New insights in peroxisomal beta-oxidation
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Citation for published version (APA):

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Download date: 30 Dec 2018
Chapter 10

Identification of the peroxisomal β-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid.

Identification of the peroxisomal β-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid

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Abstract
Docosahexaenoic acid (DHA, C22:6n-3) is an important polyunsaturated fatty acid (PUFA) implicated in a number of (patho)physiological processes. For a long time, the exact mechanism of DHA formation has remained unclear, but now it is known that it involves the production of C24:6n-3 from dietary linolenic acid (C18:3n-3) via a series of elongation and desaturation reactions, followed by β-oxidation of C24:6n-3 to C22:6n-3. Although DHA is deficient in patients lacking peroxisomes, the intracellular site of retroconversion of C24:6n-3 has remained controversial. Making use of fibroblasts from patients with defined mitochondrial and peroxisomal fatty acid oxidation defects, we show in this paper that peroxisomes, and not mitochondria, are involved in DHA formation by catalyzing the β-oxidation of C24:6n-3 to C22:6n-3. Additional studies in fibroblasts from patients with X-linked adrenoleukodystrophy (XALD), straight-chain acyl-CoA oxidase (SCOX) deficiency, D-bifunctional protein (DBP) deficiency and rizomelic chondrodysplasia punctata (RCDP) type 1, and fibroblasts from L-bifunctional protein (LBP) and sterol carrier protein X (SCPx) knockout mice, show that the main enzymes involved in β-oxidation of C24:6n-3 to C22:6n-3 are SCOX, DBP and both 3-ketoacyl-CoA thiolase and SCPx. These findings are of importance for the treatment of patients suffering from a defect in peroxisomal β-oxidation.

Introduction
For years, it was generally assumed that the biosynthesis of polyunsaturated fatty acids (PUFAs) takes place in the endoplasmic reticulum, which is also the main site for phospholipid biosynthesis (1). Docosahexaenoic acid (DHA) (C22:6n-3), the major PUFA in adult mammalian brain and retina, was believed to be synthesized from dietary linolenic acid (C18:3n-3) in a pathway consisting of a series of elongation and desaturation reactions. This pathway required that C22:5n-3 becomes desaturated at position 4 by a microsomal acyl-CoA dependent Δ4-desaturase to form C22:6n-3. Several studies in the past few years, however, have indicated that such a Δ4-desaturase does not appear to exist (2-5). Instead, it was found that a 24-carbon n-3 fatty acid is synthesized, which is desaturated at position 6 to produce C24:6n-3, followed by one round of β-oxidation with C22:6n-3 as final product. Although still disputed, the peroxisome is the likely site of C24:6n-3 β-oxidation. After its formation, DHA is transported back to the endoplasmic reticulum where it is esterified into membrane lipids (6,7). Fig. 1 shows the
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Fig. 1 Pathway of DHA biosynthesis. DHA is synthesized from dietary linolenic acid (C18:3n-3) in a series of microsomal elongation and desaturation reactions, followed by retroconversion of C24:6n-3 to C22:6n-3 in the peroxisome via one round of β-oxidation.

revised pathway for the biosynthesis of DHA. The synthesis of arachidonic acid (C20:4n-6) and C22:5n-6 from dietary linoleic acid (C18:2n-6) follows a similar pathway (1).

The β-oxidation step in the revised pathway of PUFA biosynthesis requires a considerable exchange of unsaturated fatty acids between different subcellular compartments (6). Several lines of evidence suggest that peroxisomes are the intracellular site of this β-oxidation step. First, patients suffering from Zellweger syndrome (a peroxisome biogenesis disorder), who lack functional peroxisomes, have clearly reduced levels of DHA, especially in brain and retina but also in liver, kidney (8) and blood (9). In addition, in newborn PEX5 knockout mice, a mouse model for Zellweger syndrome, the DHA concentration in the brain is also strongly reduced (40% as compared with levels in normal littermates) (10). In an extensive study on n-3 fatty acid metabolism, Moore et al. reported that control human fibroblasts metabolized [1-14C]18:3n-3 to labeled C24:5n-3, C24:6n-3 and C22:6n-3 (5). In contrast, fibroblasts from patients suffering from Zellweger syndrome metabolized [1,14C]18:3n-3 to C24:5n-3 and C24:6n-3, but not to C22:6n-3. Likewise, [3-14C]-labeled 22:5n-3, 24:5n-3 and 24:6n-3 were all metabolized to C22:6n-3 in control, but not in Zellweger fibroblasts. Similar results were obtained by Petroni et al., who incubated control and Zellweger fibroblasts with [1-14C]20:5n-3 (11). In a recent paper, it was demonstrated that peroxisomes are required for biosynthesis of DHA from linolenic acid in livers from neonatal piglets (12). This was concluded from the observation that isotope-labeled DHA, and all the intermediates of the pathway, were formed only when combined microsomal and peroxisomal fractions were incubated with [13C-U]18:3n-3.

