New insights in peroxisomal beta-oxidation
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Plasma analysis of di- and trihydroxycholestanoic acid diastereomers in peroxisomal \( \alpha \)-methylacyl-CoA racemase deficiency

Plasma analysis of di- and trihydroxycholestanoic acid diastereomers in peroxisomal α-methylacyl-CoA racemase deficiency

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Abstract
Recently, we identified a new peroxisomal disorder caused by a deficiency of the enzyme α-methylacyl-CoA racemase. Patients with this disorder show elevated plasma levels of pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), which are all substrates for the peroxisomal β-oxidation system. α-Methylacyl-CoA racemase plays an important role in the β-oxidation of branched-chain fatty acids and fatty acid derivatives since it catalyzes the conversion of several (2R)-methyl-branched-chain fatty acyl-CoAs to their (2S)-isomers. Only stereoisomers with the 2-methyl group in the (S)-configuration can be degraded via β-oxidation. In this study we used liquid chromatography/tandem mass spectrometry (LC/MS/MS) to analyze the bile acid intermediates which accumulate in plasma from patients with a deficiency of α-methylacyl-CoA racemase and, for comparison, in plasma from patients with Zellweger syndrome and patients suffering from cholestatic liver disease. We found that racemase-deficient patients accumulate exclusively the (R)-isomer of free and taurine-conjugated DHCA and THCA, whereas in plasma of patients with Zellweger syndrome and patients suffering from cholestatic liver disease both isomers were present. Based on these results we describe an easy and reliable method to diagnose α-methylacyl-CoA racemase-deficient patients by plasma analysis. Our results also show that α-methylacyl-CoA racemase plays a unique role in bile acid formation.

Introduction
Peroxisomes play an important role in the biosynthesis of bile acids from cholesterol since the peroxisomal β-oxidation is responsible for chain shortening of the C₂₇-bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), which results in formation of the primary bile acids chenodeoxycholic acid and cholic acid respectively. The enzymes involved in this process do not only handle DHCA and THCA as substrates but also other 2-methyl branched-chain fatty acids, like pristanic acid (see Fig. 1). The first step of the β-oxidation is catalyzed by branched-chain acyl-CoA oxidase (1,2), which converts the 2-methyl branched-chain acyl-CoAs into their enoyl-CoA ester. These are subsequently hydrated into a hydroxyacyl-CoA and then dehydrogenated into a β-ketoacyl-CoA. Both these steps are catalyzed by D-bifunctional protein (3-6). Finally, sterol carrier protein X (SCPx) is responsible for the thioyltic cleavage of the β-ketoacyl-CoA esters of pristanic acid as well as DHCA and THCA (7-11).

It has been demonstrated that the peroxisomal β-oxidation system is stereospecific, because the first enzyme, branched-chain acyl-CoA oxidase, can only handle (2S)-isomers.
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(12,13). For this reason, a racemase called α-methylacyl-CoA racemase, identified by Conzelmann and coworkers (14,15), is also involved in the β-oxidation of branched-chain fatty acids. This enzyme is able to convert (2R)-pristanoyl-CoA, (25R)-DHC-CoA and (25R)-THC-CoA into their (S)-isomers (14,15) (Fig. 1). This conversion is essential for degradation of these substrates, because naturally occurring pristanic acid is a mixture of two diastereomers, (2R,6R,10R) and (2S,6R,10R) (16), whereas in case of DHCA and THCA only the (25R)-isomers are produced from cholesterol (17-20). As a consequence, patients who are unable to convert the (R)-isomer of pristanoyl-CoA and the C_{27}-bile acyl-CoAs to their respective (S)-isomers, which are the true substrates for the β-oxidation system, are predicted to accumulate these compounds in their plasma. We have recently identified three patients with a complete α-methylacyl-CoA racemase deficiency due to mutations in the encoding gene as shown by expression studies in E. coli (21). The main clinical symptom in these patients was an adult-onset sensory motor neuropathy. As expected, plasma analysis in these patients revealed an accumulation of both pristanic acid and the bile acid intermediates DHCA and THCA.

