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Biochimica et Biophysica Acta 1763 (2006) 1427–1440

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Review

Peroxisomes and bile acid biosynthesis

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Received 28 April 2006; received in revised form 24 August 2006; accepted 1 September 2006
Available online 14 September 2006

Abstract

Peroxisomes play an important role in the biosynthesis of bile acids because a peroxisomal β-oxidation step is required for the formation of the mature C_{24}-bile acids from C_{27}-bile acid intermediates. In addition, de novo synthesized bile acids are conjugated within the peroxisome. In this review, we describe the current state of knowledge about all aspects of peroxisomal function in bile acid biosynthesis in health and disease. The peroxisomal enzymes involved in the synthesis of bile acids have been identified, and the metabolic and pathologic consequences of a deficiency of one of these enzymes are discussed, including the potential role of nuclear receptors therein.
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Keywords: Peroxisomal β-oxidation; Nuclear receptors; C_{27}-bile acid intermediates; Peroxisome deficiency disorders

1. Introduction

In the early 1980s, the first clues were obtained indicating the importance of peroxisomes in the biosynthesis of bile acids. In 1978, Hansen et al. were the first to report a defect in bile acid synthesis in Zellweger syndrome [1]. At the time, however, this defect was attributed to the mitochondrial abnormalities associated with this disorder. Later it became clear that these mitochondrial abnormalities are only secondary and that the bile acid biosynthesis deficiency in these patients is caused by the primary defect in this disorder, i.e. the absence of functional peroxisomes. The accumulation of C_{27}-bile acid intermediates showed that Zellweger patients were not able to cleave the side-chain of these precursors and thus could not form mature C_{24}-bile acids. After the identification of a fatty acid oxidizing system in peroxisomes, the subcellular localization of this side-chain cleavage was reinvestigated and was shown to occur in peroxisomes [2,3]. Many reports on bile acid abnormalities in patients with a peroxisome deficiency followed and even prenatal diagnosis of Zellweger syndrome by measurement of bile acids in amniotic fluid can be done reliably [4,5]. In the 1990s, next to the classical set of fatty acid oxidation enzymes, a second set of fatty acid oxidation enzymes was identified in the peroxisome and many studies were performed to resolve the role of the individual enzymes in the different peroxisomal functions, including bile acid biosynthesis. Not only in vitro studies with purified and recombinant enzymes attributed to the resolution but also the identification of patients with single enzyme deficiencies associated with bile acid abnormalities. In this paper, the current knowledge about the peroxisomal steps in bile acid biosynthesis, including the role of nuclear receptors (NRs) in their control, but also the gaps in this knowledge will be reviewed. In addition, the bile acid abnormalities in peroxisomal disorders and mouse models for peroxisomal disorders are discussed, including the consequences of these bile acid abnormalities.

2. Bile acid biosynthesis

Cholesterol is converted into bile acids via multiple pathways which involve many different enzymes. Many of these enzymes are predominantly expressed in the liver and are localized in several different subcellular compartments. Approximately 500 mg of cholesterol is converted into bile acids each day in the adult human liver. Bile acid biosynthesis involves modification of the ring structure of cholesterol, oxidation and shortening of the side chain, and finally conjugation of the bile acid with an amino acid. All these steps and the enzymes involved are reviewed in detail by

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doi:10.1016/j.bbamcr.2006.09.001

Russell [6]. In the classic pathway, bile acid biosynthesis begins with the conversion of cholesterol into 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (CYP7A1). This is a microsomal cytochrome P450 enzyme localized exclusively in the liver. Its expression is highly regulated and shows a strong diurnal rhythm. The classic pathway involving CYP7A1 is the major pathway in bile acid biosynthesis because its contribution to total bile acid synthesis is ~ 90% humans and ~ 75% in mice [6]. A schematic overview of the classic pathway is depicted in Fig. 1. In the acidic pathway, oxysterols rather than cholesterol serve as substrates for 7α-hydroxylation. Sterol 27-hydroxylase, a mitochondrial cytochrome P450 (CYP27A1), forms predominantly 27-hydroxycholesterol but can also hydroxylate cholesterol at carbons 24 and 25. Alternatively, 24- and 25-hydroxycholesterol are also the product of separate cholesterol 24- and 25-hydroxylases. To be converted into bile acids, these oxysterols must undergo 7α-hydroxylation. Oxysterol 7α-hydroxylase (CYP7B1) catalyzes the conversion of 27-hydroxycholesterol and 25-hydroxycholesterol, and is highly expressed in liver [6]. The CYP39A1 oxysterol 7α-hydroxylase acts on 24-hydroxycholesterol [6]. The acidic pathway is responsible for the remaining bile acid synthesis (~10% in humans and ~25% in mice) [6].

The next step in the ring structure modifications involves isomerization of the double bond from the 5 to the 4 position and the oxidation of the 3β-hydroxyl-group to a 3-oxo-group. This step is catalyzed by the microsomal 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (HSD3B7), which can only handle 7α-hydroxylated intermediates derived from both cholesterol and oxysterols [6]. At this point in the biosynthesis pathway, the intermediates can be acted upon by sterol 12α-hydroxylase (CYP8B1), and this will decide the fate of the produced bile acid [6]. If handled by CYP8B1, another microsomal cytochrome P450, the final product will be cholic acid (CA), whereas otherwise chenodeoxycholic acid (CDCA) (in humans) or muricholic acid (mCA) (in mice) will be formed. Subsequently, the products of HSD3B7, whether or not 12α-hydroxylated, are subject to reduction of the double bond by the enzyme Δ^4-3-oxosteroid 5β-reductase (AKR1D1), which is a cytosolic enzyme [6]. The final step of the ring modifications involves reduction of the 3-oxo-group to an alcohol-group and is catalyzed by 3α-hydroxysteroid dehydrogenase (AKR1C4) [6].

After the ring modifications, the sterol side chain is oxidized, activated and shortened by cleaving off propionyl-CoA. The first steps of this process are catalyzed by CYP27A1, which is the same mitochondrial enzyme that can initiate bile acid biosynthesis through formation of 27-hydroxycholesterol. The enzyme introduces a hydroxyl-group at carbon 27 and then oxidizes this group to an aldehyde and then to a carboxylic acid

