Role of CYP27A in cholesterol and bile acid metabolism

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Abstract **The CYP27A gene encodes a mitochondrial cytochrome P450 enzyme, sterol 27-hydroxylase, that is expressed in many different tissues and plays an important role in cholesterol and bile acid metabolism. In humans, CYP27A deficiency leads to cerebrotendinous xanthomatosis. To gain insight into the roles of CYP27A in the regulation of cholesterol and bile acid metabolism, cyp27A gene knockout heterozygous, homozygous, and wild-type littermate mice were studied. In contrast to homozygotes, heterozygotes had increased body weight and were mildly hypercholesterolemic, with increased numbers of lipoprotein particles in the low density lipoprotein size range. Cyp7A expression was not increased in heterozygotes but was in homozygotes, suggesting that parts of the homozygous phenotype are secondary to increased cyp7A expression and activity. Homozygotes exhibited pronounced hepatomegaly and dysregulation in hepatic cholesterol, bile acid, and fatty acid metabolism. Hepatic cholesterol synthesis and synthesis of bile acid intermediates were increased; however, side chain cleavage was impaired, leading to decreased bile salt concentrations in gallbladder bile. Expression of Na-taurocholate cotransporting polypeptide, the major sinusoidal bile salt transporter, was increased, and that of bile salt export pump, the major canalicular bile salt transporter, was decreased. Gender played a modifying role in the homozygous response to cyp27A deficiency, with females being generally more severely affected. Thus, both cyp27A genotype and gender affected the regulation of hepatic bile acid, cholesterol, and fatty acid metabolism.**—Dubrac, S., S. R. Lear, M. Ananthanarayanan, N. Balasubramaniyan, J. Bollineni, S. Shefer, H. Hyogo, D. E. Cohen, P. J. Blanche, R. M. Krauss, A. K. Batta, G. Salen, F. J. Suchy, N. Maeda, and S. K. Erickson. **Role of CYP27A in cholesterol and bile acid metabolism.** ***J. Lipid Res.*** **2005.** 46: **76–85.**

Supplementary key words liver • lipoproteins • lipid synthesis • fatty acids • bile alcohols • cyp7A • transporters • receptors • gender

CYP27A encodes a mitochondrial P450 enzyme, sterol 27-hydroxylase, that is expressed in a wide variety of tissues and cell types. In the liver, sterol 27-hydroxylase catalyzes the first step in the acidic or "alternative" bile acid biosynthetic pathway from cholesterol; it also plays a key role in side chain cleavage of bile acid synthetic intermediates. In peripheral tissues, it catalyzes the conversion of cholesterol to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. Both compounds can be transported to the liver via "reverse cholesterol transport" and converted to bile acids (for recent reviews, see 1, 2). Both compounds have been proposed to play a role in the regulation of lipid metabolism, either by direct intracellular modulation (3) or by acting as ligands for the nuclear transcription factor, liver X receptor (LXR) (4).

In humans, CYP27A deficiency leads to cerebrotendinous xanthomatosis (CTX). CTX is associated with the accumulation of cholesterol and cholestanol in many organs, especially the brain and nervous system, excretion of bile alcohols, and low fecal bile acid excretion (for overview, see 5). Among common clinical symptoms of CTX are early-onset cataracts, tendon xanthomas, neurological manifestations, and an increased tendency to develop premature atherosclerosis despite plasma lipid levels in the normal range.

Cyp27A gene knockout mice were reported to lack classic symptoms of CTX, at least in young males (6). They had either normal plasma lipid levels (6), similar to CTX

Manuscript received 14 June 2004 and in revised form 27 September 2004 and in re-revised form 18 October 2004.

Published, JLR Papers in Press, November 1, 2004.
DOI 10.1194/jlr.M400219-JLR200

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patients, or were hyperlipidemic (7). Like CTX patients, the mice had increased levels of liver microsomal bile alcohols (8) and increased hepatic cholesterol 7α-hydroxylase activity or cyp7A mRNA levels (7, 9). They also had decreased levels of fecal bile acids and biliary bile salts (6, 7). In contrast, overexpression of human CYP27A in mice led to little apparent change in lipid metabolism, at least in young adults (10).

Despite differences between the effects of CYP27A deficiency in humans and mice, it is clear that dysregulation of bile acid and sterol metabolism occurs in both species. Gender differences in lipid and biliary homeostasis have been reported in wild-type mice (11); thus, it was important to determine how cyp27A deficiency affected these differences. Therefore, to gain additional insights into the role of cyp27A in the regulatory mechanisms of lipid and bile acid homeostasis, we examined the effects of cyp27A deficiency in heterozygous and homozygous male and female mice relative to their wild-type littermate controls. A preliminary report of some of the work described here was published previously in abstract form (12).

METHODS

Animals

A closed breeding colony was set up at the San Francisco Department of Veterans Affairs Medical Center (VAMC) in 1999, with one founder pair of heterozygous cyp27A gene knockout mice on a predominant (>99%) C57BL/6J background from the colony of Dr. Nobuyo Maeda. These mice were generated as described elsewhere (6) and transferred onto the C57BL/6J background by backcrossing more than six generations ($>N_6$). The genotype was maintained in heterozygous animals, and heterozygote breeding was used to study homozygotes, heterozygotes, and their wild-type littermates in parallel. The colony at VAMC San Francisco was expanded by brother-sister matings, followed by random matings from the same generation. Pups were weaned at 3 weeks and maintained on normal rodent chow and water ad libitum. Expected gender and Mendelian genotype distributions were observed (>10 generations). Overall survival was ~95%, with little apparent bias by genotype or gender. Mice were studied at 4–6 months of age. All were killed in the midlight period. Animals were studied at different times throughout the year to minimize environmental effects. Protocols were approved by the Animal Studies Subcommittee, VAMC San Francisco. The Animal Research Facility at VAMC San Francisco is specific pathogen free and is Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) accredited.

Assays

Liver histology. Liver histology was assessed by hematoxylin and eosin stain as described previously (13).

Plasma. Triglycerides and cholesterol were determined as described previously (13). Lipoprotein profiles were determined by nondenaturing gradient gel electrophoresis (14).

Fecal analyses. Feces were collected individually over 3 days and freeze dried. Fecal total bile acids, fatty acids, and neutral sterols were determined according to Boehler et al. (15).

