Peroxisomes Contain a Specific Phytanoyl-CoA/Pristanoyl-CoA Thioesterase Acting as a Novel Auxiliary Enzyme in α- and β-Oxidation of Methyl-branched Fatty Acids in Mouse*

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Phytanic acid and pristanic acid are derived from phytol, which enter the body via the diet. Phytanic acid contains a methyl group in position three and, therefore, cannot undergo β-oxidation directly but instead must first undergo α-oxidation to pristanic acid, which then enters β-oxidation. Both these pathways occur in peroxisomes, and in this study we have identified a novel peroxisomal acyl-CoA thioesterase named ACOT6, which we show is specifically involved in phytanic acid and pristanic acid metabolism. Sequence analysis of ACOT6 revealed a putative peroxisomal targeting signal at the C-terminal end, and cellular localization experiments verified it as a peroxisomal enzyme. Subcellular fractionation experiments showed that peroxisomes contain by far the highest phytanoyl-CoA/pristanoyl-CoA thioesterase activity in the cell, which could be almost completely immunoprecipitated using an ACOT6 antibody. Acot6 mRNA was mainly expressed in white adipose tissue and was co-expressed in tissues with the target gene of the peroxisome proliferator-activated receptor α (PPARα) and is up-regulated in mouse liver in a PPARα-dependent manner.

Methyl-branched fatty acids such as phytanic acid (3,7,11,15-tetramethylexadecanoic acid) and pristanic acid (2,6,10,14-tetramethyldodecanoic acid) are found in ruminant fats, such as dairy products and beef, but are also found in chlorophyll-containing plants and are ingested in the diet. The body cannot metabolize chlorophyll itself, but instead intestinal flora, mainly present in ruminant animals, can cleave off the side chain of chlorophyll, producing phytol. Phytol is then converted to phytanic acid or phytanoyl-CoA depending on if the phytol is metabolized further directly in the intestine or after uptake into cells, respectively (for review, see Ref. 1). Phytanic acid is then hydroxylated to phytanoyl-CoA 2-hydroxylase (PHYH)2 in the first step. In the second step formyl-CoA is cleaved off by the 2-hydroxy-phytanoyl-CoA lyase, forming pristanal. The formyl-CoA produced is hydrolyzed into formate and oxidized to CO₂ in the cytosol. In the third and last step of α-oxidation pristanal is converted into pristanic acid by an aldehyde dehydrogenase (5).

The 2-methyl-branched pristanoyl-CoA formed by α-oxidation, or indeed pristanic acid directly ingested via the diet, is naturally present in two stereoisomers, 2S and 2R, where the peroxisomal pristanoyl-CoA oxidase (ACOX3) is specific for the S stereoisomer. Therefore, the 2R-pristanic acid must first undergo isomerization into the 2S form, which then undergoes β-oxidation. This racemization step is carried out by the 2-methylacyl-CoA racemase (AMACR) (6, 7). The first step in β-oxidation of pristanic acid is the oxidation by ACOX3, and the two subsequent steps, hydration and dehydrogenation, are catalyzed by multifunctional protein-2, with the last step, the thiolytic cleavage, carried out by sterol carrier protein x, with the release of propionyl-CoA in the first and third cycle of β-oxidation (for review, see Ref. 8). After 3 rounds of β-oxidation 4,8-dimethylnonanoyl-CoA (DMN-CoA) is produced, which can then either be hydrolyzed to the free acid or esterified to carnitine for transport to mitochondria for further β-oxidation (9, 10). These pathways are well described in the literature; however, subcellular localization, activation, and transport of substrates and products are still somewhat unclear. Both α-oxidation and the first three cycles of β-oxidation are entirely peroxisomal processes (8, 11, 12), with the possible exception of the last step of α-oxidation argued to take place either in the endoplasmic reticulum or peroxisomes (5, 13).

The importance of α-oxidation and β-oxidation is underscored by the diseases affecting either of the pathways, such as...
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Localization of ACOT6 in Peroxisomes—The full-length open reading frame encoding ACOT6 was amplified by reverse transcription-PCR from mouse kidney using the following primers 5'-CAT ATG GCG GCG ACA CTG A-3' and 5'-CAT ATG TTA CAG TTT GCT GTG-3' (Cybergene AB, Huddinge, Sweden). This phytanoyl-CoA/pristanoyl-CoA thioesterase (Acot6) gene is a member of a gene family of acyl-CoA thioesterases, localized in a condensed cluster on chromosome 12 D3 in mouse, which codes for acyl-CoA thioesterases with localizations in cytosol, mitochondria, and peroxisomes (16–18).