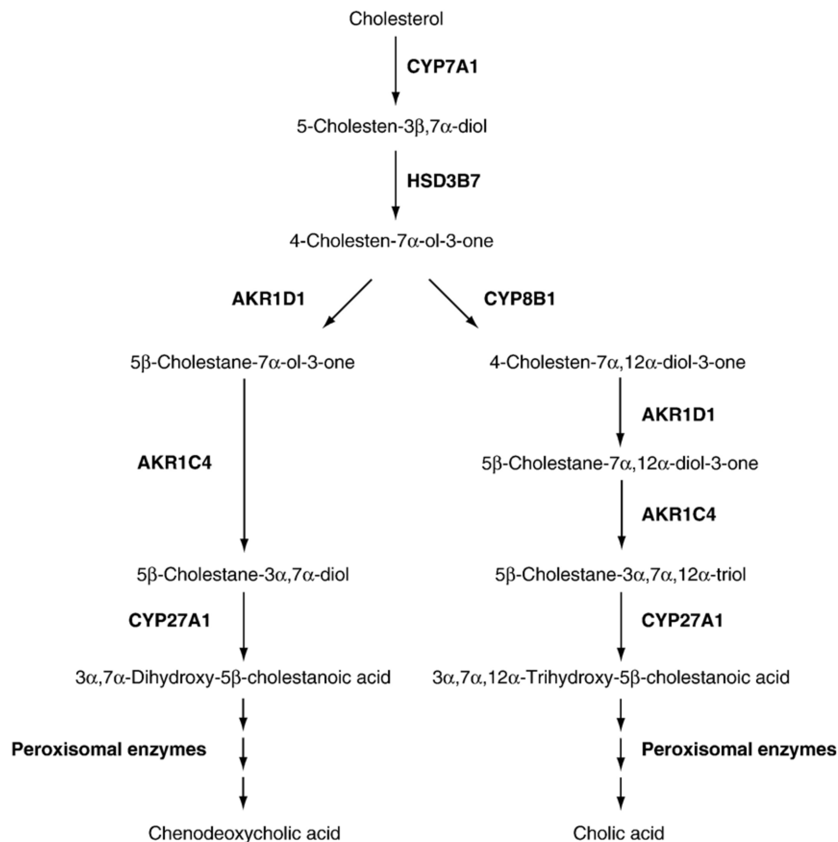

Fig. 1. Schematic representation of the classic bile acid biosynthesis pathway which is responsible for ~ 90% of the total bile acid synthesis in humans and ~ 75% of total bile acid synthesis in mice. The peroxisomal steps of the pathway are shown in Fig. 2. CYP7A1, cholesterol 7α-hydroxylase; HSD3B7, 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase; CYP8B1, sterol 12α-hydroxylase; AKR1D1, Δ^4-3-oxosteroid 5β-reductase; AKR1C4, 3α-hydroxysteroid dehydrogenase; CYP27A1, sterol 27-hydroxylase.

[6]. The products of this reaction are the bile acid intermediates 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA) (Fig. 2), which subsequently have to be activated to their CoA-ester. Two enzymes have been identified which can perform this reaction. The first is the very long-chain acyl-CoA synthetase (VLCS, encoded by *SLC27A2*) localized at the endoplasmic reticulum and in the peroxisome, and the second is the bile acyl-CoA synthetase (BACS, also called the very long-chain acyl-CoA synthetase homolog 2, encoded by *SLC27A5*) exclusively localized at the

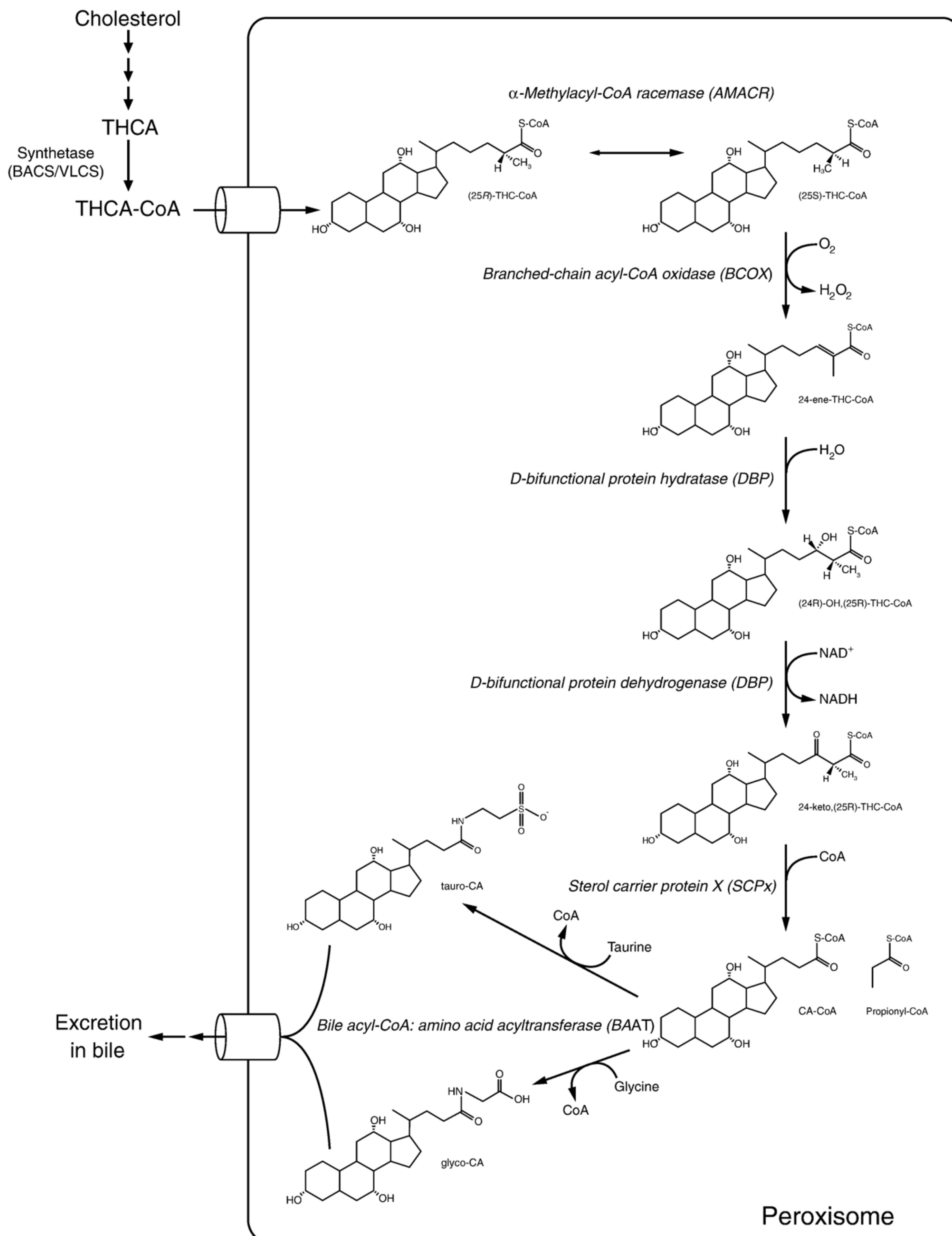

Fig. 2. Schematic representation of the peroxisomal steps involved in bile acid biosynthesis.

endoplasmic reticulum [7]. BACS, which is only present in liver, also displays activity towards C_{24}-bile acids and is thought to be involved in the activation of the bile acids which return to the liver via the enterohepatic circulation after deconjugation in the small intestine [7]. At the moment, it is unclear which of the two synthetases capable of activating C_{27}-bile acids is in vivo responsible for the CoA-ester formation of DHCA and THCA, and whether this occurs at the endoplasmic reticulum or in the peroxisome. Although activation within the peroxisome is an attractive hypothesis because the subsequent shortening of the side-chain also occurs within the peroxisome, only a very small portion of VLCS is localized in the peroxisome increasing the likelihood of microsomal activation [8]. To date, no THC-CoA synthetase activity has been demonstrated in the peroxisome [9,10]. This would imply that DHC-CoA/THC-CoA have to be transported into the peroxisome. ATP-dependent transport of THC-CoA, and not of THCA, across rat liver peroxisomal membranes has been shown by demonstration of subsequent production of CA. These studies, however, were performed with peroxisomes purified by differential centrifugation followed by isopycnic centrifugation in sucrose [11] and it is well known that purified peroxisomes are leaky. Unequivocal evidence for transport of the C_{27}-bile acid intermediates, either as free acid or as CoA-ester, is therefore still lacking and the responsible transporter remains to be identified.

