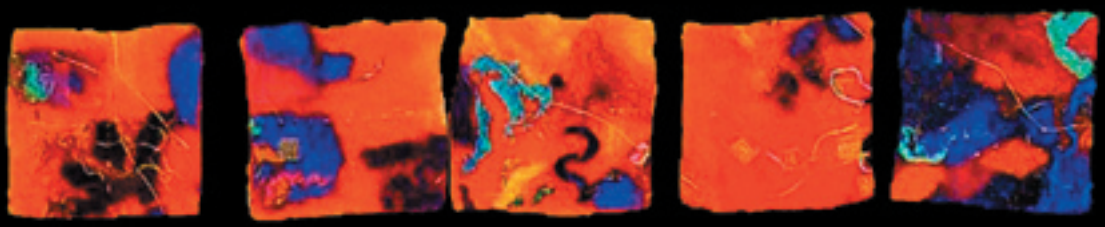
Metabolic consequences of branched-chain fatty acid accumuation in health and disease

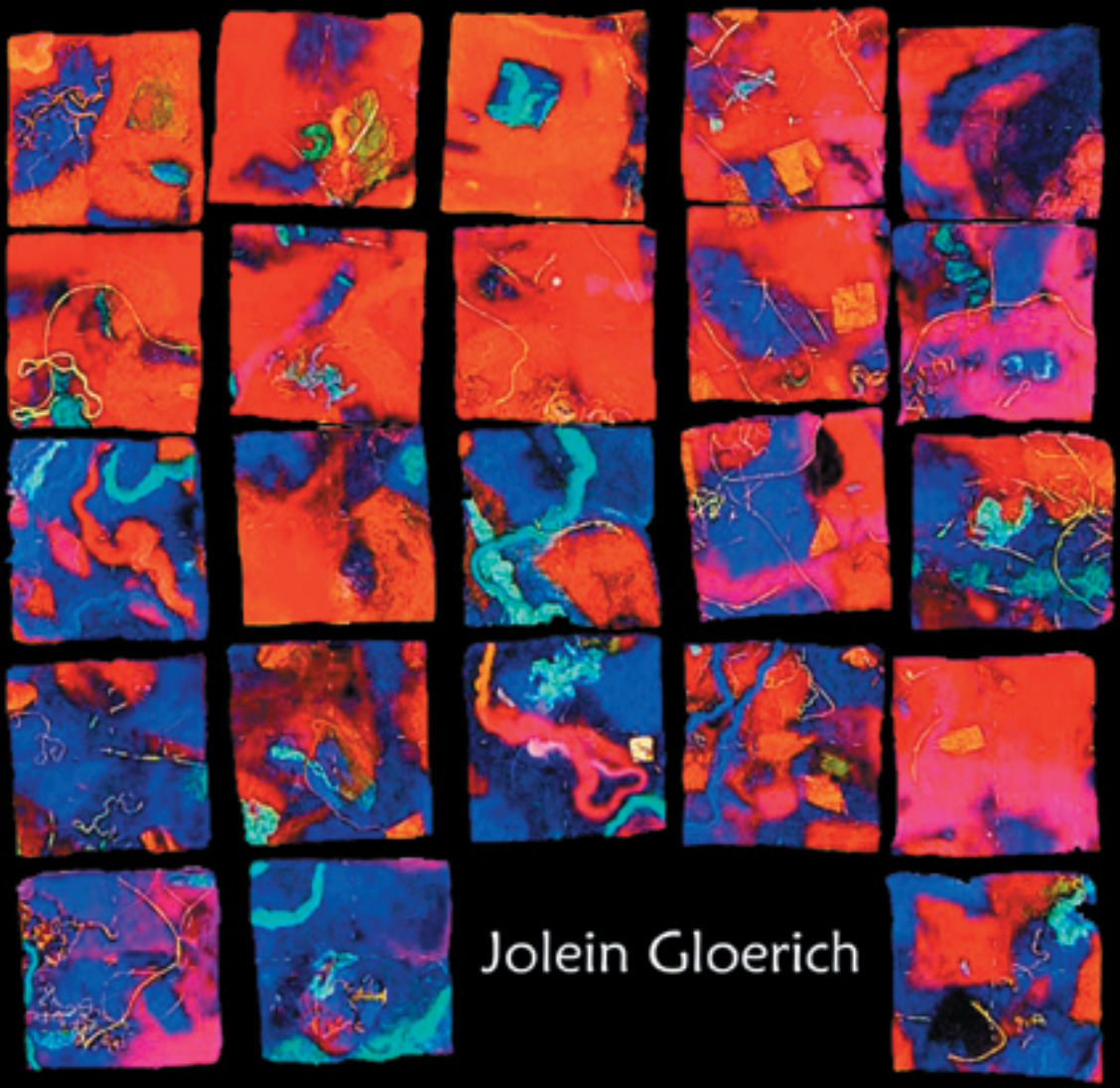
Gloerich, J.

