New insights in peroxisomal beta-oxidation
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Mutations in the gene encoding peroxisomal α-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy.

Mutations in the gene encoding peroxisomal α-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy


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Abstract
Sensory motor neuropathy is associated with various inherited disorders including Charcot-Marie-Tooth disease (1,2), X-linked adrenoleukodystrophy/adrenomyeloneuropathy (3) and Refsum disease (4). In the latter two, the neuropathy is thought to result from the accumulation of specific fatty acids. We describe here three patients with elevated plasma concentrations of pristanic acid (a branched-chain fatty acid) and C27-bile acid intermediates. Two of the patients suffered from adult-onset sensory motor neuropathy. One patient also had pigmentary retinopathy, suggesting Refsum disease, whereas the other patient had upper motor neuron signs in the legs, suggesting adrenomyeloneuropathy. The third patient was a child without neuropathy. In all three patients we discovered a deficiency of α-methylacyl-CoA racemase. This enzyme is responsible for the conversion of pristanoyl-CoA and C27-bile acyl-CoAs to their (S)-stereoisomers (5,6), which are the only stereoisomers that can be degraded via peroxisomal β-oxidation (7,8). Sequence analysis of the α-methylacyl-CoA racemase cDNA from the patients identified two different mutations that are likely to cause disease, based on analysis in Escherichia coli. Our findings have implications for the diagnosis of adult-onset neuropathies of unknown etiology.

We analyzed the plasma of two patients with adult-onset sensory motor neuropathy and additional clinical signs suggesting Refsum disease (patient 1) or X-linked adrenoleukodystrophy/adrenomyeloneuropathy (patient 2), and found a similar profile. Very long-chain fatty acids (VLCFAs) were not elevated, which excluded X-linked adrenoleukodystrophy/adrenomyeloneuropathy (Table 1). Phytanic acid was marginally elevated, but, in contrast to patients with Refsum disease, the levels of pristanic acid and the C27-bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA) were markedly increased (Table 1). This suggested a specific defect in the peroxisomal β-oxidation of branched-chain fatty acids and not a defect in the α-oxidation system, the first enzyme step of which is defective in Refsum disease (9,10) (Fig. 1). This was confirmed by a reduced pristanic acid β-oxidation activity in cultured skin fibroblasts of the patients (Table 2). When we subsequently measured the activities of the enzymes directly involved in the β-oxidation of branched-chain fatty acids (namely, branched-chain
<table>
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Table 1: Analysis of plasma VLCFAs' branched-chain fatty acids and bile acids

All concentrations are given in µM. XALD-X-linked adrenoleukodystrophy.
acyl-CoA oxidase, D-bifunctional protein and sterol carrier protein X), however, we found them all to be normal.

\[(\text{3R})-\text{Phytanoyl-CoA} \quad \text{and} \quad (\text{3S})-\text{Phytanoyl-CoA} \quad \text{Cholesterol} \]

\[(\text{2R})-\text{Pristanoyl-CoA} \quad \text{and} \quad (\text{2S})-\text{Pristanoyl-CoA} \quad (\text{25S})-\text{THC-CoA} \quad (\text{25R})-\text{THC-CoA} \]

**Fig. 1** Schematic representation of the steps involved in the oxidation of (3R)- and (3S)-phytanic acid as derived from dietary sources and (25R)-THCA produced from cholesterol in the liver. After the activation of (3R)- and (3S)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal α-oxidation system, which produces (2R)- and (2S)-pristanoyl-CoA. Because branched-chain acyl-CoA oxidase, the first enzyme of the β-oxidation system, can only handle (S)-stereoisomers, (2R)-pristanoyl-CoA needs to be converted by α-methylacyl-CoA racemase into its (2S)-isomer. The bile acid intermediates DHCA and THCA are exclusively produced as (25R)-stereoisomers. To be β-oxidized, the CoA esters of the (25R)-stereoisomer also need to be converted by α-methylacyl-CoA racemase into their (25S)-isomers.

