PHYTANIC ACID OF GLYCEROLIPIDS IN SERA FROM TWO SIBLING PATIENTS WITH REFSUM'S DISEASE AND THEIR PARENTS

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ABSTRACT

Phytanic acid, an unusual 20-carbon, branched chain fatty acid was found not only in the serum lipids of two siblings with Refsum's disease, but also in those of their parents. The family is of European ethnic origin. The serum lipids of the proband, the eldest daughter, her sister, mother and father contained 6.8, 20.2, 8.1, and 0.3 mg of phytanic acid/100 ml of serum, respectively, while healthy Japanese persons have only trace amounts. It was noted that phytanic acid was detectable mainly in glycerolipids such as triacylglycerol, phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine, but not in non-glycerolipids such as cholesteryl ester and sphingomyelin. The specific incorporation of exogenous phytanic acid into these glycerolipids was analyzed in terms of the biosynthesis of both glycerolipids and non-glycerolipids and the pathogenesis of Refsum's disease.

Refsum's disease, or hereditary ataxia polyneuritis (10), is a rare inborn error causing defective α -oxidation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). Its inheritance pattern is autosomal recessive. Refsum's disease has not, to date, identified in the Japanese population. This disease is known to be due to phytanic acid accumulation in blood and tissues (10). The usual dietary intake of phytanic acid is about 50–100 mg/day. Thus, Refsum's disease patients are treated with phytanate-free diets to reduce blood and tissues levels and thereby improve the peripheral nerve dysfunction caused by phytanic acid accumulation. The dietary treatment should be initiated as early as possible and continued throughout life. For the purpose of assessing dietary treatment, we analyzed phytanic acid levels in the sera of two siblings and compared with the results with those of their parents and healthy Japanese persons. It was noteworthy that this acid was found in glycerolipids, but not in non-glycerolipids. The specific incorporation of phytanic acid into glycerolipids

was investigated in terms of the biosynthesis of both glycerolipids and non-glycerolipids and the pathogenesis of this disease. The findings are presented in this paper.

MATERIALS AND METHODS

Case Report

The proband, the eldest of two daughters, appeared normal at birth. She was found to have cataracts at age 4 years. Laboratory tests revealed no signs of hepatic or renal dysfunction. Clinical investigations of her sera yielded the following results: triacylglycerol, 75 mg/100 ml; phospholipids, 214 mg/100 ml; β -lipoprotein, 294 mg/100 ml; NEFA, 0.11 mEq/l; HDL-cholesterol, 63 mg/100 ml; CPK, 149 mIU; β 2-microglobulin, 1.6 mg/l; iron, 38 μ g/100 ml; and copper, 144 μ g/100 ml. Counts of leukocytes, erythrocytes and platelets, and levels of serum protein and α -globulin were all within normal range. Enzymatic α -oxidation of phytanic acid was very low (Table 1).

Her sister also appeared normal at birth, but cataracts, delayed speech, poor comprehension and other developmental disabilities were appeared by

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Table 1 *Enzymatic Activities of Phytanic Acid-Oxidation in Leukocytes and Total Phytanic Acid and Cholesterol Contents in Sera from Two Sibling Patients, Their Parents and Healthy Controls*

Assays	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Control (n=4)
Enzymatic activity (pmol/h/mg protein)	0.14	0.12	—	—	4.60
Phytanic acid (mg/100 ml of sera)	6.8	20.2	0.3	8.1	trace
Cholesterol (mg/100 ml of sera)	172	137	184	170	174

age three. She was subsequently enrolled in a special training course for handicapped children. Clinical investigations of her sera yielded the following results: triacylglycerol, 129 mg/100 ml; phospholipids, 179 mg/100 ml; β -lipoprotein, 327 mg/100 ml; NEFA, 0.1 mEq/l; HDL-cholesterol, 38 mg/100 ml; β 2-microglobulin, 1.8 mg/l; CPK, 126 mIU; iron, 97 μ g/100 ml; and copper, 133 μ g/100 ml. Enzymatic α -oxidation of phytanic acid was also very low (Table 1).

