New insights in phytanic acid metabolism and toxicity

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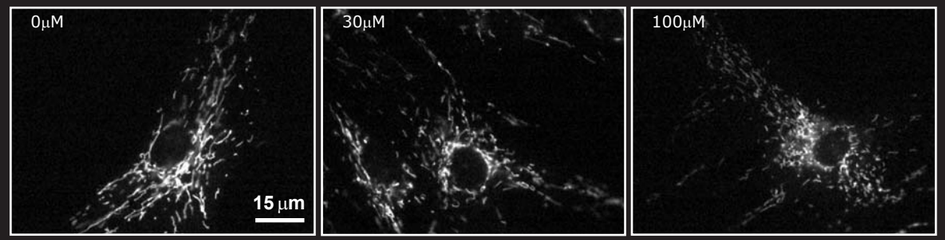

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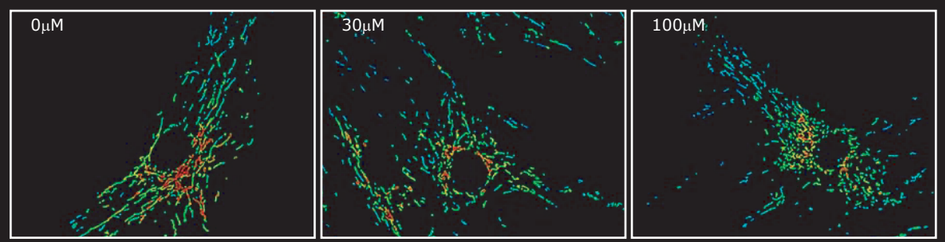

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van het proefschrift
van

Jasper Komen

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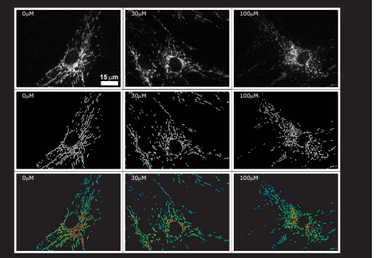

Receptie ter plaatse
na afloop van de promotie

Paranimfen

Bas Westhoff
(06-48073000)

Marc Engelen
(06-14868432)

NEW INSIGHTS IN PHYTANIC ACID METABOLISM AND TOXICITY

Cover Illustration:
Effect of phytanic acid on mitochondrial membrane potential. Shown are confocal images of TMRM-loaded fibroblasts incubated with different concentrations of phytanic acid. See chapter 6 for a detailed description of the complete method. Top row: background-corrected images. Middle row: binary mitochondrial masks. Bottom row: masks on top of background-corrected images (used for mitochondrial membrane potential calculations).

NEW INSIGHTS IN PHYTANIC ACID METABOLISM AND TOXICITY

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam op gezag van de
Rector Magnificus prof.dr. D.C. van den Boom
ten overstaan van een door het college voor
promoties ingestelde commissie, in het openbaar
te verdedigen in de Aula der Universiteit op
woensdag 12 december 2007, te 10.00 uur

door

Johannes Cornelis Komen

geboren te Hoorn

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Faculteit der Geneeskunde

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Abbreviations (1/2)

ACS1	long-chain acyl-CoA synthetase (Acyl-CoA synthetase 1)
Ah receptor	aryl or aromatic hydrocarbon receptor
ALDH3A2	aldehyde dehydrogenase 3A2
ANT	adenine nucleotide translocase
APAD	3-acetylpyridine adenine dinucleotide
ARD	adult Refsum Disease
ATRA	all-trans retinoic acid
BSTFA	N,O-bis-(trimethylsilyl)trifluoroacetamide
CAR	constitutive androstane receptor,
Complex I	NADH:ubiquinone oxidoreductase
Complex II	succinate:ubiquinone oxidoreductase
CPT	carnitine palmitoyltransferase
CYP450	cytochrome P450
CYPOR	cytochrome P450 oxidoreductase
DDC	diethyldithiocarbamate
Delta (Δ) ψ	mitochondrial membrane potential
DNP	dinitrophenol
FALDH	fatty aldehyde dehydrogenase
FCCP	carbonyl cyanide p-trifluoromethoxy-phenylhydrazone
FFAs	free fatty acids
GC/MS	gas chromatography/mass spectrometry
GOT	glutamate-oxaloacetate-transaminase
2-HACL1	2-hydroxyacyl-CoA lyase 1
20-HETE	20-hydroxyeicosatetraenoic acid
HPCL	2-hydroxyphytanoyl-CoA lyase
HPLC	high performance liquid chromatography
IL	interleukin
L/B ratio	liver/body weight ratio
L-FABP	liver fatty acid binding protein
LTB4	leukotriene B4
MDH	malate dehydrogenase
MPT	mitochondrial permeability transition
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide
17ODYA	17-octadecynoic acid
OXPHOS	oxidative phosphorylation

Abbreviations (2/2)

PAHs	polycyclic aromatic hydrocarbons
PHYH	phytanoyl-CoA hydroxylase
PPAR	peroxisome-proliferator activated receptor
PPRE	peroxisome proliferator response element
PTS	peroxisomal targeting signal
PXR	pregnane X receptor
9-cis-RA	9-cis retinoic acid
RAR	retinoic acid receptor
RCDP	rhizomelic chondrodysplasia punctata
ROS	reactive oxygen species
RXR	retinoid X receptor
SCP2	sterol carrier protein 2
SCPx	sterol carrier protein X
SIM	single ion monitoring
SLS	Sjögren-Larsson syndrome
SREBPs	Sterol Regulatory Element Binding Proteins
TER	*trans*-2-enoyl-CoA reductase
TMRM	tetramethyl rhodamine methyl ester
TMS	trimethylsilyl
TMCS	trimethylchlorosilane
TNF-α	tumor necrosis factor-α
VLCFA	very long-chain fatty acid

Contents

CHAPTER 1

Introduction

Introduction:

Phytanic acid is a branched-chain fatty acid present in the diet of humans

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a saturated branched-chain fatty acid (figure 1). The backbone of phytanic acid consists of 16 carbon atoms and contains 4 methyl groups. These 4 methyl branches make this fatty acid structurally different from other fatty acids which predominantly consist of only a straight carbon backbone. In 1910, Willstätter et al [1] synthesized 3,7,11,15-tetramethylhexadecanoic acid from phytol (3,7,11,15-tetramethylhexadec-trans-2-en-l-ol) and named it "phytansäure" (i.e. phytanic acid). 4 Decades thereafter the scientific world developed new interest in phytanic acid when phytanic acid was discovered as one of the branched-chain fatty acids present in butterfat [2]. The structure of phytanic acid was later confirmed [3] and additional sources of phytanic acid were found e.g. ox-plasma lipids, ox-periphrenic fat, milk lipids, cow rumen, and rumen bacterial lipids [4-7]. However, the origin of this unusual fatty acid remained unknown. One possibility was that phytanic acid was endogenously biosynthesized by coupling of an isopentenylpyrophosphate unit to farnesylpyrophosphate. Subsequent saturation of the double bonds and reduction of the alcohol moiety to a carboxyl group would yield phytanic acid [8]. This theory was discarded by Steinberg et al. who showed that phytanic acid cannot be synthesized endogenously in mammals [9], and that phytanic acid in mammals is derived from exogenous sources as phytanic acid itself or as its precursor, i.e. the fatty alcohol phytol.

Phytol (figure 1) is a widely abundant molecule in nature. As a constituent of chlorophyll it is present in green leaves of plants and trees. Chlorophyll is anchored into the thylakoid membrane of chloroplasts through the phytol side chain. Phytol is linked via an ester bond to the porphyrin ring of chlorophyll and accounts for one third of its molecular weight. The human digestive system is not able to degrade chlorophyll and cleave off phytol from the porphyrin ring [10], but bacteria in the rumen of ruminant animals are able to perform this action [5]. The released phytol can further be converted to phytanic acid in the ruminant. Other animals including humans are also able to convert phytol into phytanic acid [9;11-13]. Therefore, meat and dairy products originating from ruminant animals are rich sources of phytanic acid, either directly via phytanic acid or indirectly by means of the precursor phytol. Phytoplankton species, e.g. algae, also contain chlorophyll molecules. Phytoplankton chlorophyll is degraded to pheophorbide a in the gut of zooplankton after herbivorous grazing of the zooplankton, thereby releasing phytol into the marine

system [14;15]. Hence, humans are also able to obtain phytanic acid and its precursor phytol by means of eating seafood.

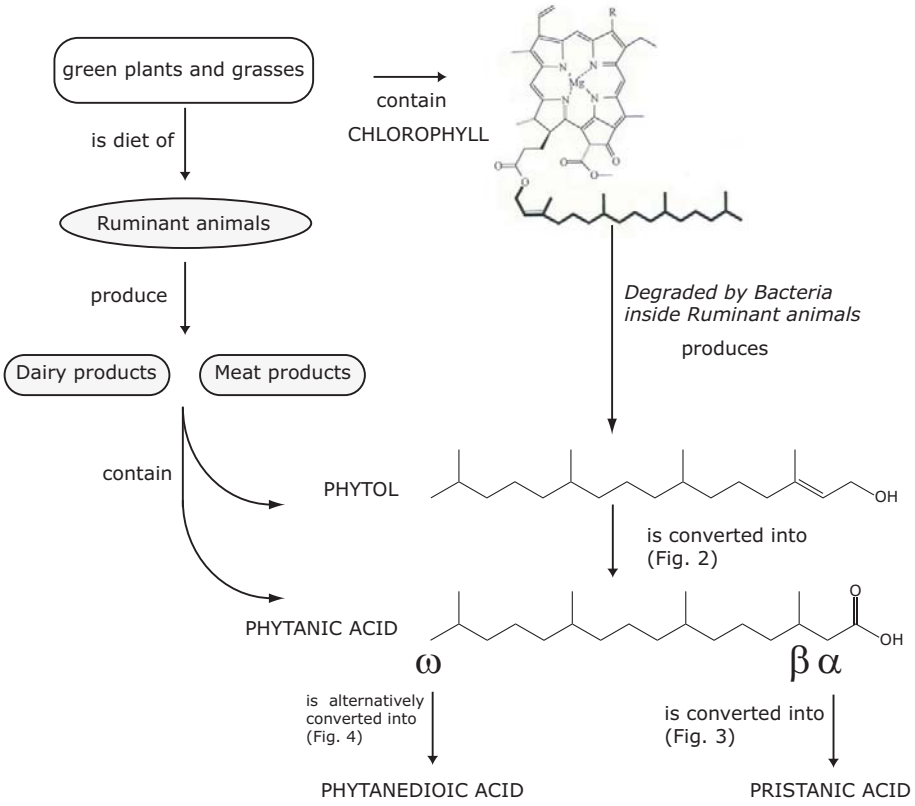

Figure 1: The origin of phytanic acid. Phytanic acid is a metabolite of chlorophyll. Chlorophyll is abundantly present in green leafs of plants and in grasses, but is also present in phytoplankton (see text). The green plants and grasses form the diet of ruminant animals. Bacteria in the digestive system of these animals can degrade chlophyll which results in the release of phytol. Phytol can subsequently be converted into phytanic acid in ruminant animals, or eventually in humans (figure 2) who obtain phytol and phytanic acid by consuming dairy products and meat from ruminant animals. Phytanic acid is normally degraded by the α-oxidation pathway which results in the formation of pristanic acid (shown in detail in figure 3). Alternatively, phytanic acid might undergo ω-oxidation which produces a dicarboxylic acid, i.e. phytanedioic acid (shown in detail in figure 4).

Phytanic acid is formed from the chlorophyll metabolite phytol

Although phytol was known to be a precursor of phytanic acid through phytol feeding studies performed 4 decades ago [9;11-13;16], the biochemical pathway responsible for the conversion of phytol into phytanic acid was discovered only recently (figure 2, and reviewed in [17]). First, phytol is oxidized into phytenic acid. It is speculated that this

occurs through the initial conversion of phytol into an aldehyde named phytenal by an alcohol dehydrogenase. Phytenal has never been detected as an intermediate of the pathway in mammals, but has been detected in the degradation pathway of phytol in marine bacteria [18;19]. Subsequently, phytenal is converted into a carboxylic acid (phytenic acid) by an aldehyde dehydrogenase. This aldehyde dehydrogenase was identified as fatty aldehyde dehydrogenase (FALDH) encoded by the *ALDH3A2* gene which was concluded from studies in fibroblasts from Sjögren Larsson syndrome (SLS, MIM# 270200) patients [20]. These patients have mutations in the *ALDH3A2* gene and Van den Brink et al. showed that SLS fibroblasts cannot convert phytol into phytanic acid [20;21].

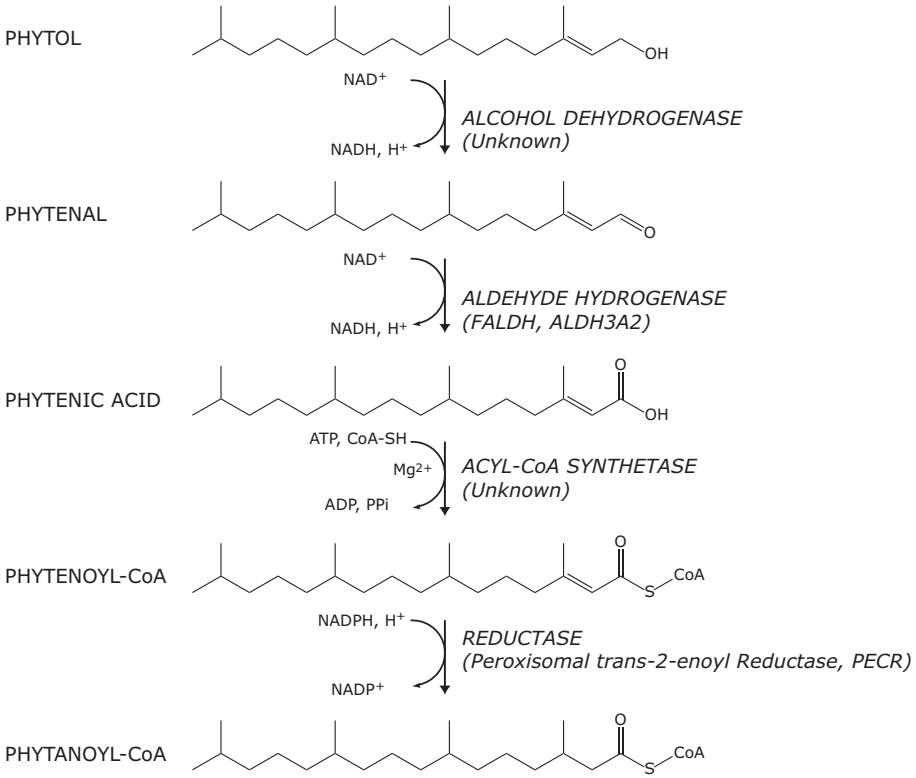

Figure 2: Conversion of phytol into phytanic acid. Phytol, originating from the diet, is first converted into phytenic acid via the intermediate phytenal due to the subsequent action of an unknown alcoholdehydrogenase and the fatty aldehyde dehydrogenase (FALDH, encoded by *ALDH3A2*). Next, phytenic acid is activated to its CoA-ester, i.e. phytenoyl-CoA, by an acyl-CoA synthetase. This step and the previous steps occur at the endoplasmic reticulum, but may possibly also occur at/in the peroxisome. Phytenoyl-CoA is reduced inside the peroxisome by peroxisomal trans-2-enoyl reductase (encoded by *PECR*) which results in the formation of phytanoyl CoA. Phytanoyl-CoA is subsequently further degraded via α-oxidation (figure 3).

The final step in the conversion of phytol to phytanic acid, i.e. the reduction of the double bond in phytenic acid, was characterized also by Van den Brink et al. [22]. They discovered that it is not phytenic acid but rather its CoA-ester intermediate, i.e. phytenoyl-CoA, which is readily reduced. Phytenoyl-CoA synthetase activity was detected in the peroxisomal and microsomal fractions of a rat liver density gradient, whereas reductase activity was present in the mitochondrial and in the peroxisomal fractions. Further investigations on the reduction of phytenoyl-CoA revealed that the peroxisomal trans-2-enoyl reductase (TER) encoded by the *PECR* gene converts phytenoyl-CoA into phytanoyl-CoA [23]. TER has a peroxisomal targeting signal 1 (PTS1) and no mitochondrial targeting signal, and was shown to be localized in peroxisomes [24]. These results indicate that a reductase other than TER is responsible for the mitochondrial reductase activity. In addition, preliminary results suggest that mitochondrial import of phytenoyl-CoA may be relatively inefficient because phytenoyl-CoA is a poor substrate for carnitine palmitoyltransferase I (CPT I). CPT I is the enzyme that would convert phytenoyl-CoA to its carnitine ester, a process which is necessary for mitochondrial import of fatty acids [23].

The formation of phytanoyl-CoA in the peroxisome by TER makes it readily available for the enzymes of the α-oxidation pathway which are all localized in this organelle as described in the next paragraph.

Phytanic acid is degraded via alpha-oxidation in peroxisomes

The discovery of the accumulation of phytanic acid in patients diagnosed with Refsum disease ([25], and in the next paragraph) resulted not only in an increased interest about the origin of phytanic acid, but also in the pathway responsible for degradation of this branched-chain fatty acid. Branched-chain fatty acids are usually broken down by β-oxidation in peroxisomes (reviewed [26;27]). However, phytanic acid contains a methyl group at the 3-position which blocks β-oxidation. In 1965, Eldjarn [28] proposed that breakdown of phytanic acid occurred from the ω-end of the fatty acid by a pathway known as ω-oxidation (discussed later). This conclusion was based on a study which showed a reduced output of sebacic acid in patients with Refsum disease after oral administration of tricaprin. However, subsequent studies of Eldjarn et al. [29;30] showed that Refsum patients have a similar ω-oxidation capacity as compared to control subjects towards branched-chain compounds. From these studies they concluded that there had to be an alternative degradation route for branched-chain fatty acids. This route was soon identified by Avigan et al. who showed that pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) was a metabolite of phytanic acid in rats [31].

Pristanic acid has almost the same structure as phytanic acid, but differs in the length of its carbon-backbone. Pristanic acid is one carbon atom shorter than its precursor

phytanic acid due to oxidative decarboxylation at the α-carbon atom of phytanic acid by a process called α-oxidation (figure 3), which was already described for straight-chain fatty acids in plants and mammalian brain [32;33]. Pristanic acid is further degraded in rodents by means of successive beta-oxidation steps [34] which ultimately lead to the formation of acetyl-CoAs, propionyl-CoAs and isobutyryl-CoA [35].

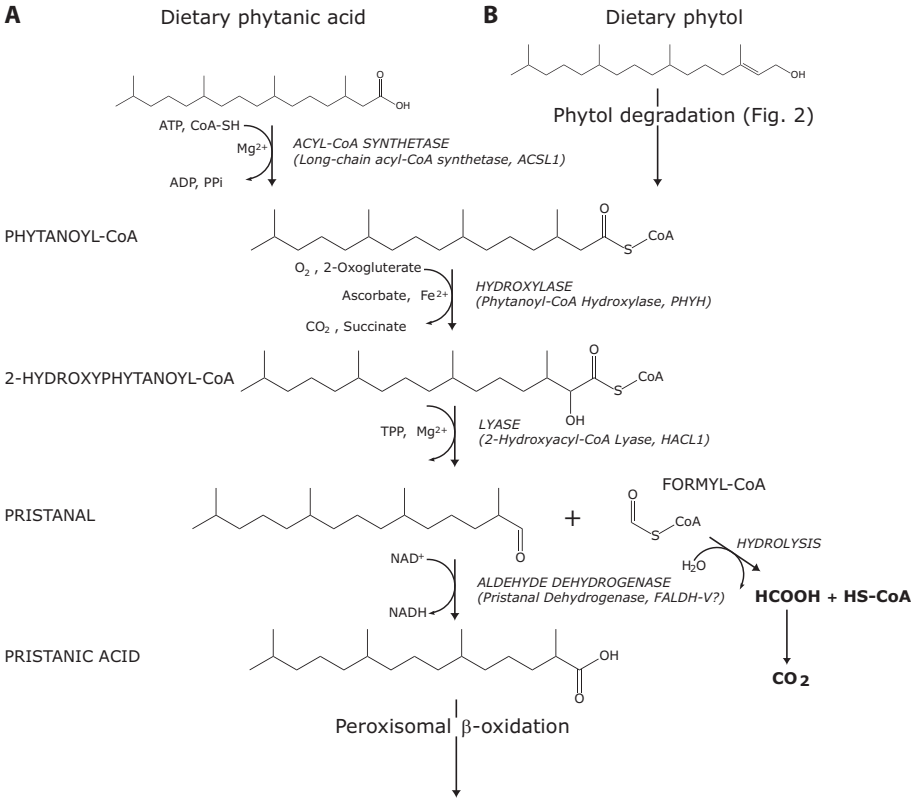

Figure 3: Schematic representation of phytanic acid α-oxidation. The actual substrate for the α-oxidation pathway is phytanoyl-CoA, which is produced from phytol **(B)** or is formed from the activation of dietary phytanic acid **(A)** by the long-chain acyl-CoA synthetase. Phytanoyl-CoA is subsequently hydroxylated at position 2 of the fatty acid by phytanoyl-CoA hydroxylase. 2-Hydroxyphytanic acid is cleaved by the action of 2-hydroacyl-CoA lyase 1 which leads to the production of pristanal and formyl-CoA. Pristanal is converted into pristanic acid by an aldehyde dehydrogenase (possibly a specific splice form of FALDH, i.e. FALDH-V), and formyl-CoA is converted into carbondioxide via hydrolysis into formic acid and free CoA. Pristanic acid is a substrate for the β-oxidation pathway in peroxisomes which is responsible for further degradation of this fatty acid.

The identification of 2-hydroxyphytanic acid as an intermediate of the α-oxidation pathway led to new insights in the enzymatic steps involved in the

breakdown of phytanic acid [36]. The hydroxylation at position 2 of phytanic acid was similar as occurring in the α-oxidation pathway of straight-chain fatty acids in brain and plants [37;38]. Nevertheless, the enzyme catalyzing the 2-hydroxylation as well as the enzymes involved in the other steps of the α-oxidation pathway remained obscure until the 1990s.

A major breakthrough in this respect was the discovery of Watkins et al. [39] and Mihalik et al. [40] that α-oxidation required the formation of phytanoyl-CoA from phytanic acid, which was catalyzed by the long-chain acyl-CoA synthetase (ACS1) [41]. Furthermore, the same study of Mihalik et al. [40] showed that phytanic acid 2-hydroxylation in rat liver required cofactors associated with a dioxygenase catalyzed hydroxylation mechanism in which 2-oxoglutarate acts as the co-substrate and Fe^{2+} and ascorbate are required co-factors. Studies from Jansen et al. [42] confirmed these results in human liver and revealed a deficiency of phytanic acid alpha-hydroxylation in liver samples of patients with Zellweger syndrome [MIM# 214100]. Since Zellweger patients do not have any functional peroxisomes, as a consequence of mutations in one of the many genes coding for proteins involved in peroxisomal biogenesis, this finally ended the controversy on the subcellular localization of the α-oxidation pathway.

These discoveries in the mid 1990s soon led to the identification of the enzyme catalyzing the hydroxylation of phytanoyl-CoA, i.e. phytanoyl-CoA hydroxylase (PHYH), encoded by the *PHYH* gene [43;44]. PHYH contains a peroxisomal targeting sequence 2 (PTS 2) and requires the PTS2 receptor (Pex7) to become imported into peroxisomes. After the discovery of PHYH the investigations continued on the remaining unidentified steps in the α-oxidation pathway of phytanic acid. Verhoeven et al. [45] showed that pristanal was an intermediate in the decarboxylation step that was hypothesized to occur after the formation of 2-hydroxyphytanic acid. Croes et al. [46] discovered that the decarboxylation step involved the release of a one-carbon-unit as formyl-CoA which upon hydrolysis yields formic acid as previously found [40;47;48]. The enzyme involved in this step was identified as a thyamine pyrophosphate dependent lyase and named 2-hydroxyphytanoyl-CoA lyase (2-HPCL) [49], later renamed 2-hydroxyacyl-CoA lyase (2-HACL1) encoded by the *HACL1* gene. This enzyme contains a PTS1 signal and depends on the Pex5 receptor for its correct localization in the peroxisomal matrix. Pristanal formed by 2-HPCL is further metabolized into pristanic acid by a NAD+ dependent aldehyde dehydrogenase present in peroxisomes [50]. Until now, this enzyme has remained unidentified although recent studies strongly suggest that a specific splice variant of FALDH (FALDH-V), located in the peroxisome is the pristanal dehydrogenase [51].

In summary, alpha-oxidation of phytanic acid takes place inside peroxisomes. After the formation of phytanoyl-CoA by the ACS1 enzyme, 2-hydroxyphytananoyl-

CoA is formed by PHYH. Subsequently, this intermediate is converted into pristanal and formyl-CoA by 2-HPCL. Formyl-CoA is hydrolysed to formic acid in the cytosol and is eventually converted into CO_2 while pristanal is oxidized into pristanic acid in peroxisomes for subsequent β-oxidation (reviewed by Verhoeven et al. [52]).

Recent studies have shown that PHYH and 2-HPCL have a broader substrate spectrum than phytanic acid and 2-hydroxyphytanic acid respectively [53;54]. In case of 2-HPCL the affinity towards 2-hydroxy long-chain fatty acids, constituents of brain cerebrosides and sulfatides, indicates that at least 2-HPCL may have a much broader function than just the metabolism of phytanic acid, hence this protein is now called 2-HACL1. However, in patients with Refsum disease who are completely PHYH deficient, the only accumulating metabolite found until now is phytanic acid indicating that *in vivo* phytanic acid seems the only important substrate for PHYH.

Refsum disease: deficiency of phytanic acid alpha-oxidation

Dietary phytol and its metabolites phytanic and pristanic acid can be degraded in healthy individuals via peroxisomal alpha-oxidation and subsequent β-oxidation as described in the previous paragraphs. There are, however, a number of inherited errors of metabolism in which this is not the case. In 1946, Dr. Sigvald Refsum described a new neurological syndrome in two Norwegian families and entitled it heredopathia atactica polyneuritiformis (MIM# 266500) [55]. The main clinical symptoms of this syndrome (later referred to as Refsum disease) include: progressive night blindness leading to retinitis pigmentosa and finally a total loss of vision, peripheral neuropathy, ataxia, increased level of cerebrospinal fluid protein with a normal cell count (reviewed in [56;57]). Additional symptoms that are found in Refsum disease patients are deafness, anosmia, cataracts, ichthyosis, and in some cases bone abnormalities are found in hands and feet of patients [58]. Cardiac arrhytmias are the main cause of death and related to times of metabolic stress. During stress, phytanic acid is released from intracellular stores which leads to a sudden increase in phytanic acid plasma levels (reviewed by Wanders et al. [59]). Almost 20 years after the discovery of the disease by Dr. Refsum, Klenk and Kahlke analyzed post-mortem tissues of a 7 years old girl diagnosed as affected by Refsum disease [25] and found markedly elevated amounts of a fatty acid in liver which they identified as phytanic acid. Findings of elevated phytanic acid levels in other patients suggested that Refsum disease was a metabolic disorder affecting phytanic acid metabolism. Indeed, phytanic acid has been used as the biochemical marker for the disease ever since.