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Chemical Synthesis of Pristanoyl-CoA and Phytanoyl-CoA—Phytanoyl-CoA and pristanoyl-CoA were synthesized chemically from the respective free acids (Sigma-Aldrich) by first forming the anhydride and in the next step the CoA ester (20). Phytanoyl-CoA and pristanoyl-CoA were then purified by reversed phase high performance liquid chromatography using a C18 Ultrasphere ODS 5-μm (4.6 × 250 mm) column (Beckman Coulter, Inc., Fullerton, CA) with a mobile phase containing 50 mM potassium phosphate buffer, pH 5.4, and 38% isopropanol. After 10 min the mobile phase was changed to 58% isopropanol for 40 min. Purified products were then verified by mass spectrometry as described in Westin et al. (18).

Expression of Recombinant ACOT6 Protein—The full-length open reading frame for Acot6 was amplified by reverse transcription-PCR from mouse kidney using the One-Step RNA PCR kit (Takara Biomedicals, Shiga, Japan) using the primers 5'-GAA TTC GTC GAC GTC GAC-3' and 5'-GTC GAC TTA CAG TTT GCT GTG-3' (Cybergene AB, Huddinge, Sweden). The addition of an EcoRI and a SalI site (indicated in bold) were used for cloning into the pMAL-C2x vector (New England Biolabs, Beverly, MA), which results in expression of the protein as a fusion protein with maltose-binding protein. The construction was transformed into BL21(DE3) pLysS (Novagen Inc., Madison, WI), and protein expression was induced by the addition of 0.3 mM isopropyl-β-D-galactopyranoside (Sigma-Aldrich Inc.) for 2 h at 37 °C in LB-media supplemented with 2% glucose, 50 μg/ml ampicillin, and 34 μg/ml chloramphenicol. Bacteria were harvested by centrifugation at 5000 × g for 10 min, washed with 20 mM Tris-HCl, pH 7.4, and frozen over-night in 50 ml of column buffer (20 mM Tris, 200 mM NaCl, and 1 mM EDTA, pH 7.4). Bacteria were lysed by sonication for 1 min at 5-s intervals and centrifuged at 16,000 × g for 30 min. Recombinant protein was purified by affinity chromatography using amylose resin (New England Biolabs), by elution with 10 mM maltose in column buffer. Purity and size of the eluted protein was checked by SDS-PAGE and Coomassie Brilliant Blue staining, and protein concentration was determined using the Bradford assay (21).

Measurement of Acyl-CoA Thioesterase Activity—Acyl-CoA thioesterase activity was measured spectrophotometrically at 232 nm in phosphate-buffered saline (PBS) pH 7.4, measuring the cleavage of the thioester bond as a decrease in the absorb-

Phytanoyl-CoA undergoes α-oxidation to pristanoyl-CoA, and the 2R stereoisomer must undergo racemization to the C-terminal vector (Invitrogen), which expresses the protein as an N-terminal fusion protein, leaving the C-terminal SKL of ACOT6 accessible. The construct was transfected into human skin fibroblasts from a control subject and a Zellweger syndrome (15). In this paper we describe the identification of a novel gene that encodes a peroxisomal acyl-CoA thioesterase specific for phytanoyl-CoA and pristanoyl-CoA that can hydrolyze these compounds to phytanic acid, pristanic acid, and coenzyme A. This phytanoyl-CoA/pristanoyl-CoA thioesterase (Acot6) gene is a member of a gene family of acyl-CoA thioesterases, localized in a condensed cluster on chromosome 12 D3 in mouse, which codes for acyl-CoA thioesterases with localizations in cytosol, mitochondria, and peroxisomes (16–18).

