Prenatal and postnatal development of peroxisomal lipid-metabolizing pathways in the mouse

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The ontogeny of the following peroxisomal metabolic pathways was evaluated in mouse liver and brain: α-oxidation, β-oxidation and ether phospholipid synthesis. In mouse embryos lacking functional peroxisomes (PEX5$^{-/-}$ knock-out), a deficiency of plasmalogens and an accumulation of the very-long-chain fatty acid $C_{26:0}$ was observed in comparison with control littermates, indicating that ether phospholipid synthesis and β-oxidation are already active at mid-gestation in the mouse. Northern analysis revealed that the enzymes required for the β-oxidation of straight-chain substrates are present in liver and brain during embryonic development but that those responsible for the degradation of branched-chain substrates are present only in liver from late gestation onwards. The expression pattern of transcripts encoding enzymes of the α-oxidation pathway suggested that α-oxidation is initiated in the liver around birth and is not active in brain throughout development. Remarkably, a strong induction of the mRNA levels of enzymes involved in α-oxidation and β-oxidation was observed around birth in the liver. In contrast, enzyme transcripts that were expressed in brain were present at rather constant levels throughout prenatal and postnatal development. These results suggest that the defective ether phospholipid synthesis and/or peroxisomal β-oxidation of straight-chain fatty acids might be involved in the pathogenesis of the prenatal organ defects in peroxisome-deficient mice and men.

Key words: α-oxidation, β-oxidation, ontogeny, peroxisome, Zellweger syndrome.

INTRODUCTION

Peroxisomes harbour several enzyme systems that are involved in lipid metabolism including the pathways of α-oxidation and β-oxidation, ether phospholipid and isoprenoid synthesis.

Four enzymic steps are involved in peroxisomal β-oxidation: (1) desaturation catalysed by an oxidase with the generation of H_2O_2, (2) hydration and (3) dehydrogenation, both catalysed by a multifunctional protein (MFP), and finally (4) a thiolytic cleavage with the formation of acetyl-CoA (or propionyl-CoA) and a fatty acyl-CoA shortened by two carbon atoms [1]. In recent years it has been demonstrated that each step of peroxisomal β-oxidation can be catalysed by at least two different enzymes, to accommodate the different substrates [1]. In the mouse, palmitoyl-CoA oxidase (ACO) desaturates straight-chain acyl-CoAs, whereas branched-chain acyl-CoA oxidase acts on 2-methyl branched-chain acyl-CoAs and on dihydroxycoprostanoyl-CoA and trihydroxycoprostanoyl-CoA. The latter two compounds are intermediates in the formation of bile acids from cholesterol. Pristanoyl-CoA oxidase, a third oxidase that was isolated from rat liver, does not seem to be expressed in the mouse [2]. By analysing MFP-2-deficient mice we demonstrated recently that hydration and further dehydrogenation of the enoyl-CoAs of very-long-chain fatty acids (VLCFAs), branched-chain fatty acids and trihydroxycoprostanic acid occurs primarily by MFP-2 [3]. MFP-1 might be responsible for the degradation of medium and long straight-chain fatty acids and eicosanoids. The thiolytic cleavage is catalysed by either 3-oxo-acyl-CoA thiolase or sterol carrier protein x (SCPx) [1]. The latter enzyme was shown to be responsible for the cleavage of branched-chain compounds [4–7].

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a fatty acid formed from phytol, is degraded by α-oxidation because β-oxidation is prevented by the presence of the 3-methyl group. The consecutive enzymic steps in the α-oxidation pathway have been resolved. After the activation of phytanic acid, phytanoyl-CoA is hydroxylated on the second carbon atom by phytanoyl-CoA hydroxylase (PAHX) [8,9] and then split by 2-hydroxyphytanoyl-CoA lyase (2-HPCL) [10], with the formation of formyl-CoA and pristanal, a 2-methyl fatty aldehyde. The latter product is oxidized to pristanic acid, which is then further degraded by peroxisomal β-oxidation. The subcellular localization of the α-oxidation pathway has not been fully elucidated but a peroxisomal involvement has been inferred from the accumulation of phytanic acid in peroxisome deficiency disorders [11]. Furthermore, rat and human PAHXs [12] were shown to contain an N-terminal peroxisome-targeting signal, and mouse and human 2-HPCL seem to have a C-terminal peroxisome-targeting signal [10].

