ω-Hydroxylation of phytic acid in rat liver microsomes: implications for Refsum disease

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Abstract The 3-methyl-branched fatty acid phytanic acid is degraded by the peroxisomal α-oxidation route because the 3-methyl group blocks β-oxidation. In adult Refsum disease (ARD), peroxisomal α-oxidation is defective, which is caused by mutations in the gene coding for phytanoyl-CoA hydroxylase in the majority of ARD patients. As a consequence, phytanic acid accumulates in tissues and body fluids. This study focuses on an alternative route of phytanic acid degradation, ω-oxidation. The first step in ω-oxidation is hydroxylation at the ω-end of the fatty acid, catalyzed by a member of the cytochrome P450 multi-enzyme family. To study this first step, the formation of hydroxylated intermediates was studied in rat liver microsomes incubated with phytanic acid and NADPH. Two hydroxylated metabolites of phytanic acid were formed, ω- and (ω-1)-hydroxyphytanic acid (ratio of formation, 5:1). The formation of ω-hydroxyphytanic acid was NADPH dependent and inhibited by imidazole derivatives. These results indicate that phytanic acid undergoes ω-hydroxylation in rat liver microsomes and that an isoform of cytochrome P450 catalyzes the first step of phytanic acid ω-oxidation.—Komen, J. C., M. Duran, and R. J. A. Wanders. ω-Hydroxylation of phytanic acid in rat liver microsomes: implications for Refsum disease. J. Lipid Res. 2004. 45: 1341–1346.

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid that originates from phytol, a breakdown product of the chlorophyll molecule. Mammals are able to take up free phytol from the diet and convert it into phytanic acid or directly take up phytanic acid. In general, branched-chain fatty acids are degraded by the β-oxidation pathway in peroxisomes and mitochondria (1). However, this is only true for 2-methyl branched-chain fatty acids. Phytanic acid contains a methyl group at the third carbon atom that blocks β-oxidation. As with other 3-methyl branched fatty acids, phytanic acid degradation proceeds primarily via α-oxidation. During α-oxidation, phytic acid is shortened by one carbon atom (the α-carbon) at the carboxyl end to produce its n-1 analog, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). This fatty acid has a 2-methyl group and can be further degraded by β-oxidation (2–4).

In adult Refsum disease (ARD), the α-oxidation pathway is deficient. In the majority of ARD patients this is attributable to mutations in the gene coding for the first enzyme in this pathway, phytanoyl-CoA hydroxylase (5–7). As a consequence, phytanic acid accumulates in ARD patients. This accumulation of phytic acid is generally used as the biochemical marker of ARD and is believed to be the major cause of the pathology. The symptoms include retinitis pigmentosa, peripheral neuropathy, and cerebellar ataxia (2). Treatment of ARD consists of a diet low in phytic acid, which may be accompanied by plasmapheresis.

Fatty acids may not only undergo α- and/or β-oxidation but also ω-oxidation. In this pathway, the carbon atom at the ω-end of the fatty acid is hydroxylated by a member of the cytochrome P450 enzyme family. This hydroxylated fatty acid is then converted into an aldehyde by an alcohol dehydrogenase, and subsequently this aldehyde is converted into a carboxyl group by an aldehyde dehydrogenase. As a consequence, the final product of the pathway is a dicarboxylic fatty acid, which can be degraded further by β-oxidation from the ω-end.

ω-Oxidation of fatty acids, prostaglandins, and leukotrienes by members of the cytochrome P450 family has been extensively investigated (8, 9), but there is hardly any data on the ω-oxidation of phytic acid except from a single report dating from 1968 (10). In previous studies, presumed metabolites of phytic acid ω-oxidation have been identified in urine of patients suffering from ARD, including 3-methylhexanedioic acid (3-methyladipic acid) and 2,6-dimethyloctanedioic acid (2,6-dimethylsuberic acid) (11–14). These metabolites are virtually undetectable in urine from normal individuals.

The fact that phytic acid ω-oxidation may occur in ARD patients could be a starting point of an alternative treatment of the disease based on the induction of phytic acid ω-oxidation. This hypothesis is all the more at-
tractive because a general characteristic of the cytochrome P450 family is its inducibility by a wide variety of drugs. For this reason, we have studied phytanic acid ω-oxidation, paying particular attention to the first step: the hydroxylation of phytanic acid. The results are described below.