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ance. Various acyl-CoAs were used as substrates, including acetyl-CoA, propionyl-CoA, acetoacetyl-CoA, butyryl-CoA, heptanoyl-CoA, decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, oleoyl-CoA, linoleoyl-CoA, linolenoyl-CoA, arachidoyl-CoA, arachidonoyl-CoA, behenoyl-CoA, branched-chain substrates including pristanoyl-CoA, phytanoyl-CoA, DMN-CoA, 2-methylcetadecanoyl-CoA, the bile acid intermediate trihydroxycholoyl-CoA, and the primary bile acid choloyl-CoA. The pristanoyl-CoA, phytanoyl-CoA, trihydroxycholoyl-CoA, and choloyl-CoA were synthesized in our laboratory, whereas 4,8-dimethylnonanoyl-CoA, behenoyl-CoA, and an aliquot of phytanoyl-CoA were a kind gift from Dr. Ronald Wanders. The other acyl-CoAs were obtained from Sigma-Aldrich. The effect of free CoASH on ACOT6 activity was tested at concentrations up to 500 μM. The kinetic parameters were calculated using Prism Enzyme Kinetics program using an extinction coefficient of E_232 = 4.25 mmol⁻¹ cm⁻¹ to calculate the specific activities.

Subcellular Fractionation and Isolation of Peroxisomes—For subcellular fractionation experiments, adult male mice on a pure C57Bl6 background (The Jackson Laboratory, Bar Harbor, ME) were used. Mice were fed either a normal chow diet or a diet containing 0.5% (w/w) clofibrate (ICI Pharmaceuticals, Jeffery Peters) were used for RNA isolation and for preparation of protein homogenates. Mice were fed either a standard chow diet or a diet containing 0.1% Wy-14,643 (Calbiochem-Novabiochem) for 1 week before sacrifice. Mice were sacrificed by CO₂ asphyxiation, and cervical dislocation, liver and kidneys were excised and frozen in liquid nitrogen, and stored at −70 °C. Synthesis of total RNA and real-time PCR (Q-PCR)—Total RNA from various mouse tissues was isolated using TRIzol Reagent (Invitrogen) and was DNase-treated using RQ1 RNase-free DNase (Promega Corp., Madison, WI). The quality of the RNA was checked on a 1% agarose-formaldehyde gel.

Tissue expression was investigated in liver, kidney, heart, lung, spleen, brain, proximal (first 10 cm of small intestine) and distal intestine (last 10 cm of small intestine), brown adipose tissue and white adipose tissue using total RNA pooled from three individual animals. For regulation by Wy-14,643 treatment in liver, three individual animals in each group were used. Synthesis of cDNA was performed with 1 μg of total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). For Acot6, a specific amplification was designed using the Primer Express software spanning the exon 2/exon 3 boundary using the primers 5’-ACG CCA TCC TCA GGT GAA AG-3’ and 5’-TCG AAG CCA TCG A-3’ and a probe with a 5’-6-carboxyfluorescein and 3’-dabcyl with the sequence 5’-TGT CTC CAA AGG TGC TGA TCT GTG CC-3’. Q-PCR was performed in single-plex and in triplicate with 18 S as an endogenous control as described previously (18).

For tissue expression of Phyh, Amacr, and Acox3, SYBR Green (Applied Biosystems) was used as well as for Acot6 in the comparative tissue expression experiment. Specific primers for Phyh were designed over the exon 4/exon 3 boundary, for Amacr over the exon2/exon 3 boundary, and for Acox3 over the exon 5/exon 6 boundary using the primers shown in Table 1. As an endogenous control, a specific amplification of mouse hypoxanthine guanine phosphoribosyltransferase (Hprt1) spanning the exon6/exon7 boundary was used, with primers also shown in Table 1. Q-PCR was run as described in Westin et al. (18) with the change of master mix to SYBR Green Power master mix (Applied Biosystems) and with the addition of a dissociation step to check the specificity of the products. The PCR products were checked by agarose gel electrophoresis. The efficacy of all primer pairs was checked by running Q-PCR on dilutions of the template cDNA, verifying that tissue expression was analyzed in the linear range of the PCR. The average threshold (Ct) val-
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RESULT

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Identification of ACOT6 as a Peroxisomal Phytanoyl-CoA/ Pristanoyl-CoA Thioesterase—Expression of ACOT6 as a fusion protein with a histidine tag or as a fusion protein with thioredoxin failed to produce soluble protein. However, expression of ACOT6 as a fusion protein with maltose-binding protein resulted in soluble protein. After purification by affinity chromatography using an amylose resin, acyl-CoA thioesterase activity was measured spectrophotometrically in PBS at 232 nm with various concentrations of pristanoyl-CoA and acyl-CoA thioesterase activity was measured spectrophotometrically in PBS at 232 nm with various concentrations of pristanoyl-CoA. $V_{max}$ and $K_m$ were calculated using the Prism Enzyme Kinetics software. The activity was measured in duplicate on one protein preparation, and data are shown as the mean activity.

