Inactivation of the Peroxisomal Multifunctional Protein-2 in Mice Impedes the Degradation of Not Only 2-Methyl-branched Fatty Acids and Bile Acid Intermediates but Also of Very Long Chain Fatty Acids*

According to current views, peroxisomal β-oxidation is organized as two parallel pathways: the classical pathway that is responsible for the degradation of straight chain fatty acids and a more recently identified pathway that degrades branched chain fatty acids and bile acid intermediates. Multifunctional protein-2 (MFP-2), also called D-bifunctional protein, catalyzes the second (hydration) and third (dehydrogenation) reactions of the latter pathway. In order to further clarify the physiological role of this enzyme in the degradation of fatty carboxylates, MFP-2 knockout mice were generated. MFP-2 deficiency caused a severe growth retardation during the first weeks of life, resulting in the premature death of one-third of the MFP-2−/− mice. Furthermore, MFP-2-deficient mice accumulated VLCFA in brain and liver phospholipids, immature C_{27} bile acids in bile, and, after supplementation with phyto- and pristanic acid in liver triacylglycerols. These changes correlated with a severe impairment of peroxisomal β-oxidation of very long straight chain fatty acids (C_{24}α), 2-methyl-branched chain fatty acids, and the bile acid intermediate trihydroxycoprostanic acid in fibroblast cultures or liver homogenates derived from the MFP-2 knockout mice. In contrast, peroxisomal β-oxidation of long straight chain fatty acids (C_{16}α) was enhanced in liver tissue from MFP-2−/− mice, due to the up-regulation of the enzymes of the classical peroxisomal β-oxidation pathway. The present data indicate that MFP-2 is not only essential for the degradation of 2-methyl-branched fatty acids and the bile acid intermediates di- and trihydroxycoprostanic acid but also for the breakdown of very long chain fatty acids.

In mammals, β-oxidation is confined to two organelles, mitochondria and peroxisomes. Whereas mitochondrial β-oxidation is primarily involved in the catabolism of short, medium, and long chain fatty acids supplementing energy to the cell, peroxisomal β-oxidation seems to be responsible for the degradation of a number of less abundant carboxylates of different molecular structure (1, 2). The substrates for peroxisomal β-oxidation known to date include very long straight chain fatty acids (containing more than 20 carbon atoms); 2-methyl-branched chain fatty acids (e.g. pristanic acid that is formed after α-oxidation of the 3-methyl-branched fatty acid phytanic acid; prostanoids; dicarboxylic acids; and the C_{27} bile acid intermediates di- and trihydroxycoprostanic acid, which are converted to the mature C_{24} bile acids via peroxisomal β-oxidation.

Similar to mitochondrial β-oxidation, peroxisomal β-oxidation consists of four steps: 1) a desaturation leading to a 2-enoyl-CoA; 2) a hydration of the enoyl-CoA to a 3-hydroxyacyl-CoA; 3) a dehydrogenation with the formation of a 3-ketoacyl-CoA; and 4) a thiolic cleavage yielding an acyl-CoA shortened by two carbon atoms. In recent years, it has become clear that the peroxisomal reactions are catalyzed by different acyl-CoA oxidases (3–5), which form H_{2}O_{2} (desaturation), different multifunctional proteins (hydration plus dehydrogenation) (6–10), and different thiolases (thiolatic cleavage) (11, 12).

Based on the substrate specificity and stereoselectivity of the newly discovered enzymes, different β-oxidation pathways have been proposed (2, 13). The classical pathway catalyzed by palmitoyl-CoA oxidase, multifunctional protein-1 (also denoted as L-bifunctional protein because the hydrated species it generates has the L-configuration), and peroxisomal thiolase is generally accepted to be responsible for the oxidation of straight chain fatty acids. All enzymes of this pathway are found in different species and can be strongly induced in rodents by ligands of the peroxisome proliferator-activated receptor α (PPARα). The oxidation of 2-methyl-branched fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acid, is believed to occur via a second pathway. In humans, this pathway consists of branched chain acyl-CoA oxidase (5), multifunctional protein-2 (the β-specific multifunctional protein, which is identical to 17β-estradiol dehydrogenase type IV and which generates the β-hydroxy intermediate (9, 14)), and sterol carrier protein-x (SCPx), the N-terminal part of which exerts thiolatic activity (12). In rats, the desaturation of bile acid intermediates is executed by trihydroxycoprostanoyl-CoA oxidase (3), which is the counterpart of the human branched chain acyl-CoA oxidase (15, 16). A third acyl-CoA

