Refsum disease: a defect in the α-oxidation of phytanic acid in peroxisomes

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Abstract The oxidation of phytanic acid to pristanic acid was previously demonstrated to be deficient in monolayer cultures of skin fibroblasts (Herndon et al. 1969. *J. Clin. Invest.* **48:** 1017–1032). However, identification of subcellular organelle with deficient enzyme activity has not been established. To define the subcellular organelle with deficient enzyme activity in the catabolism of phytanic acid, we measured the oxidation of [1-^{14}C] phytanic acid to $^{14}CO_2$ and pristanic acid in different subcellular organelles isolated from cultured skin fibroblasts from control and Refsum patients. The rates of oxidation of phytanic acid in peroxisomes, mitochondria, and endoplasmic reticulum were 37.1 ± 2.65, 1.9 ± 0.3, and 0.4 ± 0.07 pmol/h per mg protein, respectively, from control fibroblasts. The phytanic acid oxidation activity in mitochondria (2.04 ± 0.7 pmol/h per mg protein) and endoplasmic reticulum (0.43 ± 0.2 pmol/h per mg protein) from Refsum fibroblasts was similar to control fibroblasts. However, phytanic acid oxidation in peroxisomes from Refsum fibroblasts was not detected at all the protein concentrations tested. On the other hand, the peroxisomes from Refsum fibroblasts had normal rates of activation and oxidation of palmitic and lignoceric acids, suggesting that the peroxisomes isolated from Refsum fibroblasts were metabolically active. The phytanoyl-CoA ligase, the first enzyme in the α-oxidation pathway, had activity similar to that in peroxisomes from control (9.86 ± 0.09 nmol/h per mg protein) and Refsum (10.25 ± 0.31 nmol/h per mg protein) fibroblasts. The data described here clearly demonstrate that pathognomonic accumulation of phytanic acid in patients with Refsum disease is due to the deficient activity of peroxisomal α-oxidation enzyme system.—**Singh, I., K. Pahan, A. K. Singh, and E. Barbosa.** Refsum disease: a defect in the α-oxidation of phytanic acid in peroxisomes. *J. Lipid Res.* 1993. **34:** 1755–1764.

Supplementary key words phytanic acid α-oxidation • phytanoyl-CoA ligase • fibroblasts • pristanic acid

Refsum disease (heredopathia atactica polyneuritiformis), a rare neurological autosomal inherited metabolic disorder, is characterized by retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia, and pathognomonic accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in body fluids and tissues (1–3) due to a defect in the α-oxidation of phytanic acid (3–5). The α-oxidation of phytanic acid to pristanic acid involves the formation of α-hydroxyphytanic acid, an intermediate, which is then decarboxylated to pristanic acid. The defect in Refsum disease was identified to the formation of α-hydroxyphytanic acid in cultured monolayer cells (6, 7). However, the subcellular organelle responsible for the defective catabolism of phytanic acid has not yet been resolved (8–15).

The findings in monkey (12) and human liver (12, 13) indicated that phytanic acid α-oxidation to pristanic acid occurs in mitochondria but not peroxisomes. As a result, it was proposed that phytanic acid oxidation is a mitochondrial function and Refsum disease is a mitochondrial disorder (12, 13). The conclusion that phytanic acid is oxidized in mitochondria is not consistent with excessive accumulation and defective oxidation of phytanic acid (16, 17) as well as of pristanic acid in diseases in which there is a lack of peroxisomes (18–20). In addition to the observations of oxidation of phytanic acid in isolated mitochondria from rat (8–10) and human liver (12, 13) tissues, recent studies have provided evidence that phytanic acid is also oxidized to pristanic acid in endoplasmic reticulum (ER) from rat liver (14) and in peroxisomes from human cultured skin fibroblasts (15). To understand the basis for these discrepancies in various studies, we studied the phytanic acid oxidation in different subcellular organelles isolated from human tissues (liver and cultured skin fibroblasts) and rat tissues (liver and cultured skin fibroblasts). We found that phytanic acid is predominantly α-oxidized to pristanic acid in peroxisomes in humans and in mitochondria in rodents (15, 21). We have also found that Nycodenz, a gradient material used for isolation of subcellular organelles, has an inhibitory effect on the oxidation of phytanic acid to pristanic acid (21) and this inhibition in peroxisomes may have contributed to the conclusion by other investigators (12, 13) that, in humans, the

Abbreviations: POCA, 2-[-5-(chlorophenyl)-pentyl] oxiran-2-carboxylic acid; CPT, carnitine palmitoyl transferase.

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oxidation of phytanic acid is a mitochondrial function.

The rate of oxidation of [1-^{14}C]phytanic acid to pristanic acid in peroxisomes was 26-times higher than that in mitochondria and 130-times higher than that in the endoplasmic reticulum (15, 21). It raises an interesting question as to whether the excessive accumulation of phytanic acid in Refsum disease is due to deficient activity in peroxisomes and/or mitochondria and/or ER or any combination. To define the subcellular site for α-oxidation of phytanic acid which is deficient in Refsum disease, we studied the oxidation of phytanic acid in subcellular organelles isolated from cultured skin fibroblasts from patients with Refsum disease and from controls. In this study, we report that phytanic acid is predominantly oxidized in peroxisomes and its oxidation in peroxisomes isolated from cultured human skin fibroblasts from patients with Refsum disease is absent, whereas activities in mitochondria and ER from Refsum fibroblasts were similar to the mitochondria and ER activities in control fibroblasts. Therefore, we propose that the accumulation of phytanic acid in Refsum disease is due to an abnormality in the α-oxidation of phytanic acid in peroxisomes and that phytanic acid catabolism in humans is an important function of peroxisomes.

