatography and normalized to recovery by comparison with the amount of stigmastanol.

RESULTS

To disrupt the murine oxysterol 7α-hydroxylase gene, the targeting vector illustrated in Fig. 1A was constructed. Electroporation of linearized vector DNA into 129SvEv-derived 11C embryonic stem cells followed by positive/negative selection and screening produced 10 lines that harbored the desired deletion mutation (Fig. 1A). Four of these cell lines were injected into recipient C57BL/6J blastocysts to produce chimeric males, two of which subsequently transmitted the mutation through the germ line to produce heterozygous mice (Cyp7b1+/−). Crossing heterozygous mice produced a mendelian distribution of wild-type and mutant genotypes. Homozygous Cyp7b1−/− mice were indistinguishable in terms of survival, gross physical appearances, and behaviors from heterozygous and wild-type animals. Backcrossing chimeric males with 129Sv females produced a line of Cyp7b1−/− congenic mice that also did not differ from wild-type mice in these descriptive parameters.

1 The abbreviation used is: ES, embryonic stem.
To confirm that deletion of exon 6 from the Cyp7b1 gene eliminated enzyme activity, hepatic lysates were prepared from wild-type and knockout mice of both sexes and assayed for oxysterol 7α-hydroxylase enzyme activity. As shown in Fig. 1B, wild-type mice expressed an activity that converted 25-hydroxycholesterol into several 7α-hydroxylated products whose chemical identities were determined previously (13). This activity was dependent on the inclusion of NADPH in the reaction mixture and was more abundant in males than in females. In contrast, extracts prepared from Cyp7b1−/− mice contained little or no oxysterol 7α-hydroxylase activity, which suggests that the introduced mutation created the expected null allele at the Cyp7b1 locus.

RNA blotting of poly(A)⁺-enriched mRNA isolated from male and female wild-type mice with an oxysterol 7α-hydroxylase cDNA probe revealed a single hybridizing mRNA of 2.2 kilobase pairs that was more prevalent in males than in females (Fig. 1C). This mRNA was not detected in RNA isolated from the livers of knockout mice, where instead a series of larger mRNAs were found (Fig. 1C). These aberrant mRNAs may be the products of abnormal splicing from the mutated Cyp7b1 locus since splice acceptor sequences in the last intron of the gene were removed by the introduced deletion (Fig. 1A).

The effects of the mutation on the expression of the CYP7B1 protein and the ability of the abnormal mRNAs to encode the protein were tested by immunoblotting. Homogenates were prepared from the livers of male and female wild-type and mutant mice, separated by gel electrophoresis, and then blotted with a polyclonal antibody directed against amino acids 266–281 of the protein. The CYP7B1 protein has a mass of ~53 kDa.

The results of Fig. 1 suggested a sexual dimorphism in the expression of hepatic oxysterol 7α-hydroxylase that could impact on the phenotype of Cyp7b1−/− mice. To confirm these observations, we examined CYP7B1 protein levels in different
tissues of male and female mice and the effects of the hormonal environment on these levels. As shown in Fig. 2, immunoblotting of total protein extracts prepared from liver and kidney showed higher levels of CYP7B1 protein in male (lanes 1 and 9) versus female (lanes 2 and 10) tissues. The amount of protein was reduced in the liver and kidney upon castration of wild-type males (lanes 3 and 11) and was reduced to almost undetectable levels in male mice with a mutation in the androgen receptor (Tfm) (lanes 6 and 14). The apparent effects of androgens on CYP7B1 expression were confirmed by administration of dihydrotestosterone to castrated males: this androgen increased the levels of CYP7B1 protein in the tissues of previously castrated males (lanes 4 and 5 and lanes 12 and 13). The administration of estradiol to intact male mice elevated the enzyme level in the liver (lane 7), but not the kidney (lane 15); however, castration of female mice did not affect CYP7B1 expression levels in either tissue (lanes 8 and 16).

The experiments summarized in Table I were carried out to determine how loss of oxysterol 7α-hydroxylase affected cholesterol and bile acid metabolism. Plasma cholesterol and triglyceride levels were not significantly different between adult mice of sundry Cyp7b1 genotypes and sexes (Table I), nor were differences detected in the lipoprotein profiles of pooled plasmas from these animals (data not shown). The cholesterol contents of 12 tissues (adrenal gland, brain, carcass, colon, gonad, heart, small intestine, kidney, liver, lung, spleen, and stomach) were also not significantly different between wild-type and mutant mice. The bile acid pool sizes were similar in control and knockout male mice (Table I). Female mice of both genotypes had identical pool sizes, which were ~50% larger than those of their male counterparts (Table I). Deletion of the Cyp7b1 gene did not change the compositions of the bile acid pools in male and female mice, nor did it affect intestinal cholesterol absorption (Table I).