3. Peroxisomal steps in bile acid biosynthesis

After the discovery that THCA is metabolized in the peroxisome to CA by side-chain cleavage, all investigations were aimed at identifying the underlying mechanism. Especially since 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was identified as likely intermediate [1,12], a mechanism similar to fatty acid oxidation seemed very attractive. Indeed, the conversion of THCA to CA in peroxisomes was shown to be dependent on all the co-factors for fatty acid β-oxidation: ATP, Mg^{2+}, CoA, FAD and NAD^+[2,3,13]. Studies with $^{18}O_2$ and ^2H_2O revealed that the reactions involved desaturation followed by hydration, and were consistent with a reaction mechanism analogous to that of fatty acid β-oxidation [13]. With the identification and characterization of all the enzymes involved in peroxisomal β-oxidation, the role of the individual enzymes in bile acid synthesis has become clear. See for details on the peroxisomal β-oxidation enzymes Wanders and Waterham [14].

The peroxisomal steps in bile acid biosynthesis are depicted in Fig. 2. The first peroxisomal enzyme required for the conversion of DHC-CoA and THC-CoA to chenodeoxycholoyl-CoA (CDC-CoA) and choloyl-CoA (CA-CoA), respectively, is α-methylacyl-CoA racemase (AMACR) [15,16]. Its activity is necessary for this conversion, because only (25*R*)-stereoisomers are formed by CYP27A1 [17,18], whereas the peroxisomal β-oxidation system can only handle (25*S*)-isomers [19,20]. Thus, AMACR racemizes (25*R*)-DHC-CoA and (25*R*)-THC-CoA to their respective (*S*)-isomers, allowing subsequent β-oxidation of these substrates. In mouse and rat, THC-CoA oxidase (encoded by *Acox2*) oxidizes the CoA-esters of the C_{27}-bile acid intermediates [21,22]. Its ortholog in humans is the branched-chain acyl-CoA oxidase (BCOX, encoded by *ACOX2*) [23], which is involved in the oxidation of the C_{27}-bile acid intermediates and of pristanoyl-CoA that has a methyl-group at the 2 position just like the side-chains of DHCA and THCA. The acyl-CoA oxidase desaturates (25*S*)-THC-CoA to 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA (24(*E*)-ene-THC-CoA). Because β-oxidation of the side-chain of DHC-CoA is analogous to that of THC-CoA, only the intermediates of THC-CoA will be described here. The two subsequent reactions are catalyzed by D-bifunctional protein (DBP, also called multifunctional protein 2, multifunctional enzyme II, or D-peroxisomal bifunctional enzyme, encoded by *HSD17B4*). DBP consists of multiple functional units and is a stereospecific enzyme. Its hydratase unit converts 24(*E*)-ene-THC-CoA exclusively into (24*R*,25*R*)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA (24-OH-THC-CoA) [24–27], which is converted into 3α,7α,12α-trihydroxy-24-keto-5β-cholestanoyl-CoA (24-keto-THC-CoA) by the dehydrogenase unit of DBP [24–26]. Finally, sterol carrier protein X (SCPx) cleaves 24-keto-THC-CoA to CA-CoA and propionyl-CoA [28–30].

The last step in bile acid biosynthesis involves conjugation of CDC-CoA and CA-CoA with an amino acid. This reaction is catalyzed by the bile acyl-CoA: amino acid *N*-acyltransferase (BAAT), which is predominantly expressed in liver and is localized in peroxisomes and the cytosol [31–36]. This dual localization suggests that the peroxisomal BAAT is responsible for conjugation of the newly formed primary bile acids within the peroxisome, whereas the cytosolic BAAT will be involved in reconjugation of the recycled primary and secondary bile acids that have been deconjugated in the gut. Mouse BAAT is only active with taurine [37], but human and rat BAAT can conjugate bile acids with both taurine and glycine [31,32,38,39]. The ratio of glycine to taurine conjugated bile acids in humans is solely dependent on the relative abundance of the two amino acids [6]. After conjugation, the bile acids will have to be transported from the peroxisome by a yet unidentified transporter and will then be excreted from the hepatocyte into the bile.

4. Alternative pathways for bile acid biosynthesis

Studies on bile acid abnormalities in patients with a deficiency of one of the enzymes involved in bile acid biosynthesis have shown that alternative pathways for bile acid formation must exist. Although the relative contribution of these pathways will be minimal if the major pathway functions normally, they can contribute substantially to bile acid synthesis in case of defects. There is both a peroxisomal alternative pathway and a pathway that does not require any peroxisomal enzymes at all.

The proposed alternative pathway localized in peroxisomes involves the other peroxisomal bifunctional protein, LBP (also called multifunctional protein 1, multifunctional enzyme I, or L-peroxisomal bifunctional enzyme, encoded by *EHHADH*) and AMACR [40] (see Fig. 3). 24(*E*)-ene-THC-CoA is normally hydrated to (24*R*,25*R*)-24-OH-THC-CoA by DBP, but can also be converted to (24*S*,25*S*)-24-OH-THC-CoA by

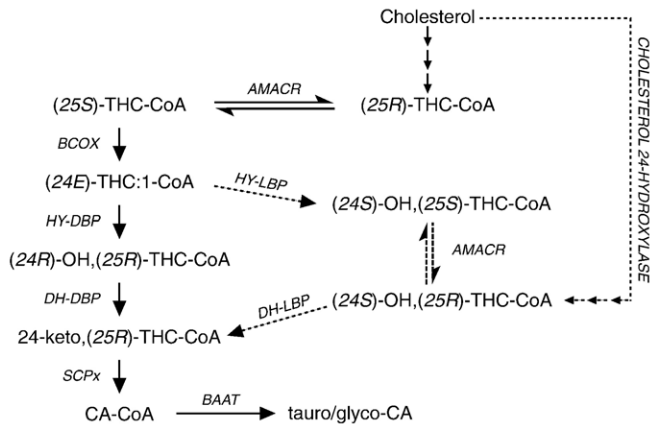

Fig. 3. Schematic representation of the peroxisomal steps in bile acid biosynthesis, including potential alternative pathways indicated by dashed arrows. The major peroxisomal pathway involves AMACR, BCOX, DBP (both the hydratase and dehydrogenase unit of DBP) and SCPx. LBP and AMACR take over the function of DBP in the alternative pathway. DBP activity is also not necessary when (24*S*)-OH, (25*R*)-THC-CoA is formed via 24-hydroxylation of cholesterol.