MATERIAL AND METHODS

Experimental procedures

Bovine calf serum, trypsin, and tissue culture media were purchased from Gibco (Grand Island, NY). NADH, NADP, NADPH, L-carnitine, cytochrome c, and α-cyclodextrin were purchased from Sigma Chemical Co. (St. Louis, MO). ATP and CoASH were obtained from P-L Biochemicals (Milwaukee, WI). Nycodenz was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). [1-^{14}C]palmitic acid (58.7 mCi/mmol) and [1-^{14}C]phytanic acid (55 mCi/mmol) were purchased from Amersham International (Arlington Heights, IL). [U-^{14}C]glycerol-3-phosphate (27 mCi/mmol) was purchased from ICN Radiochemicals (Irvine, CA). [U-^{14}C]dihydroxyacetone phosphate was prepared from [U-^{14}C]glycerol-3-phosphate by the methods of Davis and Hajra (22) as modified by Schutgens et al. (23). [1-^{14}C]lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with K[^{14}CN] as described (24).

Isolation of peroxisomes from cultured skin fibroblasts

Fibroblast cell lines from control and Refsum disease patients were cultured and fractionated as described previously (25). The cellular homogenates were centrifuged at 500 *g* for 5 min and the supernatant (post-nuclear fraction) was further fractionated by isopycnic equilibrium centrifugation in continuous Nycodenz gradients. Tubes (39 ml) for a JV-20 Beckman rotor were layered with 4 ml of 35% (w/v) Nycodenz and 28 ml of a continuous gradient consisting of 0–30% (w/v) Nycodenz in homogenization medium. The fractions with highest catalase (peroxisomal peak), cytochrome c oxidase (mitochondrial peak), and NADPH cytochrome c reductase (ER peak) from the gradient were pooled and dialyzed against the homogenization buffer for 2 h with two changes to lower the concentration of Nycodenz, as described previously (25).

Marker enzymes

Gradient fractions from human skin fibroblasts (control and Refsum) were analyzed for the following subcellular fractions: catalase (26) and DHAP acyltransferase (22) as modified by Schutgens et al. (23) for peroxisomes, cytochrome c oxidase for mitochondria (27), and NADPH-cytochrome c reductase for microsomes (28). The free catalase activity was measured in homogenates without the addition of exogenous Triton X-100 (1% final concentration) and total catalase activity in the presence of 1% Triton X-100 (26). Protein concentration was determined by the procedure of Bradford (29).

Enzyme assay for phytanic acid oxidation

The α-oxidation of phytanic acid to pristanic acid was measured as CO_2 released from [1-^{14}C]phytanic acid. [1-^{14}C]phytanic acid (12 μM) suspended in α-cyclodextrin (30) was added to the fatty acid medium as described previously (31). The reaction was started by the addition of 10–50 μg of enzyme protein and was stopped with 200 μl of 4 N H_2SO_2 after 2 h. The $^{14}CO_2$ was collected in KOH-wetted cotton by shaking the tubes overnight at 37°C. The cotton was transferred into a scintillation vial and the radioactivity was measured.

RESULTS

Specific activities in fibroblast homogenates

Table 1 shows the specific activities of marker enzymes for different subcellular organelles in the post-nuclear fraction from control and Refsum fibroblasts. There was no difference in enzyme activities for peroxisomes (catalase and dihydroxyacetonephosphate acyltransferase), mitochondria (cytochrome c oxidase), and ER (NADPH-cytochrome c reductase) between control and Refsum fibroblasts. The recovery of marker enzymes and enzymes for oxidation of different fatty acids (palmitic, lignoceric, and phytanic acids) in post-nuclear fraction from control and Refsum fibroblasts was between 85 and 90%. The specific activities of enzymes for oxidation of palmitic and lignoceric acids were similar in Refsum and control fibroblasts. However, the activity for oxidation of phytanic acid in post-nuclear fraction from Refsum disease fibroblasts was only 6% of the activity in control fibroblasts.

TABLE 1. Specific activities of marker enzymes and oxidation of fatty acids in post-nuclear fraction from cultured skin fibroblasts

Enzymes	Control	Refsum	Refsum/Control
	mU/mg protein		
Catalase	6.01 ± 0.45	5.97 ± 0.53	0.99
Cytochrome c oxidase	0.92 ± 0.21	0.89 ± 0.31	0.97
NADPH cytochrome c reductase	4.59 ± 0.59	4.77 ± 0.66	1.04
Dihydroxyacetone phosphate acyltransferase (n = 2)	12.71	11.52	0.91
	nmol/h/mg protein		
Palmitic acid oxidation	1.6 ± 0.4	1.8 ± 0.4	1.12
Palmitoyl-CoA ligase	8.5 ± 1.7	7.9 ± 1.9	0.93
Lignoceric acid oxidation	0.07 ± 0.01	0.08 ± 0.01	1.14
Lignoceroyl-CoA ligase	0.29 ± 0.08	0.33 ± 0.06	1.14
Phytanic acid oxidation	0.011 ± 0.002	0.00066 ± 0.00012	0.06
Phytanoyl-CoA ligase	1.86 ± 0.25	1.92 ± 0.3	1.03
Percent distribution of catalase			
Membrane bound	76,73	69,74	
Free	24,27	31,26	

Enzyme activities are expressed as mean ± SD in three different lines of control and Refsum cells.