Publication date
2006

Document Version
Final published version



Metabolic consequences of branched-chain fatty acid accumulation in health and disease



Jolein Gloerich

Metabolic consequences of branched-chain fatty acid accumulation in health and disease

Metabolic consequences of branched-chain fatty acid accumulation in health and disease

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof.mr. P.F. van der Heijden
ten overstaan van een door het
college voor promoties ingestelde commissie,
in het openbaar te verdedigen
in de Aula der Universiteit

op woensdag 6 december 2006, te 10.00 uur

door

Jolein Gloerich

geboren te Heerlen

Promotiecommissie:

Promotoren:	Prof. dr. R.J.A. Wanders
	Prof. dr. K.W.A. Wirtz
Co-promotor:	Dr. S. Ferdinandusse
Overige leden:	Prof. dr. F.A. Wijburg
	Prof. dr. B.T. Poll-The
	Prof. dr. C.J.F. van Noorden
	Prof. dr. W.H. Lamers
	Dr. M. Duran
	Dr. M.A.A.P. Willemsen

Faculteit der Geneeskunde

The work described in this thesis was carried out at the laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics (Emma's Children's Hospital), Academic Medical Center, University of Amsterdam, The Netherlands and was supported financially by a grant from the Netherlands Organization for Scientific Research (NWO 901-03-097).

Nobody said it was easy

The Scientist - Coldplay

voor mam, pap en Marcel

Table of contents

Abbreviations

ALDH3A2	aldehyde dehydrogenase 3A2
AMACR	α-methylacyl-CoA racemase
BB	γ-butyrobetaine
BBD	γ-butyrobetaine dioxygenase
BCOX	branched-chain acyl-CoA oxidase
BTP	bis-tris propane
CACT	carnitine-acylcarnitine translocase
CAT	carnitine acetyltransferase
COT	carnitine octanoyltransferase
CPT	carnitine palmitoyltransferase
CYP	cytochrome P450 hydroxylase
DBP	D-bifunctional protein
DBP DH	D-bifunctional protein 3-hydroxyacyl-CoA dehydrogenase
DBP HY	D-bifunctional protein enoyl-CoA hydratase
DHCA	dihydroxycholestanoic acid
DMSO	dimethylsulfoxide
Elovl	long-chain fatty acid elongase
ESI-tMS	electrospray ionization tandem mass spectrometry
FALDH	fatty aldehyde dehydrogenase
FAO	fatty alcohol:NAD^+ oxidoreductase
GC-MS	gas chromatography-mass spectrometry
HPCL	2-hydroxyphytanoyl-CoA lyase
HPLC	high performance liquid chromatography
IPTG	isopropyl-1-thio-β-D-galactopyranoside
IRD	infantile Refsum disease
LBP	L-bifunctional protein
LCAD	long-chain acyl-CoA dehydrogenase
MBP	maltose-binding protein
MCAD	medium-chain acyl-CoA dehydrogenase
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide
MTP	mitochondrial trifunctional protein
NALD	neonatal adrenoleukodystrophy
ORF	open reading frame
PBD	peroxisome biogenesis disorder
PhyH	phytanoyl-CoA hydroxylase
PMP70	peroxisomal membrane protein 70
PPAR	peroxisome-proliferator activated receptor
PPRE	peroxisome proliferator response element
PTS	peroxisomal targeting signal
RCDP	rhizomelic chondrodysplasia punctata
RXR	retinoid-X receptor
SBCHAD	short branched-chain 3-hydroxyacyl-CoA dehydrogenase
SCAD	short-chain acyl-CoA dehydrogenase
SCHAD	short-chain 3-hydroxyacyl-CoA dehydrogenase
SCOX	straight-chain acyl-CoA oxidase
SCPx	sterol carrier protein X
SLS	Sjögren-Larsson syndrome
TER	*trans*-2-enoyl-CoA reductase
THCA	trihydroxycholestanoic acid
THIO	peroxisomal 3-ketoacyl-CoA thiolase
TMABADH	trimethylaminobutyraldehyde dehydrogenase
TML	trimethyllysine
VLCAD	very long-chain acyl-CoA dehydrogenase
VLCFA	very long-chain fatty acid
ZS	Zellweger syndrome