the hydratase unit of LBP [41]. (24*S*,25*S*)-24-OH-THC-CoA is, however, not a substrate for the dehydrogenase unit of LBP or DBP [25,41], but AMACR can convert it to the (24*S*,25*R*)-isomer [40] which can be handled by the dehydrogenase unit of LBP [41]. In addition, LBP will be involved in the synthesis of bile acids from (24*S*)-hydroxycholesterol as formed by cholesterol 24-hydroxylase which is predominantly expressed in mammalian brain. The (24*S*)-hydroxycholesterol is excreted across the blood–brain barrier into the circulation and is important for the elimination of cholesterol from the brain [42,43]. However, humans produce 6–7 mg of (24*S*)-hydroxycholesterol per day, of which ~3.5 mg is catabolized to bile acids, whereas ~500 mg of bile acids are synthesized per day [6]. This indicates that, although this pathway is important for the turnover of cholesterol in the brain, it contributes little to overall bile acid synthesis. In case of a deficiency of DBP or AMACR the alternative pathways involving LBP could be significant however. Attempts to determine the importance of this pathway in vivo by studying the bile acids abnormalities in LBP:DBP double knockout mice have been unsuccessful until now, because these animals turned out to have a peroxisomal import defect [34]. Details about this study will be discussed below.

In addition to the major pathways involving the mitochondrial CYP27A1 and the peroxisomal β-oxidation enzymes, there is an alternative microsomal plus cytosolic pathway for the cleavage of the steroid side-chain of 5β-cholestane-3α,7α,12α-triol [44–47]. The pathway starts with microsomal 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol followed by an additional 24-hydroxylation to yield 5β-cholestane-3α,7α,12α,24*S*,25-pentol. This compound subsequently is oxidized to a ketone and the 3-carbon side-chain is cleaved off as acetone by cytosolic enzymes. This 25-hydroxylase pathway is only active on 12α-hydroxylated compounds, thus CDCA is never formed, and its contribution to overall bile acid biosynthesis is less than 5% in human and rat [45–47].

5. Bile acid abnormalities in peroxisomal disorders

5.1. Peroxisome deficiency disorders

Analysis of plasma bile acids from children with a peroxisome deficiency disorder, including Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease, invariably shows the presence of C_{27}-bile acid intermediates and a C_{29}-dicarboxylic acid [48,49]. This dicarboxylic acid (3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestane-26-oic acid) was first identified by Parmentier et al. [50,51] and is thought to be formed by chain-elongation of THC-CoA. Van Eldere et al. [49] performed an extensive study on serum bile acids among 33 patients with a peroxisome deficiency disorder and 100 control subjects. They found that total serum bile acids ranged from normal to fifty times increased values which reveals the presence of cholestasis in many of these patients. The sum of DHCA, THCA and C_{29}-dicarboxylic acid as a percentage of the total sum of serum bile acids ranged from less than 5% to more than 90%. The patients with a less severe clinical presentation (Zellweger patients with a survival of more than 1 year and patients with neonatal adrenoleukodystrophy and infantile Refsum disease) had, on average, less cholestasis and a lower percentage of bile acid precursors [49]. This implies that the extent of the peroxisome deficiency corresponds to the extent of the deficiency in bile acid biosynthesis in these patients. The C_{24}-bile acids were normally conjugated, but the C_{27}-bile acid intermediates were only partially conjugated [49], which is most likely caused by the low affinity of BAAT towards C_{27}-bile acyl-CoAs [33,52].

In urine, the C_{27}-bile acids are also detectable, but very striking, especially in young children, is the presence of tetrahydroxy-5β-cholestanoic acid and sometimes even pentahydroxy-5β-cholestanoic acid (Fig. 4A). These hydroxyl-groups can be present on the steroid nucleus and have been identified as the 1β- and 6α-hydroxylated derivatives of THCA [53,54], but microsomal hydroxylases can also form C24-, C25- or C26-hydroxylated derivatives of THCA [55]. Hydroxylation of THCA is an important mechanism for increasing the polarity and thereby the urinary excretion of the C_{27}-bile acids. The C_{29}-dicarboxylic acid is not always present in urine or bile, because it is poorly excreted [51,56].

In contrast to the increased bile acid levels in serum due to cholestasis, the C_{24}-bile acids were strongly decreased in duodenal fluids of three patients with Zellweger syndrome [51]. In vivo studies on the formation of bile acids in patients with Zellweger syndrome by intravenous administration of labeled precursors have shown that the pool size of CDCA and CA was markedly reduced [12,56,57].

5.2. AMACR deficiency

AMACR deficiency can be diagnosed by the exclusive accumulation of (25*R*)-THCA and (25*R*)-DHCA [58,59] in plasma. For comparison, the ratio (25*S*/25*R*)-THCA was 0.23±0.05 for free THCA and 0.26±0.03 for tauro-THCA in plasma of four patients with Zellweger syndrome [58].

The presence of both isomers shows that AMACR is enzymatically active in case of a peroxisome deficiency disorder. Most patients with AMACR deficiency only present after adolescence, but one child presented at 2 weeks of age with a deficiency of fat-soluble vitamins and cholestasis. The bile acid abnormalities have been very well documented in this case [59]. Consistent with cholestasis the serum bile acid concentration and the urinary bile acid excretion were elevated, whereas the biliary bile acid concentration measured in the duodenal aspirate was decreased (0.9 mmol/L; normal > 10 mmol/L). Overall the C_{27}-bile acids accounted for 82.2%, 57.6% and 39.3% of the total bile acids in serum, urine and bile, respectively. THCA was also the major bile acid found in the feces and accounted for 72.2% of the total fecal bile acids excreted. Just like in patients with Zellweger syndrome, many polyhydroxylated C_{27}-bile acids were present in urine.

