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PEROXISOMES CONTAIN A SPECIFIC PHYTANOYL-CoA/PRISTANOYL-CoA THIOESTERASE ACTING AS A NOVEL AUXILIARY ENZYME IN ALPHA- AND BETA-OXIDATION OF METHYL-BRANCHED FATTY ACIDS IN MOUSE

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Running title: A novel phytanoyl-CoA/pristanoyl-CoA thioesterase in peroxisomes

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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 *Acox3* (the pristanoyl-CoA oxidase). Furthermore, Acot6 was identified as a target gene of the PPARα and is upregulated in mouse liver in a PPARα dependent manner.

Methyl branched fatty acids such as phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and pristanic acid (2,6,10,14-tetramethylpentadecanoic 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 (1)). Phytanic acid containing a methyl group in the third position is first converted to a 2-methyl branched fatty acid, pristanic acid, via α-oxidation (2), which can then undergo β-oxidation as outlined in Figure 1. Before β-oxidation can take place, the phytanic acid is activated to the CoA ester, which can occur either outside the peroxisome by the long-chain acyl-CoA synthetase or inside the peroxisome by the very long-chain acyl-CoA synthetase (3,4).

α-Oxidation consists of three steps where the phytanoyl-CoA is hydroxylated into 2-hydroxyphytanoyl-CoA by phytanoyl-CoA 2-hydroxylase (PHYH) in the first step. In the second step, formyl-CoA is cleaved off by the 2-hydroxyphytanoyl-CoA lyase, forming pristanal. The formyl-CoA produced is hydrolyzed into formate and oxidized to CO_2 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, 2R and 2S, 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 (MFP-2), with the last step, the thiolytic cleavage, carried out by sterol carrier protein x (SCPx), with the release of propionyl-CoA in the first and third cycle of β-oxidation (for review see (8)). After three 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 β-oxidation argued to take place either in the ER or peroxisomes (5,13).
The importance of α-oxidation and β-oxidation are underscored by the diseases affecting either of the pathways, such as Refsum's disease, AMACR deficiency and MFP-2 deficiency (for reviews see (8,14)). Also the importance of the breakdown of phytol to phytanic acid is demonstrated by the occurrence of a disease affecting the microsomal fatty aldehyde dehydrogenase (FALDH) catalyzing the oxidation of phytenal to phytenic acid, causing the Sjögren-Larsson 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, which 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 code for acyl-CoA thioesterases with localizations in cytosol, mitochondria and peroxisomes (16-18).

MATERIALS AND METHODS

Localization of ACOT6 in peroxisomes- The full-length open reading frame encoding ACOT6 was amplified by RT-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). The product was cloned into the pcDNA3.1/NT-GFP vector (Invitrogen Corp., Carlsbad, CA), which expresses the protein as an N-terminal green fluorescent 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 patient using the calcium phosphate method. Immunofluorescence microscopy was carried out as described previously (19).

Chemical synthesis of pristanoyl-CoA and phytanoyl-CoA- Phytanoyl-CoA and pristanoyl-CoA were synthesized chemically from the respective free acids (Sigma-Aldrich Inc., St. Louis, MO) 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 5μm (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 (18).

Expression of recombinant ACOT6 protein- The full-length open reading frame for *Acot6* was amplified by RT-PCR from mouse kidney using the One-Step RNA PCR kit (Takara Biomedicals, Shiga, Japan), using the following primers: 5'- **GAA TTC** ATG GCG GCG ACA CTG AGC G -3' and 5'- **GTC GAC** TTA CAG TTT GCT GTG CCT G -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 construct was transformed into BL21(DES3) pLysS (Novagen Inc., Madison, WI) and protein expression was induced by the addition of 0.3 mM isopropyl-β-D-galactopyranoside (Sigma-Aldrich Inc., St. Louis, MO) 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 overnight in 50 ml of column buffer (20 mM Tris, 200 mM NaCl and 1 mM EDTA, pH 7.4). Bacteria were lyzed by sonication for 1 min at 5s intervals and centrifuged at 16 000× g for 30 min. Recombinant protein was purified by affinity chromatography using amylose resin (New England Biolabs, Beverly, MA), 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 absorbance. 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-methyloctadecanoyl-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 lab, while 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 Inc., St. Louis, MO. 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 $mM^{-1}cm^{-1}$ to calculate the specific activities.

Subcellular fractionation and isolation of peroxisomes- For subcellular fractionation experiments, adult male mice on a pure C57Bl6 background (Jackson Laboratories, Maine, USA) were used. Mice were fed either a normal chow diet or a diet containing 0.5% (w/w) clofibrate (ICI Pharmaceuticals, Macclesfield, Cheshire, U.K.) for 1 week and all mice were fasted overnight before sacrifice. Following sacrifice by CO_2 asphyxiation and cervical dislocation, liver and kidneys were excised and used directly for subcellular fractionation. Tissues were weighed and placed in ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, 15 mM MES, pH 6.5, minced finely, homogenized and diluted to a 10% homogenate. Homogenates were centrifuged for 10 min at 500 g_{av} and pellets were re-homogenized and re-centrifuged. Post-nuclear supernatants were combined and centrifuged at 4000 g_{av} for 10 min to sediment mitochondria, which were saved for subsequent measurements. The supernatants were centrifuged at 30 000 g_{av} for 20 min to obtain the light mitochondrial fraction containing the bulk of peroxisomes. The pellets were washed once by centrifugation and finally dissolved in ice-cold 0.25 M sucrose and 15 mM Hepes pH 7.4. These fractions were then layered on top of linear 20-45% Optiprep (Sigma-Aldrich Inc., St. Louis, MO) gradients, resting on a 50% Optiprep cushion, and centrifuged for 90 min at 40 000 g_{av} in a fixed angle rotor and 1 ml fractions were collected from the bottom of the gradient. The fractions were analyzed for the peroxisomal marker catalase as described in (22). Supernatants obtained after preparation of the light-mitochondrial fractions were centrifuged at 40 000 g_{av} for 2h and supernatants were saved as cytosolic fractions and pellets were dissolved in 0.25 M sucrose and 15 mM Hepes, pH 7.4, and saved as microsomal fractions for subsequent measurements.

Immunoprecipitation of ACOT6 in purified peroxisomes- ACOT6 anti-sera were produced in rabbits immunized with a peptide with the following sequence: CQKYLNGEKPARH, which corresponds to amino acids 406-417 of the ACOT6 protein and a C-terminal Cys for coupling of the peptide to Keyhole Limpet Haemocyanin (KLH). The antibody was affinity purified by chromatography as described previously (17). Pre-immune serum or affinity purified ACOT6 antibody in various concentrations (in PBS/0.1% Triton) was added to Protein A sepharose beads and incubated at 4°C overnight. The protein A sepharose suspension was centrifuged at 10 000 g_{av} in a microfuge for 10 min, the supernatants were removed and 100 μg of peroxisomal protein in PBS/0.1% Triton was added and incubated for 2 h at 4°C in an orbital shaker. The protein A sepharose suspension was spun down and the supernatants were used for acyl-CoA thioesterase activity measurements as described above.