These data suggested that loss of the CYP7B1 oxysterol 7α-hydroxylase and hence of the alternate pathway of bile acid synthesis was compensated for by increased output from the cholesterol 7α-hydroxylase pathway or perhaps another pathway. To test this hypothesis, we determined the expression levels of cholesterol 7α-hydroxylase as a readout of the classical pathway and of sterol 27-hydroxylase as a measure of the alternate pathway. The levels of cholesterol 7α-hydroxylase mRNA were not changed between normal and knockout mice of both sexes as judged by blotting (Fig. 3A). However, Cyp7b1−/− knockout mice had elevated levels (30–40%) of cholesterol 7α-hydroxylase protein (Fig. 3B). There were no changes in sterol 27-hydroxylase mRNA and protein between wild-type and mutant mice (Fig. 3A and B), although males of both genotypes had ~2-fold more sterol 27-hydroxylase protein than females (Fig. 3B).

A chemical phenotype was revealed in Cyp7b1−/− mice when isotope dilution-gas chromatography-mass spectrometry was used to measure levels of side chain oxysterols in the plasma, livers, and kidneys of wild-type and knockout animals of both sexes. As shown in Fig. 4A, the levels of free and esterified 24-hydroxycholesterol in the plasma were not significantly different between animals of various Cyp7b1 genotypes. In contrast, the plasma levels of 25- and 27-hydroxycholesterol were markedly increased in both male and female knockout mice. These increases were larger in Cyp7b1−/− males than in Cyp7b1−/− females, and both free and esterified pools of these two oxysterols were elevated (Fig. 4A). The liver and kidney contents of 25- and 27-hydroxycholesterol also were increased (Fig. 4, B and C, respectively), suggesting that plasma levels dictated tissue oxysterol content. These data also revealed marked differences in the distribution of free and esterified forms of the oxysterols in different tissues. In the plasma, the distribution of free and esterified forms was roughly equal among the three oxysterols (Fig. 4A), whereas in the liver, 24- and 25-hydroxycholesterol were largely present in free form and 27-hydroxycholesterol in an esterified form (Fig. 4B). A

![Fig. 2. Sexual dimorphism in hepatic and renal CYP7B1 expression.](http://www.jbc.org/) Microsomal membranes were prepared from the livers or kidneys (n = 3) of male or female mice treated in the indicated ways. Aliquots (75 μg) of protein were subjected to immunoblotting using a polyclonal anti-peptide antibody directed against amino acids 266–281 of the CYP7B1 oxysterol 7α-hydroxylase. Lanes 1 and 9, extracts from untreated male mice; lanes 2 and 10, extracts from untreated female mice; lanes 3 and 11, extracts from male mice castrated 7 days prior to harvest; lanes 4 and 12, extracts from male mice castrated 7 days prior to harvest and then treated with dihydrotestosterone (DHT) for 1 day; lanes 5 and 13, extracts from male mice castrated 7 days prior to harvest and then treated with estradiol (E) for 3 days; lanes 6 and 14, extracts from male mice harboring a mutation in the X-linked androgen receptor gene (Tfm); lanes 7 and 15, extracts from male mice treated with estradiol (E) for 7 days; lanes 8 and 16, extracts from female mice castrated 7 days prior to harvest. Bip, immunoglobulin heavy chain-binding protein.

### Table I

| Parameter                                      | Wild-type Male | Cyp7b1−/− Male | Wild-type Female | Cyp7b1−/− Female |
|------------------------------------------------|----------------|----------------|------------------|------------------|
| Plasma cholesterol (mg/dl)                     | 76 ± 16        | 75 ± 12        | 61 ± 12          | 59 ± 11          |
| Plasma triglyceride (mg/dl)                    | 51 ± 20        | 61 ± 29        | 41 ± 18          | 43 ± 15          |
| Bile acid pool size (μmol/100 g body wt)       | 82 ± 12        | 74 ± 9         | 116 ± 23         | 116 ± 15         |
| Bile acid pool composition (mg/dl)              | 48 ± 3         | 48 ± 6         | 53 ± 5           | 55 ± 6           |
| % cholic acid                                   | 33 ± 3         | 32 ± 7         | 32 ± 5           | 29 ± 7           |
| % β-muricholic acid                            | 18 ± 4         | 20 ± 5         | 16 ± 7           | 16 ± 3           |
| Intestinal cholesterol absorption (mg/dl)      | 38 ± 4         | 38 ± 3         | 52 ± 5           | 51 ± 4           |
| (n = 10; %)                                     |                |                |                  |                  |

