Developmental Changes of Bile Acid Composition and Conjugation in L- and d-Bifunctional Protein Single and Double Knockout Mice*

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Sacha Ferdinandusse‡§, Simone Denis‡, Henk Overmars‡, Lisbeth Van Eeckhoudt‡, Paul P. Van Veldhoven, Marinus Duran‡, Ronald J. A. Wanders‡, and Myriam Baes‡

From the ‡Academic Medical Center, Laboratory of Genetic Metabolic Diseases, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands and §Katholieke Universiteit Leuven, Laboratory of Clinical Chemistry, and the Department of Pharmacology, 49 O/1 Herestraat, Leuven B 3000, Belgium

Peroxisomal β-oxidation is an essential step in bile acid synthesis, since it is required for shortening of C27-bile acid intermediates to produce mature C24-bile acids. d-Bifunctional protein (DBP) is responsible for the second and third step of this β-oxidation process. However, both patients and mice with a DBP deficiency still produce C24-bile acids, although C27-intermediates accumulate. An alternative pathway for bile acid biosynthesis involving the peroxisomal l-bifunctional protein (LBP) has been proposed. We investigated the role of LBP and DBP in bile acid synthesis by analyzing bile acids in bile, liver, and plasma from LBP, DBP, and LBP:DBP double knock-out mice. Bile acid biosynthesis, estimated by the ratio of C27:C24-bile acids, was more severely affected in double knock-out mice as compared with DBP∥/∥ mice but was normal in LBP∥/∥ mice. Unexpectedly, trihydroxycholestanoyl-CoA oxidase was inactive in double knock-out mice due to a peroxisomal import defect, preventing us from drawing any firm conclusion about the potential role of LBP in an alternative bile acid biosynthesis pathway. Interestingly, the immature C27-bile acids in DBP and double knock-out mice remained unconjugated in juvenile mice, whereas they occurred as taurine conjugates after weaning, probably contributing to the minimal weight gain of the mice during the lactation period. This correlated with a marked induction of bile acyl-CoA:amino acid N-acyltransferase expression and enzyme activity between postnatal days 10 and 21, whereas the bile acyl-CoA synthetases increased gradually with age. The nuclear receptors hepatocyte nuclear factor-4α, farnesoid X receptor, and peroxisome proliferator receptor α did not appear to be involved in the up-regulation of the transferase.

Bile acids play an important role in the solubilization and digestion of dietary lipids and in the excretion of cholesterol. Bile acids are synthesized from cholesterol in the liver via a series of reactions involving many different enzymes located throughout the cell (1). The nonpolar steroid nucleus of cholesterol is converted into a considerably more polar steroid by enzymes located in the endoplasmic reticulum and cytosol. In the mitochondrion, a carboxyl group is formed on the aliphatic side chain of the sterol molecule, which is shortened by β-oxidation in the peroxisome (Fig. 1). As a consequence, bile acid synthesis is disturbed in peroxisome biogenesis disorders and also in peroxisomal DBP† deficiency. DBP, alternatively called multifunctional protein 2, is involved in the second and third step of the β-oxidation of 2-methyl branched-chain fatty acids or fatty acid derivatives like the C27-bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA) (2). After cleavage of the side chain, the primary C24-bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are formed. In mice, CDCA is only a minor bile acid, since most of it is converted via hydroxylation into α/β-ω-muri-CA. After synthesis, bile acids are conjugated with taurine or glycine by the bile acyl-CoA:amino acid N-acyltransferase (BAAT) (1). In mice, almost exclusively taurine-conjugates are present, because murine BAAT does not use glycine as substrate (3).

Patients with a deficiency of DBP accumulate C27-bile acid intermediates, but there are still mature C24-bile acids present in their plasma and bile (4, 5). This suggests that an alternative pathway for bile acid biosynthesis exists, which does not involve DBP. Indeed, there is an alternative microsomal pathway for cleavage of the side chain that does not involve any peroxisomal enzymes. This 25-hydroxylation pathway accounts for less than 5% of bile acid synthesis in several species studied (6–8). Recently, another alternative pathway has been proposed involving the other peroxisomal bifunctional enzyme, LBP (also called multifunctional protein 1), and α-methylacyl-CoA racemase (AMACR) (Fig. 1) (9). During peroxisomal β-oxidation of THC-CoA, first (24E)-trihydroxycholesteryl-CoA (THC:1-CoA) is formed by the branched-chain acyl-CoA oxidase in humans and by trihydroxycholestanoyl-CoA oxidase (AOCX2) in mice. THC:1-CoA is then converted to (24R,25R)-24OH-THC-CoA by the hydratase part of DBP, and subsequently this is converted into 24-keto-THC-CoA by the dehydrogenase part of DBP. Finally, this is thiolytically cleaved by sterol carrier protein X (10). Alternatively, THC:1-CoA can be converted to (24S,25S)-24OH-THC-CoA by the hydratase part

† The abbreviations used are: DBP, d-bifunctional protein; ACOX1, straight-chain acyl-CoA oxidase; ACOX2, trihydroxycholestanoyl-CoA oxidase; ACOX3, pristanoyl-CoA oxidase; AMACR, α-methylacyl-CoA racemase; BACS, bile acyl-CoA synthetase; BAAT, bile acyl-CoA:amino acid N-acyltransferase; CA, cholic acid; CDCA, chenodeoxycholic acid; DHCA, dihydroxycholestanolic acid; DKO, double knock-out; FXR, farnesoid X receptor; HNF4α, hepatocyte nuclear factor-4α; LBP, l-bifunctional protein; PPARα, peroxisome proliferator receptor α; THCA, trihydroxycholestanolic acid; THC, trihydroxycholesteryl-CoA; THC:1-CoA, (24E)-trihydroxycholestanoyl-CoA; VLCS, very long-chain acyl-CoA synthetase; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; Pa, postnatal day n; MES, 4-morpholineethanesulfonic acid; THC, trihydroxycholestanoyl-CoA.
The peroxisomal enzymes involved in this part of the pathway: AMACR, branched-chain acyl-CoA oxidase (BCOX) in humans and ACOX2 in mice, both the enoyl-CoA hydratase part of β-fumaprotein (HY-DBP) and the hydroxylac-CoA dehydrogenase part (DH-DBP), and sterol carrier protein X (SCPX). A proposed alternative pathway involving LBP and AMACR is indicated by dashed arrows. The formed cholesterol (CA-CoA) is conjugated with taurine or glycine by BAAT.