The deficiency of phytanic acid oxidation is also observed in diseases with defects in biogenesis of peroxisomes (e.g., Zellweger syndrome) (16, 19, 20, 32) but the normal activity of dihydroxyacetone phosphate acyltransferase and normal oxidation of palmitic and lignoceric acids and deficient oxidation of phytanic acid clearly identify these cell lines as fibroblasts from patients with classical Refsum disease. Moreover, the similar distribution of catalase in peroxisomes (membrane-bound activity) and cytosol in Refsum and control fibroblasts (Table 1) is consistent with previous observation that Refsum fibroblasts have cytochemically normal peroxisomes (33). The post-nuclear fractions from control and Refsum fibroblasts were used for isolation of subcellular organelles by density gradient centrifugation.

Distribution of marker enzymes for different organelles and enzyme activities for oxidation of fatty acids in isopycnic gradient of post-nuclear fraction from Refsum and control fibroblasts

To identify the subcellular organelles with deficient enzyme activity for oxidation of phytanic acid, the subcellular organelles from cultured skin fibroblasts from control and Refsum were prepared in Nycodenz gradient according to a procedure described previously from this laboratory (25). This procedure allows us to isolate intact peroxisomes (34) that exhibit complex metabolic functions such as α- and β-oxidation of fatty acids (15, 21, 25, 34, 35). The distribution of marker enzymes for different organelles is shown in **Fig. 1.** The relative specific activities of marker enzymes for different subcellular organelles in mitochondria, peroxisomes, and ER and percent contamination of these fractions by other organelles are shown in **Table 2.** The specific activities for oxidation of fatty acids in mitochondrial, ER, and peroxisomal peak fractions after removal of Nycodenz by dialysis (21) are summarized in **Table 3.** The recovery of phytanic acid oxidation in the gradients from control and Refsum cells was 56.3% and 83.7%, respectively. The recovery of phytanic acid oxidation activity was lower in controls than Refsum because the peroxisomal activity, observed in control cells, is relatively unstable as compared to the mitochondrial and ER activities, as observed from control and Refsum cells. The phytanic acid oxidation in control cells was localized in gradient regions corresponding to marker enzymes for peroxisomes (62%), mitochondria (31%), and microsomes (6.6%). Based on the distribution of catalase in the mitochondrial and ER regions of the gradient, a significant amount of phytanic acid oxidation activity observed in mitochondria and ER can be accounted for by peroxisomal contamination of these fractions.

Nycodenz is the material of choice for separation of subcellular organelles by isopycnic density gradient centrifugation (12, 13, 15, 21, 25, 34, 35). We have recently reported that Nycodenz has an inhibitory effect on the oxidation of phytanic acid (21). This inhibition of phytanic acid was reversible, i.e., the inhibition of Nycodenz can be decreased or eliminated by dialysis against isotonic solution (0.25 M sucrose, 3 mM imidazole, and 1 mM Na_2 EDTA buffer, pH 7.4) (**Fig. 2-A**) and addition of Nycodenz to already dialyzed peroxisomes reproduced the inhibition observed in peroxisomes directly from the gradient without dialysis (Fig. 2-B). The rates of oxidation of phytanic acid in different subcellular organelles