Gallbladder bile analyses. Gallbladder bile cholesterol, phospholipids, and bile salts were analyzed as described previously (13). Bile salt hydrophobicity index was assessed according to Heuman (16), and cholesterol saturation index was calculated according to Carey (17). Bile alcohols were determined according to Batta et al. (18).

Liver cholesterol and triglycerides. Whole liver homogenate total, free, and esterified cholesterol and triglycerides were determined as described previously (13).

Assay of HMG-CoA reductase and cholesterol 7α-hydroxylase. Microsomes were prepared from flash-frozen liver, and enzyme activities were determined as described previously (13). Briefly, cholesterol 7α-hydroxylase activity was assayed with consideration of endogenous cholesterol using [4-^{14}C]cholesterol and an NADPH-generating system followed by product extraction and separation by column and thin layer chromatography. HMG-CoA reductase was assayed using [^{14}C]HMG-CoA and an NADPH-generating system followed by product separation by thin layer chromatography.

In vivo synthesis of cholesterol and fatty acids. The ^{3}HOH method was used as described previously by Erickson et al. (19) except that 40 mCi of ^{3}HOH/mouse was used.

Western blotting. LDL receptors and scavenger receptor class B type I (SR-BI) were determined as described previously (13). The sterol-regulatory element binding proteins (SREBPs), both the nuclear and microsomal forms, were determined in whole liver homogenates after low-speed centrifugation to remove whole cells and debris, using antibodies specific for SREBP-1 or SREBP-2 from Santa Cruz Biotechnology, Inc. Plasma apolipoproteins were determined as described previously (20).

Determination of hepatic bile salt and sterol transporters. Expression of the bile salt basolateral transporter, Na-taurocholate cotransporting polypeptide (Ntcp; Slc10a1), the canalicular bile salt export pump (Bsep; Abcb11), the canalicular organic anion transporter, multidrug resistance-associated protein 2 (Mrp2; Abcc2), the basolateral cholesterol transporter, Abca1, and the canalicular sterol transporters, Abcg5 and Abcg8, and the nuclear protein, small heterodimer protein (Shp), were determined by Northern blot. The following cDNA probes were used: Ntcp, a 0.9 kb *Eco*RI fragment (GenBank M77479) isolated from the full-length cDNA cloned into pBluescript; Bsep, a 4.0 kb *Xho*I fragment (GenBank U69487) isolated from the full-length cDNA cloned into pcDNA3; Mrp2, a 2.5 kb fragment encoding the C-terminal half of the cDNA (GenBank L49379) amplified by PCR and cloned into pCR2.1; Abca1, a 1.8 kb fragment of rat cDNA amplified by RT-PCR from rat liver mRNA and cloned into pCR 2.1 using primers derived from the mouse Abca1 sequence (submitted to GenBank; accession number AY208182); Abcg5 and Abcg8, a 1.6 kb rat ABCG5 cDNA (GenBank AF312714) and a 1.5 kb rat ABCG8 cDNA (AF351785) amplified by RT-PCR from rat liver mRNA and cloned into pCR 2.1; Shp, a 0.8 kb fragment released by digestion of pCMX-mSHP plasmid (a kind gift of Dr. D. Mangelsdorf) with *Bam*HI/*Nhe*I. All amplified RT-PCR products were verified by automated fluorescence sequencing and Basic Local Alignment Search Tool analysis against the GenBank database.

Liver total RNA was prepared using Trizol (GIBCO-BRL) according to the manufacturer's directions. Poly(A)$^+$ RNA was isolated by binding to biotinylated oligo-T followed by absorption onto streptavidin paramagnetic particles using PolyATract Sytem IV (Promega). Five micrograms of poly(A)$^+$ RNA from each sample was fractionated on 1% formaldehyde-agarose by standard techniques. mRNA was blotted to nylon membranes by capillary transfer and probed with cDNAs labeled with [α-^{32}P]dCTP. Hybridization and washing conditions were as described by Hardikar, Ananthanarayanan, and Suchy (21). Blots were exposed to PhosphorImager screens, and the signals were quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA). All blots were

normalized to GAPDH mRNA detected using a 1.3 kb *Pst*I fragment isolated from full-length GAPDH cDNA cloned into pGEM3 (Promega Corp., Madison, WI).

Other hepatic mRNA expression levels. RNA was extracted from flash-frozen livers, equal amounts pooled from five samples of each gender and genotype, and mRNAs were determined by Northern blot as described previously (22) using 1 μg of total RNA or 350 ng of poly(A)$^+$ RNA. In some cases, five RNA samples for each gender and genotype were analyzed individually. The following cDNA probes were used: rat cyp27A from N. Avadhani; rat cyp7A from John Chiang; mouse cyp7B, cyp8B, and C(27)3β-Hsd from David Russell; a rat LDL receptor fragment from John Trawick; hamster SREBP-1 and SREBP-2 from the ATCC (numbers 87012 and 87030, respectively); rat LXRα, peroxisome proliferator-activated receptor α (PPARα), and PPARγ from Tony Bass; mouse retinoid X receptor (RXRα) and farnesoid X receptor (FXR) from Barry Forman; mouse SR-BI from Monty Krieger; mouse Cpt-1 from Sonia Najjar; rat cyp4A and Acyl Coenzyme A oxidase (AOX)1 from Deanna Kroetz; LPL from Rick Kraemer; and multiple drug resistant protein Mdr2 (Abcb4) from Richard Green. The 28S RNA band or cyclophilin mRNA was used to correct for RNA loading.

Genotyping

Tail tip DNA was extracted using the Dneasy Tissue Kit from Qiagen (#69504) and analyzed by PCR using primers and conditions as described by Rosen et al. (6) and the Advantage cDNA PCR kit from Clontech (#K1905-1).

Statistics

Statistical comparisons were made according to ANOVA followed by a Student-Neuman-Keuls or a Dunn post hoc test for the genotype effect within male and female groups. A value of $P < 0.05$ was considered significant. Unless otherwise stated, all values are means ± SEM.

RESULTS

Hepatic expression of cyp27A was decreased in the heterozygous gene knockouts (~50%) compared with their wild-type littermate controls (**Fig. 1**). This allowed the study of the effects of different levels of cyp27A expression: wild-type or control (100%), heterozygous knockout (~50%), and homozygous knockout (not detectable).