FIGURE 2. ACOT6 is a peroxisomal protein. An ACOT6/pcDNA3.1 NT-GFP construct was transfected into human fibroblasts from a control subject (A) and a Zellweger patient (B), and localization was detected by immunofluorescence microscopy using a TRITC-labeled anti-GFP antibody. The punctate staining in control fibroblasts was abolished in the fibroblasts from the Zellweger patient, demonstrating that ACOT6 is a peroxisomal protein.

FIGURE 3. Recombinant ACOT6 is highly active on pristanoyl-CoA. ACOT6 was expressed in the pMAL-C2x vector as a fusion protein with maltose-binding protein. Recombinant protein was purified by affinity chromatography, and acyl-CoA thioesterase activity was measured spectrophotometrically in PBS at 232 nm with various concentrations of pristanoyl-CoA. $V_{max}$ and $K_m$ were calculated using the Prism Enzyme Kinetics software. The activity was measured in duplicate on one protein preparation, and data are shown as the mean activity.

| TABLE 1  |
|----------------|
| Sequences of SYBR Green primers for mouse phytanoyl-CoA hydroxylase (Phyh), α-methylacyl-CoA racemase (Amacr), acyl-CoA oxidase 3 (Acox3), acyl-CoA thioesterase 6 (Acot6), and hypoxanthine guanine phosphoribosyltransferase (Hprt1) for Q-PCR |
| **Phyh**  |
| Forward 5'-ACT GCC TTT CCC CGG AGA TT-3'  |
| Reverse 5'-TGG CTC CAA TGG AAC ACT CCA-3'  |
| **Amacr**  |
| Forward 5'-CTA TGT GGC TTT ATC AGG COT TC-3'  |
| Reverse 5'-TTC TCA CGG CCT CTG CCA AT-3'  |
| **Acot3**  |
| Forward 5'-TGG AGA AGA TCT ATA GCC TGG AGA TT-3'  |
| Reverse 5'-ATT TCG GTG AGA GCA AACAC G C-3'  |
| **Acot6**  |
| Forward 5'-GGT GAA AAG GAC CTT CGC AAG TG-3'  |
| Reverse 5'-ATG TCA GAG GGC ATA TCC AAC AA-3'  |
| **Hprt1**  |
| Forward 5'-GGT GAA AAG GAC CTT CGC AAG TG-3'  |
| Reverse 5'-ATG TCA GAG GGC ATA TCC AAC AA-3'  |
|  

ues per triplicate were used to calculate the relative amounts of mRNA using the 2 $^{-\Delta\Delta C_t}$ method according to Applied Biosystems guidelines.

Western Blot Analysis—Liver homogenates were prepared from control and Wy-treated male mouse, and 50 μg of protein was used for SDS-PAGE and Western blotting as described previously (24). ACOT6 antibody was produced and purified as described above, and the antibody reactivity was checked against recombinant ACOT6 protein.

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min, and results showed that ACOT6 hydrolyzed almost 100% of the added substrate, suggesting that ACOT6 has no stereospecificity for the 2R and 2S isomers (data not shown). Increasing concentrations of free CoASH up to 500 μM showed that ACOT6 activity is not regulated by free CoASH (data not shown), similar to the other members of this gene family.