In spite of the many experiments which show that peroxisomes are involved in the biosynthesis of PUFAs, Infante et al. propose that synthesis of these fatty acid occurs in the outer mitochondrial membrane via a channeled carnitine-dependent pathway (13,14).
Although there is not much direct experimental evidence to support the existence of such a mitochondrial pathway, a role for the mitochondrion in the biosynthesis of DHA cannot be ruled out with absolute certainty. We therefore set out to study the role of peroxisomes and mitochondria, and their fatty acid oxidation systems, in DHA synthesis in more detail.

Fig. 2 shows a schematic representation of the peroxisomal β-oxidation system. There are two complete sets of β-oxidation enzymes present in the peroxisome (15). Straight-chain acyl-CoA oxidase (SCOX) is responsible for the initial oxidation of very long-chain fatty acyl-CoAs, while branched-chain acyl-CoA oxidase (BCOX) oxidizes branched-chain fatty acyl-CoAs. The enoyl-CoA esters of both straight- and branched-chain fatty acids are then hydrated and subsequently dehydrogenated by the same enzyme: D-bifunctional protein (DBP). The function of the second multifunctional protein present in the peroxisome, L-bifunctional protein (LBP), is still unknown. The last step of the β-oxidation process, the thiolytic cleavage, is performed by sterol carrier protein X (SCPx) in case of the branched-chain substrates, while straight-chain substrates most likely are handled by both SCPx and the classical 3-ketoacyl-CoA thiolase.

![Diagram of peroxisomal β-oxidation system]

Fig. 2 Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA) and branched-chain fatty acyl-CoAs (pristanoyl-CoA and THC-CoA). Oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), while oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP and SCPx (see (15) for review).

Until now, only patients with an isolated defect of SCOX and DBP have been identified. In addition, patients suffering from Rizomelic Chondrodysplasia Punctata (RCDP) type 1 lack 3-ketoacyl-CoA thiolase in their peroxisomes. This is, however, not the only deficiency in these patients. Due to a defect in PEX7, the gene encoding the peroxisome targeting signal 2 (PTS2) receptor, their peroxisomes lack all proteins imported via this receptor, including alkyl-dihydroxyacetonephosphate synthase, an enzyme of the
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plasmalogen biosynthetic pathway, and phytanoyl-CoA hydroxylase, the first enzyme of the peroxisomal α-oxidation pathway (16-18). No patients have been identified with a deficiency of BCOX, LBP and SCPx, but knockout mice have been created for the latter two enzymes (19,20).

To elucidate the role of both the peroxisome and mitochondrion, we studied the biosynthesis of DHA from [l-14C]18:3n-3, [l-14C]20:5n-3 and [3-14C]24:6n-3 in fibroblasts from patients with a peroxisome biogenesis disorder and from patients with a deficiency of one of the following mitochondrial enzymes: carnitine palmitoyltransferase 1 (CPT I), carnitine acylcarnitine translocase (CACT), carnitine palmitoyltransferase 2 (CPT II) and very long-chain acyl-CoA dehydrogenase (VLCAD). The first three enzymes are necessary for the transport of activated fatty acids across the inner mitochondrial membrane (21) and the last enzyme is part of the mitochondrial β-oxidation system (22). In addition, we investigated the role of the various peroxisomal β-oxidation enzymes in DHA biosynthesis by incubating fibroblasts from patients with a deficiency of SCOX and DBP, patients suffering from RCDP type 1, and from LBP and SCPx knockout mice with 14C-labeled precursors. We also studied DHA synthesis in fibroblasts from a patient suffering from X-linked adrenoleukodystrophy (XALD). These patients accumulate very long-chain fatty acids because of an impaired peroxisomal β-oxidation of these fatty acids. However, this is not caused by a deficiency of one of the enzymes of the β-oxidation system, but by a defect of the peroxisomal membrane protein ALDP (adrenoleukodystrophy protein) (23,24).

Materials and Methods

Materials
Radiolabeled [1-14C]18:3n-3, [1-14C]20:5n-3 and [1-14C]22:6n-3 were purchased from New England Nuclear (DuPont, Boston, MA). [3-14C]24:6n-3 was synthesized as described previously (2). Each radiolabeled fatty acid had a specific activity between 50 and 55 mCi/mmol.