Because pristanic acid β-oxidation activity was reduced but not fully deficient, we next examined whether the patients were deficient in α-methylacyl-CoA racemase activity. α-methylacyl-CoA racemase is not directly involved in the β-oxidation itself, but it is important in the β-oxidation of branched-chain fatty acids and C_{27}-bile acids. This peroxisomal enzyme catalyzes the interconversion of (R)- and (S)-stereoisomers of α-methyl-branched-chain fatty acyl-CoA esters (5,6,11), including pristanoyl-CoA, which naturally occurs as a mixture of two different stereoisomers ((2R)- and (2S)-pristanoyl-CoA, see Fig. 1) (12,13). In addition it catalyzes the interconversion of the CoA esters of DHCA and THCA (DHC-CoA and THC-CoA, respectively), which are exclusively produced as (25R)-stereoisomers (14), into their respective (25S)-stereoisomers. Although α-methylacyl-CoA racemase is able to convert both the (R)- and (S)-stereoisomers, its physiological function is to produce the (S)-stereoisomers, because only these serve as substrate for branched-chain acyl-CoA oxidase (7,8), the first enzyme of the peroxisomal β-oxidation system of branched-chain fatty acids (Fig. 1). We therefore predicted that a deficiency of α-methylacyl-CoA racemase would result in a partially reduced pristanic acid
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β-oxidation in cultured skin fibroblasts because the pristanic acid used in the assay is a racemic mixture.

Furthermore, we predicted that only the (R)-stereoisomers of pristanic acid, DHCA and THCA would accumulate in plasma from these patients. Therefore we further analyzed the plasma of patient 1 and 2 by liquid chromatography/tandem mass spectrometry (LC/MS/MS) and detected an accumulation of only (25R)-THCA (data not shown). In addition we measured α-methylacyl-CoA racemase activity in fibroblasts from both patients and found it to be fully deficient (Fig. 2 and Table 2).

During the course of this study a third patient, diagnosed with Niemann-Pick type C (NPC) (15), was identified. This patient, in addition to typical NPC features, showed biochemical abnormalities similar to those of the other two patients, suggesting a second genetic defect (Table 1). α-Methylacyl-CoA racemase activity was also fully deficient in fibroblasts from this patient (Table 2).

A search of the EST database of the National Center of Biotechnology Information with the amino acid sequences of mouse and rat α-methylacyl-CoA racemase identified one human EST clone that was mapped at chromosome 5p13.2–5q11.1 and predicted to contain the entire ORF. On the basis of the DNA sequence of this EST clone, we amplified the human α-methylacyl-CoA racemase cDNA by RT-PCR. Human α-methylacyl-CoA racemase showed 81% and 77% identity, respectively, with the amino acid sequence of rat and mouse α-methylacyl-CoA racemase (16) (Fig. 3).

Fig. 2 Measurement of α-methylacyl-CoA racemase activity in fibroblast homogenates from a control subject (A,B) and patient 1 (C). Activity was measured by monitoring the production of (25R)-THC-CoA (peak 2) from (25S)-THC-CoA (peak 1) using HPLC. Homogenates were incubated with (25S)-THC-CoA at 37°C for 60 min (B,C) or, as a control, for 0 min (A). α-Methylacyl-CoA racemase activity was detectable in the fibroblast homogenate of the control (B), but no activity was measured for patient 1 (C) or patients 2 and 3 (data not shown).
Fig. 3 Alignment of the amino acid sequences of mouse, rat and human α-methylacyl-CoA racemase. Black boxes indicate identical amino acids and gray boxes represent similar amino acids. Amino acid changes frequently observed in human control alleles are indicated below the human sequence. The human sequence contains an additional 21 aa at its amino terminus compared with the published sequences of the mouse and rat α-methylacyl-CoA racemase (the arrowhead indicates the initiation methionine of the published mouse and rat α-methylacyl-CoA racemase sequence (16)). Translation of the presumed 5' noncoding regions of the rat and mouse cDNA sequence, however, indicated that the latter two must represent 5' truncated cDNA species. The human α-methylacyl-CoA racemase contains a putative carboxy-terminal peroxisomal targeting signal type 1 (ASL), like the rat and the mouse proteins (ANL).

