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Phytanoyl-CoA Hydroxylase Is Present in Human Liver, Located in Peroxisomes, and Deficient in Zellweger Syndrome: Direct, Unequivocal Evidence for the New, Revised Pathway of Phytanic Acid α-Oxidation in Humans

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid which accumulates in a number of inherited diseases in human. Because β-oxidation is blocked by the methyl group at C-3, phytanic acid first undergoes decarboxylation via an α-oxidation mechanism. The structure and subcellular localization of the phytanic acid α-oxidation pathway have remained enigmatic through the years, although they have generally been assumed to involve phytanic acid and *not* its CoA-ester. This view has recently been challenged by the findings that in rat liver phytanic acid first has to be activated to its CoA-ester before α-oxidation and by the discovery of a new enzyme, phytanoyl-CoA hydroxylase, which converts phytanoyl-CoA to 2-hydroxyphytanoyl-CoA. We now show that this newly discovered enzyme is also present in human liver. Furthermore, we show that this enzyme is located in peroxisomes and deficient in liver from Zellweger patients who lack morphologically distinguishable peroxisomes, which provides an explanation for the long-known deficient oxidation of phytanic acid in these patients. These results suggest that phytanic acid α-oxidation is peroxisomal and that it utilizes the coenzyme A derivative as substrate, thus giving further support in favour of the new, revised pathway of phytanic acid α-oxidation.

Phytanic acid is a branched-chain fatty acid (3,7,11,15-tetramethylhexadecanoic acid) which cannot undergo straightforward β-oxidation due to the presence of a methyl-group at the 3-position. Instead phytanic acid first has to be decarboxylated via an α-oxidation mechanism to yield pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) which has the methyl-group in the 2-position, thus allowing β-oxidation.

The mechanism and subcellular localization of the phytanic acid α-oxidation pathway has remained obscure despite intensive efforts to elucidate it [1]. Resolution of this pathway is important because phytanic acid accumulates in a number of inherited diseases in man, including classical Refsum disease, Zellweger syndrome and rhizomelic chondrodysplasia punctata [1,2].

Based on early studies by Steinberg and coworkers and others [3-8], most authors favoured an α-oxidation pathway in which free phytanic acid would be converted to pristanic acid via 2-hydroxyphytanic acid and presumably 2-ketophytanic acid [6], although this viewpoint was disputed by some [7]. A major problem with these studies was that none of the presumed

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enzymes had been identified. Furthermore, there was also ambiguity with regard to the subcellular localization of the α-oxidation pathway. Early studies [8] suggested that α-oxidation is primarily mitochondrial. The finding that phytanic acid oxidation is deficient in cells from patients lacking peroxisomes led to the hypothesis that peroxisomes are the location for phytanic acid α-oxidation, at least in man. Subsequent studies, however, provided no experimental support for this postulate. Indeed, α-oxidation determined as $^{14}CO_2$ formation was found to be mitochondrial both in rat [9] as well as in man [10], whereas others found it in the endoplasmic reticulum [11]. Yet another view was held by Singh and coworkers [12] who claimed that α-oxidation is peroxisomal in man but mitochondrial in the rat.

Recent studies have shed new light on the phytanic acid α-oxidation pathway [13,14]. Indeed, it now seems that the pathway of phytanic acid α-oxidation involves phytanoyl-CoA and *not* free phytanic acid. Furthermore, we recently demonstrated that the next enzyme in the pathway is a Fe^{++} and 2-oxoglutarate requiring hydroxylase which converts phytanoyl-CoA to 2-hydroxyphytanoyl-CoA [14].

In the present study we have investigated whether phytanoyl-CoA hydroxylase is found in human liver. Furthermore, we have determined the subcellular localization of this enzyme and have measured its activity in liver from patients who lack morphologically distinguishable peroxisomes (Zellweger syndrome) (see [15] for review). Taken together, the results described in this paper provide strong evidence in favour of a new, revised pathway for phytanic acid α-oxidation in man.

