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Review

Phytanic acid: production from phytol, its breakdown and role in human disease

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Abstract. Phytanic acid is a branched-chain fatty acid that accumulates in a variety of metabolic disorders. High levels of phytanic acid found in patients can exceed the millimolar range and lead to severe symptoms. Degradation of phytanic acid takes place by α-oxidation inside the peroxisome. A deficiency of its breakdown, leading to elevated levels, can result from either a general peroxisomal dysfunction or from a defect in one of the enzymes involved in α-oxidation. Research on Refsum disease, belonging to the latter group of disorders and characterized by a deficiency of the first enzyme of α-oxidation, has extended our knowledge of phytanic acid metabolism and pathology of the disease greatly over the past few decades. This review will centre on this research on phytanic acid: its origin, the mechanism by which its α-oxidation takes place, its role in human disease and the way it is produced from phytol.

Keywords. Phytanic acid, Refsum disease, phytol, α-oxidation, peroxisome biogenesis disorder, rhizomelic chondrodysplasia punctata, peroxisome.

Introduction

Phytanic acid is a branched-chain fatty acid that plays an important role in a number of metabolic diseases. Research on phytanic acid started in the 1950s when dairy products were being investigated for their fatty acid content. These studies revealed that phytanic acid is a major constituent of a variety of food products in the human diet, especially in products derived from grazing animals. In the following decade it was found that phytanic acid is a C_{20} fatty acid, made up of a C_{16} backbone and four additional methyl groups, which makes it a saturated branched-chain fatty acid (Fig. 1) (reviewed in [1]). The interest in phytanic acid increased considerably when elevated phytanic acid levels were reported in tissues of a patient suffering from Refsum disease [2]. Once it had been established that phytanic acid was also massively elevated in plasma of this patient, an easy biochemical diagnostic test became available. Since then, phytanic acid is generally regarded as the most important pathognomonic marker of the disease. However, an accumulation of phytanic acid is observed not only in Refsum disease, but also in patients suffering from peroxisomal biogenesis disorders and rhizomelic chondrodysplasia punctata type 1 [3, 4]. The elevated levels of phytanic acid are thought to contribute or directly lead to the symptoms observed in these patients. In addition, recent evidence indicates that phytanic acid is involved in the induction of apoptosis in astrocytes [5, 6] as well as in the onset of heart pathology [7]. The vital role phytanic acid plays in these processes warrants a good understanding of the origin and behaviour of this molecule inside the body and inside the cell. This role will be addressed in this review together with a brief general overview of peroxisomal functions and disorders.

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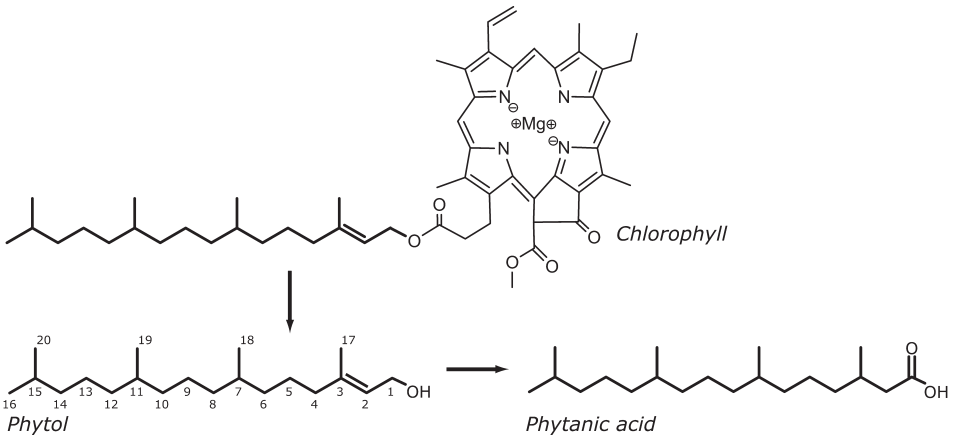

Figure 1. The structures of chlorophyll, phytanic acid, or 3,7,11,15-tetramethylhexadecanoic acid, and its precursor phytol.

Role of peroxisomes in human physiology

After the first morphological description in the early 1950s, peroxisomes were identified as distinct subcellular organelles in the 1960s by DeDuve and coworkers. It took until the 1980s for researchers to realize that peroxisomes play an indispensable role in human physiology, as concluded from studies on a rare genetic disease called Zellweger syndrome (ZS), characterized by the absence of peroxisomes. These studies led to the identification of a number of essential metabolic functions in peroxisomes, including: (i) fatty acid β-oxidation; (ii) etherphospholipid biosynthesis; (iii) fatty acid α-oxidation; and (iv) glyoxylate detoxification.

Fatty acid β-oxidation

Fatty acid β-oxidation in peroxisomes proceeds via a mechanism similar to that in mitochondria via four subsequent steps of dehydrogenation, hydratation, dehydrogenation again and thiolytic cleavage, as catalyzed by distinct peroxisomal and mitochondrial β-oxidation enzymes (Fig. 2a). Importantly, the two systems are involved in the oxidation of different sets of substrates, with peroxisomes catalyzing the β-oxidation of very long chain fatty acids, pristanic acid, and di- and trihydroxycholestanoic acid. The latter compounds are involved in the formation of cholic acid and chenodeoxycholic acid in the liver. Another compound handled by the peroxisomal system only is tetracosahexaenoic acid (C24 : 6), which after one cycle of β-oxidation in peroxisomes produces docosahexaenoic acid (C22 : 6). The enzymology of peroxisomal β-oxidation has been worked out in detail, and it is now clear that multiple enzymes are involved in the β-oxidation of these compounds [8, 9]. Nevertheless, much remains to be learned about the peroxisomal β-oxidation system, which includes the transport of fatty acids across the peroxisomal membrane and export of the products of peroxisomal β-oxidation, as well as reoxidation of intraperoxisomal NADH (reduced nicotinamide adenine dinucleotide).