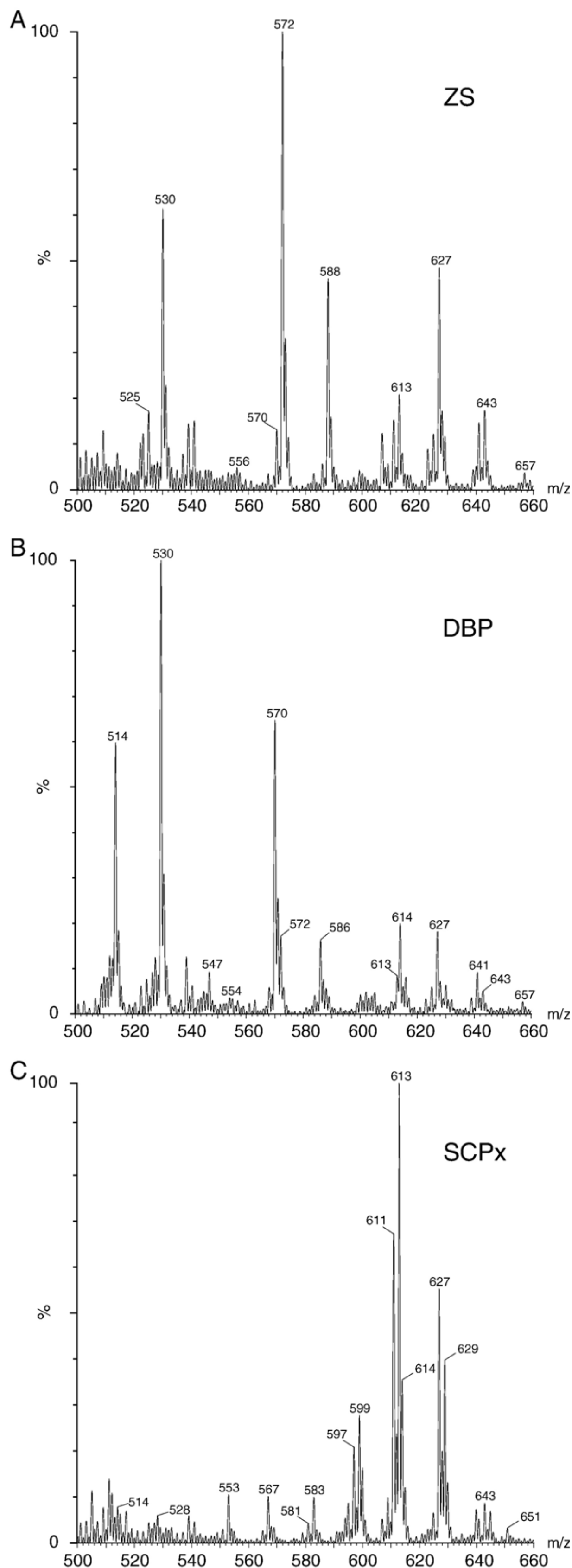

Fig. 4. Negative ion electrospray tandem mass spectrometric analysis of urine bile acids and bile alcohols from A, a patient with Zellweger syndrome, B, a patient with DBP deficiency and C, a patient with SCPx deficiency. Very characteristic is the presence of tauro-OH-THCA (tetrahydroxy-5β-cholestanoic acid) (*m*/*z* 572) and taurine conjugated pentahydroxy-5β-cholestanoic acid (*m*/*z* 588) in urine of the patient with Zellweger syndrome, the presence of the taurine conjugate of hydroxylated 24-ene-THCA (*m*/*z* 570) and dihydroxylated 24-ene-THCA (*m*/*z* 586) in the patient with DBP deficiency, and the presence of bile alcohol glucuronides in urine of the SCPx-deficient patient. These compounds are not present in the urine of a control subject. The (postulated) identity of the metabolites is as follows: *m*/*z* 514, tauro-CA; *m*/*z* 530, tauro-OH-CA; *m*/*z* 570 tauro-OH-24-ene-THCA; *m*/*z* 572, tauro-OH-THCA; *m*/*z* 586, tauro-diOH-24-ene-THCA; *m*/*z* 588, pentahydroxy-5β-cholestanoic acid; *m*/*z* 611, pentahydroxy-27-nor-5β-cholestane-24-one or 5β-cholestanetetrol glucuronides; *m*/*z* 613, 27-nor-5β-cholestanepentol glucuronide; *m*/*z* 627, hexahydroxy-27-nor-5β-cholestane-24-one or 5β-cholestanepentol glucuronides; *m*/*z*, 629 27-nor-5β-cholestanehexol glucuronide; *m*/*z* 643, heptahydroxy-27-nor-5β-cholestane-24-one or 5β-cholestanehexol glucuronides.

5.3. DBP deficiency

The bile acid abnormalities associated with DBP deficiency differ between the different types of DBP deficiency. Patients with a type III deficiency, i.e. an isolated 3-hydroxyacyl-CoA dehydrogenase deficiency, accumulate predominantly 24-OH-THCA. Studies on the isomeric configuration revealed that the main accumulating isomer was (24*R*,25*R*)-24-OH-THCA, which is formed by the hydratase unit of DBP, but is not catabolized further because of the deficiency of the dehydrogenase unit [60,61]. (24*R*,25*S*)- and (24*S*,25*S*)-isomers of 24-OH-THCA were also present [60,61]. (24*R*,25*S*)-24-OH-THCA can be formed by AMACR from the accumulating (24*R*,25*R*)-isomer [40], but cannot be degraded because this isomer is not handled by the dehydrogenase unit of LBP [41]. (24*S*,25*S*)-24-OH-THCA is formed by the hydratase unit of LBP from 24-ene-THC-CoA but also cannot be degraded further [41]. In type I-deficient patients (i.e. combined deficiency of both the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase unit) and presumably also in type II-deficient patients (i.e. isolated deficiency of the enoyl-CoA hydratase unit), although this has not been studied in detail for type II-deficient patients, only the (24*S*,25*S*)-isomer of 24-OH-THCA was present, formed by LBP. DHCA, THCA and the direct substrate of DBP, 24(*E*)-ene-THCA was present in all types of DBP deficiency [60,61]. In urine, the presence of the taurine conjugate of hydroxylated 24-

ene-THCA (*m*/*z* 570) and dihydroxylated 24-ene-THCA (*m*/*z* 586) is characteristic for patients with DBP deficiency (Fig. 4B). A recent study among a large cohort of DBP-deficient patients revealed however, that in 26% of the patients no C_{27}-bile acids were present in plasma [62]. Similar to the situation in the peroxisome deficiency disorders, it was shown that patients with a longer survival tended to accumulate less THCA and DHCA, whereas their levels of CA and CDCA were higher. This suggests that residual DBP activity most likely contributes to the extent of bile acid abnormalities in the patients. This was further substantiated by structure-based analysis of the mutated proteins in patients with mild biochemical abnormalities [63].

5.4. SCPx deficiency

Only one patient with SCPx deficiency has been described so far [64]. Only trace amounts of DHCA and THCA were present in plasma and urine of this patient. Interestingly, tandem mass spectrometric analysis of urine consistently showed large peaks with *m*/*z* 611 and 627, which are most likely pentahydroxy-27-nor-5β-cholestane-24-one and hexahydroxy-27-nor-5β-cholestane-24-one glucuronides, respectively (Fig. 4C). These compounds could be formed by hydroxylation and decarboxylation of the substrate of SCPx, 3α,7α,12α-trihydroxy-24-keto-cholestanoyl-CoA [65]. In addition, compounds with *m*/*z* 613 and 629 were abundantly present, which are most likely 27-nor-5β-cholestanepentol and 27-nor-5β-cholestanehexol glucuronides, respectively. The presence of large amounts of bile alcohols in urine of this patient suggests that the block in bile acid biosynthesis at the level of SCPx may initiate the alternative pathway for bile acid biosynthesis not requiring peroxisomal β-oxidation; i.e. the microsomal 25-hydroxylase pathway [45,46].