Animals and treatments for investigation of tissue expression and regulation of expression- Adult male wild-type (+/+) mice or PPARα-null (-/-) mice on a pure Sv/129 genetic background (23) (kindly provided by Dr. Frank Gonzalez and Dr. Jeffrey 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 International, La Jolla, CA) for 1

week before sacrifice. Mice were sacrificed by CO_2 asphyxiation and cervical dislocation and tissues were excised, frozen in liquid nitrogen and stored at –70°C.

Isolation of total RNA and Real-Time-PCR (Q-PCR)- Total RNA from various mouse tissues was isolated using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA) 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 amplicon was designed using the Primer Express Software spanning over the exon 2/exon 3 boundary using the following primers: 5'-ACG CCA TCC TCA GGT GAA AG-3' and 5'-TCA GGA AAG CAG CCA TCG A-3', and a probe with a 5'-FAM and 3'-dabcyl with the following sequence 5'-TGT CTC CAA AGG TGC TGA TCT GTG CC-3'. Q-PCR was performed in single-plex and in triplicate with 18S as an endogenous control as described previously (18). For tissue expression of *Phyh*, *Amacr* and *Acox3*, SYBRgreen (Applied Biosystems, Foster city, CA) was used, as well as for *Acot6* in the comparative tissue expression experiment. Specific primers for *Phyh* were designed over the exon 4/exon 5 boundary, for *Amacr* over the exon2/exon3 boundary and for *Acox3* over the exon 5/exon 6 boundary, using the primers shown in Table I. As an endogenous control, a specific amplicon of mouse hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) spanning over the exon6/exon7 boundary was used, with primers also shown in Table I. Q-PCR was run as described in (18) with the change of master mix to SYBRgreen Power master mix (Applied Biosystems, Foster City, CA) and with 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) values per triplicate were used to calculate the relative amounts of mRNA using the $2^{-\Delta\Delta CT}$ 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.

RESULTS

Identification of ACOT6 as a novel peroxisomal acyl-CoA thioesterase- We have previously identified a cluster of genes on mouse chromosome 12 D3, which codes for acyl-CoA thioesterases. To date we have characterized 5 of these genes, named *Acot1-5*. *Acot3*, *Acot1* and *Acot2* encode long-chain acyl-CoA thioesterases localized in peroxisomes, cytosol and mitochondria, respectively (17,25,26). *Acot5* and *Acot4* encode a medium-chain acyl-CoA thioesterase and a succinyl-CoA thioesterase, respectively (17,18). This cluster contains a further gene, named *Acot6*, which shows an identical gene organization to the other genes in this cluster. Based on the genomic sequence, primers were designed to amplify the open reading frame of *Acot6* by PCR and the product was completely sequenced and deposited under Accession Number AY999300. The resulting protein contains 419 amino acids, with a calculated molecular mass of 46.7 kDa and shows approximately 70% sequence identity to the other ACOTs in this gene family. Notably the C-terminal ends serine, lysine, leucine (-SKL), strongly suggesting a peroxisomal localization. To examine whether this –SKL results in targeting of the protein to peroxisomes, we expressed the protein as an N-terminal fusion protein with green fluorescent protein (GFP), leaving the C-terminal -SKL accessible. The Acot6-GFP plasmid was transfected into human skin fibroblasts and showed a punctate expression indicative of a peroxisomal localization (Fig. 2A). A peroxisomal localization was then confirmed by transfection of the same plasmid into fibroblasts from a Zellweger patient. These fibroblasts lack

functional peroxisomes since they have a generalized import defect, and in these cells ACOT6-GFP expression showed a diffuse cytosolic localization (Fig. 2B), confirming that the punctate expression visible in the control fibroblasts is indeed peroxisomal.

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 on a variety of acyl-CoAs (see MATERIALS AND METHODS), which showed ACOT6 to be active only on pristanoyl-CoA (2,6,10,14-tetramethylpentadecanoyl-CoA) with a V_{max} of 3.20 μmol/min/mg and a K_m of 24 μM (Fig. 3). The enzyme was not active on any other methyl branched substrates, i.e. 2-methyloctadecanoyl-CoA and DMN-CoA, although at this stage phytanoyl-CoA was not available, demonstrating that ACOT6 is highly specific for pristanoyl-CoA. The 2-methyl group in pristanic acid is present in both the 2R and 2S configuration, and it has been shown that the peroxisomal pristanoyl-CoA oxidase (ACOX3) is only active on the 2S-stereoisomer (8). Therefore the α-methylacyl-CoA racemase (AMACR) is required as an auxiliary enzyme for complete oxidation of pristanic acid (6). Since the commercial pristanic acid was chemically synthesized, it is presumed to contain a racemic mixture of 2R and 2S stereoisomers. We therefore tested if ACOT6 was active on both stereoisomers by incubation of ACOT6 with the presumed racemic mixture of synthesized pristanoyl-CoA for 30 min, and results showed that ACOT6 hydrolyzed almost 100% of the added substrate, suggesting that ACOT6 has no stereo-specificity 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. In order 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. 4A-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 pre-immune 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 (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 protein, while the activity was only 3-11 nmol/min/mg 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 as high as 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 peroxisome proliferator-activated receptor alpha (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 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 the 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 upregulated (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 upregulation 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. Pristanoyl-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 phytanoyl-CoA 2-hydroxylase (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. 7C). The α-methylacyl-CoA racemase (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, although different expression compared to *Acot6* and *Acox3*.