Steinberg et al. discovered that phytanic acid can not be synthesized endogenously and originates from dietary sources [9]. Hence, the metabolic defect causing Refsum disease was suggested to occur in the degradation pathway of phytanic acid. This was confirmed in 1967 when the same group performed both *in*

vivo and *in vitro* studies which showed that Refsum patients had a decrease in the capacity to metabolize phytanic acid compared to healthy controls [60]. The exact origin of the defect remained obscure until Mihalik et al. [40] identified phytanoyl-CoA hydroxylase as the enzyme responsible for the first step of the α-oxidation pathway, shortly followed by Jansen et al. and Croes et al. [42;48]. Subsequently, Mihalik et al. and Jansen et al. identified *PHYH* as the gene encoding phytanoyl-CoA hydroxylase in 1997 [43;44;61]. Most Refsum patients were found to have mutations in the *PHYH* gene resulting in a deficiency of phytanoyl-CoA hydroxylase or an inactive protein, and hence, a deficient α-oxidation pathway (reviewed by Jansen et al. [62]). However, in a subset of patients no mutations were found in *PHYH* indicating that mutations in other genes might also give rise to the clinical spectrum of Refsum disease. Deficient phytanic acid α-oxidation was already known to occur in various other diseases such as Zellweger syndrome [MIM# 214100], neonatal adrenoleukodystrophy [MIM# 202370], infantile Refsum disease [MIM# 266510] and rhizomelic chondrodysplasia punctata (RCDP) type 1 [MIM# 215100]. However, multiple other peroxisomal metabolic pathways are affected in the diseases mentioned above. Hence, the phenotype of these diseases is more severe compared to Refsum disease.

RCDP type 1 is caused by mutations in the *PEX7* gene encoding the Pex7 protein which is responsible for targeting proteins containing a PTS2 signal, among which PHYH, into peroxisomes [63-65]. Van den Brink et al. found that mild mutations in the *PEX7* could also lead to the clinical manifestations of Refsum disease [66]. These mutations affected the phytanic acid α-oxidation capacity to such an extent that the patients had the symptoms of Refsum disease. Although plasmalogen biosynthesis in fibroblasts of these patients, which also depends on correct functioning of the Pex7 protein, was lowered as well when compared to healthy controls, the residual activity seems enough to lead to the milder Refsum disease symptoms instead of RCDP type 1 clinical manifestations.

Refsum disease: Treatment

The enormous accumulation of phytanic acid in plasma and tissues in patients is thought to be the only cause of Refsum disease and is used as a diagnostic marker [67]. In patients, phytanic acid is stored in adipose tissue, liver, kidney, muscle, plasma, and nerve tissues predominantly in the form of triglycerides, and to a lesser extent in phospholipids and cholesterol esters [25;68-70]. When it was found that phytanic acid originated from exogenous sources patients were soon treated by a diet low in phytanic acid ([71], and reviewed in [56]). Importantly, the diet should be well controlled and high in calories to prevent weight loss in the patient and the subsequent mobilization of phytanic acid from intracellular stores [72]. Additionally,

during times of crisis the diet can be accompanied by plasma exchange to decrease the sudden rise in the phytanic acid plasma level (reviewed by Gibberd et al. [73]). The diet induced decrease of phytanic acid levels and the subsequent decreased progression of the disease showed that phytanic acid accumulation is the underlying cause of the pathogenesis in Refsum disease.

The toxicity of phytanic acid

Multiple theories have arisen on the toxic effects of phytanic acid (reviewed in [56]). The molecular distortion hypothesis is based on the idea that the branched chain fatty acid phytanic acid replaces the straight chain fatty acids present in membrane lipids, thereby changing the structure of the membrane. This would distort highly ordered membrane structures such as myelin to such an extent that this would ultimately lead to the clinical signs. Furthermore, changes in membrane structure might also influence the function of membrane proteins by an alteration of their interaction with membrane lipids. The anti-metabolite theory suggests that phytanic acid may act as a substrate in cellular pathways involving structurally related compounds. Among these compounds are vitamins A, E and K, and other isoprenoid (-like) compounds. However, convincing evidence in favor of the anti-metabolite theory has been lacking so far.

The discovery that phytanic acid is a ligand for the nuclear hormone receptors RXRα/β and PPARα provided new thoughts on the pathophysiological role of phytanic acid [74-76]. It suggested that the accumulation of phytanic acid may lead to changes in gene expression, which might contribute to the pathology of the disease. Moreover, Idel et al. found that phytanic acid could induce iNOS dependent apoptosis triggered by Tumor Necrosis Factor-α (TNF-α) activation and secretion in smooth muscle cells by a so far unknown signalling pathway which appeared to be independent of the previously mentioned nuclear hormone receptors PPARα and RXR [77].

Recent studies have focused on the effect of phytanic acid on mitochondrial functioning. Mitochondria are key players in both cellular metabolism and apoptosis. Furthermore, incorrect functioning of mitochondria has been implicated in cell death and ageing related neurodegenerative diseases (reviewed in [78;79]). Free fatty acids (FFAs) are known to cause uncoupling of oxidative phosphorylation in mitochondria due to their protonophoric properties. In addition, FFAs can cause changes in Ca^{2+} homeostasis and are thought to be involved in the opening of the mitochondrial permeability transition (MPT) pore (reviewed in [80]). Schönfeld et al. have demonstrated that free phytanic acid causes cell death of hippocampal astrocytes isolated from rat brain [81]. Other studies in isolated rat brain mitochondria and astrocytes further showed that phytanic acid decreases the membrane potential

without opening the MPT pore and changes Ca^{2+} homeostasis [82]. In addition, Schönfeld et al. found also that phytanic acid increases state 4 (resting state) respiration and decreases state 3 (phosporylating state) and (FCCP) uncoupled respiration in isolated mitochondria [81;83-85]. The results provided in these studies suggest that the effects of phytanic acid on respiration are caused by interactions of this fatty acid at multiple sites in mitochondria. It was hypothesized that phytanic acid acts primarily as an uncoupler on state 4 respiration, while in state 3 or uncoupled mitochondria phytanic acid would exert its effects by direct interactions with proteins in the respiratory chain such as the adenine nucleotide translocase (ANT), cytochrome c, and complex I thereby decreasing the electronflux through the respiratory chain, at least under conditions of maximal respiration induced by ADP or FCCP [81;83;85]. The direct interaction of phytanic acid with complex I would also be the cause of phytanic acid induced ROS production [85].

The studies of Schönfeld et al. described in the previous paragraph showed that phytanic acid may exert a multitude of different effects on mitochondrial functioning. In order to get more insights on these effects we performed our own studies in human skin fibroblasts (described in Chapter 6). One part of the studies was performed in digitonin permeabilized fibroblasts. The concentration of digitonin used in these experiments causes permeabilization of the cell membrane only while keeping the mitochondrial membrane intact [86]. Hence, the respiratory chain can be stimulated with mitochondrial substrates like malate (in the presence of glutamate) and succinate (with rotenone added to inhibit complex I). Consequently, the inhibition of complex I could be studied with intact mitochondria. Our results showed that phytanic acid acted primarily as an uncoupler on state 3 respiration in our system (Chapter 6). Furthermore, experiments performed on intact fibroblasts showed that phytanic acid was capable of decreasing the membrane potential while under the same conditions the NAD(P)H autofluorescence levels in mitochondria were decreased. These data suggest that the results obtained in our *in vitro* system are representative for the action of phytanic acid in intact cells, at least in cultured fibroblasts, and that the protonophoric action of phytanic acid predominates.

Further studies in our mouse model for Refsum disease (*Phyh* -/- mouse, described in Chapter 5) should be undertaken to see if the protonophoric effect of phytanic acid actually contributes to the symptoms observed in Refsum disease.

ω-Oxidation of fatty acids: overview

In previous paragraph phytanic acid α-oxidation and subsequent β-oxidation have been discussed. In the early 1930s, however, another pathway for the degradation of fatty acids was identified [87;88]. It is known as the third oxidation pathway for fatty acids and consists of the formation of a second carboxyl group at the ultimate methyl-

group (ω-end) of the fatty acid (depicted in figure 4). Hence, this third route of fatty acid degradation was named the ω-oxidation pathway [87].

The ω-oxidation pathway consists of three consecutive steps (figure 4). First, the fatty acid is hydroxylated at the ω-end of the molecule. This reaction is catalyzed by a cytochrome P450 enzyme (CYP450) which will be discussed in more detail in the next paragraphs. Second, the formed ω-hydroxy-fatty acid is further converted into a ω-oxo-fatty acid by an alcohol dehydrogenase. Subsequently, an aldehyde dehydrogenase converts the ω-oxofatty acid into a dicarboxylic acid. Alternatively, the ω-hydroxy-fatty acid can also be converted into a dicarboxylic acid by a CYP450 catalyzed route, which is suggested to consist of two more hydroxylations at the ω-end and the elimination of water from the dihydroxy-fatty acid intermediate (route B in figure 4) [89;90]. While the CYP450s involved in ω-hydroxylation of fatty acids are localized in the ER, the subsequent dehydrogenation steps were found to occur in both the cytosol and ER [91].

The dicarboxylic acids formed during ω-oxidation are subsequently activated by a microsomal dicarboxylyl-CoA synthetase and further broken down in mitochondria and peroxisomes by β-oxidation to C6-C8 dicarboxylic acids [92-94]. These C6-8 dicarboxylic acids are water soluble and can be excreted via urine.The ω-oxidation pathway is only a minor degradation route for most fatty acids [95-97]. This contribution may increase under certain circumstances, including diabetes, starvation, and via administration of hypolipidemic drugs [98-104].

In literature several hypotheses have been advanced concerning the physiological function of the ω-oxidation of fatty acids and the consequent formation of dicarboxylic acids. The first hypothesis is that ω-oxidation of long- and medium-chain fatty acids followed by subsequent β-oxidation leads to an increase in succinyl-CoA formation which can then be used for the net formation of glucose from fatty acids [99]. Furthermore, the increased formation of succinyl-CoA and subsequent hydrolysis into succinate might give rise to an increased oxidation of acetyl-CoA, since the succinate produced may be converted into oxaloacetate and could thus play an anaplerotic role in the citric acid cycle [98]. Another hypothesis is that the ω-oxidation pathway is a detoxification pathway for unesterified fatty acids. Indeed, the ω-oxidation pathway might work as a sink for fatty acids in times when the input of fatty acids in the regular β-oxidation system exceeds its capacity, e.g. in times of starvation, diabetes, or lipolysis induced by hypolipidemic drugs. In addition, ω-oxidation is also required for the degradation of other fatty acid-like compounds, such as vitamins E, and multiple eicosanoids. Consequently, ω-oxidation forms an important process in the regulation of these biologically active compounds (recently summarized in a number of reviews [105-108], and also discussed in more detail later.

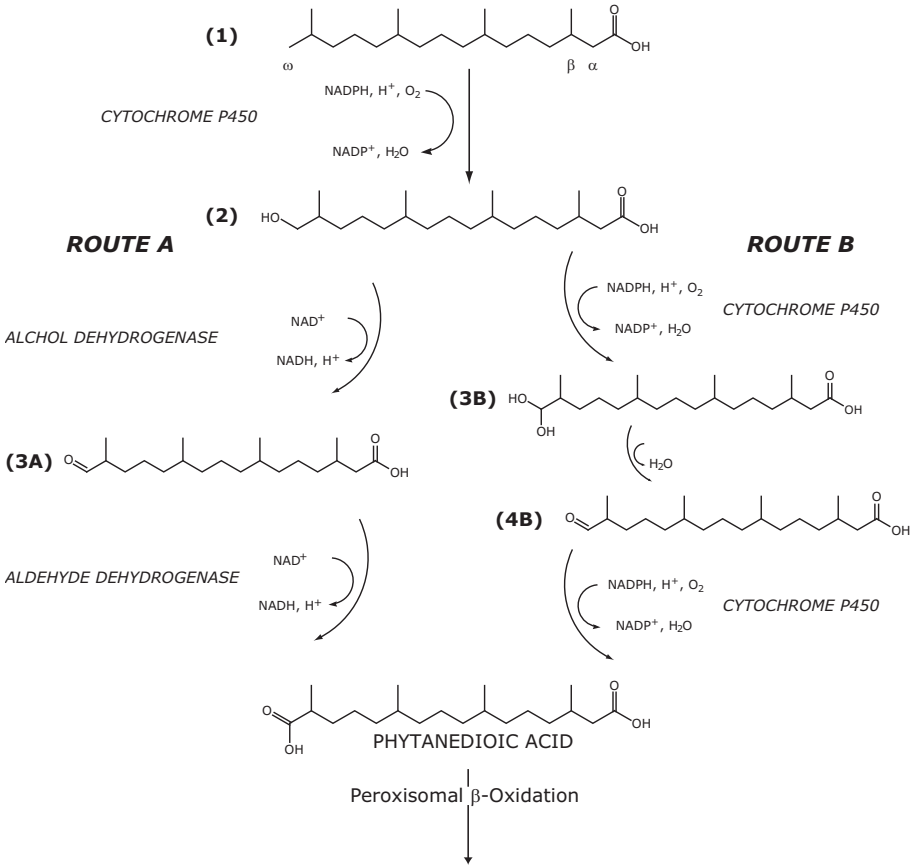

Figure 4: Schematic representation of the ω-oxidation of phytanic acid. The first step of the ω-oxidation of phytanic acid is performed by cytochrome P450 enzymes. This step consists of the hydroxylation of a methyl group at the ω-end of phytanic acid **(1)** and produces ω-hydroxyphytanic acid **(2)**. Subsequently, ω-hydroxyphytanic acid is converted into a dicarboxylic acid which may occur via 2 different routes. **(A)** First, ω-Oxophytanic acid **(3A)** is formed by an alcohol dehydrogenase. Next, ω-oxophytanic acid is oxidized by an aldehyde dehydrogenase and consequently phytanedioic acid is formed. **(B)** Alternatively, ω-Hydroxyphytanic acid undergoes 2 more hydroxylations by cytochrome P450s with the release of water in between. Intermediates of this alternative route are ω-dihydroxyphytanic acid **(3B)** and ω-oxophytanic acid **(4B)**. Phytanedioic acid has a methyl group on position 2 (seen from the newly formed carboxyl group). Hence, phytanedioic acid can be broken down via the β-oxidation pathway in the peroxisome.

Alternative degradation of phytanic acid via ω-oxidation

As discussed in previously, the low-phytanic acid diet decreases phytanic acid levels in Refsum patients despite the fact that some of the Refsum patients had a full block in

the α-oxidation pathway [109]. This suggests that there must be another pathway for the degradation of phytanic acid in humans. In 1965, Eldjarn et al. already found that phytanic acid degradation via ω-oxidation does occur in humans, although a defect in this pathway was not the underlying cause of Refsum disease [28-30]. Analysis of urine from Refsum patients provided new evidence for the breakdown of phytanic acid via ω-oxdidation [110-112]. The compounds 3-methyladipic acid and 3,6-dimethyloctanoic acid were identified in Refsum patients whereas they were not present in urine from healthy individuals. These compounds could in theory be formed by ω-oxidation of phytanic acid followed by subsequent β-oxidation from the ω-end of the dicarboxylic acid formed (phytanedioic acid). Furthermore, Wierzbicki et al. showed that the level of 3-methyladipic acid in urine correlated with the phytanic acid plasma levels in Refsum patients [113]. This suggests that phytanic acid accumulation in patients leads to an increased flux through the ω-oxidation pathway resulting in the formation of 3-methyladipic acid, which is excreted in urine.

The indirect evidence indicating that the flux of phytanic acid through the ω-oxidation pathway is increased in Refsum patients fits nicely with the previously mentioned hypothesis that the ω-oxidation pathway may act as an alternative detoxification pathway in times when levels of fatty acids, especially non-esterified fatty acids, are high. Direct evidence for the ω-oxidation of phytanic acid was provided by Try [114] who showed that phytanic acid could give rise to more polar metabolites when incubated with human, rat, or guinea pig microsomal liver fraction and NADPH *in vitro*. However, the exact structures of these metabolites were not identified in this study. In theory, phytanic acid ω-oxidation may lead to various different polar metabolites since phytanic acid can be hydroxylated on each of the five methyl groups during the first step of the ω-oxidation pathway in contrast to straight-chain fatty acids which only have one methyl group available at the ω-end of the molecule. Therefore, in our studies we attempted to characterize the ω-oxidation pathway for phytanic acid in more detail in rat and human liver microsomal fractions (Chapter 2 & 3). In addition, we identified the enzymes capable of performing the first step of the ω-oxidation pathway, i.e. ω-hydroxylation, as members of the CYP450 superfamily (Chapter 4). The CYP450 proteins are known for their inducibility by a wide variety of drugs and may form a new therapeutical target for Refsum disease. Induction of the first step of the ω-oxidation pathway might increase the flux through the pathway on the whole and therefore might provide a sufficient alternative breakdown pathway for phytanic acid in Refsum patients.

The ω-hydroxylation of other fatty acids by CYP450 proteins has been the topic of many studies over the years. The next paragraphs will give an introduction in the large and complex field of CYP450 research with the focus on fatty acid ω-hydroxylation.

ω-Hydroxylation of fatty acids by Cytochrome P450 proteins

The early studies on the fatty acid ω-oxidation pathway during the 1930s focused on the formation of dicarboxylic acids after administration of considerable quantities of triglycerides containing medium-chain fatty acids to healthy individuals [87;88], or rats [115]. It took until the 1960s before enzymatic assays were performed in guinea pig, rat and human liver tissues [116-118] and the detected intermediates found in these studies gave new insights into the mechanism of the ω-oxidation pathway of fatty acids.

Soon after these studies the enzyme system responsible for the first step of the pathway, the ω-hydroxylation of the fatty acid, was identified by Lu and Coon in rabbit liver microsomes [119]. They found that this enzyme system belonged to the family of CYP450s which were discovered a decade earlier by Omura and Sato [120]. Subsequent studies revealed that this enzyme system consisted of a CYP450, cytochrome P450 oxidoreductase (CYPOR), and a heat stable fraction later identified as phosphatidylcholine [121-123].

This microsomal hydroxylation system appeared to be reactive with a multitude of substrates and was inducible with a wide variety of compounds. This suggested that there were multiple CYP450 iso-enzymes present in the microsomal fraction, each with a specific substrate specificity and regulation (reviewed in [124]). However, difficulties in purification of CYP450 proteins were not solved until 1975 when Haugen et al. isolated multiple isoforms of CYP450 proteins from rabbit liver microsomes [125]. Soon after this initial discovery more CYP450 proteins were isolated from rabbit, rat, pig, and human tissues (reviewed by Lu and West [124]).

The development in molecular biology led to an enormous increase in the number of CYP450 proteins discovered in many different organisms and tissues since the early 1980s. Characterization of these enzymes showed that CYP450 enzyme systems were involved in many diverse biological systems by acting on a huge array of substrates. Among these substrates are endogenous compounds, such as fatty acids, eicosanoids, and vitamins as previously mentioned. Other important endogenous substrates for CYP450s are sterols and steroids, bile acids, and vitamin D3 derivatives. However, CYP450s are also important for the metabolism and detoxification of exogenous compounds, among which are multiple environmental chemicals, drugs, and natural plant products.

The enormous number of CYP450 iso-enzymes required a well structured nomenclature. Nebert et al. [126;127] suggested to use the three-letter code CYP as abbreviation for any cytochrome P450, followed by an Arabic number for the specific family (enzymes sharing ≥ 40% identity) and a letter designating the subfamily (enzymes sharing ≥ 55% identity). Subsequently, the individual gene within the subfamily should be indicated with an Arabic numeral. The internet has become a

major help in organizing CYP450 data during the years. A major contribution with regard to this is the website of Dr. David R. Nelson (*http://drnelson.utmem.edu/CytochromeP450.html*) which reports all CYP450 genes known in all species and serves as a source for sequence alignment and provides information on CYP450 protein structure. There are 57 human CYP450 genes arranged into 18 families and 42 subfamilies [128]. Resolution of the physiological role of most proteins encoded by these genes is still ongoing and the subject of intense research in many laboratories across the world. This manuscript mainly focuses on the CYP450s involved in ω-hydroxylation of fatty acids most of which are members of the CYP4 family.

Fatty acid ω-hydroxylation by CYP450 proteins in humans

The CYP4A subfamily

During the early 1970s Hamberg and Bjorkhem already showed that lauric acid (dodecanoic acid) hydroxylation not only occurred at the ω-position of the fatty acid but also at the (ω-1)-position in the rat liver microsomal fraction [129]. Further studies on the ω- and (ω-1)-hydroxylation suggested that different enzymes were involved in each type of hydroxylation (reviewed in [124]). The (ω-1)-hydroxylation of lauric acid in rat liver and kidney was found to be catalyzed mainly by the alcohol inducible CYP2E1 [130;131], while the ω-hydroxylation of lauric acid was found to be catalyzed by a different enzyme which was highly inducible in rat by administration of hypolipodemic drugs, such as clofibrate [132]. The same enzyme was also able to ω-hydroxylate arachidonic acid [133]. This clofibrate inducible arachidonic acid/lauric acid ω-hydroxylase from rat liver was subsequently cloned, and according to the nomenclature rules was shown to belong to a new family, i.e. family 4, and was named CYP4A1 [134]. Subsequently, the human homologue of this enzyme was identified and named CYP4A11 [135;136].

CYP4A11 has a broad substrate spectrum. CYP4A11 is able to ω-hydroxylate the saturated fatty acids lauric acid, myristic acid (tetradecanoic acid), palmitic acid (hexadecanoic acid), and the unsaturated fatty acids oleic acid ((Z)-octadec-9-enoic acid) and arachidonic acid (*all-cis*-5,8,11,14-eicosatetraenoic acid) [137]. Recently, another CYP4A subfamily member in humans was identified and designated CYP4A22 [138]. This protein is highly homologous with CYP4A11 and not abundantly present in tissues. Kawashima et al. have expressed the CYP4A22 protein in *E. coli*, and showed that this protein has lauric acid ω-hydroxylase activity [139], but erroneously reported it to be CYP4A11.

The CYP4F subfamily

The CYP4A subfamily is not the only subfamily of CYP4 proteins in humans that are capable of ω-hydroxylation of fatty acids. During the early 1980s Hansson et al. reported on the ω-oxidation of leukotriene B4 (LTB4) in human leukocytes [140]. The ω-oxidation pathway is necessary for the degradation (and thereby inactivation) of this compound which plays an important role in the inflammation process. The CYP450 involved in this pathway belonged to a thus far unidentified subfamily, the CYP4F subfamily (reviewed in [106;141]), and was designated as CYP4F3. Later it was found that the *CYP4F3* gene could give rise to two different transcripts by alternative promoter usage and tissue specific gene splicing which results in two different proteins [142;143]. The isoform originally detected in leukocytes was designated CYP4F3A and the other, which was detected in liver, was designated CYP4F3B. These proteins differ from each other due to the alternative use of only one exon. However, this causes a substantial difference in substrate specificity with CYP4F3A being specific for LTB4, whereas CYP4F3B has a higher specificity towards arachidonic acid [142;144].

Shortly after the cloning of CYP4F3A from human leukocytes in 1993, Kikuta et al. identified a novel LTB4-hydroxylating CYP450 in human liver [145]. This isoform was named CYP4F2 and has a high homology with the CYP4F3B protein. CYP4F2 was shown to be the major arachidonic acid ω-hydroxylase in human liver and kidney with a higher substrate specificity for arachidonic acid than the already established arachidonic acid ω-hydroxylase CYP4A11 [146;147]. The formation of ω-hydroxylated arachidonic acid (20-hydroxyeicosatetraenoic acid, 20-HETE) plays an important role in the regulation of the cardiovascular system as it is a known vasoconstrictor (reviewed by Roman [148]). CYP4F2 was also shown to ω-hydroxylate LTB4 in liver which suggests that this protein might play a role in the inflammatory system [149]. Furthermore, CYP4F2 is responsible for ω-hydroxylation of the phytyl tail of the tocopherols and tocotrienols that are collectively called vitamin E [150]. ω-Hydroxylation is the initial step for the degradation of vitamin E via ω-oxidation and subsequent β-oxidation [150;151].

There are three more members in the CYP4F subfamily identified in humans (reviewed by Kalsotra and Strobel [106]). These were recently discovered and only partly characterized. CYP4F8 is present in epithelial linings and catalyzed the (ω-1)-hydroxylation of prostaglandin H2. CYP4F11 is mainly expressed in liver, followed by kidney, heart, brain and skeletal muscle. No endogenous substrates have been found for CYP4F11 until now but it has been shown that recombinant CYP4F11 is quite active in hydroxylating some xenobiotics. Finally, the CYP4F12 protein detected in human liver, heart, gastrointestinal and urogenital epithelia is active towards both eicosanoids and xenobiotics.

Other CYP4 homologues

The CYP4B1 protein, which is predominantly expressed in lung, forms another subfamily of ω-hydroxylases. However, this protein has no clear substrate spectrum, but it is capable of ω-hydroxylating medium-chain fatty acids and xenobiotics (reviewed in [152]). Other CYP450s belonging to family 4 have been identified in human. Their homology with the known CYP4 subfamilies suggests that these orphans (i.e. CYPs with unknown substrate specificity) might be able to ω-hydroxylate fatty acid and/or fatty acid-like compounds [153].

The most important and well-characterized ω-hydroxylases, the CYP450s belonging to the CYP4A and 4F subfamilies, are not only present in humans. CYP4A/F homologues are well represented in other animals, such as mouse, rat, and rabbit [106;154;155]. Moreover, these animals contain more CYP4A and CYP4F subfamily members than humans may make interpretation of results found in studies with these animals difficult.

Induction of fatty acid ω-hydroxylases

CYP4A gene regulation and the role of PPARa in CYP4A induction

The induction of drug metabolizing enzymes by foreign compounds has been the topic of many research studies during the last several decades. Halfway the 20th century it was already known that the enzymatic activity in the liver microsomal fraction could be increased when laboratory animals were administrated certain kinds of xenobiotics, e.g. the classical types of inducers: polycyclic aromatic hydrocarbons (PAHs) and barbiturates, as described in the review of Conney [156]. A third type of metabolic enzyme inducers are the hypolipidemic drugs of the fibrate class, which were already in use since the early 1960s, and were found to upregulate the ω-hydroxylation of lauric acid [103;104]. All of these compounds are able to induce one or more CYP450s, however, the precise process leading to this induction remained unclear for some decades. The discovery that a receptor (the aryl or aromatic hydrocarbon (Ah) receptor) was involved in the induction by PAHs of the CYP450s responsible for the hydroxylation of PAHs (CYP1 family) was the first step in unraveling the complex mechanism of CYP450 regulation (reviewed in [157]). Another breakthrough in CYP450 regulation was the finding that peroxisome proliferator-activated receptor alpha (PPARα) was involved in the induction of CYP4A enzymes (reviewed by Johnson et al. [158]). PPARα is a member of the steroid hormone receptor superfamily of ligand-activated transcription factors (also referred to as the nuclear receptor family) [159]. Moreover, many members of this superfamily were found to be involved in the regulation of multiple CYP450s [160;161].

PPARα is a member of the larger family of PPARs which consists beside the α-isoform of a β (δ), and a γ-isoform. All isoforms play important roles in physiological processes as lipid sensors, and regulators of lipid and glucose homeostasis. However, the different PPARs have specific substrate specificities, tissue distribution and are controlling specific subsets of transcriptional profiles (reviewed in [162;163]). Activation of the PPARs by the so called peroxisome proliferators (a structurally unrelated class of compounds among which are fatty acids, plasticizers, herbicides and the fibrate class of hypolipidemic drugs) enables the receptor to dimerize with another nuclear receptor, the retinoid X receptor (RXR) [164]. The ligand activated heterodimer can bind to specific sequences of DNA known as peroxisome proliferators responsive elements (PPRE) in the promoter regions of target genes, thereby inducing gene expression of the target gene. Most of these target genes are involved in lipid metabolism. Especially pronounced is the induction of proteins involved in peroxisomal fatty acid metabolism which leads to an increase in peroxisomal number (i.e. peroxisome proliferation) and size [165]. Induction of hepatic peroxisome proliferation by PPARα activation in rodents ultimately leads to hepatomegaly and hepatocarcinogenesis (reviewed by Gonzalez [166]). Fortunately, these toxic effects of PPARα ligands are not present in humans [167]. Therefore, fibrates are still in use as important drugs for the treatment of patients with dyslipidemia and / or metabolic syndrome (reviewed in [168;169]).