Patient cell lines
Cell lines were used from several patients suffering from various peroxisomal and mitochondrial fatty acid β-oxidation disorders. The fibroblasts from patients with a peroxisome biogenesis disorder studied in this paper were from four patients with Zellweger syndrome and one patient with neonatal adrenoleukodystrophy (NALD), which is a less severe form of a peroxisome biogenesis defect. These patients all had the clinical and biochemical abnormalities described for patients with a peroxisome biogenesis disorder, including deficient C26:0 and pristanic acid β-oxidation and phytanic acid α-oxidation (25). The fibroblasts from the XALD patient had impaired C26:0 β-oxidation, which is caused by a mutation in the gene encoding the peroxisomal membrane protein ALDP (23,24). The SCOX and DBP deficient patients all had mutations in the encoding gene and no enzyme activity could be measured in fibroblasts of these patients (26-28). Peroxisomes from the patients with RCDP type 1 we studied, lack 3-ketoacyl-CoA thiolase due to a mutation in the PEX7 gene encoding the PTS2 receptor. Immunoblot studies
performed with an antibody raised against 3-ketoacyl-CoA thiolase revealed that only the unprocessed protein of 44 kDa is present in fibroblast homogenates. It is known that 3-ketoacyl-CoA thiolase is synthesized as a precursor protein and is proteolytically cleaved to its mature form of 41 kDa inside the peroxisome (29). Cultured skin fibroblasts from an SCPx knockout mouse were obtained from Dr. U. Seedorf (Westphalian Wilhelms-University Münster, Germany) (19) and fibroblasts of an LBP knockout mouse were generated by Qi et al. (20). Both knockout mice have been fully characterized and completely lack SCPx or LBP gene function, respectively. The fibroblasts from patients with a mitochondrial β-oxidation disorder used in this study were from patients with a confirmed deficiency of CPT I, CACT, CPT II or VLCAD due to mutations in the encoding genes (see (22) for review). These mutations result in a deficiency of mitochondrial fatty acid oxidation as established by individual enzyme activity measurements in cultured skin fibroblasts.

All patient cell lines used in this study were taken from the cell repository of the Laboratory for Genetic Metabolic Diseases (Academic Medical Center, University of Amsterdam, the Netherlands) and were derived from patients diagnosed in this center. Informed consent was obtained from parents or guardians of the patients whose fibroblasts were studied in this paper and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

**Experimental protocol**

DHA synthesis from [1-14C]18:3n-3, [1-14C]20:5n-3 and [3-14C]24:6n-3 was studied in cultures of fibroblasts grown in tissue culture flasks (25 cm²). Incubations were carried out in minimal essential medium (MEM) supplemented with penicillin/streptomycin, and containing 10% fetal calf serum (fatty acid free), 20 mM HEPES and 14C-labeled fatty acid. In case of [1-14C]18:3n-3 and [1-14C]20:5n-3 the incubation was carried out with 2 μM labeled fatty acid, while [3-14C]24:6n-3 was used at a concentration of 0.2 μM. The fibroblasts were kept in an incubator at 37°C for 96 h except for the incubations with [3-14C]24:6n-3, which were terminated after 24 h. Parallel incubations were performed to determine the amount of protein.

**Lipid analyses**

Lipids were extracted from the incubation medium as described by Moore et al. (5). Briefly, the lipids were extracted with a 2:1 (v/v) mixture of chloroform-methanol containing 1% acetic acid (v/v). The chloroform phase was dried under N₂, the residue resuspended in 2 ml 1.5 N HCl/methanol and heated to 90°C for 2 h to produce fatty acid methyl esters. After extraction of the methyl esters in heptane, the heptane phase was dried under N₂ and the residue resuspended in 150 μl 70% acetonitrile, which was stored at -20°C until analysis. Seventy microliters of the sample was subjected to high performance liquid chromatography (HPLC) analysis as described below.

To isolate cellular lipids, the incubation medium was removed and the fibroblasts were scraped into 1 ml of methanol and transferred to a screw-top glass vial. The tissue culture flasks was washed with 1 ml 3N HCl/methanol, which also was transferred to the glass vial. Finally, fatty acid methyl esters were produced and extracted as described above.
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**HPLC analysis**

Radioactive methyl esters prepared from the cell lipids or incubation medium were separated by reversed-phase HPLC. A reversed-phase C\(_{18}\)-column (4.6 x 150 mm; Beckman, Fullerton, CA) with 5 \(\mu\)m spherical packing was used with a mobile phase of water and acetonitrile in a two-step isocratic elution (76% acetonitrile for 50 min, 90% acetonitrile for 10 min), followed by an equilibration period of 10 min at 76% acetonitrile. The effluent was mixed with scintillation solution at a 1:1 ratio, and the radioactivity was measured by passing the mixture through an online Radiomatic Instruments (Packard, Meriden, CT) Flo One-\(\beta\) radioactivity detector. The system was standardized with methyl esters of the following radiolabeled fatty acids: C18:3n-3, C20:5n-3, C24:6n-3 and C22:6n-3.