Effects on body weight

Cyp27A gene knockout heterozygous mice had higher body weights than wild-type mice ($P < 0.01$), whereas homozygotes were similar to wild-type mice (**Table 1**). Food consumption was similar in all groups regardless of genotype (~0.18 g/day/g body weight).

Effects on the liver

Hepatomegaly was found in homozygotes relative to wild-type mice ($P < 0.0001$), with little effect noted in heterozygotes (Table 1). Despite their increased size, the homozygous livers appeared grossly normal by histology (data not shown). No changes in liver protein, DNA, or cholesterol contents expressed per gram of liver were observed. Liver triglycerides were increased in male homozygotes (32.2 ± 5.1 mg/g liver, n = 6, vs. 9.9 ± 2.2 mg/g for wild-type mice, n = 6; $P < 0.001$); however, no changes were observed in male heterozygotes or in female heterozygotes or homozygotes.

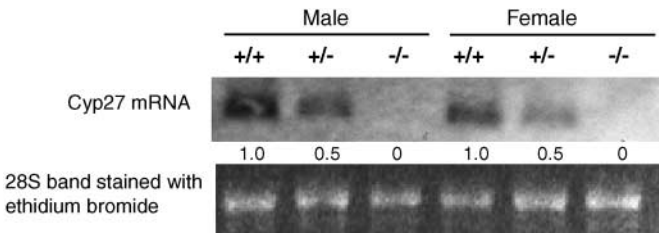

Fig. 1. Cyp27A expression in control (+/+), heterozygous (+/−), and homozygous (−/−) cyp27A gene knockout mice. Equal amounts of RNA were pooled from five livers of each gender, and genotype and levels of cyp27A mRNA expression were determined by Northern blot. Blots were normalized to the 28S RNA band. A representative blot is shown.

Effects on plasma lipids and lipoproteins

In heterozygotes, plasma cholesterol was increased, whereas plasma triglycerides were similar to wild-type levels (**Table 2**). The differences in plasma cholesterol correlated with increased levels of lipoprotein particles in the heterozygote mouse plasmas, as determined by nondenaturing gradient gel electrophoresis (**Fig. 2**). The average size of lipoproteins in the LDL size range also was increased. Plasma levels of both apolipoprotein B-100 (apoB-100) and apoB-48 were increased by approximately twofold ($P < 0.05$), with little effect on apoE, apoA-IV, or apoA-I.

In contrast, in homozygotes, both plasma cholesterol and triglycerides were decreased (Table 2). These changes correlated with the decreased levels of lipoproteins in the homozygous plasma (Fig. 2) and a decrease in apoB-100 (~20%; $P < 0.02$). No changes were found in plasma levels of apoB-48 or in apoE, apoA-IV, or apoA-I.

In homozygous livers, expression of lipoprotein lipase was increased by 2.4- ± 0.2-fold ($P < 0.01$), suggesting that decreased plasma triglycerides in homozygotes are secondary to increased activity of lipoprotein lipase in the liver.

Effects on fecal bile acid, neutral sterol, and fatty acid excretion

Both male heterozygotes and homozygotes excreted greater amounts (~2-fold) of neutral sterols and fatty acids than controls, whereas only female homozygotes showed

TABLE 1. Effects of Cyp27A genotype on body weight and liver

Sex and Genotype	Body Weight	Liver
	g	*% body weight*
Males +/+ (n = 29)	30.6 ± 0.5	4.9 ± 0.1
Males +/− (n = 45)	34.3 ± 0.7[a]	4.8 ± 0.1
Males −/− (n = 24)	30.4 ± 0.7	7.0 ± 0.2[b]
Females +/+ (n = 26)	24.5 ± 0.4	4.6 ± 0.1
Females +/− (n = 40)	28.6 ± 1.0[a]	4.3 ± 0.1
Females −/− (n = 34)	24.2 ± 0.4	7.0 ± 0.2[b]

+/+, control; +/−, heterozygous; −/−, homozygous.
[a] $P < 0.001$ vs. +/+.
[b] $P < 0.0001$ vs. +/+.

TABLE 2. Effects of Cyp27A genotype on plasma cholesterol and triglycerides

Sex and Genotype	Cholesterol	Triglycerides
	mg/dl	
Male +/+ (n = 16)	74.1 ± 4.7	70.2 ± 9.4
Male +/− (n = 31)	91.1 ± 4.4[a]	60.7 ± 4.7
Male −/− (n = 21)	61.1 ± 3.2[b]	24.2 ± 3.1[c]
Female +/+ (n = 15)	60.0 ± 1.7	31.2 ± 3.4
Female +/− (n = 25)	73.4 ± 3.5[a]	28.6 ± 3.2
Female −/− (n = 29)	50.1 ± 1.9[a]	13.2 ± 1.8[c]

[a] $P < 0.01$ vs. +/+.
[b] $P < 0.03$ vs. +/+.
[c] $P < 0.0001$ vs. +/+.

greater sterol and fatty acid excretion (∼1.5-fold) (**Table 3**). Fecal bile acid contents were similar in male wild-type and homozygote animals, although they were decreased modestly in heterozygotes. In contrast, in females, fecal bile acid content was similar in wild-type and heterozygote animals, but it was decreased ∼70% in homozygotes.

Effects on gallbladder bile composition

Cyp27A genotype had no statistically significant effect on cholesterol or phospholipid concentrations in gallbladder bile (**Table 4**). Biliary bile salt concentrations were similar in wild-type mice and heterozygotes, but they were significantly lower in homozygotes: for males, ∼50% ($P < 0.01$); for females, ∼70% ($P < 0.001$). In female homozygotes, this resulted in an increased cholesterol saturation index (0.56 ± 0.06 vs. 0.28 ± 0.03 for wild-type mice; $P < 0.01$). Little change in bile salt spectrum or in bile salt hydrophobicity index was noted (data not shown) in heterozygotes or homozygotes of either sex.