MATERIALS AND METHODS

Materials
Phytanic acid and 3-hydroxyheptadecanoic acid were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD⁺ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonazole, and miconazole were purchased from Sigma (St. Louis, MO). Methyl-β-cyclodextrin was from Fluka (Buchs, Switzerland).

Preparation of rat liver microsomes
Microsomes were isolated from rat livers by differential centrifugation essentially as described by Baudhuin et al. (15). To this end, male Wistar rats fed a standard laboratory diet were fasted overnight before killing and removal of the liver. The livers were rapidly chilled and washed several times in buffer containing 250 mM sucrose, 0.5 mM EDTA, and 2 mM MOPS/KOH (final pH 7.4). Subsequently, the livers were minced and homogenized with a Potter S homogenizer (B. Braun) with a Teflon pestle at 500 rpm (five strokes), followed by centrifugation of the homogenate for 10 min at 550 g. The obtained postnuclear supernatant was subjected to centrifugation at 22,500 g for 10 min to remove mitochondria and lysosomes. Finally, the microsomal fraction was obtained by centrifugation of the supernatant for 3 h at 32,000 g. The microsomal pellet fraction was taken up in PBS containing 5 mM DTT and divided into small aliquots, which were stored at −80°C. The microsomes were sonicated three times for 10 s at 8 W before each experiment. The protein concentration of the microsomal fraction was determined with the method described by Bradford (16).

Phytic acid ω(1)-hydroxylase assay
The standard reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, and rat liver microsomes (1 mg/ml end concentration) plus phytanic acid dissolved in DMSO (200 μM end concentration, unless indicated otherwise). Reactions were initiated by the addition of NADPH at a final concentration of 1 mM. The final reaction volume was 0.2 ml. Reactions were terminated by the addition of 0.2 ml of 1 M HCl. Subsequently, 1 ml of PBS was added followed by the addition of 0.1 ml of 12.1 M HCl. The internal standard (10 nmol of 3-hydroxyheptadecanoic acid in 20 μl of ethanol) was added to this aqueous mixture. The samples were extracted twice with 6 ml of ethylacetate-diethyl ether (1:1, v/v). The organic layer was collected, and the solvents were evaporated under vacuum using a rotary evaporator at room temperature. The residue was dissolved in 4 ml of ethylacetate and further dried with MgSO₄. After spinning down the MgSO₄, the solution was transferred to 4 ml reaction vials, and the solvent was evaporated under nitrogen. To enable GC-MS analysis, the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson (17). TMS ester/ether formation was performed with 40 μl of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and 10 μl of pyridine. The vials were sealed with a Teflon-lined screw cap and incubated at 80°C for 1 h. After the incubation, the solution could be directly used for GC-MS analysis.

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-bled mass spectrometer column (25 m × 0.30 μm) (Chrompack, Middelburg, The Netherlands). The oven temperature was programmed as follows: 70°C for 2 min, 5°C/min to 120°C, 7°C/min to 260°C, 3.5 min hold at 260°C, 15°C/min to 275°C, and hold for 10 min. The identities of the substrate, the internal standard, and the reaction product(s) were verified by taking mass spectra of the pertrimethylsilylated derivatives in the scanning electron impact mode. The single ion monitoring 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 done by integration of the peaks followed by dividing the analyte peak areas with the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion m/z 233).

RESULTS AND DISCUSSION

Hydroxylation of phytanic acid by rat liver microsomes
To study the ω-oxidation of phytanic acid, rat liver microsomes were incubated in a phosphate-buffered medium containing phytanic acid. When NADPH was added,
two products appeared in the chromatogram, one with a retention time of 29.1 min and the other with a retention time of 29.7 min (Fig. 1). The major peak was identified as \( \omega \)-hydroxyphytanic acid (16-hydroxyphytanic acid) according to its mass spectrum (Fig. 2A). Mass spectral analysis of this peak revealed the presence of a fragment \( \text{m/z} \) 103 characteristic for \( \omega \)-hydroxy acids, representing the terminal \( \text{CH}_2\text{OSi(CH}_3\text{)}_3 \) moiety (18). The minor peak corresponds to \(( \omega - 1 \)-hydroxyphytanic acid (15-hydroxyphytanic acid) (Fig. 2B), the product of \(( \omega - 1 \)-hydroxylation. A general characteristic of the TMS derivatives of the hydroxy fatty acids is cleavage of the molecule adjacent to the hydroxyl group (19). It was deduced from the mass spectrum in Fig. 2B that the base peak at \( \text{m/z} \) 131 represents the \( \text{(CH}_3\text{)}_2\text{COSi(CH}_3\text{)}_3 \) moiety, in analogy with the mass spectrum of 3-hydroxyisovaleric acid.