**Results**

**Peroxisomal versus mitochondrial involvement in DHA biosynthesis**

**Linolenic acid (C18:3n-3) utilization**

After a 96-h incubation of control human skin fibroblasts with [\(^{1}\text{C}\)18:3n-3, substantial amounts of the radiolabeled fatty acids contained in the cells consisted of C22:6n-3 (mean value in 5 different control fibroblast cell lines was 15.5%) (see Table 1). In addition, radioactivity was detected in almost all intermediates of the proposed pathway of DHA biosynthesis (Fig. 1), including radiolabeled C24:5n-3 and C24:6n-3 (Fig. 3). Similar results were obtained with fibroblasts from patients with a deficiency of mitochondrial fatty acid oxidation. Fibroblasts from patients with a deficiency in one of the steps involved in the mitochondrial carnitine shuttle (CPT I, CACT or CPT II) as well as from a patient with a defect of the first enzyme of the mitochondrial fatty acid oxidation system, VLCAD, revealed normal synthesis of DHA from radiolabeled linolenic acid compared to the synthesis observed in control fibroblasts (Table 1). In contrast, no radiolabeled C22:6n-3 was formed in fibroblasts from patients with Zellweger syndrome, although the [\(^{1}\text{C}\)18:3n-3 was converted to other intermediates in the biosynthetic pathway. In addition, increased amounts of radiolabeled C24:6n-3, the precursor of DHA, were found. Fibroblasts from a patient with NALD, a milder variant of Zellweger syndrome characterized by a less severe peroxisomal deficiency, synthesized some radiolabeled DHA but only 1% of the radioactivity was converted to C22:6n-3 after the incubation period (Table 1).

Analysis of the radiolabeled fatty acids excreted in the medium revealed a similar pattern as the analysis of the fatty acids contained in the cells. This was true for both control skin fibroblasts as well as fibroblasts from patients with a mitochondrial or peroxisomal defect (data not shown). This is in agreement with the findings by Moore et al. (5), who concluded that these mitochondrial and peroxisomal defects do not cause selective retention or release of certain radiolabeled fatty acids. Therefore, all results shown are obtained by analysis of the cells only.
<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>C18:3n-3</th>
<th>C20:4n-3</th>
<th>C20:5n-3</th>
<th>C22:5n-3</th>
<th>C24:5n-3</th>
<th>C24:6n-3</th>
<th>C22:6n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 5)</td>
<td>3,470 ± 407&lt;sup&gt;′&lt;/sup&gt;</td>
<td>1,269 ± 554</td>
<td>41,264 ± 10,159</td>
<td>39,346 ± 13,977</td>
<td>803 ± 604</td>
<td>1,057 ± 762</td>
<td>16,265 ± 6,117</td>
</tr>
<tr>
<td>Zellweger (n = 4)</td>
<td>7,570 ± 2,055&lt;sup&gt;′&lt;/sup&gt;</td>
<td>3,500 ± 775</td>
<td>33,305 ± 5,612</td>
<td>78,883 ± 20,305</td>
<td>2,696 ± 361</td>
<td>8,081 ± 2,185</td>
<td>0 ± 0</td>
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<td>NALD</td>
<td>3,092</td>
<td>1,727</td>
<td>13,255</td>
<td>60,404</td>
<td>755</td>
<td>4,834</td>
<td>917</td>
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<td>CPT I</td>
<td>8,393</td>
<td>3,786</td>
<td>74,214</td>
<td>79,215</td>
<td>2,557</td>
<td>2,068</td>
<td>15,488</td>
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<td>CACT (n = 3)</td>
<td>8,696 ± 2,999&lt;sup&gt;′&lt;/sup&gt;</td>
<td>4,215 ± 1,852</td>
<td>60,030 ± 9,776</td>
<td>99,987 ± 28,947</td>
<td>3,814 ± 818</td>
<td>2,624 ± 800</td>
<td>18,419 ± 4,478</td>
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<td>CPT II</td>
<td>7,199</td>
<td>2,845</td>
<td>55,798</td>
<td>84,113</td>
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<td>VLCAD</td>
<td>6,599</td>
<td>3,319</td>
<td>54,998</td>
<td>60,449</td>
<td>2,781</td>
<td>2,024</td>
<td>14,501</td>
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<td>XALD</td>
<td>10,560</td>
<td>2,211</td>
<td>61,678</td>
<td>58,828</td>
<td>2,482</td>
<td>1,635</td>
<td>13,498</td>
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<td>SCOX (n = 3)</td>
<td>7,861 ± 1,706&lt;sup&gt;′&lt;/sup&gt;</td>
<td>4,625 ± 2,104</td>
<td>54,654 ± 15,350</td>
<td>94,991 ± 42,300</td>
<td>3,783 ± 1,124</td>
<td>6,310 ± 2,505</td>
<td>1,791 ± 55</td>
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<tr>
<td>DBP (n = 3)</td>
<td>4,137 ± 2,590&lt;sup&gt;′&lt;/sup&gt;</td>
<td>2,203 ± 1,199</td>
<td>42,769 ± 21,368</td>
<td>54,065 ± 21,838</td>
<td>1,892 ± 814</td>
<td>3,090 ± 1,028</td>
<td>3,078 ± 1,518</td>
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<td>RCDP type 1 (n = 3)</td>
<td>3,262 ± 1,093&lt;sup&gt;′&lt;/sup&gt;</td>
<td>2,698 ± 1,013</td>
<td>36,447 ± 10,017</td>
<td>66,120 ± 23,755</td>
<td>2,626 ± 2,670</td>
<td>2,688 ± 137</td>
<td>18,015 ± 2,724</td>
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<td>Control mouse</td>
<td>1,671</td>
<td>773</td>
<td>56,858</td>
<td>70,228</td>
<td>882</td>
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<td>10,050</td>
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<td>LBP -/- mouse</td>
<td>2,669</td>
<td>0</td>
<td>122,890</td>
<td>78,476</td>
<td>1,187</td>
<td>3,065</td>
<td>18,273</td>
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<td>SCPX -/- mouse</td>
<td>2,005</td>
<td>1,912</td>
<td>88,115</td>
<td>48,912</td>
<td>1,439</td>
<td>1,671</td>
<td>12,588</td>
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</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. n = number of different cell lines; all incubations were performed in triplicate; 'mean value ± standard deviation.
Peroxisomal β-oxidation enzymes involved in DHA biosynthesis