Even using the maltose-binding protein expression system it was very difficult to obtain active ACOT6 protein, probably due to improper folding. To verify that ACOT6 is indeed a peroxisomal pristanoyl-CoA thioesterase, we also performed complementary experiments utilizing purified peroxisomes from mouse liver and kidney from control and clofibrate-treated animals. Peroxisomes were isolated using standard procedures with the final step being gradient centrifugation in Optiprep. When performing the biochemical characterization of recombinant ACOT6 (described in Fig. 3), we did not have access to phytanoyl-CoA, which is not commercially available. Based on the structural similarity to pristanoyl-CoA, we therefore subsequently synthesized phytanoyl-CoA and tested the activity in the purified organelle fractions. Indeed, the activity in purified peroxisomes is very similar with phytanoyl-CoA and pristanoyl-CoA, with K_m values of 32 and 35 μM and V_max values of 176 and 215 nmol/min/mg, respectively, suggesting that ACOT6 could be similarly active on both these substrates (Fig. 4, A and B). To establish whether the peroxisomal phytanoyl-CoA and pristanoyl-CoA thioesterase activities in peroxisomes are catalyzed by ACOT6, we immunoprecipitated the peroxisomal fraction with an ACOT6 peptide antibody using preimmune serum as a control. The anti-ACOT6 IgG immunoprecipitated almost all of the peroxisomal phytanoyl-CoA and pristanoyl-CoA thioesterase activity, demonstrating that ACOT6 is the major phytanoyl-CoA/pristanoyl-CoA thioesterase in peroxisomes (Fig. 4C). As expected, no lauroyl-CoA thioesterase activity was immunoprecipitated with the ACOT6 antibody. Taken together, these data strongly suggest that ACOT6 is a novel peroxisomal thioesterase with a function in regulation of phytanic acid and pristanic acid metabolism.

Phytanoyl-CoA and Pristanoyl-CoA Thioesterase Activity Is Highest in Peroxisomes—The important role of peroxisomes in the metabolism of phytanic acid and pristanic acid has been well established (for review, see Ref. 27). If the physiological function of ACOT6 is to regulate phytanic acid and pristanic acid levels, we would expect the activity to be highest in peroxisomes. Measurement of phytanoyl-CoA/pristanoyl-CoA thioesterase activity in different subcellular fractions showed that this is indeed the case. The activity in the peroxisomal fractions was 60–70 nmol/min/mg of protein, whereas the activity was only 3–11 nmol/min/mg of protein in the cytosolic, mitochondrial, and microsomal fractions (Fig. 5). In addition, ACOT6 activity appears to be high in liver since the thioesterase activity with these substrates is about double the thioesterase activity with lauroyl-CoA (C12-CoA) (Fig. 4C), which in peroxisomes is due to the combined actions of ACOT3, ACOT5, and ACOT8 (17, 19).

Expression of Acot6 mRNA, Protein, and Activity Is Increased via the PPARα—Peroxisomes from liver and kidney were isolated from control and clofibrate-treated mice, and comparison of the activity between peroxisomes from liver and kidney showed that the activity was higher in liver than in kidney.
showed that kidney peroxisomes contain 2–3 times higher activity with lauroyl-CoA, phytanoyl-CoA, and pristanoyl-CoA than liver peroxisomes (Fig. 6A). Clofibrate treatment did not change the specific activity to any noticeable extent, suggesting that the expression of ACOT6 is induced by the clofibrate treatment in parallel with peroxisome proliferation. The expression of many genes involved in peroxisomal β-oxidation as well as all the other members of this thioesterase family are regulated via PPARα. We, therefore, investigated the regulation of Acot6 at mRNA and protein level by treatment of wild type and PPARα-null mice with the peroxisome proliferator Wy-14,643. Q-PCR on liver RNA from male mice treated with Wy-14,643 showed that Acot6 mRNA is highly up-regulated (about 11-fold) in liver in a PPARα-dependent manner (Fig. 6B). We also investigated the regulation of ACOT6 in liver at protein level using Western blotting, which also showed a strong up-regulation of ACOT6 in a PPARα-dependent manner (Fig. 6C).

Peroxisome proliferation is evident in mouse liver after treatment with peroxisome proliferators, which has been reported to increase the cytoplasmic area of peroxisomes about 5–8-fold (28, 29). The magnitude of the increased expression of ACOT6 is, therefore, compatible with the activity data showing that the treatment increases the expression in parallel with peroxisome proliferation and thereby maintains a similar specific activity in peroxisomes after proliferation by fibrate treatment.