In an inter-tissue mRNA analysis, a few selected tissues were analyzed by Q-PCR in parallel using SYBRgreen probes for *Acot6, Acox3, Amacr* and *Phyh*. Since primer efficacy was confirmed to be 100% for all primer pairs as described in MATERIALS AND METHODS, it should be possible to compare expression of different genes analyzed in parallel. Such Q-PCR analysis suggest 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 methyl-branched fatty acids phytanic acid and pristanic acid. The significance of this activity is evident from the subcellular fractionation experiments, which showed that phytanoyl-CoA/pristanoyl-CoA thioesterase activity is clearly highest in peroxisomes. The data further show that the activity with these substrates in peroxisomes isolated from liver and kidney is 2-3 fold higher than the activity with lauroyl-CoA, a straight chain acyl-CoA, suggesting that it represents a major thioesterase activity in peroxisomes. Immunoprecipitation using an ACOT6 antibody immunoprecipitated almost all phytanoyl-CoA/pristanoyl-CoA thioesterase activity, and taken together these data strongly connect ACOT6 to metabolism of phytanic and pristanic acid in peroxisomes. Furthermore, recombinant ACOT6 showed no activity with saturated or unsaturated straight-chain acyl-CoAs of varying chain length, or with other branched-chain substrates such as DMN-CoA and 2-methyloctadecanoyl-CoA, demonstrating that ACOT6 is apparently specific for phytanoyl-CoA/pristanoyl-CoA. These data raise the question of the physiological function of ACOT6. Elucidation of the metabolism of phytanic acid during the last few years has demonstrated the importance of peroxisomes, which is obvious from the severe elevation in phytanic acid and pristanic acid seen in various peroxisomal disorders (30). Branched fatty acids are widely distributed in foods at low amounts, where humans typically ingest about 50-100 mg/day (8), of which about 50% is absorbed and eventually metabolized or stored in triglycerides. Human plasma contains detectable amounts of phytanic and pristanic acid (31), and it was also demonstrated that human and bovine tissues contain comparable amounts of pristanic acid and phytanic acid with the highest levels found in white adipose tissue (32). Studies in rats fed pristanic acid suggested that about 10% of the absorbed pristanic acid is still stored after one week, preferentially in white adipose tissue and liver (33,34). Therefore, evidently a considerable amount of the ingested branched fatty acids are stored in white adipose tissue and liver. Earlier investigations have suggested α-oxidation and β-oxidation to occur mainly in liver and kidney (35-37), which is in line with our data showing that the expression of some selected genes involved in these pathways are strongly expressed in these tissues. However, our data also show a remarkable co-expression of *Acox3* and *Acot6* with the highest expression in white adipose tissue, suggesting a different metabolism in this tissue. It should be kept in mind that the β-methyl group in pristanic acid is present in both the 2R- and 2S- isomers. However, 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 (CROT) and transported to the mitochondria for further degradation to 2,6-dimethylheptanoyl-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 following 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, promoting temporary storage of the 2R isomer in white adipose tissue before being further metabolized in other tissues.

The identification of *cot6* as a novel PPAR target gene is in line with the previously described PPAR regulation of all the other genes of the thioesterase gene cluster, and suggests a role for PPAR also in the metabolism of branched-chain lipids. Phytol feeding in mice has a lipid-lowering effect in plasma and results in upregulation of genes in straight-chain -oxidation, together with the MFP2 (D-bifunctional protein) and SCPx involved in branched chain -oxidation, and the PHYH involved in -oxidation, although the latter enzyme is induced in a PPAR-independent manner (41). It would be likely that *Acot6* would also be upregulated by phytol feeding, via PPAR and would therefore contribute to the - and -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 PPAR and RXR, 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 PPAR 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 -oxidation of pristanoyl-CoA. Our data suggests a function for ACOT6 in hydrolyzing in particular 2R-pristanoyl-CoA in white adipose tissue peroxisomes to remove it from the -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 -oxidized in liver following racemization.

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Key words: Pristanic acid, phytanic acid, peroxisome proliferator-activated receptor, white adipose tissue, lipid metabolism.

FIGURE LEGENDS

Figure 1. **Peroxisomal β-oxidation, racemization and α-oxidation of branched fatty acids**. Phytanoyl-CoA undergoes α-oxidation to pristanoyl-CoA and the 2R stereoisomer must undergo racemization to 2S-pristanoyl-CoA, which can be β-oxidized. Boxed enzymes are studied in this paper.*The aldehyde dehydrogenase is argued to be localized either in peroxisomes or endoplasmic reticulum.

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 immunofluoresence microscopy using a Tritc-labeled anti-GFP antibody. The punctate staining in control fibroblasts is 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 phosphate buffered saline (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 is shown as the mean activity.

Figure 4. **ACOT6 is a specific phytanoyl-CoA/pristanoyl-CoA thioesterase.** Acyl-CoA thioesterase activity for pristanoyl-CoA (**A**) and phytanoyl-CoA (**B**) was measured in highly purified peroxisomes from liver of control mice. Acyl-CoA thioesterase activity was measured spectrophotometrically in PBS at 232 nm with various concentrations of phytanoyl-CoA and pristanoyl-CoA. Enzyme kinetics were calculated using the Prism Enzyme Kinetics software. Activity measurements were carried out in duplicate and data is shown as the mean activity.
(**C**) Immunoprecipitation of purified peroxisomes was carried out with an ACOT6 antibody, using pre-immune serum as a control. Following immunoprecipitation, the remaining acyl-CoA thioesterase activity was measured in the supernatants with 25 μM phytanoyl-CoA, pristanoyl-CoA or lauroyl-CoA in PBS at 232 nm and the specific activities were calculated. The experiment was performed twice and data are shown ± range.

Figure 5. **Peroxisomes contain the highest phytanoyl-CoA/pristanoyl-CoA thioesterase activity**. Subcellular fractionation of male mouse liver was performed and acyl-CoA thioesterase activity was measured spectrophotometrically in PBS at 232 nm with 25μM phytanoyl-CoA and pristanoyl-CoA and the specific activity was calculated. Cyt; cytosol, Mic; microsomes, Mito; mitochondria, Px; peroxisomes. The subcellular fractionation was carried out on pooled livers and the activity measurements were performed in duplicate and one representative experiment is shown.

Figure 6. **Expression of *Acot6* mRNA, protein and activity is increased via the peroxisome proliferator-activated receptor alpha (PPARα).** (**A**) Peroxisomes were prepared by subcellular fractionation of pooled liver and kidney of control mice and mice fed 0.5% clofibrate in the diet for one week. Acyl-CoA thioesterase activity was measured spectrophotometrically in duplicate with 25 μM of lauroyl-CoA, pristanoyl-CoA and phytanoyl-CoA and the specific activity was calculated for each substrate. (**B**) Wild type (+/+) and PPARα-null (-/-) mice were fed either a standard chow diet or a diet containing 0.1% Wy-14,643 for one week. Regulation of *Acot6* expression was investigated at mRNA level in liver by Q-PCR using *Hprt1* as an endogenous control, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The data are means ± S.E.M., *n=3* (**C**) Western blotting was carried out on 50 μg liver homogenates from wild type (+/+) and PPARα- null (-/-) mice using an ACOT6 antibody.

Figure 7. ***Acot6* and *Acox3* are co-expressed in white adipose tissue.** Tissue expression of *Acot6* (**A**), *Acox3* (**B**), *Phyh* (**C**), and *Amacr* (**D**) was investigated at mRNA level using Q-PCR in Sv129 male mice and an amplicon for *Hprt1* was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression levels and presented as percentage of the tissue with highest expression for each gene. Real time PCR was carried out on two cDNA preparations and data are shown ± range. Prox. I.; proximal intestine, Dist. I.; distal intestine, BAT; brown adipose tissue, and WAT; white adipose tissue.