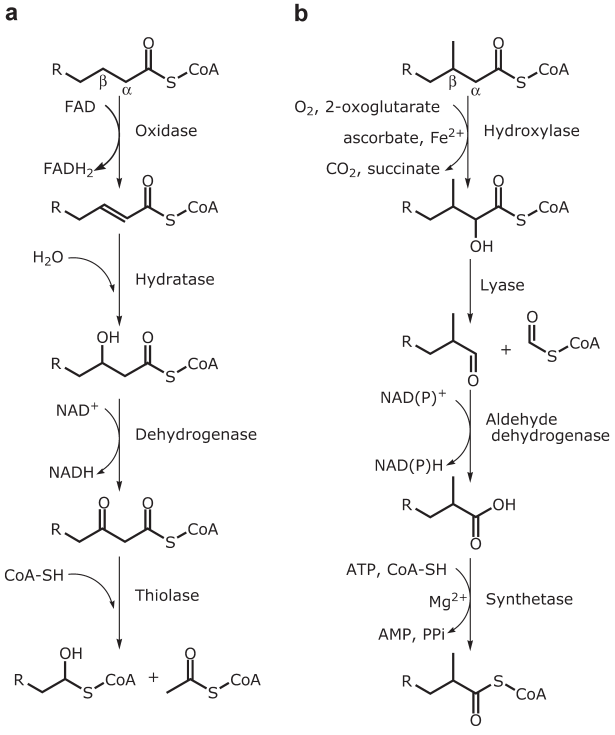

Figure 2. Mechanism of α- and β-oxidation. (*a*) Each cycle of β-oxidation consists of an oxidation, a hydratation, a second oxidation and finally thiolysis. In the last step an acetyl-CoA is released, and the length of the fatty-acid carbon chain is reduced by two carbon atoms. The resulting acyl-CoA is then ready for another cycle of β-oxidation. (*b*) α-Oxidation is initiated by hydroxylation of the 3-methyl acyl-CoA. A lyase reaction releases a formyl-CoA from the molecule, which can be converted into CO_2. Next, an aldehyde dehydrogenase converts the fatty aldehyde into the corresponding acid, which is then activated to its CoA-ester by a synthetase reaction. The resulting 2-methyl acyl-CoA can be further β-oxidized.

Etherphospholipid biosynthesis

Peroxisomes also play a crucial role in etherphospholipid synthesis, as concluded initially from the finding that plasmalogens, the main end product of etherphospholipid biosynthesis in mammals, including humans, are markedly deficient in tissues of ZS patients [10]. The first two enzymes of the etherphospholipid biosynthetic route, i.e. dihydroxyacetone phosphate acyltransferase and alkyldihydroxyacetone phosphate synthase (ADHAPS), are localized exclusively in peroxisomes, with the remainder of the pathway being localized in the endoplasmic reticulum.

Glyoxylate detoxification

Glyoxylate is a toxic metabolite produced from different sources and is rapidly converted into glycine under normal conditions by the enzyme alanine glyoxylate aminotransferase, which is strictly peroxisomal in humans. In case of a deficiency of alanine glyoxylate aminotransferase, as in hyperoxaluria type 1, glyoxylate accumulates and is rapidly converted into oxalate, which precipitates as calcium oxalate in multiple tissues, notably the kidneys, with loss of kidney function in time [11].

Another essential function of peroxisomes is fatty acid α-oxidation. Phytanic acid is the main substrate for α-oxidation, and its metabolism will be discussed in detail below.

Origin of phytanic acid

Work by Steinberg and colleagues demonstrated that endogenous synthesis of phytanic acid does not take place, although this would be quite conceivable because its poly-isoprenoid structure is similar to that of farnesol and geranylgeraniol. These latter compounds can be synthesized in mammalian cells by assembly of multiple mevalonate units [12]. Phytanic acid only differs from geranylgeraniol by the absence of three double bounds (at positions Δ^6, Δ^{10} and Δ^{14}), and a carboxyl instead of an alcohol group. However, it was observed that radiolabelled mevalonate administered to rats did not lead to incorporation of label into phytanic acid [13, 14]. Therefore, it was concluded that all phytanic acid is derived from dietary sources.

Dietary intake of phytanic acid

Phytanic acid is especially abundant in ruminant animals, including cows and sheep, in various tissues such as fat, liver, plasma, milk and rumen content. However, it is also present in some non-ruminants such as rats, pigs and humans. The amount of phytanic acid found ranges from about 0.01 to 0.3% of the total fatty acid pool, but can exceed the 10% mark in milk from cows fed on ensilage, the fermented grass that is used as winter feed for cattle. High amounts were also detected in Antarctic krill (1.4%), the plankton that forms the basis of the oceanic food chain, which accounts for the presence of phytanic acid in, for example, molluscs, fish oil, whale oil and whale milk [1]. In addition, high levels of phytanic acid are present in earthworms (up to 3.5%). This raised the hypothesis that phytanic acid was actually derived from a precursor molecule that is very abundant in nature, namely phytol.

Phytol, the precursor of phytanic acid

Normally, phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) is a constituent of the chlorophyll molecule (Fig. 1), a biomolecule that is involved in the production of energy from light. Since almost all photosynthetic organisms use chlorophyll, phytol is also abundantly present in nature. As a consequence of the consumption of large quantities of grasses, ruminant animals take in a lot of chlorophyll. On being digested, the phytol moiety is released and can then be converted into phytanic acid.

Experiments where radioactively labelled chlorophyll was administered orally to rats or human subjects showed that in both cases around 95% of the chlorophyll passed through the digestive system intact, leaving only 5% of the phytol available for conversion to phytanic acid [15, 16]. The implication of this finding is that the major source of phytanic acid for humans does not come from chlorophyll-bound phytol, but rather from phytanic acid present in food products. In surveys of the phytanic acid content of a variety of food products, high levels were indeed found in goods such as milk, butter and cheese and meat from cows and sheep, while in meat from pigs and poultry hardly any could be detected. Almost no phytanic acid is present in vegetables, while some species of fish and fish oils were found to contain high amounts [17–19].