*Intestinal cholesterol absorption* for different genotypes and sexes (Table I), nor were differences detected in the lipoprotein profiles of pooled plasmas from these animals (data not shown). The cholesterol contents of 12 tissues (adrenal gland, brain, carcass, colon, gonad, heart, small intestine, kidney, liver, lung, spleen, and stomach) were also not significantly different between wild-type and mutant mice. The bile acid pool sizes were similar in control and knockout male mice (Table I). Female mice of both genotypes had identical pool sizes, which were ~50% larger than those of their male counterparts (Table I). Deletion of the Cyp7b1 gene did not change the compositions of the bile acid pools in male and female mice, nor did it affect intestinal cholesterol absorption (Table I).

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majority of each oxysterol was present in free form in the kidney (Fig. 4C).

The concentrations of serum 24- and 27-hydroxycholesterol measured in male wild-type mice (Fig. 4A) were comparable to those reported in a previous study (24). However, the values determined for liver oxysterol contents (Fig. 4B) were substantially lower (16–30-fold) than those measured in two earlier studies (25, 26). These differences may reflect dietary influences (the mice used here were fed normal chow, whereas those in the earlier study (25) were fed normal chow supplemented with 20% peanut oil) or differences in the detection methods (26). Although precautionary measures to exclude autoxidation of cholesterol were not taken during sample workup in the experiments of Fig. 4, the three oxysterols measured here do not form spontaneously or are minor autoxidation products of cholesterol (21). Thus, the nonenzymatic formation of these sterols contributed very little to the final values measured.

To determine the consequences of hyperoxysterolemia in the Cyp7b1−/− mice for cholesterol metabolism, we measured in vivo sterol synthetic rates by following the incorporation of [3H]water into digitonin-precipitable sterols. The data of Fig. 5A show that there were no significant differences in sterol synthetic rates between male wild-type and knockout mice in the adrenal gland, brain, heart, lung, and spleen. However, in the experiment shown, a 46% reduction in de novo sterol synthesis was detected in the kidneys of male Cyp7b1−/− mice. In a second experiment involving six animals per group, sterol synthesis was reduced 34% in the knockout male kidney (data

Fig. 3. Cholesterol 7α-hydroxylase and sterol 27-hydroxylase levels in mice of different Cyp7b1 genotypes. A, equal amounts of hepatic poly(A)+-enriched mRNA from six animals of the indicated sexes and genotypes were pooled, and aliquots (5 μg) were separated by electrophoresis through glyoxal-agarose gels. After transfer to nylon filters, cholesterol 7α-hydroxylase mRNA (CYP7A1; left panel) and sterol 27-hydroxylase mRNA (CYP27; right panel) were detected by hybridization using radiolabeled cDNA probes. The filters were stripped and reprobed with a second radiolabeled cDNA to detect the cyclophilin mRNA (lower panels). The relative amounts of CYP7A1 and CYP27 mRNAs (Level) in each lane were determined by phosphoimaging after normalization to the cyclophilin signal. B, equal amounts of liver were pooled from six animals, and microsomal (CYP7A1) and mitochondrial (CYP27) proteins were prepared as described under “Experimental Procedures.” Aliquots (150 μg of protein) were separated on SDS-polyacrylamide gels, transferred to membranes, and blotted with anti-peptide antibodies directed against cholesterol 7α-hydroxylase (CYP7A1; left panel) or sterol 27-hydroxylase (CYP27; right panel). Control (Ctl) lanes contained aliquots of hepatic microsomal or mitochondrial proteins isolated from Cyp7a1−/−/Cyp27−/− double knockout mice. Membranes were stripped and reprobed with an antibody against the immunoglobulin heavy chain-binding protein (BIP; left panel) or against carnitine palmitoyltransferase-2 (CPT2; right panel) for normalization purposes (Level). kb, kilobase pairs.