* These studies were supported by grants from Geconcerteerde Onderzoeksaacties K.U. Leuven (GOA/99/08). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Received for publication, March 9, 2000
Published, JBC Papers in Press, March 15, 2000, DOI 10.1074/jbc.M001994200

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oxide, pristanoyl-CoA oxidase, is very active in rat liver and is thought to be involved in pristanic acid breakdown (4, 17). In mice, as in humans, pristanoyl-CoA oxidase is not detectable at the protein level (17, 18).2

Several in vitro and in vivo studies indicate that the separation between the two pathways may not be as strict as generally believed and that intermediates produced in one of these β-oxidation pathways can be shuffled to the other system. In particular, in vitro experiments have shown that the multifunctional proteins (MFP-1 and MFP-2) have a broad substrate spectrum; they are both capable of hydrating the enoyl-CoA esters of straight chain and 2-methyl-branched fatty acids and of the bile acid intermediates and they can both dehydrogenate straight chain and branched chain 3-hydroxyacyl-CoAs (6–10, 19, 20). However, it was concluded from stochochemical studies that MFP-2 and not MFP-1 is responsible for the degradation of bile acid intermediates and pristanic acid. Indeed, only the hydroxylated intermediate produced by MFP-2 has the right configuration to be further dehydrogenated by the same enzyme (7, 9, 10, 19).

Besides this involvement in the degradation of branched compounds, there are indications that MFP-2 might also be physiologically important for the oxidation of straight chain fatty acids. During studies on linoleic acid (21) and palmitic acid3 breakdown by purified peroxisomes, generation of both 3-hydroxyacyl-CoA stereoisomers was observed. In human fibroblasts, which contain more MFP-2 than MFP-1, MFP-2 was suggested to play a major role in the peroxisomal oxidation of long chain and very long chain fatty acids (VLCFA) (22). Furthermore, mice with an inactivated MFP-1 gene display normal levels of VLCFA (23), whereas a patient with a genetic defect in the MFP-2 gene was reported to accumulate, besides branched fatty acids and bile acid intermediates, also VLCFA (24). However, the latter observation could also be explained by an inhibitory effect of accumulating branched chain β-oxidation intermediates on MFP-1 (24).

The aim of the present study was to further investigate the in vivo role of MFP-2 by generating a mouse model with MFP-2 deficiency. Inactivation of the MFP-2 gene caused a severe growth retardation and death of one-third of the mice during the first postnatal weeks. Overall peroxisomal β-oxidation activity toward different substrates, individual enzyme activities, and accumulation of substrates for peroxisomal β-oxidation were examined. The results demonstrate that MFP-2 is of prime importance not only in the degradation of 2-methyl-branched fatty acids and bile acid intermediates but also in the degradation of very long chain fatty acids.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector and Generation of MFP-2−/− Mice**—A P1 genomic clone encompassing 80 kilobase pairs of the mouse MFP-2 gene was obtained through Genome Systems (St. Louis, MO) by polymerase chain reaction screening using as primers AgCATgggAC- CTATTTTAAAGAAGACAg (forward) and TTCGgTCATCGTgACCACggCg- TTg (reverse). These oligonucleotides are located at base pairs 399–423 and 582–606 of the published mouse cDNA (25) and encompass a 1.5-kilobase pair genomic DNA fragment. The P1 clone was partially mapped by restriction enzyme digestion and Southern hybridization and by polymerase chain reaction analysis to locate intron/exon boundaries. The targeting vector was constructed by subcloning a 3.5-kilobase pair BamHI/HindIII fragment located in the 5′-upstream region of the gene as the 5′-flank and a 6.0-kilobase pair SspI/KpnI fragment (lo-2 The mouse acyl-CoA oxidase acting on the CoA-esters of bile acid intermediates and pristanic acid will be named branched chain acyl-CoA oxidase, given the similarity between the mouse and human enzyme.

3 M. Dieuaide-Noubhani, G. P. Mannaeerts, and P. P. Van Veldhoven, unpublished data.

ated in intron 3) as the 3′-flank in the pNT vector (26). Linearization of the targeting vector and electroporation in R1 ES cells were done as described previously (27). Southern analysis of 280 clones resistant to both G418 and ganciclovir revealed four clones that had undergone the correct homologous recombination (Fig. 1). Aggregation of these recombinant ES cell clones with embryonic stem cells resulted in several chimeric mice, one of which transferred the MFP-2−/− genotype to the offspring.