In addition, the first two steps in ether phospholipid synthesis, catalysed by the enzymes dihydroxyacetonephosphate acyltransferase (DHAPAT) and alkyldihydroxyacetonephosphate syn-

Abbreviations used: ACO, palmitoyl-CoA oxidase; DHAPAT, dihydroxyacetonephosphate acyltransferase; E, embryonic day; 2-HPCL, 2-hydroxyphytanoyl-CoA lyase; MFP, multifunctional protein; PAHX, phytanoyl-CoA hydroxylase; SCPx, sterol carrier protein x; VLCFA, very-long-chain fatty acid.

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thase, take place in peroxisomes [13]. Important members of the ether phospholipid family are platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycerophosphocholine) and the plasmalogens, which contain a double bond adjacent to the ether function. The latter are very abundant in brain but their physiological role has not been elucidated.

Several enzymes involved in the synthesis of isoprenoids have been shown to be located in peroxisomes [14]. However, it has not been established whether these enzyme activities are essential or whether they are supplementary to the activities in other cell compartments. The enzymes of this pathway will not be considered further here.

Peroxisome biogenesis disorders (incidence 1 in 25000 to 1 in 50000) such as Zellweger syndrome are caused by a deficient import of peroxisomal matrix enzymes [15,16]. At birth, Zellweger patients are characterized by hypotonia, psychomotor retardation, facial dysmorphism and convulsions. Several defects including an impairment of neuronal migration causing malformation of the cortex, cerebellum and the olivary nucleus, and renal cysts occur during fetal development, whereas liver fibrosis develops only postnatally [17]. In Zellweger patients there is impairment of peroxisomal lipid metabolism, resulting in a decreased tissue content of plasmalogens, depletion of docosahexaenoic acid, and an accumulation of VLCFAs, branched-chain fatty acids (pristanic acid and phytanic acid) and bile acid intermediates [11]. Two transgenic mouse models of Zellweger syndrome have been generated (PEX5 [18] and PEX2 [19] knockout mice), lacking functional peroxisomes, in which the neuronal migration disorder and biochemical abnormalities of Zellweger patients were present. The pathogenesis of Zellweger syndrome has hitherto not been explained. In particular, the link between peroxisome dysfunction and the characteristic neuronal migration disorder is still unclear. To identify the pathogenic factors it would be relevant to document which peroxisomal metabolic pathways are already active at mid-gestation. Only fragmentary information is currently available on the appearance of peroxisomes and peroxisomal enzymes in different tissues.

The present study focuses on the development of the different peroxisomal metabolic pathways in liver and brain tissue of normal mice. These tissues were chosen because of the abnormalities in Zellweger syndrome and because liver is the organ most enriched in peroxisomes. The results indicate that the β-oxidation of straight-chain fatty acids and the synthesis of ether phospholipids are active at mid-gestation in liver and brain. In contrast, α-oxidation and β-oxidation of branched-chain fatty acids seem to start around birth in liver and are probably absent from brain.

EXPERIMENTAL

Tissue preparation

Pregnant mice were killed by cervical dislocation at 9.5 (E9.5), 11.5, 14.5 and 18.5 days after detection of the vaginal plug. Brain and liver tissue were excised from E14.5, E18.5, newborn (postnatal day 0.5) mice and 1-week-old, 3-week-old and 6–8-week-old mice after decapitation or cervical dislocation of the animals. The head and trunk were used from the E11.5 mice, and E9.5 embryos were used as a whole. Tissues were snap-frozen in liquid nitrogen.

Northern analysis

For the isolation of total RNA, 20–100 mg of each frozen tissue was homogenized in 1 ml of TRIzol Reagent (Gibco BRL Life Technologies) with a Polytron tissue homogenizer. All subsequent steps were performed in accordance with the manufacturer's specifications. For Northern blot analyses, 30 μg of total RNA was separated on 1 % (w/v) agarose/formaldehyde gels and transferred to nylon membranes (Hybond-N$^{+\text{TM}}$; Amersham).

The probes used to detect mRNA of ACO, MFP-2, SCPx and PAHX corresponded to nt 761–1601 (GenBank accession no. AF006688), nt 1042–2113 [20], nt 172–703 (thiolase domain) or nt 1514–2037 (SCP2 domain) [21] and nt 345–1126 [22] respectively of the published mouse cDNA sequences. These probes were obtained by oligo(dT)-primed reverse-transcriptase-mediated PCR with total RNA extracted from the liver of an adult mouse (first-strand cDNA synthesis kit; Amersham Pharmacia Biotech). The gene-specific primers used in the PCR contained a *Bam*HI site flanked by 6 bp at the 5′ end (forward primers, tagtagGGATCC) or an *Eco*RI site (reverse primers, gatgatGAATTC) for cloning purposes.