5.5. BAAT deficiency

Four members of two Amish families have been reported with a homozygous mutation in the *BAAT* gene [66]. The patients had increased serum bile acid levels and the bile acids in serum were unconjugated. Because unconjugated bile acids are not efficiently excreted in the bile, the bile acid concentrations in the intestine were hypothesized to be very low resulting in fat malabsorption, failure to thrive and a vitamin-K dependent coagulopathy. Another boy had been described previously with no glyco- or tauro-conjugated C_{24}-bile acids, but at that time a specific BAAT deficiency was not established with certainty [44]. This patient had a similar clinical presentation with cholestasis associated with high serum and urine bile acid levels and low levels of fat-soluble vitamins.

6. Bile acid abnormalities in mouse models for peroxisomal disorders

6.1. Mouse models for Zellweger syndrome

No studies on bile acid abnormalities in mouse models for Zellweger syndrome have been reported yet. Reduction of the C_{24}-bile acid levels and accumulation of C_{27}-bile acid intermediates have been reported as unpublished observations in both the *Pex2* knockout mouse [67] and in the hepatocyte-selective *Pex5* knockout mouse [68].

6.2. Amacr knockout mouse

Negative ion mass spectrometry was used to analyze bile acids in liver, bile and plasma from adult (4.5 months) *Amacr* knockout mice [69]. Compared to wild type animals, *Amacr*−/− mice accumulated C_{27}-bile acid precursors, whereas the levels of the C_{24}-bile acids were strongly decreased in liver, bile and plasma. The major accumulating precursors were THCA and tauro-THCA. In wild type livers, the amount of C_{24}-bile acids and C_{27}-bile acid intermediates was 349 ± 181 and 21.5 ± 8.6 μmol/kg, respectively, whereas in knockout livers this was 61.9 ± 28.1 and 111 ± 45 μmol/kg. Thus, the total amount of bile acids was also reduced in knockout mice (370 ± 189 μmol/kg in wild type livers and 173 ± 17 μmol/kg in knockout livers).

6.3. LBP, DBP and LBP:DBP knockout mice

No bile acid abnormalities have been observed in LBP knockout mice. In contrast, the major bile acid in liver, bile and plasma from DBP−/− mice was 24(*E*)-ene-THCA [34,70]. The severity of the bile acid synthesis defect in adult DBP−/− mice (6–10 weeks old) was similar to that in *Amacr*−/− mice. In knockout livers the amount of C_{24}-bile acids and C_{27}-bile acid intermediates was 43.5 ± 10.1 and 154.1 ± 53.6 μmol/kg, respectively, and in wild type livers 347.2 ± 176.6 and 2.74 ± 0.74 μmol/kg [34]. A surprising observation was that the accumulating C_{27}-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. This age-dependent increase in conjugation of C_{27}-bile acid intermediates was in sharp contrast to the situation with the C_{24}-bile acids, which were already fully conjugated at postnatal day 2. This conjugation defect of C_{27}-bile acid was caused by a very low expression of BAAT in mouse pups, in combination with a high K_m for C_{27}-bile acyl-CoAs [33,34,52].

To investigate the possible role of LBP in an alternative pathway for bile acid biosynthesis, bile acid profiles of LBP:DBP double knockout mice were compared with those of DBP and LBP single knockout mice [34]. Since almost no double knockout mice survive beyond weaning, comparative analysis in mice from all the different genotypes could only be performed before postnatal day 21. The deficiency of C_{24}-bile acids was more pronounced in double knockout mice than in DBP−/− mice, and the C_{27}/C_{24}-bile acid ratio was even higher. The major accumulating C_{27}-bile acid in DKO mice, however, was not the direct substrate of the bifunctional proteins, 24(*E*)-ene-THCA, like in DBP−/− mice, but THCA and OH-THCA. This was the result of a deficiency of the THC-CoA oxidase in livers from double knockout mice due to a peroxisomal import defect [34,71]. Unfortunately, this inability to oxidize THC-CoA prevented any conclusions to be drawn about the potential role of LBP in a rescue pathway for bile acid biosynthesis.

6.4. Scpx knockout mouse

Biliary levels of bile acids were ~20% lower in adult *Scpx* knockout mice compared to wild type mice, but these differences were not statistically significant [65]. In serum C_{24}-bile acids were decreased 40–60%, and an unusual bile alcohol and some unusual bile acids were abundantly present. These bile acids were 23-nor-CA, 23-nor-CDCA and 23-nor-ursodeoxyCA, and they are most likely formed via α-oxidation and 23-hydroxylation from their normal counterparts. Human patients with cerebrotendinous xanthomatosis (CTX), which is caused by CYP27A1 deficiency, also have elevated levels of 23-nor-bile acids [72]. The bile alcohol was identified as 3α,7α,12α-trihydroxy-27-nor-5β-cholestane-24-one, which is formed after decarboxylation of the substrate of SCPx 24-keto-THC-CoA, and was also present as glucuronide-conjugate in urine from the human SCPx-deficient patient [64]. Treatment of *Scpx*−/− mice with cholestyramine, which induces bile acid synthesis, increased the concentration of 3α,7α,12α-trihydroxy-27-nor-5β-cholestane-24-one and of the 23-nor-bile acids. No THCA or OH-THCA was detected in bile or serum of *Scpx*−/− mice [65].