**Southern, Northern, and Western Analysis**—Mice were genotyped by extraction of tail DNA, digestion with EcoRV, and hybridization of the Southern blot with the 3′ external probe (Fig. 1). For Northern analysis, 25 μg of total RNA extracted from adult mouse livers by the Chomczynski procedure (28) was loaded on a 1.1% (w/v) agarose gel containing 3% (w/v) formaldehyde. A 1071-base pair probe corresponding to the 1042–2113 base pair fragment of the mouse MFP-2 cDNA was generated by reverse transcription-polymerase chain reaction from mouse liver RNA and used for Northern hybridization in the presence of formamide.

For Western blot analysis, liver tissue from 1-, 2-, or 6-week-old mice was homogenized in 0.25 M sucrose, 5 mM MOPS, pH 7.2, 1 mM EDTA. Thirty μg of protein were separated by SDS-polyacrylamide gel electrophoresis (10% gels) and blotted. The following antibodies raised in rabbits were used: the 53-kDa subunit of rat palmitoyl-CoA oxidase (17), rat trihydroxycoprostanoyl-CoA oxidase (29), rat MFP-1 (obtained from Prof. W. Just, Heidelberg), rat MFP-2 (7), rat peroxisomal thiolase (11), rat PMP70 (obtained from Prof. D. Fahimi, Heidelberg), CYP4A (obtained from Affinity Bioreagents), cholesterol 7α-hydroxylase (obtained from Dr. D. Russell, Dallas, TX), and mouse Pex5p (30).

**β-Oxidation of 14C-Labeled Substrates in Liver Homogenates and in Fibroblast Cultures**—Liver homogenates from 6–8-week-old mice were freshly prepared in 0.25 M sucrose, 5 mM MOPS, pH 7.2, and incubated with 0.1 mM [1-14C]palmitoyl-CoA under conditions favoring either peroxisomal or mitochondrial β-oxidation (31). Incubations with 0.1 mM [26,26,3,7,12-trihydroxycoprostanic acid were performed under cytochrome conditions as described previously (32), but dithiothreitol was omitted, and the specific activity was raised to 5 μCi/μmol. Formation of CO2 was negligible in homogenates, and oxidation rates were calculated from the generation of acid soluble products as described.

Fibroblasts were prepared by trypsinization of skin tissue of newborn mice and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. Passage 3 cells were plated in 25-cm2 flasks, grown to 80% confluency, washed with PBS, and incubated in closed flasks for approximately 24 h with 3 ml of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 0.2% Ultroser H and 5 μM cholesterol and 10 μM 3-methyl[1-14C]hexadecanoic acid. The generation of [1-14C]lignoceric acid, or 3-methyl-[1-14C]hexadecanoic acid. The generation of 14C-labeled CO2, formate, and acid-soluble substances was quantified as described (33, 34).

**Measurement of Enzyme Activities—Acyl-CoA oxidase activities in liver homogenates from 6–8-week-old mice were analyzed as described (35). For the measurements of palmitoyl-CoA oxidase, homogenates were pretreated with N-ethylmaleimide, and 100 μM palmitoyl-CoA was used as substrate in the presence of 10 μM bovine serum albumin. For the determination of branched chain acyl-CoA oxidase, the N-ethylmaleimide pretreatment was omitted, and 100 μM 2-methylhexadecanoyl-CoA, in the presence of 40 μM bovine serum albumin, was used as substrate. Due to the apparent absence of pristanoyl-CoA oxidase in mouse liver (17), this substrate is used by branched chain acyl-CoA oxidase and results in higher activities than those obtained with trihydroxycoprostanoyl-CoA.4

Multifunctional proteins were measured by following the hydroxylation of 3-hydroxy-3-phenylpropionyl-CoA, a substrate introduced by Schulz and co-workers (36). For the CoA-ester synthesis, the hydroxy-succinimide esters (150 μmol) (37) of the 3-hydroxy-3-phenylpropionic acid isomers (Acros, crystallized from ethanol, were dissolved in 2.5 ml of tetrahydrofuran and mixed with an equal volume of 0.2 M NaHCO3 containing 50 μmol CoA and stirred for 3 h at room temperature. After removal of the organic solvent by a stream of nitrogen, pH was brought to 4 with acetic acid, 2 ml of methanol was added, and remaining ester was extracted with heptane/ethyl acetate (1:1, v/v). Insoluble material was removed by centrifugation, and the supernatant was adjusted to pH 7 with NaHCO3. For the assay, a 50-μl aliquot of the homogenates, appropriately diluted, was added to cuvettes, followed by the addition of 400 μl of reaction medium and 50 μl of 1 mM CoA-ester (either the 3E-isomer for the measurement of MFP-1 or the 3Z-isomer for the measurement of MFP-2).
Mendelian inheritance of the MFP-2 ligible in liver homogenates derived from MFP-2-deficient mice. Lipids were further separated in neutral lipids, fatty acids, and phospholipids by ion exchange chromatography (Bond Elut NH$_2$, 500 mg, Varian). The fractions containing phospholipids were subjected to acidic methanolysis in the presence of internal standards (heptadecanoic acid and heptadecanoic acid) followed by gas chromatography of the fatty acid methyl esters on a BPX70 column (40). Triglycerides were isolated from the fractions containing neutral lipids by TLC (solvent heptane/methanol, and 1:1 or 1:2 of ethanol). After boiling for 5 min, the mixture was centrifuged, and the precipitate was washed once with 600 μl of ethanol. The combined supernatants, after adding 1 ml of water, were extracted twice with 2 ml of heptane and then acidified with 50 μl of 1 N HCl, and bile acids were extracted with 2 × 2 ml of diethylether. The ether phases were dried under nitrogen and dissolved in 20 μl of 70% methanol, and 1 μl was mixed with glycerol and analyzed by negative ion fast atom bombardment mass tandem mass spectrometry as described by Libert et al. (42).