Figure 8. **Relative expression of *Acot6*, *Amacr* and *Acox3* in various tissues**. The expression levels of *Acot6*, *Amacr* and *Acox3* were compared by Q-PCR of the three amplicons in liver, kidney, proximal intestine and white adipose tissue on the same plate using SYBRgreen for detection. An amplicon for *Hprt1* was used as an endogenous control and the $2^{-\Delta\Delta Ct}$ method was used to calculate the expression levels that are presented as percentage of the *Amacr* expression in kidney (the gene with highest expression). Prox. I.; proximal intestine and WAT; white adipose tissue. The data are the means of triplicate determinations of each sample.

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 (TG's) 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 dimethylnonanoyl-CoA (DMN-CoA). However, due to the stereospecificity of ACOX3 for the 2S-stereoisomer, the racemization of the 2R to the 2S stereoisomer by α-methyacyl-CoA racemace (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 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 phytanic acid) is also in gene regulation, acting as ligands for the PPARα and RXRα in the nucleus of liver or brown adipose tissue.

TABLE I. Sequences of SYBRgreen primers for mouse phytanoyl-CoA hydroxylase (*Phyh)*, □-methylacyl-CoA racemase (*Amacr*), acyl-CoA oxidase 3 (*Acox3*), acyl-CoA thioesterase 6 (*Acot6*) and hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) for Q-PCR.

Phyh	Fwd 5'- ACT GCC TTC TCC CCG AGA TT -3'
	Rev 5'- TGG GTC CAG TGA AAC ACT CCA -3'
Amacr	Fwd 5'-CTA TTT GGC TTT ATC AGG CGT TC -3'
	Rev 5'-TTC TCA CCG CTT CTG CCA AT-3'
Acox3	Fwd 5'- TTG AGA AGA TCT ATA GCC TGG AGA TTT -3'
	Rev 5'- AGT TCG GTG AGA GCA AAACAG C -3'
Acot6	Fwd 5'- GGT GAA AAG GAC CTC TCG AAG TG -3'
	Rev 5'- ATA GTC AAG GGC ATA TCC AAC AAC A -3'
Hprt1	Fwd 5'- GGT GAA AAG GAC CTC TCG AAG TG -3'
	Rev 5'- ATA GTC AAG GGC ATA TCC AAC AAC A -3'

Fig. 1
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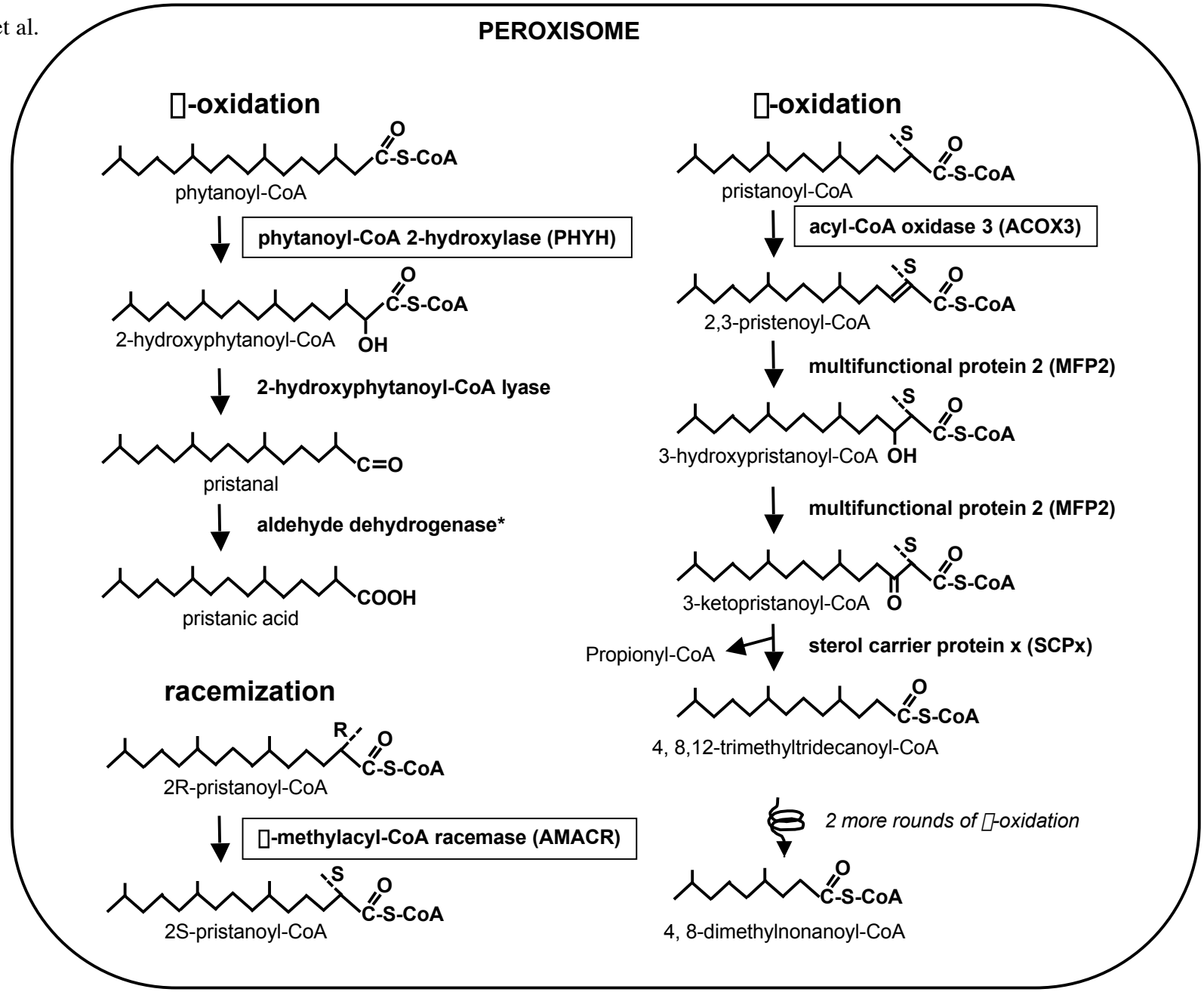