Breakdown of phytanic acid

In Refsum disease patients, phytanic acid accumulates, while other fatty acids are apparently broken down without difficulty. The branched-chain structure of phytanic acid was proposed to be the reason for this difference. Normal degradation of fatty acids takes place by β-oxidation, but in the case of phytanic acid, the presence of a methyl group at the 3-position (Fig. 1) makes this mechanism impossible.

α-Oxidation

Insight into how phytanic acid is degraded came from the detection of pristanic acid in rats that were administered a dose of phytol [20]. Pristanic acid (2,6,10,14-

tetramethylpentadecanoic acid) possesses a carbon chain that is one carbon atom shorter than phytanic acid, which led to the hypothesis that phytanic acid undergoes one round of α-oxidation. The process of α-oxidation consists of the removal of one carbon atom instead of the two that are removed by β-oxidation (Fig. 2). The hypothesis was supported by experiments with human subjects that received an artificial branched-chain substrate, 3,6-dimethyloctanoic acid. It was established that this substrate undergoes α-oxidation and furthermore that its degradation is deficient in Refsum disease patients [21]. Production of pristanic acid was also found in human fibroblasts cultured in the presence of radiolabelled phytanic acid [22]. Although it was first believed that the degradation of phytanic acid did not involve its co-enzyme A (CoA)-ester, it is now clear that the opposite is true. The true pathway is shown in Figure 2b.

β-Oxidation is initiated by a dehydrogenation reaction that forms a double bond at the 2–3 position. It was shown that this does not occur for the α-oxidation of phytanic acid, since no phytenic acid (3,7,11,15-tetramethylhexadec-2-enoic acid) could be detected in rats injected with radiolabelled phytanic acid [13]. Instead, 2-hydroxyphytanic acid formation was seen in these rats, which led to the hypothesis that hydroxylation of phytanic acid at the 2 position was the first step of α-oxidation. This supposition was supported by *in vivo* studies that showed that in plasma of human subjects who had been administered [1-^{13}C]phytanic acid, 2-hydroxy [1-^{13}C]phytanic acid could be detected [23, 24].

Activation of phytanic acid

The enzyme responsible for the activation of phytanic acid to its CoA ester was first reported to be a distinct phytanoyl-CoA ligase and thought to be present in peroxisomes in human liver and in microsomes and mitochondria in rat liver [25]. However, later research showed that phytanic acid can be activated by long-chain acyl-CoA synthetase, which is a known enzyme present in peroxisomes that also has affinity for straight-chain fatty acids [26].

Hydroxylation of phytanic acid

The first true step of α-oxidation of phytanoyl-CoA is its hydroxylation into 2-hydroxyphytanoyl-CoA by the enzyme phytanoyl-CoA hydroxylase (PAHX or PhyH) as first discovered by Mihalik and coworkers [27]. PAHX is a non-haem iron(II) and 2-oxoglutarate-dependent oxygenase. Iron(II) functions in the active site of these enzymes, and the presence of ascorbate helps to keep the iron in the +2 valence. 2-Oxoglutarate functions as a co-substrate and is converted into succinate [28, 29]. The finding that PAHX is deficient not only in classical Refsum patients but also in patients lacking peroxisomes, i.e. ZS patients, immediately suggested that PAHX is a peroxisomal enzyme. Subsequent subcellular localization studies confirmed this supposition [30].

Decarboxylation of phytanic acid

The second step of phytanic acid α-oxidation involves shortening the molecule by one carbon atom. Originally, it was presumed that CO_2 was produced in this reaction, but this hypothesis was refuted by Poulos et al. [31], who observed that >90% of the radioactivity coming from [1-^{14}C]phytanic acid α-oxidation in fibroblasts was present in the water-soluble, instead of the gaseous fraction. They and others [28] showed that the radioactivity came from formate, or, rather, as was discovered somewhat later, from formyl-CoA, which is now known to be the true product of the decarboxylation of phytanic acid [32] (Fig. 2b). Formyl-CoA is unstable at neutral pH and gives rise to free coenzyme A and formate non-enzymatically [32]. Subsequently, formate is converted into CO_2.

With the release of formyl-CoA, 2-hydroxyphytanoyl-CoA is converted into the aldehyde pristanal (2,6,10,14-tetramethylpentadecanal).

The enzyme catalyzing this reaction was defined as 2-hydroxyphytanoyl-CoA lyase (HPCL) and was further characterized using a specific enzyme assay in rat liver homogenates. These studies revealed that HPCL is localized in peroxisomes, which is in line with the emerging hypothesis that the complete process of α-oxidation was localized within the peroxisomes [33].

Shortly thereafter, the enzyme was purified from rat liver peroxisomes and cloned. The human complementary DNA (cDNA) was expressed from which a dependence on thiamine pyrophosphate and Mg^{2+} was discovered [34]. In further studies it was observed that HPCL has specificity for substrates that possess a 2-hydroxy and a CoA-moiety, but a 3-methyl group is not necessary for activity [35]. Affinity for hydroxyl substrates is quite unusual for lyases, since in most cases a 2-keto carboxyl compound is used as a substrate [36].