Fig. 4. Plasma and tissue levels of oxysterols. A, a pool of plasma was prepared from mice (n = 6) of the indicated sex and Cyp7b1 genotype, and levels of oxysterols were determined by isotope dilution-gas chromatography-mass spectrometry as described under “Experimental Procedures.” White bars on the histogram indicate total oxysterol levels (free plus esterified), and shaded bars indicate free oxysterol levels. The levels of free and total 25-hydroxycholesterol in the livers of male Cyp7b1−/− mice were the same. B, same as in A, except oxysterols were extracted from the livers of mice (n = 3) of the indicated Cyp7b1 genotype and sex. C, same as in A, except oxysterols were extracted from the kidneys of mice (n = 3) of the indicated Cyp7b1 genotype and sex. In several cases, the levels of free oxysterols slightly exceeded or were identical to those of total oxysterols.
with mice that overexpress the cholesterol 25-hydroxylase gene (29) are currently testing this hypothesis by crossing transgenic higher concentration may be required for an
thesis is decreased by
rolemia induced in
Cyp7b1
lar levels does not appear to adversely affect cholesterol syn-
pecific, as renal sterol synthetic rates were similar in wild-type animals, the rates of sterol synthesis in the male kidney (but not female) kidney.2 These data imply that the increased sterol synthesis in the normal male may thus be related to the need to replace cholesterol catabolized by the oxysterol 7α-hydroxylase enzyme. In agreement with this idea, deletion in mice of the sterol 27-hydroxylase enzyme, which synthesizes a substrate for the CYP7B1 oxysterol 7α-hydroxylase enzyme, also causes a 50% reduction in sterol synthesis in the male (but not female) kidney.2 These data imply that the male kidney in the mouse plays a unique and as yet to be determined role in cholesterol metabolism, which we speculate may be related to the synthesis and excretion of pheromones in the urine.

The absence of a bile acid phenotype in Cyp7b1<sup>−/−</sup> mice is easier to explain. The output from the classical pathway is regulated by the level of bile acids (2). When this level is decreased, the activity of cholesterol 7α-hydroxylase and the synthesis of bile acids increase to restore homeostasis. Thus, a decrease in the output from the alternate pathway by inactivation of the CYP7B1 oxysterol 7α-hydroxylase should be compensated for by an increase in synthesis via the classical pathway. The alternate pathway synthesizes ~20% of the bile acid

2 S. D. Turley, M. Schwarz, D. W. Russell, and J. M. Dietschy, unpublished observations.
pool in the male mouse (9, 16), meaning that the level of cholesterol 7α-hydroxylase activity should be increased by a corresponding amount in Cyp7b1−/− animals. In agreement with this general hypothesis, the bile acid pool size in knockout mice is the same as that in wild-type controls (Table I), and male and female Cyp7b1−/− mice have slightly elevated levels of cholesterol 7α-hydroxylase enzyme (Fig. 3B). This elevation is more evident in males than in females (Fig. 3B), suggesting that the CYP7B1 pathway contributes more to the bile acid pool in males. Furthermore, the hepatic level of CYP7B1 in wild-type mice is higher in males (Figs. 1 and 2), as is the level of sterol 27-hydroxylase (Fig. 3B). We have not been able to consistently demonstrate an increase in cholesterol 7α-hydroxylase mRNA in knockout mice. This failure is probably due to the small size of the change and to large intra-animal variation in cholesterol 7α-hydroxylase mRNA levels.

The accompanying paper (33) reports a second oxysterol 7α-hydroxylase whose expression is unchanged between wild-type and Cyp7b1−/− mice. The results of these two studies thus suggest that the cholesterol 7α-hydroxylase pathway is the major regulated pathway in mice, whereas the oxysterol 7α-hydroxylases pathways are constitutively active.