MATERIALS AND METHODS

Phytanoyl-CoA hydroxylase activity measurements. Incubations were done essentially as described before for rat liver peroxisomes [14] with some modifications. These changes include the use of [1-^{14}C] phytanoyl-CoA to replace [1-^{14}C] phytanic acid in the presence of ATP, Mg^{++} and coenzyme A. [1-^{14}C]-phytanoyl-CoA was synthesized enzymatically using purified acyl-CoA synthetase and [1-^{14}C] phytanic acid and subsequently purified by radio-HPLC (see [14]). Phytanoyl-CoA hydroxylase activity measurements were performed in the following medium: 25 mM Tris-HCl, 0.25 mM DTT, 10 mM ATP, 5 mM $MgCl_2$, 0.2 mM coenzyme A, 0.5 mM $NH_4Fe\ SO_4$, 1 mM 2-oxoglutarate, 1 mM ascorbate plus 25 μM [1-^{14}C] phytanoyl-CoA. Final pH=7.5. Incubations were allowed to proceed for 30 min at 37°C and the different acyl-CoA esters separated by HPLC analysis (see [14] for details).

Other enzyme activity measurements. Glutamate dehydrogenase [16], acyl-CoA: dihydroxyacetonephosphate acyltransferase [17], L-pipecolate oxidase [18], catalase [19], esterase [19] and phosphoglucose isomerase [19] were determined as described.

Differential centrifugation of human liver postnuclear supernatants. Pieces of human liver tissue obtained from patients undergoing liver resection were immediately chilled in a medium containing 250 mM sucrose, 0.5 mM EDTA and 2 mM MOPS (final pH 7.4), finely minced and subjected to differential centrifugation exactly as described before [19].

Patients. Liver samples were obtained from patients showing all the clinical and biochemical abnormalities described for the Zellweger syndrome including a deficiency of acyl-CoA: dihydroxyacetone phosphate acyltransferase (DHAPAT) [19] and L-pipecolate oxidase [19] (see Table II; for further details see [15]). Unless they were used for differential centrifugation, tissue specimens were immediately frozen at −80°C and stored at this temperature until used.

RESULTS

Earlier we reported that rat liver peroxisomes contain a phytanoyl-CoA hydroxylase which catalyses conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA via a dioxygenase reaction requiring Fe^{++} and 2-oxoglutarate as cofactors. In an initial experiment we investigated whether this enzyme is also present in human liver homogenates. Using preformed, enzymatically synthesized [1-^{14}C] phytanoyl-CoA rather than phytanic acid in the presence of ATP, Mg^{++} and Coenzyme A as was done in our earlier studies [14], we found that 2-hydroxyphytanoyl-CoA is readily formed if the appropriate cofactors are present. Omission of 2-oxoglutarate or

TABLE I
Cofactor Dependence of Phytanoyl-CoA Hydroxylase in Human Liver Homogenates

Omission from reaction medium	Phytanoyl-CoA hydroxylase activity (nmol/hr · mg protein)
None	2.45 ± 0.88 (11)
$FeNH_4SO_4$	⩾0.05 (3)
2-oxoglutarate	⩽0.05 (3)
O_2 ($+N_2$)	0.17 ± 0.10 (3)

Note. For experimental details see Materials and Methods. Values represent the mean ± S.D. with the number of liver specimens analyzed in parentheses.

Fe^{2+} from the incubation medium leads to a complete loss of enzyme activity (Table I). Furthermore, if measurements were performed in the absence of molecular oxygen, activity was also very low, further suggesting a true dioxygenase mechanism.

The next set of experiments was performed to identify the subcellular site of phytanoyl-CoA hydroxylase. To this end a fresh human liver homogenate was subjected to differential centrifugation to prepare nuclear, heavy mitochondrial, light mitochondrial, microsomal and cytosolic fractions. When these fractions were assayed for marker enzyme activities as well as phytanoyl-CoA hydroxylase activity, the relative specific activity of phytanoyl-CoA hydroxylase was greatest in the light mitochondrial fraction which is enriched in peroxisomes (Fig. 1E). Furthermore, the pattern of relative specific activities found for phytanoyl-CoA hydroxylase closely mirrors that of catalase, a peroxisomal marker enzyme (Fig. 1B), whereas glutamate dehydrogenase (mitochondria), esterase (endoplasmic reticulum) and phosphoglucose isomerase (cytosol) showed a different activity profile (Fig. 1A, C, and D, respectively).