Dehydrogenation of pristanal to pristanic acid

The third step of phytanoyl-CoA α-oxidation concerns the conversion of pristanal into pristanic acid. This reaction was first measured in human liver [37] and subsequently in cultured human fibroblasts [38]. Since it had previously been suggested that the conversion of 2-hydroxyphytanoyl-CoA into pristanic acid takes place at the endoplasmatic reticulum (ER) [39], a candidate enzyme for the dehydrogenation of pristanal was fatty aldehyde dehydrogenase (FALDH, ALDH10), present on the ER membrane. To test this, fibroblasts derived from patients suffering from Sjögren-Larsson syndrome (SLS), who are deficient in FALDH, were investigated for their abil-

ity to degrade phytanic acid. It was observed that oxidation of [2,3-^{3}H]phytanic acid was reduced to 25% in SLS fibroblasts. In addition, in SLS cells that were incubated in the presence of [2,3-^{3}H]phytanic acid, a 4-fold increase was detected in the accumulation of radioactivity in N-alkylphosphatidyl ethanolamine [40]. Both these findings indicate a possible role of FALDH in the breakdown of phytanic acid. However, such a mechanism would imply that the pathway of α-oxidation is not completely peroxisomal, but would rely on the translocation of pristanal to the ER to be converted into pristanic acid, which would then have to be transported back to the peroxisomes for further β-oxidation. This unlikely mechanism sparked detailed investigation into the possible involvement of FALDH in α-oxidation.

Incubations using pristanal as a substrate revealed that there was a >25% residual activity of pristanal dehydrogenase in SLS fibroblasts, while with other substrates, such as octadecanal, this residual activity was <10% [40, 41]. This finding suggests that another aldehyde dehydrogenase different from FALDH (ALDH10) is involved in the conversion of pristanal into pristanic acid. Importantly, it was shown that a distinct peroxisomal pristanal dehydrogenase activity exists inside the peroxisome [32, 41].

Because of the large number of aldehyde dehydrogenases known in man (more than 10, reviewed in [42]) it does not come as a great surprise that more than one of these can use pristanal as a substrate. Interestingly, none of the aldehyde dehydrogenases described in the review by Yoshida et al. [42] has a peroxisomal localization. It cannot definitively be ruled out, however, that FALDH is involved in the breakdown of pristanal, since some evidence exists that it has a double localization inside the cell, namely in peroxisomes as well as in the ER, at least in rats [43, 44], although later studies indicated an exclusive ER localization of FALDH [45, 46]. Interestingly, our own unpublished efforts in purifying pristanal dehydrogenase activity from purified rat peroxisomes have twice resulted in the identification of FALDH. This finding might be explained by microsomal contamination of the peroxisomes that were used or, alternatively, the presence of ER-membrane proteins in the peroxisomes. Interestingly, recent evidence has been produced supporting a contentious model of peroxisome biogenesis in which newly formed peroxisomes bud off from the ER, which suggests that a direct connection may well exist between these two organelles [47, 48].

Localization studies of FALDH are further complicated by the existence of two splice variants in the corresponding *ALDH10* gene. Both in humans and in mice it was shown that the last intron is not spliced in about 10% of the *ALDH10* transcripts, which leads to an extension of the protein at the C-terminus by 27 or 26 amino acids, respectively [49–51]. The C-terminus of FALDH has been shown to be crucial for localization of the enzyme to the ER. Furthermore, a stretch of hydrophobic amino acids was shown to be essential for anchoring the protein to the ER, since deletion of this domain resulted in a cytosolic localization [45]. The protein resulting from variant splicing was shown to be still associated with the ER, although less strongly as compared with the full-length protein [45, 46]. Therefore, the possibility remains that this splice variant might have an alternative localization in the cell. Precise expression studies of the two human variants will have to elucidate the putative role of FALDH in α-oxidation.

Some reports in the literature indicate that a distinct aldehyde dehydrogenase inducible by clofibrate is present in rat liver peroxisomes [52]. However, since it is known that FALDH is also induced by clofibrate [53] and since the aldehyde dehydrogenase that was described by Antonenkov et al. [52] is also membrane-bound, it cannot be excluded that microsomal contamination is at the basis of these results. The true identity of the aldehyde dehydrogenase involved in α-oxidation, therefore, remains elusive, and further research will have to establish whether an additional aldehyde dehydrogenase, peroxisomal or not, metabolizes pristanal into pristanic acid.

Conversion of phytol into phytanic acid

In contrast to the well-studied breakdown mechanism of phytanic acid, the way in which phytol is broken down is a neglected topic. Phytol-feeding studies in the 1960s have shed some light on intermediates of the phytol to phytanic acid conversion, but it was not until 20 years later that a comprehensive model was proposed.

The mechanism of the conversion of phytol into phytanic acid

Phytol is an unsaturated fatty alcohol, and therefore two distinct processes are required for it to be transformed into phytanic acid, namely the reduction of the double bond at the 2,3 position and oxidation of the alcohol into a carboxyl group. Which particular intermediates are formed depends on the order in which the reactions occur, as shown in Figure 3a.

One of the possible intermediates, phytenic acid, was indeed detected in rats fed on a phytol-enriched diet, while the other, dihydrophytol, was absent [13, 54]. This suggested that at least in rats, phytol is first converted to phytenic acid, which then can be reduced to produce phytanic acid. Furthermore, an accumulation of phytanic acid was observed upon injection of phytenic acid in rats, showing that it is a bona fide intermediate of phytol degradation [13]. It has to be noted, however, that dihydrophytol can also be converted into phytanic acid [55]. Dihydrophytol production has been detected in the rumen of cows [56], but this finding might well be the result of bacterial processes.

Production of phytenic acid

The way in which phytol is converted into phytenic acid was studied by Muralidharan and Muralidharan [57]. They performed *in vitro* incubations of rat liver post-nuclear supernatant with phytol and observed production of phytenic acid, while no dihydrophytol production was detected, which confirmed the findings of the *in vivo* studies. A somewhat crude characterization of the reaction showed optimal rates of phytenic acid production in mitochondria and microsome-enriched fractions, while the supernatant showed no activity, implying that cytosolic alcohol dehydrogenase was not involved in the reaction [57, 58]. The highest activity was measured in rat liver, with about 10% of that found in kidney and spleen. Some activity was present in brain, heart and lungs, while in intestine and adipose tissue no phytenic acid production could be detected [58]. Experiments performed in our own group with human skin fibroblasts cultured in the presence of phytol showed that human cells also have the capacity to convert phytol into phytanic acid, and that phytenic acid is also an intermediate in this reaction [59].