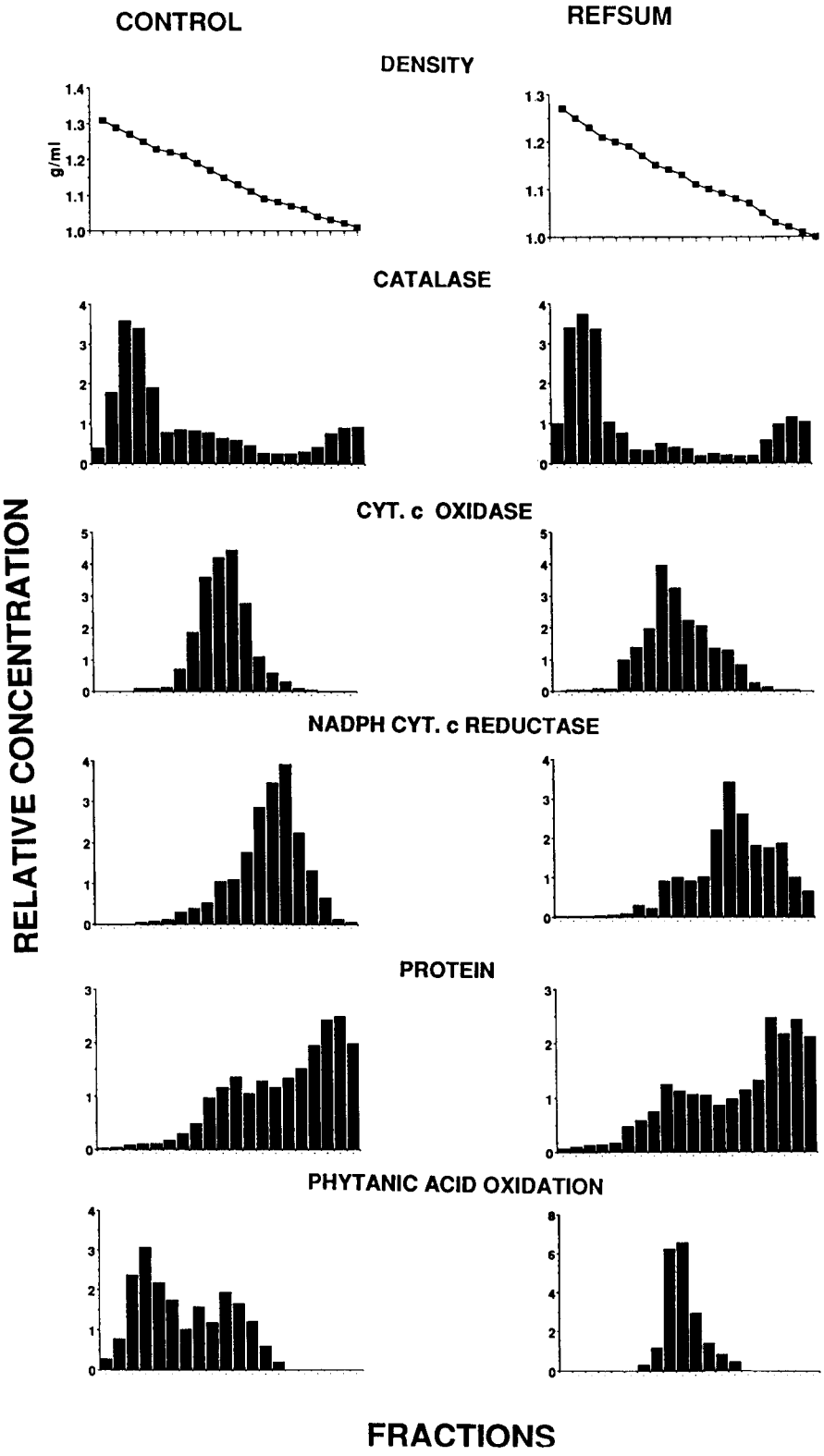

Fig. 1. Subcellular fractionation of cultured human skin fibroblasts from control and Refsum patients. Human skin fibroblasts were fractionated by differential and density gradient centrifugation as described in the text. The distribution of subcellular organelles in the gradient from cultured skin fibroblasts was identified by their marker enzymes: catalase for peroxisomes, cytochrome c oxidase for mitochondria, and NADPH cytochrome c reductase for endoplasmic reticulum. The rates of oxidation of phytanic acid in different fractions from the gradient after dialysis were measured as described in the text. These results are average of two gradients.

TABLE 2. Relative specific activities of marker enzymes and percent contamination in subcellular fractions isolated from control and Refsum cultured skin fibroblasts

	Relative Specific Activity			% Contamination		
	Peroxisomes	Mitochondria	Microsomes	Peroxisomes	Mitochondria	Microsomes
Catalase						
Control	28.76 ± 7.48	0.56 ± 0.12	0.19 ± 0.02		1.95 ± 0.30	1.63 ± 0.42
Refsum	26.32 ± 3.55	0.47 ± 0.10	0.21 ± 0.04		2.05 ± 0.26	1.47 ± 0.08
Cytochrome c oxidase						
Control	0.35 ± 0.12	3.25 ± 0.55	0.72 ± 0.21	0.85 ± 0.41		9.82 ± 2.35
Refsum	0.38 ± 0.08	3.17 ± 0.82	0.82 ± 0.15	0.81 ± 0.24		13.24 ± 3.40
NADPH cytochrome c reductase						
Control	0.21 ± 0.05	0.69 ± 0.14	2.95 ± 0.52	0.65 ± 0.15	16.5 ± 3.82	
Refsum	0.27 ± 0.05	0.77 ± 0.12	2.88 ± 0.41	0.76 ± 0.11	17.13 ± 2.54	

Relative specific activities and percent contamination are expressed as the means ± SD in different cell lines. There were no significant differences between control and Refsum fibroblasts.

(peroxisomal, mitochondrial, and ER) from control and Refsum fibroblasts before and after removal of Nycodenz by dialysis against isotonic solution were also examined at different protein concentrations (**Fig. 3**). The removal of Nycodenz by dialysis increased the rate of oxidation of phytanic acid to pristanic acid in mitochondria and ER from control and Refsum fibroblasts, respectively, to a similar degree. The activity was linear up to 100 μg peroxisomal protein in both dialyzed and nondialyzed peroxisomes and mitochondria and up to 200 μg in ER. Consistent with our previous observations (21), the dialysis of peroxisomal peak fractions increased the oxidation of phytanic acid in peroxisomes from control fibroblasts 21-fold. However, there was no detectable activity for oxidation of phytanic acid in both dialyzed and nondialyzed peroxisomes from Refsum fibroblasts at all protein levels tested (Fig. 3). The mitochondrial and ER activities for α-oxidation of phytanic acid from Refsum fibroblasts was normal at all the enzyme concentrations tested (Fig. 3). Fig. 1 shows the activity of phytanic acid oxidation in the gradient profiles from control and Refsum fibroblasts after dialysis. All the activity for oxidation of phytanic acid in Refsum fibroblasts was derived from mitochondria and ER (Table 3 and Figs. 1 and 3). The specific activity in the dialyzed mitochondrial fraction was only 1.3-times higher than in the nondialyzed sample (ref. 21 and Fig. 3) because mitochondria band at lower concentration of Nycodenz in the middle of the gradient (Fig. 1). These results demonstrate that the enzyme activity for oxidation of phytanic acid in peroxisomes from Refsum is deficient whereas mitochondrial and ER activities from Refsum fibroblast are similar to normal fibroblasts.

