Aberrant Oxidation of the Cholesterol Side Chain in Bile Acid Synthesis of Sterol Carrier Protein-2/Sterol Carrier Protein-x Knockout Mice*

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Peroxisomal β-oxidation plays an important role in the metabolism of a wide range of substrates, including various fatty acids and the steroid side chain in bile acid synthesis. Two distinct thiolases have been implicated to function in peroxisomal β-oxidation: the long known 41-kDa β-ketothiolase identified by Hashimoto and co-workers (Hijikata, M., Ishii, N., Kagamiyama, H., Osumi, T., and Hashimoto, T. (1987) J. Biol. Chem. 262, 8151–8158) and the recently discovered 60-kDa SCPx thiolase, that consists of an N-terminal domain with β-ketothiolase activity and a C-terminal moiety of sterol carrier protein-2 (SCP2, a lipid carrier or transfer protein). Recently, gene targeting of the SCP2/SCPx gene has shown in mice that the SCPx β-ketothiolase is involved in peroxisomal β-oxidation of 2-methyl-branched chain fatty acids like pristanic acid. In our present work we have investigated bile acid synthesis in the SCP2/SCPx knockout mice. Specific inhibition of β-oxidation at the thiolytic cleavage step in bile acid synthesis is supported by our finding of pronounced accumulation in bile and serum from the knockout mice of 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (which is a known bile alcohol derivative of the cholic acid synthetic intermediate 3α,7α,12α-trihydroxy-24-keto-cholestanoyl-coenzyme A). Moreover, these mice have elevated concentrations of bile acids with shortened side chains (i.e. 23-norcholesterol and 23-norcholenoxycholic acid), which may be produced via α- rather than β-oxidation. Our results demonstrate that the SCPx thiolase is critical for β-oxidation of the steroid side chain in conversion of cholesterol into bile acids.

Peroxisomes catalyze essential reactions in a number of different pathways and thus play an indispensable role in intermediary metabolism. Their importance is emphasized by the existence of a group of disorders called peroxisomal diseases, caused by an impairment of one or more peroxisomal functions. The cerebro-hepato-renal (Zellweger) syndrome is generally considered to be the prototype of this group of diseases. Zellweger patients show a large range of severe clinical anomalies, often leading to early death (1). The catabolic pathways in which peroxisomes are involved include the β-oxidation of fatty acids, the α-oxidation of phytanic acid, the degradation of glyoxylate and various xenobiotics. In addition, anabolic functions contribute to the synthesis of ether phospholipids, docosahexaenoic acid, bile acids, cholesterol, and other isoprenoids (2, 3).

Sterol carrier protein-2 (SCP2)‡ contains a PTS1 peroxisomal targeting signal at its C terminus and the protein is one of the most abundant proteins that is present in peroxisomes (4). Despite this fact, no human inherited disease is known at present that is caused by SCP2 mutations. The SCP2 gene (Scp2) codes not only for SCP2, but also for a fused protein of 60 kDa called sterol carrier protein-x (SCPx) (5, 6). The C-terminal domain of SCPX is identical with SCP2 whereas its N-terminal part consists of an active β-ketothiolase (7, 8). The two proteins are expressed from a common gene via alternative transcription initiation at two separated promoters (9). It was shown previously that SCPx is localized in peroxisomes and that its β-ketothiolase functions in a newly identified peroxisomal β-oxidation pathway that is involved in the oxidation of 2-methyl-branched chain fatty acids like pristanic acid (10). Conversely, the long known 41-kDa peroxisomal β-ketothiolase identified by Hashimoto and co-workers (11) acts preferentially on straight very long chain substrates.

In contrast, relatively little is known about the precise roles of SCP2. It has been shown that the protein binds acyl-CoAs (12, 13) and forms specific complexes in peroxisomes with acyl-CoA oxidase and other β-oxidation enzymes (14). In addition, our previous data indicated that peroxisomal α-oxidation of phytanoyl-CoA is diminished in the SCP2/SCPX knockout mice (10). These results imply that the protein may play a role as substrate carrier or regulatory factor in peroxisomal acyl-CoA metabolism. It was recently shown that the SCP2/SCPX gene disruption is associated with activation of the peroxisome proliferator activated receptor, PPARα (15), which suggests that SCP2 represents a potentially important peroxisomal regulator of PPARα signal transduction.