The experiments described above characterize the conversion of phytol into phytenic acid. This process, however, is a two-step mechanism. The enzymatic oxidation of an alcohol into an acid usually requires an alcohol dehydrogenase and an aldehyde dehydrogenase. This implies that phytol would be converted first into phytenal and subsequently into phytenic acid (Fig. 3b). Phytenal has already been shown to be a degradation product of phytol in marine bacteria, where it can also be converted into phytenic and phytanic acid [60, 61].

FALDH is required for the production of phytenic acid

Since production of phytenic acid was shown to be high in microsomes [58, and our own data], microsomal enzymes were investigated that might catalyze this reaction. Kelson et al. reported that FALDH could convert dihydrophytol into phytanic acid [62]. This prompted investigations of our own to establish whether FALDH is involved in the breakdown of phytol. Fibroblast cell lines derived from SLS patients, marked by a deficiency in FALDH, were cultured in a medium containing phytol. These cells did not show any production of phytenic acid, in contrast to the abundant formation of phytanic acid in control cells. [59, 63]. Furthermore, under incubation conditions similar to those Muralidharan and Muralidharan had used [58], homogenates from fibroblasts derived from SLS patients were clearly deficient in the breakdown of phytol [63].

FALDH is thought to be part of an enzyme complex, fatty alcohol:NAD^+ oxidoreductase (FAO), that consists of an alcohol and aldehyde dehydrogenase [64]. FAO is involved in the cycling of fatty acids to fatty alcohols, by which the

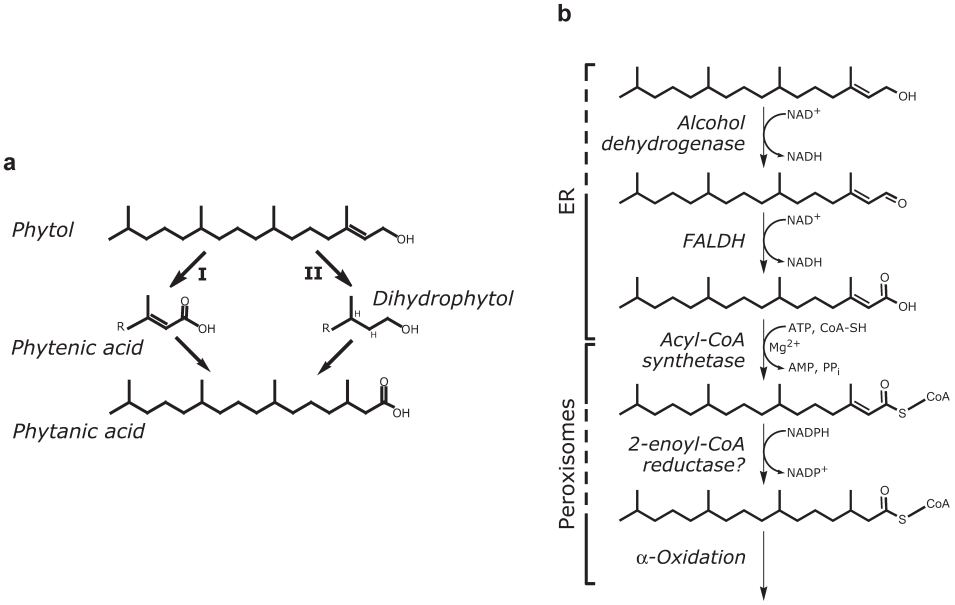

Figure 3. Pathway of phytol breakdown. (*a*) Possible reaction sequences leading to phytanic acid production. Mechanism I involves first the oxidation of phytol to phytenic acid followed by the reduction of phytenic acid, as observed in rat and humans. Mechanism II involves first the reduction of phytol to dihydrophytol and next the oxidation to phytanic acid, as proposed for cows. (*b*) The phytol breakdown pathway as proposed by our group. Indicated are the cellular compartments where the reactions take place. Dashed lines signify proposed locations: the alcohol dehydrogenase catalyzing the first step of phytol metabolism that is thought to form a complex with FALDH on the ER membrane; and the reduction of phytenoyl-CoA for which activity was found in both peroxisomes and mitochondria, with the former compartment presumed to be the most likely site for phytenoyl-CoA reduction to occur (see text). The activation of phytenic acid to phytenoyl-CoA can occur either at the ER or in peroxisomes.

availability of fatty alcohols for the incorporation in e.g. ether lipids is regulated [65]. The two enzyme activities can be separated via column purification of FAO. This approach resulted in the cloning and characterization of FALDH [62, 66, 67]. The alcohol dehydrogenase enzyme proved to be unstable and has yet to be identified [68]. It is likely, however, that the FAO complex as a whole catalyzes the conversion of phytol into phytenic acid.

Phytol and SLS

The biochemical hallmark of SLS is the accumulation of long-chain aliphatic alcohols [69], and clinical symptoms include ichthyosis, mental retardation and spastic diplegia or tetraplegia [70, 71]. The accumulation of fatty alcohols instead of fatty acids might lie in the instability of the alcohol dehydrogenase when it is separated from FALDH [68] or in the reactivity of aldehydes, but this question has not been addressed satisfactorily. Recently, it was observed that SLS patients accumulate leukotriene B_4, which is also degraded by FALDH [71]. Leukotriene B_4 is a proinflammatory cytokine and may play a role in the characteristic ichthyosis that is observed in these patients.

After it was found that the degradation of phytol is deficient in SLS patients, it was questioned whether a possible accumulation of phytol might contribute to the symptoms observed in the patients. A link was speculated between the accumulation of phytol and the rise of an unknown lipid peak that was observed using magnetic resonance imaging in some SLS patients [72]. However, investigations in our lab have shown normal levels of phytol in plasma of SLS patients [unpublished data].