of LBP; however, this is not substrate for the dehydrogenase part of LBP or DBP. In vitro studies have shown that (24S,25S)-24OH-THC-CoA is a substrate for AMACR, which can convert this isomer to the (24S,25R)-isomer, which in turn can be handled by the dehydrogenase part of LBP (9, 11). If this route would be operational in vivo, it could account for some residual bile acid synthesis in DBP-deficient patients. In addition, the (24S)-hydroxycholesterol, which is formed in order to eliminate cholesterol from the brain and is excreted across the blood-brain barrier into the circulation, can be converted into bile acids, after α-oxidation, via LBP (12). (24S,25S)-24OH-THC-CoA is present in body fluids of patients with DBP deficiency, indicating that in these patients LBP indeed forms this hydroxy-intermediate (13). The question remains, however, whether the alternative pathway via LBP really contributes to bile acid synthesis when DBP functions normally or whether it can partly take over the role of DBP in bile acid synthesis in case of a deficiency of DBP.

To answer this question, we performed bile acid analysis in LBP, DBP, and DBP/DBP double knock-out (DKO) mice. DBP−/− mice exhibit a severe postnatal growth retardation and a variable life span, with more than 50% of the mice dying before weaning, whereas the others survive into adulthood. In adult DBP−/− mice, CAβ7 bile acid intermediates have been identified, as expected, but CA24-bile acids are still present (14). Although the creation of the LBP−/− mouse has been published in 1996 and no gross phenotypic defects were described (15), no specific bile acid analysis in this mouse model has been reported. LBP/DBP DKO mice have a complete block at the level of the second step of peroxisomal β-oxidation. They exhibit severe growth retardation and postnatal mortality, with almost none surviving beyond weaning (16). It is unclear why the DKO mice are much more severely affected than the single DBP knock-out mice, because at this moment no specific function has been attributed to LBP. In order to compare bile acid composition, we collected liver, plasma, and bile of mice with the different genotypes between postnatal day 2 (P2) and adulthood. Because of the poor survival of the DKO mice, we could only analyze samples of these mice until P14–15. Since we noticed a major difference in the conjugation of bile acids in young animals compared with adult animals, a second goal was to investigate the ontogeny of hepatic bile acid conjugation.

**EXPERIMENTAL PROCEDURES**

**Animals**—Wild type and single DBP knock-out (originally named multifunctional protein 2 knock-out) mice were obtained by inbreeding LBP:DBP+/+;+− mice, whereas LBP knock-out and DKO mice were descendants of LBP:DBP−/−;−− breeding pairs (14, 15, 17). Mice were bred in the animal housing facility of the University of Leuven under conventional conditions. They had unlimited access to standard rodent chow (Muranac-G; Carfil Quality-Pavan Services, Oud-Turnhout, Belgium; for breeding pairs, this was enriched in a ratio of 1:1 with AM-II (Hope Farms, Arie Blok, Woerden, The Netherlands)) and water and were kept on a 12-h light/dark cycle. All animal experiments were approved by the Institutional Animal Ethical Committee of the University of Leuven.

Wild type and peroxisome proliferator receptor α (PPARα)−/− mice on a Sv129 genetic background, which were used for bile acyl-CoA synthetase and bile acyl-CoA:aminio acid acyltransferase activity measurements, were obtained from the Jackson Laboratory.

**Bile Acid Analysis**—Bile acids were analyzed by HPLC-negative ion electrospray tandem mass spectrometry as described (18) with only minor modifications. The HPLC column was used as a C8-column (Phenomenex Luna; 50 × 1 mm), and the bile acids were separated using a gradient from 60:40 (ammonium formiate, pH 8.1/MMeOH (v/v)) to 90:10 (acetonitrile/H2O (v/v)) at a flow rate of 60 μl/min. Sample preparation for plasma was performed as described in Ref. 18. For bile acid analysis in bile, the bile was diluted 500 times in H2O. For analysis in liver, 15–25 mg of liver (wet weight) was homogenized in 150 μl of H2O. After sonication, 150 μl of MeOH was added plus 100 μl of internal standard (2,2,4,4-2H4,tauro-CA,2,2,4,4-2H4,tauro-CDCA,2,2,4,4-2H4,glyco-CA,2,2,4,4-2H4,glyco-CDCA,2,2,4,4-2H4,SCCA, and 2,2,4,4-2H4,HCDCCA). After another round of sonication, the sample was deproteinized by the addition of 750 μl of acetonitrile followed by subsequent centrifugation for 10 min at 20,000 × g at 4 °C. The supernatant was removed and stored briefly, whereas the pellet was dissolved in 300 μl of MeOH/H2O (1:1), sonicated, and deproteinized as described above. The supernatants were pooled and evaporated under a stream of N2, and the residue was dissolved in 250 μl of MeOH/H2O (1:2). Five μl was injected into the HPLC tandem mass spectrometric system. Quantitation of the various conjugated bile acids was done using multiple reaction monitoring, whereas the various unconjugated bile acids were detected with selected ion monitoring.