Tissue Expression Profiling Shows That Acot6 Is Co-expressed with Acox3—Q-PCR analysis of mRNA levels was used to establish the tissue expression of Acot6. The data showed that Acot6 mRNA is most highly expressed in white adipose tissue followed by kidney, liver, brown adipose tissue, and brain (Fig. 7A). The identification of a specific phytanoyl-CoA/pristanoyl-CoA thioesterase with high expression in white adipose tissue is intriguing, and we therefore set out to investigate tissue expression of some other genes involved in the α-oxidation pathway and branched-chain β-oxidation pathway. Pesticoid-CoA oxidase (Acox3), the first and presumed rate-limiting step in the branched-chain β-oxidation pathway (see Fig. 1), is also most highly expressed in white adipose tissue, and the expression pattern closely resembles the expression pattern of Acot6 (Fig. 7B). The first step in the α-oxidation pathway is catalyzed by PHYH, and Q-PCR showed that Phyh mRNA is widely expressed, with highest expression in brown adipose tissue and liver and only low expression in lung, spleen, and brain (Fig.
The AMACR, the enzyme that converts the 2R form of pristanoyl-CoA into the 2S form, is most highly expressed at mRNA level in liver and kidney but also showed a rather similar expression profile to Phyh (Fig. 7D). The expression analysis, thus, revealed that Acot6 and Acox3 show similar tissue expression profiles with both genes being most highly expressed in white adipose tissue and that Phyh and Amacr also show similar expression profiles to each other but different expression compared with Acot6 and Acox3.

In an inter-tissue mRNA analysis, a few selected tissues were analyzed by Q-PCR in parallel using SYBR Green probes for Acot6, Acox3, Amacr, and Phyh. Because primer efficacy was confirmed to be 100% for all primer pairs as described under “Materials and Methods,” it should be possible to compare expression of different genes analyzed in parallel. Such a Q-PCR analysis suggests that Phyh mRNA expression is about 100 times higher in liver than for the other enzymes (data not shown). Amacr expression is also much higher than expression of Acot6 and Acox3 in liver and kidney. However, from this analysis it appears that Acot6 and Acox3 may be expressed at very similar levels in the various tissues and that Amacr is expressed at a lower level than Acot6 and Acox3 in white adipose tissue (Fig. 8).