Eicosapentaenoic acid (C20:5n-3) utilization
Similar results were obtained after incubation of fibroblasts with [1-14C]20:5n-3 (Table 2). After an incubation of 96 h, control fibroblasts produced radiolabeled C22:5n-3 and C22:6n-3, as well as small amounts of C24:5n-3 and C24:6n-3. Fibroblasts from patients with a deficiency of either CPT I, CACT, CPT II or VLCAD revealed normal synthesis of DHA from [1-14C]20:5n-3. Fibroblasts from patients with Zellweger syndrome, however, produced no radiolabeled DHA. In contrast, they accumulated large amounts of C24:6n-3 (about 10-times more than observed in control fibroblasts). Incubations of fibroblasts from a patient with NALD resulted in intermediate values. These fibroblasts produced 10% of the amount of radiolabeled DHA formed in control fibroblasts and accumulated about 6-times the normal amount of C24:6n-3.

Fig. 3 Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 2 μM [1-14C]18:3n-3 for 96 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), a patient with Zellweger syndrome (B) and a CACT-deficient patient (C).

Tetracosahexaenoic acid (C24:6n-3) utilization
The results with [1-14C]18:3n-3 and [1-14C]20:5n-3 support the finding by Moore et al. (5) that the pathway for DHA synthesis from n-3 fatty acid precursors in human fibroblasts involves the formation of 24-carbon polyunsaturated intermediates, followed by
Table 2 Radiolabeled fatty acids produced by human and mouse skin fibroblasts after a 96 h-incubation with [1-\(^{14}\)C]20:5n-3