In contrast to wild-type mice, bile alcohols were readily detectable in homozygous gallbladder bile, mainly the 24-tetrol, with lesser amounts of the 24*S* and 24*R* pentols

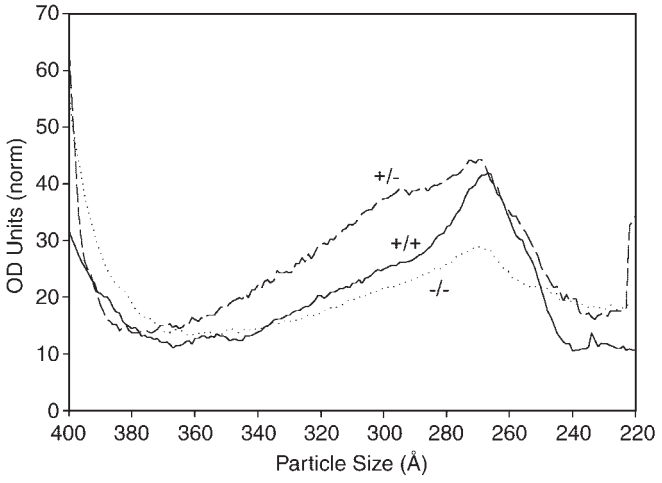

Fig. 2. Effect of cyp27A genotype on plasma lipoprotein profile. Plasma from cyp27A gene knockout wild-type, heterozygous, and homozygous littermates were analyzed by nondenaturing gradient gel electrophoresis as described in Methods. Representative profiles are shown. Å, Ångstrom; OD, optical density.

(**Fig. 3**). Of note is that levels in female homozygous bile were ∼10-fold higher than those in males.

Effects on bile acid synthesis

Expression of cyp7A was increased by ∼8-fold in male homozygotes and ∼4-fold in female homozygotes, with little effect in heterozygotes of either gender (**Fig. 4**). Cholesterol 7α-hydroxylase activity was increased by ∼4.5-fold in male homozygotes (122 ± 8 pmol/min/mg vs. 26 ± 2 for wild-type; $P < 0.0001$) and ∼3.6-fold in female homozygotes (149 ± 7 pmol/min/mg vs. 37 ± 2 for wild-type; $P < 0.0001$).

To assess downstream effects on bile acid synthesis (for review of these pathways, see 23), expression of other enzymes in the pathway was studied by Northern blot analysis of total RNA pooled from five livers of each gender and genotype (Fig. 4). Expression of cyp7B, encoding oxysterol 7α-hydroxylase, was decreased by ∼70% in female homozygotes and heterozygotes, with little effect in male heterozygotes and an ∼40% decrease in male homozygotes. Gender differences in the wild-type animals were maintained in both heterozygotes and homozygotes. Expression of cyp8B, encoding sterol 12α-hydroxylase, a key enzyme for cholic acid synthesis, was little changed in heterozygotes, but it was increased by ∼6-fold in male homozygotes and ∼1.6-fold in female homozygotes. Expression of C(27)3β-Hsd, which is common to all bile acid synthetic pathways, was affected only in the female homozygotes, in which it was decreased by ∼50%. Taken together, these results suggest that gender modulates the expression of genes encoding enzymes in the bile acid synthetic pathway in response to cyp27A deficiency.

Effects on cholesterol metabolism

Whole body and liver cholesterol synthesis were increased in homozygotes, with no statistically significant effect in heterozygotes (**Table 5**). In agreement with the effects on in vivo liver cholesterol synthesis, hepatic HMG-CoA reductase activity was increased in homozygotes relative to wild-type mice: for males, 410 ± 40 pmol mevalonate (MVA)/min/mg protein vs. 70 ± 6 ($P < 0.001$); for females, 471 ± 35 pmol MVA/min/mg protein vs. 93 ± 5 ($P < 0.001$).

Hepatic LDL receptors, which use the endocytic pathway, and SR-BI, which use the selective uptake pathway, were unaffected at either the mRNA or protein level (data not shown).

Effects on fatty acid metabolism

Whole body and liver fatty acid synthesis were unaffected in female heterozygotes and homozygotes. Whole body synthesis was increased in male heterozygotes (∼1.7-fold) (Table 5), with no effect in homozygotes. Liver fatty acid synthesis was unaffected in any genotype.

To address the potential effects on hepatic fatty acid oxidation, the expression of several key genes was determined by Northern blot using total RNA pools. Expression of Cpt1, a limiting factor in mitochondrial fatty acid β-oxidation, was increased by ∼70% in female homozygotes, with no effect in heterozygotes and no effect in

TABLE 3. Effects of Cyp27A genotype on fecal bile acids and lipids

Sex and Genotype	Bile Acids	Neutral Sterols	Fatty Acids
		mg/day/100 g body weight	
Males +/+	3.41 ± 0.20 (n = 6)	9.30 ± 0.43 (n = 6)	18.92 ± 4.52 (n = 4)
Males +/−	2.01 ± 0.31[a] (n = 5)	12.78 ± 0.94[a] (n = 5)	38.72 ± 4.95[b] (n = 5)
Males −/−	2.86 ± 0.35 (n = 4)	18.80 ± 1.18[c] (n = 4)	45.01 ± 4.00[a] (n = 3)
Females +/+	4.18 ± 1.03 (n = 8)	14.45 ± 1.92 (n = 8)	57.11 ± 6.93 (n = 4)
Females +/−	3.91 ± 0.74 (n = 5)	10.21 ± 2.36 (n = 5)	42.52 ± 5.07 (n = 5)
Females −/−	1.32 ± 0.28[b] (n = 6)	21.39 ± 1.80[b] (n = 6)	86.73 ± 9.04[b] (n = 6)

[a] $P < 0.01$ vs. +/+.
[b] $P < 0.02$ vs. +/+.
[c] $P < 0.001$ vs. +/+.

males of any genotype. Genotype had little effect on the expression of cyp4A, a microsomal ω fatty acid oxidase, or AOX1, a peroxisomal β fatty acid oxidase (data not shown).

Effects on liver bile salt transporters

Expression of Ntcp, the major sinusoidal bile salt transporter, was increased by ~3-fold in homozygotes (**Table 6**), with little effect in male heterozygotes but a significant increase in female heterozygotes. Expression of Bsep, the major canalicular bile salt transporter, was decreased in homozygotes (Table 6), with a decrease in male heterozygotes but no effect in female heterozygotes.