Extraction and Analytical Procedures for Serum Lipids

Total lipids were extracted from 0.5 ml serum samples obtained from each of the two siblings, their parents and healthy Japanese subjects, with a 5 ml mixture of hexane-isopropanol (3:2, v/v) containing 250 μ g of butylated hydroxytoluene as an antioxidant, and thoroughly re-extracted with 5 ml of the same solution without the antioxidant. The combined extracts were evaporated to dryness under a nitrogen stream. The residue was dissolved in a given volume of chloroform-methanol (2:1, v/v). An aliquot of the lipid solution was applied to TLC. Simple lipids were separated by one-dimensional TLC, in which a silica gel plate was developed with hexane-diethyl ether-acetic acid (88:22:1). Phospholipids were identified by two-dimensional TLC, in which the plate was developed with chloroform-methanol-2.5 N ammonium hydroxide (60:35:8) for the first dimension, air-dried and exposed to saturated hydrogen chloride gas for 5 min to cleave the vinyl ether of plasmalogen, and finally developed with chloroform-methanol-acetone-acetic acid-water (70:15:30:15:7.5) for the second dimension. Phosphorus contents of separated phospholipids were determined by the method of Bartlett (1, 6). After methanolysis of total lipids, individual simple lipids and phospho-

lipids, respectively, the fatty acid methylesters or free cholesterol and its derivatives thus obtained were analyzed by GLC using an OV-101 silicone capillary column (0.2 mm $\times$ 25 m), in which the column temperature was set at either 160°C to 220°C or 190°C to 260°C at 2°C/min (13). Phytanic acid methylester could be separated from normal fatty acid methylesters and also identified by GC-MS (GC-9A/OP-1000A, Shimadzu) in EI (70 eV) and CI (isobutane) modes (5), using a phytanic acid standard kindly provided by Dr M. Oshima of the Clinical Research Institute, National Medical Center, Tokyo, and by Dr Y. Seyama of the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, The University of Tokyo.

RESULTS

Total Phytanic Acid and Cholesterol Contents in Individual Sera from Two Siblings and Their Parents

After methanolysis of each total lipid extract, the fatty acid methylesters thus obtained were analyzed by GLC. As shown in Fig. 1, the phytanic acid methylester was identical to the standard, and was distinguishable from normal fatty acid methylesters. Phytanic acid and cholesterol were quantitatively estimated by GLC using constant amounts of the internal standards (C21 acid or cholestanol) (11).

As shown in Fig. 2, the phytanic acid methylester was also identified by GC-MS. The CI spectrum indicated a major ion peak at M/Z 313 (326—OCH₃+H₂O), while the EI spectrum showed ion peaks at M/Z 71, 88 (101—OCH₃+H₂O), and 101. Total phytanic acid and cholesterol contents of the sera are shown in Table 1. The younger girl's phytanic acid level was higher than that of her sister, although both showed very low enzymatic α -oxidation of phytanic acid. The accumulated amounts of

Fig. 1 Gas-liquid chromatograms of fatty acid methyl esters obtained after methanolysis of total lipids in sera from the younger sister with Refsum's disease. The chromatography was performed on an OV-101 capillary column at programmed temperatures ranging from 190°C to 220°C at 2°C/min. BHT, butylated hydroxytoluene; ★phytanic acid methyl ester

Fig. 2 Chemical structure of the phytanic acid methyl ester and its mass spectra in CI (isobutane) and EI (70 eV) modes

phytanic acid correlated roughly with clinical severity in both patients. However, their healthy parents also, unexpectedly, had phytanic acid in

their sera, although the father had a much lower level. As expected, healthy Japanese persons had trace amounts in their sera. All subjects had nor-

Table 2 *Lipid Composition in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Lipids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
CE	35.9	25.5	41.0	28.4	29.5
TG	18.8	30.0	13.8	27.3	22.0
FFA	1.3	1.3	2.6	3.2	3.9
FC	6.9	4.9	5.4	3.8	6.7
PC	24.1	23.5	22.5	21.6	24.4
SM	9.3	8.1	7.8	6.7	6.4
Lyso-PC	1.5	4.7	4.8	5.6	6.4
PE	2.2	2.0	2.1	3.4	2.1

CE, cholesteryl ester; TG, triacylglycerol; FFA, free fatty acid; FC, free cholesterol; PC, phosphatidylcholine; SM, sphingomyelin; Lyso-PC, lysophosphatidylcholine; PE, phosphatidylethanolamine

Table 3 *Fatty Acid Composition of Triacylglycerol in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Fatty acids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
C16:1	3.0	2.3	2.5	2.5	5.4 ± 1.1
C16	27.3	27.3	28.6	22.2	27.7 ± 0.1
C18:2	20.9	10.8	23.8	29.7	23.3 ± 1.7
C18:1	31.7	31.2	35.7	29.2	35.4 ± 2.4
Phytanate	9.9	17.0	0.5	8.5	—
C18	5.5	10.1	5.1	6.0	4.0 ± 0.5
C20:4	1.7	1.2	3.8	1.8	3.2 ± 1.0
C22:6	—	—	—	—	1.5 ± 1.0