We subsequently studied the activity of phytanoyl-CoA hydroxylase in liver homogenates from Zellweger patients, known to lack morphologically distinguishable peroxisomes because of a defect in peroxisome biogenesis (see [15] for review). These studies (Table II) showed that phytanoyl-CoA hydroxylase was deficient in liver from Zellweger patients. In contrast, non-peroxisomal enzyme activities such as the mitochondrial marker enzyme glutamate dehydrogenase were found to be normal in these specimens suggesting that tissues were of proper quality. On the other hand in accordance with earlier studies the activities of L-pipecolate oxidase [18] and dihydroxyacetone-phosphate acyltransferase [20] were deficient, as expected for peroxisomal biogenesis disorders.

DISCUSSION

Until recently the phytanic acid α-oxidation pathway was generally thought to involve phytanic acid and *not* phytanoyl-CoA. This view is not easy to reconcile with recent findings suggesting that phytanic acid first needs activation to its CoA-ester [13]. Furthermore, the subsequent enzyme in the new, revised pathway, phytanoyl-CoA hydroxylase, only accepts phytanoyl-CoA and *not* phytanic acid as substrate [14].

The results described in this paper show that phytanoyl-CoA hydroxylase is present in human liver peroxisomes and is deficient in liver from Zellweger patients. The latter observation provides an explanation for the long known but unexplained fact that α-oxidation of phytanic acid is deficient in patients suffering from a disorder of peroxisome biogenesis (e.g. Zellweger syndrome) (see [15]). Taken together, the results presented in this paper strongly suggest that

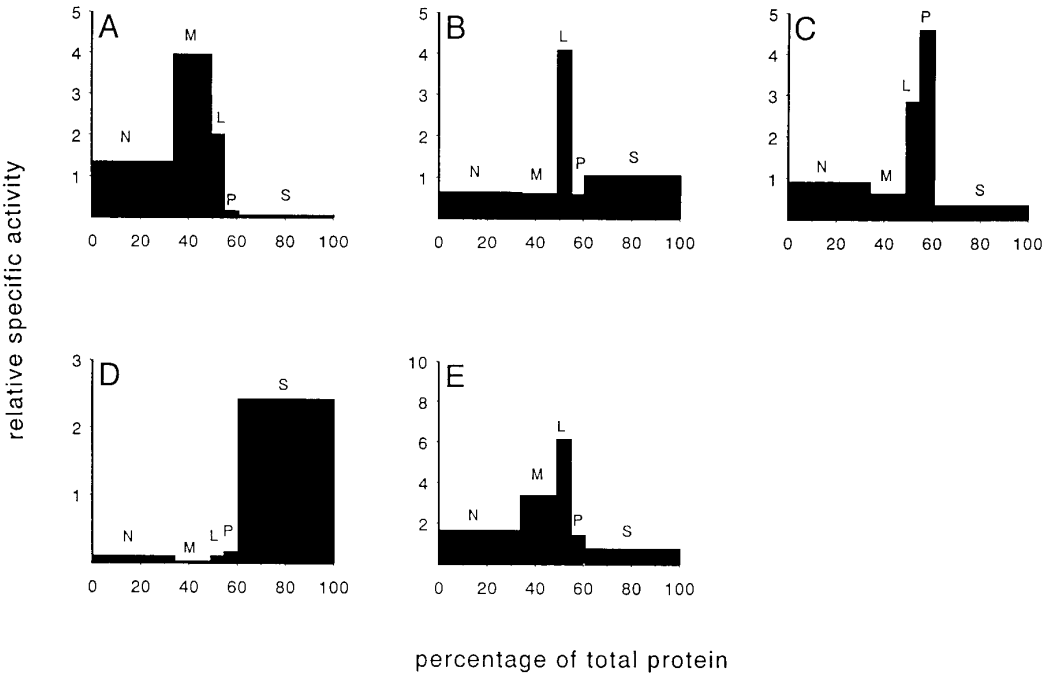

FIG. 1. Subcellular localization of phytanoyl-CoA hydroxylase in human liver. A fresh human liver specimen was homogenized and subjected to differential centrifugation to prepare a nuclear (N), heavy mitochondrial (M), light-mitochondrial (L), microsomal (P), and cytosolic (S) fraction (see [19]. In each of the fractions phytanoyl-CoA hydroxylase (Fig. 1E) and the marker enzymes were measured including glutamate dehydrogenase (Fig. 1A), catalase (Fig. 1B), esterase (Fig. 1C), and phosphoglucose isomerase (Fig. 1D).