To estimate the contribution of extra-peroxisomal sources of phytanic acid oxidation in Refsum and control cells, we examined the phytanic acid oxidation in the fibroblasts suspended in isotonic media in the absence or presence of

TABLE 3. Rates of oxidation of fatty acids in different subcellular organelles from cultured skin fibroblasts

	Peroxisomes	Mitochondria	Endoplasmic Reticulum
		nmol/h/mg protein	
Palmitic acid oxidation			
Control	14.3 ± 3.1	9.7 ± 3.0	0.865 ± 0.05
Refsum	15.4 ± 2.9	12.4 ± 2.5	1.75 ± 0.36
		pmol/h/mg protein	
Lignoceric acid oxidation			
Control	180 ± 40	4 ± 1	1.44 ± 0.11
Refsum	170 ± 5	3 ± 1	2.9 ± 1.1
Phytanic acid oxidation			
Control	37.1 ± 2.65	1.9 ± 0.3	0.4 ± 0.07
Refsum	0.0	2.04 ± 0.7	0.43 ± 0.2

The oxidation of fatty acids was measured as described in the text. The results are expressed as mean ± SD from three cell lines from control and Refsum fibroblasts.

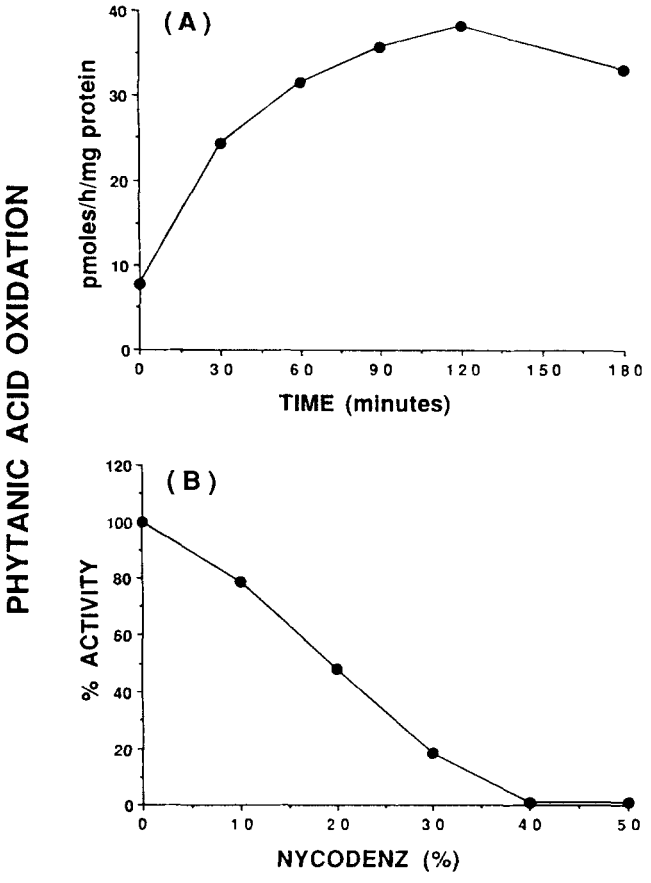

Fig. 2. Effect of Nycodenz on the enzyme activity for α-oxidation of phytanic acid. A: The phytanic acid oxidation activity was measured in peroxisomes from control human skin fibroblasts at different periods of dialysis against isotonic solution (25). B: The phytanic acid oxidation activity was measured after the addition of different concentrations of Nycodenz to peroxisomes dialyzed previously for 2 h. The specific activity of phytanic acid oxidation activity in peroxisomes dialyzed for 2 h was 39.2 ± 3.5 pmol/h per mg protein (100%).

2-[-5-(chlorophenyl)-pentyl] oxiran-2-carboxylic acid (POCA), an inhibitor of carnitine palmitoyl transferase I (CPT-I) (36). POCA inhibits the oxidation (α- and β-oxidation) of fatty acids in mitochondria whereas it has very little effect on the oxidation of fatty acids in peroxisomes (15, 21, 37). The rate of oxidation of phytanic acid in Refsum cells (0.44 ± 0.10 pmol/h per mg protein) was only 9% as compared to the control cells (4.98 ± 0.58 pmol/h per mg protein). POCA at 40 μM, the concentration at which the inhibition was maximum (15), inhibited the oxidation of phytanic acid by 12% in control cells and by 85% in cells from Refsum patients (**Fig. 4**). These results suggest that mitochondrial activity may contribute between 9 and 15% of the total cellular phytanic acid oxidation activity. Moreover, the inhibition of phytanic acid by POCA by 85% in Refsum cells suggests that most of the extra-peroxisomal activity is derived from mitochondria. These results also support the conclusion that, in human tissues, the peroxisomes are the major site of phytanic acid oxidation.