In the present study we further analyzed the C_{27}-bile acid intermediates accumulating in plasma from these patients and, for comparison, from patients with Zellweger
syndrome and patients suffering from cholestatic liver disease using liquid chromatography/tandem mass spectrometry (LC/MS/MS) to discriminate between the different diastereomers of DHCA and THCA. The results obtained indicate that α-methylacyl-CoA racemase is, indeed, indispensable for the oxidation of the bile acid intermediates and that there is no other racemase which takes over the role of the deficient enzyme. Furthermore, the plasma analysis we describe in this paper provides an easy and reliable method to diagnose α-methylacyl-CoA racemase-deficient patients.

Materials and Methods

Materials

The two diastereomers of THCA were obtained as described before (21). Taurine was purchased from Serva (Heidelberg, Germany), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide.HCl (EDC) from Sigma (St. Louis, MO) and [2,2,4,4-2H4]cholic acid was from J.H. Ritmeester BV (Utrecht, The Netherlands).

Patients

Plasma samples were obtained from three patients with a deficiency of α-methylacyl-CoA racemase, four patients with Zellweger syndrome and five patients suffering from cholestatic liver disease. The ages of the patients with cholestatic liver disease (3 males and 2 females) and Zellweger syndrome (2 males and 2 females) varied between 1 month and 3 years. The α-methylacyl-CoA racemase-deficient patients all had distinct mutations in the encoding gene and racemase activity in fibroblasts of these patients as measured with THC-CoA as substrate was completely deficient (21). Patient 1, a boy, is now 7 years old, patient 2 is a man with the age of 49 and patient 3 is a 48-year-old woman. The patients with Zellweger syndrome all had the clinical and biochemical abnormalities described for Zellweger syndrome (22). Informed consent was obtained for all patients whose plasma was studied and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

Derivatization of THCA with taurine

The two diastereomers of THCA were derivatized with taurine to be able to determine the stereospecificity of the different isomers of taurine-conjugated THCA in plasma of the patients. Derivatization of THCA was performed essentially as described by Zhang et al. (23). Briefly, 0.37 μmoles (25R)- or (25S)-THCA was dissolved in 0.2 ml 0.1 M pyridine hydrochloride (pH 5.0). Fifty μmoles EDC and 100 μmoles of taurine were added and the mixture was left for 16 h at room temperature. It was then passed through a SPE-C18-column (1.5 x 0.8 cm) (J.T. Baker, Phillipsburg, NJ). After washing the column with water, taurine-conjugated THCA was eluted with methanol. The yield was approximately 70%.

Plasma sample preparation

Fifty μl of the internal standards (IS) [2,2,4,4-2H4]cholic acid or [2,2,4,4-2H4]-taurocholic acid was added to 50 μl plasma. The mixture was deproteinized by addition of 500 μl acetonitrile followed by subsequent centrifugation for 15 min at 20,000 x g at 4°C. The
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supernatant was then evaporated under a stream of N₂ gas and the residue redissolved in 100 µl methanol/water (40/60). Twentyfive µl was injected into an LC/MS/MS system.

**Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)**

LC/MS/MS was carried out using a Hewlett-Packard (Palo Alto, CA) HP 1100 binary pump and a Micromass (Manchester, UK) Quattro II tandem Mass Spectrometer equipped with electrospray ionization (ESI). The LC separation was performed on an Altima C₁₈ reversed-phase (5 µm) column (250 mm × 2.1 mm) (Alltech, Deerfield, IL) and optimal resolution was achieved by elution with a linear gradient of methanol (70%→100%) in 5 mM ammoniumformiate buffer (pH 5.0) at a flow rate of 0.3 ml/min. MS/MS parameters were as follows: negative ion mode, capillary voltage 3.1 kV, cone voltage 70 V, collision energy 60 eV, collision pressure 0.003 mBar. Argon was used as collision gas. Taurine-conjugates were analyzed by MRM using the following transitions (IS 518.3 → 79.8; tauro-DHCA 540.3 → 79.8; tauro-THCA 556.3 → 79.8), the free compounds by SIR (IS 411.3; DHCA 433.3; THCA 449.3). The limit of detection of the bile acid intermediates was 0.05 µM.