**Peroxisomal Acyl-CoA Oxidase Measurements**—Acyl-CoA oxidase activity measurements were performed essentially as described before (19). H2O2 production by the action of the acyl-CoA oxidases was measured in mouse liver homogenates prepared in PBS with 25 μM FAD by formation of horseradish peroxidase (H2O2) unbound and horseradish peroxidase. Measurements were carried out in the following incubation mixture: 50 mM MOPS-NaOH (pH 7.6), 1 mM homovanillic acid, 18 units/ml horseradish peroxidase, 0.1 mM NaN3, 5 μM bovine serum albumin, and 200 μM C16-CoA. Fluorescence was followed at 30-s intervals for 10 min using a Coba Bio centrifugal analyzer (excitation wavelength, 327 nm; emission filter, 410–490 nm) (Hoffman-La Roche).
a final volume of 100 µl. The reactions were started by the addition of mouse liver homogenate (0.2 mg/ml) prepared in PBS and terminated after 30 min at 37 °C by the addition of 500 µl of acetonitrile. Fifty µl of [2,2,4,4-2H4]-tauro-CA and [2,2,4,4-2H4]-lCA was added as an internal standard, and the sample was centrifuged for 10 min at 20,000 × g at 4 °C. The supernatant was evaporated under a stream of N2, and the residue was dissolved in 100 µl of MeOH/H2O (1:2). Five µl was injected into the HPLC tandem mass spectrometric system. Subcellular fractions were prepared from the liver of overnight fasted mice homogenized in 0.25M sucrose, 5 mM MOPS, pH 7.2, 1 mM EDTA, and 0.1% ethanol (21) and analyzed for protein content, catalase (peroxisomal marker), lactate dehydrogenase (cytosolic marker) (21), and BAAT activity as described above.

**Northern Blotting**—RNA was extracted from liver using the TRizol® reagent (Invitrogen) and analyzed by Northern blotting as previously described (22). The blots were consecutively hybridized with radioactive probes and exposed. The probes were generated by reverse transcription-PCR on mouse liver RNA using the following primers: mBAAT (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3'), mFXR (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3'), and mHNF4α (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3'). The blots were consecutively hybridized with radioactive probes and exposed. The probes were generated by reverse transcription-PCR on mouse liver RNA using the following primers: mBAAT (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3'), mFXR (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3'), and mHNF4α (5'-GGGATTCACGATGGATGAGCT-3' and 5'-TTTATCATCAGGCTGTTAAG-3').

**Western Blotting**—Western blotting was performed with antibodies against farnesoid X receptor (FXR), hepatocyte nuclear factor-4α (HNF4α), and calregulin. Mouse liver homogenate (50 µg in case of FXR and HNF4α, 25 µg for calregulin) was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (23) and transferred to a nitrocellulose sheet. After blocking of nonspecific binding sites with 50 g/liter BSA and 10 g/liter bovine serum albumin in 1 g/liter Tween 20/PBS for 1 h, the blot was incubated for 2 h with one of the following primary antibodies: anti-FXR (diluted 1:200 in 3 g/liter bovine serum albumin; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HNF4α (diluted 1:100; Perseus Proteomics Inc., Tokyo, Japan), or anti-calregulin (diluted 1:1000; Santa Cruz Biotechnology). Goat anti-rabbit (FXR and calregulin) or goat anti-mouse (HNF4α) IgG antibodies conjugated to alkaline phosphatase were used for detection, according to the manufacturer's instructions (Bio-Rad).

**Statistical Analyses**—Data are expressed as mean ± S.D. Statistical significance was evaluated using an unpaired Student's t test. The results were considered significant when p ≤ 0.05.

### RESULTS

**Bile Acid Analysis**—Bile acids were analyzed in bile, liver, and plasma from LBP, DBP, and LBP:DBP DKO mice. Since almost no DKO mice survive beyond weaning, comparative analysis in mice from all the different genotypes could only be performed before postnatal day 21 (P21). Samples from the single knock-out mice were also analyzed at an adult age. In bile, liver, and plasma from wild type and LBP−/− mice (age <P21) the major bile acids were tauro-CA, tauro-muri-CA, and tauro-OH-CA. The mass spectra of bile from LBP−/− mice were similar to those from wild type mice (see Fig. 2), revealing only very limited amounts of bile acid biosynthesis intermediates. In contrast, C27-bile acid intermediates were the major bile acids in bile, liver, and plasma from both DBP−/− and DKO mice. DBP−/− mice accumulated mainly THCA with one double bond (THC:1), which has previously been identified by mass spectrometric analysis as the first intermediate of the peroxisomal β-oxidation process (14). DKO mice, however, accumulated predominantly THCA itself or OH-THCA. The exact position of the hydroxyl group could not be identified, but the accumulating OH-THCA is most likely a mixture of THCA hydroxylated at several different positions, because multiple peaks were observed by HPLC for the molecule with m/z 465.

In liver of both DBP−/− and DKO mice, the accumulation of C27-bile acid intermediates was accompanied by a severe deficiency of C24-bile acids (see Fig. 3). At P2−4 the liver of wild type animals contained 362 ± 88 nmol/g (wet weight) C24-bile acids, whereas the sum of all C24-bile acids in liver from DBP−/− mice was 34 ± 2 nmol/g, and in liver from the DKO mice 25 ± 10 nmol/g. This resulted in a C27/C24 ratio of 2.6 in DBP−/− mice and 5.1 in DKO mice versus a ratio of 0.01 in wild type mice. The total amount of bile acids (C24 +...
C27) was also reduced in DBP−/− and DKO mouse liver (in wild type animals 367 ± 87, in DBP−/− mice 121 ± 18, and in DKO mice 122 ± 30 nmol/g (w/w); see Fig. 3). At P10–11, the deficiency of C24-bile acids was even more pronounced in DKO mice (10 ± 3 nmol/g), resulting in a C27/C24 ratio of 13.3 in DKO mice versus 1.9 in DBP−/− mice. Surprisingly, at this age, livers from DBP−/− mice also contained reduced levels of C24-bile acids (243 ± 17 nmol/g versus 325 ± 80 nmol/g in wild type mice). In contrast to the DBP−/− and DKO mice, there was no gross accumulation of C27-bile acid intermediates in LBP−/− livers (6.2 ± 2.7 nmol/g in LBP−/− mice versus 3.2 ± 0.7 nmol/g in wild type mice, 84 ± 23 nmol/g in DBP−/− mice, and 130 ± 57 nmol/g in DKO mice). A comparative analysis of bile acid content was performed in liver from eight LBP−/−:DBP−/−/+ and seven LBP−/−:−/− mice (P10–11) to study whether subtle changes between mice of these two genotypes could help clarify the role of LBP in bile acid biosynthesis. However, no differences in bile acid levels or patterns were found (see Table I). At P2−4 and in 6–10-week-old LBP−/− mice, the amount of C24-bile acids was completely normal. This was not the case in liver of DBP−/− mice, where C24-bile acids remained very low at all ages studied (P10–11, P21, 6–10 weeks).