peroxisomes are definitely involved in phytanic acid α-oxidation in rat [14] and man (this paper). Furthermore, our data provide convincing evidence in favour of the new pathway of phytanic acid α-oxidation in humans in which phytanic acid is first activated to phytanoyl-CoA followed by hydroxylation to 2-hydroxyphytanoyl-CoA (Fig. 2). It is unclear how 2-hydroxyphytanoyl-CoA is further metabolized to pristanic acid (or pristanoyl-CoA). Studies from Poulos et al. [21] have shown that the terminal carbon atom is lost in this step as formic acid, which is a very unusual product in decarboxylation reactions. Current efforts are directed

TABLE II
Activity of Phytanoyl-CoA Hydroxylase and Other Enzymes in Liver Homogenates from Controls and Zellweger Patients

Enzyme activity measured	Zellweger patients	Controls
• Phytanoyl-CoA hydroxylase (nmol/h · mg)	$\leqslant$0.05 (3)	2.45 ± 0.88 (11)
• Glutamate dehydrogenase (μmol/min · mg)	9.1 ± 1.2 (3)	8.2 ± 1.0 (5)
• L-pipecolate oxidase (pmol/min · mg)	8.2 ± 4.1 (3)	512 ± 84 (4)
• Acyl-CoA: dihydroxyacetone phosphate acyltransferase (nmol/2h · mg)	0.12 ± 0.05 (3)	2.51 ± 0.32 (7)

Note. See Materials and Methods for experimental details. Values represent the mean ± S.D. with the number of liver specimens analyzed in parentheses.

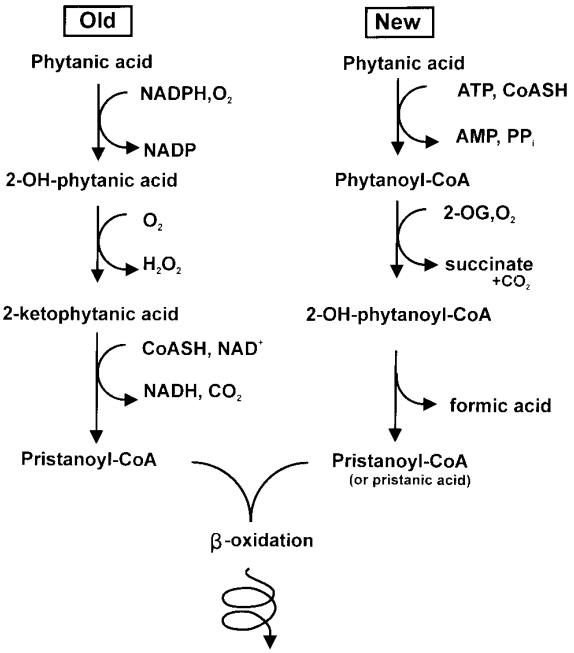

FIG. 2. Schematic representation of the phytanic acid α-oxidation pathway. According to the original scheme phytanic acid was thought to be hydroxylated to 2-hydroxyphytanic acid followed by conversion into pristanoyl-CoA probably via 2-ketophytanic acid (see [1]). Recent studies in rat liver [13, 14] and the data described in this paper argue against the original scheme and provide convincing evidence in favour of a new pathway in which phytanic acid is first converted into the CoA-ester followed by hydroxylation to 2-hydroxyphytanoyl-CoA and conversion into pristanic acid (in pristanoyl-CoA) via an enzyme which remains to be identified.

on elucidating the enzyme(s) involved in conversion of 2-hydroxyphytanoyl-CoA to pristanic acid (or pristanoyl-CoA) and formic acid.

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