Fig. 2
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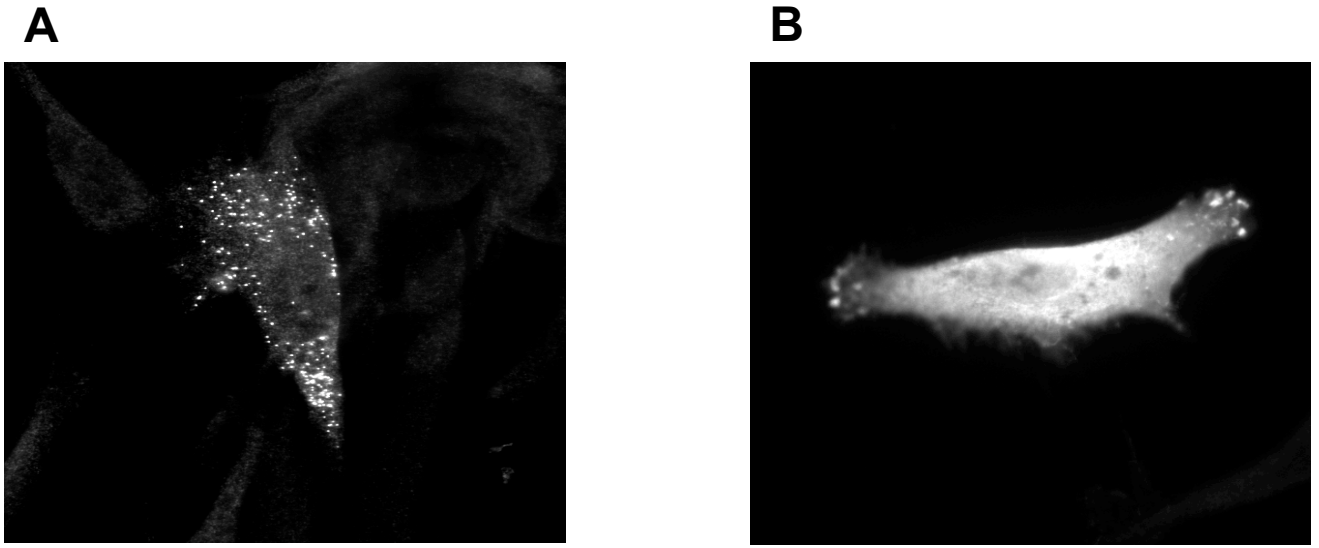
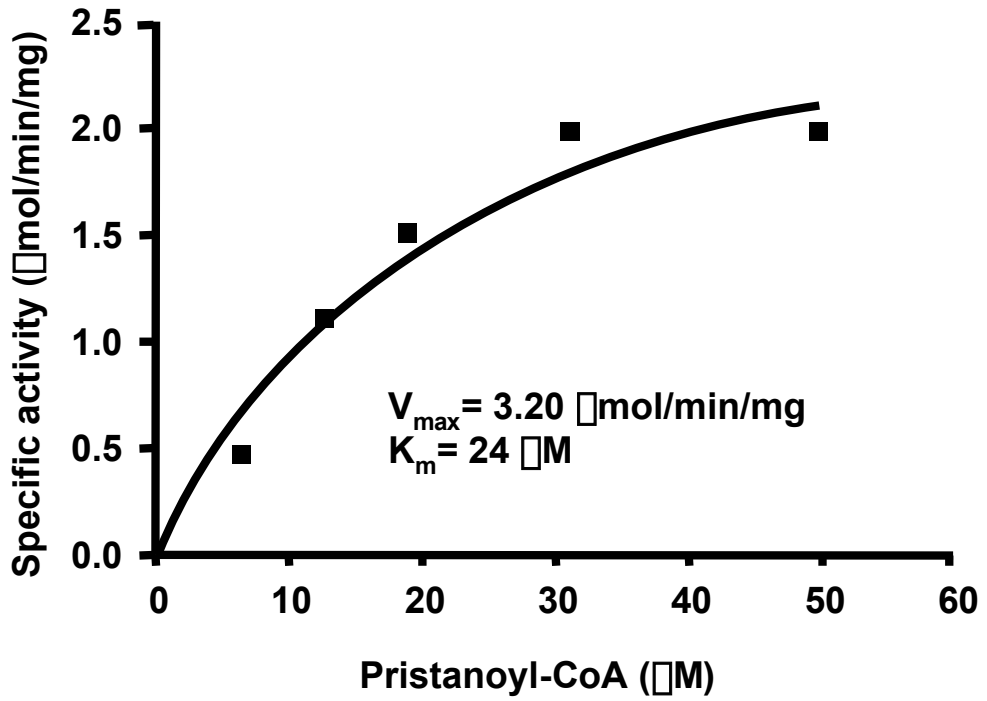


Fig. 3
Westin et al.



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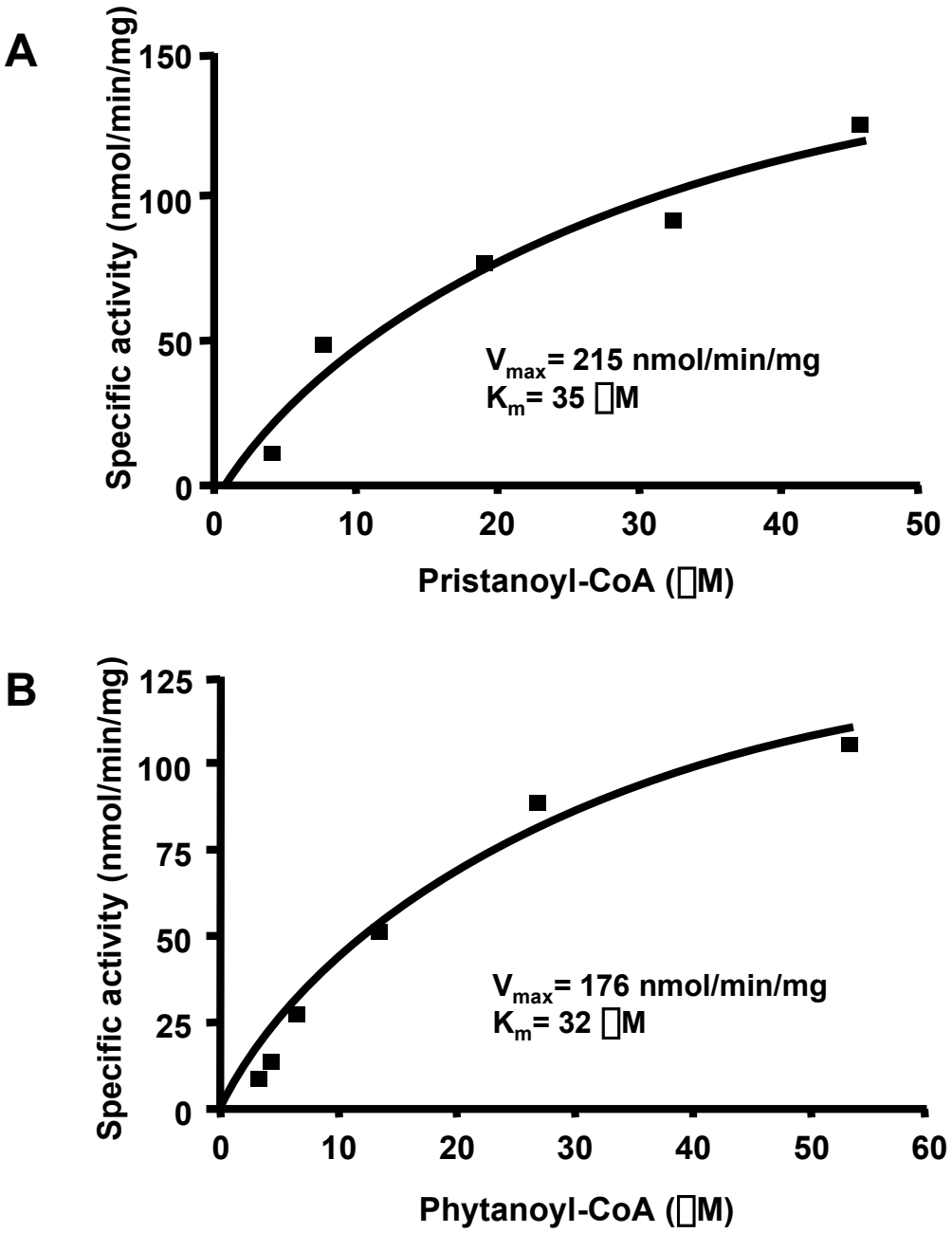