Sequence analysis of the α-methylacyl-CoA racemase cDNA amplified by RT-PCR identified three homozygous nucleotide differences in patients 1 and 2, resulting in the amino acid changes V9M, S52P and G175D. In patient 3 we found three other homozygous nucleotide differences leading to the amino acid changes L107P, S210L and K277E. We found high frequencies in 114 control alleles of the amino acid changes V9M and G175D, both identified in patients 1 and 2, and S201L and K277E, identified in patient 3, suggesting that they represent polymorphisms (Table 2). In contrast, the remaining two amino acid changes, S52P (patients 1 and 2) and L107P (patient 3), were not detected among the controls. This observation, in conjunction with the complete absence of α-methylacyl-CoA racemase activity in fibroblasts of the patients, suggested
that these two amino acid changes are disease causing. We confirmed this by expression of the two mutant and corresponding wild-type proteins as fusions to maltose binding protein (MBP) in *E. coli*. Enzyme measurements after affinity purification of the fusion proteins from *E. coli* lysates showed that both the S52P and the L107P amino acid changes resulted in inactive proteins (Fig. 4).

![Diagram](https://via.placeholder.com/150)

**Fig. 4** Expression of S52P and L107P mutant and corresponding control α-methylacyl-CoA racemase cDNAs in *E. coli*. Allele 1 contains the methionine at position 9 and the aspartic acid at position 175 (both present in patients 1 and 2). Allele 2 contains the leucine at position 201 and the glutamic acid at position 277 (both present in patient 3). The coding sequences of the various α-methylacyl-CoA racemase cDNAs were amplified by RT-PCR and expressed as a fusion with maltose binding protein (MBP) in *E. coli*. α-Methylacyl-CoA racemase enzyme activities of α-methylacyl-CoA racemase-MBP fusion proteins were measured after affinity purification from *E. coli* lysates and normalized for the amount of protein to correct for differences in expression. The results are the mean of four independent measurements.

Our results indicate that α-methylacyl-CoA racemase deficiency is associated with neurological disease in adult life. The common feature in the two adults was sensory motor neuropathy, although in one case the electrophysiology suggested an axonal neuropathy and in the other, a demyelinating neuropathy. Patient 3 contributes little to our understanding about the neurology of α-methylacyl-CoA racemase deficiency, because all of the child’s symptoms could be accounted for by NPC. The similar clinical signs associated with α-methylacyl-CoA racemase deficiency and Refsum disease (which is caused by phytanoyl-CoA hydroxylase deficiency (10,17)) indicate that sustained elevated levels of branched-chain fatty acids are progressively deleterious and result in adult-onset neuropathies.

Clinical data indicate that the symptoms associated with α-methylacyl-CoA racemase deficiency are relatively mild. This, together with the fact that routine plasma analysis in adults usually does not include analysis of bile acids and branched-chain fatty acids (18,19), implies that thus far many patients with α-methylacyl-CoA racemase deficiency may have remained undiagnosed. This stresses the importance of undertaking multiple analyses when investigating adult patients suffering from motor and sensory neuropathies.
of unknown etiology. Especially because a dietary regimen reduced in phytanic and pristanic acid may alleviate the progression of the neuropathy in α-methylacyl-CoA racemase deficiency, as in Refsum disease (20). Identification of additional patients with α-methylacyl-CoA racemase deficiency is required to appreciate the full spectrum of neurological abnormalities that can result from this enzyme deficiency.

Materials and Methods

Patients

Patient 1 was a male of European descent who exhibited a typical retinitis pigmentosa with restriction of his visual field and acuity, and primary hypogonadism when examined at 44 years of age. He also suffered from epileptic seizures and conduction studies showed a widespread axonal sensory motor neuropathy affecting the legs more severely than the arms. In childhood he showed mild developmental delay, and at the age of 18 an encephalitic illness left him temporarily blind. During the following two years his vision partially recovered, but has slowly deteriorated since then.