Optimization of the hydroxylase assay

The hydroxylation assay was further optimized for the formation of \( \omega \)-hydroxyphytanic acid. First, the influence of methyl-\( \beta \)-cyclodextrin, added to increase solubilization of the substrate, on the formation of \( \omega \)-hydroxyphytanic acid was tested (20, 21). Figure 3A shows that methyl-\( \beta \)-cyclodextrin has a positive effect on the assay, with an optimum concentration of 0.75 mg/ml. Higher methyl-\( \beta \)-cyclodextrin concentrations had a negative effect on the assay, presumably because of decreased substrate availability.

To determine the optimal pH value for our assay, a combined buffer system with 50 mM potassium phosphate and 50 mM pyrophosphate was used to cover the pH range 6.6–9.1. The result depicted in Fig. 3B shows an optimum pH of 7.6. Accordingly, all subsequent experiments were performed in 0.1 M potassium phosphate at pH 7.6.

Our next aim was to analyze the kinetics of the hydroxylation of phytanic acid under the conditions determined in the previous experiments. We already established that NADPH was an essential component of the reaction mixture (Fig. 1). The NADPH dependency of the reaction was studied in more detail by performing the assay at different NADPH concentrations. For this purpose, we included a NADPH-regenerating system (10 mM isocitrate, 10 mM MgCl\(_2\), and 0.08 unit of isocitrate dehydrogenase) in the assay mixture, because large amounts NADPH were consumed during the assay (data not shown). The formation of \( \omega \)-hydroxyphytanic acid followed simple Michaelis-
Menten kinetics (Fig. 3C). The apparent $K_m$ for NADPH derived from the Lineweaver-Burk plot (inset in Fig. 3C) was 35 μM.

Subsequently, we determined the effect of increasing phytanic acid concentrations on the formation of ω-hydroxyphytanic acid. To this end, different concentrations of phytanic acid were added in a fixed molar ratio between phytanic acid and methyl-β-cyclodextrin (Fig. 3D). An apparent $K_m$ of 114 ± 9 μM was found.

Based on the experiments described above, we selected the following assay conditions: 0.75 mg/ml methyl-β-cyclodextrin, 100 mM potassium phosphate (pH 7.6), 1 mM NADPH, and 200 μM phytanic acid. Under these conditions, the formation of ω-hydroxyphytanic acid was linear with time up to 60 min and with protein up to 1 mg/ml (data not shown).

Effect of imidazole derivatives on the formation of ω-hydroxyphytanic acid

Imidazole antifungal agents are known inhibitors of cytochrome P450 enzymes (22–24). To measure the influence of four different imidazole derivatives on the formation of ω-hydroxyphytanic acid, we studied the effects of different concentrations of these compounds on the formation of ω-hydroxyphytanic acid (Fig. 4A) and (ω-1)-hydroxyphy-
Phytic acid is hydroxylated to its \(\omega\) and (\(\omega\)-1)-hydroxy analogs in rat liver microsomes. The enzyme(s) responsible for phytic acid \(\omega\)- and (\(\omega\)-1)-hydroxylation were shown to be NADPH dependent. Therefore, the formation of phytic acid \(\omega\)- and (\(\omega\)-1)-hydroxyphytic acid is inhibited by imidazole antimycotics. The inhibition by the imidazole derivatives showed a different pattern for the two products. This strongly suggests that different members of the cytochrome P450 multienzyme family are responsible for the formation of \(\omega\)- and (\(\omega\)-1)-phytanic acid. Future work is aimed at the identification of the specific cytochrome P450s involved and the resolution of the subsequent metabolism of \(\omega\)-hydroxyphytic acid. Preliminary results have already shown the formation of the dicarboxylic acid of phytic acid. Furthermore, we will study whether phytic acid \(\omega\)-oxidation can be induced to generate a new therapeutic option for Refsum disease patients by induction of the capacity to \(\omega\)-oxidize phytic acid.

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