Besides increasing peroxisomal fatty acid oxidation PPARα is also involved in the upregulation of mitochondrial β-oxidation, fatty acid transport and the already mentioned fatty acid ω-hydroxylation via the CYP4A subfamily. Initial studies, which focused on the induction of the CYP4A subfamily in rats and mice, showed that levels of certain subtypes indeed increased in these rodent animal models after PPARα activation [170;171]. In humans, the research on the effect of the induction of the CYP4A subtypes is still not conclusive. Overexpression of PPARα in the hepatoma cell line HepG2 led to an increase of CYP4A11/A22 under specific growth conditions, suggesting the involvement of PPARα in the regulation of human CYP4A expression [172]. Another study showed that fibrates are able to induce CYP4A11 mRNA expression in primary cultures of human hepatocytes [173]. In contrast, the PPREs present in the 5' flanking regions of the CYP4A subfamily in rodents have not been identified for the human CYP4As [139].

Recently, another regulatory pathway for the CYP4A11 gene expression was discovered. Activation of a different member of the nuclear hormone receptor family, Retinoic Acid Receptor (RAR), by all-trans retinoic acid (ATRA) in the hepatoma cell line HepaRG was shown to decrease CYP4A11 gene and protein expression, ultimately leading to a decrease in catalytic activity (lauric acid hydroxylation) in this cell line [174].

In mice there are three different CYP4A genes identified. *Cyp4a10* is highly expressed in both sexes while *Cyp4a12* (consisting of two gene products, *Cyp4a12a* and *Cyp4a12b*) is predominantly male specific and *Cyp4a14* is a female specific isoform. Furthermore, the protein levels of these Cyp4a isoforms vary in different mouse strains and tissues. PPARα also plays an important role in the regulation of the expression of the different Cyp4a isoforms in mice. Fibrates are able to induce gene expression of *Cyp4a10* and *Cyp4a14* [171;175] in both liver and kidney. *Cyp4a12* is constitutively expressed in kidney and liver of male mice, whereas in kidney and liver of female mice *Cyp4a12* is expressed in low levels. Moreover, *Cyp4a12* gene expression cannot be induced by fibrates in kidney and liver of male mice, while in female mice kidney and liver *Cyp4a12* RNA levels were increased to male levels after treatment with fibrates. In addition, *Cyp4a12* gene expression is also upregulated in female mice by the treatment with androgens via a still unknown mechanism [175;176].

CYP4F induction

In contrast to the CYP4A subfamily, rather limited studies have appeared on the regulation of the genes comprising the CYP4F subfamily (reviewed in [106;107;141]). The regulation of *CYP4F2* gene has been studied most intensively of all human isoforms. Zhang et al. [177] found that the *CYP4F2* gene expression was regulated by retinoic acid and fibrates. Peroxisome proliferators suppressed *CYP4F2* promotor activity while both 9-cis retinoic acid (9-cis-RA) and all-trans retinoic acid (ATRA) induced promoter activity through activation of RAR and RXR. However, further research showed that in the hepatoma cell line HepG2 9-cis-RAincreased CYP4F2 protein content in contrast to ATRA, which only gave rise to an induction of *CYP4F2* promotor activity [178]. From these results Zhang and Hardwick concluded that *CYP4F2* gene expression is regulated by 9-cis-RA and ATRA. Activation of RXR induces gene expression (and protein content) and RAR activation results in a repression of gene expression. Recently, Hsu et al. showed that in HepG2 cells and primary hepatocytes CYP4F2 gene expression and protein content could be induced by statins, which are well known drugs used for the treatment of hypercholesterolemia [179]. Furthermore, Hsu et al. showed that the *CYP4F2* transcriptional activation is mediated through activation Sterol Regulatory Element Binding Proteins (SREBPs, reviewed in [180]) and that activation of the SREPBP-2 isoform is involved in the induction CYP4F2 by statins [179].

Parallel studies on the induction of CYP4F3 showed that this enzyme was induced HL60 cell and human leukocytes after treatment of these cells with ATRA [181;182]. However, the mechanism behind this induction has to be determined since the receptor for ATRA, RAR, seems only indirectly involved in this process.

Studies in rats and mice have shown that the expression of some isoforms of the CYP4 subfamily changes during inflammation. During an inflammatory response

an induction of CYP4F isoforms occurs in rodents needed for the breakdown of inflammatory mediators such as the eicosanoid LTB4 (reviewed in [106;107]). Recent studies by Kalsotra et al. provided evidence that specific cytokines are involved in the regulation of the CYP4F enzymes levels during inflammation. The pro-inflammatory cytokines Interleukin (IL)-1β, IL-6, and TNF-α are capable to induce CYP4Fs, while the anti-inflammatory cytokine IL-10 suppresses CYP4F expression [183].

Outline of this thesis

Refsum disease is characterized by the large accumulation of phytanic acid due to a deficiency in the regular degradation route for this fatty acid, namely α-oxidation. For a long time it has been believed that phytanic acid can also be degraded via an alternative catabolic pathway, which is suggested to be ω-oxidation. Direct and indirect evidence indicates that phytanic acid is a substrate for the enzymes of the ω-oxidation pathway. In chapter 2 we investigated whether phytanic acid was a substrate for the first step of ω-oxidation in rat liver microsomes and which metabolites are generated during this process. To this end, a novel *in vitro* assay in rat liver microsomes for the detection of ω-hydroxylated metabolites of phytanic acid was developed. In Chapter 3 we subsequently investigated the ω-hydroxylation of phytanic acid in human liver microsomes which contain different homologues of CYP450 proteins compared to rat. An effort to identify the CYP450 enzymes involved in the ω-hydroxylation step of phytanic acid was performed using specific CYP450 inhibitors and recombinant enzymes which is described in chapter 4.

In order to study the effect of upregulation of the ω-oxidation pathway in our recently generated Refsum disease mouse model (*Phyh* -/- mouse) we administered these mice the peroxisome proliferator fenofibrate. Chapter 5 describes the effect of fenofibrate on phytanic acid accumulation in *Phyh* -/- mice. Furthermore, we investigated the fenofibrate induced upregulation of Cyp4a in the mice used in this experiment.

The final chapter of this thesis handles with the toxicity of phytanic acid. The hypothesis that phytanic acid has toxic effects on the repiratory chain was further investigated in digitonin permeabilized fibroblasts and in fibroblasts in culture. We show in chapter 6 that phytanic acid exerts its toxic effects on the respiratory chain mainly by its protonophoric action.

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CHAPTER 2

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

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

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Abstract

The 3-methyl-branched fatty acid phytanic acid is degraded by the peroxisomal α-oxidation route since the 3-methyl group blocks β-oxidation. In Adult Refsum Disease (ARD) peroxisomal α-oxidation is defective, which is caused by mutations in the gene coding for phytanoyl-CoA hydroxylase in the majority of ARD patients. As a consequence, phytanic acid accumulates in tissues and body fluids. This study focuses on an alternative route of phytanic acid degradation, i.e. ω-oxidation. The first step in ω-oxidation is hydroxylation at the ω-end of the fatty acid, catalyzed by a member of the cytochrome P450 multi-enzyme family. In order to study this first step, the formation of hydroxylated intermediates was studied in rat liver microsomes incubated with phytanic acid and NADPH. Two hydroxylated metabolites of phytanic acid were formed, viz. ω- and (ω-1)-hydroxyphytanic acid (ratio of formation 5:1). Formation of ω-hydroxyphytanic acid was NADPH dependent and inhibited by imidazole derivatives. These results indicate that phytanic acid undergoes ω-hydroxylation in rat liver microsomes and that an isoform of cytochrome P450 catalyzes the first step of phytanic acid ω-oxidation.

Introduction

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

tetramethylpentadecanoic acid). This fatty acid has a 2-methyl group and can be further degraded by β-oxidation [2-4].

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

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

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

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

Material and Methods

Materials

Phytanic acid and 3-hydroxyheptadecanoic acid were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD^+ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonozole and miconazole were

purchased from Sigma (St. Louis, MO). Methyl-β-cyclodextrine was from Fluka (Buchs, Switzerland).

Preparation of rat liver microsomes

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

Phytanic acid ω(-1)-hydroxylase assay

The standard reaction mixture consisted of 100 mM potassium phosphate buffer pH 7.4 and rat liver microsomes (1 mg/ml end concentration) plus phytanic acid dissolved in DMSO (200 μM end concentration, unless indicated otherwise). Reactions were initiated by addition of NADPH at a final concentration of 1 mM. The final reaction volume was 0.2 ml. Reactions were terminated by addition of 0.2 ml 1 M HCl. Subsequently, 1 ml PBS was added followed by addition of 0.1 ml 12.1 M HCl. The internal standard (IS, 10 nmol 3-hydroxyheptadecanoic acid in 20 μl ethanol) was added to this aqueous mixture. The samples were extracted twice with 6 ml ethylacetate-diethylether (1:1 v/v). The organic layer was collected and the solvents evaporated under vacuum using a rotary evaporator at room temperature. The residue was dissolved in 4 ml ethylacetate and further dried with $MgSO_4$. After spinning down the $MgSO_4$, the solution was transferred to 4 ml reaction vials and the solvent evaporated under nitrogen. To enable gas chromatography-mass spectrometry (GS/MS) analysis the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson [17]. TMS ester/ether formation was performed with 40 μl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and 10 μl pyridine. The vials were sealed with a Teflon-lined screwcap and incubated at 80°C for 1 hour. After the incubation the solution could be directly used for GC-MS analysis.

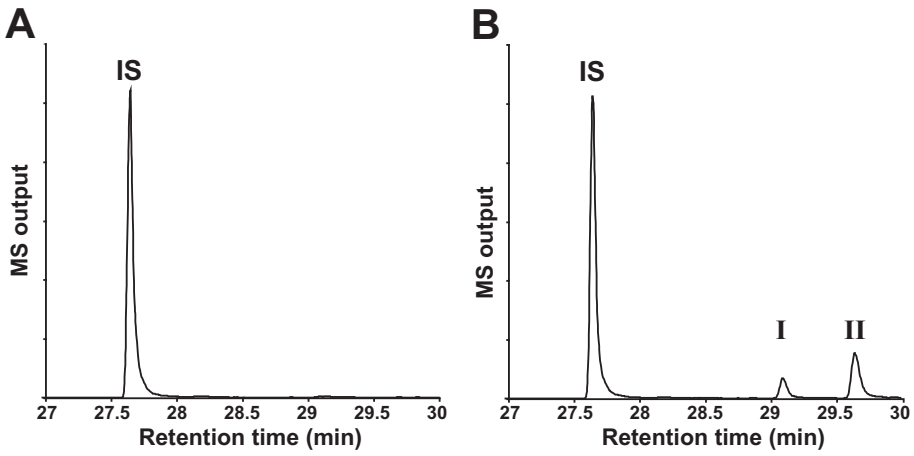

Figure 1: GC-MS (SIM mode) chromatograms of extracts from rat liver microsomes incubated with phytanic acid in the absence **(A)** or presence **(B)** of NADPH. Spectrum analysis was done as described in the methods section. (hydroxy analogues of phytanic acid). Peaks corresponding to the $(M-15)^+$ (m/z = 457) of hydroxylated phytanic acid metabolites are labelled I and II. 3-Hydroxyheptadecanoic acid (ion m/z = 233) was used as internal standard (IS).

GC/MS

GC/MS was performed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass-selective detector (Palo Alto, CA). Samples (1 µl) were injected in the splitless mode (Hewlett Packard 7683 injector) and analyzed on a CP-Sil 5 CB low bleed MS column (25 m x 0.30 µm) (Chrompack, Middelburg, The Netherlands). The oven temperature was programmed as follows: 70°C for 2 min, 5°C/min to 120°C, 7°C/min to 260°C, 3.5 min hold at 260°C then 15°C/min to 275, hold for 10 min. The identity of the substrate, the internal standard, and the reaction product(s) was verified by taking mass spectra of the pertrimethylsilylated derivatives in the scanning electron impact mode. The single ion monitoring (SIM) mode was applied for the detection of the respective $(M-15)^+$ ions (m/z 369 and 457; masses of the molecular ions minus one methyl group of the TMS derivatives of phytanic acid and ω(-1)-hydroxyphytanic acid respectively). Analyte quantification was done by integration of the peaks followed by dividing the analyte peak areas with the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion m/z 233).

Results and Discussion

Hydroxylation of phytanic acid by rat liver microsomes

In order to study the ω-oxidation of phytanic acid, rat liver microsomes were incubated in a phosphate buffered medium containing phytanic acid. When NADPH was added, two products appeared in the chromatogram, one with a retention time of 29.1 min and the other with a retention time of 29.7 min (Fig. 1).

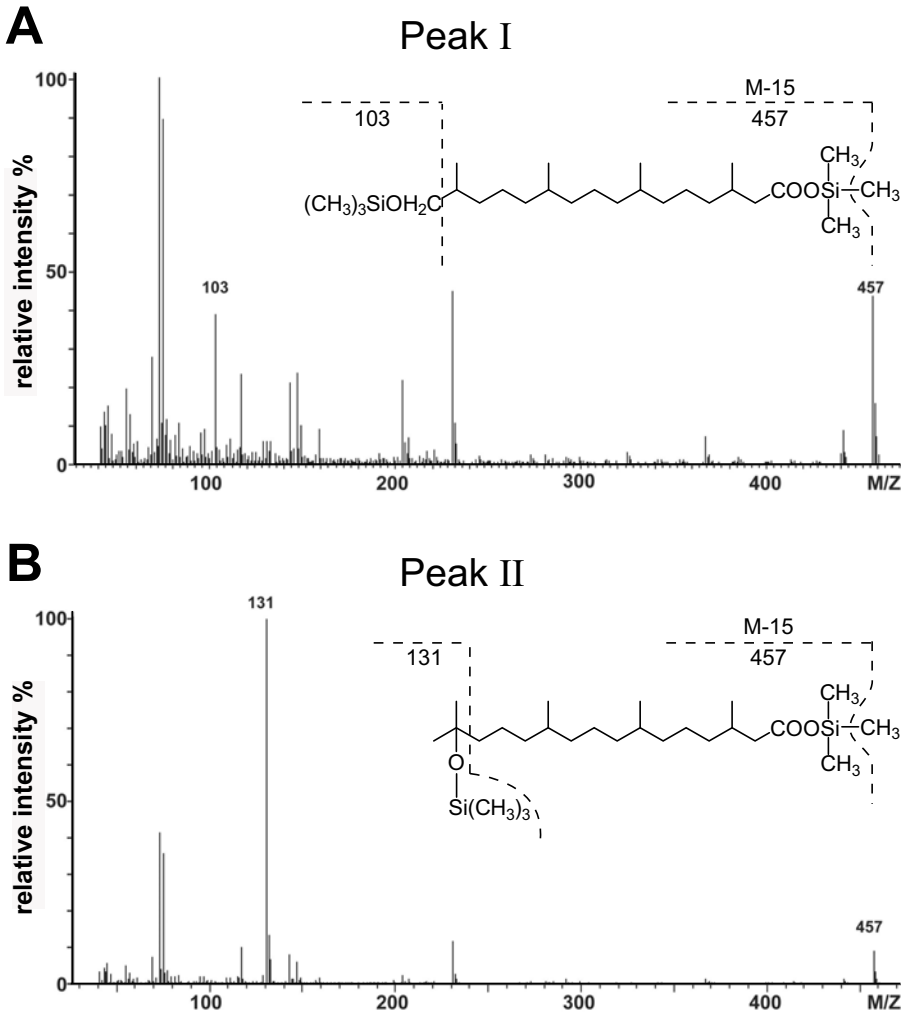

Figure 2: Mass spectra of peaks labelled I and II in Fig. 1B. Based on the fragmentation pattern shown in **(A)** and **(B)**, peak I was identified as the TMS derivative of (ω-1)-hydroxyphytanic acid, whereas peak II was identified as the TMS-derivative of ω-hydroxyphytanic acid.

The major peak was identified as ω-hydroxyphytanic acid (16-hydroxyphytanic acid) according to its mass spectrum (Fig. 2A). Mass spectral analysis of this peak revealed the presence of a fragment (m/z = 103) characteristic for ω-hydroxyacids, representing the terminal $CH_2OSi(CH_3)_3$-moiety [18]. The minor peak corresponds to (ω-1)-hydroxyphytanic acid (15-hydroxyphytanic acid) (Fig. 2B), the product of (ω-1)-hydroxylation. A general characteristic of the TMS-derivatives of the hydroxyfatty acids is cleavage of the molecule adjacent to the hydroxyl group [19]. It was deduced from the mass spectrum in Fig. 2B that the base peak at m/z = 131 represents the $(CH_3)_2COSi(CH_3)_3$-moiety, in analogy with the mass spectrum of 3-hydroxyisovaleric acid.

Optimization of the hydroxylase assay

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

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

Our next aim was to analyze the kinetics of the hydroxylation of phytanic acid under the conditions determined in the previous experiments. We already established that NADPH was an essential component of the reaction mixture (Fig. 1). The NADPH dependency of the reaction was studied in more detail by performing the assay at different NADPH concentrations. For this purpose, we included a NADPH regenerating system (10 mM isocitrate, 10 mM $MgCl_2$ and 0.08 U isocitrate dehydrogenase) in the assay mixture since large amounts NADPH were consumed during the assay (data not shown). The formation of ω-hydroxyphytanic acid followed simple Michaelis Menten kinetics (Fig. 3C). The apparent K_m for NADPH derived from the Lineweaver-Burke plot (insert in Fig. 3C) was 35 μM.

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

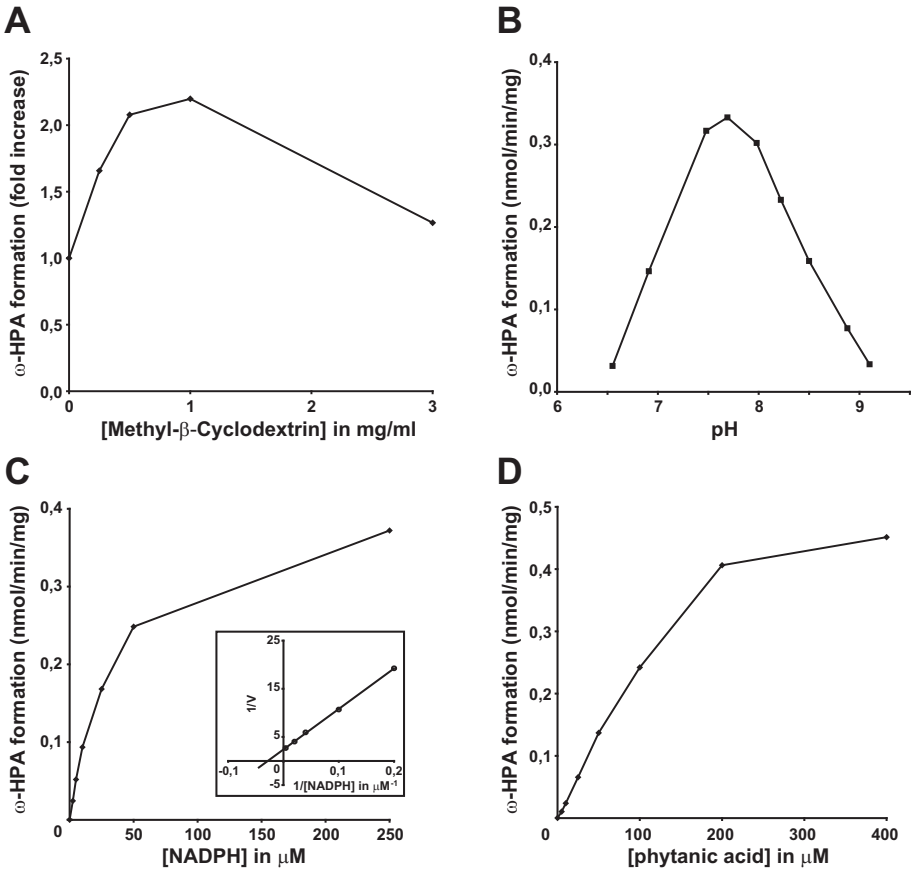

Figure 3: Optimalization of the phytanic acid hydroxylase activity in rat liver microsomes. **(A)** The effect of different concentrations of methyl-β-cyclodextrin on the formation of ω-hydroxyphytanic acid (ω-HPA) in rat liver microsomes was determined under conditions described in the methods section. The pH dependency of phytanic acid ω-hydroxylation is shown in **(B)**. The hydroxylase assay was performed as during the previous experiment **(A)**, with the exception of the use of a combined buffer containing 50 mM potassium phosphate and 50 mM pyrophosphate and 0.75 mg/ml methyl-β-cyclodextrin. In **(C)** the effect of the NADPH concentration on phytanic acid ω-hydroxylation is shown in the presence of a NADPH regenerating system (10 mM isocitrate, 10 mM $MgCl_2$ and 0.08 U isocitrate dehydrogenase). The experimental set-up was as described in the methods section with the exception of using a 100 mM potassium phosphate buffer (pH 7.6) and 0.75 mg/ml methyl-β-cyclodextrin. The K_m for NADPH was determined to be 35 μM as derived from the Lineweaver Burke plot (insert). The effect of the phytanic acid concentration on the formation of ω-hydroxyphytanic acid **(D)** was determined using the optimum experimental conditions derived from the previous experiments (100 mM potassium phosphate buffer (pH 7.6), 1 mM NADPH). The ratio methyl-β-cyclodextrin / phytanic acid was kept constant. All the data represents the mean of duplicate experiments.

Based on the experiments described above, we selected the following assay conditions: 0,75 mg/ml methyl-β-cyclodextrin,100 mM potassium phosphate (pH 7.6), 1 mM NADPH, and 200 μM phytanic acid. Under these conditions formation of ω-hydroxyphytanic acid was linear with time up to 60 min, and with protein up to 1 mg/ml (data not shown).

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

Imidazole antimycotics are known inhibitors of cytochrome P450 enzymes [22-24]. To measure the influence of four different imidazole derivatives on the formation of ω-hydroxyphytanic acid, we studied the effect of different concentrations of these compounds on the formation of ω-hydroxyphytanic acid (Fig. 4A) and (ω-1)-hydroxyphytanic acid (Fig. 4B). Fig. 4A shows that ω-hydroxyphytanic acid formation was inhibited by all four compounds with bifonazole as the most potent inhibitor, followed by ketoconazole, miconazole , and clotrimazole. Interestingly, a different picture was observed if the effect of the four imidazole derivatives was studied on the formation of the (ω-1)-compound with miconazole as most potent inhibitor, followed by ketoconazole and bifonazole. Remarkably, clotrimazole showed a stimulatory effect at low concentrations with little inhibition at the highest concentrations used (100 μM).

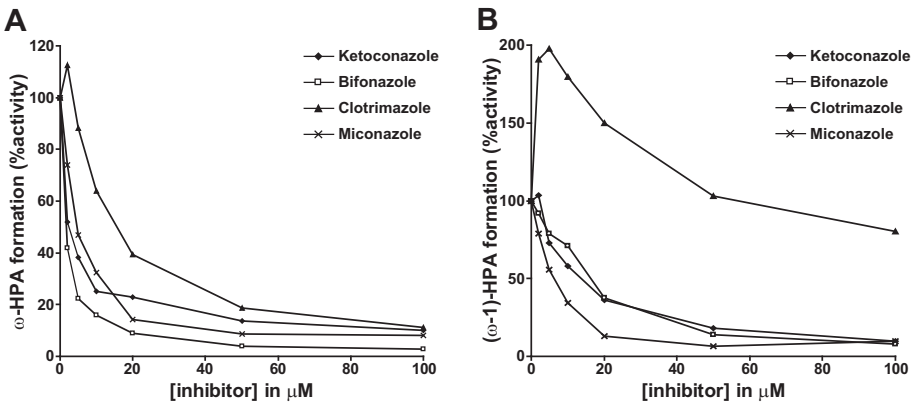

Figure 4: Effect of different imidazole antimycotics on the ω- and (ω-1)-hydroxylation of phytanic acid. Rat liver microsomes were incubated with phytanic acid in the presence of different concentrations of imidazole derivatives. The inhibitory effect of the imidazole derivatives on the formation of ω-hydroxyphytanic acid **(A)** and (ω-1)-hydroxyphytanic acid **(B)** is shown. The data represents the mean of duplicate experiments.

To summarize, phytanic acid is hydroxylated to its ω and ω-1 hydroxy analogues in rat liver microsomes. The enzyme(s) responsible for phytanic acid ω- and

(ω-1)-hydroxylation were shown to be NADPH dependent. Moreover, the formation of the ω- and ω-1 hydroxy analogues of phytanic acid was inhibited by imidazole antimycotics. The inhibition by the imidazole derivatives showed a different pattern for the two products. Hence, this strongly suggests that different members of the cytochrome P450 multi-enzyme family are responsible for the formation of ω- and (ω-1)-phytanic acid. Future work is aimed at the identification of the specific cytochrome P450s involved and the resolution of the subsequent metabolism of ω-hydroxyphytanic acid. Preliminary results have already shown the formation of the dicarboxylic acid of phytanic acid. Furthermore, we will study whether phytanic acid ω-oxidation can be induced in order to generate a new therapeutic option for Refsum disease patients by induction of the capacity to ω-oxidize phytanic acid.

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CHAPTER 3

Characterization of phytanic acid ω-hydroxylation in human liver microsomes

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Characterization of phytanic acid ω-hydroxylation in human liver microsomes

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Abstract

Phytanic acid is a 3-methyl-branched-chain fatty acid which originates from dietary sources. Since the 3-methyl group blocks regular β-oxidation, it is broken down by peroxisomal α-oxidation. Adult Refsum Disease patients accumulate phytanic acid as a result of an impairment in peroxisomal α-oxidation, caused by the deficient activity of the enzyme phytanoyl-CoA hydroxylase in the majority of patients. In this paper we studied an alternative degradation route for phytanic acid, namely ω-oxidation. During ω-oxidation a fatty acid is hydroxylated at its ω-end by a member of the cytochrome P450 multi-enzyme family. Subsequently, an alcohol dehydrogenase converts the formed hydroxyl group into an aldehyde, which is then converted into a carboxyl group by an aldehyde dehydrogenase. In case of phytanic acid ω-hydroxylation would lead to the formation of phytanedioic acid, which can be degraded by β-oxidation from the ω-end.
Here we show that phytanic acid indeed undergoes ω- and (ω-1)-hydroxylation in pooled human liver microsomes in an NADPH-dependent manner with a ratio of 15:1. Studies with imidazole antimycotics indicate that these reactions are catalyzed by one or more cytochrome P450 enzymes. Induction of the cytochrome P450 involved in phytanic acid ω-hydroxylation may increase the flux through the ω-oxidation pathway, causing increased clearance of phytanic acid in ARD patients. Hence, this alternative catabolic pathway is of potential therapeutic relevance.

Introduction

Adult Refsum Disease (ARD) is an autosomal recessive disorder caused by deficient α-oxidation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). In the majority of patients this is due to mutations in the gene encoding phytanoyl-CoA hydroxylase, a peroxisomal enzyme [1-3]. In a subset of patients mutations in the *PEX7* gene have been found [4]. Phytanoyl-CoA hydroxylase catalyzes the first step in the α-oxidation pathway of 3-methyl branched-chain fatty acids. These fatty acids require α-oxidation

for their degradation since the 3-methyl group blocks breakdown by regular β-oxidation. During α-oxidation, phytanic acid is converted into its n-1 analogue pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can readily be degraded by peroxisomal β-oxidation [5;6].