To generate DHAPAT and MFP-1 mouse cDNA probes, mouse EST sequences were screened (GenBank accession numbers AI286730, MM178728 and AA270967) and aligned respectively to the human DHAPAT cDNA (AF043937) and the rat MFP-1 cDNA (K03249). Primers were selected that amplified cDNA probes corresponding to the human DHAPAT (761–1601 bp) sequence and the rat MFP-1 (1490–2114 bp) sequence.

Other probes were generated by restriction-enzyme digestion of the following plasmids: 3-oxoacyl-CoA thiolase (1.2 kb *Eco*RI fragment of the pTW11 plasmid [23]), pristanoyl-CoA oxidase (455 bp *Eco*RI fragment of the PRNP-plasmid [24]), 2-HPCL {453 bp *Not*I/*Eco*RI fragment of mouse expressed sequence tag 720583 obtained from the I.M.A.G.E. consortium [25] (GenBank AA261575) (V. Foulon, M. Casteels and P. P. Van Veldhoven, unpublished work)} and branched-chain acyl-CoA oxidase [560 bp *Eco*RI/*Nco*I fragment of mouse EST 747544 (GenBank AA268709) (P. P. Van Veldhoven, unpublished work)].

As a control for equal loading and transfer, the blots were probed with a mouse cDNA of the ubiquitously expressed hypoxanthine phosphoribosyltransferase mRNA.

All probes were labelled by random priming with [α-^{32}P]dCTP (ReadyToGo dCTP labelling kit; Pharmacia) and 4×10^6 c.p.m. were added/ml of hybridization solution composed of 0.25 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7 % (w/v) SDS, 1 mM Na_2EDTA, 50 % (v/v) formamide, 5 % (w/v) dextran sulphate [average molecular mass 500000 kDa (Pharmacia)] and 0.01 % (w/v) fish sperm DNA (Roche). After a 24 h hybridization period, all blots were washed at high stringency (5 min at 25 °C in 2 × SSC/0.5 % SDS, 30 min at 65 °C in 0.5 × SSC/0.5 % SDS) and exposed overnight to autoradiographic film or a PhosphorImager screen. Before being reprobed, the blot was stripped by immersion in 0.5 % SDS at 100 °C and monitored.

For each tissue, four independent Northern blots were prepared containing all the time points. Two blots were exposed to X-ray film; the two others were quantified by phosphorimaging (Molecular Dynamics). For each probe the signal intensities of the scanned blots were normalized to the hybridization signals of the hypoxanthine phosphoribosyltransferase probe and are presented as means ± S.E.

SDS/PAGE and immunoblotting

Tissues were homogenized in 5 vol. of 0.25 M sucrose/1 mM EDTA/5 mM Mops (pH 7.2). Protein content was measured with the Bio-Rad Protein Assay Kit with BSA as standard. For immunoblotting, 20 μg of protein was separated on 10 % (w/v) polyacrylamide gels and transferred to a PVDF membrane (Hybond P; Amersham) by means of a semi-dry multigel

Table 1 Biochemical alterations in embryonic PEX5$^{-/-}$ and PEX5$^{+/-}$ mice

The plasmalogen and $C_{26:0}$ contents were determined in the phospholipid fraction of liver and brain. Results are means ± S.E.M.; the numbers of samples are given in parentheses.

		Content (nmol/100 nmol of phospholipids)			
		Liver		Brain	
Substance	Embryonic day	PEX5$^{+/-}$	PEX5$^{-/-}$	PEX5$^{+/-}$	PEX5$^{-/-}$
Plasmalogens	14.5	2.52 ± 0.46 (4)	0.47 ± 0.04 (4)	6.24 ± 0.42 (4)	0.42 ± 0.04 (4)
$C_{26:0}$	16.5	0.08 ± 0.05 (5)	0.60 ± 0.11 (5)	1.14 ± 0.08 (5)	2.17 ± 0.30 (5)

Table 2 Peroxisomal β-oxidation in liver homogenates throughout development

Liver homogenates were incubated with the indicated radioactive substrates. Results are means ± S.E.M.; the numbers of samples are given in parentheses. Abbreviation: n.d., not detectable.