It has once been proposed that SCP2 functions in cytosolic cholesterol transport in liver and adrenals by virtue of its ability to act as a cholesterol carrier (16–18). Bile acid synthesis starts from cholesterol and proceeds via a complex sequence of enzymatic steps that are compartmentalized in cytoplasm, endoplasmic reticulum, mitochondria, and peroxisomes (19). Therefore, this pathway represents the prototype that would

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1 The abbreviations used are: SCP, sterol carrier protein; CA, cholic acid; 23-NCA, 23-norcholesterol acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; 23-NCDCA, 23-norchenodeoxycholic acid; 23-NUDCA, 23-norursodeoxycholic acid; 27-NC-CoA, 27-norcholanoyl-CoA; 3α,7α,12α-trihydroxy-27-nor-5β-cholestanoyl-CoA; 3α,7α,12α-trihydroxycholestanoyl-CoA; GC-MS, gas chromatography-mass spectrometry; THCA, 3α,7α,12α-trihydroxycholestanoyl-CoA; THCA, 3α,7α,12α-trihydroxycholestanoyl-CoA.

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depend on target-specific cholesterol trafficking and thus on SCP2. In the present work, we therefore investigated whether the Scp2 gene disruption affects bile acid synthesis in vivo in order to elaborate more precise information about the roles of SCP2 and SCPx in metabolism.

EXPERIMENTAL PROCEDURES

Materials—Most bile acid standards were obtained from Sigma (Deisenhofen, Germany) except for the muricholic acids and oxo-bile acids, which were purchased from Steraloids (Newport, RI). All nor-bile acids that were used in this study were synthesized by side chain degradation of the corresponding bile acids (20). 3α,7α,12α-Trihydroxy-5β-nor-5β-cholestane-24-one was synthesized essentially as described in references (21, 22). All solvents and high performance liquid chromatography-grade water were obtained from Baker (Griesheim, Germany). Organic solvents were dried over molecular sieves (Merck, Darmstadt, Germany, 0.4 nm). Cholylglycine hydrolase (EC 3.5.1.24) from Clostridium perfringens, β-glucuronidase (EC 3.2.1.31), and sulfatase (EC 3.1.6.1) from Helix pomatia and all other chemicals were of analytical grade and supplied by Sigma (Deisenhofen, Germany). Reverse-phase octadecylsilane bonded silica cartridges were purchased from Baker (Baker-bond RP-18, 3 ml, 500-mg beds). The cartridges were washed with 5 ml of methanol and primed with 5 ml of water (pH 3, equilibrated with hydrochloric acid) before use. Glassware was silanized with 1% (v/v) dimethylchlorosilane in toluene to prevent adsorption of the bile acids (23).

Serum Analysis of Bile Acids—Internal standard (nordeoxycholic acid, 250 ng) was added to 100 µl of serum followed by simultaneous enzymatic hydrolysis and saponification at 45 °C for 3 h as described previously (24). Bile compounds were extracted from a serum sample using a Baker-bond RP-18 cartridge, which was washed with 5 ml of water (pH = 3, equilibrated with hydrochloric acid), cholesteryl esters were removed with 10 ml of petroleum ether (b.p. 40–60 °C), and free cholesterol was removed with 8 ml of petroleum ether/ethyl acetate 9:1 (v/v). The bound fraction was eluted with 5 ml of methanol and 5 ml of diethyl ether. The solvents were evaporated, and the residue containing the bile acids was derivatized into the methyl esters with 1 n trimethylsilylchlorosilane in methanol (400 µl) at 60 °C for 30 min. After removal of excess reagents, derivatization of the bile compounds into the trimethylsilyl ethers was carried out using a solution of pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, v/v/v; 200 µl) at 60 °C for 30 min. Excess reagents were evaporated and the derivatives of the bile compounds were dissolved in 100 µl n-hexane/pyridine (99:1, v/v). The mixture was vortexed and centrifuged at 2000 x g for 3 min. A 20 µl of the supernatant were subjected to further analyses by GC-MS.