Although the concentration of bile acids in plasma is low, the situation in the liver is relatively well reflected in plasma (Fig. 4). DBP−/− and DKO mice accumulate C27-bile acids, whereas

day they have a deficiency of C24-bile acids. This deficiency is most pronounced before weaning, but also at an adult age DBP−/− mice have statistically lower levels of C24-bile acids in their plasma. At P7–9, the sum of all C24-bile acids in plasma from DBP−/− mice is 3.5% from the C24-bile acids present in plasma from wild type animals. At P14–15, this is 7.7%; at P21 it is 27.6%; and at the age of 6–10 weeks it is 41.2%. This increasing percentage is not only due to a small increase in C24-bile acids of about 1.3-fold in DBP−/− mice but is mostly because of a strong decrease of bile acids in plasma from wild type animals (and also LBP−/− mice). Age-dependent changes in bile acid concentrations were even more pronounced in bile of DBP−/− mice (Fig. 5). C24-bile acids gradually increased with age, equaling levels in wild type mice at 5–6 months. At this age, bile from DBP−/− mice could only be distinguished.

C27-bile acids in plasma from wild type animals (P10–11) to study whether subtle changes between mice of these two genotypes could help clarify the role of LBP in bile acid biosynthesis. However, no differences in bile acid levels or patterns were found (see Table I). At P2−4 and in 6–10-week-old LBP−/− mice, the amount of C24-bile acids was completely normal. This was not the case in liver of DBP−/− mice, where C24-bile acids remained very low at all ages studied (P10–11, P21, 6–10 weeks).

Although the concentration of bile acids in plasma is low, the situation in the liver is relatively well reflected in plasma (Fig. 4). DBP−/− and DKO mice accumulate C27-bile acids, whereas

### Table I

Bile acid levels in liver from LBP:DBP−/−:+/+ and LBP−/−:−/− mice (P10–11)

|             | Total C24-bile acids | Total C27-bile acids | Total bile acids |
|-------------|-----------------------|----------------------|-------------------|
| LBP:DBP−/−:+/+ (n = 8)* | 233 ± 92 | 5.9 ± 3.5 | 239 ± 94 |
| LBP−/−:−/− (n = 7) | 256 ± 46 | 6.6 ± 1.5 | 262 ± 46 |

* n = number. All measurements were performed in duplicate.
from wild type and LBP−/− bile because of the presence of C27-bile acids. Together with a simultaneous age-dependent increase of C27-bile acids in bile of DBP−/− mice, this gave rise to elevated total bile acid levels in bile of 5–6-month-old DBP−/− mice. At all ages, the ratio of C27/C24-bile acids in bile of DBP−/− mice was much lower than in plasma and liver (at 6–10 weeks of age, the ratio was 1.2 in bile compared with 3.5 and 9.0 in liver and plasma, respectively).

A surprising observation was that the accumulating C27-bile acids in DBP−/− mice were predominantly unconjugated before weaning in liver, bile, and plasma, whereas they were mostly present as taurine-conjugates at adult age (the ratio of taurine-free C27-bile acids in liver of DBP−/− mice at P2–4 was 0.13 ± 0.06; at P10–11, 0.53 ± 0.34; at P21, 1.34 ± 0.63; and 3.37 ± 1.05 in 6–10-week-old animals; see Figs. 3–5). This age-dependent increase in conjugation of C27-bile acid intermediates was in sharp contrast to the situation with the C24-bile acids, which were already fully conjugated at P2–4 both in wild type and DBP−/− mice. Even in adult DBP−/− mice, about 23% of the C27-bile acids were unconjugated, whereas only 5% of the C24-bile acids were unconjugated in the same animals.

**Peroxisomal Acyl-CoA Oxidase Measurements**—Because, in contrast to our expectation, DKO mice accumulated mainly THCA and hydroxylated THCA instead of THC:1, we measured peroxisomal acyl-CoA oxidase activities in liver from wild type, LBP−/−, DBP−/−, and LBP:DBP−/− mice at P10–11 with THC-CoA, pristanoyl-CoA, and, for comparison, with C16-CoA (see Table II). The oxidation rate of pristanoyl-CoA and THC-CoA was markedly increased in DBP−/− as compared with wild type livers, which is in agreement with earlier results demonstrating increased protein levels for ACOX2 (14). In contrast, in DKO mice, acyl-CoA oxidase activity measured with pristanoyl-CoA was strongly reduced and was even not detectable with THC-CoA as substrate. The residual conversion of pristanoyl-CoA could very well be due to the activity of ACOX3. The lack of THC-CoA oxidase activity explains the absence of THC:1 in liver, bile, and plasma from DKO mice. Straight-chain acyl-CoA oxidase (ACOX1) activity, measured with C16-CoA as substrate, was normal in LBP−/− mice, whereas it was up-regulated in DBP−/− and DKO mice. Previous studies have shown that the protein level of ACOX1 is increased in DBP−/− and DKO mice, although in the DKO mice only the unprocessed form of the protein is present, indicative of a disruption of the peroxisomal matrix protein import (16).