Activation and reduction of phytenic acid

The enzymatic conversion of phytenic acid into phytanic acid had not received any investigation until our own recent studies [73]. According to the model depicted in Figure 3b, phytenic acid is directly converted into phytanic acid, a process which is usually NADPH (reduced nicotinamide adenine dinucleotide phosphate)-dependent. However, the rate of phytenic acid reduction was found to be very low, and detailed characterization of the enzyme activity resulted in serious doubts about its relevance for the *in vivo* situation [73]. Therefore, other ways of converting phytenic acid into phytanic acid were investigated, and in this way we discovered that, similar to phytanic acid, phytenic acid is a good substrate for a synthetase reaction. After phytenic acid is converted to its CoA ester, reduction is much more efficient and rapid, indicating that phytenoyl-CoA and not the free acid is the true substrate for this reaction. Further proof that phytenoyl-CoA is a bona fide intermediate of phytol breakdown came with the detection of the CoA ester in cells cultured in the presence of phytol [73].

This new insight implicates an additional step in the pathway of phytol degradation, catalyzed by an acyl-CoA synthetase (Fig. 3b). According to this modified pathway, phytol is still converted to phytenic acid, as mediated by FALDH. However, for the reaction to proceed further, phytenic acid first needs to be converted into phytenoyl-CoA before it can be reduced to phytanoyl-CoA. In this way, the phytanoyl-CoA can be directly α-oxidized and further degraded.

Subcellular location of phytol degradation

As described above, conversion of phytol into phytenic acid takes place in microsomes, where the FAO-enzyme complex is located. The phytenic acid produced in the FAO reaction can be activated either at the ER or the peroxisomal membrane [73], which resembles the data obtained for the formation of phytanoyl-CoA [25, 74].

For the reduction of phytenoyl-CoA, activity was found in both mitochondria- and peroxisome-enriched fractions [73]. Under *in vivo* conditions reduction of phytenoyl-CoA to phytanoyl-CoA may well be strictly peroxisomal, since phytenoyl-CoA appears unable to traverse the mitochondrial membrane [D. M. van den Brink and R. J. A. Wanders, unpublished observations]. Interestingly, a peroxisomal 2-enoyl-CoA reductase with unknown function was described fairly recently [75] that might be responsible for the peroxisomal reduction of phytenoyl-CoA. Taken together, although definite proof is still lacking, conversion of phytenic acid into phytanic acid is likely peroxisomal.

In this regard, as with the dehydrogenation of pristanal in α-oxidation, it is interesting to speculate on a possible peroxisomal localization of FALDH or another aldehyde dehydrogenase. Production of phytenic acid would then also take place in this organelle, compartmentalizing the complete breakdown of phytol in one single organelle.

Disorders with a deficiency in α-oxidation

As referred to at the beginning of this review, there are several metabolic disorders that are characterized by an accumulation of phytanic acid. Most of these show other deficiencies besides that in α-oxidation, and the clinical phenotype of the disorders differs significantly.

Peroxisome biogenesis disorders

The peroxisome biogenesis disorders (PBDs) are both clinically and genetically heterogeneous. They comprise ZS, neonatal adrenoleukodystrophy and infantile Refsum disease, with the former being the most and the latter the least severe phenotype. Common features are liver

disease, neurodevelopmental delay, retinopathy and deafness. Often dysmorphic facial features can be observed at birth, together with a hypotonic state of the affected infant. For ZS, death usually occurs in the first year, while for infantile Refsum disease patients may survive beyond infancy, with some reaching adulthood [76].

At the cellular level, PBDs are caused by defects in the genes encoding proteins responsible for either peroxisomal membrane assembly or import of peroxisomal matrix proteins (see [77]). These mutations result in the absence of functional peroxisomes and affect all peroxisomal functions. This result leads to deficiencies in the synthesis of plasmalogens, polyunsaturated fatty acids and bile acids, as well as a deficiency in β-oxidation of very long chain and branched-chain fatty acids and a deficiency in α-oxidation [78]. Although α-oxidation is deficient in PBD patients, the levels of phytanic acid in serum and tissues of PBD patients are much lower than what found in patients suffering from Refsum disease. This finding is most likely due to the fact that phytanic acid is taken in via the diet and only accumulates gradually over time. Therefore, the contribution of phytanic acid to the phenotype of the PBDs is likely to be of relatively minor importance.

Rhizomelic chondrodysplasia punctata

Related to the PBDs is rhizomelic chondrodysplasia punctata (RCDP) type 1, a disorder marked by growth retardation, profound developmental delay, cataracts and dystoses in patients who often die in their first year of life [79]. A defect in peroxin 7 lies at the basis of RCDP type 1 and results in a deficiency in the import of some, but not all, peroxisomal matrix proteins.

Peroxisomal matrix protein import

Peroxisomal matrix proteins are imported into peroxisomes via two routes. As a general rule they contain either a peroxisomal targeting signal (PTS)1 or a PTS2, which can be recognized by receptor proteins in the cytosol [80], although occasional exceptions have been described [81]. The receptor for PTS1 proteins is peroxin 5, and the receptor for PTS2 proteins is peroxin 7. Both these receptors function in a similar way. As depicted in Figure 4, the protein that needs to be imported is bound to the receptor in the cytosol. This complex is then able to dock on protein complexes present on the peroxisomal membrane, after which translocation into the peroxisomal lumen can take place [82, 83]. Both receptor peroxins function by recognizing certain amino acids present in the target protein. However, the location of these amino acids in the protein as well as the consensus sequence that makes up the PTS differs between PTS1 and PTS2 sequences (reviewed in [84]).