Table 4 *Fatty Acid Composition of Free Fatty Acid Fraction in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Fatty acids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
C16:1	1.8	1.9	1.6	1.2	2.3 ± 0.5
C16	34.9	26.8	43.2	28.0	40.5 ± 2.4
C18:2	12.0	13.6	11.4	20.4	15.9 ± 1.9
C18:1	21.4	25.3	17.8	22.5	16.2 ± 1.7
Phytanate	9.0	13.1	0.4	9.5	—
C18	18.1	12.7	22.2	17.7	16.5 ± 1.4
C20:4	2.0	5.6	2.7	0.7	6.4 ± 0.4
C20:3	0.8	1.0	0.7	—	1.3 ± 0.4
C22:6	—	—	—	—	1.1 ± 1.1

mal total serum cholesterol levels.

Serum Lipid Compositions and the Fatty Acid Compositions of Individual Lipids

Simple lipids separated by one-dimensional TLC

were quantitatively estimated by GLC, whereas phospholipids separated by two-dimensional TLC were quantitatively estimated by phosphorus contents. Based on these results, the lipid compositions of sera were determined as shown in Table 2. The two siblings showed no abnormalities in the com-

Table 5 *Fatty Acid Composition of Phosphatidylcholine in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Fatty acids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
C16:1	0.7	0.7	0.6	0.6	0.7 ± 0.3
C16	34.3	30.3	32.2	35.3	33.7 ± 2.8
C18:2	23.3	19.2	25.0	26.4	23.9 ± 4.0
C18:1	8.1	11.1	10.8	11.9	12.3 ± 1.3
Phytanate	4.9	4.1	—	—	—
C18	19.0	18.1	15.9	13.0	17.1 ± 2.9
C20:4	7.4	11.5	11.4	8.5	8.8 ± 0.3
C20:3	2.6	3.9	3.5	3.5	2.8 ± 0.6
C20:1	0.5	0.4	0.5	0.7	0.6 ± 0.1

Table 6 *Fatty Acid Composition of Cholesteryl Ester in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Fatty acids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
C16:1	2.2	3.9	2.6	2.3	2.4 ± 0.5
C16	11.3	12.7	11.5	10.6	11.5 ± 1.7
C18:2	60.6	46.8	57.6	62.5	56.8 ± 4.1
C18:1	16.1	26.0	17.2	16.2	19.0 ± 1.5
C18	0.6	1.0	0.7	0.7	1.2 ± 0.2
C20:4	9.2	9.6	10.4	7.0	8.4 ± 1.6
C20:3	—	—	—	0.8	0.7 ± 0.1
C22:6	—	—	—	—	0.1 ± 0.1

Table 7 *Fatty Acid Composition of Sphingomyelin in Sera from Two Sibling Patients, Their Parents and Healthy Controls (%)*

Fatty acids	Eldest sister (Patient 1)	Younger sister (Patient 2)	Father	Mother	Controls (n=4)
C16	33.2	36.3	27.9	35.0	30.8 ± 1.2
C18	9.5	12.4	9.7	9.5	9.5 ± 0.9
C20	5.2	4.6	5.4	6.6	5.6 ± 0.3
C22	15.3	16.3	15.9	18.2	18.6 ± 3.1
C23	7.7	6.7	6.5	7.6	6.1 ± 0.3
C24:1	14.6	10.3	19.4	7.7	15.0 ± 2.6
C24	13.0	12.0	14.0	13.8	12.8 ± 0.6
C25	1.2	1.0	0.8	1.1	1.1 ± 0.2
C26	0.3	0.4	0.4	0.5	0.5 ± 0.2

positions of their serum lipids. Fatty acid compositions of these individual lipids were determined by GLC and the results are shown in Tables 3–7. Triacylglycerol, free fatty acids, phosphatidylcholine, and other minor glycerophospholipids (data not

shown) in the sera of these two siblings definitely contained phytanic acid. In the parents, however, only triacylglycerol and free fatty acids contained phytanic acid. In contrast, cholesteryl ester and sphingomyelin contained no phytanic acid

in any of the sera.