		β-Oxidation rate (nmol/min per mg of protein)	
Developmental stage	Radioactive substrate ...	Palmitoyl-CoA	2-Methylhexadecanoate
E14.5		0.14 ± 0.05 (4)	n.d.
E16.5		0.20 ± 0.20 (3)	n.d.
E18.5		0.93 ± 0.28 (4)	n.d.
Newborn		1.06 ± 0.31 (8)	0.054 ± 0.022 (4)
Adult		2.91 ± 0.92 (4)	0.085 ± 0.040 (3)

electroblotter. After blocking for 1 h with PBS containing 5% (w/v) Protifar (Nutricia, Bornem, Belgium) and 0.05% (v/v) Tween 20, blots were incubated in the same solution for 1 h with rabbit polyclonal antibodies directed against the following antigens: the 52 kDa subunit of rat ACO [2], the 79 kDa rat MFP-2 subunit [26] and the 41 kDa rat 3-oxo-acyl-CoA thiolase [4]. After incubation with anti-rabbit IgG alkaline phosphatase (Roche), blots were stained with 0.033% Nitro Blue Tetrazolium and 0.0165% 5-bromo-4-chloroindol-3-yl phosphate in 100 mM diethanolamine buffer, pH 9.5, containing 5 mM $MgCl_2$. Each Western blot analysis was repeated four times with independent tissue samples.

Peroxisomal α-oxidation and β-oxidation

To examine peroxisomal β-oxidation activity, liver homogenates (5%, w/v) were prepared in modified Krebs–Henseleit bicarbonate buffer [27]. Oxidation of [1-^{14}C]palmitoyl-CoA and 2-methyl-[1-^{14}C]hexadecanoate was measured under conditions favouring peroxisomal β-oxidation as described previously [28]. α-Oxidation of 3-methyl-[1-^{14}C]hexadecanoate was measured as described previously [29].

Determination of fatty acid methyl esters by GLC

Brain and liver of PEX5$^{+/-}$ and PEX5$^{-/-}$ mice at E16.5 were homogenized in 3 ml of methanol/chloroform (2:1, v/v) with a Polytron homogenizer, and the total lipid fraction was isolated as described [30], dried and dissolved in 0.5 ml of chloroform.

Neutral lipids, fatty acids and phospholipids were separated with an NH_2 column (Bond-Elut; Varian, Zaventem, Belgium). The fractions containing the phospholipids were subjected to acidic methanolysis in the presence of internal standards (heptadecanoic and heptacosanoic acid) followed by GLC of the fatty acid methyl esters on a BPX70 column as described previously [31].

Determination of plasmalogen content

Phospholipids of brain and liver of E14.5 PEX5$^{+/-}$ and PEX5$^{-/-}$ mice were analysed for plasmalogen content by measuring the amount of acid-released fatty aldehyde [32]. Plasmalogen levels are expressed relative to the total amount of phospholipids in the extracts.

RESULTS

β-Oxidation in liver

Because $C_{26:0}$ levels are the most widely used parameter for evaluating the activity of the peroxisomal β-oxidation pathway, we quantified $C_{26:0}$ in liver phospholipids of embryonic (E16.5) wild-type and peroxisome-deficient mice. In PEX5 heterozygous and wild-type mice, $C_{26:0}$ was hardly detectable but in PEX5-deficient littermates the levels were reliably measurable (Table 1), indicating that peroxisomal β-oxidation of these straight-chain fatty acids is already operative at this age in the normal mouse. For various reasons, the activity of peroxisomal β-oxidation towards branched-chain substrates cannot be investigated by analysing substrate accumulations in peroxisome-deficient mice during development: first, bile acids are analysed in bile, plasma or urine, which are not available in sufficient quantities from embryonic mice, and secondly, pristanic acid is unlikely to accumulate in embryos because its precursors, phytol and phytanic acid, are present in only small amounts in mouse chow and will probably be degraded by the mother (PEX5$^{+/-}$), who has normal peroxisomal activities.

Overall peroxisomal β-oxidation activity was analysed in liver homogenates from wild-type mice by using [1-^{14}C]palmitoyl-CoA and 2-methyl-[1-^{14}C]hexadecanoate as substrates. The oxidation of the straight-chain fatty acid was measurable from E14.5 onwards and increasing activities were observed into adulthood (Table 2). In contrast, the oxidation of 2-methyl-hexadecanoate (Table 2) or 2-methyl-hexadecanoyl-CoA (results not shown) could be measured reliably only from birth onwards.