**Ontogeny of Bile Acid Conjugation in Mice**—Because we observed an age-dependent increase in conjugation of C27-bile acids in liver, plasma, and bile of DBP−/− mice (see Figs. 3–5), we decided to study bile acid conjugation in liver homogenates from mice during their development from the neonatal period until adulthood. Total C24-bile acids is the sum of t-(m)CA and (m)CA; total C27-bile acids is the sum of t-THCA, t-THC:1, OH-THCA, OH-THC:1, THCA, and THC:1; total bile acids is the sum of all C24- and C27-bile acids described above; the taurine/free C27 ratio is the ratio of the taurine-conjugated C27-bile acids over the free C27-bile acids described. The number of animals per group has been indicated in or just above the bars. *p < 0.05; **p < 0.005.

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**FIG. 4.** Bile acids measured in plasma from wild type, LBP−/−, DBP−/− and LBP:DBP−/− mice during development from the neonatal period until adulthood. Total C24-bile acids is the sum of t-(m)CA and (m)CA; total C27-bile acids is the sum of t-THCA, t-THC:1, OH-THCA, OH-THC:1, THCA, and THC:1; total bile acids is the sum of all C24- and C27-bile acids described above; the taurine/free C27 ratio is the ratio of the taurine-conjugated C27-bile acids over the free C27-bile acids described. The number of animals per group has been indicated in or just above the bars. *p < 0.05; **p < 0.005.
intermediate, were studied separately. With CA as substrate, the synthetase activity increased steadily with age from 411 ± 60 pmol/min/mg at P1 to 2212 ± 201 pmol/min/mg at 6–10 weeks in liver homogenates from wild type animals (see Fig. 6). For THCA as substrate, there was a sharp increase in synthetase activity between P1 and P2–4 (from 1180 ± 166 to 3391 ± 1106 pmol/min/mg), after which it remained constant until weaning, whereas in livers from 6–10-week-old mice, the activity was slightly lower (2557 ± 630 pmol/min/mg). Comparable activities were measured in liver homogenates of DBP−/− mice. The observed difference in activity pattern during development for these two substrates suggests that activation of CA and THCA might be mediated by different enzymes. For this reason, we studied mRNA expression of both very long-chain acyl-CoA synthetase (VLCS), which has been shown to be active with THCA in in vitro studies, and bile acyl-CoA synthetase (BACS), which uses CA and THCA, as substrate (24). Northern blot analysis revealed increasing amounts of VLCS and BACS mRNA with age; however, the amount of BACS transcript increased steadily, whereas in comparison there was a sharper increase in the amount of VLCS transcript between P0 and P10. If in vivo THCA is activated by VLCS, whereas CA is predominantly activated by BACS, the mRNA expression corresponds well to the enzyme activity measurements with both substrates.

Northern blot analysis for BAAT revealed very low levels of mRNA in 1–2-week-old mice, whereas the BAAT transcript was abundantly present at P20 (Fig. 6). The transferase activity, measured with CA-CoA as substrate, started to increase after P7−8 from 0.8 ± 0.2 to 4.5 ± 0.8 nmol/min/mg at P21 and 7.4 ± 0.6 nmol/min/mg in 6–10-week-old mice. Comparable activities were measured in liver homogenates of DBP−/− mice. Subcellular fractionation experiments on 14-day-old and 6-week-old mouse livers revealed that the age-dependent increase in BAAT activity is due to a rise in both peroxisomal and cytosolic activities (see Table III). BAAT activity, normalized for the content of catalase, was 3.2- and 3.7-fold higher, respectively, in the light mitochondrial (L) fraction enriched in per-

### TABLE II

Peroxisomal acyl-CoA oxidase activities in liver from wild type, LBP−/−, DBP−/−, and LBP:DBP−/− mice

| Activity | Wild type  | LBP−/−  | DBP−/−  | LBP:DBP−/− |
|----------|-----------|---------|---------|------------|
| Pristanoyl-CoA | 6178 ± 60 | 901 ± 180 | 557 ± 161 | ND* |
| THC-CoA   | 481 ± 335 | 914 ± 179 | 437 ± 108 | ND* |
| Wild type | 16,839 ± 6195 | 1182 ± 43 | 771 ± 124 |
| LBP−/−    | 8808 ± 66 | 155 ± 93 | ND* |

* Not detectable. Measurements were performed in two mice per group in triplicate.
measurements are indicated at the bars as substrate (synthetase and transferase activity in liver from wild type and PPARα knock-out mice at P2, P9, P16, P23, P30, and 5 and 6 weeks. The pattern for both enzyme activities during development was similar in the PPARα−/− mice and in the wild type mice (data not shown).

**DISCUSSION**

The presence of mature C24-bile acids in patients and mice with a deficiency of DBP raises the question of whether these can be generated via an alternative pathway involving LBP. This possibility was investigated by comparing bile acid profiles and phenotypes of LBP:DBP DKO mice with those of DBP and LBP single knock-out mice. If LBP would indeed be involved in such an alternative pathway, this would lead to a more extensive accumulation of potentially toxic C27-bile acid intermediates and a greater shortage of C24-bile acids in DKO mice and could be an explanation for the more severe phenotype of DKO mice when compared with DBP−/− mice.

In liver, bile, and plasma from LBP−/− mice, we found a normal pattern of bile acids. This was in line with the absence of a gross phenotype in these mice (15) and the fact that defects in bile acid biosynthesis do cause clear cut symptoms in patients and mice (28, 29). Unexpectedly, we found significantly reduced levels of C24-bile acids in liver of LBP−/− mice, but only at P10–11 and not at P2–4 or in 6–10-week-old mice. In theory, this could point to a higher dependence on a potential alternative bile acid biosynthesis pathway involving LBP at this stage in development. However, if the reduction of C24-bile acids is a direct consequence of the inactivity of LBP, one would expect accumulation of C27-bile acids. Although the amount of C27-intermediates was doubled in LBP−/− mice compared with wild type mice, their contribution to the total bile acid pool in liver was only 2.5%, whereas this was 65 and 93% in livers from DBP−/− and DKO mice, respectively. Comparative analysis of bile acids in LBP:DBP−/−/+ and LBP:DBP−/−:−/− mice did not reveal any additional insights into a potential role for LBP in bile acid biosynthesis, since no differences were found in bile acid levels or patterns between mice of these two genotypes. We therefore hypothesize that the temporarily reduced levels of C24-bile acids in LBP knock-out mice are rather due to a dysregulation of bile acid homeostasis by thus far unknown factors.