Gas Chromatography-Mass Spectrometry Analyses—GC-MS was performed on a Finnigan GCQ (Thermoquest, Egelsbach, Germany) equipped with an ion trap mass analyzer and a HT-5 fused silica capillary column (30 m, inner diameter 0.32 mm, film thickness 0.25 µm) (Chromatographic Service GmbH, Langerwehe, Germany). The temperature of the column oven was maintained at 55 °C for 1 min and increased with 30 °C/min to 280 °C. The temperature of the injection port and the detection unit was at 300 °C. Helium at a flow rate of 1 ml/min was used as the carrier gas. The split ratio was 1:10. Peak areas were quantified by comparing the ion current of the sum of characteristic quantitation masses with the GCQ data processing software 2.2.

Analyses of Bile Samples—Murine bile was collected by puncture of the gall bladder and analyzed according to a modification of the method of Chiжиwa and Nakayama (26). Briefly, 10 µg of epiprostanol was added as internal standard per 5 µl of bile, followed by protein precipitation with ethanol and enzymatic hydrolysis with cholylglycine hydrolase. Bile acids were extracted with ethyl acetate and derivatized into ethyl esters dimethylsilyl ethers. Alternatively, derivatization into methyl esters trimethylsilyl ethers was carried out as described above (23). The final sample was analyzed by gas chromatography, performed on a SP-7100 (Spectra Physics, Darmstadt, Germany) equipped with an flame ionization detector and a S554-CB fused silica capillary column (30 m, inner diameter 0.32 mm, film thickness 0.25 µm) (Chromatographic Service GmbH, Langerwehe, Germany). The temperature of the column oven was maintained at 100 °C for 1 min, then increased with 20 °C/min to 200 °C, maintained for 5 min and finally increased with 5 °C/min to 280 °C. The temperature of the injection port and the detection unit was at 300 °C. Helium at a flow rate of 1 ml/min was used as the carrier gas. The split ratio was 1:10. Peak areas were quantified by comparing the ion current of the sum of characteristic quantitation masses with the GCQ data processing software 2.2.

RESULTS

Any abnormality leading to decreased production of bile acids is likely to result in an up-regulation of the cholesterol 7α-hydroxylase as a result of reduced negative feedback suppression of this enzyme (29). To clarify whether this mechanism operates in SCP2/SCPx knockout mice, we compared the expression of cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase in liver from SCP2/SCPx knockout mice with C57Bl/6 controls. As is shown in Fig. 1, cholesterol 7α-hydroxylase was expressed ~4-fold higher in the knockout mice compared with controls.

![Fig. 1. Expression of cholesterol-7α-hydroxylase (CYP7a) and sterol-27-hydroxylase (CYP27) mRNA in liver from SCP2/SCPx knockout mice and C57Bl/6 controls.](image)
knockout mice than in C57Bl/6 controls. In contrast, no difference between the two strains could be observed concerning the mitochondrial sterol 27-hydroxylase.

Methyl ester-trimethylsilylether derivatives of bile acids and other relevant compounds were analyzed by GC-MS in bile collected from SCP2/SCPx knockout mice and age- and sex-matched C57Bl/6 controls. As is shown in Fig. 2, all major bile acids, including cholic acid and the three muricholic acids (α, β, and ω), were present in bile from homozygous knockout mice. In addition, the primary bile acid CDCA, which represents only a minor component of murine bile, could be detected as ethyl ester-dimethylethylsilylether derivative in the samples from the knockout mice (data not shown). Based on an analysis including six mice in each experimental group, we found that the mean biliary levels of bile acids were 20% lower in homozygotes compared with C57Bl/6 controls, although the differences were not significant statistically. Moreover, the relative fractions of the common bile acids did not differ significantly between both strains (data not shown). We noted, however, two additional peaks in the chromatograms from knockout bile (marked X1 and X2 in Fig. 2). Both were detected in every sample from homozygous transgenex but were not detectable in heterozygotes or C57Bl/6 controls. When homozygotes were treated with the bile acid sequestrating drug cholestyramine, known to stimulate bile acid synthesis, both peaks increased pronouncedly, whereas cholic acid remained at a lower level in homozygotes compared with controls (Fig. 3). After 14 days of treatment with cholestyramine, the two extra peaks did not appear in analyses with the samples from C57Bl/6 controls (Fig. 3).