RESULTS

Targeting of the Mouse MFP-2 Gene—With the aim of abolishing both enzymatic activities (hydratase and dehydrogenase) of the MFP-2 gene product, a targeting vector was constructed such that after homologous recombination the first three exons were deleted (Fig. 1). The successful inactivation of the MFP-2 gene in MFP-2$^{-/-}$ mice was demonstrated by Northern and Western blotting showing the total absence of MFP-2 transcripts and protein (Fig. 1). This was further confirmed by analyzing the dehydration of 3S-hydroxy-3-phenylpropionyl-CoA, catalyzed by the α-specific hydratase domain of MFP-2. The dehydration activity was easily measurable in liver homogenates from wild type and MFP-2$^{-/-}$ mice but was negligible in liver homogenates derived from MFP-2-deficient mice (Table 1).

Growth Retardation and Survival of MFP-2 Knockout Mice—Genotyping 158 pups from 15 litters demonstrated a normal Mendelian inheritance of the MFP-2$^{-/-}$ allele (22% MFP-2$^{+/+}$; 52% MFP-2$^{-/-}$; 26% MFP-2$^{+/-}$). At birth, the MFP-2$^{-/-}$ mice were indistinguishable from their wild type and heterozygous littermates. However, from postnatal day 2 on, a marked growth retardation of the MFP-2-deficient mice was observed (Fig. 2, A and B). Weight gain was particularly low during the lactation period resulting in an average 50% reduction of body weight in comparison with wild type littermates. Approximately 30% of MFP-2$^{-/-}$ mice were more severely affected, grew only marginally, and died between postnatal days 2 and 12 (Fig. 2C). After weaning at 3 weeks of age, weight gain of MFP-2$^{-/-}$ mice resumed, and their body weight was on average 10% lower than controls in the adult stage. The growth survival of a representative litter in which all pups survived into adulthood is shown in Fig. 2B. Macroscopic and light microscopic inspection of the major MFP-2$^{-/-}$ organs did not reveal any changes with the exception of the colon content of suckling mice. These feces had a more yellow fluid appearance and contained more undigested residues as compared with those from age-matched controls. No fat droplets were identifiable by Sudan red staining. Female MFP-2-deficient mice were fertile, but males had a strongly reduced fertility.

Peroxisomal and Mitochondrial β-Oxidation of $^{14}$C-Labeled Substrates—The consequences of inactivation of the MFP-2 gene on the catabolism of different substrates for peroxisomal β-oxidation were tested by incubating fibroblast cultures and liver homogenates with $^{14}$C labeled substrates (Tables II, III). Palmitic acid was degraded to the same extent in fibroblast cultures derived from MFP-2$^{-/-}$ and MFP-2$^{-/-}$ mice. However, in intact cells a major mitochondrial contribution in the breakdown of this long chain fatty acid can be expected. When oxidation of palmitoyl-CoA was studied in liver homogenates under conditions favoring the peroxisomal pathway (presence of cyanide; absence of carnitine and albumin) (31), activities in homogenates of MFP-2$^{-/-}$ mice were enhanced 2.5-fold. In contrast, the mitochondrial β-oxidation of palmitate was not affected by the absence of MFP-2. When fibroblasts were incubated with lignoceric acid, a specific substrate for peroxisomal β-oxidation, degradation was 10 times lower in MFP-2$^{-/-}$ fibroblasts than in controls, strongly suggesting that MFP-2 is more important than MFP-1 for VLCFA breakdown. As expected, the oxidation of a 2-methyl-branched fatty acid, 2-methylhexadecanoic acid, was markedly reduced in MFP-2-deficient fibroblasts (80% reduction as compared with wild type fibroblasts). Trihydroxycoprostanic acid was not β-oxidized by fibroblasts, presumably because of lack of uptake or activation. In liver homogenates prepared from MFP-2-deficient mice, the degradation rate of trihydroxycoprostanic acid was 5 times lower than in homogenates from wild type mice.
MFP-2 Knockout Mice