7. Pathological consequences of a peroxisomal bile acid biosynthesis defect

Bile acid abnormalities are thought to contribute to the liver disease and growth impairment associated with peroxisome deficiency disorders. Patients with Zellweger syndrome have hepatomegaly, elevated serum liver enzymes, excessive hepatocyte iron stores, cholestasis and steatorrhea. Lesions of cholangioles, inflammation and bile duct proliferation can be present upon histological examination of the liver [73]. Many patients with Zellweger syndrome develop fibrosis and the patients that survive longer may develop cirrhosis [73]. Bile acids are crucial for bile flow and absorption of dietary lipids and fat-soluble vitamins [74]. C_{27}-bile acids have altered physical properties and as a consequence they might not function properly as bile acids. They are excreted in bile, but the low C_{27}/C_{24}-bile acid ratio in bile from DBP and *Amacr* knockout mice compared to the ratio in liver shows that they are not excreted as efficiently as C_{24}-bile acids [34,69]. This is probably at least in part due to the fact that C_{27}-bile acids are only partly conjugated. It has been suggested that the C_{27}-bile acids intermediates are especially toxic, but currently there is no direct proof for this. It is known, however, that conjugated bile acids are more soluble, less toxic and better promoters of dietary lipid absorption. The contribution of the overall bile acid abnormalities to the liver disease has been clearly established [73,75]. Oral treatment of a 6-month-old Zellweger patient with CA and CDCA (100 mg each per day) normalized the serum liver enzymes and serum bilirubin, and a histological improvement in the extent of inflammation, bile duct proliferation and cholestasis was observed [75]. Steatorrhea improved and was accompanied by an improvement in growth. Plasma and urinary C_{27}-bile acids decreased after treatment [75]. The rationale behind this treatment was i) to inhibit the endogenous synthesis of bile acids by down-regulation of CYP7A1 and thereby preventing further synthesis and accumulation of potentially hepatotoxic bile acid intermediates (see the next paragraph for detailed explanation of this feedback mechanism), ii) to increase bile flow by replenishing the decreased levels of C_{24}-bile acids, and iii) to increase the intraluminal bile acid concentration, thereby facilitating the absorption of fats and fat-soluble vitamins resulting in improvement in growth. In two Japanese patients with Zellweger syndrome treatment with bile acids also decreased THCA levels and the serum liver enzymes [76]. Although the success of this treatment clearly shows the involvement of the bile acid abnormalities in the liver pathology, it remains difficult to establish the direct effects of these bile acid alterations because in peroxisome deficiency disorders and also in DBP deficiency multiple peroxisomal functions are deficient.

Most patients with AMACR deficiency [77] and the SCPx-deficient patient [64] presented at an adult age with a neuropathy. The neuropathy is believed to be caused by the progressively increasing concentration of branched-chain fatty acids, because the clinical presentation is similar to the one observed in Refsum disease where accumulation of phytanic acid is the only biochemical abnormality [78]. Two siblings with AMACR deficiency, however, presented with cholestatic liver disease in the first neonatal weeks [59]. The patients had clear bile acid abnormalities described above, whereas the pristanic acid levels were only minimally elevated due to their young age. Liver pathology in the sister revealed giant cell transformation, the prevalence of necrotic hepatocytes and periportal hemosiderosis, and these changes resembled the changes seen in several other bile acid synthetic defects [59,73]. Cholic acid treatment reduced C_{27}-bile acids in urine and markedly improved liver histology [59]. This supports the results obtained with treatment of the patient with Zellweger syndrome and shows that in peroxisomal disorders the defect in bile acid biosynthesis contributes to the development of cholestatic liver disease. It has also been postulated that the accumulation of C_{27}-bile acid intermediates may play a role in the neuronal migration defect in Zellweger syndrome [79,80], but this remains to be investigated.

8. Nuclear receptors involved in the peroxisomal steps of bile acid biosynthesis

Bile acid homeostasis is controlled by NRs. NRs are a family of transcription factors, which contains 48 members in human, and are involved in the control of numerous processes, including development and metabolism. NRs typically consist of a DNA-binding domain and a ligand-binding domain. The DNA binding domain binds specific so-called response elements in the promoter of target genes. Upon ligand binding, NRs change conformation which induces dissociation of corepressors and the recruitment of transcriptional cofactors, resulting in the activation of transcription. This property makes NRs on the one hand interesting pharmaceutical targets and on the other hand potential mediators in the pathology of disorders in which potential ligands accumulate. In this review we will

focus on the NRs that are relevant for the peroxisomal steps in bile acid biosynthesis, and discuss their possible role in the pathology and their therapeutic potential in peroxisomal disorders with a bile acid synthesis defect.

The identification of bile acids as the endogenous ligands of farnesoid X receptor α (FXRα, NR1H4) [81–83] was the first proof that NRs control bile acid homeostasis. This discovery suggested a function for FXRα in the enterohepatic recycling of bile acids and the feedback regulation of bile acid biosynthesis, which is in line with the reported expression pattern of FXRα in liver and intestine [84]. In these tissues, FXRα activation protects against accumulation of bile acids, which is toxic, by affecting the expression of genes involved in bile acid transport, binding and synthesis via mechanisms that have been reviewed recently [6,85,86]. With respect to the peroxisomal part of the bile acid biosynthesis pathway, FXRα activation has been shown to induce the expression of BACS and BAAT in primary rat hepatocytes and rat liver. Analysis of the human BACS and BAAT promoter revealed FXR response elements, suggesting that FXRα activation leads to increased conjugation of bile acids [87]. As discussed in previous sections, BACS is localized at the endoplasmic reticulum and is responsible for the reactivation of free bile acids, which have been deconjugated in the intestinal lumen. The BAAT activity localized in the cytosol is most likely necessary for the subsequent reconjugation, whereas the BAAT activity in peroxisomes is responsible for conjugation of newly synthesized bile acids. Thus the coordinate FXRα-dependent induction of BACS and BAAT is necessary for the reconjugation of bile acids. Whether this mechanism plays a role in the peroxisomal conjugation of newly synthesized bile acids, is questionable.

Of particular interest is short heterodimer partner (SHP, NR0B2), which is also an FXRα target gene. SHP is an atypical NR that only has a ligand-binding domain but no DNA-binding domain, and inhibits the activity of several nuclear receptors. FXRα-mediated SHP induction underlies the negative feedback regulation of bile acid biosynthesis [88–93]. SHP binds and interferes with the activity of liver X receptor α (LXRα, NR1H3) [88] and liver receptor homolog-1 (LRH-1, NR5A2) [90,91], both necessary in mice for transcriptional activation of CYP7A1, the rate-limiting enzyme step in bile acid biosynthesis. SHP induction is also the basis for the downregulation of CYP8B1 by bile acids [94]. This elegant feed back regulation of bile acid synthesis is the molecular mechanism behind the decrease in plasma and urinary C_{27}-bile acids after oral treatment of a Zellweger patient with CA and CDCA [75,76].

Both conjugated and unconjugated bile acids are FXRα ligands. This has been explained by analysis of the crystal structure of FXRα. The steroid nucleus of the bile acid molecule occupies the ligand binding pocket with the carboxylate group oriented towards the solvent. Conjugated amino acids will be positioned completely out of the pocket and are solvent exposed [95,96]. It is therefore no surprise that C_{27}-bile acid biosynthesis intermediates are also FXRα ligands (see for the structure of C_{27}-bile acids Fig. 2). Of particular interest are THCA and DHCA, which accumulate in several peroxisomal disorders [97]. As discussed earlier, cholestasis is frequently observed in peroxisome deficiency disorders. The pathogenesis of this symptom is unknown. FXRα stimulates expression of canalicular bile acid transporters and the bile acid conjugating enzymes, and thereby promotes excretion of bile acids into the bile and bile flow. A defect in FXRα signaling due to decreased levels of C_{24}-bile acids could therefore in theory offer an explanation for the cholestasis. In FXRα knockout mice, however, bile formation is not affected [98]. Combined with the fact that THCA and DHCA are bonafide FXRα ligands [97], FXRα does not seem to play a prominent role in the pathogenesis of cholestasis in peroxisome deficiency disorders.