Of the multitude of peroxisomal matrix enzymes in mammals, the vast majority are known to possess a PTS1. A defect of peroxin 5 therefore results in a deficiency of most peroxisomal processes and leads to a PBD phenotype, while peroxin 7 deficiency leads to RCDP. In hu-

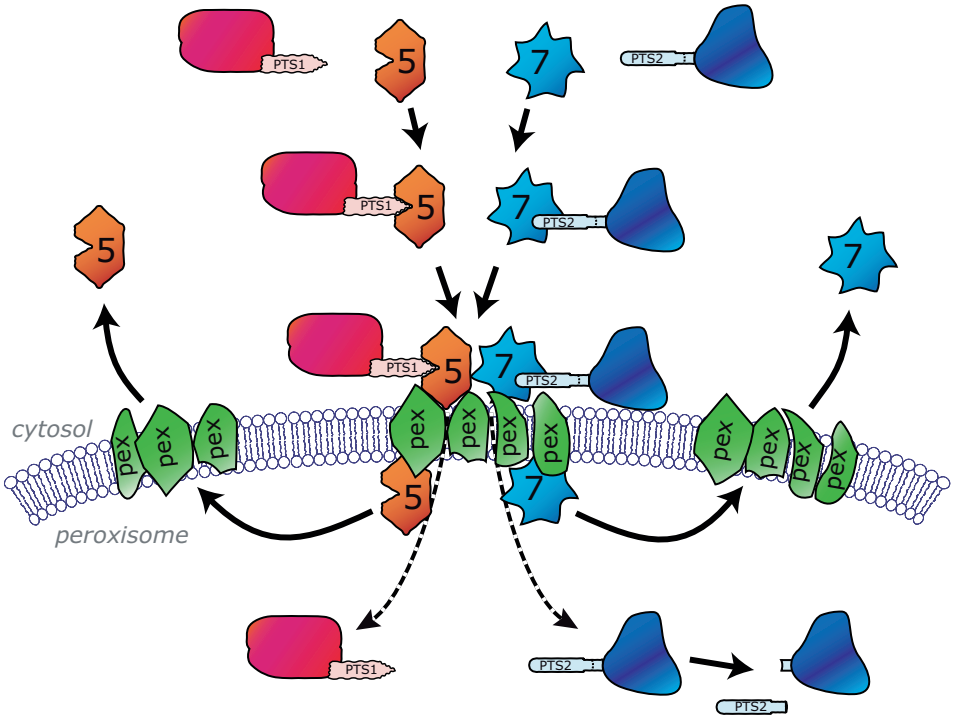

Figure 4. Simplified cartoon of the import of peroxisomal matrix proteins. See text for details.

mans, only three proteins have been identified that contain a PTS2 and are imported by peroxin 7. These include (i) peroxisomal thiolase, one of the enzymes involved in β-oxidation, which is most likely functionally redundant with another thiolase present in peroxisomes. A deficiency of this enzyme therefore has no consequence for the peroxisomal β-oxidation of different fatty acids; (ii) PAHX, mislocalization of which leads to a deficiency of α-oxidation and accumulating levels of phytanic acid; and (iii) ADHAPS, which leads to the inability to synthesize plasmalogens as a consequence of its mislocalization [85]. As with the PBDs, phytanic acid is thought to be of minor importance in the development of clinical symptoms in RCDP, except in milder cases that survive for longer periods. These latter patients are managed by a low phytanic acid diet, which prevents onset of additional symptoms.

Although mutations in *PEX7*, the gene encoding for peroxin 7, lead to RCDP [86, 87], mutations in two other genes can also lead to the same disease. Depending on its molecular basis, RCDP is divided into three types. Type 1 corresponds to mutations in *PEX7*, while types 2 and 3 correspond to mutations in one of the two genes encoding the peroxisomal enzymes required for plasmalogen synthesis [88]. The fact that RCDP types 2 and 3 share a common phenotype with RCDP type 1 further suggests that the deficiency in α-oxidation bears no consequence for the phenotypic presentation of RCDP patients, at least in the first years of life.

Refsum disease

Heredopathia atactica polyneuritiformis, or Refsum disease, was first described by Sigvald Refsum, a Norwegian neurologist, in 1946 [89]. The symptoms characteristic for Refsum disease include retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia and elevated protein concentrations in the cerebrospinal fluid in the absence of an increased number of cells [90]. Additionally, in a recent evaluation, anosmia was found in nearly all Refsum disease patients, while deafness, ichthyosis and cardiac arrhythmias were also regularly present [91–93].

The onset of symptoms is usually relatively late in life, with most patients presenting in adolescence, which is thought to be due to the gradual accumulation of phytanic acid from the diet. The first clinical manifestations often start with night blindness followed by further deterioration of vision. Although onset of disease may occur much earlier in life, the subtle nature of the first symptoms makes it difficult to delineate the precise age at which the disease started. However, in most cases the symptoms will progress to retinitis pigmentosa, leading to tunnel vision or even complete loss of sight. Occurrence of cardiac arrhythmias can sometimes lead to death or necessitate a cardiac transplant. To prevent the accumulation of phytanic acid, a diet is prescribed which in most patients leads to a reduction of phytanic acid levels and usually reversal of at least some symptoms.

Although accumulation of phytanic acid has been observed for many decades in Refsum disease patients, the precise biochemical basis of Refsum disease was not described until 1997, when the enzyme defect was identified in Refsum disease patients at the level of phytanoyl-CoA hydroxylase, which was soon followed by the resolution of the molecular basis of Refsum disease [94, 95].

While there has been speculation regarding other substrates for PAHX [96], none of these has been shown to be relevant for the *in vivo* situation aside from phytanic acid. Therefore, all the Refsum disease symptoms are likely to be the consequence of the accumulation of phytanic acid. This makes Refsum disease distinct from all the other phytanic acid storage syndromes where other functions are affected as well, as described above.