**TABLE I**

| Enzyme                        | Substrate                             | MFP-2+/− | MFP-2−/− | MFP-2+/− |
|-------------------------------|---------------------------------------|----------|----------|----------|
| Palmitoyl-CoA oxidase         | Palmitoyl-CoA                          | 7.00 ± 1.70 (3) | 7.83 ± 0.74 (4) | 27.6 ± 2.13 (4) |
| Branched chain acyl-CoA oxidase | 2-Methylhexadecanoyl-CoA                | 0.271 ± 0.025 (3) | 0.223 ± 0.044 (3) | 0.722 ± 0.136 (4) |
| MFP-1 (dehydratase)           | 3R-Hydroxy-3-phenylpropionyl-CoA        | 123 ± 11.3 (3) | 115 ± 7.32 (7) | 234 ± 21.6 (7) |
| MFP-2 (dehydratase)           | 3S-Hydroxy-3-phenylpropionyl-CoA        | 71.4 ± 6.04 (3) | 41.4 ± 1.25 (7) | 0.21 ± 0.10 (7) |
| Urate oxidase                 | Uric acid                             | 10.7 ± 0.93 (3) | 10.3 ± 0.69 (4) | 25.4 ± 2.03 (4) |

Values represent the mean ± S.E. of the activities expressed as nmol · min · mg protein (n = number of samples).

FIG. 2. Retarded somatic growth of MFP-2 knockout mice. A, photograph of a wild type (upper) and a MFP-2−/− (lower) littermate on postnatal day 15. B, growth curve of MFP-2 knockout offspring (triangles, four mice) and wild type or heterozygous offspring (squares, eight mice) of the same litter. Mean ± S.E. of body weights are shown. C, survival curve of MFP-2 knockout mice; out of 65 newborn MFP-2−/− mice, 25 died before the age of 3 weeks.

Induction of Peroxisomal β-Oxidation Enzymes—As already mentioned, no MFP-2 dehydration activity could be measured in liver homogenates of MFP-2−/− mice. In contrast, the activity of MFP-1, palmitoyl-CoA oxidase, and urate oxidase appeared to be induced 2–4-fold as compared with wild type controls (Table I). Western blot analysis confirmed the induction of palmitoyl-CoA oxidase, MFP-1, and peroxisomal thiolase (Fig. 3). The peroxisomal membrane protein PMP70 and the microsomal CYP4A ω-hydroxylase were induced to a lesser extent, whereas the expression of PEX5p, the import receptor of most peroxisomal matrix proteins, was unaltered as compared with controls (data not shown). These protein inductions were continuously present from the first postnatal week into adulthood.

Curiously, also branched chain acyl-CoA oxidase, an enzyme that is hardly influenced in rat by fibrate treatment (3), was induced. The activity of this enzyme was hardly measurable in wild type mice, but it could easily be detected in liver homogenates of the MFP-2−/− mice (Table I).

Fatty Acid Analysis in Tissues—In view of the reduced oxidation rates of VLCFA, accumulations of these carboxylates could be expected in tissues of MFP-2 knockout mice. C26 levels were quantified in the phospholipid fraction of brain and liver using GC analysis of the fatty acid methyl esters. In livers and brains of adult MFP-2−/− mice, C26 levels were increased 3–6-fold as compared with age-matched controls (Fig. 4A). Up until 6 weeks of age, no accumulation of the 2-methyl-branched fatty acid pristanic acid was found in the liver phospholipid or triglyceride fraction. However, supplementation of mouse chow with phytol (5 mg/g), as was previously done for SCPx knockout mice (43), led to increased levels of pristanic acid and to a greater extent of its precursor phytanic acid in the phospholipid and triglyceride fraction of MFP-2−/− livers (Fig. 4B). The increased levels of branched chain fatty acids in MFP-2-deficient mice caused weight loss, cataract, and ataxia very similar to the observations in SCPx knockout mice treated with this dose of phytol (43).