Expression of Mrp2, an important canalicular transporter for organic anions, including bile alcohol glucuronides, was reduced by 60% in male heterozygotes, whereas in female heterozygotes, Mrp2 message levels were increased by 5.2-fold ($P < 0.05$); however, levels in homozygotes were similar to those in wild-type mice (Table 6).

Effects on hepatic sterol transporters

Expression of the basolateral cholesterol transporter, Abca1, was unchanged in males; however, in females, levels decreased in both heterozygotes and homozygotes (Table 6).

As previously reported for mouse liver (24), two messages for Abcg5, at 2.3 and 3.3 kb, and for Abcg8, at 2.6 and 3.7 kb, were seen. The variation in size appears to be attributable to differences in length of the 3′-untranslated regions of the mRNA. Changes in levels of the more abundant, smaller message for each were quantitated. Expression of Abcg5 was similar in males regardless of genotype but was increased in female homozygotes (Table 6). Expression of Abcg8 was decreased in male heterozygotes and homozygotes; in contrast, expression in females was increased by 3.5-fold in heterozygotes and 1.4-fold in homozygotes (Table 6). If Abcg5 and Abcg8 are acting as heterodimers, it seems likely that these changes will result in a neutral effect on biliary cholesterol transport.

TABLE 4. Effects of Cyp27A genotype on gallbladder bile composition

Sex and Genotype	Bile Salts	Phospholipids	Cholesterol
		mM	
Males +/+ (n = 6)	311 ± 35	17.31 ± 0.81	2.54 ± 0.42
Males +/− (n = 4)	268 ± 46	12.36 ± 1.98	2.54 ± 0.39
Males −/− (n = 7)	165 ± 28[a]	14.18 ± 3.46	2.02 ± 0.38
Females +/+ (n = 3)	290 ± 34	19.74 ± 8.08	4.25 ± 1.34
Females +/− (n = 7)	238 ± 38	18.60 ± 2.08	3.08 ± 0.41
Females −/− (n = 6)	90 ± 19[b]	17.08 ± 1.49	3.38 ± 0.30

[a] $P < 0.01$ vs. +/+.
[b] $P < 0.001$ vs. +/+.

Effects on biliary phospholipid transport

Expression of Mdr2, the major canalicular protein responsible for biliary phospholipid secretion and for a significant portion of biliary cholesterol secretion (25), was increased by ~2-fold in heterozygotes and ~4-fold in homozygotes (Table 6).

Effects on nuclear transcription factors and expression of their target or sentinel genes

Because changes in the expression of nuclear transcription factors or in their action could contribute to the dysregulation of cholesterol, bile acid, and fatty acid metabolism observed in cyp27A-deficient mice, effects on selected key transcription factors and their target or sentinel genes were studied by Northern blot analysis of total hepatic

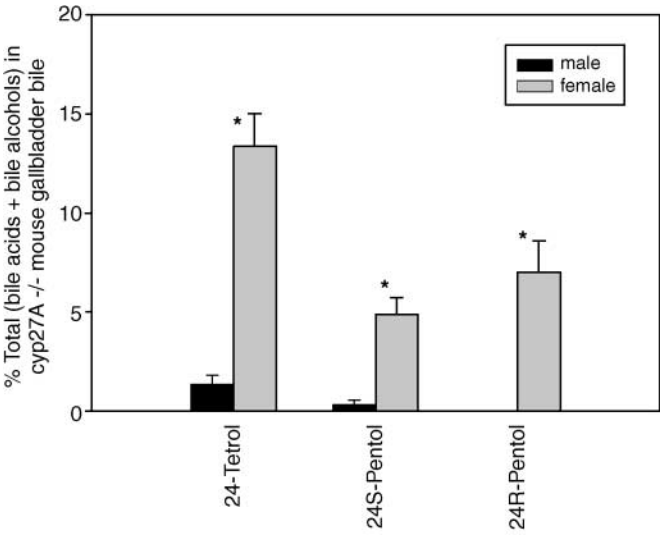

Fig. 3. Effect of cyp27A homozygous gene knockout on gallbladder bile alcohol content in male and female mice. Gallbladders were removed and snap frozen, and bile alcohols were analyzed as described in Methods. The amounts of each bile alcohol are expressed as a percentage of the sum of the total bile salts and bile alcohols in bile. *$P < 0.001$. Error bars indicate ± SEM.

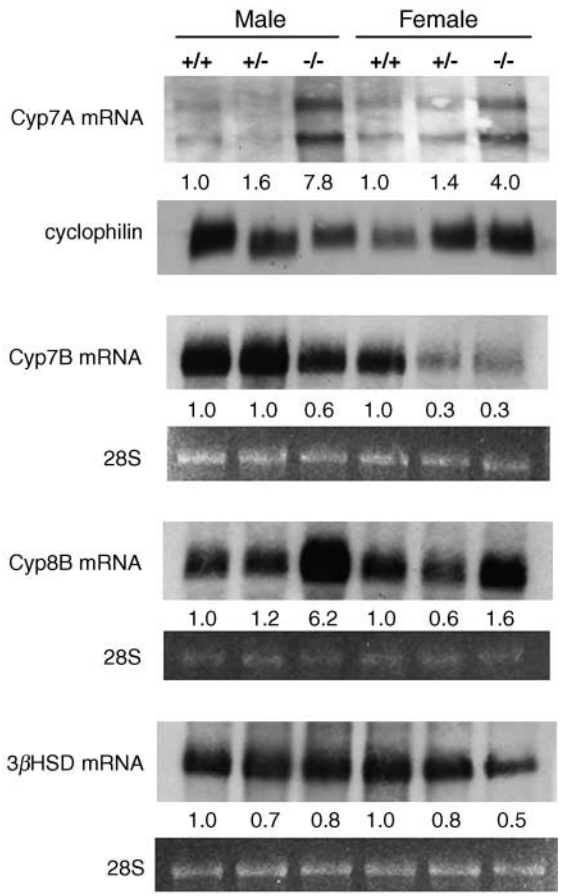

Fig. 4. Effect of cyp27A deficiency on expression of genes for enzymes in the bile acid biosynthetic pathway. mRNA levels were determined by Northern blots of pooled total or poly(A)$^+$ RNA from each gender and genotype as described in Methods. Values were normalized to the 28S band except for cyp7A, which was normalized to cyclophilin. Within each male or female group, wild-type values were set as 1.0 and the heterozygous and homozygous values were normalized to this.