DBP−/− and LBP:DBP DKO pups did accumulate large amounts of C27-bile acids in liver, bile, and plasma, accompanied by a severe reduction of C24-bile acids. These changes were more pronounced in DKO mice, resulting in an even higher C27/C24-bile acid ratio. However, we could not conclude from these findings that LBP is indeed involved in a minor rescue pathway in case of DBP deficiency, because the major accumulating C27-bile acids in DKO mice were THCA and OH-THCA instead of the expected THC:1. We demonstrated that this was due to the inability of liver homogenates from DKO mice to oxidize THC-CoA. These results are in agreement with previous studies showing that DKO mice lack catalase-containing peroxisomes in their hepatocytes (16) and contain unprocessed ACOX1, compatible with a defect in the import of peroxisomal matrix proteins. Furthermore, it was reported that in humans with peroxisome biogenesis disorders, branched-chain acyl-CoA oxidase is not active when mislocalized to the cytosol, whereas ACOX1 can be normally active in its unprocessed form, which was also confirmed in our studies (Table II) (30, 31). Unfortunately, this lack of THC-CoA oxidase activity in liver from LBP:DBP DKO mice prevents us from drawing any conclusion about the potential role of LBP in a rescue pathway for bile acid biosynthesis. The nearly complete absence of mature bile acids in DKO mice does stress the importance of peroxisomes for bile acid biosynthesis in mice, since the alternative microsomal pathway clearly cannot com-

![Image](96x595 to 271x623)

![Image](96x627 to 271x664)

![Image](96x668 to 272x719)

**FIG. 6. A** Northern blot analysis of the ontogeny of the enzymes involved in bile acid conjugation. Thirty μg of total liver RNA from wild type mice from embryonic day 16.5, P0, P10, P20, and 6 weeks of age was loaded, and the blot was probed for BACS, VLCS, and BAAT. Northern blot analysis for β-actin was performed as a control for RNA loading. B, measurement of bile acyl-CoA synthetase activity in mouse liver during development with CA as substrate (black bars) and THCA as substrate (gray bars). The ages of the mice are indicated below the bars. The number of animals used per group for the enzyme activity measurements are indicated at the bottom of the figure (n). C, measurement of bile acyl-CoA amino acid acyltransferase activity with CA-CoA and taurine as substrate (white bars).

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The nuclear hormone receptors FXR and HNF4 play roles in liver development. The expression of calregulin was studied as a control.

addition of the activities in all fractions (nuclear, mitochondrial, light mitochondrial, microsomal, and cytosolic fractions).

DBP were even further reduced in DKO mice. Interestingly, in mice was 14-fold lower than in control mice, and these levels normally developed.

The active resorption of bile acids in the intestine is still not attained by 1 week of age (33). Therefore, at P14–15, our first time and glucuronidated dianionic bile salts, have reached adult levels by 6–10-week-old mice. The presence of large amounts of C27-bile acids in bile indicates that they are a substrate for hepatic bile acid transporters in the canalicular membrane. The ratio of C27/C24-bile acids, however, was only 1.2 in bile from adult DBP/−/− mice, whereas it was 3.5 in liver, suggesting that the transporters have less affinity for the C27-bile acids than for the mature C24-bile acids. The same might be true for the basolateral bile salt transporters in hepatocytes responsible for the uptake of bile acids from the blood stream, since in plasma the ratio was 9.

While studying the differences in bile acid composition during development, we noticed that in contrast to the C24-bile acid pool, which was almost fully conjugated at all ages, the C27-bile acids were predominantly unconjugated before weaning and that conjugation increased with age. In rats, CA conjugation has been reported to be significantly lower in suckling than in adult rats (34), which was accounted for by a sharp increase of CA synthetase activity at P21. The authors concluded that the synthetase activity was rate-limiting for bile acid conjugation. Our studies in mice did not confirm these observations, since we found a steady increase in expression of the mRNAs of BACS and VLCS during postnatal development, which correlated well with the enzyme activities measured. In contrast, a marked induction of BAAT expression was seen between P10 and P21 in mice, which was paralleled by increasing transferase activity using CA-CoA as a substrate. This also coincided well with the transition of primarily unconjugated C27-bile acids in liver, bile, and plasma of DBP/−/− mice during the lactation period to mainly conjugated C27-bile acids from P21 on. We were only able to measure transferase activity with THC-CoA as substrate in a peroxisome-enriched fraction prepared from adult liver, albeit the activity was very low. This is most likely caused by the substrate specificity of BAAT. The effect of bile acid structure on the activity of bovine BAAT has been studied, and it was found that extending the side chain by one methylene group caused a 30-fold decrease in activity at V_{max} (35). THC-CoA itself was not tested in this study, but in a later study, recombinant human BAAT was reported to have very low activity toward THC-CoA (36). We therefore conclude that C27-bile acids remain predominantly unconjugated in mouse pups because of the very low expression of BAAT and a high K_{m} for C27-bile acyl-CoAs. Only in adult mice, when BAAT is abundantly present in liver, a large portion of the C27-bile acids become conjugated. In contrast, the K_{m} for the mature C24-bile acids is low enough to allow almost complete conjugation of the C24-bile acid pool even when the expression of BAAT is very low. Thus, in addition to the extremely low amounts of bile acids in bile of lactating DBP/−/− and DKO mice, they are mostly present in their unconjugated state, which will have a further negative impact on the resorption of lipids in the intestine, since unconjugated bile acids are much