The deficiency of α-oxidation in ARD patients leads to the gradual accumulation of phytanic acid. Elevated phytanic acid in the absence of abnormalities in any of the other peroxisomal parameters including plasma very long chain fatty acids, bile acid intermediates and erythrocyte plasmalogens is suggestive for ARD. Classical symptoms are: progressive retinitis pigmentosa, peripheral neuropathy, anosmia, and cerebellar ataxia [7-9]. The only treatment available at the moment is a diet low in phytanic acid, which may be preceded by plasmapheresis. This alleviates the phytanic acid accumulation and slows down the progression of the disease [9].

The diet-induced decrease of phytanic acid levels in ARD patients suggested the existence of an alternative degradation pathway for this molecule. ω-Oxidation is a known breakdown pathway for fatty acids and therefore might be an alternative pathway for phytanic acid degradation as well [10;11]. ω-Oxidation consists of three successive steps. Initially the fatty acid is hydroxylated by a member of the cytochrome P450 enzyme family at the carbon atom localized at the ω-end. Subsequently, the hydroxyl group is converted to an aldehyde by an alcohol dehydrogenase, which in turn can be oxidized to a carboxyl-group by an aldehyde dehydrogenase. In case of phytanic acid this leads to 1,16-phytanedioic acid, which, in principle, can be further degraded by β-oxidation from the ω-end. The existence of the ω-oxidation pathway for phytanic acid degradation is supported by several reports that describe elevated levels of 3-methyladipic acid (3-methylhexanedioic acid) and 2,6-dimethylsuberic acid (2,6-dimethyloctanedioic acid) in urine of ARD patients, which are believed to be products of ω-oxidation of phytanic acid and the subsequent β-oxidation of phytanedioic acid [12-15]. Recently, we have shown that rat liver microsomes are able to ω-hydroxylate phytanic acid [16]. This reaction is catalyzed by a member of the cytochrome P450 enzyme family and resulted in the formation of two metabolites, ω- and (ω-1)-hydroxyphytanic acid. Cytochrome P450 enzymes are readily inducible by a variety of drugs [17;18]. Induction of the cytochrome P450 involved in phytanic acid ω-hydroxylation might lead to an increased clearance of phytanic acid in Refsum patients with obvious implications for the treatment of these patients. In this paper, we have extended our studies from rat liver microsomes to human liver microsomes. Species-specific differences in expression levels of the cytochrome P450 enzymes and the existence of a difference in number of isoforms in certain cytochrome P450 subfamilies between rat and human, e.g. CYP4A and CYP4F [10;19], emphasize the importance of this study.

Materials and Methods

Materials

Phytanic acid was obtained from the VU University Medical Center Metabolic Laboratory (Dr H.J. ten Brink, Amsterdam, the Netherlands). 3-Hydroxyheptadecanoic acid was from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD^+ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonozole and miconazole were obtained from Sigma (St. Louis, MO, USA). Methyl-β-cyclodextrine was from Fluka (Buchs, Switzerland). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Pooled human liver microsomes were obtained from BD Gentest™ (Woburn, MA, USA). Rat liver microsomes were prepared from Male Wistar rats by differential centrifugation as described in detail [16]. Other chemicals used were of the highest quality possible.

Phytanic acid ω- and ω-1-hydroxylation assay

Essentially the same conditions were used as previously described [16], except for the final reaction volume which was now 0.1 ml. In brief, phytanic acid dissolved in DMSO was added to a solution of microsomes (1 mg/ml final concentration) in 100 mM potassium phosphate buffer (pH 7.7), containing phytanic acid at a final concentration of 200 μM, unless indicated otherwise. Reactions were initiated by adding NADPH (final concentration 1 mM) and terminated by addition of 0.1 ml 1 M HCl. Subsequently, 0.5 ml phosphate-buffered saline (PBS) was added followed by 50 μL 12.1 M HCl. After addition of the internal standard (IS, 2 nmol 3-hydroxyheptadecanoic acid in 20 μl ethanol) the samples were extracted twice with 6 ml ethylacetate-diethylether (1:1 v/v). The organic layers were collected and the solvents evaporated. The residue was dissolved in 4 ml ethylacetate and further dried with anhydrous $MgSO_4$ and again evaporated. To enable gas chromatography-mass spectrometry (GS/MS) analysis the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson [20]. TMS ester/ether formation was performed by incubating the samples with 40 μl BSTFA containing 1% TMCS and 10 μl pyridine at 80°C for 1 hour. After the incubation the solution was directly used for GC-MS analysis.

GC/MS

GC/MS was performed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass-selective detector (Palo Alto, CA). Samples (1 μl) were injected in the splitless mode (Hewlett Packard 7683 injector) and analyzed on a CP-

Sil 5 CB low bleed MS column (25 m x 0.30 μm) (Chrompack, Middelburg, The Netherlands). The oven temperature program used was described previously [16]. The single ion monitoring (SIM) mode was applied for the detection of the respective $(M-15)^+$ ions (m/z 369 and 457; masses of the molecular ions minus one methyl group of the TMS derivatives of phytanic acid and ω(-1)-hydroxyphytanic acid respectively). Analyte quantification was done by integration of the peaks followed by dividing the analyte peak areas by the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion m/z 233).

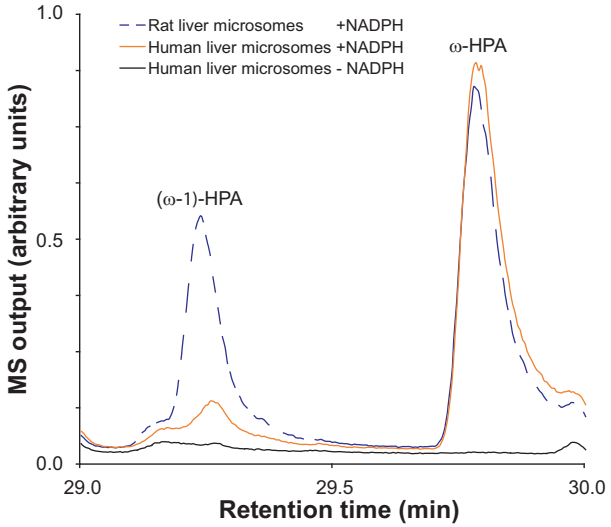

Figure 1: GC-MS (SIM mode) chromatograms of extracts of human liver microsomes incubated with phytanic acid in the absence (solid black line) or presence (solid grey line) of NADPH. For comparison, a chromatogram of an extract of rat liver microsomes incubated with phytanic acid in the presence of NADPH (dashed line) is shown. The peaks are the $(M-15)^+$ ions (m/z = 457) of trimethyl-silylated ω- and (ω-1)-hydroxyphytanic acid (ω- and (ω-1)-HPA).

Results

Phytanic acid ω-hydroxylation in pooled human liver microsomes

Our previous studies showed that phytanic acid undergoes NADPH-dependent ω- and (ω-1)-hydroxylation in rat liver microsomes [16]. To test whether this also occurs in human liver microsomes the same assay was performed under the optimum conditions described for rat liver microsomes (see Materials and Methods section). Under these conditions, the human liver microsomes indeed showed the capacity to produce ω- and (ω-1)-hydroxyphytanic acid (Fig. 1). The identity of the ω- and (ω-1)-hydroxylated products was confirmed by their corresponding fragmentation patterns as was done earlier when using rat liver microsomes [16]. The major difference between rat and human microsomal systems is the ratio between the two products formed, as can be seen in Fig. 1. In pooled human microsomes the ratio ω : (ω-1) was 15.4 ± 0.7, whereas in rat liver microsomes this ratio was found to be 2.2 ± 0.2 as measured in four separate experiments.

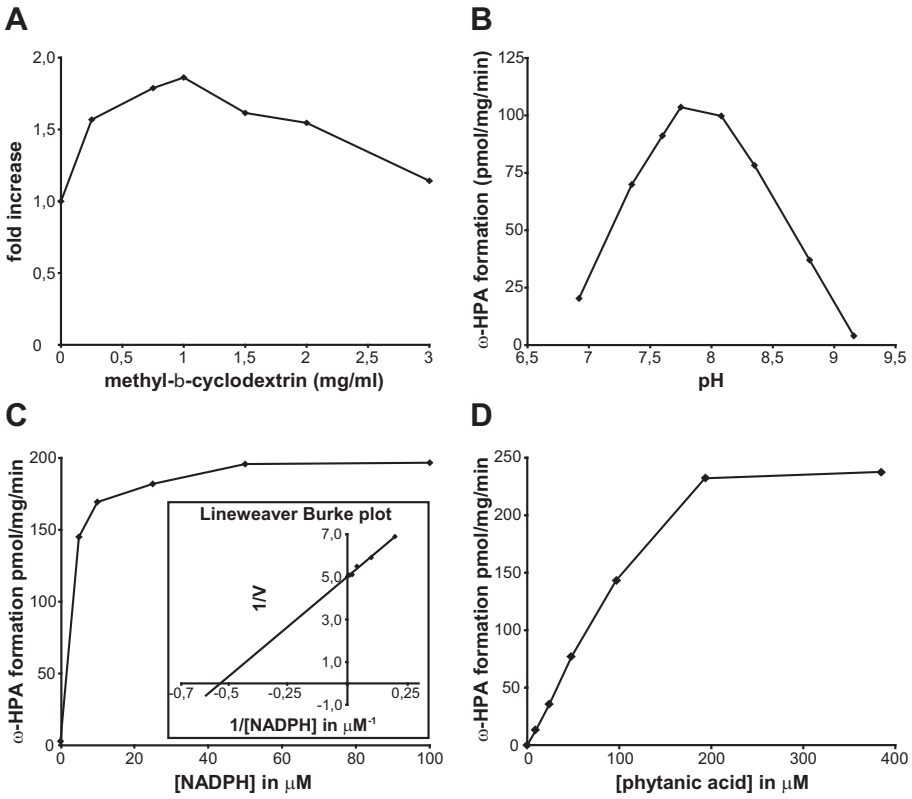

Figure 2: Optimization of phytanic acid ω-hydroxylation in pooled human liver microsomes. **(A)** The effect of different concentrations of methyl-β-cyclodextrin on the formation of ω-hydroxyphytanic acid (ω-HPA) in rat liver microsomes was determined. The pH dependency of phytanic acid ω-hydroxylation is shown in **(B)**. The hydroxylase assay was essentially performed as in **(A)**, with the exception of the use of a combined buffer containing 50 mM potassium phosphate and 50 mM pyrophosphate and 0.75 mg/ml methyl-β-cyclodextrin. The effect of the NADPH concentration on phytanic acid ω-hydroxylation in the presence of an NADPH-regenerating system (10 mM isocitrate, 10 mM $MgCl_2$ and 0.08 U isocitrate dehydrogenase) is shown in **(C)**. The experimental set-up was as described in the methods section with the exception of the use of a 100 mM potassium phosphate buffer (pH 7.7) and 0.75 mg/ml methyl-β-cyclodextrin. The K_m for NADPH was 2 μM as determined from the Lineweaver Burke plot (insert). The effect of the phytanic acid concentration on the formation of ω-hydroxyphytanic acid **(D)** was determined using the optimum experimental conditions derived from the previous experiments (100 mM potassium phosphate buffer (pH 7.7), 1 mM NADPH). The ratio methyl-β-cyclodextrin/phytanic acid was kept constant. All the data represents the mean of duplicate experiments.

Optimization of the phytanic acid ω(-1)-hydroxylase assay

The marked difference between the ratios of product formation between human and rat liver microsomes (Fig. 1) led us to optimize the hydroxylation assay in human liver microsomes for ω-hydroxyphytanic acid formation. First, we tested the effect of methyl-β-cyclodextrin, which was used in the assay to increase the solubilization of phytanic acid [21]. As shown in Fig. 2A, methyl-β-cyclodextrin had a positive influence on the formation of ω-hydroxyphytanic acid up to approximately 1 mg/ml. A further increase of methyl-β-cyclodextrin in the assay had a negative effect on the rate of product formation, presumably caused by a decrease in the availability of the substrate. The same phosphate-based buffer system as used in our earlier work on rat liver microsomes (50 mM potassium phosphate/50 mM pyrophosphate) was used for the determination of the optimum pH of the reaction [16]. The pH optimum of the reaction was 7.7 (Fig. 2B). All subsequent experiments were done at this particular pH.

In order to determine the Km for NADPH, the NADPH concentration was varied in the assay in combination with the use of an NADPH-regenerating system (10 mM isocitrate, 10 mM $MgCl_2$ and 0.08 U isocitrate dehydrogenase). The reaction followed simple Michaelis Menten kinetics (Fig. 2C) and from the Lineweaver Burke plot (insert Fig. 2C) a Km of 2 μM (mean of duplicate experiments) could be deduced. This Km is considerably lower than the Km determined in rat liver microsomes (35 μM) [16].

Subsequently, the Km of the enzyme for phytanic acid was determined. Different concentrations of phytanic acid were used with a fixed ratio between phytanic acid and methyl-β-cyclodextrin. The v versus [S] plot did not follow Michaelis-Menten kinetics so the calculation of the respective Km from the Lineweaver Burke plot could not be done. Consequently, we estimated the Km for phytanic acid as the substrate concentration that shows half maximal ω-hydroxylation activity. Based on the data in Fig. 2D, an apparent Km of 80 μM was determined (Fig. 2D). The nonlinear kinetics may, at least in part, be caused by inefficient solubilization of phytanic acid, although methyl-β-cyclodextrin was used in the assay. In vivo, this problem may be overcome by a carrier protein which provides the substrate to the P450 enzyme, similar to sterol carrier protein 2 (SCP2) acting as a carrier protein for phytanoyl-CoA during α-oxidation [22]. Liver fatty acid binding protein (L-FABP) is a possible candidate to play such a role during ω-hydroxylation based on the notion that L-FABP has a high affinity for phytanic acid outside of the peroxisome. L-FABP is already known to be required for regular branched-chain fatty acid uptake and metabolism by having a role in cytoplasmic fatty acid transport [23;24].

Effect of azole antimycotics

The formation of ω-hydroxyphytanic acid was shown to be inhibited by imidazole derivatives in rat liver microsomes, indicating that the reaction is catalyzed by a

member of the cytochrome P450 enzyme family [25-27]. To establish whether ω-hydroxylation of phytanic acid is also catalyzed by a cytochrome P450 protein in human liver microsomes, we performed activity measurements in the presence of different concentrations of the imidazole derivatives bifonazole, clotrimazole, ketoconazole and miconazole, which were all found to inhibit product formation (Fig. 3A). Ketoconazole appeared to be the most potent inhibitor and not bifonazole which was most potent in rat liver microsomes [16]. As shown in Fig. 3 the effect of the inhibitors on (ω-1)-hydroxyphytanic acid formation was much more pronounced as compared to the inhibitory effect on ω-hydroxyphytanic acid formation. This is also clear from the IC50 values in Table 1.

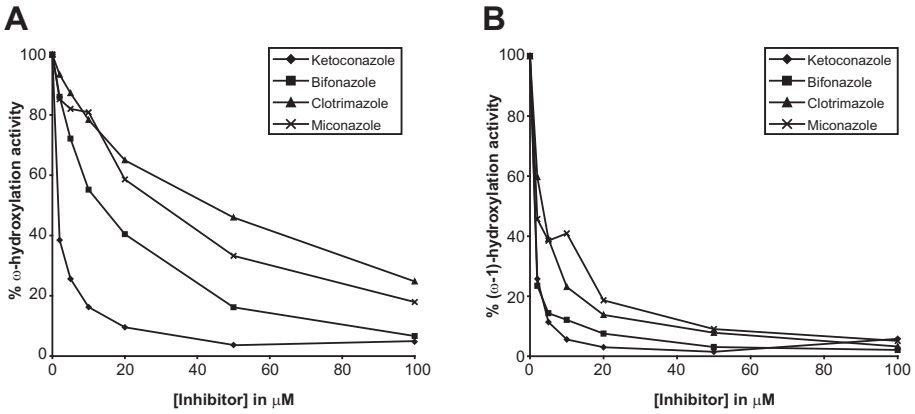

Figure 3: Effect of different imidazole antimycotics on the ω- and (ω-1)-hydroxylation of phytanic acid. Pooled human liver microsomes were incubated with phytanic acid in the presence of different concentrations of imidazole derivatives. The inhibitory effect of the imidazole derivatives on the formation of ω-hydroxyphytanic acid **(A)** and (ω-1)-hydroxyphytanic acid **(B)** is shown. The data represents the mean of duplicate experiments.

Discussion

The results described in this paper show that human liver microsomes are able to ω-hydroxylate phytanic acid. The formation of the products ω- and (ω-1)-hydroxyphytanic acid was NADPH dependent and inhibited by imidazole antimycotics. Compared to rat liver microsomes, less (ω-1)-hydroxyphytanic acid was formed in human liver microsomes, whereas the rate of formation of ω-hydroxyphytanic acid was similar in the two types of microsomes. This finding agrees with literature data which have shown the same phenomenon for other fatty acids [28]. However, the difference in the ratio of the rate of ω- and (ω-1)-hydroxyphytanic

acid formation between human and rat is markedly greater than for the fatty acids studied earlier [28]. In human liver microsomes, phytanic acid is mainly hydroxylated at the ω-position which is a favourable event, as ω-hydroxyphytanic acid can be metabolized further to 1,16-phytanedioc acid which is a substrate for β-oxidation, in contrast to (ω-1)-hydroxyphytanic acid.

Another difference between rat and human liver microsomes is the pattern of inhibition by imidazole derivatives which are known to be nonspecific P450 inhibitors [26]. The difference in inhibition of hydroxylation by the compounds used may be explained by the structural differences of the various antimycotics. Bifonazole was found to be the most potent inhibitor in rat liver microsomes, whereas ketoconazole is the most potent in human liver microsomes (Fig. 3). Furthermore, inhibition of (ω-1)-hydroxyphytanic acid formation differed markedly between human and rat liver microsomes.

Our future work is aimed at the identification of the human cytochrome P450(s) involved in the ω-hydroxylation of phytanic acid. It may well be that multiple P450s are involved in phytanic acid ω-hydroxylation as shown for other fatty acids such as lauric acid, arachidonic acid and leukotrienes, which are all hydroxylated by multiple P450s [10;28-30]. Induction of one of the P450s involved in phytanic acid ω-hydroxylation could be a possible therapeutic target for the treatment of ARD since upregulation of this P450 would lead to accelerated clearance of phytanic acid. In this respect it is important to mention that the expression of a variety of different P450s is controlled by members of the nuclear receptor superfamily. Indeed, CAR, PXR, and PPAR have been shown to mediate the induction of hepatic P450s belonging to the CYP2, CYP3, and CYP4 families respectively [17;18]. If the P450 responsible for phytanic acid ω-hydroxylation is under the control of such a receptor, induction of this P450 would be feasible as ligands for these receptors have already been identified and in many cases approved as drugs (see [17;18] for detailed information).

Table 1: IC50 values for the inhibition of phytanic acid ω- and (ω-1)- hydroxylation by imidazole derivatives as calculated from Fig. 3

Inhibitor	**IC50 value***	
	ω-hydroxyphytanic acid	**(ω-1)-hydroxyphytanic acid**
Bifonazole	13 μM	<2 μM
Clotrimazole	43 μM	3 μM
Ketoconazole	<2 μM	<2 μM
Miconazole	30 μM	<2 μM

*Data represents the mean of duplicate experiments.

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CHAPTER 4

Identification of the cytochrome P450 enzymes responsible for the ω-hydroxylation of phytanic acid

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Identification of the cytochrome P450 enzymes responsible for the ω-hydroxylation of phytanic acid

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Abstract

Patients suffering from Refsum disease have a defect in the α-oxidation pathway which results in the accumulation of phytanic acid in plasma and tissues. Our previous studies have shown that phytanic acid is also a substrate for the ω-oxidation pathway. With the use of specific inhibitors we now show that members of the cytochrome P450 family 4 class are responsible for phytanic acid ω-hydroxylation. Incubations with microsomes containing human recombinant CYP450s (Supersomes™) revealed that multiple cytochrome P450 enzymes of the family 4 class are able to ω-hydroxylate phytanic acid with the following order of efficiency: CYP4F3A > CYP4F3B > CYP4A11 > CYP4F2.

Introduction

The degradation of the majority of fatty acids occurs via β-oxidation in mitochondria and peroxisomes. 3-Methyl-branched-chain fatty acids, however, are not substrates for regular β-oxidation but first need to undergo one round of β-oxidation in peroxisomes. During α-oxidation a one-carbon unit is removed from the carboxy-terminus of the fatty acid yielding the n-1 analogue with the methyl group at position 2. The 2-methyl-branched-chain fatty acid thus formed, can then undergo normal β-oxidation [1].

The most abundant 3-methyl-branched chain fatty acid, at least in humans, is phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). Phytol, a fatty alcohol and a metabolite of chlorophyll, is the precursor of phytanic acid. Mammals cannot absorb chlorophyll and release phytol, but are able to convert free phytol into phytanic acid and to absorb phytanic acid itself. Fats of dairy products and meat are rich in phytanic acid and phytol since chlorophyll degradation and the subsequent conversion of

phytol to phytanic acid occurs effectively by bacteria present in the rumen of ruminants.

Phytanic acid accumulates in patients with adult Refsum disease (ARD, MIM 266500) which is due to a defect in the α-oxidation pathway caused by mutations in one of two genes including the *PAHX* gene which codes for phytanoyl-CoA hydroxylase [2;3], and the *PEX7* gene which codes for the PTS2 receptor [4]. The majority of ARD patients have mutations in the *PAHX* gene. The increased levels of phytanic acid in plasma and tissues are thought to be the direct cause for the pathology of the disease. Among the symptoms are: progressive retinitis pigmentosa, peripheral neuropathy, anosmia, cerebellar ataxia and ichthyosis [5]. The accumulation of phytanic acid is gradual because phytanic acid originates from dietary sources. The only known treatment of ARD consists of a diet low in phytanic acid, which may be combined with plasmapheresis. The diet causes the phytanic acid levels to decrease in patients and reduces the progression of the disease [5]. This decrease in phytanic acid rather than its stabilization indicates that there is another pathway involved in phytanic acid breakdown besides α-oxidation because in many ARD patients the α-oxidation pathway is completely defective.

There is a third degradation pathway known for fatty acids besides α- and β-oxidation, namely ω-oxidation, which leads to the formation of a carboxyl group at the ω-end of the fatty acid [6;7]. The first step involves hydroxylation of the fatty acid at the ω-end of the molecule. The ω-hydroxylated fatty acid is then converted into the corresponding dicarboxylic acid, which may either be catalysed by the subsequent action of an alcohol dehydrogenase and an aldehyde dehydrogenase, or catalysed by a cytochrome P450 (CYP450) enzyme [8]. In the case of phytanic acid, ω-oxidation will introduce a new carboxyl group on the ω-end with a methyl-group at position 2. The formed ω-dicarboxylic acid of phytanic acid, i.e. phytanedioic acid, can undergo β-oxidation after activation of the ω-carboxyl group to its CoA-ester in the same manner as 2-methyl-branched fatty acids.

Earlier studies have provided evidence suggesting that phytanic acid does undergo ω-oxidation under in vivo conditions [9-11]. More recently we have shown that the first step of the ω-oxidation pathway, i.e. the ω-hydroxylation of phytanic acid, takes place in rat and human liver microsomes and is catalyzed by one or more members of the CYP450 enzyme superfamily [12;13]. The expression of many of the enzymes which belong to this family is known to be induced by a large variety of drugs [14;15]. Hence, induction of the CYP450 responsible for the ω-hydroxylation of phytanic acid may increase the flux through the ω-oxidation pathway, thereby increasing the clearance of phytanic acid in ARD patients. As the CYP450 enzymes have multiple roles in cellular metabolism, the induction of the CYP450 involved should be as specific as possible to reduce unwanted side effects from the administered drugs. For this reason it is important to identify the specific CYP450

involved in the ω-hydroxylation of phytanic acid. In this paper we report our studies on the identification of the CYP450 enzyme(s) involved in the ω-hydroxylation of phytanic acid in humans.

Materials and Methods

Materials

Phytanic acid was obtained from the VU University Medical Center, Metabolic Laboratory (Dr H.J. ten Brink, Amsterdam, the Netherlands). 3-Hydroxyheptadecanoic acid was from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD^+ were obtained from Roche (Mannheim, Germany). Methyl-β-cyclodextrin was from Fluka (Buchs, Switzerland). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Pooled human liver microsomes and human recombinant CYP450 isoforms (Supersomes™) were obtained from BD Gentest™ (Woburn, MA, USA). The Supersomes™ used contained also CYP450 reductase and cytochrome b5 in addition to the CYP450. The CYP450 inhibitors diethyldithiocarbamate (DDC), furafylline, ketoconazole, 17-octadecynoic acid (17-ODYA), omeprazole, quinidine, sulphaphenazole, trimethoprim, and troleandomycin were purchased from Sigma Aldrich (St Louis, MO, USA). All other chemicals used were of the highest quality possible.

Phytanic acid ω-hydroxylase assay

The ω-hydroxylation of phytanic acid was measured as described earlier [12;13] with slight modifications. In brief, phytanic acid dissolved in DMSO was added to a reaction mixture (final volume 0.2 ml) containing human liver microsomes (1 mg/ml final concentration), and 100 mM potassium phosphate buffer (pH 7.7) containing methyl-β-cyclodextrin (0.75 mg/ml final concentration). When Supersomes™ (25 pmol P450/ml) were used in experiments, the protein concentration of the reaction mixture was kept constant at 0.75 mg/ml by adding Insect Control Supersomes™. The final concentration of phytanic acid was 0.2 mM unless indicated otherwise. Reactions were initiated by the addition of NADPH (final concentration 1 mM) and terminated after 30 min by adding 0.2 ml of 1 M HCl to the mixture. Subsequently, 1 ml phosphate-buffered saline was added, followed by 100 µl 12.1 M HCl, and 20 µl 0.2 mM 3-hydroxyheptadecanoic acid in DMSO as internal standard. The reaction mixtures were extracted twice with 4 ml ethylacetate-diethylether (1:1 v/v). Both fractions were collected and the solvents were evaporated under N_2. The extracted fatty acids were derivatized to their corresponding trimethylsilyl derivatives for gas chromatography/mass spectrometry analysis as described previously [12].

Inhibitor studies

During the inhibitor studies the ω-hydroxylation assay was performed as described above except that incubations were performed in the presence of the following inhibitors: trimethoprim (CYP2C8), sulfaphenazole (CYP2C9), omeprazole (CYP2C19), quinidine (CYP2D6), DDC (CYP2E1), and ketoconazole (CYP3A4) [16-19]. Furafylline (CYP1A2), DDC (diethyldithiocarbamate) (CYP2E1), 17-ODYA (17-octadecynoic acid) (CYP4), and troleandomycin (CYP3A4) were pre-incubated with human liver microsomes for 15 minutes as following the protocol of Eagling et al. [16] in the presence of NADPH before adding phytanic acid to the mixture and subsequent incubation (30 min). Stock solutions were prepared in DMSO. Inhibitor concentrations are given in Fig. 1.

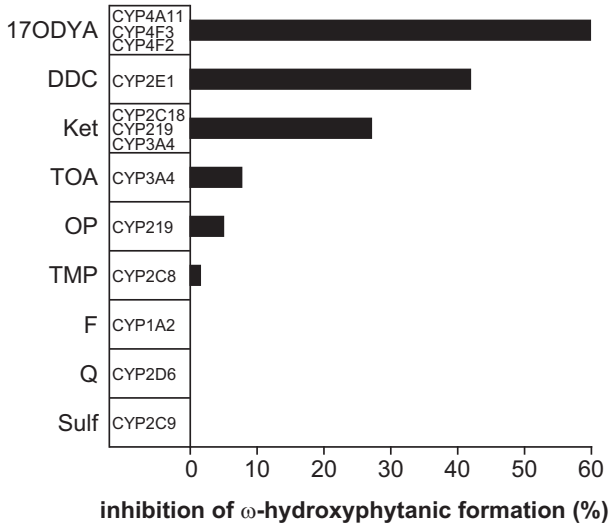

Figure 1: Inhibition of ω-hydroxyphytanic acid formation in human liver microsomes by several CYP450 isoform specific inhibitors. Pooled human liver microsomes were incubated with phytanic acid in the presence of the following inhibitors: TMP, trimethoprim (100 μM); Ket, ketoconazole (1 μM); OP, omeprazole (10 μM); Sulf, sulfaphenozole (10 μM); Q, quinidine (10 μM); F, furafylline (20 μM); TOA, troleandomycin (100 mM); DDC, diethyldithiocarbamate (100 μM); 17ODYA, 17-octadecynoic acid (1 μM). The bars represent the mean of duplicate experiments.