RNA pools. Any differences observed were then confirmed by Northern blot analysis of RNA from five individual livers for each gender and genotype. No differences were observed for LXRα or FXR. RXRα, a heterodimer partner for a number of nuclear transcription factors implicated in the regulation of genes related to the cholesterol, fatty acid, and bile acid metabolic pathways, was little affected in males (1.00 ± 0.07 units for wild-type mice vs. 1.06 ± 0.12 for heterozygotes vs. 1.12 ± 0.25 for homozygotes), but it was increased ($P < 0.001$) in female homozygotes (1.00 ± 0.07 units for wild-type mice vs. 1.42 ± 0.28 for heterozygotes vs. 2.44 ± 0.17 for homozygotes). PPARα and PPARγ were unaffected. Increased mRNA level of Cpt1, a PPAR target gene, was observed in female homozygotes only (see above). Expression of Shp, a FXR target gene, was little affected except in male heterozygotes, in which mRNA level was decreased by ~40%, although this was not mirrored by an effect on Shp target genes. Expression of the Shp target genes Ntcp and cyp7A was increased in homozygotes (see above), supporting a lack of effect of cyp27A deficiency on Shp action. SREBP-1 and SREBP-2 mRNA levels and amounts of their respective nuclear proteins were unchanged, as were effects on hepatic fatty acid synthesis, a pathway including multiple SREBP-1 target genes, and on expression of LDL receptors, a SREBP-2 target gene (see above).

DISCUSSION

This work was designed to gain additional insight into the roles of CYP27A in the regulation of lipid and bile acid metabolism. Key results are summarized in **Table 7**.

Effect of genotype

Cyp27A gene knockout heterozygosity resulted in increased body weight in both males and females, despite similar food consumption. The heterozygotes had higher levels of LDL-size lipoproteins than either wild-type mice or homozygotes; this likely reflected increased numbers of particles because both apoB-100 and apoB-48 were increased, as was plasma cholesterol. This suggests that decreased hepatic expression of cyp27A may be a greater risk factor for the development of atherosclerosis than its absence. In contrast to the homozygotes, the heterozygotes did not show an increase in cyp7A expression, suggesting that the heterozygous phenotype reflects the consequences of cyp27A deficiency uncomplicated by effects secondary to cyp7A increases.

Cyp27A homozygous deficiency in our colony was associated with essentially normal to mild hypolipidemia, as described by Rosen et al. (6) for the first cyp27A gene

TABLE 5. Effects of Cyp27A genotype on in vivo cholesterol and fatty acid synthesis in liver and in the whole body

	Liver		Whole Body	
Sex and Genotype	Cholesterol	Fatty Acids	Cholesterol	Fatty Acids
		μmol ^{3}HOH/h/g		
Male +/+ (n = 3)	4.70 ± 1.72	49.54 ± 15.21	17.6 ± 4.3	294.8 ± 8.7
Male +/− (n = 5)	3.50 ± 0.44	83.95 ± 8.51	26.9 ± 2.8	518.9 ± 36.4[a]
Male −/− (n = 5)	15.17 ± 0.61[a]	70.76 ± 7.46	44.1 ± 5.4[b]	318.3 ± 31.5
Female +/+ (n = 5)	5.10 ± 0.5	40.22 ± 3.95	13.3 ± 1.1	336.0 ± 62.5
Female +/− (n = 4)	9.51 ± 2.20	67.73 ± 17.30	19.1 ± 1.9	274.0 ± 20.6
Female −/− (n = 5)	18.65 ± 2.47[a]	36.13 ± 3.51	41.9 ± 6.6[b]	255.7 ± 4.1

[a] $P < 0.001$ vs. +/+.
[b] $P < 0.01$ vs. +/+.

TABLE 6. Effects of Cyp27A genotype on expression of hepatic bile salt, cholesterol, and phospholipid transporters

	Bile Salt			Cholesterol			Phospholipid
Sex and Genotype	Ntcp	Mrp2	Bsep	Abca1	Abcg5	Abcg8	Mdr2
				units ± *SE*			
Male +/+	6.0 ± 0.8	7.6 ± 0.6	1.3 ± 0.1	2.9 ± 0.6	6.4 ± 0.4	3.6 ± 0.3	1.00 ± 0.06
Male +/−	5.0 ± 0.8	3.1 ± 0.6[a]	0.6 ± 0.1[a]	1.6 ± 0.8	6.0 ± 0.4	2.8 ± 0.6[a]	1.89 ± 0.30[b]
Male −/−	19.2 ± 4.0[a]	7.4 ± 1.0	0.8 ± 0.04[a]	1.7 ± 0.4	7.1 ± 0.04	1.5 ± 0.2[a]	3.29 ± 0.67[b]
Female +/+	7.9 ± 0.2	6.2 ± 0.8	7.6 ± 0.1	5.4 ± 0.2	4.1 ± 0.7	1.2 ± 0.1	1.00 ± 0.26
Female +/−	38.9 ± 2.0[a]	32.8 ± 10.2[a]	7.1 ± 1.4	2.4 ± 0.4[a]	5.1 ± 0.8	4.2 ± 0.1[a]	3.72 ± 0.48[a]
Female −/−	19.3 ± 1.9[a]	7.2 ± 0.6	5.4 ± 0.6[a]	0.6 ± 0.04[a]	11.0 ± 0.3[a]	1.7 ± 0.1[a]	4.11 ± 0.89[a]

Bsep, bile salt export pump; Mdr, multiple drug reistant protein; Mrp2, multidrug resistance-associated protein 2; Ntcp, Na-taurocholate cotransporting polypeptide. N = 5 in each group.

[a] $P < 0.01$ relative to +/+.

[b] $P < 0.02$ relative to +/+.

knockout colony and as found in human CYP27A deficiency. This is in marked contrast to the findings of Repa et al. (7), who reported hyperlipidemia. This difference may in part reflect genetic background; the colony at Dallas is on a mixed C57Bl/6:129Sv genetic background of uncertain proportion (7), whereas our colony is on a predominant (>99%) C57Bl/6J background. The importance of genetic background as a critical determinant of phenotype is emphasized in an excellent review by Leiter (26).