1 General Introduction

General introduction

Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) has long been known as a chemical derivative of phytol,[1] which is the alcohol moiety of the chlorophyll molecule. In the 1950s phytanic acid was first reported as a naturally occurring substance after studies on the fatty acid content of butterfat.[2,3] These studies revealed that branched-chain fatty acids, like phytanic and pristanic acid, are major constituents of a variety of food products, especially those derived from grazing animals and fish.

Research on phytanic acid increased considerably after the discovery that phytanic acid levels were elevated in tissues and plasma of patients suffering from Refsum disease.[4] It became clear that phytanic acid degradation takes place in the peroxisome by subsequent α- and β-oxidation reactions. In addition to Refsum disease, other disorders with defects in branched-chain fatty acid degradation have been identified, leading to elevated levels of phytanic acid and its metabolite, pristanic acid, in plasma and tissues of patients.[5] The accumulation of branched-chain fatty acids is thought to play a role in the pathogenesis of these diseases. Recent studies indicate that phytanic acid is involved in apoptotic pathways in astrocytes and vascular smooth muscle cells[6-8] and the onset of heart problems.[9] In addition, phytanic and pristanic acid have been identified as ligands for the transcription factors retinoid-X receptor (RXR) and peroxisome-proliferator activated receptor α (PPARα).[10,11] Via activation of these transcription factors, the branched-chain fatty acids are able to regulate their own metabolism by induction of fatty acid oxidation. This will be discussed in more detail in this chapter.

Origin of branched-chain fatty acids

Endogenous synthesis of branched-chain fatty acids

Unlike many straight-chain fatty acids, phytanic and pristanic acid are not endogenously synthesized in mammals. This is remarkable, because other branched-chain compounds, with structures closely resembling that of phytanic acid, like farnesol and geranylgeraniol (**Figure 1**), can be endogenously synthesized from mevalonate units.[12] Studies in which radiolabelled mevalonate was administered to rats did not show any incorporation of the label in phytanic acid,[13,14] suggesting that all phytanic acid present in mammalian cells is derived from direct dietary intake or dietary intake of its precursor, i.e. phytol.

Dietary branched-chain fatty acids and phytol

After the discovery of phytanic acid as a constituent of butterfat,[2,3] it was detected in a large variety of food products in the human diet. In fish and food products derived from marine animals, such as whale oil and milk, relatively high levels of phytanic acid are detected, which is thought to originate from the high amount of phytanic acid found in krill. In addition, phytanic acid is particularly abundant in various tissues of ruminant animals, such as liver and fat, but also in dairy products.[15]

The precursor of phytanic acid, phytol, forms the alcohol side-chain of the chlorophyll molecule, which is essential for photosynthesis in green plants. Studies in which a dose

of radiolabelled chlorophyll was orally administered to rats and even to human subjects, revealed that about 95% of the radiolabelled chlorophyll remained intact in the digestive system, and that only 5% of the phytol was released and converted to phytanic acid.[16,17] In contrast, in ruminants, phytol is effectively cleaved from the chlorophyll molecule by micro-organisms in the gut.[18] Free phytol can then be further metabolized to phytanic acid, hence the high levels of phytanic acid in food products derived from ruminants. Together with the fact that mammals cannot synthesize phytanic acid endogenously, this shows that phytanic acid in mammals originates from direct absorption from food products or the absorption of free phytol and subsequent degradation to phytanic acid.