In agreement with previous observations (30, 34, 35), palmitic acid oxidation had bimodal distribution in mitochondria and peroxisomes and palmitoyl-CoA ligase had trimodal distribution in ER, mitochondria, and peroxisomes from control fibroblasts. This distribution was similar in subcellular organelles from Refsum and control fibroblasts (Table 3 and **Table 4**). The enzyme activities for activation of lignoceric acid in ER and peroxisomes and oxidation of lignoceric acid in peroxisomes were also similar in Refsum and control fibroblasts (Tables 3 and 4). These results suggest that peroxisomes isolated from Refsum fibroblasts were metabolically functional. The distribution of enzyme activity for phytanoyl-CoA ligase had a trimodal distribution in mitochondria, ER, and peroxisomes from both control and Refsum fibroblasts and the specific activities of phytanoyl-CoA ligase in ER, mitochondria, and peroxisomes from control and Refsum fibroblasts were similar (Table 4). The different specific activity of phytanoyl-CoA ligase than palmitoyl-CoA and lignoceroyl-CoA ligases in different subcellular organelles suggests that phytanoyl-CoA ligase activity, at least in peroxisomes, may be derived from an acyl-CoA ligase other than palmitoyl-CoA and lignoceroyl-CoA ligases (Table 4). The activation of phytanic acid to phytanoyl-CoA by phytanoyl-CoA ligase is the first required step in the oxidation of phytanic acid (15, 21). The normal activity of phytanoyl-CoA ligase activity in peroxisomes suggests that the defect in oxidation of phytanic acid in Refsum patients is due to an abnormality in the subsequent step(s) in the α-oxidation of phytanic acid.

DISCUSSION

The results described here clearly demonstrate that the defect in the oxidation of phytanic acid in cultured skin fibroblasts from Refsum patients is in peroxisomes. The activities observed in ER and mitochondria from Refsum fibroblasts were similar to those of control fibroblasts. This defect in the peroxisomal oxidation of phytanic acid may be responsible for the observed accumulation of excessive amounts of phytanic acid in tissues from Refsum patients (1–3). In the plasma of Refsum patients, phytanic acid may account for as much as 5–30% of the total fatty acid, whereas normal plasma generally contains undetectable amounts (less than 0.3 mg/dl) of phytanic acid (3, 38). Based on the ability of a diet deficient in phytanic acid to lower the levels of phytanic acid in plasma and tissue of Refsum patients (3, 39, 40), as well as the inability to observe biosynthesis of phytanic acid in animal tissue (3, 41), it is believed that phytanic acid originates from dietary sources either as phytanic acid or phytol which is readily converted into phytanic acid (3, 41, 42). The daily

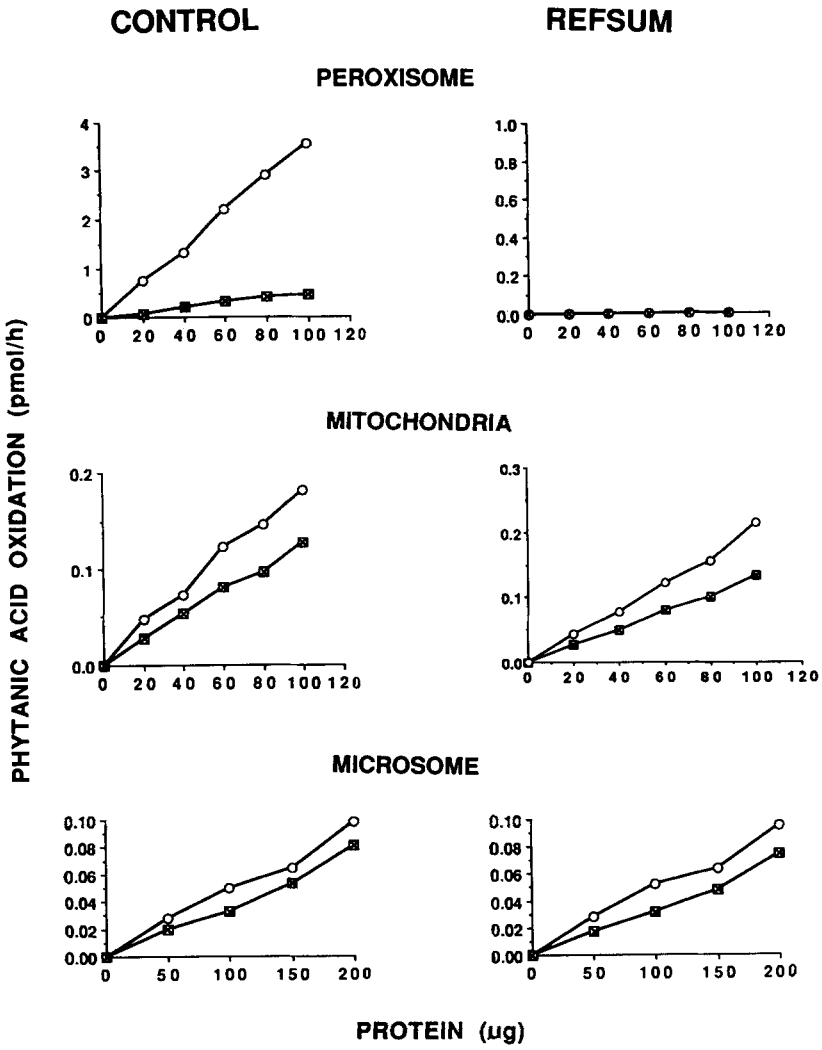

Fig. 3. The oxidation of phytanic acid in different subcellular organelles from control and Refsum fibroblasts. The rates of oxidation of [1-^{14}C]phytanic acid in peroxisomes, mitochondria, and endoplasmic reticulum from control and Refsum fibroblasts were measured at different protein concentrations before (⊠) and after (○) dialysis according to the procedure described in the text. The results are average of two experiments in duplicate from two cell lines of control and Refsum fibroblasts.

intake of phytanic acid from an ordinary diet has been estimated to be somewhere between 50 and 100 mg/day (39).