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REFERENCES
1. Schwarz, M., Lund, E. G., and Russell, D. W. (1998) Curr. Opin. Lipidol. 9, 1–6
2. Russell, D. W., and Setchell, K. D. R. (1992) Biochemistry 31, 4737–4749
3. Wang, H., Chen, J., Hollister, K., Sewers, L. C., and Forman, B. M. (1999) Mol. Cell 3, 543–553
4. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hall, M. V., Lustig, K. D., Mangelersdor, D. J., and Shan, B. (1999) Science 284, 1362–1365
5. Parks, D. J., Blanchard, S. G., Bleidsee, R. K., Chandra, G., Conser, T. G., Kiefer, S. A., Stimmel, J. B., Wilson, T. M., Zavacki, A.-M., Moore, D. D., and Lehmann, J. M. (1999) Science 284, 1365–1368A
6. Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R., and Mangelsdor, D. J. (1999) Nature 383, 728–731
7. Lehmann, J. M., Kiefer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J.-L., Sundseth, S. S., Vinegar, D. A., Blanchard, D. E., Spencer, T. A., and Willson, T. M. (1997) J. Biol. Chem. 272, 3137–3140
8. Forman, B. M., Ruan, B., Chen, J., Schroepfer, G. J., Jr., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10588–10593
9. Schwarz, M., Russell, D. W., Dietschy, J. M., and Turley, S. D. (1998) J. Lipid Res. 39, 1833–1843
10. Turley, S. D., Schwarz, M., Spady, D. K., and Dietschy, J. M. (1998) Hepatology 28, 1088–1094
11. Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J.-M. A., Hamner, R. E., and Mangelsdor, D. J. (1998) Cell 93, 693–704
12. Vlachasaki, Z., Rairath, S. K., Heuman, D. M., Struvitz, R. T., Hylemon, P. B., Avadhani, N. G., and Pandak, W. M. (1996) Am. J. Physiol. 270, G646–G652
13. Schwarz, M., Lund, E. G., Lathe, R., Bjorkhem, I., and Russell, D. W. (1997) J. Biol. Chem. 272, 23995–24001
14. Xu, G., Salen, G., Shefer, S., Tint, G. S., Nguyen, L. B., Chen, T. S., and Greenblatt, D. (1999) J. Clin. Invest. 103, 89–95
15. Lammert, F., Wang, D. Q.-H., Paigen, B., and Carey, M. C. (1999) J. Lipid Res. 40, 2080–2090
16. Schwarz, M., Lund, E. G., Setchell, K. D. R., Kayden, H. J., Zerwekh, J. E., Bjorkhem, I., Herz, J., and Russell, D. W. (1996) J. Biol. Chem. 271, 18024–18031
17. Setchell, K. D. R., Schwarz, M., O’Connell, N. C., Lund, E. G., Davis, L. D., Lathe, R., Thompson, H. R., Tsyan, R. W., Sokol, R. J., and Russell, D. W. (1998) J. Clin. Invest. 102, 1695–1703
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Mansour, S. L., Thomas, K. R., and Capecci, M. R. (1988) Nature 336, 348–352
20. Dzeloetic, S., Breuer, O., Lund, E., and Diczfaluys, U. (1995) Anal. Biochem. 225, 73–80
21. Smith, L. L. (1987) Chem. Phys. Lipids 44, 87–125
22. Mahendroo, M. S., Cala, K. M., and Russell, D. W. (1996) Mol. Endocrinol. 10, 380–392
23. Turley, S. D., Daggy, B. P., and Dietschy, J. M. (1994) Gastroenterology 107, 444–452
24. Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shipizen, A., Triger, L., Eggertsen, G., Bjorkhem, I., and Leitersdor, E. (1998) J. Biol. Chem. 273, 14805–14812
25. Lund, E., Breuer, O., and Bjorkhem, I. (1992) J. Biol. Chem. 267, 25992–25997
26. Sauzier, S. E., Kandutsch, A. A., Gayen, A. K., Swahn, D. K., and Spencer, T. A. (1989) J. Biol. Chem. 264, 6863–6869
27. Kandutsch, A. A., and Chen, H. W. (1974) J. Biol. Chem. 249, 6057–6061
28. Brown, M. S., and Goldstein, J. L. (1974) J. Biol. Chem. 249, 7306–7314
29. Lund, E. G., Kaur, T. S., Sakai, J., Li, W.-P., and Russell, D. W. (1998) J. Biol. Chem. 273, 34516–34548
30. Taylor, F. R., Sauzier, S. E., Shown, E. P., Parish, E. J., and Kandutsch, A. A. (1984) J. Biol. Chem. 259, 12382–12387
31. Dawson, P. A., van der Westhuizen, D., Goldstein, J. L., and Brown, M. S. (1989) J. Biol. Chem. 264, 9046–9052
32. Cases, S., Novak, S., Zheng, Y.-W., Myers, H. M., Lear, S. R., Sande, E., Welch, C. B., and Kandutsch, A. A. (1999) J. Biol. Chem. 274, 18024–18031
33. Li-Hawkins, J., Lund, E. G., Bronson, A. D., and Russell, D. W. (2000) J. Biol. Chem. 275, 16543–16549