Bile Acid Analysis—The degradation of cholesterol into C24 bile acids involves two or three hydroxylations of the ring structure and shortening of the side chain by ω-oxidation followed by peroxisomal β-oxidation. As revealed by mass spectrometric analysis of the bile acids extracted from the bile, the taurine conjugate of a trihydroxylated C24 bile acid (m/z 514) is the most abundant species in control mice, followed by a dihydroxylated C24 compound (Fig. 5, A and C). In mice, the trihydroxylated C24 bile acids consist predominantly of ω- and β-muricholic acid and to a lesser extent of cholic acid. In bile of the MFP-2 knockout mice, these C24 bile acids were still present, but various other bile acids with larger masses are seen (Fig. 5, B and D). By daughter ion analysis, the major species with m/z 554 was shown to consist of taurine conjugates of dihydroxylated C27 bile acids possessing a double bond in the side chain (Fig. 5E). To further evaluate bile acid levels, the expression of cholesterol 7α-hydroxylase, which is under a negative feedback inhibition by bile acids (44), was analyzed by Western blotting. In microsomes of MFP-2 knockout mice, cholesterol 7α-hydroxylase was only slightly up-regulated as compared with wild type mice (data not shown), probably reflecting the presence of considerable levels of normal C24 bile acids in adult MFP-2 knockout mice.
The impaired degradation of VLCFA under conditions of MFP-2 deficiency (46, 47) suggests that peroxisomal thiolase and not SCPx is responsible for cleavage of the 3-keto derivatives of the VLCFA-CoAs. These results call for a reconsideration of the organization of peroxisomal β-oxidation; instead of a strict separation of the two pathways, substrates desaturated by acyl-CoA oxidase can be further converted by either MFP-1 (long chain fatty acids) or MFP-2 (VLCFA), and the reaction products of the latter enzymes can converge again for the thiolytic cleavage by thiolase.

The occurrence of immature C27 bile acids, carrying an unsaturated side chain, in the bile of MFP-2-deficient mice confirms the importance of MFP-2 (6, 7, 10) in the formation of bile acids. However, normal C24 bile acids were also present, similar to the observation in a patient with complete MFP-2 deficiency (48, 49). This suggests that alternative pathways, which bypass MFP-2, convert cholesterol into mature bile acids. The sterol 25-hydroxylase pathway involving cleavage of acetone from the side chain could account for some of the bile acid synthesis, although it was demonstrated that the activity of the microsomal 25-hydroxylase is relatively low in mouse liver (50). It has been recently reported that in brain, excess cholesterol is eliminated via the liver (51, 52). It was suggested that this intermediate is metabolized to bile acids in the liver, which implies ring hydroxylations and oxidation of the side chain. This would lead to the formation of 24S,25R-3,7,12,24-tetrahydroxycoprostanoyl-CoA, which has the correct stereochemical configuration for further dehydrogenation by MFP-1. As an additional explanation for the occurrence of normal bile acids, it can be proposed that trihydroxycoprostanoyl-CoA is hydrated by MFP-1 with the formation of 24S,25R-3,7,12,24-tetrahydroxycoprostanoyl-CoA, which after conversion by a racemase to the 24S,25R-stereoisomer (53) can again be dehydrogenated by MFP-1.

**FIG. 3. Induction of peroxisomal enzymes in MFP-2 knockout mice.** Western blots containing in each lane 30 µg of liver protein of either wild type, MFP-2+/−, or MFP-2−/− mice (in duplicate) were incubated with the indicated enzyme antibodies. ACO and Br-CoA ox, palmitoyl-CoA oxidase and branched chain acyl-CoA oxidase, respectively.

**TABLE II**

Activity of peroxisomal β-oxidation in MFP-2 knockout mice: Fibroblast cultures

| Substrate | AS  
|-----------|
| [1-14C]Palmitic acid (n = 2) | 2.59  
| 2-Methyl-[114C]Hexadecanoic acid | 1.03  
| [1-14C]Lignoceric acid (n = 4) | 0.027  

*ASM, acid-soluble material: sum of CO 2 and acid-soluble products, expressed in nmol/mg of protein per 24 h. Mean ± S.E. is presented.

**TABLE III**

Activity of peroxisomal β-oxidation in MFP-2 knockout mice: Liver homogenates

| Substrate | MFP-2+/− | MFP-2−/− |
|-----------|----------|----------|
| [1-14C]Palmitoyl-CoA (peroxisomal) (n = 6) | 2.59  
| [1-14C]Palmitoyl-CoA (mitochondrial) (n = 3) | 1.03  
| [1-14C]Trihydroxycoprostanic acid (n = 5) | 0.027  