The mass spectra that were obtained for both compounds are shown in Fig. 4. They point to normal 3α,7α,12α-hydroxylated ring structures that are combined with abnormal side chains. Whereas the side chain of X1 appeared 14 mass units smaller than the carboxymethylated side chain of cholic acid, it was 2 mass units smaller in X2. Comparative analyses performed on methyl ester-trimethylsilyl ether and ethyl ester-dimethylethylsilyl ether modified biliary samples showed that X1 was an unusual bile acid whereas X2 lacked the carboxylic function and thus represented an unusual bile alcohol (data not shown). Comparison with a number of chemically synthesized standards led to the identification of X1 as 23-NCA and X2 as 27-NC-24-one. Whereas 23-NCA differs from cholic acid by lack of one methylene group in the side chain, 27-NC-24-one represents an unusual bile alcohol carrying a keto-function at C-atom 24, shortened by one methylene group compared with trihydroxycholestanol (Fig. 4).
Due to small sample sizes and variable secretion rates of different biliary constituents, analytical measurements of murine bile have limitations. Therefore, we next performed analyses of bile acids and the related alcohols in serum. On the basis of their mass spectra, we identified 23-NCDCA and 23-NUDCA as additional nor-bile acids which were present besides 23-NCA in sera but were absent from bile of SCP2/SCPx knockout mice (Fig. 5). As demonstrated in Fig. 6, the sera from SCP2/SCPx knockout mice also contained high concentrations of 27-NC-24-one, which was not detected in C57Bl/6 controls. Conversely, significantly lower concentrations of all common bile acids were found in the knockout mice compared with C57Bl/6 controls. The decrease found for cholic acid, CDCA, the muricholic acids (α, β, ω), DCA, and UDCA consisted of 40–60% (p < 0.001). In contrast, extremely high concentrations of 23-NCA, 23-NCDCA, 23-NUDCA, and 27-NC-24-one could be demonstrated in SCP2/SCPx knockout mice treated with cholestyramine (Fig. 6). Conversely, these compounds were essentially absent from serum of identically treated C57Bl/6 controls.

We did neither detect varanic acid, known to accumulate in patients with d-peroxisomal bifunctional enzyme deficiency (30), nor THCA, present in Zellweger disease (1), or complex bile alcohols consisting of 27 C-atoms and cholestanol (known to accumulate in the human disease cerebrotendinous xanthomatosis) (19) in bile or serum of the knockout mice (data not shown).

Our results show for the first time that the SCP2/SCPx gene is critical for the synthesis of normal bile acids in vivo. Although the sequence of enzymatic steps in bile acid synthesis are diverse from different species, almost all mammals produce 7-α-hydroxylated bile acids that consist of 24 C-atoms (19). These compounds play important roles in cholesterol and lipid metabolism, intestinal lipid and vitamin absorption, and in diseases like atherosclerosis, cholestatic liver disease, or gall stone formation. Unlike in normal mice, a large fraction of bile acids consist of only 23 C-atoms (i.e. 23-NCA and 23-NCDCA) in SCP2/SCPx knockout mice. These bile acids, which lack one methylene group from their side chains, accumulate preferentially in serum of chow- or cholestyramine-fed knockout mice, whereas their concentrations in bile are relatively low. Thus, it may be assumed that the 23-nor-bile acids are less efficiently secreted in bile than their normal counterparts. In particular, 23-NCDCA showed extremely high concentrations in serum, whereas it appeared much less prominent in bile. This is in line with previous data showing that 23-NCA and 23-NCDCA are conjugated ineffectively in rats (31, 32). Moreover, hepatic uptake and secretion into bile of 23-NCA was shown to be much lower compared with cholic acid in the perfused rat liver (33).