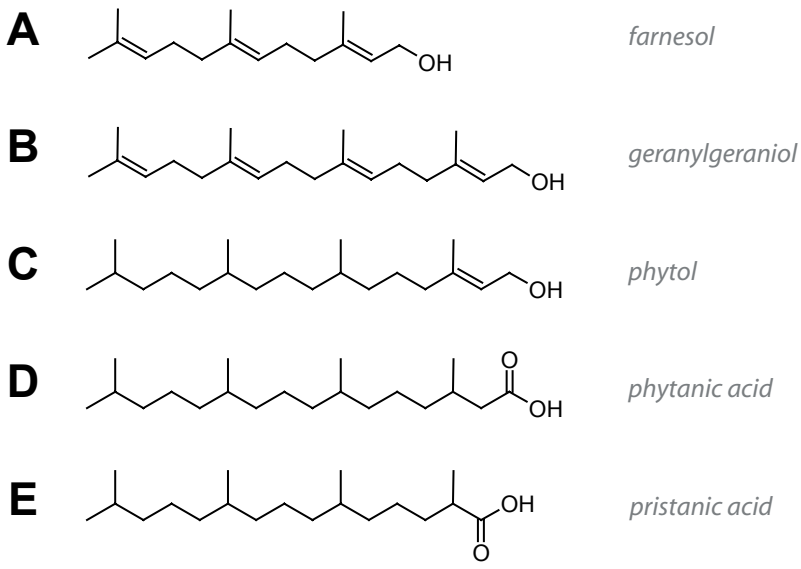


Figure 1: Chemical structure of several branched-chain compounds found in human tissues and plasma. A: farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol). B: geranylgeraniol (3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol). C: phytol (3,7,11,15-tetramethyl-hexadec-2-en-1-ol). D: phytanic acid (3,7,11,15-tetratmethylhexadecanoic acid). E: pristanic acid (2,6,10,14-tetramethylpentadecanoic acid).

Degradation of phytol to phytanic acid

The degradation of phytol to phytanic acid has long been a neglected topic in phytanic acid research. Although addition of phytol to the diet has been used in a number of animal studies to investigate the accumulation of phytol metabolites in tissues and plasma,[10,19-21] the enzymes and mechanisms involved in the breakdown of phytol to phytanic acid have long been unknown.

Mechanism of phytol degradation

In early studies on the origin of phytanic acid in mammals, it was shown that both phytol and dihydrophytol could be converted to phytanic acid in the rat.[22] In addition, phytol was shown to be converted to phytenic acid. From these results, two possible mechanisms for

the conversion of phytol to phytanic acid were proposed (**Figure 2**). In the first pathway, the 2,3-double bond of phytol is first reduced to form the intermediate dihydrophytol, then the alcohol-group of this compound is oxidized into a carboxyl-group to form phytanic acid. In the second pathway, the oxidation of the alcohol moiety is the first step, forming phytenic acid as intermediate, which is subsequently reduced to phytanic acid.

Further investigations on the degradation of phytol in the rat showed that after addition of phytol to the diet, phytenic acid, but not dihydrophytol, could be detected.[13,18] In addition, accumulation of phytanic acid was observed after injection of phytenic acid in rats.[13] These results indicate that, at least in rats, phytenic acid, and not dihydrophytol, is the *in vivo* intermediate in phytol degradation. These findings were confirmed *in vitro*, because after incubation of a rat liver post-nuclear supernatant fraction with phytol, production of phytenic acid, and not of dihydrophytol, was observed.[23,24]

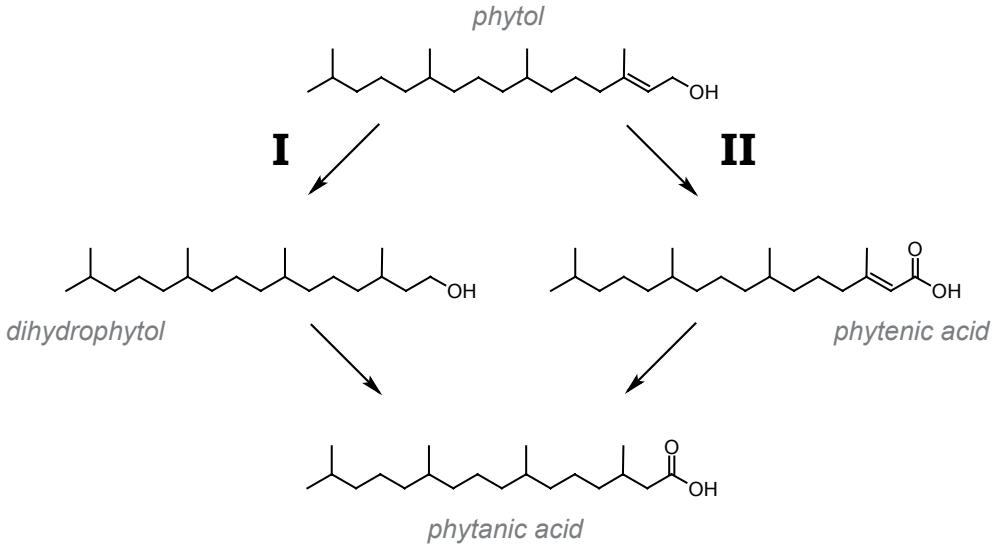


Figure 2: Two possible reaction mechanisms for the conversion of phytol to phytanic acid. I: phytol is first converted to dihydrophytol by reduction of the 2,3-double bond and subsequently oxidized to phytanic acid. II: phytol is oxidized to phytenic acid and subsequently the 2,3-double bond is reduced to form phytanic acid.