Fig. 4
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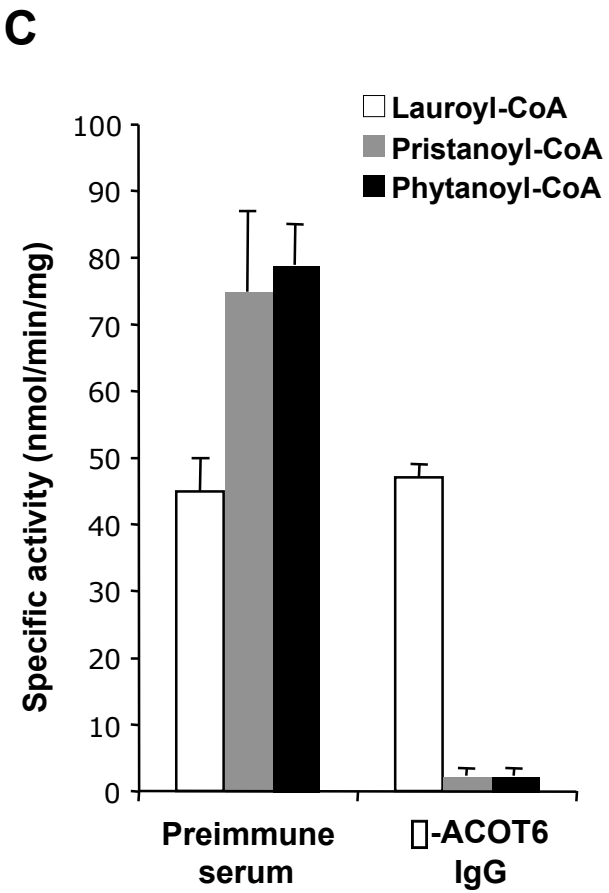

Fig. 5
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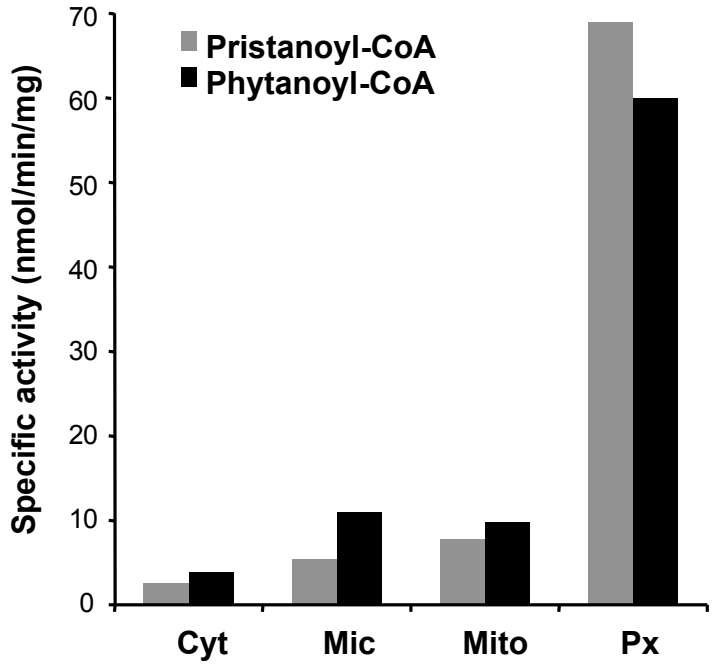

Fig. 6
Westin et al.

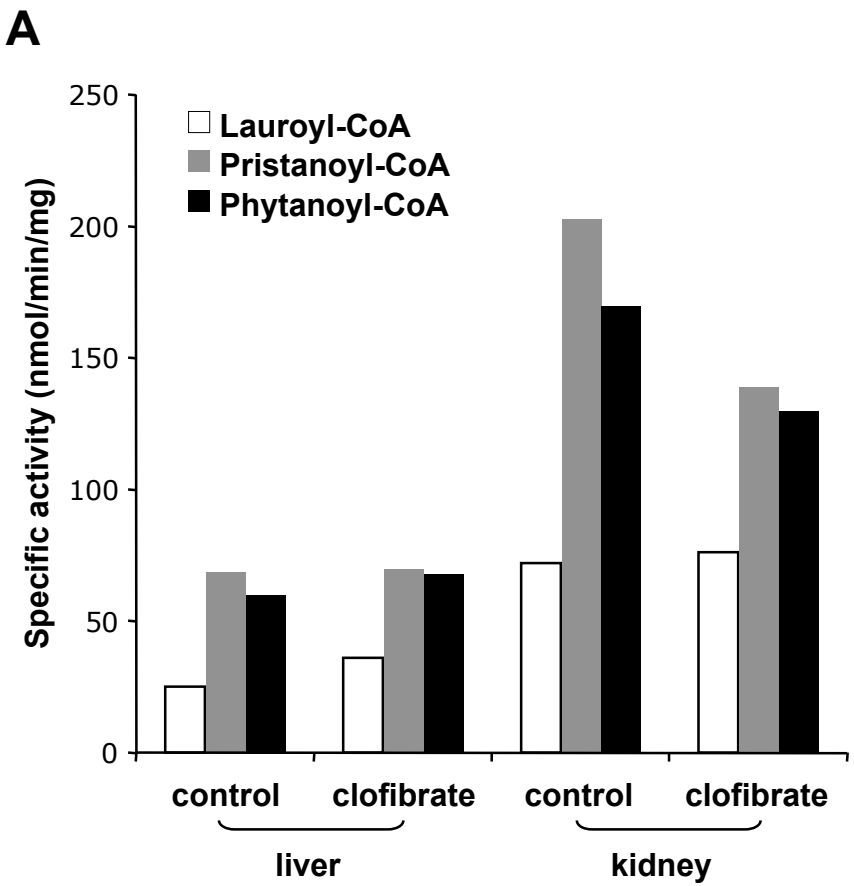

Fig. 6
Westin et al.