**DISCUSSION**

Based on detailed substrate specificity studies of the different enzymes involved, peroxisomal β-oxidation was proposed to be organized in two parallel pathways: one pathway responsible for the degradation of straight chain fatty acids and a second pathway, whose enzymes were more recently identified, responsible for the breakdown of pristanic acid and bile acid intermediates. However, the present study demonstrates that straight VLCFAs accumulate, besides branched chain fatty acids and bile acid intermediates, in mice lacking MFP-2, an enzyme belonging to the second pathway. These findings are in accordance with the increased levels of VLCFAs recently observed in MFP-2-deficient patients (24). It can be argued that the impaired degradation of VLCFA under conditions of MFP-2 deficiency is due to the accumulating branched chain enoyl-CoAs that competitively inhibit MFP-1. However, in the present study it was unequivocally demonstrated that the degradation of VLCFA was also impaired in fibroblast cultures of the MFP-2 knockout mice, which are not exposed to pristanic acid or bile acid intermediates. In addition, the oxidation of long chain fatty acids was even enhanced in liver homogenates of MFP-2-deficient mice as compared with wild type, rather supporting increased activity of MFP-1. Taking further into account the fact that VLCFA levels are not increased in MFP-1-deficient mice (23), it can be concluded that MFP-2 and not MFP-1 is the principal enzyme responsible for the degradation of VLCFA. Consequently, the function of MFP-1 seems to be restricted to the oxidation of long chain fatty acids and probably eicosanoids and dicarboxylic acids.

These results raise the question of which oxidase and which thiolase are involved in the degradation of VLCFA. In vitro, palmitoyl-CoA oxidase, branched chain acyl-CoA oxidase, and pristanoyl-CoA oxidase can desaturate lignoceryl-CoA (5, 45). The importance of palmitoyl-CoA oxidase in vivo conditions is supported by the fact that VLCFA accumulate in patients and in mice with a deficiency of this enzyme (46, 47). No changes in VLCFA levels were observed in SCPx knockout mice (43), suggesting that peroxisomal thiolase and not SCPx is responsible for cleavage of the 3-keto derivatives of the VLCFA-CoAs. These results call for a reconsideration of the organization of peroxisomal β-oxidation; instead of a strict separation of the two pathways, substrates desaturated by acyl-CoA oxidase can be further converted by either MFP-1 (long chain fatty acids) or MFP-2 (VLCFA), and the reaction products of the latter enzymes can converge again for the thiolytic cleavage by thiolase.

**TABLE II**

Activity of peroxisomal β-oxidation in MFP-2 knockout mice: Fibroblast cultures

| Substrate | MFP-2+/− | MFP-2−/− |
|-----------|----------|----------|
| [1-14C]Palmitic acid (n = 2) | 2.05  
| 2-Methyl-[114C]Hexadecanoic acid (n = 3) | 9.08  
| [1-14C]Lignoceric acid (n = 4) | 1.11  

*ASM, acid-soluble material: sum of CO 2 and acid-soluble products, expressed in nmol/mg of protein per 24 h. Mean ± S.E. is presented.

**TABLE III**

Activity of peroxisomal β-oxidation in MFP-2 knockout mice: Liver homogenates

| Substrate | MFP-2+/− | MFP-2−/− |
|-----------|----------|----------|
| [1-14C]Palmitoyl-CoA (peroxisomal) (n = 6) | 2.59  
| [1-14C]Palmitoyl-CoA (mitochondrial) (n = 3) | 1.04  
| [1-14C]Trihydroxycoprostanic acid (n = 5) | 0.004  

**FIG. 3. Induction of peroxisomal enzymes in MFP-2 knockout mice.** Western blots containing in each lane 30 µg of liver protein of either wild type, MFP-2+/−, or MFP-2−/− mice (in duplicate) were incubated with the indicated enzyme antibodies. ACO and Br-CoA ox, palmitoyl-CoA oxidase and branched chain acyl-CoA oxidase, respectively.
The essential role of MFP-2 in the degradation of branched chain fatty acids was demonstrated by supplementing phytol, a tetramethyl-branched fatty alcohol, to the diet of MFP-2-deficient mice. Standard mouse chow indeed contains very few precursors of branched chain fatty acids, which is in contrast to the daily dietary intake by humans of phytol and phytanic acid present in dairy products. Phytol is converted to phytanic acid, which is subsequently shortened by α-oxidation with the formation of pristanic acid. The latter 2-methyl-branched fatty acid is further degraded by peroxisomal β-oxidation. After a few weeks of phytol treatment, accumulations of pristanic acid and its precursor phytanic acid were found in liver triglycerides and phospholipids of the MFP-2−/− mice, causing severe weight loss and neurological symptoms. Since wild type and MFP-2−/− mice tolerated this diet well, it is evident that MFP-2 is necessary for the degradation of branched chain fatty acids.