23-Nor-bile acids are most likely produced via α-oxidation from their normal counterparts. It has been shown that 23-hydroxylated cholic acid and CDCA are converted rapidly to the respective 23-nor-bile acids in several rodents (34). Increased 23-hydroxylation has also been shown in human pa-
apylation from 3-oxoisomal bile acid metabolism). Model analyses have shown that oxidation of the steroid side chain that is present in 3α,7α,12α-trihydroxy-24-keto-cholestanolic acid and 3α,7α,12α-trihydroxy-24-cholestenoic acid in a human patient who may have had thiolase deficiency (36). Thus, we think that our results show convincingly that side chain oxidation is inhibited in vivo in SCP2/SCPx knockout mice at the thiolytic step that takes place in peroxisomal β-oxidation of THCA-CoA. This leads us to conclude that the thiolase that is associated with SCPx is essential for the thiolytic step involved in bile acid synthesis.

In a previous study, we showed that the SCP2/SCPx knockout mice have a defect in peroxisomal α- and β-methyl-branched fatty acid metabolism (10). The metabolic block occurred at two levels of peroxisomal metabolism: first, inefficient α-oxidation, presumably via inhibition of import of 3-methyl-branched fatty acids into peroxisomes, and second, inhibition of the thiolytic cleavage of the 2-methylated 3-ketoacyl-CoA derivatives. The structures of these 3-keto substrates are in fact very similar as the 24-keto substituted steroid side chain that is present in 3α,7α,12α-trihydroxy-24-keto-cholestanolic acid. Thus, it appears plausible to us that both substrates use the same thiolase for their β-oxidation. This conclusion is furthermore in accordance with the substrate specificity that has been studied in vitro for the recombinant rat SCPx protein (37–39). In contrast, the substrate for peroxisomal β-oxidation, which is THCA, did not accumulate in SCP2/SCPx knockout mice. This negative finding shows that import of THCA-CoA into peroxisomes may not depend on the presence of SCP2 or SCPx. Alternatively, accumulation of THCA may be prevented by induction of efficient alternative side chain oxidation pathways. Such extraperoxisomal pathways could also explain why SCP2/SCPx knockout mice are still capable of synthesizing normal bile acids. One known alternative side chain oxidation pathway that is active in mice is the microsomal 25-hydroxylase pathway (19). This or a 24-hydroxylase pathway was made previously responsible for residual bile acid synthesis in sterol 27-hydroxylase knockout mice (29). Alternatively, the long known 41-kDa peroxisomal β-ketothiolase identified by Hashimoto and co-workers (11) could be responsible for the residual synthesis of normal bile acids in the SCP2/SCPx knockout mice. Although this enzyme is known to act preferentially on straight long chain fatty acid substrates (37), it cannot be excluded that overlap of substrate specificity exists between the two peroxisomal thiolases.

The hydroxylation steps which occur at the β-cholestanole nucleus are apparently not affected in our model. We think that this is an interesting finding, because SCP2, which is missing in the model, has once been proposed to act as cytoplasmic cholesterol carrier (16–18). Considering the physicochemical properties and asymmetric intracellular distribution of cholesterol one would expect that target-specific intracellular cholesterol transport would be required for bile acid synthesis. According to current concepts, the initial steps that use cholesterol as the substrate proceed via two alternative pathways. The first and presumably major pathway consists of 7α-hydroxylation of cholesterol catalyzed by cholesterol-7α-hydroxylase (called CYP7a) at the endoplasmic reticulum (19). The second, which is called the acidic pathway, is initiated by 27-hydroxylation of cholesterol in mitochondria and the 7α-hydroxylation step proceeds later via a newly identified oxysterol 7α-hydroxylase called CYP7b1 (40–42). Thus, the recent studies showed that there are two enzymes that are capable of 7α-hydroxylation, the classical cholesterol 7α-hydroxylase, and the newly identified oxysterol 7α-hydroxylase. The precise met-

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Role of SCP2/SCPx in Murine Bile Acid Synthesis

![Graph](image_url)

**Fig. 6.** Serum concentrations of bile acid metabolites in chow- and cholestyramine-fed mice. Serum samples were collected from adult male SCP2/SCPx knockout mice (hatched bars) and C57Bl/6 controls (filled bars). Analyses of bile acid metabolites in serum were performed as described under “Experimental Procedures.” The values represent means ± S.E. of six animals. Identification of the compounds is based on comparison of GC-MS analyses with appropriate standards. 7αxoxo-CA, 7-oxocholic acid; MCA, muricholic acid.