Results

Recently, we showed that phytanic acid is ω-hydroxylated in (pooled) human liver microsomes and that this reaction is catalyzed by one or more CYP450 enzymes [13]. Furthermore, we found that phytanic acid is not exclusively hydroxylated at the ω-position but also at the (ω-1)-position in a 15:1 ratio. In the present study we have focused only on the formation of ω-hydroxyphytanic acid because this compound is formed predominantly and can be converted into 1,16-phytanedioic acid, a substrate for subsequent β-oxidation.

To determine which CYP450 contributes to phytanic acid ω-hydroxylation in human liver microsomes, we have incubated human liver microsomes with a number

of Cytochrome P450 subfamily selective inhibitors as described in the materials and methods section. Concentrations used were derived from previous studies [16-19]. Substantial inhibition of phytanic acid ω-hydroxylation was only found with ketoconazole, DDC, and 17-ODYA, which are specific inhibitors of CYP3A4, CYP2E1 and CYP4 family members, respectively. Troleandomycin, omeprazole, and trimethoprim showed minor effects, while with sulphaphenazole, quinidine, and furafylline no inhibitory effect was observed at all (Fig. 1). Inspection of the results of Fig.1 for ketoconazole reveals that the formation of ω-hydroxyphytanic acid is inhibited by 30% at a final concentration of 1 μM of ketoconazole. This indicates that ketoconazole is a relatively poor inhibitor of phytanic acid ω-hydroxylation especially since the IC50 value of ketoconazole for the CYP3A4 enzyme has been established to be in the nanomolar range [16]. The inhibitory effect of ketoconazole at higher concentrations as observed in Fig. 1 is probably due to the fact that at these concentrations ketoconazole also inhibits other CYP450s, as indeed described in literature [16;20].

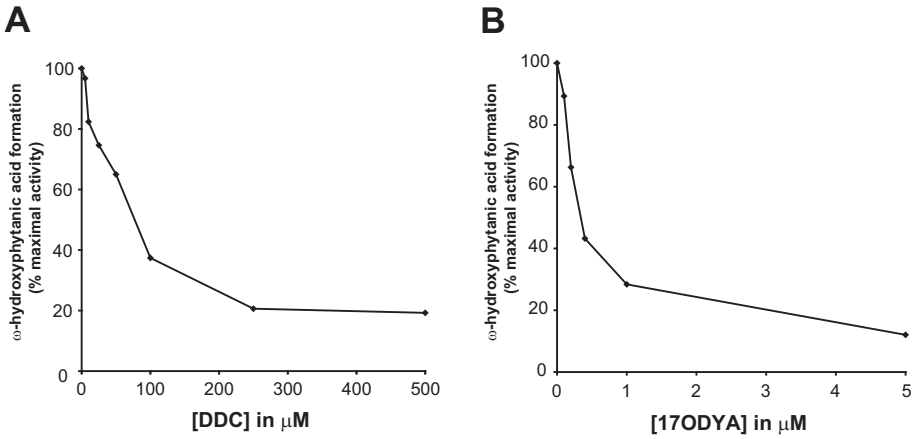

Figure 2: Inhibition of the formation of ω-hydroxyphytanic acid by different concentrations of DDC **(A)** and 17-ODYA **(B)**. The inhibitory effect of DDC **(A)** and 17-ODYA **(B)** on the formation of ω-hydroxyphytanic acid in pooled human liver microsomes is shown. The data represent the mean of duplicate experiments.

In order to estimate the IC50 values for the inhibitors DDC and 17-ODYA, we incubated human liver microsomes in the presence of phytanic acid and different concentrations of inhibitor (Fig. 2). From the data of Fig 2A, an IC50 value of DDC of 90 μM can be calculated (Fig. 2A). Although DDC is often used as a selective inhibitor of CYP2E1 in literature [21;22], DDC is actually a more potent inhibitor of CYP2A6 and CYP2B6 catalyzed reactions (IC50 ≈ 125 μM for CYP2E1 vs. IC50 ≤

10 μM for CYP2A6 and CYP2B6 [21]). It can be concluded from these IC50 values that the contribution of CYP2A6 and 2B6 to the ω-hydroxylation of phytanic acid is only minor, whereas it cannot be excluded that CYP2E1 possesses phytanic acid ω-hydroxylation activity. However, since DDC is known to inhibit multiple CYP450 isoforms when used in high concentrations [16;21] it may well be that multiple CYP450s are involved in the inhibitory action of DDC in addition to CYP2E1. As shown in Fig. 2B, 17-ODYA was found to be a potent inhibitor of phytanic acid ω-hydroxylation with an IC50 < 400 nM. 17-ODYA is a fatty acid analogue which acts through suicide inhibition and is selective for the CYP4 family of ω-hydroxylases (IC 50 < 100 nM [23]) which are involved in the ω-hydroxylation of long-chain fatty acids, arachidonic acid and leukotrienes [6;7;23-26].

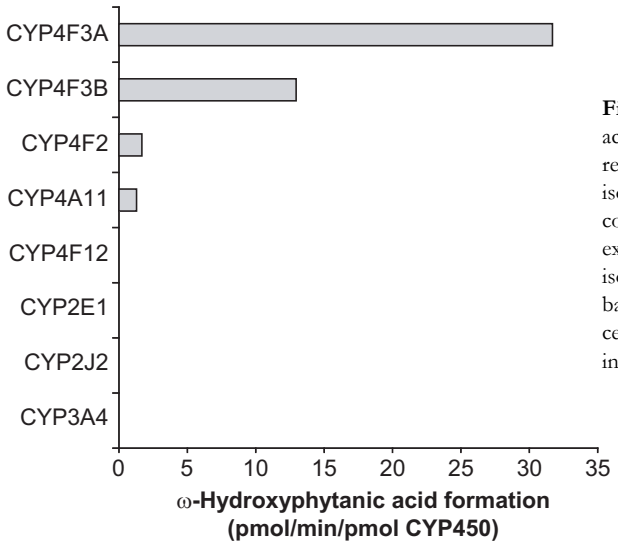

Figure 3: ω-Hydroxyphytanic acid formation by human recombinant CYP450 isoforms. Microsomes containing individually expressed human CYP450 isoforms prepared from baculovirus-infected insect cells (Supersomes™ were incubated with phytanic acid

The results described in the previous paragraph indicate that one or more members of the CYP4 family most likely are involved in the ω-hydroxylation of phytanic acid because the IC50 value found for 17-ODYA is comparable with previously reported values for this family of CYP450 enzymes. In order to substantiate this conclusion, we studied phytanic acid ω-hydroxylation in microsomes containing individually expressed CYP4s prepared from baculovirus-infected insect cells (Supersomes™). We tested all commercially available members of the human CYP4 family. Furthermore, we also performed incubations with Supersomes™ containing CYP3A4 and CYP2E1 to verify the conclusion drawn above that the inhibition observed with ketoconazole and DDC was indeed due to the non-specificity of these inhibitors at higher concentrations (see previous paragraph).

Indeed, as shown in Fig. 3, microsomes expressing CYP3A4 and CYP2E1 were completely devoid of phytanic acid ω-hydroxylation activity. Among the CYP4s tested, there were four CYP450s displaying phytanic acid ω-hydroxylation activity: CYP4F3A, CYP4F3B, CYP4A11 and CYP4F2 (Fig. 3). In order to determine the kinetic parameters of the different CYP4s we varied the substrate concentration in the incubation mixture with the four active CYP4s. Kinetic parameters were determined by non-linear regression analysis using a single-component Michaelis-Menten model and are depicted in table 1. The results obtained show that CYP4F3A has highest catalytic efficiency of the CYP4s tested as concluded from the V_{max}/Km ratios.

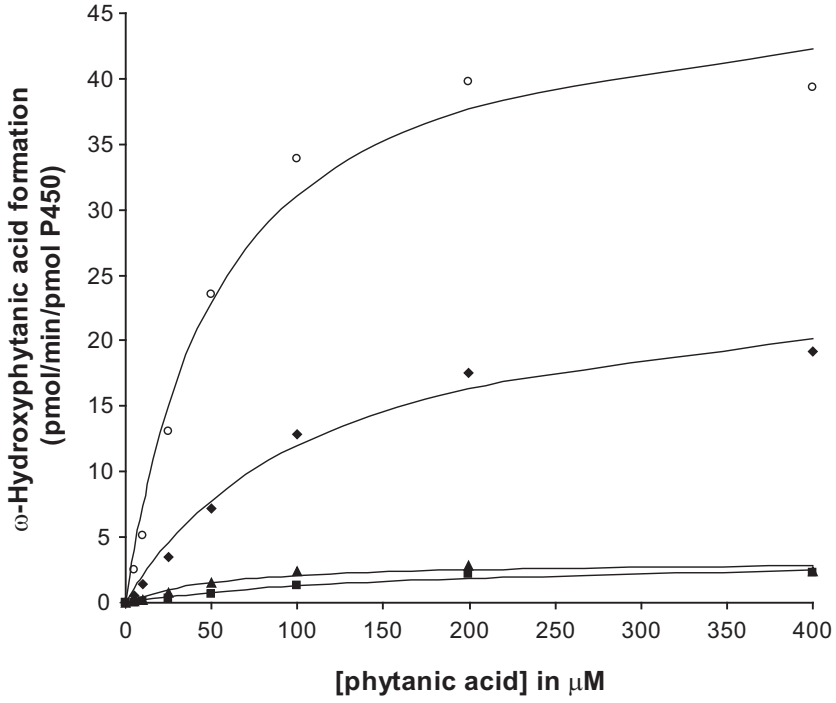

Figure 4: ω-Hydroxyphytanic acid formation as a function of the amount of substrate in the assay. Supersomes™ were incubated in the presence of different concentrations of phytanic acid and NADPH under conditions described in the Methods section. The ratio methyl-β-cyclodextrin / phytanic acid was kept constant. Lines represent rates predicted using Michaelis-Menten kinetic parameters derived from non-linear regression analysis of the results. The data points represent average values of three experiments. (CYP4F3A (○), CYP4F3B (♦), CYP4F2 (▲), CYP4A11 (■))

Discussion

The results described in this paper indicate that among the CYP4 family members tested in this study, CYP4F3A is the most efficient enzyme in ω-hydroxylating phytanic acid followed by CYP4F3B (table 1). *CYP4F3B* is a splice variant of the *CYP4F3* gene in liver and differs in only one exon from *CYP4F3A* [27]. This has been shown to cause a difference in substrate specificity with CYP4F3A having the highest affinity for leukotriene B4 (LTB4) and CYP4F3B for arachidonic acid [28]. In this paper the difference in substrate specificity between the two splice variants is shown by using phytanic acid as a substrate.

The CYP4F3A enzyme is expressed in polymorphonuclear leukocytes and was found to be involved in LTB4 ω-hydroxylation, a process required for the degradation of this inflammatory agent [29]. Since CYP4F3A is not expressed in liver, this enzyme cannot be responsible for the phytanic acid ω-hydroxylation activity observed in human liver microsomes [28;30]. Based on the results described in this paper other liver CYP4 enzymes able to ω-hydroxylate phytanic acid in addition to CYP4F3B are CYP4A11 and CYP4F2 [31;32]. Although CYP4A11 shows little phytanic acid ω-hydroxylation activity as compared to CYP4F3A and CYP4F3B, the CYP4A family is known to be induced by hypolipidemic drugs and peroxisome proliferators (PP) via PPARα [33], and can therefore be considered as a potential target for further research aimed to induce the activity of the α-oxidation pathway of phytanic acid in ARD patients. Preliminary experiments in which phytanic acid ω-hydroxylation capacity was measured in liver homogenates of mice fed a diet containing Wy14643 (0.1%) have shown that phytanic acid ω-hydroxylation is induced approximately 8-fold as compared with normal fed mice.

Table 1: Kinetic parameters for the formation of ω-hydroxyphytanic acid by CYP4A11, CYP4F2, CYP4F3A and CYP4F3B

	K*m** ***μM	V_{max} ***pmol/min/pmol P450***	V_{max}**/ *K*m**
CYP4A11	191	3.7	0.02
CYP4F2	56	3.2	0.06
CYP4F3A	55	48.1	0.87
CYP4F3B	119	26.1	0.22

Km and V_{max} values were estimated by non-linear regression analysis of the data points presented in Fig. 4 in which each data point is the mean of 3 separate experiments. A single-component Michaelis-Menten model was used for calculation.

Acknowledgements

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CHAPTER 5

The effect of Fenofibrate on phytanic acid ω-hydroxylation and phytanic acid accumulation in Phytanoyl-CoA hydroxylase knockout mice

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The effect of Fenofibrate on phytanic acid ω-hydroxylation and phytanic acid accumulation in Phytanoyl-CoA hydroxylase knockout mice

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Abstract

Refsum disease is an autosomal recessive neurodegenerative disorder caused by a defect in the α-oxidation pathway which is responsible for the one-carbon decarboxylation of the dietary fatty acid phytanic acid. As a result of this defect, phytanic acid accumulates in plasma and tissues of these patients which is believed to be the main cause of the pathology in Refsum disease. Previous studies described in literature have provided evidence for the existence of an alternative degradation pathway for phytanic acid, i.e. ω-oxidation. Recently, it was shown that enzymes of the CYP4A subfamily of cytochrome P450s were able to catalyze the first step of phytanic acid ω-oxidation. CYP4A enzymes are known to be upregulated by fibrates. Such an upregulation might increase the capacity of the total phytanic acid ω-oxidation pathway, which would be beneficial for patients with Refsum disease. In order to study the effect of fibrates on phytanic acid ω-oxidation and phytanic acid accumulation in Refsum disease we treated *Phyh* -/- mice (mice lacking phytanoyl-CoA hydroxylase) with fenofibrate. This resulted in the decreased accumulation of phytanic acid in plasma and extra-hepatic tissues, except in cerebellum. Phytanic acid levels in livers of fenofibrate treated mice were increased. Taken together, these data point to a shift of phytanic acid from extrahepatic tissues to the liver. The ω-hydroxylation capacity and CYP4A content in the livers of fenofibrate treated mice were increased and, in addition, a possible ω-oxidation product of phytanic acid was found to be increased in fenofibrate treated livers. These results suggest that the degradation of phytanic acid via ω-oxidation is increased in the liver. In conclusion, the results in this paper show that fenofibrate causes a decrease in phytanic acid levels in extra-hepatic tissues by increased transport of phytanic acid to the liver and subsequent breakdown of phytanic acid via increased hepatic ω-oxidation. Hence, fenofibrate treatment is possibly a new therapeutical approach for the treatment of Refsum disease.

Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid present in dietary sources, such as meat and dairy products. Phytanic acid is degraded via decarboxylation at the α-carbon by a pathway known as the α-oxidation pathway. This results in the formation of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and formyl-CoA. Subsequently, pristanic acid can be degraded via β-oxidation which ultimately leads to the formation of multiple acetyl-CoA and propionyl-CoA units as well as isobutyryl-CoA [1].

In patients suffering from Refsum disease (heredopathia atactica polyneuritiformis, MIM# 266500) the α-oxidation pathway is deficient. The disease is primarily caused by mutations in the *PHYH* gene encoding the first enzyme of the α-oxidation pathway, i.e. phytanoyl-CoA hydroxylase (PHYH) [2;3]. In a subset of patients specific mutations in the *PEX7* gene, encoding Peroxin 7 (the transport protein required for the correct localization of PHYH in the peroxisome), are the underlying cause of Refsum disease [4]. Refsum patients accumulate phytanic acid in plasma and tissues as a result of the deficiency in the α-oxidation pathway [5]. The accumulation of phytanic acid is believed to be the cause of the symptoms and is also used as the biochemical marker for the disease. The major symptoms consist of progressive retinitis pigmentosa, leading to night blindness and ultimately a total loss of vision, peripheral neuropathy, ataxia, anosmia, and deafness (reviewed by Steinberg [6]). At present, the only therapeutical intervention consists of a diet low in phytanic acid, which may be combined with plasmapheresis in times of metabolic crisis. The diet has been shown to decrease plasma phytanic acid levels and consequently may result in a decelerated progression of the disease [7-9].

The diet-induced decrease of phytanic acid levels suggests that phytanic acid can be broken down via another fatty acid degradation pathway than the α-oxidation pathway. Data from different studies indicates that phytanic acid is a substrate for the ω-oxidation pathway, which involves the conversion of a fatty acid into a dicarboxylic acid by means of a multi-step reaction mechanism. First, the ultimate methyl group of the fatty acid (at the ω-end) is hydroxylated by a cytochrome P450 (CYP450) enzyme. The formation of the ω-hydroxyl group is followed by the consequent action of an alcohol dehydrogenase and an aldehyde dehydrogenase which convert the ω-hydroxyfatty acid into a dicarboxylic acid via an aldehyde intermediate. Alternatively, the ω-hydroxyfatty acid may be converted into a carboxylic acid via a CYP450 catalyzed route consisting of additional hydroxylations at the ω-end and the subsequent release of water [10;11]. Phytanic acid ω-oxidation would result in the formation of phytanedioic acid (2,6,10,14-tetramethylhexadecanedioic acid) which is a substrate for peroxisomal β-oxidation from the ω-end. Potential phytanedioic acid β-oxidation metabolites have been identified in urine from patients with Refsum disease,

among which are 3-methyladipic acid, and 2, 6-dimethyloctanedioic acid, suggesting that the dicarboxylic acid of phytanic acid is indeed formed in patients [12-14]. Conversely, these potential phytanedioic acid β-oxidation metabolites are not found in urine of healthy individuals. Furthermore, Wierzbicki et al. [15] showed that 3-methyladipic acid excretion in urine of Refsum patients correlates with phytanic plasma levels.

Recently, we performed *in vitro* studies which were focused on the first step of the ω-oxidation of phytanic acid, i.e. the ω-hydroxylation step. Rat and human liver microsomal fractions were able to form ω-hydroxyphytanic acid from phytanic acid when supplemented with NADPH, the cofactor required for CYP450 enzymes [16;17]. In addition, we showed that the phytanic acid ω-hydroxylases in humans belong to the CYP450s of family 4. CYP4F3A was the most efficient member in catalyzing the ω-hydroxylation of phytanic acid followed by CYP4F3B, CYP4F2, and CYP4A11 [18]. Upregulation of phytanic acid ω-hydroxylating CYP450s could increase the flux of phytanic acid through the entire ω-oxidation pathway which could lead to an increased breakdown of phytanic acid via β-oxidation of the formed phytanedioic acid. Hence, upregulation of these CYP450s may be considered as a potential new treatment of Refsum disease.

A large number of CYP450 enzymes are readily inducible by registrated drugs [19-21]. However, there is hardly any data present in literature on the induction of enzymes belonging to the CYP4F subfamily, which are the most efficient phytanic acid ω-hydroxylases. On the other hand, it is well known that CYP4A enzymes are readily induced by hyperlipidemic drugs of the fibrate class in rodents and humans (reviewed in [22;23]). Fibrates are ligands for the peroxisome proliferator-activated receptor alpha (PPARα), a member of the nuclear hormone receptor superfamily. Activation of PPARα by ligands enables this receptor to form a heterodimer with the Retinoic X Receptor (RXR) [24]. The PPARα/RXR heterodimer is able to bind to Peroxisome Proliferator Response Elements (PPREs) in promoter regions of several genes involved in lipid metabolism, including the *CYP4A* genes, resulting in induction of gene-expression (reviewed by Yu and Reddy [25]). Mice have multiple CYP4A enzymes, including Cyp4a10, Cyp4a12a and b, and Cyp4a14. Cyp4a10 and Cyp4a14 are inducible by fibrates in liver and kidney. Cyp4a12 enzymes cannot be induced by fibrates in liver and kidney of male mice, but in female mice fibrates are able to induce Cyp4a12 [26-28].

Recently a mouse model for Refsum disease (phytanoyl-CoA hydroxylase knock-out mouse, *Phyh* -/-) has been generated. When phytanic acid, or its precursor phytol, is supplemented to the diet, *Phyh* -/- mice accumulate phytanic acid in plasma and tissues (unpublished data). This indicates that, at least at the metabolical level, this is a good model for Refsum disease. In this study we investigated the effects of fenofibrate on phytanic acid metabolism in *Phyh* -/- mice. To this end, male *Phyh* -/-

mice were subjected to four different diets. The control group received standard mouse chow (1), while the other groups were fed standard chow enriched with (2) phytanic acid, (3) fenofibrate, or (4) a combination of both. Afterwards, the levels of phytanic acid were determined in multiple tissues. The *in vitro* phytanic acid ω-hydroxylation capacity was determined in liver homogenates and the expression of the CYP4A and CYP4F enzymes in liver and kidney tissue was studied. Furthermore, acylcarnitine levels were determined in liver.

Materials and Methods

Materials

Fenofibrate was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Phytanic acid, [$^2H_3$]carnitine and [$^2H_3$]C_3-, C_8- and C_{16}-acylcarnitine internal standards were obtained from Dr Herman J. ten Brink (VU Medical Hospital, Amsterdam, The Netherlands). NADPH was from Roche (Mannheim, Germany). Methyl-β-cyclodextrin was from Fluka (Buchs, Switzerland). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). The internal standard for GS-MS analysis, 3-hydroxyheptanoic acid, was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Other chemicals used were of highest quality possible.

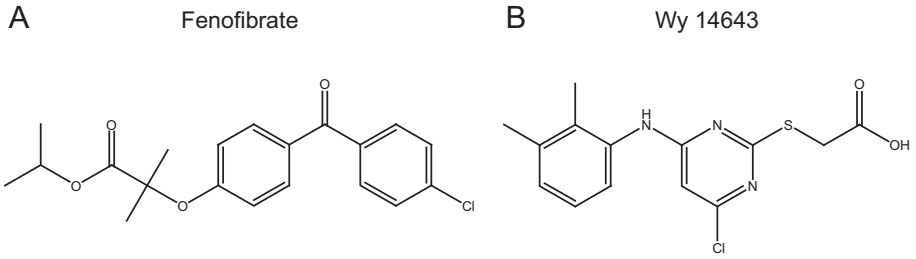

Figure 1: Chemical structures of **(A)** fenofibrate (propan-2-yl 2-[4-(4-chlorobenzoyl)-phenoxy]-2-methylpropanoate) and **(B)** Wy14643 (pirinixic acid, 2-[4-chloro-6-[(2,3-dimethylphenyl)amino]pyrimidin-2-yl]sulfanylacetic acid).

Animals

Eight week old *Phyh* -/- mice on a Swiss genetic background were fed standard mouse chow (Special Diets Services, UK). The enriched diets consisted of the standard mouse chow supplemented with 0.01% (w/w) phytanic acid, 0.1% (w/w) fenofibrate, or a combination of both. The different groups consisted of four male mice which were kept on the different diets for 2 weeks with water and food ab libitum until they

were sacrificed. The weight of the animals was monitored twice a week. At the end of the experiment the mice were anesthetized using ketamine (100 mg/kg) in combination with xylazine (10 mg/kg), blood was collected by cardiac puncture, and subsequently tissues were collected and liver weight determined. Plasma was immediately prepared by centrifugation. Tissues and plasma were snap-frozen in liquid nitrogen and stored at -80°C until use. All experiments were approved by the University of Amsterdam Animals Experiments Committee. The liver tissues of wild-type and PPARα KO mice untreated and treated with Wy14643 were a kind gift of Dr. N. van Vlies [29].

Phytanic acid ω-hydroxylation assay

The assay was performed essentially as described previously [17]. Briefly, tissue was homogenized by pottering and subsequent sonication (3 times 8 W) in ice-cold PBS. The protein concentration of the homogenates was determined according to Smith et al. [30]. The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.6) containing 1 mg/ml protein, 0.75 mg/ml methyl-β-cyclodextrin, 200 μM phytanic acid (all end-concentrations). The reaction was initiated by the addition of 1 mM (end-concentration) NADPH. Total reaction volume was 200 μl. After 30 min the reaction was stopped by the addition of 200 μl 1 M HCl. The internal standard (4 nmol 17-hydroxyheptadecanoic acid) was added and subsequently fatty acids were extracted three times with 4 ml ethylacetate/diethylether (1:1, v/v). The solvent was evaporated under N_2. Fatty acids were derivatized to their corresponding trimethylsilyl derivatives for gas chromatography/mass spectrometry (GC/MS) analysis [16].

Imunnoblot

Tissues were homogenized in ice-cold PBS containing protease inhibitors (Roche, Basel, Germany). 15 μg of protein was loaded on a 10% (w/v) SDS-polyacrylamide gel and subjected to elecrophoresis. Subsequently, proteins were transferred electrophoretically on a nitrocellulose membrane (Schleicher and Shuell, Dassel, Germany). Non-specific binding sites were blocked using a solution containing 3% (w/v) non-fat dried milk protein (Protifar, Nutricia, Zoetermeer, the Netherlands) and 1% (w/v) BSA in 0.1% (w/v) Tween-20/PBS. Next, the blots were incubated with specific primary antibodies against CYP4A11 (rabbit polyclonal, US Biological Swampscott, MA), or CYP4F2 (rabbit polyclonal, Research Diagnostics Inc., Concord, MA). The blots were incubated with secondary antibodies conjugated to alkaline phoshatase for detection (Goat-anti-Rabbit AP-conjugated, Bio-Rad, Hercules, CA). Proteins were detected using a BCIP/NBT Color Development Substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Promega, Madison, WI).

Fatty acid analysis

Phytanic acid, pristanic acid, and the very-long-chain fatty acids (VLCFAs) were analyzed in plasma and multiple tissues of the mice. To this end, small amounts of tissue were taken and weighed (wet-weight). Homogenates were made in ice-cold PBS by pottering and subsequent sonication (3 times 10s at 8W). Protein concentration was determined [30] and an amount of homogenate corresponding to 0.5 mg protein was used for analysis. Plasma analysis was performed with 100 µl of plasma. The fatty acid content was determined using a GC/MS based method [31].

Acylcarnitine analysis

Acylcarnitine analysis was performed essentially as described by Van Vlies et al. [32]. Briefly, approximately 30 mg of liver tissue (wet-weight) was freeze-dried overnight. Subsequently, the samples were kept on ice and the tissues were grounded to powder. The weight of the powder was determined while keeping the samples on ice in between weighing. The following internal standards were added: [$^2H_3$]Carnitine (16.25 nmol) for free carnitine, [$^2H_3$]C_3-acylcarnitine (0.125 nmol) for C_2–C_5-acylcarnitines, [$^2H_3$]-C_8-acylcarnitine (0.05 nmol) for C_6–C_{10}-acylcarnitines and [$^2H_3$]C_{16}-acylcarnitine (0.05 nmol) for C_{12}–C_{22} acylcarnitine. The C_3-C_5-dicarboxylic acylcarnitines were quantified with [$^2H_3$]-C_8-acylcarnitine as internal standard, all the higher dicarboxylic acylcarnitines were analyzed with [$^2H_3$]C_{16}-acylcarnitine. The samples were sonicated twice for 20 s at 2.5W in 1 ml of an 4:1 (v/v) acetonitrile/water solution (80% acetonitrile). The suspensions were centrifuged at 16,000 x *g* for 5 min. The supernatants were evaporated under nitrogen at 40°C. Next, the samples were propylated by the addition of the propylation reagent 100 µl volume (a 4:1 (v/v) mixture of propan-2-ol and acetylchloride), followed by vortex-mixing, and incubation of the samples at 60°C for 15 min. The propylation mixture was evaporated under nitrogen and the samples were taken up in 100 µl acetonitril for HPLC/tandem-MS analysis [32].