Genetic heterogeneity within Refsum disease

Once the gene causing Refsum disease had been identified, mutational analysis of *PAHX* was performed in many Refsum disease patients. A recent review summarizes *PAHX* sequence analysis in 31 unrelated affected families, where 29 different variant alleles were observed [97], and elucidation of the PAHX structure has provided insight into how these mutations affect the function of the protein [98]. Somewhat surprisingly, however, it was found that mutations were not present in all patients, despite a clear deficiency in PAHX activity. A possible explanation for this finding was provided by the finding of genetic heterogeneity, as concluded from linkage analysis studies in Refsum disease patients' families [99]. Four out of eight families tested in this study did not show significant linkage to the chromosome 10p13 locus where *PAHX* is located. This finding led to the conclusion that in up to 50% of patients diagnosed with Refsum disease, the *PAHX* gene itself is not affected; the true molecular defect lies at another locus. This percentage may well be an overestimation, however, since from the above-mentioned review of *PAHX* sequence analyses it appears that mutations in *PAHX* have been found in more than 80% of Refsum disease patients [97].

After analysis of the four families failed to show linkage to the *PAHX* locus, investigations were continued to identify the locus that did show significant linkage. Further research in two affected families established linkage to a locus on chromosome 6q22-24 [100]. This locus contains the *PEX7* gene, and subsequent analysis showed that patients from these families had a defect in peroxisomal import of PTS2 proteins. Mutational analysis identified mutations in the *PEX7* gene, which indicated that these patients had the same molecular defect as RCDP type 1

patients [100]. Most likely, the mild phenotype observed in these patients, as compared with the much more severe phenotype normally found in RCDP type 1 patients, can be explained by the fact that the mutations do not result in complete deficiency of peroxin 7 function but allow some residual PTS2-protein import. Moser et al. [88] provided the first evidence for genetic heterogeneity within Refsum disease through complementation analysis studies. Interestingly, the patient described was one of the first patients to be diagnosed and described by Sigvald Refsum [89]. In their study, Moser et al. [88] observed that the cell line was able to complement another Refsum disease cell line, showing that *PAHX* was not the gene involved in this patient. Instead, the cells did not show complementation with a RCDP type 1 cell line, and mutations were found in the *PEX7* gene [86]. Since these findings were part of a larger screen of molecular defects of patients suffering from peroxisome biogenesis defects, the implications for Refsum disease were not appreciated.

Taken together, these data show that Refsum disease is caused by mutations in *PAHX* in the majority of patients and by mild mutations in *PEX7* in a subgroup of patients. However, there still remain a small number of patients in whom no mutations in either of these two genes can be found, leaving open the possibility of additional loci for Refsum disease.

Toxicity of phytanic acid and its effects on nuclear receptors

Of great interest to elevations in phytanic acid levels in the disorders summarized above is the role of phytanic acid in the activation of specific nuclear receptors. These nuclear receptors function as transcription factors that activate gene transcription when in a ligand-bound state. It was shown that phytanic acid can activate the nuclear retinoid-X receptor (RXR), which is involved in a variety of cellular processes, including regulation of fatty acid metabolism [101, 102]. In addition, phytanic acid was found to be a ligand for peroxisome proliferator-activated receptor (PPAR)α [103], while at the same time it had no apparent effect on PPARβ or PPARγ.

Upon ligand binding, PPARα forms a heterodimer with RXR, which allows it to bind to promoter elements of its target genes and so activates their transcription. The targets consist mainly of genes encoding for proteins that function in lipid and glucose metabolism. Since, besides phytanic acid, a number of other fatty acids were also shown to act as natural PPARα ligands, transcriptional regulation by PPARα is postulated to play a central role in the regulation of cellular fatty acid levels.

The EC_{50} (mean effective concentration) of phytanic acid for PPARα has been reported to be about 40 μM [104], which is above levels normally found in healthy individuals. However, the phytanic acid levels found in Refsum disease patients are much higher, amounting even to >1 mM. Such levels perturb normal lipid homeostasis significantly through this mechanism. Increased expression through PPARα of genes involved in lipid degradation leads to increased breakdown of fatty acids, which might explain the loss of adipose tissue observed in patients. Interestingly, mice fed a phytol diet showed a decrease in levels of long-chain fatty acids, which was not observed in PPARα-deficient mice used in the same experiment [105]. Many proteins involved in lipid degradation were found to be induced in a PPARα-dependent manner, showing the important role phytanic acid might exert in pathological conditions.

In addition, direct toxic effects of phytanic acid have been described. There is some evidence that these toxic effects are mediated by increases in intracellular Ca^{2+} levels and accompanied by a slow decrease in the membrane potential of mitochondria. This would lead to a decrease in ATP production and an increase in reactive oxygen species, both of which have been shown to occur in cultured cells and isolated mitochondria of neuronal origin [5, 6].

Concluding remarks

In the past decade much has been learned about the breakdown of phytanic acid. What remains is the question of pristanal dehydrogenation and whether, if α-oxidation is to be completely peroxisomal, it is catalyzed by FALDH present in the peroxisome or whether there is a true peroxisomal aldehyde dehydrogenase.

At the same time however, the mechanism by which phytanic acid is produced from phytol is still not completely resolved. Although in the last few years the basic mechanism has been elucidated, only one of the enzymes involved has been identified so far. Also, the subcellular localization needs further study before any firm conclusions can be drawn. Furthermore, it will be important to look at other possible substrates for this pathway. Good candidates might be isoprenoid alcohols such as geranylgeraniol and farnesol, which possess a branched-chain carbon chain similar to phytol and about the degradation of which almost nothing is known.

Another challenge will be to link phytanic acid accumulation directly to the pathology found in patients suffering from the various disorders that have been described. Now that more data on the toxicity of phytanic acid are becoming available through research in cell culture, the knowledge so gained might be used to perform studies in mouse models that have been produced for these disorders. Ultimately, this research might provide tools to clinicians treating these patients to reduce or prevent the damage done by phytanic acid.

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