**DISCUSSION**

The Acot6 gene is located in a dense cluster of six genes coding for acyl-CoA thioesterases, of which five genes have been characterized previously (17, 18, 25, 26). One gene codes for a cytosolic thioesterase (Acot1), one gene codes for a mitochondrial thioesterase (Acot2), and three genes code for peroxisomal thioesterases (Acot3-5). Of these thioesterases, only ACOT4 shows a narrow substrate specificity, hydrolyzing succinyl-CoA, and to a low extent glutaryl-CoA (18). Here we have characterized in detail the sixth gene in this cluster, Acot6, and show that this gene also codes for a peroxisomal thioesterase, which specifically hydrolyzes the CoA esters of the methylbranched fatty acids phytanic acid and pristanic acid. The significance of this activity is evident from the subcellular fraction-
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**FIGURE 9. Putative functions for ACOT6 in the metabolism of branched fatty acids.** Pristanic acid may enter white adipose tissue (WAT) from the circulation or be released from triglycerides (TGs) as a result of lipolysis within WAT and is present as both the 2R and 2S stereoisomer. Subsequent metabolism of pristanic acid involves the activation to the CoA ester (pristanoyl-CoA) and three cycles of β-oxidation to form DMN-CoA. However, because of the stereospecificity of ACOX3 for the 2S stereoisomer, the racemization of the 2R to the 2S stereoisomer by AMACR is required for complete β-oxidation of pristanic acid. In WAT peroxisomes, the AMACR is expressed at very low levels, which would result in the accumulation of the 2R-pristanoyl-CoA. Therefore, ACOT6 is required to hydrolyze the 2R-pristanoyl-CoA to 2R-pristanic acid, which may then either be esterified/reesterified into triglycerides and stored in WAT or, alternatively, be transported to the liver (or kidney). The 2R-pristanic acid can then enter peroxisomes and be racemized to the 2S isomer by AMACR, resulting in β-oxidation to DMN-CoA. The figure also depicts a role for acyl-CoA thioesterase 5 or 8 (ACOT5 or ACOT8) in hydrolysis of DMN-CoA to release free DMN for excretion or the action of carnitine octanoyltransferase (CROT) in the formation of DMN-carnitine, which would be transported to the mitochondria for further oxidation. An alternative role for pristanic acid (or phytic acid) is also in gene regulation, acting as ligands for the PPARα and retinoid X receptor α (RXRα) in the nucleus of liver or brown adipose tissue.
ever, only the 2S stereoisomer can be degraded via β-oxidation (38) and, therefore, racemization becomes an important step in the β-oxidation of pristanoyl-CoA, which proceeds for three cycles in peroxisomes with the formation of DMN-CoA, as outlined in Fig. 9. DMN-CoA is transferred to the corresponding carnitine ester by peroxisomal carnitine octanoyltransferase and transported to the mitochondria for further degradation to 2,6-dimethylheptanoic-CoA (39). Alternatively the DMN-CoA could be hydrolyzed to free DMN by ACOT8 or ACOT5 for transport out of the peroxisome (10, 19). Interestingly, as shown in Fig. 8 (in which we have attempted to compare the expression levels of the Amacr, Acox3, and Acot6), the expression of the racemase, Amacr, seems to be quite low in peroxisomes in white adipose tissue, especially when considering that this enzyme has a dual localization in peroxisomes and mitochondria (40). These data may be interpreted in such a way that the apparently low level of Amacr becomes rate-limiting in white adipose tissue in the β-oxidation of branched fatty acids, and therefore, excess 2R-pristanoyl-CoA produced in white adipose tissue peroxisomes may be hydrolyzed by ACOT6 (Fig. 9). Hence, ACOT6 may have a function in hydrolyzing 2R-pristanoyl-CoA into 2R-pristanic acid, which can exit the peroxisome and after activation to the corresponding CoA-ester be esterified into triacylglycerols in white adipose tissue or, alternatively, be transported to, for example, the liver or kidney for further metabolism. This hypothesis is also supported by our observation that ACOT6 does not show selectivity for the 2R and 2S isomers, which would allow for the hydrolysis of accumulating 2R-pristanoyl-CoA. It would, therefore, be of interest to analyze triacylglycerols in white adipose tissue for the relative content of 2R- and 2S-pristanic acid. Taken together, our data suggest that ACOT6 can function as an auxiliary enzyme in the oxidation of pristanic acid, preventing temporary storage of the 2R isomer in white adipose tissue before being further metabolized in other tissues.

The identification of Acot6 as a novel PPARα target gene is in line with the previously described PPARα regulation of all the other genes of the thioesterase gene cluster and suggests a role for PPARα also in the metabolism of branched-chain lipids. Phytol feeding in mice has a lipid-lowering effect in plasma and results in up-regulation of genes in straight-chain β-oxidation together with the multifunctional protein-2 (β-bifunctional protein) and sterol carrier protein x involved in branched chain β-oxidation and the PHYH involved in α-oxidation, although the latter enzyme is induced in a PPARα-independent manner (41). It would be likely that Acot6 would also be up-regulated by phytol feeding via PPARα and would, therefore, contribute to the α- and β-oxidation of branched fatty acids. Both products of the activity of ACOT6, phytanic acid and pristanic acid, have previously been shown to function in gene regulation, probably acting as ligands for nuclear receptors. In cell experiments pristanic acid and phytanic acid have been shown to be potent activators of PPARα and retinoid X receptor α, respectively (42, 43). Therefore, ACOT6 may also function in recruiting or maintaining ligands for these nuclear receptors and thereby also be involved in gene regulation.

In summary we have identified a novel peroxisomal PPARα-regulated phytanoyl-CoA/pristanoyl-CoA thioesterase in mouse.

This enzyme is most highly expressed in white adipose tissue, similar to Acox3, the presumed rate-limiting enzyme in β-oxidation of pristanoyl-CoA. Our data suggest a function for ACOT6 in hydrolyzing in particular 2R-pristanoyl-CoA in white adipose tissue peroxisomes to remove it from the β-oxidation pathway, and the produced 2R-pristanic acid may be esterified into triacylglycerols (which may explain why pristanic acid accumulates in white adipose tissue) or be β-oxidized in liver after racemization.

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