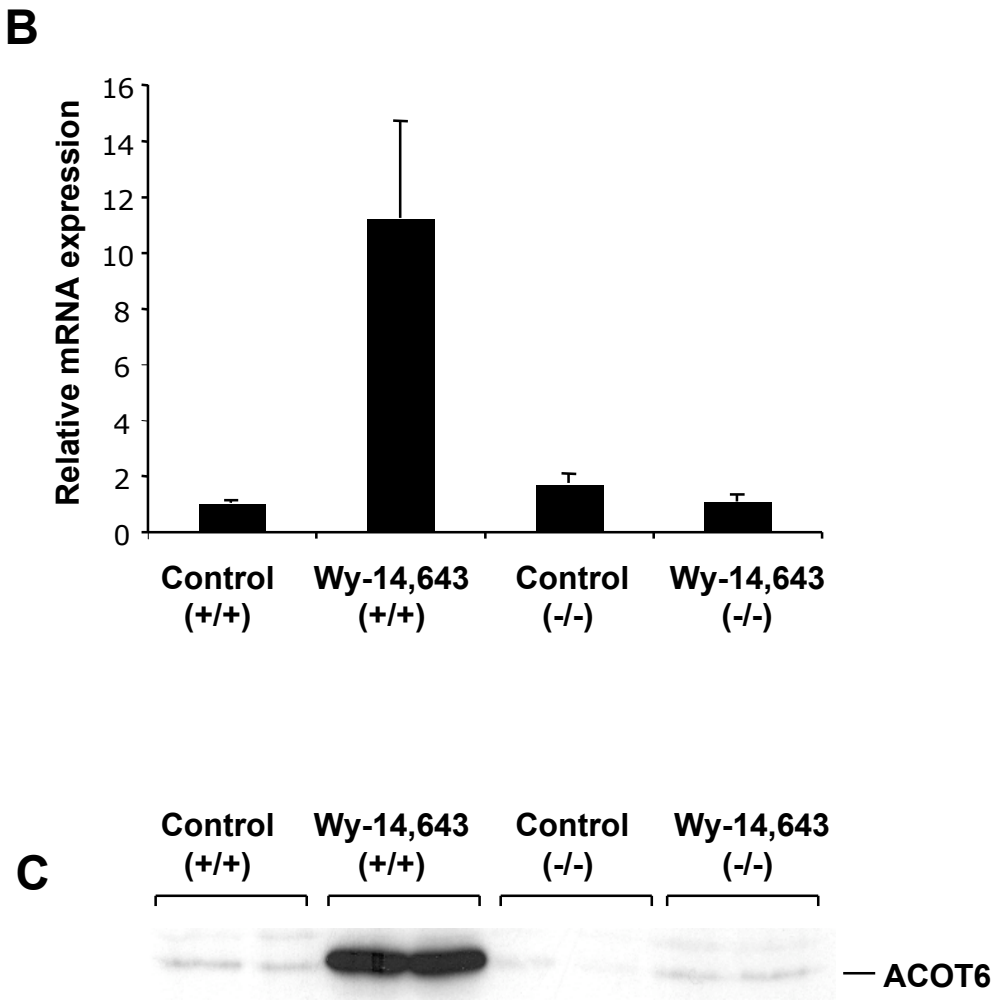

Fig. 7
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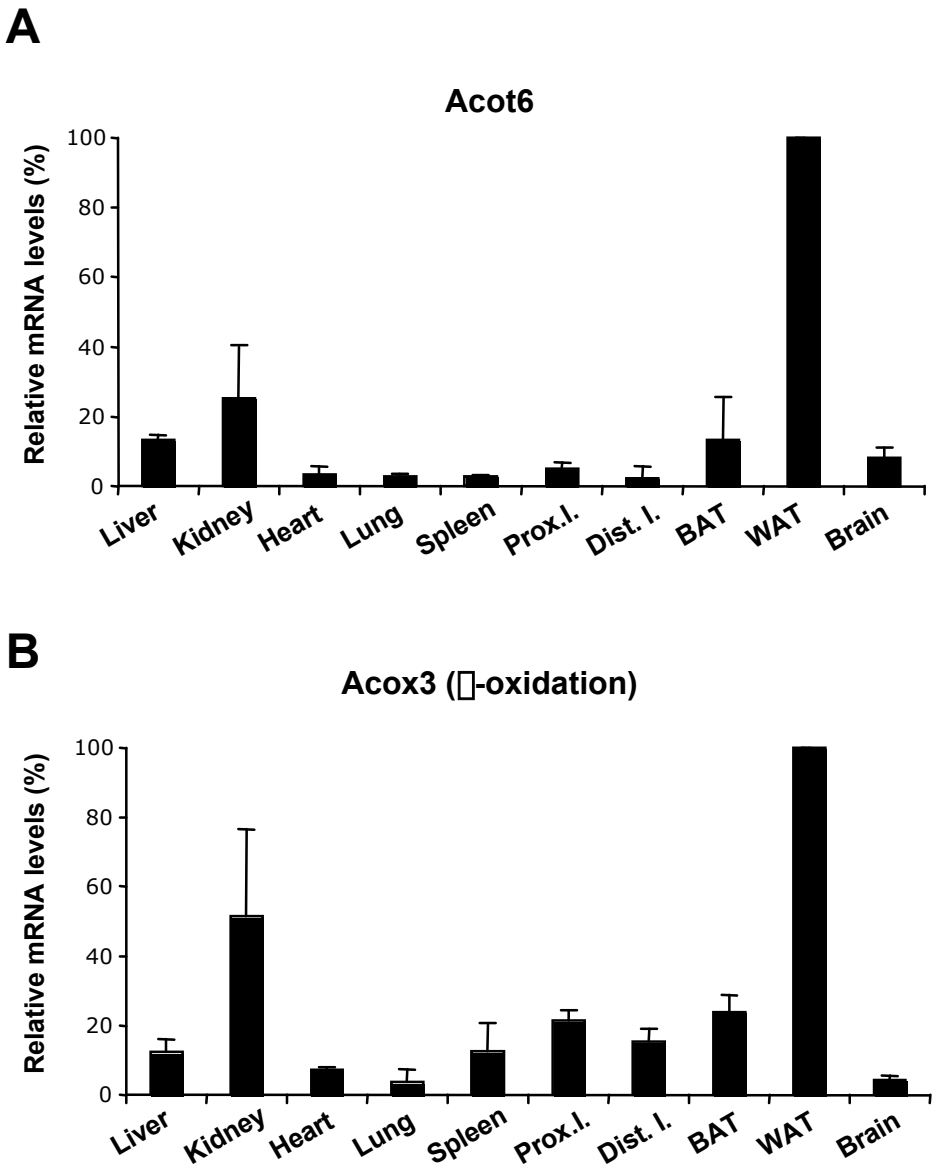