Of interest was that LPL expression in the liver was increased in our homozygotes, suggesting that liver nascent lipoproteins were acted upon by LPL in the liver itself before circulation to the periphery, providing an explanation for the observed hypolipidemia. Because hepatic LDL receptors were unaffected, increased hepatic uptake of locally produced VLDL remnants and free fatty acids would lead to increased hepatic VLDL production, albeit likely as a "futile cycle." Other changes in fat metabolism within the liver likely also reflect this.

The net effects of cyp27A heterozygous and homozygous deficiency on the major hepatic input and output pathways for cholesterol appeared neutral. The lack of effect on hepatic LDL receptors and SR-BI suggested that uptake pathways were unaffected. Despite the apparent decrease in bile salt transport into bile, both cholesterol and phospholipid concentrations in gallbladder bile remained within the littermate wild-type ranges. Studies of single gene knockouts of the canalicular sterol transporters Abcg5 and Abcg8 suggest that Abcg5/Abcg8 hetero-

TABLE 7. Summary of the effects of cyp27A deficiency

	Heterozygous		Homozygous	
Element	Male	Female	Male	Female
Plasma				
Cholesterol	↑	↑	→	→
Triglycerides	→	→	↓	↓
LDL-size particles	↑	↑	→↓	→↓
Feces				
Bile acids	→↓	→	→	↓
Cholesterol	↑	→	↑	↑
Fatty acids	↑	→	↑	↑
Gallbladder bile				
Bile salts	→	→	↓	↓↓
Cholesterol	→	→	→	→
Phospholipid	→	→	→	→
Bile alcohols	ND	ND	↑	↑↑↑
Liver				
Hepatomegaly	No	No	Yes	Yes
Triglyceride mass	→	→	↑	→
Cholesterol synthesis	→	→	↑	↑
Fatty acid synthesis	→	→	→	→
Bile acid intermediate synthesis	→	→	↑	↑
Biliary transport	→Ntcp, ↓Mrp2, ↓Bsep, ↑Mdr2	↑Ntcp, ↑Mrp2, →Bsep, ↑Mdr2	↑Ntcp, →Mrp2, ↓Bsep, ↑Mdr2	↑Ntcp, →Mrp2, ↓Bsep, ↑Mdr2
LDL receptors and scavenger receptor class B type I	→	→	→	→
Nuclear receptors	→	→	→	↑retinoid X receptor-α, →others

Arrow pointing up, increase; arrow pointing down, decrease; horizontal arrow, no change; ND, not detected.

dimers are not essential for biliary cholesterol excretion: Abcg5 single gene knockout had little effect on biliary cholesterol secretion (27), whereas Abcg8 single gene knockout decreased it (28). Thus, it is difficult to predict the effects of the observed changes in mRNA levels for these two transporters, which clearly responded differently depending on cyp27A genotype and on gender. In female homozygotes, expression of Abca1, a basolateral sterol transporter, was decreased, perhaps reflecting an altered hierarchy for free cholesterol use in the female livers.

Expression of Mdr2, which is responsible for biliary phospholipid secretion and which also has a significant influence on biliary sterol secretion (25), was increased in both male and female homozygotes. This likely explains the maintenance of nearly normal amounts of phospholipids and cholesterol in bile, despite an apparent decrease in bile salt secretion, as indicated by decreased bile salt concentrations in gallbladder bile.

Although liver cholesterol levels were similar to control levels in heterozygotes and homozygotes, hepatic cholesterol synthesis in the homozygotes was increased, as was HMG-CoA reductase activity, a rate-limiting enzyme for sterol synthesis. This suggested that cyp27A homozygous deficiency led to dysregulation in coordinated regulatory mechanisms of hepatic cholesterol metabolism.

The increased hepatic cholesterol synthesis in the homozygotes may be related to decreased cholesterol absorption, as was reported for male cyp27A gene knockout homozygotes on a mixed genetic background (7) and as indicated in the present work by increased fecal neutral sterol excretion. This would lead to less cholesterol entering the liver from the intestine and, potentially, relative upregulation of sterol synthesis. However, increased cholesterol synthesis in the homozygous livers likely reflects the increased cholesterol 7α-hydroxylase activity, reported here and previously, in both cyp27A-deficient mice (9) and humans (9 and references therein). Both HMG-CoA reductase and cholesterol 7α-hydroxylase reside in the endoplasmic reticulum (ER). Reductase is sensitive to ER cholesterol levels (see 29 for a review); local depletion of ER cholesterol as a result of increased cholesterol 7α-hydroxylase activity would stabilize HMG-CoA reductase, ensuring an adequate supply of de novo synthesized cholesterol. The lack of effect on levels of SREBP proteins (nuclear and total), also sensitive to ER cholesterol levels (29), and on their target genes lends support for such a posttranslational mode of regulation.

Hepatic bile salt transporters were variably affected by cyp27A deficiency (for reviews of the regulation of these transporters, see 30–33). In agreement with the decreased bile salt concentrations in gallbladder bile in the homozygotes, expression of Bsep, the major canalicular bile salt transporter, was modestly decreased. Despite the massive increases in gallbladder bile alcohols in the homozygotes, expression of Mrp2, a canalicular organic anion transporter that transports glucuronides, the major excretory form of bile alcohols, was unaffected, suggesting no transcriptional regulatory pressure on this transporter.

Homozygotes had increased expression of Ntcp, the major hepatic sinusoidal bile salt transporter; in other work (34), we found that expression of Oatp2, a second sinusoidal bile salt transporter, also was increased. It is likely that these changes reflect a response to alteration in the enterohepatic circulation induced by cyp27A homozygous deficiency. In preliminary studies (35), we found that expression of intestinal bile salt transporters also was increased, likely as part of a compensatory mechanism to maintain the enterohepatic circulation.