The catabolic pathways of phytanic acid in animals as well as in humans have been extensively studied by Steinberg and co-workers (3–8, 38–44). Due to the methyl group at the β-position, the phytanic acid cannot be degraded by normal β-oxidation pathway but rather it first undergoes an α-oxidation to pristanic acid followed by β-oxidation to 3 moles of acetate, 3 moles of propionate, and 1 mole of butyrate. Although this catabolic sequence of phytanic acid may be the same in humans (3, 7) and rodents (8), different organelles are responsible for this function (21). We recently studied the oxidation of phytanic acid in subcellular organelles from human (liver and cultured skin fibroblasts) and rat (liver and cultured skin fibroblasts) tissues and found that phytanic acid is predominantly oxidized to pristanic acid in humans in peroxisomes and in rodents in mitochondria (21). The rate of oxidation of phytanic acid to pristanic acid in human peroxisomes from human skin fibroblasts was 26- to 29-times and 110- to 130-times higher in peroxisomes than mitochondria and ER, respectively, and in rodent fibroblasts the rate was 15- and 9-times higher in mitochondria than in peroxisomes and ER, respectively (15, 21). This observation of peroxisomes as the major site of phytanic acid oxidation in humans is consistent with the excessive accumulation and defective oxidation of phytanic acid in diseases with abnormalities in the biogenesis of peroxisomes (e.g., Zellweger syndrome) (16–19, 31). Recent

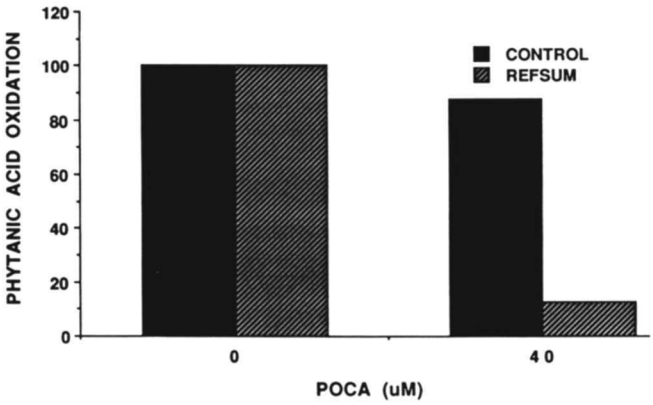

Fig. 4. Effect of 2-[-5-(chlorophenyl)-pentyl]oxiran-2-carboxylic acid in cultured skin fibroblasts from control and Refsum patients. The rates of oxidation of [1-^{14}C]phytanic acid in cultured skin fibroblasts suspended in isotonic media (0.25 M sucrose in 3 mM imidazole buffer, pH 7.4, and 1 mM EDTA) were measured as described in the text. These results are the average of two experiments in duplicate from two cell lines from control and Refsum patients. The rates of phytanic acid oxidation in control and Refsum cultured skin fibroblast of 4.98 ± 0.58 and 0.44 ± 0.10 pmol/h per mg protein, respectively, represent 100% activity.

studies of Watkins, Mihalik, and Skjeldal (12) and Wanders et al. (13) with subcellular organelles purified by Nycodenz gradient from monkey and human liver demonstrated that phytanic acid in these species is oxidized in mitochondria but not in peroxisomes. Based on these observations it was suggested that Refsum disease is not a peroxisomal disorder (12, 45). Nycodenz has been the material of choice for purification of subcellular organelles, especially peroxisomes. We have found that Nycodenz has a strong inhibitory effect on the oxidation of phytanic acid (Fig. 2 and ref. 21). Greater inhibition was observed in samples with higher Nycodenz to protein ratios. This inhibition of α-oxidation of phytanic acid is reversible and can be decreased or eliminated by removal of Nycodenz by dialysis (21). The dialysis of ER, mitochondrial, and peroxisomal fractions from human skin fibroblasts against isotonic solution increased the phytanic acid oxidation activity by 1.3-, 1.3-, and 5-fold, respectively (21). The activity for oxidation of phytanic acid was absent in peroxisomes from Refsum fibroblasts whereas ER and mitochondrial activities in Refsum fibroblasts were similar to ER and mitochondrial activities from control fibroblasts (Table 3 and Fig. 3). The higher specific activity of phytanic acid oxidation in peroxisomes than in mitochondria and ER from human tissues (ref. 21 and Table 3) and absence of phytanic acid oxidation activity only in peroxisomes from Refsum fibroblasts as compared to normal activities in mitochondria and ER (Fig. 1 and Table 3) clearly demonstrate that excessive accumulation of phytanic acid in tissues from patients with Refsum disease is caused by the absence of α-oxidation of phytanic acid to pristanic acid in peroxisomes.