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**TABLE III**

|                  | LDH  | BAAT | Catalase | Ratio BAAT/Catalase | Ratio 6 weeks/14 days |
|------------------|------|------|----------|--------------------|-----------------------|
|                  | units/g liver | milliunits/g liver | units/g liver |                     |                       |
| 14 days          |      |      |          |                    |                       |
| L fraction       | 8.8  | 126  | 19.6     | 6.5                |                       |
| S fraction       | 828  | 47   | 3.4      | 14.1               |                       |
| Total recovered activity | 889  | 495  | 40.5     |                     |                       |
| 6 weeks          |      |      |          |                    |                       |
| L fraction       | 1.3  | 300  | 14.3     | 21.0               | 3.2                   |
| S fraction       | 238  | 404  | 7.7      | 51.6               | 3.7                   |
| Total recovered activity | 261  | 1747 | 60.5     |                     |                       |

FIG. 7. A, Northern blot analysis of the expression of FXR in mouse liver during development. B, Western blot analysis of the expression of the nuclear hormone receptors FXR and HNF4α in mouse liver during development. The expression of calregulin was studied as a control.

compensate for the loss of peroxisomal activity.

In wild type mice, the amount of bile acids in bile was higher before weaning than in adulthood, which could very well reflect their importance for the absorption of the large amount of milk triacylglycerols ingested during the suckling period. This high amount of bile acids in bile before weaning could be caused by an imbalance between bile acid secretion and resorption. In rats, it has been shown indeed that the expression of two components of ileal bile acid absorption, the apical sodium-dependent bile acid cotransporter and the ileal bile acid-binding protein, is very low during the first postnatal weeks and is markedly increased at the time of weaning. This time is not only characterized by a transition from milk to solid food but also by many hormonal changes that have been shown to be involved in the ontogenic expression of apical sodium-dependent bile acid cotransporter and ileal bile acid-binding protein (32). The low expression of apical sodium-dependent bile acid cotransporter and ileal bile acid-binding protein leads to malabsorption of bile acids in the intestine and might be compensated by a higher synthesis and excretion of bile acids in bile. Recently, it has been demonstrated that the protein level for the canalicular bile salt export pump and the multidrug resistance-associated protein 2, responsible for excretion of sulfated and glucuronidated dianionic bile salts, have reached adult levels by 1 week of age (33). Therefore, at P14–15, our first time point of bile acid measurements in bile, the canalicular excretion of bile acids already functions at an adult level, whereas the active resorption of bile acids in the intestine is still not normally developed.

The concentration of bile acids in bile of lactating DBP/−/− mice was 14-fold lower than in control mice, and these levels were even further reduced in DKO mice. Interestingly, in DBP/−/− mice, the amounts of C24- and C27-bile acids increased with age, probably as a result of increased synthesis, reaching normal levels in 6–10-week-old mice. The presence of large amounts of C27-bile acids in bile indicates that they are a substrate for hepatic bile acid transporters in the canalicular membrane. The ratio of C27/C24-bile acids, however, was only 1.2 in bile from adult DBP/−/− mice, whereas it was 3.5 in liver, suggesting that the transporters have less affinity for the C27-bile acids than for the mature C24-bile acids. The same might be true for the basolateral bile salt transporters in hepatocytes responsible for the uptake of bile acids from the blood stream, since in plasma the ratio was 9.
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less efficient in emulgating lipids. This certainly contributes to the small to minimal weight gain of the lactating pups of both genotypes.

Recently, the nuclear hormone receptors FXR, HNF4α, and PPARα have been shown to play a regulatory role in bile acid conjugation. Both BACS and BAAT expression are induced by FXR in rat liver (25), and HNF4α−/− mice exhibited a markedly decreased expression of VLCS and BAAT, which was associated with strongly elevated levels of unconjugated bile acids in bile (26). In addition, it was found that a decreased level of FXR in the ileum during the suckling period might account, in part, for the lack of ileal bile acid-binding protein expression at that time (37). Also the PPARα ligand WY-14,643 was shown to down-regulate BAAT expression in a PPARα-dependent way (27). However, we found no differences in bile acyl-CoA synthetase or bile acyl-CoA:amino acid acyltransferase activities between wild type and PPARα−/− livers during development, indicating the PPARα is most likely not involved in the ontogenic regulation of BACS, VLCS, and/or BAAT. We did find an increase of FXR and HNF4α protein at P7−8 in mouse liver, suggesting that the low expression of FXR and HNF4α in liver in the first postnatal days of life could, at least in part, play a role in the low expression of BAAT. In a very recent study in rat liver, it was demonstrated that the expression of nuclear receptors during rat liver development could control the regulation of bile acid and cholesterol metabolic pathways (38). However, the expression patterns of BACS, VLCS, and BAAT do not parallel the expression of the nuclear receptors, and especially the marked up-regulation of BAAT at weaning strongly suggests the involvement of hormonal and dietary factors.

In conclusion, the deficiency of mature bile acids in LBP−/− DBP DKO mice is more extensive than in DBP−/− mice, which could play a role in their worse prognosis. However, the comparative analysis of bile acid biosynthesis in these mouse models is complicated by abnormalities in the import of peroxisomal enzymes in DKO mice, resulting in strongly reduced activities of THC-CoA oxidase, the first enzyme involved in the peroxisomal steps of bile acid biosynthesis. For more insight into the role of the bile acid synthesis defect in the development of their severe phenotype, further experiments should be performed by feeding the mice bile acids to replenish the reduced levels of C24-bile acids and to inhibit the synthesis of the potentially toxic C27-bile acids. Furthermore, this study documented extensive changes in bile acid content in bile of wild type and DBP−/− mice and a strong increase in bile acid conjugation capacity both in vivo and in vitro during development.

