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Characterization of phytanic acid ω-hydroxylation in human liver microsomes

J.C. Komen, M. Duran, R.J.A. Wanders *

Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma Childrens Hospital, University of Amsterdam, Academic Medical Center, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands

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Abstract

Phytanic acid is a 3-methyl branched-chain fatty acid which originates from dietary sources. Since the 3-methyl group blocks regular β-oxidation, it is broken down by peroxisomal α-oxidation. Adult Refsum disease patients accumulate phytanic acid as a result of an impairment in peroxisomal α-oxidation, caused by the deficient activity of the enzyme phytanoyl-CoA hydroxylase in the majority of patients. In this paper, we studied an alternative degradation route for phytanic acid, namely ω-oxidation. During ω-oxidation a fatty acid is hydroxylated at its ω-end by a member of the cytochrome P450 multi-enzyme family. Subsequently, an alcohol dehydrogenase converts the formed hydroxyl group into an aldehyde, which is then converted into a carboxyl-group by an aldehyde dehydrogenase. In case of phytanic acid ω-hydroxylation would lead to the formation of phytanedioic acid, which can be degraded by β-oxidation from the ω-end. Here, we show that phytanic acid indeed undergoes ω- and (ω-1)-hydroxylation in pooled human liver microsomes in an NADPH-dependent manner with a ratio of 15:1. Studies with imidazole antimycotics indicate that these reactions are catalyzed by one or more cytochrome P450 enzymes. Induction of the cytochrome P450 involved in phytanic acid ω-hydroxylation may increase the flux through the ω-oxidation pathway, causing increased clearance of phytanic acid in ARD patients. Hence, this alternative catabolic pathway is of potential therapeutic relevance.

Keywords: Phytanic acid; Refsum disease; Branched-chain fatty acids; ω-Oxidation; Cytochrome P450; ω-Hydroxylation

1. Introduction

Adult Refsum disease (ARD) is an autosomal recessive disorder caused by deficient α-oxidation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). In the majority of patients this is due to mutations in the gene encoding phytanoyl-CoA hydroxylase, a peroxisomal enzyme [1–3]. In a subset of patients mutations in the *PEX7* gene have been found [4]. Phytanoyl-CoA hydroxylase catalyzes the first step in the α-oxidation pathway of 3-methyl branched-chain fatty acids. These fatty acids require α-oxidation for their degradation since the 3-methyl group blocks breakdown by regular β-oxidation. During α-oxidation, phytanic acid is converted into its $n-1$ analog pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can readily be degraded by peroxisomal β-oxidation [5,6].

The deficiency of α-oxidation in ARD patients leads to the gradual accumulation of phytanic acid. Elevated phytanic acid in the absence of abnormalities in any of the other peroxisomal parameters including plasma very long chain fatty acids, bile acid intermediates, and erythrocyte plasmalogens is suggestive for ARD. Classical symptoms are: progressive retinitis pigmentosa, peripheral neuropathy, anosmia, and cerebellar ataxia [7–9]. The only treatment available at the moment is a diet low in

* Corresponding author. Fax: +31 20 6962596.
E-mail address: r.j.wanders@amc.uva.nl (R.J.A. Wanders).

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phytanic acid, which may be preceded by plasmapheresis. This alleviates the phytanic acid accumulation and slows down the progression of the disease [9].