It is unclear at this point whether the severe growth retardation during the first postnatal weeks is caused by a low food intake or by malabsorption. In addition, it has not been resolved whether this deficit in weight gain is related to the accumulation of VLCFA, reduced levels of normal bile acids, or to the presence of immature bile acids. Since the low growth rate of MFP-2-deficient mice occurs in particular during the period of high fat intake, it seems likely that it is related to the abnormalities of the bile acid composition in the intestines. In adult MFP-2-deficient mice, high levels of normal bile acids seem to be formed by alternative pathways, but whether these pathways are also operative in the early postnatal period needs to be evaluated. Possibly, the C27 bile acids could be toxic for the intestines, which might be alleviated when solid food is ingested. Further analysis of fat and bile acid content in the intestines during the lactation period and treatment of adult MFP-2-deficient mice with high fat diets might clarify this issue. Interestingly, two other mouse models with inactivation of enzymes involved in the side chain degradation of cholesterol have been generated. In mice lacking sterol 27-hydroxylase, the first enzyme in the pathway, the bile acid pool in intestines and bile was strongly reduced, but only traces of C27 bile alcohols (50) were found. SCPx-deficient mice were shown to accumulate C25 bile acids (including norcholic acid) in bile and in serum and to a much lesser extent an unusual C26 bile alcohol carrying a keto function at C-24 (54). Remarkably, no growth abnormalities were described for these two mouse models (43, 50), whereas mice with inactivation of cholesterol 7α-hydroxylase (55) displayed a severe growth retardation and postnatal death. These observations suggest that not only the reduced levels of regular bile acids but also the type and levels of abnormal bile acids might be responsible for influencing growth. Strikingly, palmitoyl-CoA oxidase knockout mice, which presumably have normal bile acids but increased VLCFA, displayed a growth retardation starting from the second postnatal week (47). A mechanism for this impaired growth was not reported.

The absence of MFP-2 causes an up-regulation of the peroxisomal β-oxidation enzymes of the classical pathway, palmitoyl-CoA oxidase, MFP-1, and thiolase, which is already apparent 1 week postnatally. Since the expression of the microsomal enzyme CYP4A, the peroxisomal membrane protein PMP70, and urate oxidase was also increased, this pattern of protein induction seems to coincide with the gene induction pattern of the nuclear receptor PPARα. In palmitoyl-CoA oxidase knockout mice, similar enzyme inductions were reported (47). It was further demonstrated that PPARα activation underlay the enzyme up-regulation, since double knockout mice lacking both palmitoyl-CoA oxidase and PPARα failed to increase expression of these genes (56). Also in SCPx knockout mice, PPARα-regulated genes were induced, and it was suggested that the accumulating phytanic acid acts as the activating PPARα ligand under these circumstances (57). It can be expected that also the inactivation of MFP-2 impairs the degradation of a PPARα activator, but the nature of this ligand needs to be investigated. Unexpectedly, also the protein levels and activity...
of branched chain acyl-CoA oxidase were induced in MFP-2-deficient mice. In rats, a lack of responsiveness of this enzyme to PPARα activators has been reported (3, 16). Possibly, the mouse gene contains PPAR-responsive elements, since clofibrate treatment also up-regulated this enzyme in this species.4

In conclusion, the present study has unequivocally demonstrated that in mice MFP-2 is essential for the degradation of saturated very long chain and branched chain fatty acids and for the formation of mature bile acids.

Acknowledgments—We thank B. Das, L. Pauwels, E. Meyhi, and R. Rozenberg for excellent technical assistance and Prof. D. Russell, Prof. W. Just, and Prof. D. Fahimi for providing antisera. We greatly appreciate the help of Prof. M. Dewerchin with the generation of the chimeric mice and Prof. E. de Hoffmann (CICO, UCL, Louvain-la-Neuve, Belgium) with the mass spectrometric analysis of the bile samples.

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FIG. 5. Bile acid analysis of MFP-2 knockout mice. A and B show the negative ion fast atom bombardment-mass spectrum of the mouse bile extracts. In C and D, the presence of taurine conjugates is analyzed by parent scans for atomic mass units 124 (A and C, MFP-2+/−; B and D, MFP-2−/−). E, a daughter scan of the major bile acid seen in B (m/z 554) is shown. This peak probably consists of a mixture of taurine-conjugated Δ24-trihydroxylated cholestanolic acids (fragmentation patterns in F) with hydroxyl groups in different positions of the ring structure, which are the precursors of α/β-isomers of muricholic acids (specific fragments m/z 413) or of cholic acid (specific fragment m/z 399). Also, a dihydroxylated Δ24-analogue (m/z 538) and the saturated trihydroxylated C27 bile acid (m/z 556) are present in bile of MFP-2−/− mice.
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