Statistical analysis

Data are expressed as means ± standard deviation. Statistical significance was evaluated using one-way ANOVA followed by Tukey-Kramer multi-comparison test using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were considered statistically significant at $P < 0.05$.

Results

Previous studies have shown that phytanic acid is a substrate for the first step of the ω-oxidation pathway in human and rat liver microsomes. It was found that the human enzymes involved in this first step, i.e. phytanic ω-hydroxylation, belong to the CYP450 family 4. One of the phytanic acid ω-hydroxylases, i.e. CYP4A11, is known to be induced by compounds of the fibrate class of peroxisome proliferators including Wy14643 (Fig. 1A) and fenofibrate (Fig. 1B) (reviewed by Johnson et al. [23]). The inducibility of CYP4A enzymes by fibrates is a common characteristic of this subfamily throughout other organisms as well. Indeed, the CYP4A subfamily in mice is readily induced by fibrate treatment [26]. To test whether phytanic acid ω-hydroxylation enzymes are induced in mice by fibrate treatment we studied the *in vitro* ω-hydroxylation capacity in mouse liver homogenates of wildtype and *PPARa* -/- mice which were administered either a control diet, or a diet supplemented with 0.1% Wy14643 [29]. Figure 2 shows that the diet supplemented with Wy14643 induced phytanic acid ω-hydroxylation capacity in liver homogenates of wild type mice. This induction was PPARα-dependent as liver homogenates of *PPARa* -/- mice showed no induction of phytanic acid ω-hydroxylation.

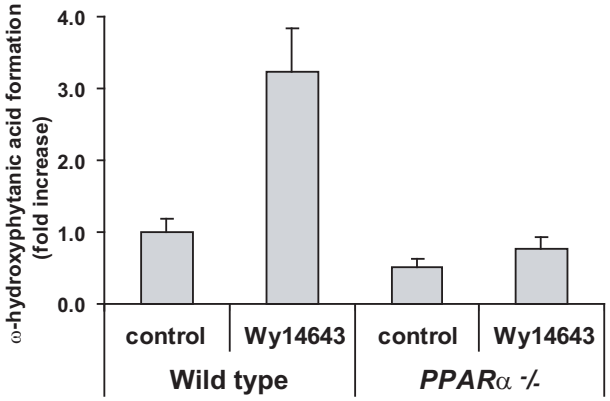

Figure 2: Phytanic acid ω-hydroxylation in mouse liver homogenates. Phytanic acid ω-hydroxylation was measured in liver homogenates of wild type mice and PPARα KO (*PPARa* -/-) mice on either a control diet, or a Wy14643-enriched diet for 2 weeks. Results are normalized against the mean values of wild type mice on control diet. Values represent the mean of three animals per group ± SD.

These results show that fibrates are able to upregulate the first step of ω-oxidation of phytanic acid. The induction of the first step of the ω-oxidation pathway for phytanic acid may increase the overall ω-oxidation capacity and thereby decrease phytanic acid levels which would be very benificial in case of an accumulation of phytanic acid such as in Refsum disease. Moreover, fibrate treatment influences several aspects of fatty acid metabolism besides induction of ω-oxidation, which could affect the accumulation of phytanic acid. In order to gain more insights into the *in vivo* effects of fibrates on phytanic acid accumulation, we studied the effect of fenofibrate

treatment in the recently generated *Phyh* -/- mice. These mice accumulate phytanic acid in plasma and tissues as a result of a defect in the α-oxidation pathway when phytanic acid is present in the diet. In this study 8 weeks old male *Phyh* -/- mice were divided into four different groups (four mice per group) and each group was fed a different diet for 2 weeks. These diets consisted of (1) standard mouse chow (control group), standard mouse chow supplemented with (2) 0.01% phytanic acid (phytanic acid group), (3) 0.1% fenofibrate (fenofibrate group) and (4) a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination group).

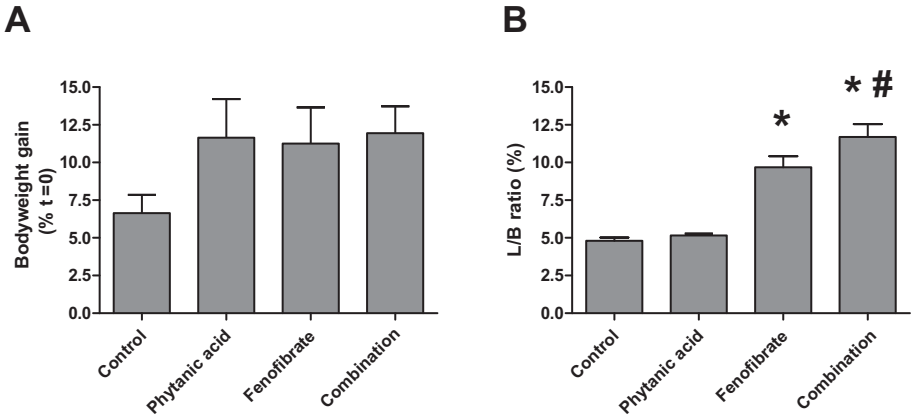

Figure 3: Body weight gain **(A)** and liver/body weight (L/B) ratio **(B)** of *Phyh* -/- mice on standard diet (control), standard diet supplemented with 0.01% phytanic acid (phytanic acid), 0.1% fenofibrate (fenofibrate) and a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination) for 2 weeks. **(A)** The body weight was monitored during the 2 weeks of diet. Data in the graph represent the mean body weight gain during that period ± SD expressed in percentage of the weight of the animals at the start of the experiment. **(B)** L/B ratio expressed in percentage. Values represent the mean of four animals per group ± SD. * $P < 0.001$ between the fenofibrate/combination group and the control group. # $P < 0.01$ between the fenofibrate group and the combination group.

The weight of the mice was monitored during the period of treatment. All groups gained weight during the period of treatment (Fig. 3A). After two weeks the mice were dissected and the weight of the livers was measured. The different diets caused significant differences in the average liver/body weight (L/B) ratio between the groups (Fig. 3B). Although in the phytanic acid group the L/B ratio did not change significantly when compared to the control group, both the fenofibrate group and the combination group had a significantly increased L/B ratio when compared to the control group (>200% increased). The group on the combination diet had the highest L/B ratio which was significantly higher than the L/B ratio of the fenofibrate group

(11.7 ± 0.9 vs. 9.7 ± 0.7, P < 0.01). Hence, phytanic acid enhanced the effect of fenofibrate on L/B ratio in the combination group.

Next, plasma and tissue phytanic acid levels were determined by GC/MS analysis (Fig. 4). Plasma phytanic acid levels were increased in the phytanic acid group when compared to the control group and fenofibrate group, which is in good agreement with our previous data (unpublished). Interestingly, the phytanic acid plasma levels were significantly lower in plasma of mice in the combination group when compared to the phytanic acid treated group (48 ± 5 μM vs. 102 ± 13 μM, respectively, P< 0.001) (Fig. 4A).

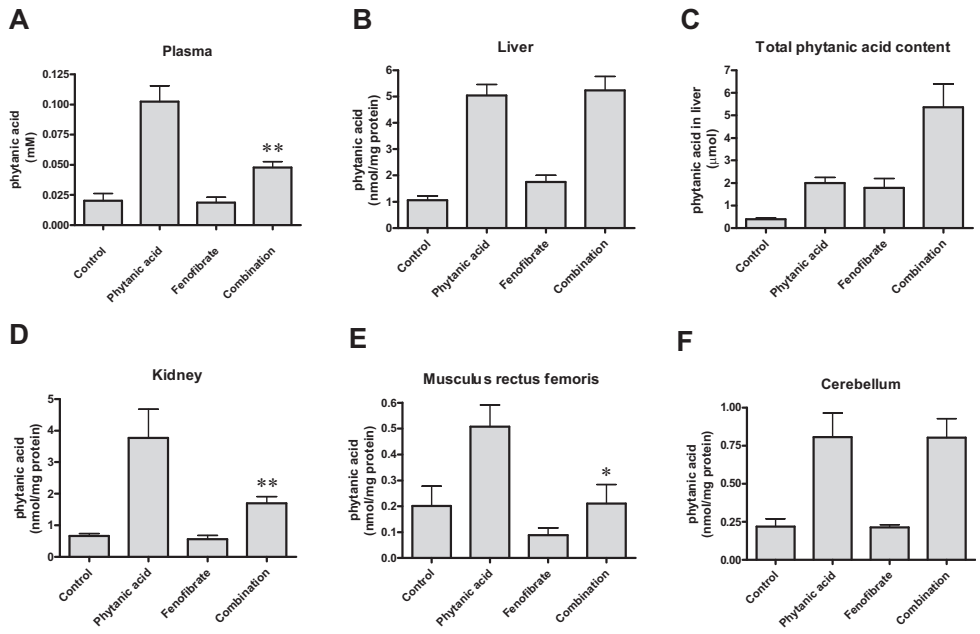

Figure 4: Phytanic acid analysis in plasma and different tissues of *Phyh* -/- mice on standard diet (control), standard diet supplemented with 0.01% phytanic acid (phytanic acid), 0.1% fenofibrate (fenofibrate) and a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination) for 2 weeks. Phytanic acid accumulation was measured in **(A)** plasma, **(B)** liver, **(D)** kidney, **(E)** muscle, and **(F)** cerebellum. Total liver phytanic acid content was determined by correcting for the liver weight **(C)**. Values represent the mean of four animals per group ± SD. * P < 0.01 between the combination group and the phytanic acid group. ** P < 0.001 between the combination group and the phytanic acid group.

Liver analysis showed that phytanic acid concentrations increased (approximately 5- fold) in livers of mice on the phytanic acid diet (Fig. 4B). The fenofibrate group also showed an increased average concentration of phytanic acid in liver when compared to the control group, although the increase was not statistically

significant. Taken into account the size of the livers, which was increased in the fenofibrate group and the combination group, the total liver phytanic acid content in the combination group was highest of all the groups (Fig. 4C). As a result of the increased liver size the phytanic acid content of livers in the fenofibrate group did not differ from the phytanic acid group, and was significantly higher than the phytanic acid content in liver of the control group. Next to the liver, the kidney is a key organ involved in ω-oxidation of fatty acids (reviewed in [22;33;34]). Phytanic acid concentration increased in kidney of mice on diets supplemented with phytanic acid (Fig. 4D). In contrast to liver, but similar to the situation in plasma, phytanic acid concentrations in kidney were lower in the group on the combination diet than on the phytanic acid diet. This was also observed in the muscle (musculus rectus femoris) (Fig. 4E). In contrast, no effect of fenofibrate on phytanic acid level was observed in cerebellum (Fig. 4F).

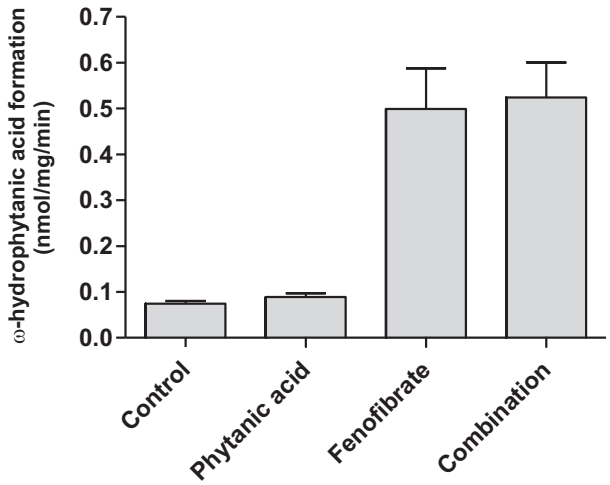

Figure 5: Phytanic ω-hydroxylation in mice liver homogenates of *Phyh* -/- mice on standard diet (control), standard diet supplemented with 0.01% phytanic acid (phytanic acid), 0.1% fenofibrate (fenofibrate) and a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination) for 2 weeks. Values represent the mean of four animals per group ± SD.

One reason for the diet induced differences in phytanic acid levels might be increased ω-oxidation of phytanic acid. In order to study whether fenofibrate caused an induction of the first step of the ω-oxidation of phytanic acid in *Phyh* -/- mice, the phytanic acid ω-hydroxylation was measured *in vitro* in liver homogenates [17]. The results of the measurements clearly show that the ω-hydroxylation of phytanic acid is induced by fenofibrate (Fig. 5). The induction of phytanic acid ω-hydroxylation capacity is the same for liver homogenates of both the fenofibrate and the combination group.

Previously we have shown that the CYP450 enzymes responsible for the ω-hydroxylation of phytanic acid in humans belong to the CYP4A and the CYP4F subfamilies [18]. To investigate which of these enzyme families was responsible for the induction of phytanic acid ω-hydroxylation capacity caused by fenofibrate, we

performed western blot analysis with antibodies raised against human CYP4A11 and CYP4F2. The fenofibrate and combination group showed a clear increase in CYP4A levels in both liver and kidney, whereas CYP4F protein levels were not increased and even seemed to be decreased in liver (Fig. 6). This strongly suggests that the increase in phytanic acid ω-hydroxylation was caused by an induction of CYP450 proteins of the 4A subfamily.

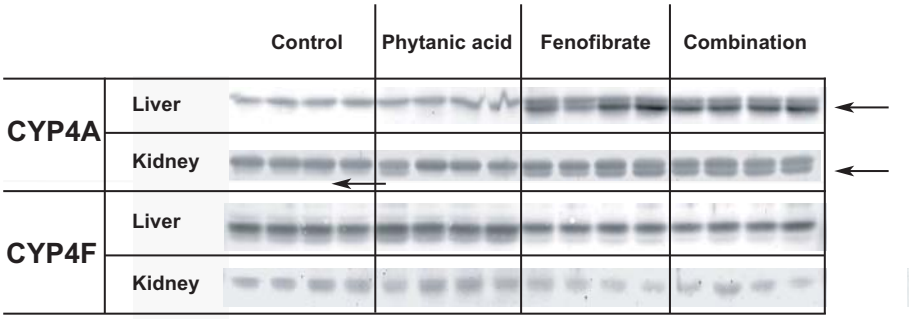

Figure 6: Immunoblot analysis of CYP4A and CYP4F in liver and kidney homogenates of *Phyh* -/- mice on standard diet (control), standard mouse chow supplemented with 0.01% phytanic acid (phytanic acid), 0.1% fenofibrate (fenofibrate) and a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination) for 2 weeks (four mice per group).

The induction of the ω-oxidation pathway by fenofibrate treatment (shown in Figs. 5 and 6) should result in an increase of dicarboxylic acid production under *in vivo* conditions. However, in our analysis of very long-chain fatty acids we could not detect the possible phytanic acid ω-oxidation product, i.e. phytanedioic acid. Hence, another approach was taken to study the levels of dicarboxylic acids. To this end, acyl-carnitine analysis was performed in freeze-dried liver tissue (Fig. 7). In agreement with previous studies which showed that fibrates increase carnitine levels in a PPARα-dependent manner in rodents ([29;35]), the carnitine levels in *Phyh* -/- mice increased as a result of the fenofibrate present in the diet (control group 749 ± 74, phytanic acid group 852 ± 223, fenofibrate group 4013 ± 695, combination group 5126 ± 400 (in pmol/mg dry-weight), $P < 0.001$ between fenofibrate / combination group and the control group). The levels of the saturated fatty acyl-carnitines with a carbon-chain of C_2, C_4, C_5, C_{10}, C_{12}, C_{16}, and C_{20} were increased in livers of mice belonging to the fenofibrate and combination group (data not shown).The levels of other saturated acyl-carnitines did not differ between the groups. Notably, the mice receiving phytanic acid accumulated C_{20}-acyl-carnitine compared to the control. Moreover, mice on the combination diet had the highest C_{20}-acyl-carnitine levels of all groups (control group 1.58 ± 0.53, phytanic acid group 9.27 ± 6.36, fenofibrate group 16.39 ± 5.44, combination group 60.84 ± 8.94 (in pmol/mg dry-weight)). Since C_{20}-acylcarnitine

corresponds to the mass of phytanoyl-carnitine, this indicates that phytanoyl-carnitine is accumulating as a result of dietary phytanic acid supplementation. Measurement of the dicarboxylic acylcarnitines showed a clear increase of C_3, C_5, C_8, C_{10}, C_{15}, C_{16} dicarboxylic acylcarnitines when fenofibrate was present in the diet. Of the dicarboxylic acylcarnitines accumulating in livers of mice on a diet supplemented with fenofibrate the C_3, C_5, C_7, C_{10}, C_{12}, C_{15} dicarboxylic acylcarnitines correspond to possible phytanedioic acid β-oxidation metabolites (Fig. 7).

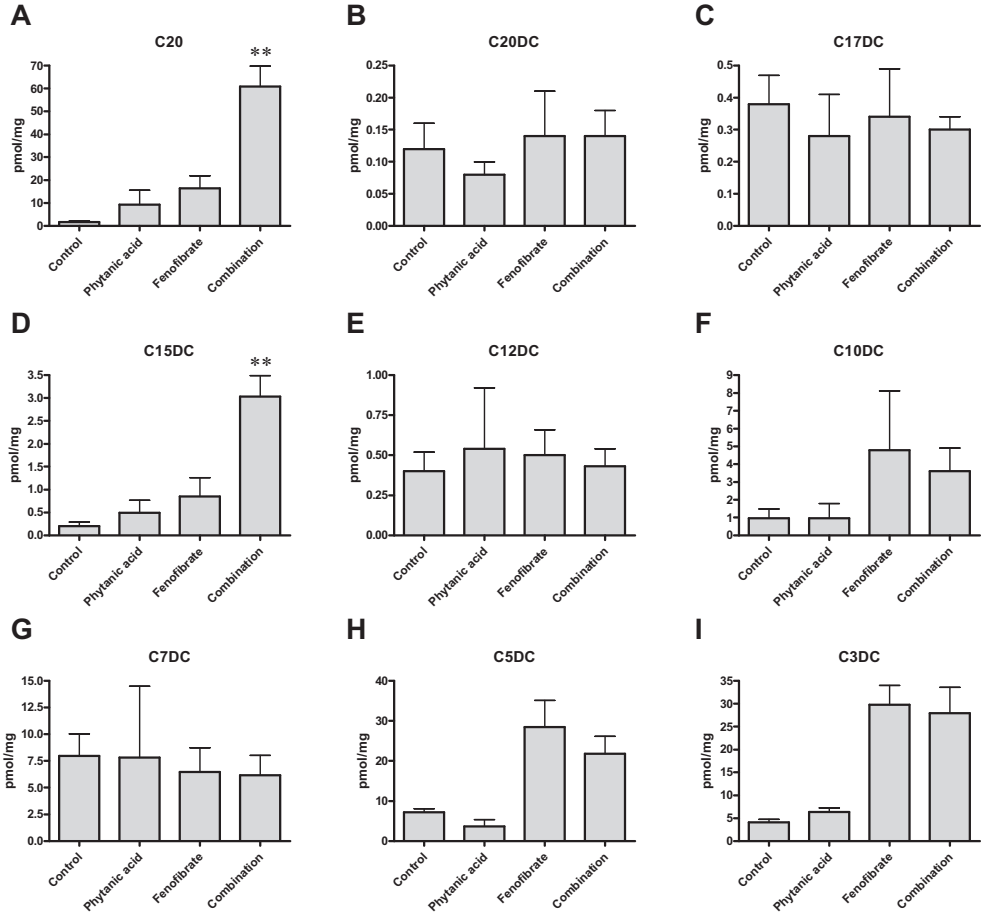

Figure 7: Acylcarnitine levels of C_{20}-acylcarnitine and potential dicarboxylic acylcarnitine metabolites originating from phytanic acid ω-oxidation in liver tissue of *Phyh* -/- mice on standard diet (control), standard diet supplemented with 0.01% phytanic acid (phytanic acid), 0.1% fenofibrate (fenofibrate) and a combination of 0.01% phytanic acid and 0.1% fenofibrate (combination) for 2 weeks. **(A)** C_{20}-acylcarnitine, **(B)** C_{20}-dicarboxylyl carnitine, **(C)** C_{17}-dicarboxylyl carnitine, **(D)** C_{15}-dicarboxylyl carnitine, **(E)** C_{12}-dicarboxylyl carnitine, **(F)** C_{10}-dicarboxylyl carnitine, **(G)** C_7- dicarboxylyl carnitine, **(H)** C_5- dicarboxylyl carnitine, **(I)** C_3-dicarboxylyl carnitine. Results are shown in pmol/mg dry liver weight. Values represent the mean of four animals per group ± SD. ** $P < 0.001$ between the combination group and the phytanic acid group.

However, only the C_{15} DC-acyl-carnitine was significantly increased in the combination group compared to the fenofibrate group, suggesting that this compound actually originates from phytanic acid ω-oxidation (control group 0.20 ± 0.09, phytanic acid group 0.49 ± 0.28, fenofibrate group 0.85 ± 0.41, combination group 3.03 ± 0.46 (in pmol/mg dry-weight)). The increased acyl-carnitine levels in livers of fenofibrate treated animals are in agreement with the well-known effect of fenofibrate on lipid catabolism. The presence of increased levels of C_{15} DC-acyl-carnitine strongly suggests increased ω-oxidation of phytanic acid in *Phyh* -/- mice after treatment with fenofibrate.

Discussion

Recent studies performed with recombinant human CYP4 enzymes have shown that CYP4A11 is able to ω-hydroxylate phytanic acid [18;36]. Homologues of this enzyme are present in rat and mice. Recombinant rat CYP4A enzymes have also been shown to ω-hydroxylate phytanic acid. The CYP4A family is known to be induced by peroxisome proliferators of the fibrate class. Moreover, recent data has shown that induction of the CYP4A enzymes in rat by clofibrate increased phytanic acid ω-hydroxylation capacity in liver microsomes [36]. We demonstrated that fibrates upregulate phytanic acid ω-hydroxylation activity in a PPARα-dependent manner in mouse liver (Fig. 2). This increase in phytanic acid ω-hydroxylation capacity could lower the phytanic acid levels in Refsum disease as it might provide an alternative breakdown pathway of phytanic acid via ω-oxidation and subsequent β-oxidation. In this study, we examined the effect of upregulation of Cyp4A enzymes on phytanic acid accumulation in *Phyh* -/- mice, a recently generated mouse model for Refsum disease, in which *Phyh*, the gene encoding phytanoyl-CoA hydroxylase is disrupted (unpublished data). To this end, *Phyh* -/- mice were treated with fenofibrate which is already in use in the clinic for the treatment of hypercholesterolemia, mixed dyslipidaemia and hypertriglyceridaemia in humans (reviewed in [37]).

The phytanic acid ω-hydroxylation capacity was increased in liver homogenates of the *Phyh* -/- mice that were treated with fenofibrate (Fig. 5). This corresponded with increased expression of CYP4A proteins which suggests that these enzymes are indeed responsible for the increased phytanic acid ω-hydroxylation capacity. The antibodies used to study the protein expression level do not distinguish between the different Cyp4a homologues. However, since male mice were used in the experiment, the homologues as induced by fenofibrate are most likely Cyp4a10 and Cyp4a14 [26-28]. The increased ω-hydroxylation of phytanic acid as a result of fibrate induced expression of CYP4A enzymes could increase the overall ω-oxidation capacity. This is strongly supported by the finding of increased C_{15} DC-acyl-carnitine in livers of mice receiving both phytanic acid and fenofibrate. The mass of this

acylcarnitine species corresponds to that of the carnitine ester of a metabolite (2,6,10-trimethyldodecanedioic acid) of the phytanic acid ω-oxidation product phytanedioic acid.

Treatment with fenofibrate had no effect on the phytanic acid levels in liver of *Phyh* -/- mice. The total amount of phytanic acid in liver even increased when taking into account the liver weight. L/B ratio increased strongly due to the fenofibrate treatment, which is a known phenomenon. When chronically treated this could lead to liver tumor formation (reviewed by Lake [38]). In our experiments no liver tumors were visible probably due to the relatively short treatment period. In humans, tumor formation by fibrates does not occur because PPARα functions differently in man than in mice (reviewed in [39;40]). The L/B ratio was highest in the group receiving both fenofibrate and phytanic acid, suggesting that since phytanic acid is a natural ligand for PPARα and RXR, phytanic acid caused additional hepatocyte proliferation via activation of these nuclear receptors. The lack of effect on liver phytanic acid levels may be explained by the increased fat catabolism caused by fenofibrate. Fibrates induce lipoprotein lipase-mediated lipolysis, increase fatty acid uptake by the liver, decrease triglyceride synthesis, and induce hepatic β-oxidation [41]. The decrease of phytanic acid in the liver due to the increased ω-oxidation capacity most likely did not exceed the increase in phytanic acid concentration due to increased lipolysis and increased fatty acid uptake in the liver and a decreased triglyceride synthesis at the same time. The decrease in phytanic acid concentration in plasma, kidney and muscle due to the fenofibrate treatment are in agreement with this hypothesis. The increased uptake of phytanic acid in liver is important since the liver is the major site for fatty acid ω-oxidation and subsequent peroxisomal β-oxidation of the produced dicarboxylic acids. Interestingly, C_{15}-dicarboxylyl-carnitine, which most likely is a metabolite of the dicarboxylic acid of phytanic acid, i.e. phytanedioic acid, strongly increased in livers of mice receiving fenofibrate and phytanic acid.

Cerebellum was the only extra-hepatic tissue where no effect of fenofibrate treatment on phytanic acid concentration was observed. This could be explained by the fact that fenofibrate is actively removed from the brain by the multi-drug transporter p-glycoprotein [42]. Moreover, fatty acid metabolizing cytochrome P450s and PPARα are hardly expressed in brain [43;44]. The phytanic acid levels in the nervous system will probably only decrease after long-term fibrate treatment when phytanic acid input is restricted. Most Refsum disease patients only develop symptoms during adolescence due to the slow accumulation of phytanic acid over the years. Long-term fibrate treatment would result in a maximal catabolism of phytanic acid via ω-oxidation in the liver and prevent incorporation of phytanic acid in triglycerides and phospholipids at the same time. This is important since disruption of normal membrane structure by incorporation of phytanic acid in different lipid classes is thought to be one of the most important pathogenic effects of phytanic acid.

Future studies should focus on the effects of long-term fibrate treatment on phytanic acid accumulation in *Phyh* -/- mice. In this study we have shown that phytanic acid ω-oxidation is increased in liver by fenofibrate, but the hepatic phytanic acid levels did not decrease due to the large input of phytanic acid originating from other tissues. In time, the phytanic acid levels in liver might decrease due to increased degradation via ω-oxidation. More importantly, phytanic acid plasma levels were decreased after treatment with fenofibrate which prevents further accumulation of phytanic acid in extra-hepatic tissue, including the nervous tissue, in the long run. For this reason, longer treatment with fenofibrate may result in lower phytanic acid levels in brain of *Phyh* -/- mice as well. In order to prevent carcinogenesis in the liver during long-term treatment with fenofibrate a lower treatment dose than used in this study (0.1% w/w) should be considered.

In summary, the results described in this paper show that fenofibrate has multiple effects on phytanic acid metabolism in *Phyh* -/- mice. Fenofibrate induces phytanic acid transport from extra-hepatic tissues to the liver resulting in a decrease of phytanic acid accumulation in these tissues (except for the cerebellum). Furthermore, fenofibrate increased phytanic acid ω-oxidation capacity in the liver due to induced expression of CYP4A enzymes. This increased ω-oxidation capacity will increase the rate of phytanic acid degradation via this pathway and fenofibrate also induces the subsequent β-oxidation of the phytanedioc acid formed. Long-term treatment of mice will have to show whether these processes will eventually lead to decreased phytanic acid concentrations in liver and nervous tissue.