Conversion of phytol to phytenic acid

In vitro experiments have shown that the presence of the cofactor NAD^+ is required for the enzymatic conversion of phytol to phytenic acid.[24] In rat, highest phytenic acid production was observed in liver, followed by kidney and spleen (both about 10% of the activity found in liver). Some activity was detected in brain, heart and lungs, whereas no phytenic acid production was observed in intestine, adipose tissue and muscle.[23] More recently, it was shown that phytenic acid is also formed in human fibroblasts after incubation with phytol.[25] In subcellular fractionation studies, high phytenic acid production was detected in microsome-enriched and mitochondrial fractions, whereas no conversion was observed in cytosolic fractions.[24]

Enzymatic oxidation of an alcohol to an acid usually takes place in two steps. First, the alcohol is converted into an aldehyde by the action of an alcohol dehydrogenase. This intermediate is then further oxidized by an aldehyde dehydrogenase. Recent studies have shown that indeed phytol is first converted into phytenal and subsequently into phytenic acid.[25] A schematic overview of the conversion of phytol to phytenic acid is shown in **figure 3**. A microsomal aldehyde dehydrogenase, fatty aldehyde dehydrogenase (FALDH), was shown to catalyze the second step in the conversion of phytol to phytenic acid. This was investigated using human fibroblasts of control subjects and of patients suffering from Sjögren-Larsson Syndrome (SLS), a metabolic disorder that is characterized by a deficiency in FALDH. When fibroblasts of SLS patients were cultured in the presence of phytol no formation of phytenic acid was observed, whereas formation of phytenic acid was detected in control fibroblasts.[25] In addition, the conversion of phytol to phytenic acid was deficient in fibroblast homogenates of SLS patients, whereas phytenic acid formation was readily detected in control subject fibroblast homogenates.[25,26]

The alcohol dehydrogenase responsible for the conversion of phytol to phytenal remains to be identified. A likely candidate is the alcohol dehydrogenase that, together with FALDH, forms the fatty alcohol:NAD^+ oxidoreductase (FAO) enzyme complex.[27] FAO is involved in the oxidation of many different medium- and long-chain alcohols to the corresponding fatty acids. Attempts to characterize the alcohol dehydrogenase of FAO via purification of the enzyme complex have been unsuccessful, because the enzyme proved to be unstable.[28]

Conversion of phytenic acid to phytanic acid

In a recent study on the reduction of phytenic acid in rat liver, NADPH-dependent conversion of phytenic acid into phytanic acid was observed, although at a slow rate.[26] Because of this low activity, an alternative mechanism for the conversion of phytenic acid was investigated. A possible mechanism would be activation of phytenic acid to its CoA-ester, phytenoyl-CoA, prior to reduction of the double bond. Indeed, formation of phytenoyl-CoA was observed both in rat liver homogenates and in rat hepatocytes after incubation with phytol. In addition, reductase activity measurements in rat liver showed much higher rates of phytanoyl-CoA formation from phytenoyl-CoA than formation of phytanic acid from phytenic acid.[26]

Subcellular fractionation experiments in rat liver revealed that phytenoyl-CoA synthetase activity is localized both in microsomes and peroxisomes. Because phytenic acid formation, by the action of FALDH, takes place on the outside of the ER-membrane, activation of phytenic acid could occur directly after synthesis at the ER-membrane or at the peroxisomes. The enzyme responsible for the activation of phytenic acid is still unknown, but a likely candidate is the long-chain acyl-CoA synthetase, which has been shown to activate phytanic acid.[29]

Phytenoyl-CoA reductase activity was detected both in mitochondria and in peroxisomes.[30] Because phytanoyl-CoA is degraded via α-oxidation in the peroxisome, it is tempting to speculate that the reduction of phytenoyl-CoA also takes place in the peroxisome. In chapter 6 of this thesis the peroxisomal *trans*-2-enoyl-CoA reductase (TER) was shown to catalyze the reduction of phytenoyl-CoA to phytanoyl-CoA.[31] A schematic representation of the conversion of phytenic acid to phytanic acid is shown in figure 3.