<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>C20:5n-3</th>
<th>C22:5n-3</th>
<th>C24:5n-3</th>
<th>C24:6n-3</th>
<th>C22:6n-3</th>
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<tr>
<td>Controls (n = 5)</td>
<td>52,198 ± 8,570(^{1})</td>
<td>57,325 ± 10,241</td>
<td>113 ± 179</td>
<td>1,782 ± 489</td>
<td>25,740 ± 5,801</td>
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<tr>
<td>Zellweger (n = 4)</td>
<td>35,375 ± 5,460(^{1})</td>
<td>173,244 ± 47,838</td>
<td>454 ± 114</td>
<td>18,217 ± 6,319</td>
<td>0 ± 0</td>
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<td>NALD</td>
<td>12,434</td>
<td>127,834</td>
<td>254</td>
<td>10,424</td>
<td>2,709</td>
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<td>CPT I</td>
<td>75,871</td>
<td>104,389</td>
<td>62</td>
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<td>25,703</td>
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<td>CACT (n = 3)</td>
<td>65,920 ± 3,356(^{1})</td>
<td>122,591 ± 39,373</td>
<td>608 ± 216</td>
<td>2,607 ± 430</td>
<td>22,156 ± 7,377</td>
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<td>753</td>
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<td>VLCAD</td>
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<td>824</td>
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<td>XALD</td>
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<td>422</td>
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<td>SCOX (n = 3)</td>
<td>54,264 ± 7,102(^{1})</td>
<td>147,870 ± 19,946</td>
<td>540 ± 208</td>
<td>10,791 ± 1,018</td>
<td>2,610 ± 564</td>
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<td>DBP (n = 3)</td>
<td>53,579 ± 26,796(^{1})</td>
<td>99,505 ± 40,835</td>
<td>0</td>
<td>5,544</td>
<td>5,545 ± 2,990</td>
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<tr>
<td>RCDP type 1 (n = 3)</td>
<td>38,706 ± 121(^{1})</td>
<td>71,072 ± 10,403</td>
<td>618 ± 105</td>
<td>3,164 ± 588</td>
<td>21,911 ± 354</td>
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<tr>
<td>Control mouse</td>
<td>63,244</td>
<td>92,878</td>
<td>0</td>
<td>2,865</td>
<td>15,382</td>
</tr>
<tr>
<td>LBP +/- mouse</td>
<td>111,787</td>
<td>105,147</td>
<td>569</td>
<td>4,607</td>
<td>28,837</td>
</tr>
<tr>
<td>SCPX +/- mouse</td>
<td>106,583</td>
<td>63,424</td>
<td>0</td>
<td>2,398</td>
<td>18,505</td>
</tr>
</tbody>
</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. n = number of different cell lines; all incubations were performed in triplicate; \(^{1}\) mean value ± standard deviation.
Peroxisomal \(\beta\)-oxidation enzymes involved in DHA biosynthesis

The retroconversion of C24:6n-3 to C22:6n-3. To study this retroconversion reaction more directly, fibroblasts were incubated with \( [3^{14}C]24:6n-3 \) (Table 3). Control fibroblasts converted almost all radiolabeled C24:6n-3 to C22:6n-3, which also was observed in fibroblasts from patients with a CACT deficiency. The rate of \(\beta\)-oxidation of this substrate in these cell lines was 10 pmol/h/mg. Fibroblasts from patients with Zellweger syndrome, however, produced no radiolabeled DHA (Fig. 4).

<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>Amount of radiolabeled fatty acid detected (counts per min) per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>C24:6n-3</td>
</tr>
<tr>
<td></td>
<td>3,609</td>
</tr>
<tr>
<td></td>
<td>2,945</td>
</tr>
<tr>
<td>Zellweger</td>
<td>9,692</td>
</tr>
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<td></td>
<td>10,023</td>
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<tr>
<td>CACT</td>
<td>2,321</td>
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<td></td>
<td>2,876</td>
</tr>
<tr>
<td>SCOX</td>
<td>11,091</td>
</tr>
<tr>
<td></td>
<td>11,068</td>
</tr>
<tr>
<td>DBP</td>
<td>8,356</td>
</tr>
</tbody>
</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. All incubations were performed in triplicate.

Role of the peroxisomal fatty acid \(\beta\)-oxidation enzymes in DHA biosynthesis

Linolenic acid (C18:3n-3) utilization

The results obtained in fibroblasts from patients lacking functional peroxisomes and patients with a mitochondrial fatty acid oxidation defect confirmed that the peroxisome, and not the mitochondrion, is the site for retroconversion of C24:6n-3 in the pathway for C22:6n-3 synthesis. Since the peroxisomal \(\beta\)-oxidation system consists of two separate sets of enzymes, the question was which enzymes would be responsible for the \(\beta\)-oxidation process in the pathway of DHA synthesis. To investigate this, we studied DHA synthesis from radiolabeled precursors in fibroblasts from patients with a deficiency of one of the \(\beta\)-oxidation enzymes or from knockout mice lacking one of the enzymes. After 96-h incubation of human skin fibroblasts deficient for SCOX activity with \( [1^{14}C]18:3n-3 \), 1% of the radiolabeled fatty acids contained in the cells consisted of C22:6n-3 compared to 15.5% in control fibroblasts (see Table 1 and Fig. 5). In fibroblasts from patients with a deficiency of DBP 2.6% of the radiolabeled fatty acids was C22:6n-3 (Table 1 and Fig. 5). Both SCOX- and DBP-deficient fibroblasts accumulated relatively large amounts of C24:6n-3 (about 6- and 3-times more than observed in control fibroblasts, respectively). In contrast, DHA synthesis from \( [1^{14}C]18:3n-3 \) was normal in fibroblasts from patients with RCDP type 1, characterized by the absence 3-ketoacyl-CoA thiolase in their peroxisomes, and from an XALD patient. The results obtained after incubation of skin fibroblasts from a control mouse with \( [1^{14}C]18:3n-3 \), were comparable with the results in control human
skin fibroblasts. In fibroblasts from both the LBP and SCPx knockout mouse normal synthesis of C22:6n-3 was observed (Table 1).