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CHAPTER 6

Phytanic acid impairs mitochondrial respiration through protonophoric action

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Phytanic acid impairs mitochondrial respiration through protonophoric action

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Abstract:

Refsum Disease is a rare inherited neurodegenerative disorder characterized by accumulation of the dietary branched-chain fatty acid phytanic acid in plasma and tissues as caused by a defect in the alpha-oxidation pathway. The accumulation of phytanic acid is believed to be the main pathophysiological cause of the disease. However, the exact mechanism(s) by which phytanic acid exerts its toxicity have not been resolved. In this study, the effect of phytanic acid on mitochondrial respiration was investigated. The results show that in digitonin-permeabilized fibroblasts phytanic acid decreases ATP synthesis, whereas substrate oxidation per se is not affected. Importantly, studies in intact fibroblasts revealed that phytanic acid decreases both the mitochondrial membrane potential and NAD(P)H autofluorescence. Taken together, the results described in this manuscript show that unesterified phytanic acid exerts its toxic effect mainly through its protonophoric action, at least in human skin fibroblasts.

Introduction

Patients with the rare inherited neurological disorder Refsum disease (heredopathia atactica polyneuritiformis, MIM #266500) present with markedly increased levels of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in their plasma and tissues. The precursor of this branched-chain fatty acid, the chlorophyll side chain phytol (3,7,11,15-tetramethylhexadec-trans-2-en-l-ol), is produced by bacteria in the stomach of ruminants [1-3]. Hence, the main sources of phytanic acid for humans are dairy products and meats of these animals, whereas, in addition, certain kinds of seafood has been found to contain large amounts of phytol and phytanic acid [4;5].

In healthy individuals, phytanic acid undergoes oxidative decarboxylation by a process called alpha-oxidation, resulting in the formation of formyl-CoA and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). Pristanic acid is subsequently degraded by peroxisomal β-oxidation (reviewed in [6]). In Refsum disease, the alpha-oxidation pathway is deficient due to mutations in either the *PHYH* gene, which encodes phytanoyl-CoA hydroxylase (PHYH), the first enzyme of the alpha-oxidation pathway [7;8], or the *PEX7* gene, which encodes peroxin 7, a protein required for correctly targeting PHYH to the peroxisomes [9]. Consequently, patients with Refsum disease accumulate significant amounts of phytanic acid in plasma and tissues, which are thought to be the major cause of the pathology of the disease. Multiple clinical symptoms are observed, the most pronounced being retinitis pigmentosa, which leads to progressive visual loss and ultimately to blindness. Additional symptoms include anosmia, peripheral neuropathy, ataxia, cardiac arrhythmias and ichthyosis [10;11]. Although a diet low in phytanic acid slows down the progression of the disease, little is known about the mechanism(s) linking the clinical manifestations to the increased accumulation of phytanic acid (reviewed in [12]).

Recent studies employing rat hippocampal astrocytes revealed that phytanic acid can cause massive cell death within a few hours of exposure [13-15]. Furthermore, these studies showed that cell death was preceded by an increased release of Ca^{2+} from internal stores, leading to a rise in the cytosolic free Ca^{2+} concentration, increased generation of reactive oxygen species (ROS) and a marked depolarisation of the mitochondrial membrane potential. Currently, research on the cytotoxic mechanism of phytanic acid is focused predominantly on mitochondria, which are among the most vulnerable organelles in the cell. Mitochondrial dysfunction impairs cellular and tissue integrity and has been implicated in a wide range of disorders including age-related neurological diseases, cardiovascular disease, cancer and diabetes (reviewed in [16-18]). Mitochondria are central not only to cellular energy and Ca^{2+} homeostasis but also to cell death and work of the past years has shown that fatty acids can affect each of these functions (reviewed in [19]). As far as phytanic acid is concerned, studies in intact rat brain mitochondria revealed that it can decrease the rate of phosphorylating respiration, decrease the oxidative production of ATP and decrease the activity of adenine nucleotide translocase (ANT) [14;15;20;21], suggesting that phytanic acid primarily acts through inhibition of ADP/ATP exchange. In addition, these studies showed that phytanic acid can increase the rate of non-phosphorylating respiration, indicating an additional effect at the level of the oxidative phosphorylation (OXPHOS) system. Recent work in permeabilized mitochondria revealed that phytanic acid can decrease the activity of NADH:ubiquinone oxidoreductase (complex I), suggesting that phytanic acid can also act by directly inhibiting the fuelling of the OXPHOS system [21]. Finally, studies in intact mitochondria showed that phytanic acid can decrease the NAD(P)H level under non-

respiring conditions but that, unexpectedly, this effect of phytanic acid was reversed by the complex I inhibitor rotenone, suggesting that phytanic acid can also act as a protonophore [21]. The latter conclusion, however, contrasts with the finding that phytanic acid can decrease carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) -stimulated respiration ([21], but see also [20]).

In order to gain insight into the mechanism underlying the inhibitory effect of phytanic acid on phosphorylating respiration, we assayed its effect on the production of ATP in digitonin-permeabilized human skin fibroblasts respiring on substrates specific for either complex I or complex II (succinate:ubiquinone oxidoreductase). Importantly, parallel measurement of the consumption of these substrates allowed us to assess the fuelling state of the OXPHOS system. For comparison, we included the complex-specific inhibitors rotenone (complex I) and malonate (complex II), expected to decrease ATP production and substrate consumption with the same potency, and the protonophore 2,4-dinitrophenol (DNP), expected to decrease the production of ATP much more potently than the consumption of the substrate. In addition, we performed life cell analysis to study the effects of phytanic acid on the mitochondrial membrane potential and intracellular NAD(P)H levels using digital imaging microscopy. Finally, the specificity of the effect of phytanic acid was evaluated using phytol, phytanic acid methyl ester, pristanic acid and palmitic acid. Collectively, the results indicate that phytanic acid decreases mitochondrial respiration primarily through its protonophoric action and that both the carboxylgroup and the methyl groups are required for this activity.

Materials and Methods

Chemicals

Phytanic acid and pristanic acid were purchased from the VU University Medical Center Metabolic Laboratory (Dr. H.J ten Brink, Amsterdam, the Netherlands). Phytol and palmitic acid were from Merck (Darmstadt, Germany) and phytanic acid methyl ester was from Ultra Scientific Inc. (North Kingstown, RI). DMSO was obtained from Sigma-Aldrich (St. Louis, MO). Cell culture material, media and TMRM for life cell imaging were from Invitrogen (Breda, The Netherlands). All other chemicals and enzymes were of the highest quality available.

Cell culture for enzymatic assays

Human skin fibroblasts from control individuals were cultured at 37° C (5% CO_2) in nutrient mixture Ham's F-10 with L-glutamine and 25 mM HEPES supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 250 ng/mL amphotericin B.

Cell culture for life cell imaging

Fibroblasts were obtained from a healthy individual (#5120) according to the relevant Institutional Review Boards and cultured in medium 199 with Earle's salt supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37° C. For microscopy analysis, cells were seeded on glass coverslips (Ø 24 mm) and cultured to ~70% confluence.

Enzymatic assays

ATP synthesis assay in digitonin-permeabilized fibroblasts

The ATP synthesis assay was performed essentially as described by Wanders et al. [22] with slight modifications in the protocol. In brief, human skin fibroblasts were grown to confluency followed by detachment from the culture flask by trypsinization as described before [23]. The protein content of the fibroblast suspension was estimated by light scattering (OD_{600} = 0.14 corresponds to ~ 0.05 mg/ml protein). Subsequently, the suspension was centrifuged (500x g_{av}) and cells were taken up in fresh medium. From this suspension aliquots (30 μg of protein) were taken and seeded per well in a 24 wells plate. The next day, the cells were washed twice with PBS followed by addition of the reaction medium (containing 150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 10 mM potassium phosphate, 1 mM ADP, 0.1% (w/v) bovine serum albumin, 40 μg/ml digitonin and either 10 mM malate (plus 10mM glutamate), or 10 mM succinate (plus 50 μM rotenone)). Phytanic acid, 2,4-dinitrophenol, rotenone, malonate, and the phytanic acid analogues were all added to the reaction mixture from DMSO stock solutions. Reactions were allowed to proceed for 30 min at room temperature. When samples were prepared for aspartate measurements, the cells were harvested from culture flasks and incubations were performed with the cells in solution (0.5 mg/ml in 100 μl). Reactions were terminated with perchloric acid, protein was removed by centrifugation and the acidic protein-free supernatants were neutralized [22]. Subsequently, ATP was measured fluorimetrically as described previously [24] using a Cobas Fara centrifugal analyzer. Afterwards, the same samples were used for aspartate, or malate measurements.

Aspartate measurements

Aspartate was measured using the end-point method described in Bergmeyer et al. [25]. This method uses the subsequent conversion of aspartate to malate via oxaloacetate in a reaction mixture containing glutamate-oxaloacetate-transaminase (GOT), malate dehydrogenase (MDH), NADH and α-ketogluterate. The decrease in NADH as a consequence of the reduction of oxaloacetate to malate by MDH was measured fluorimetrically with a Cobas Fara centrifugal analyzer. Quantification was done by means of a standard curve.

Malate measurements

Malate was determined fluorimetrically (excitation at 365 nm, emission at 450 nm) on a Cobas Fara centrifugal analyzer by the end-point measurement of the reduction of the NAD^+ analogue APAD (3-acetylpyridine adenine dinucleotide) to APADH by MDH. The reaction was performed in a 100 mM Tris/HCl buffer at pH 9 containing 0.1% Triton X-100 and 1mM APAD. Malate was quantified by means of a standard curve.

Fluorescence measurements in living fibroblasts:

Quantification of mitochondrial membrane potential:

Fibroblasts were cultured to ~70% confluence on glass coverslips, and were incubated with 100 nM tetramethyl rhodamine methyl ester (TMRM; Invitrogen) for 30 min at 37° C. After loading, the cells were washed and incubated for another 30 min at 37° C in medium containing either the vehicle (DMSO) or the compound of interest. After incubation, the medium was replaced by a HEPES-Tris buffered medium (132 mM NaCl, 4.2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM D-glucose, 10 mM HEPES, pH 7.4) containing either DMSO or the compound of interest. Next, the coverslips were mounted in a temperature-controlled (37° C) incubation chamber attached to the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) equipped with a x63, 1.25 NA Plan NeoFluar objective. TMRM was excited at 540 nm using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany) and fluorescence emission light was directed by a 560DRLP dichroic mirror (Omega Optical Inc., Brattleboro, VT, USA) through a 565ALP emission filter (Omega Optical Inc.) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific Photometrics, Vianen, The Netherlands). Routinely, 25 fields of view were analyzed per coverslip using an image capturing time of 100 ms. Mitochondrial TMRM fluorescence was quantified using an automated image-processing algorithm described in detail before [26-28]. Briefly, a mitochondrial mask is calculated from the original TMRM image and then superimposed on this image to determine the mean mitochondrial pixel intensity for each individual cell as an estimate of the average mitochondrial membrane potential. Image processing and analysis were performed using Image Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD). In each experiment, the average value obtained with DMSO-treated cells was set at 100%, to which all other values were related. Numerical results were visualized using Origin Pro 7.5 (Originlabs, Northampton, MA) and values from multiple experiments were expressed as means ± SE. Statistical significance (Bonferroni corrected) was assessed using Student's t-test.

Quantification of intracellular NAD(P)H autofluorescence:
Intracellular NAD(P)H autofluorescence was measured as described previously [29]. Briefly, fibroblasts, cultured to ~70% confluence on glass coverslips, were preincubated for 30 min at 37° C in medium containing either DMSO or the compound of interest. Immediately before imaging, the medium was replaced by HEPES-Tris medium containing either the vehicle or the compound of interest. Coverslips were transferred to the incubation chamber on the stage of the inverted microscope equipped with a Zeiss 40x/1.3 NA Plan NeoFluar objective. The cells were excited at 360 nm using the monochromator and fluorescence emission light was directed by a 415DCLP dichroic mirror (Omega Optical Inc.) through a 510WB40 emission filter (Omega Optical Inc.) onto the CCD-camera. Routinely, 10 fields of view were analyzed per coverslip using an image capturing time of 1 second. The mean fluorescence intensity was determined in an intracellular region of interest and, for purpose of background correction, an extracellular region of identical size. Quantitative image analysis was performed with Metamorph 6.0. In each experiment, the average value obtained with vehicle-treated cells was set at 100%, to which all other values were related. Numerical results were visualized using Origin Pro 7.5 and values from multiple experiments were expressed as means ± SE. Statistical significance (Bonferroni corrected) was assessed using Student's t-test.

Results

Effect of phytanic acid on ATP synthesis via complex I
In order to examine the effect of phytanic acid on mitochondrial ATP synthesis we used digitonin-permeabilized fibroblasts [22] rather than isolated mitochondria. Malate (plus glutamate) or succinate (plus rotenone) were used as respiratory chain substrates. Incubations were performed in the absence and presence of either phytanic acid or established mitochondrial inhibitors and uncouplers including rotenone, a well known inhibitor of complex I (reviewed in [30]), malonate, an inhibitor of complex II, and the protonophore 2,4-dinitrophenol (DNP).

Figure 1 depicts the effects on ATP synthesis when malate was used as a substrate for the respiratory chain in combination with glutamate. Malate is converted by malate dehydrogenase in the mitochondrial matrix to oxaloacetate with the simultaneous reduction of the cofactor NAD^+ to NADH. Consequently, NADH is readily available to be used as a substrate for complex I of the OXPHOS system, while oxaloacetate and the added glutamate are converted by glutamate-oxaloacetate transaminase to α-ketoglutarate and aspartate, thereby eliminating the product inhibition of malate dehydrogenase by oxaloacetate. Phytanic acid gradually decreased ATP synthesis when its concentration was increased up to 100 μM (Fig. 1A). A similar result was obtained with DNP when using concentrations up to 50 μM (Fig. 1B).

Rotenone appeared much more potent in decreasing ATP synthesis than either DNP or phytanic acid (Fig. 1C). The complex I inhibitor exerted its inhibitory effect in the nanomolar range, while phytanic acid and the uncoupler were effective in the micromolar range.

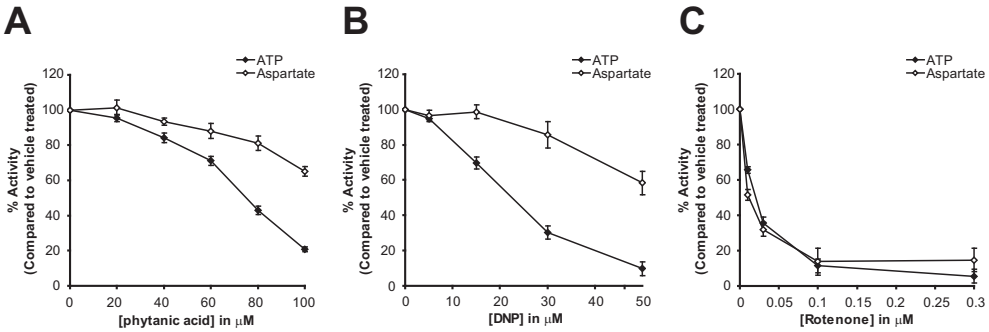

Figure 1: The effect of phytanic acid on ATP and aspartate synthesis in digitonin permeabilized fibroblasts. Digitonin permeabilized fibroblasts were incubated with different concentrations of **(A)** phytanic acid, **(B)** 2,4-dinitrophenol (DNP), and **(C)** rotenone for 30 min in the presence of malate (plus glutamate). Subsequently, the amount of ATP and aspartate was measured as described in the Methods section. Vehicle- (DMSO) treated cells were set at 100%. Data represents average values of separate measurements depicted in % of vehicle-treated cells ± SEM (Phytanic acid, N=6; DNP, N=8; Rotenone, N=7).

Next, we determined the effect of phytanic acid, DNP and rotenone on the formation of aspartate, which is the end-product of malate oxidation, at least when malate oxidation is studied in the presence of glutamate. Aspartate was measured in the same samples used for ATP measurement. Phytanic acid (Fig. 1A) and DNP (Fig. 1B) inhibited aspartate synthesis considerably less potently than ATP synthesis, while, in sharp contrast, rotenone decreased both processes equipotently (Fig. 1C). The inhibition pattern of rotenone is in agreement with a reduction in complex I-mediated NADH oxidation, decreasing both the enzymatic conversion of malate to oxaloacetate and the proton motive force-driven synthesis of ATP. The inhibition pattern of phytanic acid is similar to that of DNP but clearly different from that of rotenone, thus disfavouring the idea that it acts by inhibiting complex I and suggesting that it rather acts as a protonophore.

Effect of phytanic acid on ATP synthesis via complex II

In order to demonstrate that the inhibitory effect of phytanic acid was not restricted to complex I-mediated ATP synthesis, we next determined its effect on ATP synthesis when the complex II substrate succinate was used as electron donor for the repiratory chain. Rotenone was included in the reaction medium to inhibit the electron flow

coming from complex I. Under these conditions, malate is the end-product of succinate oxidation via the formation of fumarate and its amount was determined in the same samples used for ATP measurement as a measure of complex II activity. Both phytanic acid (Fig. 2A) and DNP (Fig. 2B) inhibited ATP synthesis in a dose-dependent manner, while decreasing the formation of malate much less potently. As observed for complex I, specific inhibition of respiratory chain complex II by malonate decreased both processes equipotently (Fig. 2C). Taken together, the results obtained for complex II-mediated ATP synthesis are in agreement with the notion that phytanic acid inhibits ATP synthesis primarily through its protonophoric action.

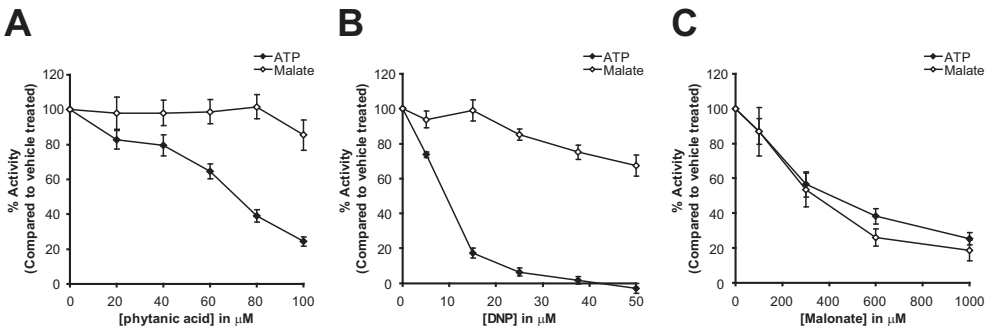

Figure 2: The effect of phytanic acid on ATP and malate synthesis in digitonin permeabilized fibroblasts. Digitonin permeabilized fibroblasts were incubated with different concentrations of (**A**) phytanic acid, (**B**) 2,4-dinitrophenol (DNP), (**C**) malonate in the presence of succinate (plus rotenone) for 30 min. Subsequently, the amount of ATP and malate was measured as described in the Methods section. Vehicle- (DMSO) treated cells were set at 100%. Data represents average values of separate measurements depicted in % of vehicle-treated cells ± SEM (Phytanic acid, N=9; DNP, N=8; malonate, N=6).

To assess the specificity of the inhibitory effect of phytanic acid, we subsequently determined the effect of different analogues of phytanic acid on complex I-mediated ATP synthesis. Of the analogues tested, phytanic acid and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) inhibited ATP synthesis equipotently (Fig. 3). Neither the methyl-ester of phytanic acid nor its precursor phytol (3,7,11,15-tetramethylhexadec-trans-2-en-l-ol) influenced malate-dependent ATP synthesis, suggesting that the carboxyl group is important for the inhibitory effect of phytanic acid. Furthermore, the straight-chain fatty acid palmitic acid (hexadecanoic acid) inhibited ATP synthesis, but much less efficiently than phytanic acid and pristanic acid. This suggests that in addition to the carboxylgroup, methyl branches contribute to the capacity of phytanic acid and pristanic acid to decrease ATP synthesis.

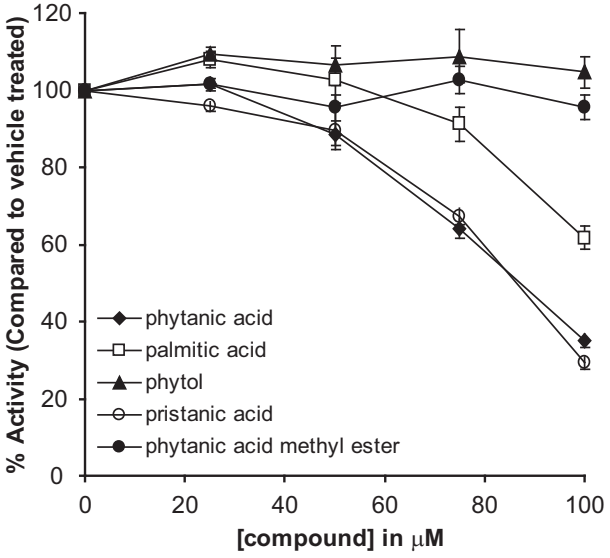

Figure 3: The effects of analogues of phytanic acid on ATP synthesis in digitonin permeabilized fibroblasts. Digitonin permeabilized fibroblasts were incubated with different concentrations of phytanic acid (♦), palmitic acid (□), phytol (▲), pristanic acid (○), and phytanic acid methyl ester (●) using malate as a substrate for the respiratory chain (plus glutamate) for 30 min. The ATP formation was measured as described in the Methods section. Vehicle-(DMSO) treated cells were set at 100%. Data represents average values of separate measurements depicted in % of vehicle-treated cells ± SEM (all compounds, N=4).

Effect of phytanic acid and its analogues on mitochondrial membrane potential

The results described above were obtained in experiments performed in an artificial cell system in which mitochondrial oxidative phosphorylation was studied after the plasma membrane was disrupted by digitonin. In order to investigate the action of phytanic acid under more physiological conditions, i.e. in intact cells, we performed experiments with intact living human skin fibroblasts. To substantiate the notion that phytanic acid decreases mitochondrial ATP synthesis primarily by acting as a protonophore, we next investigated the effect of phytanic acid on the mitochondrial membrane potential ($\Delta\psi$). To this end, fibroblasts were loaded with the lipophilic fluorescent cation TMRM for 30 min and subsequently incubated with increasing concentrations of phytanic acid for another 30 min. The accumulation of TMRM in the mitochondrial matrix follows the Nernst equation and, therefore, its fluorescence intensity is a sensitive readout of $\Delta\psi$ [31]. Video imaging microscopy and subsequent quantification of the mean mitochondrial pixel intensity per cell revealed a dose-dependent decrease of $\Delta\psi$ with increasing concentrations of phytanic acid (PA; Fig. 4A). To obtain information on the relative magnitude of the decrease in $\Delta\psi$, TMRM-loaded fibroblasts were treated with 100 nM rotenone for 30 min. This treatment significantly decreased the mitochondrial fluorescence intensity to 90.5±0.96% of the value obtained with untreated control cells (n=82 cells for both the untreated and rotenone-treated condition; $p<0.001$). This value was slightly lower than that obtained with 100 μM phytanic acid.

Next, we investigated the effect of the different phytanic acid analogues. Based upon the phytanic acid dose-inhibition curve (Fig. 4A), each analogue was tested at a concentration of 30 μM. At this concentration, all analogues caused a decrease in mitochondrial TMRM staining (Fig. 4B). However, in agreement with the findings in the permeabilized cell system (Fig. 3), the effect was largest for phytanic acid and pristanic acid and lowest for phytanic acid methyl ester and phytol. These results demonstrate that phytanic acid can also alter the driving force for mitochondrial ATP production when added to the intact cell.

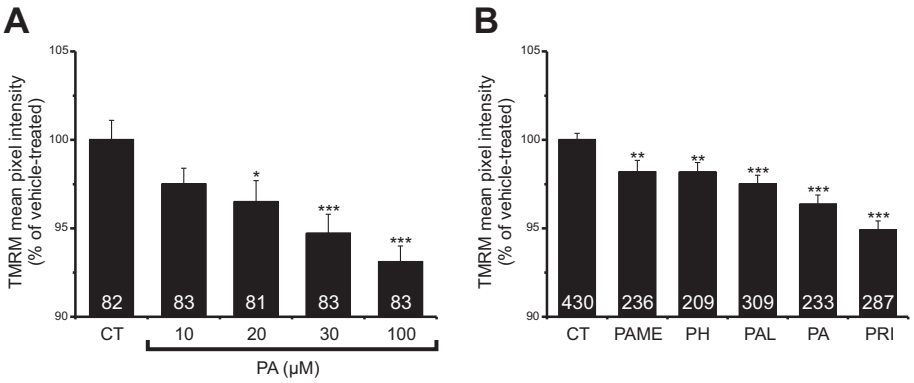

Figure 4: The dose-dependence of the phytanic acid effect and the effect of phytanic acid analogues on mitochondrial membrane potential. **(A)** Fibroblasts were incubated with TMRM for 30 min and subsequently with the indicated concentrations of phytanic acid for another 30 min. Images were collected by video-imaging microscopy and processed to the masked images. Next, these images were used to calculate the mean TMRM fluorescence intensity per mitochondrial pixel as an estimate of the mitochondrial membrane potential. In each experiment, the average value obtained with DMSO-treated control fibroblasts (CT) was set at 100%, to which all other values were related. **(B)** The effect of phytanic acid and its analogues on the mean TMRM fluorescence intensity. Cells were loaded with TMRM for 30 min and subsequently incubated with 30 μM of the indicated compound for another 30 min. After calculation of the mean TMRM fluorescence intensity per mitochondrial pixel, the average value obtained with DMSO-treated control fibroblasts (CT) was set at 100%, to which all other values were related. Numerals in **(A)** and **(B)** represent the number of individual cells analysed. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ significantly different from vehicle-treated control. PA = phytanic acid, PAME = phytanic acid methyl ester, PH = phytol, PAL = palmitic acid, PRI = pristanic acid.

Effect of phytanic acid, DNP and rotenone treatment on NAD(P)H autofluorescence in healthy fibroblasts

A depolarized $\Delta\psi$ can result from uncoupling and/or inhibition of the electron transport chain. Therefore, it is not possible to differentiate between these mechanisms by measuring the mitochondrial TMRM fluorescence intensity. We recently demonstrated that mitochondrial uncoupling by FCCP (carbonyl cyanide p-

trifluoromethoxy-phenylhydrazone) decreased cellular NAD(P)H levels in living fibroblasts [29]. Similarly, it has been shown that DNP treatment reduces NAD(P)H levels in living rat ventricular myocytes [32]. Conversely, we observed that specific inhibition of complex I by rotenone increased intracellular NAD(P)H levels [29].

In accordance with previous results, we here show that DNP, when added at a concentration of 30 μM for 30 min, significantly reduced NAD(P)H autofluorescence, whereas, in sharp contrast, 100 nM rotenone, when present for the same period of time, markedly increased the NAD(P)H signal (Fig. 5). Treatment of the fibroblasts with either 30 μM or 100 μM phytanic acid for 30 min decreased NAD(P)H autofluorescence to the same extent as DNP. These findings, furthermore, substantiate the idea that phytanic acid acts as a protonophore to decrease the proton motive force and thus the driving force for mitochondrial ATP production.