**Results and discussion**

DHCA and THCA are obligatory intermediates in the major biosynthesis route of the primary bile acids chenodeoxycholic acid and cholic acid from cholesterol. They are produced from 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, respectively. The mitochondrial 27-hydroxylase involved in this pathway has been shown to be stereospecific, which exclusively leads to the formation of the (25R)-isomer of DHCA and THCA (17-20). Activation of DHCA and THCA occurs at the membrane of the endoplasmic reticulum followed by transport of DHC-CoA and THC-CoA into the peroxisome via a mechanism yet unknown. In the peroxisome (25R)-DHC-CoA and (25R)-THC-CoA are rapidly converted by α-methylacyl-CoA racemase (14,15) into their (25S)-isomers, that can enter the β-oxidation spiral.

Recently, three patients have been identified with a deficiency of α-methylacyl-CoA racemase due to mutations in the encoding gene. Plasma analysis revealed a marked increase in the levels of pristanic acid and of the C₂₇-bile acid intermediates DHCA and THCA (21). These compounds, however, are known to accumulate in several other peroxisomal disorders, including isolated defects in the peroxisomal β-oxidation system and defects in peroxisomal biogenesis (22,24-26). To examine the plasma C₂₇-bile acids in closer detail, we developed a method to study the different diastereomers of DHCA and THCA in plasma from patients with Zellweger syndrome and patients with an isolated α-methylacyl-CoA racemase deficiency. In addition, we studied plasma from patients suffering from cholestatic liver disease, who also accumulate bile acid intermediates in plasma but do not have a metabolic disorder affecting the oxidation of branched-chain fatty acids and fatty acid derivatives per se. The diastereomers of both free and taurine-conjugated C₂₇-bile acids could be studied with our LC/MS/MS method. To determine the elution pattern of the diastereomers of taurine-conjugated THCA, (25R)- and (25S)-THCA were derivatized with taurine. Both free and taurine-conjugated (25S)-THCA eluted at a lower concentration of methanol than the (25R)-isomer (Fig. 2). Unfortunately,
Fig. 2 Separation of the diastereomers of free and taurine-conjugated DHCA and THCA by LC/MS/MS. Analysis of the standards for (25S)- and (25R)-THCA in the free acid form and taurine-conjugated are shown (A and B, respectively). Plasma analysis in patients with Zellweger syndrome (C) revealed the presence of both diastereomers of free and taurine-conjugated THCA, whereas patients with a deficiency of α-methylacyl-CoA racemase (D) accumulate only the (25R)-isomer. No standards were available of the separate diastereomers of free and taurine-conjugated DHCA, but the exclusive accumulation of peak 2 for both compounds in racemase-deficient patients strongly suggests that peak 2 represents the (25R)-isomer.

no standards were available for DHCA. Therefore, we can only speculate about the identification of the diastereomers of free and taurine-conjugated DHCA.

Examination of plasma from four different patients with Zellweger syndrome revealed the presence of two diastereomers of both free and taurine-conjugated DHCA and THCA
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(Table 1 and Fig. 2). DHCA was mainly present as free acid, whereas in most patients more THCA was taurine-conjugated than unconjugated. The predominant peak of both free and taurine-conjugated THCA corresponded to the (25R)-isomer. The mean values (± SD) for the (25S/25R)-isomer ratios in these four patients were 0.23 (± 0.05) and 0.26 (± 0.03) for free and taurine-conjugated THCA, respectively (Table 1). These results are in agreement with the ratio (25S/25R)-THCA found in urine from an infant with Zellweger syndrome by Une and coworkers (27). The presence of both isomers indicates that α-methylacyl-CoA racemase is enzymatically active in patients with Zellweger syndrome. A residual racemase activity of 10% for pristanoyl-CoA in fibroblasts from patients with Zellweger syndrome compared to controls has indeed been reported (15), and corresponds to the results we obtained with THC-CoA as substrate in fibroblasts from patients with Zellweger syndrome (controls 97 ± 28 pmol/min/mg [n = 13]; patients with Zellweger syndrome 17 ± 5 pmol/min/mg [n = 3]). For free and taurine-conjugated DHCA, respectively, the mean values (± SD) for the ratios peak 1/peak 2 in the four patients with Zellweger syndrome were 0.19 (± 0.02) and 0.43 (± 0.08) (Table 1).

Table 1 Analysis of the diastereomers of free and taurine-conjugated DHCA and THCA in plasma from five patients with cholestatic liver disease, four patients with Zellweger syndrome and three patients with an α-methylacyl-CoA racemase deficiency.