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REFERENCES

1. Russell, D. W. (2003) Annu. Rev. Biochem. 72, 137−174

2. Wanders, R. J. A., Barth, P. G., and Heymans, H. S. A. (2001) in The Molecular and Metabolic Bases of Inherited Disease (Scrivner, C. R., Beaudet, A. L., Sly, W. S., and Valle, D. eds) pp. 3219−3256, McGraw-Hill, New York.

3. Falany, C. N., Forinbergh, H., Leiter, E. H., and Barnes, S. (1997) J. Lipid Res. 38, 1139−1148

4. Clayton, P. T., Lake, B. D., Hjem, M., Stephenson, J. B., Besley, G. T., Wanders, R. J., Schram, A. W., Tager, J. M., Schutgens, R. B., and Lawson, A. M. (1998) J. Inherit. Metab. Dis. 21, 165−168

5. Uno, M., Koshi, M., Suzuki, Y., Akaboshi, S., Yoshii, M., Kuramato, T., and Fujimura (1997) J. Biochem. (Tokyo) 122, 655−668

6. Duane, W. C., Bjorkhem, I., Hamilton, J. N., and Mueller, S. M. (1988) Hepatology 8, 613−618

7. Duane, W. C., Pooler, P. A., and Hamilton, J. N. (1988) J. Clin. Invest. 82, 82−85

8. Honda, A., Saen, G., Shefer, S., Matsuzaki, Y., Xu, G., Battu, A. K., Tint, G. S., and Tanaka, N. (2000) J. Lipid Res. 41, 442−451

9. Cuebas, D. A., Phillips, C., Schmitz, W., Aliev, E., and Novikov, D. K. (2002) Biochem. J. 363, 801−807

10. Wanders, R. J., Freken, P., Ferdinandusse, S., Jansen, G. A., Waterham, H. R., Van Roermund, C. W., and Van Grunsven, E. G. (2001) Biochem. Soc. Trans. 29, 250−267

11. Xu, R., and Cuebas, D. A. (1996) Biochem. Biophys. Res. Commun. 221, 271−278

12. Bjorkhem, I., Andersson, U., Ellis, E., Alievus, G., Elleberg, L., Dezfaluzy, U., Sovjall, J., and Ennarsson, C. (2001) J. Biol. Chem. 276, 37004−37010

13. Van Roermund, C. W., and Van Grunsven, E. G. (2001) J. Biol. Chem. 276, 16329−16336

14. Qi, C., Zhu, Y., Pan, J., Ueda, N., Mada, N., Yeldandi, A. V., Rao, M. S., Hashimoto, T., and Reddy, J. K. (1999) J. Biol. Chem. 274, 15773−15780

15. Jia, Y., Qi, C., Zhang, Z., Hashimoto, T., Rao, M. S., Huyghe, S., Suzuki, Y., Van Veldhoven, P. P., Baes, M., and Reddy, J. K. (2003) J. Biol. Chem. 278, 47232−47239

16. Baes, M., Gresse, P., Huyghe, S., De, N. K., Qi, C., Jia, Y., Mannerts, G. P., Evrard, V., Van, V. P., Declercq, P. E., and Reddy, J. K. (2002) J. Neuro-pathol. Exp. Neurol. 61, 368−374

17. Boomsma, A. H., Overmars, H., van Rooij, A. van, Lint, A. E., Wanders, R. J., Gennip, A. H., and Freken, P. (1999) J. Inherit. Metab. Dis. 22, 307−310

18. Wanders, R. J., Denis, S. W., and Dacremont, G. (1993) J. Biochem. (Tokyo) 113, 577−582

19. Ferdinandusse, S., Casteels, M., Janssens, A., Meulders, L., Mannerts, G. P., Declercq, P. E., Van Veldhoven, P. P., and Baes, M. (2001) Biochem. J. 353, 673−680

20. Baes, M., Huyghe, S., Casteels, M., Janssens, A., Meuldiers, L., Mannerts, G. P., Declercq, P. E., Van Veldhoven, P. P., and Baes, M. (2001) Biochem. J. 353, 105−106

21. Ishibashi, S., Schwarz, M., Frykman, P. K., Herz, J., and Russell, D. W. (1996) J. Biol. Chem. 271, 24771−24779

22. Frederick, P. C., Kitte, J. L., Paterson, R. J., Tazikala, R. K., Bischoff, E. D., Schulman, A. J., and Westin, S. K. (2003) J. Biol. Chem. 278, 27703−27711

23. Inoue, Y., Yu, A. M., Inoue, J., and Gonzalez, F. J. (2004) J. Biol. Chem. 279, 2480−2489

24. Solas, K., Kase, R. F., Pham, V., Bamberg, K., Hunt, M. C., and Alexson, S. E. (2004) J. Lipid Res. 45, 1051−1060

25. Nishibashi, S., Schwarz, M., Frykman, P. K., Herz, J., and Russell, D. W. (1996) J. Biol. Chem. 271, 18017−18023

26. De Vries, K. E., Daugherty, C. C., Tyson, W., Mierau, G., Heubi, J. E., Balistreri, W. F., and Setchell, K. D. (2000) Pediatr. Dev. Pathol. 3, 1−16

27. Baumgart, E., Vanhooren, J. C., Fransen, M., Marynen, P., Puye, M., Vandekerckhove, J., Leunissen, J. A., Fahimi, H. D., Mannerts, G. P., and Van Veldhoven, P. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13748−13753

28. Ferdinandusse, S., Finckh, B., de Hingh, Y. C., Stroomer, A. E., Denis, S., Kohlschutter, A., and Wanders, R. J. (2003) Mol. Genet. Metab. 79, 281−287

29. Hwang, S. T., and Henning, S. J. (2003) J. Biochem. (Tokyo) 133, 221−226

30. Baes, M., Huyghe, S., Casteels, M., Janssens, A., Meuldiers, L., Mannerts, G. P., Declercq, P. E., Van Veldhoven, P. P., and Baes, M. (2001) Biochem. J. 353, 673−680