Genes up- or downregulated via FXRα activation have been profiled by microarray analysis [87]. In the published list of genes none of the peroxisomal genes involved in bile acid biosynthesis are present. This may not come as a surprise because the main regulated enzyme in bile acid biosynthesis is the rate-limiting CYP7A1. Moreover, the peroxisomal enzymes involved in bile acid biosynthesis, AMACR, BCOX, DBP and SCPx, also exert other functions not related to bile acid synthesis. In conclusion, although FXRα is the main regulator of bile acid homeostasis, evidence that it controls the peroxisomal steps is currently lacking.

Recently, the role of hepatocyte nuclear factor 4α (HNF4α; NR2A1) in bile acid biosynthesis was studied in detail [99]. Initial studies of a liver specific HNF4α knockout mouse already demonstrated a role in bile acid homeostasis. These mice had increased serum levels of bile acids most likely caused by reduced expression of basolateral bile acid transporters [100]. Follow-up studies showed decreased expression of BACS and BAAT leading to changes in bile acid conjugation [101]. Expression of *Cyp7a1*, *Cyp7b1*, *Cyp8b1*, *Cyp27a1*, *Acox2* (encoding THC-CoA oxidase) and *Scpx*, all bile acid biosynthetic genes, was also decreased. Despite these changes, bile acid pool size and composition were only mildly affected [101]. HNF4α binds to the promoter of about 12% of all tested genes expressed in liver [102]. Most of these promoters are also occupied by RNA polymerase II. Thus HNF4α is rather a widely acting, constitutively active transcription factor in liver than a specific regulator of bile acid homeostasis.

Pregnane X receptor (PXR, NR1I2, also referred to as steroid and xenobiotic receptor, SXR) is a NR expressed in liver and intestine and is involved in the metabolism of potentially toxic foreign chemicals, often referred to as xenobiotics [103]. PXR is also involved in bile acid homeostasis. First of all lithocholic acid (LCA), a secondary bile acid derived from CDCA, activates PXR. LCA acid is a hydrophobic bile acid that causes cholestasis when administered to animals. Detoxification of LCA is achieved by 6α-hydroxylation (mediated by cytochrome P450 3A, CYP3A [104]) and conjugation, pathways known to be activated by PXR. Indeed, activation of PXR by LCA or a synthetic ligand (pregnenolone 16α-carbonitrile, PCN) stimulates uptake and metabolism of LCA in the hepatocyte via upregulation of the organic anion transporting protein 2 (OATP2, *Slc21a5*) and CYP3A [105–108]. More importantly, LCA-induced liver damage can be reduced by coadministration of PCN [107,108], but also in bile duct ligation, a more physiologic cholestasis model, *Cyp3a* upregulation via PXR

activation ameliorates cholestatic liver disease [109,110]. In addition, PXR prevents hepatorenal toxicity from cholesterol metabolites [111].

Detoxification of bile acid precursors plays an important role in *Cyp27a1* knockout mice [112]. The bile acid intermediates that accumulate in these mice, 5β-cholestane-3α,7α,12α-triol and 7α-hydroxy-4-cholesten-3-one, also activate PXR [113,114]. Interestingly, these metabolites are more potent activators of mouse PXR when compared with human PXR, which may explain why human CTX patients fail to increase CYP3A activity [113,114]. The upregulation of *Cyp3a* in *Amacr* knockout mice [69] suggests that similar to the situation in the *Cyp27a1* knockout mouse, the accumulated C_{27}-bile acids activate mouse PXR. In the *Pex2* knockout mouse, however, *Cyp3a* expression was decreased [67]. In vitro studies are therefore necessary to assess if peroxisomal bile acid metabolites can activate human and mouse PXR. As discussed before, tetrahydroxylated and pentahydroxylated C_{27}-bile acids are present in urine of patients with several different peroxisomal deficiencies. These include 6α-hydroxylated C_{27}-bile acids, which are potential products of CYP3A metabolism. The large amounts of bile alcohols in the SCPx-deficient patient may be explained by the induction of xenobiotic metabolism. It is unknown if C_{27}-bile acid hydroxylation is induced via PXR activation as a defense mechanism to reduce their toxicity. Increasing the hydroxylation will promote the urinary excretion of C_{27}-bile acids and as a consequence liver pathology might be ameliorated in peroxisome deficiency disorders.

Recently, studies with the synthetic LXR agonist T0901317 revealed that peroxisomal β-oxidation is regulated by LXRα [115]. The LXR agonist induced the genes encoding straight-chain acyl-CoA oxidase (*ACOX1*), LBP (*EHHADH*) and the classic 3-ketoacyl-CoA thiolase (*ACAA1*). Given the fact that the bile acid synthesis pathway uses BCOX, DBP and SCPx, the other peroxisomal β-oxidation enzymes, it is not expected that LXR activation directly affects the peroxisomal steps in bile acid synthesis. In addition DHCA did not activate LXR [97].

Bile acids have been reported to signal also via alternative pathways, including other NRs, such as the vitamin D receptor (VDR, NR1I1) and constitutive androstane receptor (CAR, NR1I3), and non-NR pathways including mitogen-activated protein kinase (MAPK) pathways [116,117] and the G-protein-coupled receptor (GPCR) TGR5 [118,119]. So far the effect of bile acid precursors on these signaling routes has not been studied.

9. Concluding remarks

Almost all peroxisomal steps in bile acid biosynthesis have been resolved in the past years by studies with recombinant enzymes and studies in material from patients and different mouse models. The transporters in the peroxisomal membrane, however, for the C_{27}-bile acid intermediates and the conjugated C_{24}-bile acids are still unknown. Other important questions that remain are: i) How are the residual C_{24}-bile acids formed in patients with a peroxisomal bile acid synthesis defect? Which alternative pathway is involved and can this pathway perhaps be induced? ii) Are the bile acid abnormalities also involved in the development of the neurological symptoms associated with peroxisomal disorders and not only in the pathology of the liver? Are the C_{27}-bile acid intermediates toxic in this respect? iii) Is modulation of the transcriptional control of bile acid homeostasis a potential therapy for these disorders? Induction of xenobiotic metabolism of bile acids via PXR activation might be a therapeutic option in analogy to the situation in CTX patients. However, feedback inhibition of bile acid biosynthesis via bile acid supplementation and the resulting FXRα activation seems to be far more promising. Although the peroxisomal steps in bile acid biosynthesis are not downregulated after FXRα activation, the decrease in the rate limiting CYP7A1 activity decreases the flux through the bile acid biosynthesis pathway thereby decreasing the levels of C_{27}-bile acids. The replenished C_{24}-bile acids increase bile flow and stimulate the absorption of fat and fat-soluble vitamins.

Acknowledgments

We thank Prof. Ronald Wanders and Dr. Ries Duran for critical reading of the manuscript. This work was supported by the Netherlands Organisation for Scientific Research (NWO grant 916.46.109).

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