DISCUSSION

It is known that phytanic acid is not an endogenous, but rather an exogenous substance acquired through dietary intake (10). A major mechanism of phytanic acid degradation is the α -oxidation pathway, involving an initial α -hydroxylation followed by decarboxylation to generate pristanic acid (2, 6, 10, 14-tetramethylpentadecanoic acid). Further degradation of the pristanic acid results from a series of β -oxidations of straight chain fatty acids (10). Recently, ten Brink *et al.* (14) reported that four different groups of diseases were characterized by defects in phytanic acid α -oxidation and/or pristanic acid β -oxidation: 1) Refsum's disease by a defect in phytanic acid α -oxidation; 2) rhizomelic chondrodysplasia punctata, by a defect in 2-hydroxyphytanic acid decarboxylation; 3) generalized peroxisomal disorders, with defects in 2-hydroxyphytanic acid decarboxylation and in pristanic acid β -oxidation; and 4) single peroxisomal β -oxidation enzyme deficiencies, with a defect in pristanic acid β -oxidation, resulting in an impaired phytanic acid α -oxidation secondary to inhibition. In this study, 2-hydroxyphytanic acid and pristanic acid were undetectable in the sera of two siblings with Refsum's disease, although ten Brink *et al.* (14) found very small amounts of both acids in plasma from both healthy individuals and Refsum's disease patients. The disparity in results was thought to be due to differences in the sensitivity of the measurement technique used. The finding of phytanic acid suggested that the disease might be due to a defect in phytanic acid α -hydroxylation. However, Refsum's disease does not seem to involve a systemic error in α -hydroxylation or in one-carbon degradation of fatty acids, based on the absence of any deficiency in hydroxy fatty acids or odd-numbered fatty acids in either cerebroside or sulfatide in the tissues studied by MacBrinn and O'Brien (8). Although it has been reported that trace amounts of 3,7,11,15-tetramethylhexadec-15-monoenoic acid and 3,7,11,15-tetramethylhexadec-6,10,14-trienoic acid can be identified by mass-spectrometry in sera from Refsum's disease patients (2, 3), neither we nor ten Brink *et al.* (14) were able to detect them in the patients. Although the two siblings we examined seemed to be homozygous in terms of enzymatic α -oxidation of phytanic acid, they had different clinical symptoms and serum phytanic acid contents. Their clinical severities cor-

related with the amounts of accumulated phytanate. On the other hand, their parents presented no clinical signs whatsoever, though the mother unexpectedly had a serum phytanic acid level as high as that of her eldest daughter. The father also had phytanic acid in his serum but at a much lower level. Since enzymatic α -oxidation of phytanic acid was not examined in either parent, it was not clear whether they were heterozygous. There are no recorded examples of established heterozygotes with clinical disease (10). Regarding this problem, Kahlke and Richterich (7) reported the mother of a patient who had a phytanate level as high as that of her affected son. Nevin *et al.* (9) also reported that phytanate accounted for 2.6% of total fatty acids in the asymptomatic mother of two patients. Gibberd *et al.* (4) found 4 mg of phytanate/100 ml of sera in the asymptomatic mother of a confirmed case. Phytanate was found in both triacylglycerol and phosphatidylcholine in our two sibling patients, suggesting that exogenous phytanate is incorporated into major triacylglycerol and phosphatidylcholine via the diacylglycerol metabolic pathway in the intestine or liver. However, the phytanate was found only in triacylglycerol in the parents of our patients. Thus, it would be of interest to know whether the phytanate accumulation in phosphatidylcholine gradually produces serious cell membrane damage, resulting in the disease. The finding that major cholesteryl ester contains no phytanate may suggest that the lecithin (phosphatidylcholine)-cholesterol acyltransferase in blood and acylcholesterol acyltransferase in the liver can distinguish phytanate from other normal fatty acids. Similarly, sphingosine-acyl-CoA acyltransferase for the synthesis of ceramide, the precursor of sphingomyelin may also recognize phytanate and therefore not incorporate it into sphingomyelin. It is likely that virtually all sphingolipids are devoid of phytanate, even in the tissues and organs of patients with Refsum's disease (8). These patients may thus be spared the severe neurological symptoms seen in patients with sphingolipidoses, that is, Tay-Sachs disease (13) and metachromatic leukodystrophy (12). According to a recently published review (10), the treatment of this disease involves large volume plasma exchange and dietary control, based on phytanate-free and high calorie diets. The high calorie intake prevents mobilization of phytanate from fat stores that contain exclusively triacylglycerol. Our findings concerning the distribution of phytanate in glycerolipids support the concept that dietary treatment is the most effective

form of therapy and brings about significant improvement in the peripheral nerve dysfunction characteristic of Refsum's disease.

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