![Graphs showing radiolabeled fatty acids](image)

**Fig. 4** Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 0.2 µM [3-14C]24:6n-3 for 24 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), a patient with Zellweger syndrome (B), a DBP-deficient patient (C), and a CACT-deficient patient (D).

**Eicosapentaenoic acid (C20:5n-3) utilization**

Similar results were obtained after incubation with [1,14C]20:5n-3 (see Table 2). In fibroblasts of patients with SCOX and DBP deficiency about 10% and 18%, respectively, of the amount of radiolabeled DHA synthesized by control fibroblasts was produced. In fibroblasts from patients with RCDP type 1 and XALD, however, no deficiency in DHA synthesis was found. Compared to the C22:6n-3 production from [1-14C]20:5n-3 in control mouse skin fibroblasts, the production in fibroblasts from both the LBP and SCPx knockout mouse was also normal.

**Tetracosahexaenoic acid (C24:6n-3) utilization**

Incubations with [3-14C]24:6n-3 were performed to study directly the retroconversion of this substrate to C22:6n-3 in peroxisomal ß-oxidation mutants. Both fibroblasts of patients with an SCOX deficiency and of a patient with a deficiency of DBP formed very little radiolabeled DHA, only 10% and 6% respectively of the amount produced in
control fibroblasts (Table 3, Fig. 4). The β-oxidation rate of [3-14C]24:6n-3 in these fibroblasts was 0.7 and 0.4 pmol/h/mg, respectively, compared to 10 pmol/h/mg in control fibroblasts.

Fig. 5 Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 2 μM [1-14C]18:3n-3 for 96 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), an SCOX-deficient patient (B), a DBP-deficient patient (C) and an RCDP type 1 patient (D).

Discussion
Although many experimental data have been presented in the last few years which indicate that a peroxisomal β-oxidation step is part of the biosynthetic pathway of DHA, the role of the peroxisome in this pathway has remained subject of discussion. Our studies with control fibroblasts and fibroblasts from patients with Zellweger syndrome show that synthesis of C22:6n-3 in human cells involve peroxisomal retroconversion of C24:6n-3. When fibroblasts from Zellweger patients were incubated with [1-14C]18:3n-3 or [1-14C]20:5n-3 radiolabeled C24:6n-3 was formed, while no radiolabeled C22:6n-3 could be detected. This shows that C24:6n-3 is not an elongation-product from C22:6n-3, but that C24:6n-3 is an intermediate in DHA synthesis. In fibroblasts from patients with a deficiency of one of the mitochondrial enzymes CPT I, CACT, CPT II or VLCAD, the synthesis of radiolabeled C22:6n-3 from either [1-14C]18:3n-3 or [1-14C]20:5n-3 was normal. These results support the pathway proposed by Voss et al. (2), who suggested that instead of a direct conversion of C22:5n-3 to C22:6n-3 by a microsomal Δ4-desaturase, C22:5n-3 is first elongated to C24:5n-3, which is then desaturated by a Δ6-desaturase to
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C24:6n-3, followed by retroconversion of C24:6n-3 to C22:6n-3. This is also in agreement with recent findings in a patient with a Δ6-desaturase deficiency, a newly identified disorder (30). Fibroblasts of this patient hardly formed any C22:6n-3 from C24:5n-3, while the conversion of C24:6n-3 to C22:6n-3 was normal. In this paper, we directly evaluated this last step of DHA synthesis and found that fibroblasts from peroxisome-deficient patients did not convert [3-14C]24:6n-3 to radiolabeled C22:6n-3, while this conversion was normal in fibroblasts from patients with a mitochondrial fatty acid oxidation defect. This confirms the results obtained by Moore et al. (5) that DHA biosynthesis in human fibroblasts is a peroxisome-dependent process.

DHA plays an important role in the structure of cell membranes, particularly of neuronal tissues and retinal photoreceptor cells, which suggests that the DHA deficiency observed in Zellweger patients could very well be involved in the clinical symptomatology of this syndrome (demyelination, psychomotor retardation and retinopathy). It has been claimed that supplementation of DHA might result in, at least, some clinical improvement in Zellweger patients (31,32). Since peroxisomal β-oxidation is an essential step in the biosynthesis of DHA, studies in patients with a deficiency of a single β-oxidation enzyme could shed more light on the role of DHA in the pathology of peroxisomal fatty acid oxidation disorders. Because of these possible clinical implications, it is important to know which of the β-oxidation enzymes are responsible for the oxidation of C24:6n-3.