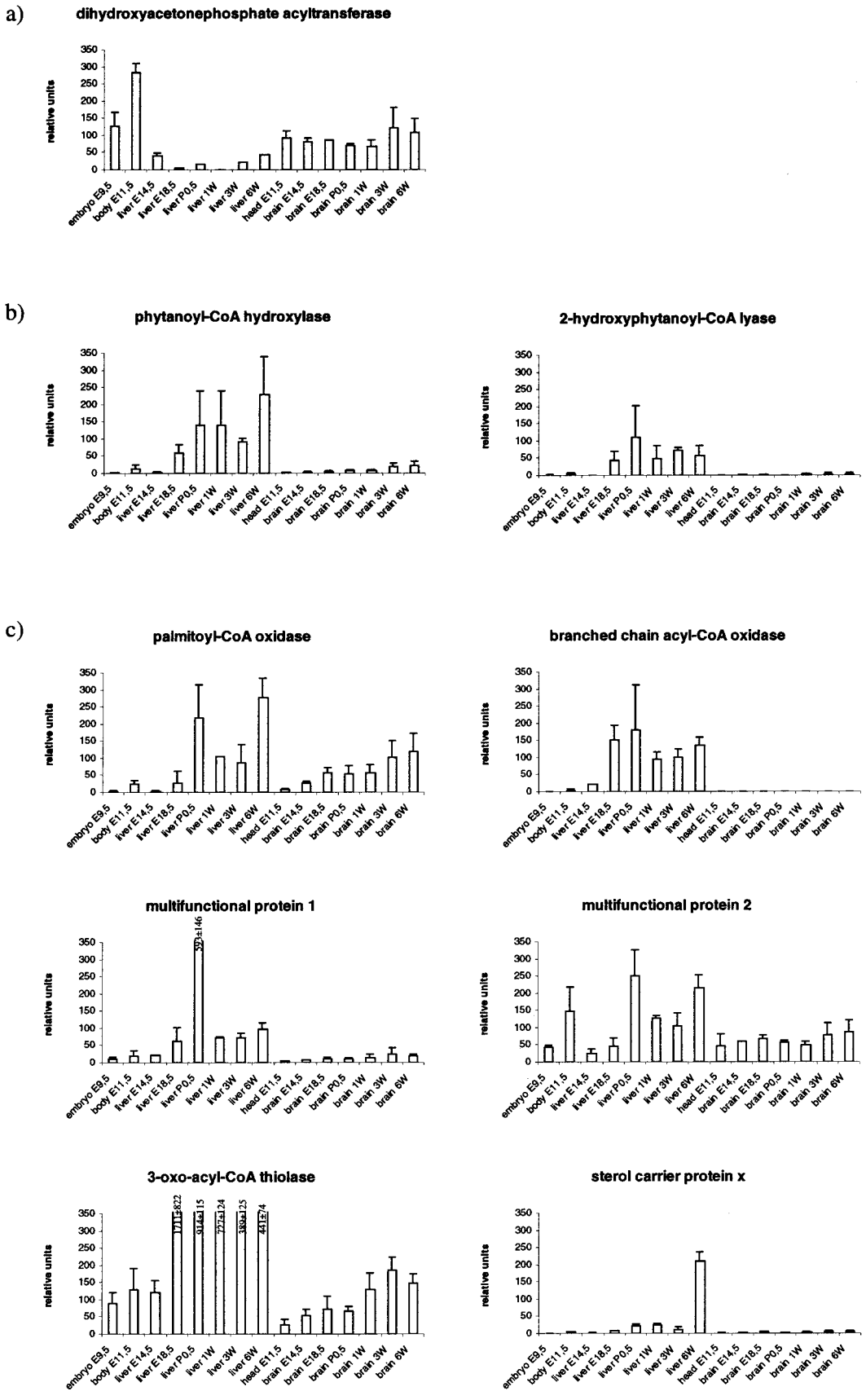

Figure 1 For legend, see opposite page

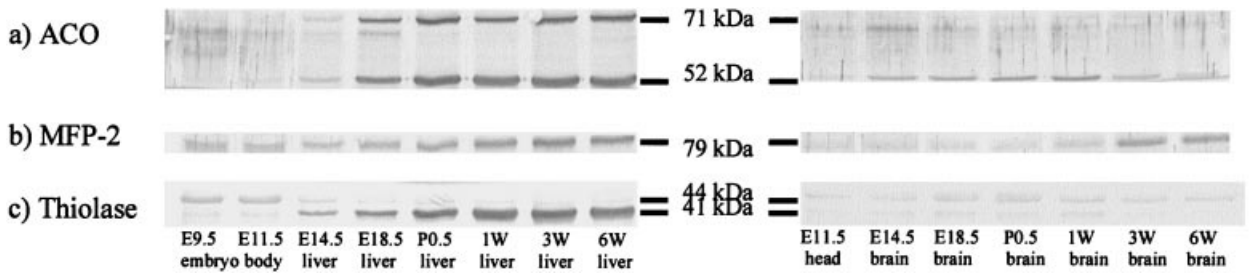

Figure 2 Western analysis of peroxisomal β-oxidation enzymes

Liver (left panels) and brain (right panels) of normal mice were analysed at different stages of development. Polyclonal antibodies raised against rat ACO recognizing the 71 and 52 kDa subunits (**a**), against rat MFP-2 recognizing the 79 kDa subunit (**b**) and the 45 and 34 kDa breakdown products (results not shown) and against rat 3-oxo-acyl-CoA thiolase recognizing the precursor form (44 kDa) and mature form (41 kDa) (**c**) were used. Abbreviations: P0.5, newborn; *n*W, *n* week(s) old.

The occurrence of the different peroxisomal β-oxidation enzymes was examined further in liver tissue by Northern (Figure 1c) and Western (Figure 2, left panels) blot analyses. The mRNA species encoding the three peroxisomal acyl-CoA oxidases were expressed differentially. ACO transcripts were detected from E11.5 onwards in the trunk and were strongly induced in liver around birth (more than 7-fold) (Figure 1c). This was well correlated with the immunoreactive detection of ACO (Figure 2a, left panel). Branched-chain acyl-CoA oxidase transcripts were detectable from E14.5 onwards and were then induced more than 10-fold (Figure 1c). As observed previously [24], pristanoyl-CoA oxidase mRNA was not detectable in liver tissue of the mouse (results not shown). Transcripts of MFP-1 and MFP-2 were present from E11.5 onwards in the trunk. MFP-2 mRNA was even clearly detectable in the whole embryo at E9.5, which was confirmed by immunoblotting, revealing expression of the 79 kDa MFP-2 (Figure 2b, left panel) and a proteolytic product of 34 kDa (results not shown) at that age and throughout development. Strong induction of the MFP-1 mRNA was observed around birth. 