Although bile acids have been implicated in their regulation (30–33, 36, 37), effects of altered bile salt pool/load on the expression of hepatic genes encoding bile salt and organic anion transporters and on the phospholipid translocase Mdr2 are not well understood. The lack of effect in the present work on the expression of FXR, a nuclear transcription factor implicated in both negative and positive transcriptional regulation in response to bile acids, or on Shp, an FXR target gene, suggests that bile acid flux, or the responsible regulatory pool, remained below a level required for Shp induction. This is reinforced by the finding that the expression of two Shp target genes, cyp7A and Ntcp, was increased, rather than decreased, in the homozygotes. The moderate decrease in expression of Bsep, the major canalicular bile salt transporter and a direct FXR target gene, also suggests that the bile acid ligand pool for FXR is relatively lower in the homozygous than in wild-type livers.

Recently, it was reported that bile alcohols can act as ligands for FXR (38). Because these compounds accumulate in the setting of cyp27A deficiency (see above and 8), the expression of FXR target genes may have been modulated by the balance between bile acids and bile alcohols.

Effects of gender

The main gender difference in response to cyp27A deficiency was that the homozygous females generally were more adversely affected. This may reflect the greater increase in cholesterol 7α-hydroxylase activity in homozygous females compared with homozygous males ($P < 0.02$). As a consequence, cholestanol production in the homozygous females may be relatively increased; liver microsomal cholestanol content was shown previously to be increased in cyp27A-deficient mice (8). The relatively greater increase in gallbladder bile alcohols and greater decrease in bile salts in female homozygotes relative to males suggest that the capacity of compensatory system(s) for side chain cleavage was limited to a greater extent in females. Among the compensatory mechanisms identified to date in cyp27A gene knockout mice is cyp3A induction (9, 34); cyp3A transcription is estrogen sensitive (39). Thus, females may be at increased risk for development of CTX, at least in mice. Although our colony, in general, does not show classic manifestations of CTX, we have had three female homozygotes that developed adolescent-onset cataracts, a frequent clinical symptom of CTX in humans. Human CTX has been reported in a female heterozygote (40), but to date, not in a male, and there is a slight bias toward development of more severe forms of CTX in females (41).

The greater increases in cholesterol 7α-hydroxylase activity in female homozygotes, coupled with decreased expression of cyp7B and C(27)3β-Hsd, which is common to both the neutral and acidic bile acid synthetic pathways, suggest regulation of these genes by 7α-hydroxycholesterol or its products. In females, cyp7B is expressed at a lower level (42); this difference was maintained in cyp27A heterozygotes and homozygotes. The decrease in C(27)3β-Hsd in the female homozygotes suggests the possibility that expression of this gene may be downregulated in response to increased bile alcohols or other bile acid synthetic intermediates, some of which are ligands for Pregnane X receptor (PXR) (34, 43) or FXR (38). Whether C(27)3β-Hsd is a target gene for either of these nuclear transcription factors is unknown. It has been reported to be unresponsive to dietary cholesterol and to changes in bile acid pool size (44).

Increased expression of cyp8B in the homozygotes suggests adaptive pressure to maintain steryl 12α-hydroxylase activity, and this increase likely is responsible for the increased bile alcohols. Cyp8B expression also is increased in cyp7A deficiency (13), suggesting that this phenotype in both cyp7A and cyp27A deficiencies reflects a response to decreased hepatic bile acid flux or bile acid concentration in a critical regulatory pool.

The nuclear transcription factor RXRα is an obligate heterodimeric partner for a number of transcription factors implicated in the regulation of cholesterol, bile acid, and fat metabolism. The availability of RXRα could modulate many metabolic pathways coordinated through this nuclear transcription factor. Its increased expression in female cyp27A homozygotes suggests that this may be an important response to cyp27A deficiency in females. Little is known about the regulation of RXRα expression. Fatty acids (45) have been implicated in its upregulation; thus, the disturbances in fat metabolism accompanying cyp27A deficiency in females may be responsible.

Sex differences in fatty acid metabolism are well known, including in the expression of PPARα, which has lower basal expression in females (46). The increase in expression of the PPAR target gene Cpt1 in female homozygous livers suggests an adaptation to cyp27A deficiency by specifically increasing hepatic mitochondrial fatty acid oxidation. In contrast, male homozygotes showed no changes in the expression of genes related to fatty acid oxidation and compensated by storing fatty acids as triglycerides, which appeared to have a relatively benign effect. Cyp7A deficiency also resulted in increased Cpt1 expression in females only (13), suggesting that this effect may be secondary to decreased bile acid synthesis/secretion and not a direct effect of either cyp27A or cyp7A deficiency.

In conclusion, cyp27A deficiency affects all three hepatic metabolic pathways: cholesterol, fatty acid, and bile acid. This suggests the following: *1*) specific compounds, formed directly by sterol 27-hydroxylase activity, or their metabolites play important roles in hepatic and whole body regulation and integration of these three pathways; *2*) compensatory mechanisms induced by cyp27A homozygous deficiency (e.g., induction of cyp7A) alter the regulatory integration of these three key metabolic pathways; and *3*) gender plays a critical role in modulating the cyp27A-deficient phenotype.

This work was supported in part by National Institutes of Health Grants HL-52069 (S.K.E.), DK-26756 (S.S.), DK-56830 (G.S.), DK-48873 and DK-56626 (D.E.C.), HD-20632 (M.A. and F.J.S.), HL-18574 (R.M.K.), and DK-26743 (University of California, San Francisco Liver Center); by Merit Awards from the Department of Veterans Affairs (S.K.E. and G.S.); by a grant from the University of Medicine and Dentistry of New Jersey Foundation (S.S.); by a Grant-in-Aid from the American Heart Association, Heritage Affiliate (A.K.B.); and by a University of California, San Francisco Research Evaluation and Allocation Committee (REAC) grant (S.K.E.). S.D. was the recipient of postdoctoral fellowships from the French Ministry of Foreign Affairs (Lavoisier Program), the French Society of Nutrition, and the Fondation Singer-Polignac. H.H. was the recipient of funds from the Japan-North America Medical Exchange Foundation and from the American Liver Foundation (Postdoctoral Research Fellowship). Dr. Haiteng Deng of the Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine (supported in part by the Albert Einstein Comprehensive Cancer Center, National Institutes of Health Grant CA-1330), is gratefully acknowledged for expert assistance with mass spectrometry measurements. The authors thank Sandra Huling of the University of California, San Francisco Liver Center Morphology Core for assistance with liver morphology. Lastly, the authors thank their many colleagues for their kind contributions to the cDNA library.

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