![Diagram of fatty acid β-oxidation machinery in human peroxisomes](image_url)

**Fig. 6** Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes involved in the retroconversion of C24:6n-3 to C22:6n-3. Our results showed that C24:6n-3 is β-oxidized by the same set of enzymes involved in the β-oxidation of the very long-chain fatty acids C26:0 and C24:0 (see Fig. 2). Oxidation of C24:6n-3 involves straight-chain acyl-CoA oxidase (SCOX), D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPX). Branched-chain acyl-CoA oxidase and L-bifunctional protein, however, are also both able to handle this substrate, but cannot maintain normal C22:6n-3 production without SCOX and DBP activity, respectively.
The results obtained in this study show that C24:6n-3 is β-oxidized by the same set of enzymes as used for the β-oxidation of the very long-chain fatty acids C26:0 and C24:0 (see Fig. 2 and Fig. 6). Current evidence holds that oxidation of C24:0 and C26:0 involves SCOX, DBP and both 3-ketoacyl-CoA thiolase and SCPx (15). Upon incubation of fibroblasts of patients with a deficiency of SCOX with [3-14C]24:6n-3, a strongly reduced rate of C24:6n-3 β-oxidation was found. However, some residual activity was present. It is not likely that the defective SCOX protein is responsible for this residual activity, since no SCOX protein can be detected when performing immunoblot analysis in any of the SCOX-deficient cell lines used in this study and it has been established that one of these patients has a large deletion of the SCOX gene (26). This suggests that the other peroxisomal oxidase, BCOX, can also handle this substrate, but that its activity is not sufficient for normal C22:6n-3 production. It is also unlikely that the mitochondrial β-oxidation system is responsible for the residual activity, because in fibroblasts of Zellweger patients, in which both SCOX and BCOX are lacking, there is no C24:6n-3 β-oxidation activity, while in patients with a deficiency of mitochondrial fatty acid β-oxidation normal activity was measured. In case of the second and third step of the β-oxidation process of C24:6n-3, our studies showed that, as for C26:0, DBP is responsible for these steps. As in SCOX-deficient patients, some residual activity was present in DBP-deficient patients. The fact that no DBP protein can be detected upon immunoblot analysis in virtually all patients studied, argues against a role of DBP in residual C22:6n-3 formation in DBP-deficient cells. The most likely explanation is that the other peroxisomal multifunctional enzyme, LBP, can also act on this substrate. Studies in fibroblasts of an LBP knockout mouse have shown, however, that LBP activity is not essential for DHA production. In the presence of either one of the peroxisomal thiolases normal amounts of DHA were produced from labeled precursors, which shows that both thiolases are able to perform the last step of C24:6n-3 β-oxidation and can maintain normal C22:6n-3 production by themselves, in the absence of the other thiolase. This also has been observed for C26:0 β-oxidation, which is normal both in patients with RCDP type 1 and in SCPx knockout mice, in contrast to pristaneic acid β-oxidation which is deficient in mice lacking SCPx function (33). It should be noted, however, that for the interpretation of our results we assume that the pathway of DHA biosynthesis is similar in man and mice.

Our results obtained with fibroblasts from an XALD patient indicate that β-oxidation of C24:6n-3 does not follow the exact same route as C26:0 and C24:0. The peroxisomal membrane protein ALDP, which is mutated in patients suffering from XALD resulting in impaired β-oxidation of very long-chain fatty acids, including C26:0 and C24:0, appears not to be involved in retroconversion of C24:6n-3. We found that DHA synthesis is normal in fibroblasts from an XALD patient, which is in agreement with results obtained by Petroni et al. (11).

Because of our results in fibroblasts from SCOX- and DBP-deficient patients, it would be interesting to investigate DHA levels in these patients. Martinez already showed that one patient with a deficiency of DBP (which was called bifunctional enzyme deficiency at that time) had low brain DHA levels, although they were not so severely reduced as in
patients with a peroxisome biogenesis disorder (8). In addition, a preliminary study performed by our group on PUFA composition in plasma samples from 10 DBP-deficient patients, revealed reduced DHA levels in 5 patients (unpublished data). This might reflect the reduced but not fully deficient DHA synthesis we observed in fibroblasts of these patients, in contrast to the complete deficiency in Zellweger patients. The presence of DHA and its precursors in the diet influences the PUFA composition of membrane lipids, and this influence probably becomes even greater in case of reduced DHA synthesis as in DBP patients.

Acknowledgments
This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands) and grant HL49264 from the National Heart Lung and Blood Institute, National Institutes of Health. The authors thank H.R. Waterham for critical reading of the manuscript.

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Peroxisomal β-oxidation enzymes involved in DHA biosynthesis


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deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein

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