3-Oxo-acyl-CoA thiolase transcripts were present in the early embryo (E9.5) and later in the body (E11.5) and liver (E14.5) and were markedly induced from E18.5 onwards (Figure 1c), which was confirmed by the immunoblotting experiments (Figure 2c, left panel). To detect SCPx transcripts, a probe directed to the thiolase domain was used initially. Two transcripts of 3.0 and 2.2 kb were detected from E18.5 onwards and the levels increased strongly into adulthood. The 3.0 kb transcript was the most abundant and is shown in Figure 1(c). When a probe was used that specifically recognized the SCP2 domain of SCPx, two additional transcripts of smaller sizes (0.9 and 1.6 kb) were found, in agreement with the different transcription initiation sites [33] and previous reports [21]. Remarkably, these SCP2 specific transcripts were already present at E11.5 (results not shown).

β-Oxidation in brain

The levels of $C_{26:0}$ doubled in brain phospholipids of embryonic (E16.5) PEX5$^{-/-}$ mice in comparison with those of wild-type mice (Table 1), indicative of an active peroxisomal β-oxidation system of straight-chain fatty acids in the normal embryonic mouse brain. For unknown reasons, peroxisomal β-oxidation activity could not be measured in brain homogenates of wild-type mice at any developmental stage. To test whether these homogenates contained substances inhibiting peroxisomal β-oxidation activity, brain preparations were added to liver homogenates. However, no evidence for an inhibitory effect on liver peroxisomal β-oxidation was found (results not shown).