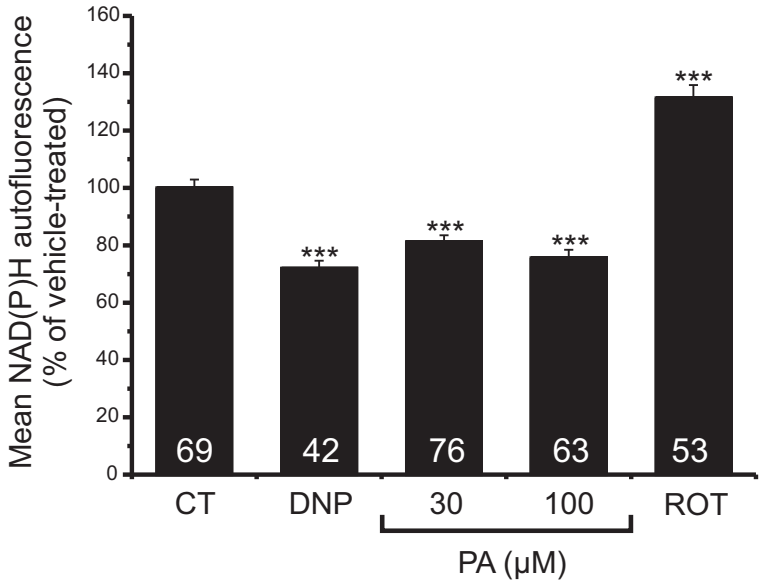

Figure 5: Phytanic acid treatment decreases NAD(P)H autofluorescence in living human fibroblasts. Cells were treated with either rotenone (100 nM), DNP (30 μM), or phytanic acid (PA; 30 and 100 μM, respectively) for 30 min and subsequently monitored for NAD(P)H autofluorescence by video-imaging microscopy. For each cell, the average fluorescence intensity per pixel was calculated for a mitochondrial-rich region as a measure of the local NAD(P)H level. The average value obtained with vehicle-treated control cells was set at 100%, to which all values were related. Numerals represent the number of individual cells analysed. *** Significantly different from vehicle-treated control cells ($p < 0.001$).

Discussion

Previous studies in isolated rat brain mitochondria indicated that phytanic acid can affect the function of mitochondria in multiple ways. Depending on the experimental conditions, evidence was provided that phytanic acid can act as a protonophore, as an inhibitor of complex I (NADH:ubiquinone oxidoreductase) and as an inhibitor of adenine nucleotide translocase (ANT) [20;21]. Using digitonin-permeabilized human skin fibroblasts, we here show that phytanic acid can effectively inhibit the production of ATP in mitochondria selectively respiring on either the complex I substrate malate (plus glutamate) or the complex II substrate succinate (plus rotenone). This finding indicates that inhibition of complex I alone cannot be the sole explanation for the inhibitory effect of phytanic acid on mitochondrial ATP synthesis. Parallel measurement of either the complex I-mediated production of aspartate or the complex II-mediated production of malate revealed that phytanic acid hardly affected the activities of these two complexes. In addition, this result excludes the possibility that phytanic acid acts solely by inhibition of the ANT. Exactly the same pattern of inhibition was obtained with the protonophore 2,4-dinitrophenol (DNP), whereas, in sharp contrast, both the complex I inhibitor rotenone and the complex II inhibitor malonate decreased the production of ATP and either aspartate (rotenone) or malate (malonate) equipotently. Only at higher concentrations, phytanic acid caused some reduction in either aspartate or malate production, but it should be noted that the same degree of reduction was observed at DNP concentrations that were equally effective in decreasing the production of ATP. Finally, phytanic acid inhibited complex I- and complex II-mediated ATP production with the same potency, demonstrating the absence of any additional inhibitory effect on complex I alone. Collectively, these results demonstrate that phytanic acid does not significantly inhibit the activity of either complex I or complex II in respiring mitochondria. Moreover, they suggest that under these conditions phytanic acid inhibits mitochondrial ATP production primarily by acting as a protonophore. This protonophoric action of phytanic acid may be caused by distortion of the packing of the lipids in the inner mitochondrial membrane (described previously in [33]).

In previous work, rat brain mitochondria were freeze-thawed in order to make complex I accessible for externally added NADH [21]. However, this procedure will also increase the availability of phytanic acid, which is normally not taken up by mitochondria. Artificially adding phytanic acid to disrupted mitochondria may, therefore, not be representative for the in vivo situation. In our experiments, we used digitonin to selectively permeabilize the plasma membrane (see [22]). This procedure leaves the mitochondria intact, which may explain the lack of effect of phytanic acid on the activity of complex I in this study.

The present work demonstrates that phytanic acid affects mitochondrial functioning also when added to the intact cell. Using a mitochondrial mask to specifically determine the amount of TMRM fluorescence from this compartment, we show that phytanic acid can readily depolarize the inner mitochondrial membrane in human skin fibroblasts (see also, [13-15]). The decrease in TMRM fluorescence obtained with 100 μM phytanic acid, the maximal concentration used in this study, was small but significant and only slightly less than that obtained with 100 nM rotenone. Previous work in rat neurons revealed that rotenone, when added at a 10-fold higher concentration of 1 μM, depolarized the inner mitochondrial membrane by only ~17 mV [34]. However, the phosphorylation state of the cytosolic ATP pool has been demonstrated to be very sensitive to small changes in mitochondrial membrane potential [35], and, indeed, rotenone was found to dramatically reduce the cellular ATP level when administered in a chronic fashion [36]. Therefore, the apparently minor depolarization of the mitochondrial membrane potential evoked by phytanic acid might have severe consequences for long-term energy homeostasis in Refsum patients.

In addition to its effect on the mitochondrial membrane potential, phytanic acid readily decreased the steady-state NAD(P)H level in living fibroblasts. The same observation was reached with the protonophore DNP, whereas, in sharp contrast, the complex I inhibitor rotenone markedly increased the steady-state NAD(P)H level (see also, [29]). These findings are in agreement with our conclusion that phytanic acid acts primarily as a protonophore, stimulating the rate of NADH consumption also in mitochondria respiring under phosphorylating conditions. In agreement with the present findings, previous work showed that phytanic acid readily decreased the NAD(P)H level in isolated rat brain mitochondria respiring under non-phosphorylating conditions and that the complex I inhibitor rotenone reversed this effect [21]. On the other hand, our conclusion that the protonophoric action of phytanic acid is much stronger than its alleged action as an inhibitor of complex I is clearly at variance with previous work showing that phytanic acid can decrease FCCP-stimulated respiration in isolated rat brain mitochondria incubated under phosphorylating conditions ([21], but see also [20]). At present we have no explanation for these contradictory findings other than possible differences between the test system: isolated rat brain mitochondria [20;21] versus digitonin-permeabilized and intact human skin fibroblasts (this study).

ATP synthesis was most potently inhibited by pristanic acid and phytanic acid. Palmitic acid was considerably less potent, whereas phytol and phytanic acid methyl ester had no inhibitory effect at all. Similarly, when added at a concentration of 30 μM, pristanic acid and phytanic acid decreased the mitochondrial membrane potential more effectively than palmitic acid, whereas phytol and phytanic acid methyl ester were the least effective. These findings suggest that the carboxyl group of

pristanic acid and phytanic acid is important for their protonophoric action. Indeed, mild uncoupling by free fatty acids is known to occur due to import of protons via their carboxyl group from the acidic cytosol to the alkaline matrix [19]. The lower potency of palmitic acid, which has the same carbon backbone as phytanic acid but lacks the methyl side chains, suggests that these latter side chains significantly contribute to the protonophoric action of pristanic acid and phytanic acid.

The present finding that pristanic acid and phytanic acid are equally effective in decreasing the mitochondrial membrane potential and inhibiting the oxidative production of ATP is readily explained by their great similarity; i.e. the carbon backbone of pristanic acid is only one C-atom shorter. Importantly, pristanic acid is known to accumulate in a number of inherited disorders of metabolism including: (1) D-bifunctional protein deficiency (MIM# 261515), (2) α-methylacyl-CoA racemase deficiency (MIM# 604489), and the recently found (3) sterol carrier protein X deficiency (MIM# 184755) (reviewed in [37]). Although pristanic acid is not the only compound accumulating in these disorders, it may contribute to the pathology of these diseases.

Previous work in isolated mitochondria revealed that phytanic acid can readily promote the formation of superoxide, increase the oxidation of the glutathione pool and decrease the activity of aconitase, suggesting that chronic elevation of phytanic acid levels may cause oxidative damage [14;15;21]. In addition, these studies showed that phytanic acid can cause opening of the permeability transition pore [20] and induce the release of cytochrome c [14;15], key events in the initiation of apoptotic cell death. In view of the present conclusion that phytanic acid acts primarily as a protonophore, decreasing the oxidative production of ATP, it remains to be established if and how this property is related to its stimulatory effect on mitochondrial superoxide production.

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Summary & Samenvatting

Summary

Fatty acids are degraded in organisms in order to produce energy. The degradation of fatty acids occurs in two cellular compartments (organelles). In mitochondria the long-, medium-, and short-chain fatty acids are broken down by a process called β-oxidation. In peroxisomes very-long-chain fatty acids, long-chain dicarboxylic acids, and long-chain fatty acids containing branches on position 2 of the fatty acid are shortened via a β-oxidation pathway resembling the one in mitochondria. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a dietary fatty acid, present in meat fat and dairy products, that has a methyl-branch on position 3 which makes this fatty acid not suitable for degradation via β-oxidation. Peroxisomes contain a specific pathway to degrade phytanic acid, i.e. the α-oxidation pathway, which shortens phytanic acid through a one-carbon decarboxylation at the α-carbon. This results in the production of the 2-methyl branched-chain fatty acid pristanic acid (2,6,10,14-tetramethyl-pentadecanoic acid) which can subsequently be broken down by sequential β-oxidation in peroxisomes and mitochondria. Patients suffering from Refsum disease have a defect in the α-oxidation pathway due to mutations in the *PHYH* gene which codes for the α-oxidation enzyme phytanoyl-CoA hydroxylase. As a consequence these patients are unable to degrade phytanic acid and accumulate phytanic acid in plasma and tissues (especially adipose tissue), which is believed to be the main reason why these patients become ill. Refsum patients have eye-problems (retinitis pigmentosa), which starts with night blindness and can progress into a total loss of vision. In addition, they have a loss of smell (hyposmia), and may suffer from an unstable gait (ataxia), numbness in arms and legs (peripheral neuropathy), hearing loss, and bone abnormalities (short metacarpals/metatarsals). Moreover, weight loss, for example during times of crisis such as a disease, is especially dangerous because of the sudden increase in plasma phytanic acid levels as a result of the release of phytanic acid from the fat stores in the body. The markedly elevated levels of phytanic acid are extremely detrimental for the heart and may lead to heart failure.

The way phytanic acid exerts its toxic effects is still not clear. One theory is that phytanic acid disturbs the function of the mitochondrial respiratory chain (the machinery that produces the energy currency in the cell). In order to resolve the way in which phytanic acid affects respiratory chain functioning, we performed studies in both digitonin-permeabilized human skin fibroblast and intact fibroblasts (Chapter 6). The results of these studies show that phytanic acid does not affect the functioning of the enzyme complexes that make up the respiratory chain but rather acts as a protonophore. The protonophoric action of phytanic acid consists of carrying protons across the mitochondrial membrane without the generation of ATP, also known as uncoupling of oxidative phosphorylation. The concentration at which phytanic acid

exerts this protonophoric effect is lower than observed in plasma of Refsum patients indicating that this effect may contribute to the pathology of the disease. However, these concentrations in plasma reflect the total phytanic acid concentrations and not the unesterified (free) phytanic acid concentration as used in our experiments. In tissue and plasma, fatty acids are readily esterified or bound to fatty acid binding proteins in order to dissolve them. So, it can be discussed whether the free phytanic acid concentrations used in Chapter 6 correspond to the free phytanic acid concentrations in Refsum patients. Furthermore, our experiments were performed in human skin fibroblasts and the effect of phytanic acid could be different in other cell types.

Despite the fact that Refsum disease has been studied quite thoroughly throughout the years there is still no cure for this disease. Patients are treated with a diet low in phytanic acid which stabilizes the progression of the disease. Moreover, phytanic acid levels in Refsum patients on this diet decreases which is thought to be due to the breakdown of phytanic acid via an alternative degradation pathway. In literature, indirect evidence has been presented that suggests that this alternative pathway is the ω-oxidation pathway in which phytanic acid is first converted into a dicarboxylic acid, which can then further be degraded by peroxisomal β-oxidation from the omega-end (shown in Figure 4, Chapter 1). In this thesis we showed that the enzymes that catalyze the first step of the ω-oxidation pathway of phytanic acid, i.e. ω-hydroxylation, are present in liver homogenates of rat and human (Chapters 2 and 3). To this end, we developed an enzyme assay to measure phytanic acid ω-hydroxylation followed by the detection of the formed metabolites by gaschromatography / mass spectrometry (GCMS). Our studies also revealed that in rat the ratio of (ω-1)- and ω-hydroxyphytanic acid was rather high compared to the situation in humans where (ω-1)-hydroxyphytanic acid was hardly formed (Figure 1 in Chapter 3).

Studies performed by others had already shown that enzymes belonging to the cytochrome P450 enzyme family were responsible for the ω-hydroxylation of fatty acids. Preliminary experiments (Figure 4 in Chapter 2, Figure 3 in Chapter 3) confirmed these results for phytanic acid. Subsequently, we established the identity of the specific human CYP450(s) catalyzing the ω-hydroxylation of phytanic acid (Chapter 4). Inhibitor studies suggested that the CYP450s belonged to the family 4 enzymes. Indeed, subsequent studies performed with recombinant human CYP450 enzymes of the family 4 showed that specifically CYP4F3A, CYP4F3B, CYP4F2, and CYPA11 could ω-hydroxylate phytanic acid.

At this moment, the underlying mechanisms regulating the expression of the CYP4F family of enzymes are still unknown. However, it is well described in literature that CYP4A11 can be upregulated via activation of the Peroxisome Proliferator-Activated Receptor alpha (PPARα). Fibrates, such as fenofibrate, are ligands of PPARα and activate this receptor by binding to it. This results in the increased

expression of numerous genes involved in fatty acid metabolism, among which CYP4A11. Homologues of the CYP4A11 enzyme are present in mice and just like in humans their expression can be upregulated by fibrates via PPARα. Upregulation of the first step of the ω-hydroxylation pathway may increase the overall ω-oxidation of phytanic acid, thereby creating a possibility for Refsum patients to get rid of phytanic acid more readily. In order to test this hypothesis we treated our recently generated *Phyh* -/- mice with fenofibrate. These mice are deficient in the phytanoyl-CoA hydroxylase enzyme just as Refsum patients. The *Phyh* -/- mice accumulate phytanic acid when phytanic acid or its precursor phytol is present in the diet and therefore they are a good biochemical model for Refsum disease. In the study described in Chapter 5 we show that fenofibrate treatment of *Phyh* -/- mice leads to a decrease in phytanic acid levels in plasma, kidney, and muscle. The total phytanic acid content in livers of mice on diets supplemented with fenofibrate increased, indicating that phytanic acid is transported from the other tissues to the liver. In cerebellum, no effect of fenofibrate on phytanic acid accumulation was observed. An explanation for this is that fenofibrate may not cross the blood-brain-barrier or is actively pumped out of the brain in combination with a low expression of PPARα and fatty acid metabolizing cytochrome P450s. The phytanic acid ω-hydroxylation capacity of the livers of *Phyh* -/- mice treated with fenofibrate increased which coincided with an increase of CYP4A enzymes. Furthermore, in the *Phyh* -/- mice treated with fenofibrate a possible metabolite originating from the dicarboxylic acid of phytanic acid (the ω-oxidation product of phytanic acid) was increased. This strongly suggests that fenofibrate increases the breakdown of phytanic acid via ω-oxidation. Increased degradation of phytanic acid through the ω-oxidation pathway might ultimately also decrease the phytanic acid levels in liver after most of the phytanic acid stored in other tissues is exported to and metabolized in the liver. In Refsum patients treated with a diet low in phytanic acid the neurological symptoms stabilize or even improve when phytanic acid plasma levels decrease. This suggests that in the *Phyh* -/- mice on the fenofibrate diet the phytanic acid levels in the brain could also decrease after a longer period of treatment. However, the study in Chapter 6 only lasted 2 weeks which was too short to see any effect of phytanic acid accumulation in brain. Further research is required to investigate the effect of long-term fenofibrate treatment.

All in all, the results from our studies on the ω-oxidation of phytanic acid (Chapters 2-5) indicate that upregulation of phytanic acid ω-oxidation by fenofibrate may be considered as a potential new approach in the treatment of Refsum disease. Furthermore, fenofibrate may be administrated to Refsum patients without any apparent problem since it is already used in the clinic for the treatment of hyperlipedemia and therefore has already undergone all the costly clinical trials.

Samenvatting voor iedereen

Vetzuren worden in ons lichaam afgebroken voor de productie van energie. Dit gebeurt in aparte compartimenten (organellen) in de cel. In het mitochondrion worden de kort-, middel-, en lang-keten vetzuren afgebroken via een proces dat β-oxidatie genoemd wordt. De zeer-lang-keten vetzuren, lang-keten dicarbonzuren en de lang-vertakt-keten vetzuren worden ook afgebroken door β-oxidatie, echter, dit gebeurt in een ander organel, namelijk in het peroxisoom. Fytaanzuur is een lang-vertakt-keten vetzuur wat veel voorkomt in vlees en zuivel producten (Figuur 1 in hoofdstuk 1). Fytaanzuur kan niet door middel van β-oxidatie in het peroxisoom worden afgebroken omdat het een vertakking op positie 3 van het molecuul heeft. Dit maakt het onmogelijk om fytaanzuur de reacties van de β-oxidatie te laten doen ondergaan. Gelukkig heeft de cel hier een oplossing voor en kan fytaanzuur 1 koolstof atoom ingekort worden door een proces dat α-oxidatie heet (Figuur 3 in hoofdstuk 1). De α-oxidatie van fytaanzuur vindt ook plaats in het peroxisoom en het vetzuur dat hierbij geproduceerd wordt, te weten pristaanzuur, heeft een vertakking op positie 2 en kan wel verder worden afgebroken door middel van β-oxidatie in het peroxisoom.

Bij patiënten met de ziekte van Refsum werkt het α-oxidatie systeem niet. Dit komt doordat één enzym van het α-oxidatie systeem (het enzym fytanoyl-CoA hydroxylase) niet functioneert of helemaal niet aanwezig is vanwege mutaties in het gen dat voor dit enzym codeert (het *PHYH* gen). Doordat het α-oxidatie systeem niet werkt kunnen patiënten met de ziekte van Refsum fytaanzuur niet afbreken en gaat dit stapelen in vetweefsel, organen en in het bloed (plasma). Het stapelen van fytaanzuur zorgt ervoor dat Refsum patiënten ziek worden. Zij krijgen eerst nachtblindheid wat later verergert tot tunnelvisie en uiteindelijk leidt tot totale blindheid (dit proces heeft de medische naam progressieve retinitis pigmentosa). Ook hebben deze patiënten een verminderd reukvermogen en kunnen zij onder andere last krijgen van een onstabiele tred (wankel lopen), gevoelloosheid in armen en benen, doofheid en hebben patiënten soms aangeboren botafwijkingen in handen en voeten. Verder kan plotseling gewichtsverlies tijdens een crisis situatie, zoals bijv. bij griep, leiden tot een plotselinge stijging van fytaanzuur in de bloedbaan doordat in situaties van gewichtsverlies de vetvoorraden van het lichaam aangesproken worden en die zitten in Refsum patiënten vol met fytaanzuur. Deze plotselinge stijging van het fytaanzuur niveau is zeer slecht voor het hart en kan leiden tot hartfalen.

Waarom stapeling van fytaanzuur zo toxisch is weet men nog niet precies. Eén theorie hierover is dat fytaanzuur het functioneren van de ademhalingsketen in mitochondrion kan verstoren. De ademhalingsketen is verantwoordelijk voor het maken van de energie (in de vorm van ATP) in de cel. Als de ademhalingsketen verstoord raakt leidt dit ertoe dat er te weinig ATP is en dat de energie-afhankelijke processen in de cel niet meer goed meer kunnen verlopen. In hoofdstuk 6 wordt het

effect van fytaanzuur op de ademhalingsketen van gekweekte huidcellen beschreven. Fytaanzuur had inderdaad een verstorend effect op de productie van ATP. De resultaten laten echter zien dat dit niet komt doordat fytaanzuur de functie van de eiwitten van de ademhalingsketen verstoort maar als ontkoppelaar van de oxidatieve fosforylering werkt. Normaal gesproken stromen protonen (H^+) door een speciaal eiwit heen het negatief geladen mitochondrion in. Dat eiwit gebruikt de energie van die stroming om ATP te maken (ongeveer zoals bij een stuwmeer). Ontkoppelaars, zoals fytaanzuur, zorgen ervoor dat protonen het mitochondrion inkomen zonder dat er ATP gemaakt wordt wat dus zorgt voor energie verspilling en slecht is voor de cel.

Ondanks het feit dat er veel onderzoek is gedaan naar de ziekte van Refsum door de jaren heen, bestaat er nog steeds geen middel tegen deze ziekte. Omdat fytaanzuur vanuit het dieet ons lichaam bereikt krijgen Refsum patiënten een speciaal fytaanzuur-arm dieet. Dit dieet zorgt ervoor dat de ernst van de symptomen in de loop der tijd niet toeneemt en in sommige gevallen zelfs afneemt. De fytaanzuur niveaus gaan omlaag op dit dieet. Men denkt dat dit komt omdat fytaanzuur ook via een ander proces dan α-oxidatie kan worden afgebroken. Studies die beschreven staan in de literatuur hebben indirecte bewijzen geleverd dat deze alternatieve afbraakroute voor fytaanzuur de ω-oxidatie route is. Bij de ω-oxidatie van fytaanzuur vindt er een aantal reactiestappen plaats aan de ω-zijde van fytaanzuur (Figuur 4 in hoofdstuk 1). Dit is aan de tegenovergestelde kant van de carbonzuur-groep. Hierdoor ontstaat een dicarbonzuur, fytaandizuur, wat door middel van β-oxidatie vanuit de voormalige ω-kant kan worden afgebroken in het peroxisoom. In de hoofdstukken 2 en 3 is onderzocht of de enzymen die de eerste stap van de ω-oxidatie van fytaanzuur verzorgen, de ω-hydroxylatie, aanwezig waren in lever van de rat en de mens. Om dit te kunnen bestuderen is er eerst een methode (enzym assay) ontwikkeld om dit te kunnen meten. Deze studies onthulden dat ω-hydroxylering van fytaanzuur plaatsvindt in de lever van zowel de mens als de rat. In de rat werd echter nog een ander bijproduct gemeten ((ω-1)-hydroxyfytaanzuur) wat niet meer verder de ω-oxidatie kan doorlopen (Figuur 1 in hoofdstuk 3). Gelukkig werd dit doodlopende product nauwelijks gevonden bij de ω-hydroxylering van fytaanzuur in de lever van de mens.

Voorgaande studies beschreven in de literatuur hadden al laten zien dat de ω-hydroxylering van rechte-keten-vetzuren wordt gekatalyzeerd door enzymen behorende bij de Cytochrome P450 (CYP450) familie. In de hoofdstukken 2 en 3 is te zien dat dit ook geldt voor het vertakt-keten vetzuur fytaanzuur. Omdat de CYP450 familie uit heel veel enzymen bestaat die betrokken zijn bij vele processen in de cel naast ω-hydroxylering van vetzuren zijn we op zoek gegaan naar de specifieke enzymen die verantwoordelijk zijn voor de ω-hydroxylatie van fytaanzuur. In hoofdstuk 4 staat beschreven dat die enzymen geïdentificeerd zijn als CYP450 enzymen behorende bij de familie 4. Er werden meerdere enzymen binnen deze

familie gevonden die de ω-hydroxylatie van fytaanzuur konden uitvoeren in de mens, namelijk CYP4F3A, CYP4F3B, CYP4F2 en CYP4A11. Er is nog nauwelijks iets bekend over de regulatie van de CYP4F enzymen. Echter, van CYP4A enzymen is bekend dat zij opgereguleerd kunnen worden via activatie van de transcriptie factor Peroxisome Proliferator-activated Receptor α (PPARα). Transcriptie factoren zijn eiwitten die de expressie van ander eiwitten kunnen reguleren (verhogen of verlagen). PPARα kan geactiveerd worden door binding aan specifieke stoffen, fibraten genaamd, en reguleert naast inductie van de CYP4A enzymen ook de expressie van vele andere enzymen die betrokken zijn bij vetzuurafbraak. Door PPARα te activeren met fibraten en daarmee de CYP4A enzym expressie te verhogen zou je de ω-hydroxylering van fytaanzuur kunnen verhogen. Dit zou er toe kunnen leiden dat de totale ω-oxidatie van fytaanzuur sneller gaat en dus dat er meer fytaanzuur kan worden afgebroken via deze alternatieve afbraakroute in Refsum patiënten.

Om deze hypothese te kunnen onderzoeken hebben we gebruik gemaakt van ons muis model voor de ziekte van Refsum. Deze muizen (*Phyh* -/- muizen) missen door middel van genetische manipulatie het enzym fytanoyl-CoA hydroxylase net als Refsum patiënten. De *Phyh* -/- muizen stapelen ook fytaanzuur wanneer je dit toevoegt aan het voer (er zit normaal gesproken nauwelijks fytaanzuur in muizen voer). Deze *Phyh* -/- muizen zijn op verschillende diëten gezet, namelijk gewoon voer, gewoon voer met fytaanzuur toegevoegd (om fytaanzuur te laten stapelen), gewoon voer met fenofibraat toegevoegd (om PPARα te activeren) of gewoon voer met zowel fytaanzuur als fenofibraat toegevoegd. Hoofdstuk 5 beschrijft de effecten van fenofibraat op de fytaanzuur stapeling en ω-oxidatie van fytaanzuur in de *Phyh* -/- muizen. Fenofibraat zorgde ervoor dat de fytaanzuur niveaus in plasma, nier en spier daalden. Daarentegen stapelde fytaanzuur zich op in de lever. Dit komt waarschijnlijk doordat fytaanzuur vanuit de andere weefsels naar de lever wordt gebracht om daar afgebroken te worden. Fenofibraat behandeling had geen effect op de fytaanzuur stapeling in de (kleine) hersenen. Dit komt mede doordat fenofibraat de hersenen niet goed kan bereiken wat al beschreven staat in de literatuur. De ω-hydroxylatie capaciteit van de levers van de muizen was verhoogd door de fenofibraat behandeling. Dit ging gepaard met een verhoogde expressie van de CYP4A enzymen in lever, maar ook in de nier was de expressie van de CYP4A enzymen verhoogd. Verder werd er een verhoging van een metaboliet van de ω-oxidatie van fytaanzuur aangetroffen in de levers van de muizen op het combinatie dieet wat suggereert dat de volledige ω-oxidatie route van fytaanzuur ook werkelijk werd verhoogd door de behandeling met fenofibraat. De lever is het belangrijkste orgaan wat betreft de ω-oxidatie van vetzuren. Het feit dat fytaanzuur zich stapelt in de lever terwijl in de andere weefsels de fytaanzuur niveaus omlaag gaan is dus niet ongunstig omdat in de lever de afbraak moet plaatsvinden via de verhoogde ω-oxidatie. Na langdurige behandeling zal de fytaanzuur stapeling in hersenen uiteindelijk hopelijk ook minder worden door de

fenofibraat behandeling, aangezien het fytaanzuur niveau in plasma lager is en daarmee ook de toevoer van fytaanzuur naar de hersenen lager is. Dit fenomeen wordt ook gezien bij Refsum patiënten op dieet waarbij de neurologische symptomen afnemen naarmate de fytaanzuur plasma niveaus gedurende de behandeling lager worden.

Al met al suggereren de fytaanzuur ω-oxidatie studies beschreven in de hoofdstukken 2 tot en met 5 dat het opreguleren van de ω-oxidatie van fytaanzuur in de toekomst misschien een bijdrage zou kunnen leveren aan de behandeling van patiënten met de ziekte van Refsum. Fenofibraat is hiervoor een goed kandidaat-geneesmiddel aangezien het al gebruikt wordt in de kliniek als vetverlagend geneesmiddel en dus al klinische trials heeft ondergaan.

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THANKS!

Dankwoord

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