Steinberg and associates (3, 6, 7) studied the oxidation of phytanic acid in two humans with Refsum disease and in cultured skin fibroblasts from Refsum patients. They found that the Refsum patients and skin fibroblasts from Refsum patients oxidized phytanic acid at a rate of only 3–5% of normal. From these results, they concluded that the degree of defect in the oxidation of phytanic acid in Refsum patients and skin fibroblasts derived from Refsum patients is similar (3). Therefore, cultured skin fibroblasts should be a suitable tissue for enzymatic studies for demonstration of deficient activity in Refsum patients. The studies reported here clearly demonstrate that defective oxidation of phytanic acid in Refsum fibroblasts is due to impaired activity of α-oxidation of phytanic acid in peroxisomes, whereas ER and mitochondrial activities are normal. This raises a question about the physiological significance of the activity observed in mitochondria and ER. The recovery of phytanic acid oxidation activity in peroxisomes, mitochondria, and ER from a gradient of control cultured skin fibroblasts was 62, 32, and 6%, respectively. The deficient activity for oxidation of phytanic acid only in peroxisomes in cell lines from patients who accumulate phytanic acid in excessive

TABLE 4. Acyl-CoA ligase activities

	Peroxisomes	Mitochondria	Microsomes
		nmol/h/mg protein	
Palmitoyl-CoA ligase			
Control	22.91 ± 3.2	17.5 ± 1.43	39.22 ± 2.24
Refsum	19.63 ± 1.85	17.28 ± 1.27	32.44 ± 4.66
Lignoceroyl-CoA ligase			
Control	0.84 ± 0.07	0.32 ± 0.04	0.72 ± 0.05
Refsum	0.74 ± 0.08	0.27 ± 0.02	0.59 ± 0.14
Phytanoyl-CoA ligase			
Control	9.86 ± 0.09	1.45 ± 0.02	2.31 ± 0.31
Refsum	10.25 ± 0.31	1.72 ± 0.36	1.96 ± 0.03

The activation of fatty acids was measured as described in the text. The results are expressed as mean ± SD from three cell lines from control and Refsum fibroblasts.

amounts, in rhizomelic chondrodysplasia punctata (21) and Refsum disease suggests that, in humans, peroxisomes are the major site of phytanic acid oxidation. The distribution of significant amounts of catalase in the mitochondrial and ER regions of the gradient suggests that contamination by peroxisomes may account for some of the phytanic acid oxidation activity observed in the mitochondrial-ER region of the gradient. The observance of only 9% activity in Refsum fibroblasts suspended in isotonic media as compared to controls and inhibition of only 12% by POCA in intact cells (Fig. 4) suggests that ER and mitochondrial activities may represent between 9 and 15% of the total cellular activity. These in vivo studies may be a better reflection of physiological status of phytanic acid oxidation in cells than the in vitro studies with isolated organelles. The oxidation of phytanic acid requires its conversion to phytanoyl-CoA by phytanoyl-CoA ligase (15, 21). The higher specific activity of phytanoyl-CoA ligase in peroxisomes than in mitochondria (7-times) and ER (4-times) also suggests that peroxisomes are the major site of phytanic acid oxidation.

The catabolism of phytanic acid involves α-oxidation to pristanic acid followed by β-oxidation of pristanic acid to 3 moles of acetate, 3 moles of propionate, and 1 mole of butyrate (3, 7, 8). The identification of an enzyme system for activation (Table 4) and for α-oxidation of phytanic acid (15, 21) and pristanoyl-CoA oxidase (13) in peroxisomes and excessive accumulation and defective oxidation of pristanic acid in patients who lack peroxisomes (e.g, Zellweger syndrome) (16-18, 32) suggest that human peroxisomes may be responsible for α-oxidation of phytanic acid as well as β-oxidation of pristanic acid. The α-oxidation of phytanic acid to pristanic involves four steps: activation of phytanic acid to phytanoyl-CoA, α-hydroxylation to α-hydroxyphytanic acid, dehydrogenation to 2-oxophytanic acid, and finally decarboxylation to pristanic acid and CO_2. The normal activities of phytanoyl-CoA ligase (Table 4) and normal oxidation of 2-hydroxyphytanic acid and pristanic acid (3, 6, 7) in Refsum fibroblasts as compared to deficient oxidation of phytanic acid (3) suggest that the defect in Refsum disease is in the hydroxylation of phytanic acid. The different specific activity of phytanoyl-CoA ligase as compared to palmitoyl-CoA and lignoceroyl-CoA ligases in different subcellular organelles suggests that phytanoyl-CoA ligase activity, at least in peroxisomes, may be derived from acyl-CoA ligases other than palmitoyl-CoA and lignoceroyl-CoA ligase. The identification of activities of phytanoyl-CoA ligase (Table 4), phytanic acid oxidase (15), and β-oxidation of pristanic acid (20) suggests that peroxisomes in human tissues contain an enzyme system for α-oxidation of phytanic acid as well as for β-oxidation of pristanic acid.

In summary, the α-oxidation of phytanic acid to pristanic acid in peroxisomes from Refsum fibroblasts is impaired whereas ER and mitochondrial activity are normal. The identification of the subcellular organelle responsible for deficient oxidation of phytanic acid in Refsum patients should enhance studies to delineate the molecular basis for the loss of phytanic acid α-hydroxylase activity in Refsum disease.

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