Subsequently, the expression of the peroxisomal β-oxidation enzymes was examined in the brain by Northern (Figure 1c) and Western (Figure 2, right) blot analyses. ACO was expressed in mouse brain from E11.5 onwards (Figure 1c), confirming a recent report by others [34]. At all ages the processed 52 kDa band of ACO was easier to detect than the unprocessed 71 kDa band in brain. In contrast, branched-chain acyl-CoA oxidase transcripts could not be detected in mouse brain throughout development (Figure 1c), as previously observed in adult rat [35] and adult human [36] brain. MFP-1 and SCPx transcripts were present in brain at extremely low levels (Figure 1c), which is in line with recent reports on MFP-1 expression in rat brain [37]. In contrast, MFP-2 and thiolase mRNA (Figure 1c) and protein (Figures 2b and 2c, right panels) could be detected in brain from E11.5 onwards. Remarkably, both the processed (41 kDa) and the unprocessed (44 kDa) forms of thiolase were present in brain at all ages, whereas in liver only the 41 kDa band was detected. In contrast with the liver, no strong induction of the expressed enzymes was observed in brain perinatally at the mRNA or protein level.

α-Oxidation

Overall α-oxidation of 3-methyl-[1-^{14}C]hexadecanoate was 0.04 ± 0.02 nmol/min per mg of protein (mean ± S.E.M., $n = 5$) in liver homogenates of newborn mice, which was at the detection limit of the assay. The α-oxidation activity increased in the liver of adult mice (0.09 ± 0.03 nmol/min per mg of protein; mean ± S.E.M., $n = 4$).

The expression of PAHX and 2-HPCL was examined at the mRNA level. Both transcripts were detectable in the body at

Figure 1 Northern analysis of peroxisomal enzymes during development in liver and brain

Levels of mRNA for peroxisomal enzymes involved in ether phospholipid synthesis (**a**), α-oxidation (**b**) and β-oxidation (**c**) are shown. RNA was extracted from the indicated tissues (full embryo/body/liver and head/brain) of normal mice at different time points during development. Values represent scanned units normalized to the band intensities of the housekeeping gene hypoxanthine phosphoribosyltransferase. Means ± S.E. for two independent experiments are shown. Abbreviations: *n*W, *n* week(s) old; P0.5, newborn.

E11.5 and were strongly induced in liver from E18.5 onwards (Figure 1b). In contrast, these transcripts were hardly detectable in brain throughout development (Figure 1b), which is comparable to human, in which no expression of hydroxylase in the adult brain was detected [29].

Ether phospholipid synthesis

To evaluate the activity of the ether phospholipid synthesis pathway during mouse development, we analysed the plasmalogen content in liver and brain from E14.5 PEX5-deficient mice. In newborn PEX5$^{-/-}$ mice, it has previously been shown that plasmalogens are depleted and that the activity of DHAPAT is undetectable [18]. A 5-fold and a 15-fold decrease in plasmalogen levels were observed in liver and brain respectively from E14.5 PEX5$^{-/-}$ mice in comparison with tissues from heterozygous littermates (Table 1). This finding of an early activity of ether phospholipid synthesis during embryonic development in the mouse was corroborated by the detection of transcripts for DHAPAT, the first enzyme in this pathway, in the E9.5 mouse embryo and in both the body and the head of E11.5 embryos (Figure 1c). At all later ages, DHAPAT mRNA was more abundantly present in brain than in liver, in agreement with lower levels of plasmalogens in the latter organ.

DISCUSSION

Peroxisome deficiency in man and in mouse causes several organ defects that occur during embryonic development. In Zellweger fetuses, neuronal migration defects, delays in neuronal maturation, renal cysts and abnormal calcifications in bone have been documented [17]. In peroxisome-deficient mice (PEX5 and PEX2 knock-out), impairment of neuronal migration, significant growth retardation and abnormalities at the ultrastructural level in the liver were observed at birth [18,19]. The aim of the present study was to document which peroxisomal metabolic pathways are active in two major organs, liver and brain, during embryonic development.

The observations that, during embryonic development (E14.5–E16.5), plasmalogen levels are significantly decreased, and that $C_{26:0}$ levels are increased in liver and brain of PEX5$^{-/-}$ mice in comparison with wild-type and heterozygous controls, demonstrate that ether phospholipid synthesis and peroxisomal β-oxidation are active in normal mice at this stage. Decreased plasmalogen levels and increased levels of VLCFA have also been observed in Zellweger fetuses [17,38]. Because the blood–brain barrier has not been established during this period of embryonic development, it could be argued that the metabolic alterations in brain are caused by the defective peroxisomal function in liver. However, Northern analysis revealed that during embryonic development, transcripts of ACO, MFP-2, thiolase and DHAPAT are present at comparable or even higher levels in brain than in liver, suggesting that both embryonic tissues might be equipped with an active peroxisomal β-oxidation and ether phospholipid synthesis pathway. It remains unexplained why β-oxidation cannot be detected in brain homogenates with [^{14}C]palmitoyl-CoA as a substrate, whereas the activity can be reliably measured in liver homogenates derived from E14.5 mice.