Patient 2 was a female of European descent who was completely well until the age of 48, when she began to tire easily and was found to be hypothyroid. She then developed heaviness of her legs on exercise, with dragging of both feet on walking. She had a spastic paraparesis, but the MRI scan of the cervical spine showed no abnormality. Nerve conduction studies showed a demyelinating sensory motor polyneuropathy. Analysis of plasma VLCFAs was undertaken to determine whether the patient had adrenomyeloneuropathy (as a symptomatic heterozygote). Phytanic acid and pristanic acid levels were analyzed simultaneously and found to be elevated.

Patient 3 was the second child born to doubly consanguineous Asian parents (15). At the age of 18 months he was diagnosed with NPC (complementation group 1). In addition to the typical biochemical features of NPC, an accumulation of pristanic acid, C27-bile acid intermediates and, to a lesser extent, phytanic acid was detected in plasma from this patient, but not in other NPC patients studied, suggesting a second genetic defect. He has shown progressive neurological signs consistent with NPC. With this background, detection of a subtle neuropathy is not possible.

Pristanic acid β-oxidation

We measured pristanic acid β-oxidation as described (21).

Synthesis of (25S)- and (25R)-THC-CoA

The CoA thioester of THCA (22) was chemically synthesized as described (23). We purified the two stereoisomers by high-performance liquid chromatography (HPLC) using a reversed-phase C18-column (Supelcosil SPLC-18-DB, 250 mm×10 mm) and determined the stereospecificity of the two isomers of THC-CoA after mild alkaline hydrolysis of the CoA thioesters and analysis of the free acids by LC-MS as described (24).
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**Enzyme assays**
We measured α-methylacyl-CoA racemase activity in fibroblast homogenates with (25S)-THC-CoA (50 μM) as substrate and monitored the production of (25R)-THC-CoA with HPLC. The incubation mixture consisted of sodium phosphate buffer (6.4 mM, pH 7.4), NaCl (70 mM), ATP (10 mM), MgCl2 (10 mM) and CoA (100 μM). Reactions were allowed to proceed for 60 min at 37°C and terminated by the addition of HCl (0.18 M), followed by resolution of the (25S)- and (25R)-THC-CoA by HPLC. We carried out HPLC with a reversed-phase C18-column (Alltima 250 mm × 4.6 mm, Alltech) and achieved optimal resolution by elution with a linear gradient of methanol in potassium phosphate buffer (50 mM, pH 5.3). We performed activity measurements of α-methylacyl-CoA racemase–MBP fusion proteins as described for the α-methylacyl-CoA racemase activity measurements in fibroblast homogenates.

**Mutation analysis of the human α-methylacyl-CoA racemase cDNA**
We prepared first-strand cDNA from total RNA isolated from cultured skin fibroblasts as described (25). Two sets of α-methylacyl-CoA racemase-specific primers with -21M13 or M13rev extensions were used to amplify the α-methylacyl-CoA racemase cDNA in two overlapping fragments by RT-PCR. We sequenced the PCR fragments in both directions by means of -21M13 and M13rev fluorescent primers on an ABI 377A automated DNA sequencer according to the manufacturer's protocol (Perkin-Elmer).

**Expression of the α-methylacyl-CoA racemase cDNA in E. coli**
We amplified by PCR the coding sequence of wild-type and mutant α-methylacyl-CoA racemase cDNAs, cloned it in-frame with the coding sequence of MBP in pMALc2 (New England BioLabs) and sequenced to exclude Taq polymerase-introduced errors. E. coli DH5α cells were transformed with the resulting expression plasmids and induced for 2 h with isopropyl-β-D-thiogalactoside (2 mM) at 37°C. Subsequently we purified α-methylacyl-CoA racemase–MBP fusion protein from the E. coli lysate by one-step affinity chromatography according to the manufacturer's protocol (New England BioLabs).

**GenBank accession numbers**
Human ESTs, H19271 (STS, WI-16117), H19272; mouse α-methylacyl-CoA racemase, U89906; rat α-methylacyl-CoA racemase, U89905; human α-methylacyl-CoA racemase, AF158378.

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Chapter 6

References