<table>
<thead>
<tr>
<th>THCA free acid</th>
<th>Cholestatic</th>
<th>Zellweger</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) - a</td>
<td>ND</td>
<td>1.9-13.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>(R) - a</td>
<td>ND</td>
<td>9.7-75.5</td>
<td>2.2</td>
<td>2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>(S/R) - b</td>
<td>0.23 ± 0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DHCA free acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(peak 1) - a</td>
<td>ND</td>
<td>4.4-14.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>(peak 2) - a</td>
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<td>22.0-76.8</td>
<td>30.9</td>
<td>21.3</td>
<td>4.4</td>
</tr>
<tr>
<td>(1/2) - b</td>
<td>0.19 ± 0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>THCA taurine-conjugated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S) - a</td>
<td>0.06 - 0.43</td>
<td>3.8-10.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(R) - a</td>
<td>0.23 - 2.39</td>
<td>16.1-34.8</td>
<td>0.6</td>
<td>9.9</td>
<td>3.8</td>
</tr>
<tr>
<td>(S/R) - b</td>
<td>0.25 ± 0.08</td>
<td>0.26 ± 0.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DHCA taurine-conjugated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(peak 1) - a</td>
<td>ND</td>
<td>0.8-3.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(peak 2) - a</td>
<td>ND</td>
<td>1.7-7.0</td>
<td>ND</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>(1/2) - b</td>
<td>0.43 ± 0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholic acid a e</td>
<td>14.1-74.8</td>
<td>0.3-13.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Chenodeoxycholic acid a e</td>
<td>32.2 - 100.9</td>
<td>4.3-37.0</td>
<td>0.2</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>
| ^range in μM. ^ratio mean ± SD. ^sum of glycine and taurine conjugated species (normal range 0.7-10 μM chenodeoxycholic acid; 0.1-4.7 mM cholic acid). ND, not detectable. Cholestatic, cholestatic liver disease patients (n=5); Zellweger, patients with Zellweger syndrome (n=4); patient 1-3, α-methylacyl-CoA racemase deficient patients.

In the patients with cholestatic liver disease the mean value (± SD) for the (25S/25R)-isomer ratios for taurine-conjugated THCA was 0.25 (± 0.08), which is similar to the ratio found in patients with Zellweger syndrome (0.26 ± 0.03; p>0.05; t-test). These results confirm that plasma from Zellweger patients can be used as a control in this assay, even though the biogenesis of peroxisomes, where the racemase is localized, is disturbed in these patients. The amount of free THCA and free and taurine-conjugated DHCA in
plasma of patients with cholestatic liver disease was too low to draw any conclusions about the distribution of the different diastereomers.

Plasma analysis of C27-bile acid intermediates in the three patients with a defined α-methylacyl-CoA racemase deficiency revealed the exclusive accumulation of the (25R)-isomer of both free and taurine-conjugated THCA (Table 1 and Fig. 2). Only one diastereomer of DHCA was present in both free acid form and in taurine-conjugated form. This strongly suggests that, as for THCA, peak 2 of free and taurine-conjugated DHCA, which elutes at a higher methanol concentration than peak 1, represents the (25R)-isomer (Fig. 2). The concentrations of the normal C24-bile acids cholic acid and chenodeoxycholic acid were in the lower part of the normal range. These bile acids could be synthesized using the alternative 25-hydroxylation pathway (28), but the lack of 25-hydroxylated bile alcohols (data not shown) in plasma of racemase-deficient patients suggest that other pathways might be responsible for the residual C24-bile acid biosynthesis.

Routine plasma analysis in adult patients with sensory motor neuropathy usually does not include analysis of bile acids and branched-chain fatty acids. This, together with the fact that the clinical symptoms associated with α-methylacyl-CoA racemase deficiency are relatively mild, implies that thus far many patients with α-methylacyl-CoA racemase deficiency may have remained undiagnosed. The method described in this paper provides a unique diagnostic tool for this disorder. Only a small amount of plasma is needed, the analysis takes little time and the exclusive accumulation of the (25R)-isomer of free and taurine-conjugated DHCA and THCA indisputably reveals a deficiency of α-methylacyl-CoA racemase in the patient. Finally, our data indicate that α-methylacyl-CoA racemase plays an indispensable role in bile acid formation.

Acknowledgments
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References