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Patients with cerebrotendinous xanthomatosis who have elevated concentrations of 23-nor-bile acids (35). Cerebrotendinous xanthomatosis is caused by defective sterol-27-hydroxylation, catalyzed by a mitochondrial enzyme that belongs to the cytochrome P-450 family. Because the defect leads to a block in normal β-oxidation of the side chain located prior to the peroxisomal import of THCA-CoA, it differs clearly from the situation in the SCP2/SCPx knockout mouse. Nevertheless, induction of α-oxidation, leading to synthesis of 23-nor-derivatives, may represent a more general mechanism of compensation that could be associated with inefficient β-oxidation of the steroid side chain. It appears, however, interesting to us that 23-nor-bile acids were apparently absent from sterol-27-hydroxylase knockout mice (29), which suggests that their production is regulated differently in human and mice.

Besides the 23-nor-bile acids, we detected accumulation of high amounts of 27-NC-24-one in SCP2/SCPx knockout mice (29). Alternatively, the long known 41-kDa peroxisomal β-hydroxylase that is associated with SCPx is essential for the thiolytic step in vivo in SCP2/SCPx knockout mice. This negative finding shows that import of THCA-CoA into peroxisomes may not depend on the presence of SCP2 or SCPx. Alternatively, accumulation of THCA may be prevented by induction of efficient alternative side chain oxidation pathways. Such extraperoxisomal pathways could also explain why SCP2/SCPx knockout mice are still capable of synthesizing normal bile acids. One known alternative side chain oxidation pathway that is active in mice is the microsomal 25-hydroxylase pathway (19). This or a 24-hydroxylase pathway was made previously responsible for residual bile acid synthesis in sterol 27-hydroxylase knockout mice (29). Alternatively, the long known 41-kDa peroxisomal β-ketothiolase identified by Hashimoto and co-workers (11) could be responsible for the residual synthesis of normal bile acids in the SCP2/SCPx knockout mice. Although this enzyme is known to act preferentially on straight long chain fatty acid substrates (37), it cannot be excluded that overlap of substrate specificity exists between the two peroxisomal thiolases.
FIG. 7. Schematic illustration of normal peroxisomal ß-oxidation of the steroid side chain and effect of the SCP2/SCPx gene disruption. Modifications at the steroid side chain: 1, 27-hydroxylation catalyzed by mitochondrial sterol-27-hydroxylase (CYP 27). 2, conversion to 3α,7α,12α-trihydroxycholestanolic acid (THCA) by cytosolic alcohol dehydrogenases. 3, formation and import of THCA-CoA into peroxisomes, followed by conversion to 24-THCA-CoA via the peroxisomal THCA-CoA oxidase. 4, formation of 24-hydroxy-THCA-CoA (varanyl-CoA) catalyzed by the enoyl-CoA hydratase activity of the ß-PBE (formerly known as 17β-hydroxysteroid dehydrogenase, type 4). 5, conversion to 24-keto-THCA-CoA by the dehydrogenase activity of the ß-PBE, 6, thiolysis cleavage of 24-keto-THCA-CoA catalyzed by the hypothetical function of SCP2 and SCPx as b-hydroxysteroid dehydrogenase, type 4). 5, conversion to 24-keto-THCA-CoA via the peroxisomal THCA-CoA oxidase. 4, formation of 24-hydroxy-THCA-CoA (varanyl-CoA) catalyzed by the enoyl-CoA hydratase activity of the ß-PBE (formerly known as 17β-hydroxysteroid dehydrogenase, type 4). 5, conversion to 24-keto-THCA-CoA by the dehydrogenase activity of the ß-PBE, 6, thiolysis cleavage of 24-keto-THCA-CoA by the hypothetical function of SCP2 and SCPx as ß-hydroxysteroid dehydrogenase, type 4).

abolic consequences in mice of inhibition of either pathway became evident from the recent characterization of CYP7a and steroid 27-hydroxylase knockout mice (29, 40, 43). In our study, we show that the abnormalities in bile acid synthesis of the SCP2/SCPx knockout mice are clearly distinct from those that have been described for the two other models. Therefore, we presume that the hypothetical function of SCP2 and SCPx as cytosolic cholesterol carrier may either be masked by efficient compensatory mechanisms in our model or that this function is not essential for murine bile acid synthesis in vivo.

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