The Northern blot experiments are also informative when considering which substrates of peroxisomal β-oxidation can already be degraded during embryonic development and could consequently accumulate under conditions of peroxisome deficiency. 2-Methyl-branched-chain acyl-CoAs and the bile-acid intermediate trihydroxycoprostanoyl-CoA need branched-chain acyl-CoA oxidase, MFP-2 and SCPx for degradation by peroxisomal β-oxidation [1,3]. Because transcripts of branched-chain acyl-CoA oxidase were not detectable in brain and those of SCPx were hardly detectable in liver until birth, it seems unlikely that the above-mentioned substrates are degraded by embryonic mice. This lack of detection during the fetal period does not depend on the probe used for Northern hybridization because intense signals for these transcripts were observed in the adult mouse liver. Furthermore, when a cDNA probe was used that recognized the 3′ end of the coding region of SCPx, SCP2-specific transcripts were detected by E11.5, whereas the transcripts specific for SCPx appeared only in newborns (results not shown).

In contrast, the degradation of VLCFAs requires ACO, MFP-2 and 3-oxo-acyl-CoA thiolase as deduced from the analysis of ACO [39], MFP-2 [3] and SCPx [6] knock-out mice. As already mentioned, the transcripts for these enzymes were identified in the body as well as in the head of normal fetuses starting at E11.5, which is in line with the observation that $C_{26:0}$ accumulates in these tissues in peroxisome-deficient embryos. Other straight-chain compounds, including dicarboxylic acids and eicosanoids, are also degraded by ACO and thiolase but it is unclear whether either MFP-1, MFP-2 or both are involved in their catabolism. Because MFP-1 deficient mice do not display any abnormalities [40], it can be hypothesized that these substances can be degraded by MFP-2, which is expressed ubiquitously. It therefore seems that all enzymes necessary to degrade straight-chain fatty carboxylates are present in liver and brain of normal embryonic mice.

Transcripts for PAHX [8,9] and 2-HPCL [10], two enzymes involved in α-oxidation, were detected from E11.5 onwards in the body but were hardly detectable in brain throughout development. This expression pattern seems to indicate that α-oxidation in the mouse occurs in liver during the postnatal period but not in brain.

A remarkable induction of most α-oxidation and β-oxidation enzyme transcripts was observed in liver just before or after birth. In view of the timing, this induction does not seem to be triggered solely by food intake. Interestingly, this induction was not observed in brain.

Together, the results are compatible with the following: (1) β-oxidation of straight-chain fatty acids occurring at mid-gestation in mouse liver and brain, and (2) α-oxidation and β-oxidation of branched-chain fatty acids and β-oxidation of bile acid intermediates starting at around birth in the mouse liver but absent from brain at all stages. The proposal that the fetal liver is unable to degrade branched-chain acyl-CoAs and bile acid intermediates is in line with the physiological occurrence and role of these compounds: bile acids are necessary for the resorption of lipids and fat-soluble vitamins when feeding has started; phytanic acid is from exogenous origin, so that the developing fetus is probably not exposed to this substrate and its degradation product, pristanic acid.

In conclusion, of the three metabolic pathways studied, only those responsible for the degradation of straight-chain fatty acids and the synthesis of ether phospholipids seem to be active at mid-gestation in liver as well as in brain. A deficiency in these specific pathways could be responsible for the organ defects that occur in Zellweger patients and in mice with peroxisome deficiencies during embryonic development. Consequently, the synthesis of ether phospholipids and the degradation of straight-chain fatty acids via peroxisomes might be necessary for normal embryonic development. However, we cannot exclude the possibility that peroxisomes might exert other metabolic functions that still need to be identified and could be essential during embryogenesis. Our results further suggest that disturbed metab-

olism of branched-chain fatty acids and bile acid intermediates is not important prenatally but might contribute to the symptoms that appear after birth. This is in line with the clinical observations in Refsum disease, which is caused by disturbed branched-chain fatty acid metabolism [41]. Refsum patients are normal at birth but develop pathology in the first or second decade of life [41]. The generation of new mouse models, in which the peroxisomal metabolic pathways are selectively blocked, will undoubtfully provide more insight in the pathogenic factors causing peroxisome biogenesis disorders.

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