Fig. 7
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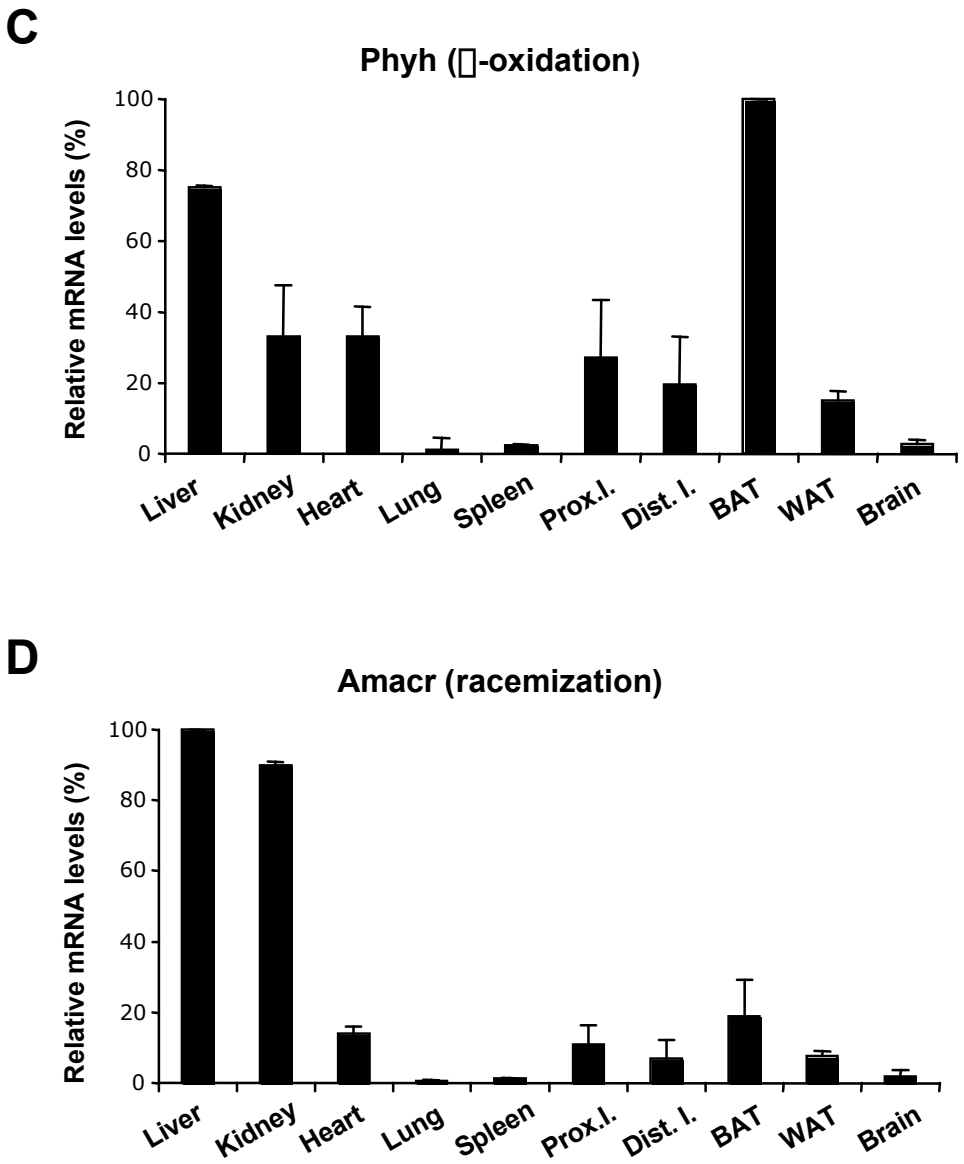

Fig. 8
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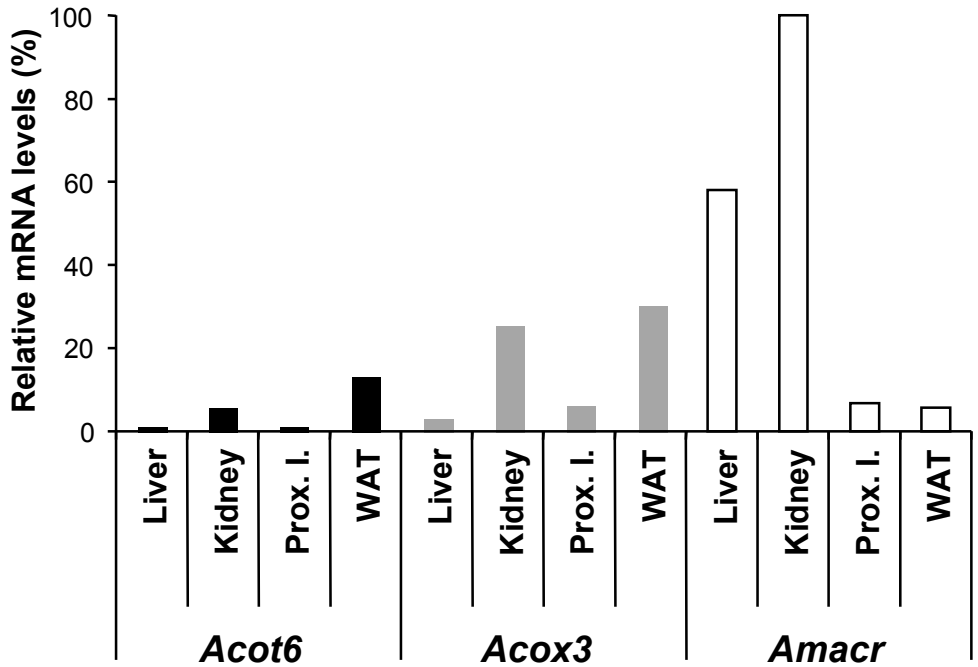

Fig. 9
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