The diet-induced decrease of phytanic acid levels in ARD patients suggested the existence of an alternative degradation pathway for this molecule. ω-Oxidation is a known breakdown pathway for fatty acids and therefore might be an alternative pathway for phytanic acid degradation as well [10,11]. ω-Oxidation consists of three successive steps. Initially the fatty acid is hydroxylated by a member of the cytochrome P450 enzyme family at the carbon atom localized at the ω-end. Subsequently, the hydroxyl group is converted to an aldehyde by an alcohol dehydrogenase, which in turn can be oxidized to a carboxyl-group by an aldehyde dehydrogenase. In case of phytanic acid this leads to 1,16-phytanedioic acid, which, in principle, can be further degraded by β-oxidation from the ω-end. The existence of the ω-oxidation pathway for phytanic acid degradation is supported by several reports that describe elevated levels of 3-methyladipic acid (3-methylhexanedioic acid) and 2,6-dimethylsuberic acid (2,6-dimethyloctanedioic acid) in urine of ARD patients, which are believed to be products of ω-oxidation of phytanic acid and the subsequent β-oxidation of phytanedioic acid [12–15]. Recently, we have shown that rat liver microsomes are able to ω-hydroxylate phytanic acid [16]. This reaction is catalyzed by a member of the cytochrome P450 enzyme family and resulted in the formation of two metabolites, ω- and (ω-1)-hydroxyphytanic acid. Cytochrome P450 enzymes are readily inducible by a variety of drugs [17,18]. Induction of the cytochrome P450 involved in phytanic acid ω-hydroxylation might lead to an increased clearance of phytanic acid in Refsum patients with obvious implications for the treatment of these patients. In this paper, we have extended our studies from rat liver microsomes to human liver microsomes. Species-specific differences in expression levels of the cytochrome P450 enzymes and the existence of a difference in number of isoforms in certain cytochrome P450 subfamilies between rat and human, e.g., CYP4A and CYP4F [10,19], emphasize the importance of this study.

2. Materials and methods

2.1. Materials

Phytanic acid was obtained from the VU University Medical Center Metabolic Laboratory (Dr. H.J. ten Brink, Amsterdam, the Netherlands). 3-Hydroxyheptadecanoic acid was from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD^+ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonazole, and miconazole were obtained from Sigma (St. Louis, MO, USA). Methyl-β-cyclodextrin was from Fluka (Buchs, Switzerland). *N*,*O*-bis-(Trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Pooled human liver microsomes were obtained from BD Gentest (Woburn, MA, USA). Rat liver microsomes were prepared from Male Wistar rats by differential centrifugation as described in detail [16]. Other chemicals used were of the highest quality possible.

2.2. Phytanic acid ω- and ω-1-hydroxylation assay

Essentially the same conditions were used as previously described [16], except for the final reaction volume which was now 0.1 ml. In brief, phytanic acid dissolved in DMSO was added to a solution of microsomes (1 mg/ml final concentration) in 100 mM potassium phosphate buffer (pH 7.7), containing phytanic acid at a final concentration of 200 μM, unless indicated otherwise. Reactions were initiated by adding NADPH (final concentration 1 mM) and terminated by addition of 0.1 ml of 1 M HCl. Subsequently, 0.5 ml phosphate-buffered saline (PBS) was added followed by 50 μl of 12.1 M HCl. After addition of the internal standard (IS, 2 nmol 3-hydroxyheptadecanoic acid in 20 μl ethanol) the samples were extracted twice with 6 ml ethylacetate–diethylether (1:1 v/v). The organic layers were collected and the solvents evaporated. The residue was dissolved in 4 ml ethylacetate and further dried with anhydrous $MgSO_4$ and again evaporated. To enable gas chromatography–mass spectrometry (GC–MS) analysis the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson [20]. TMS ester/ether formation was performed by incubating the samples with 40 μl BSTFA containing 1% TMCS and 10 μl pyridine at 80 °C for 1 h. After the incubation the solution was directly used for GC–MS analysis.

2.3. GC–MS

GC–MS was performed on a Hewlett–Packard 6890 gas chromatograph coupled to a Hewlett–Packard 5973 mass-selective detector (Palo Alto, CA). Samples (1 μl) were injected in the splitless mode (Hewlett–Packard 7683 injector) and analyzed on a CP-Sil 5 CB low bleed MS column (25 m × 0.30 μm) (Chrompack, Middelburg, The Netherlands). The oven temperature program used was described previously [16]. The single ion monitoring (SIM) mode was applied for the detection of the respective $(M-15)^+$ ions (*m*/*z* 369 and 457; masses of the molecular ions minus one methyl group of the TMS derivatives of phytanic acid and ω(-1)-hydroxyphytanic acid, respectively). Analyte quantification was performed by integration of the peaks followed by dividing the analyte peak areas by the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion *m*/*z* 233).