The site of phytol degradation in the body

Measurement of phytol levels in tissues of mice fed a phytol-enriched diet, showed phytol accumulation in liver and small intestine, but not in brain, heart and kidney. Upon longer

duration of the diet, phytol levels in liver, but not in small intestine, increased, suggesting that phytol is transported to the liver for degradation (chapter 4). This suggests that the liver is the main site of phytol degradation in the body, which is supported by the fact that enzyme activities of FALDH, phytenoyl-CoA synthetase and phytenoyl-CoA reductase are highest in liver (chapter 4).

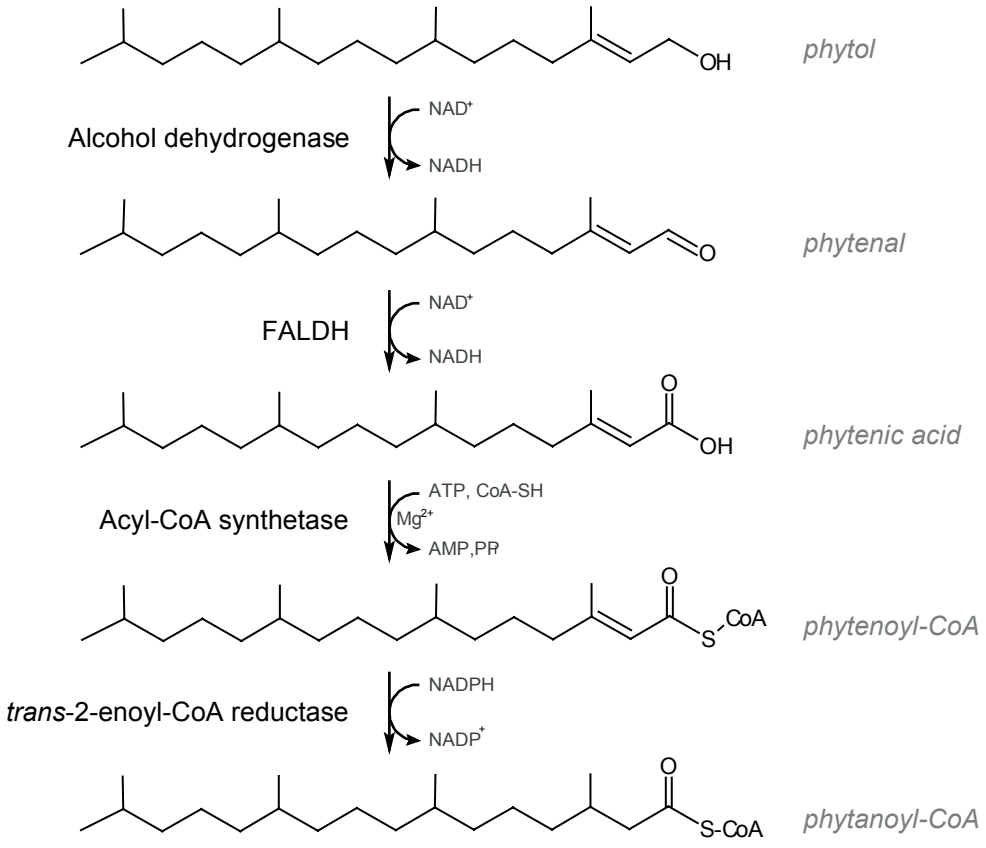


Figure 3: The degradation of phytol into phytanic acid. The alcohol group of phytol is first converted to an acid group by the subsequent action of an alcohol dehydrogenase and fatty aldehyde dehydrogenase (FALDH). Phytenic acid is then activated to its CoA-ester and reduced by *trans*-2-enoyl-CoA reductase (TER) to form phytanoyl-CoA.

Stereospecificity of phytol degradation

In studies on the degradation of phytol to phytenic acid, it was observed that only (*E*)-phytenic acid was formed after incubation of human fibroblast homogenates and mouse liver homogenates with a racemic mixture of (*E*)- and (*Z*)-phytol (chapter 4,[25]). Incubations of mouse liver homogenates with a mixture of (*E*)- and (*Z*)-phytenal also resulted in the exclusive formation of (*E*)-phytenic acid (chapter 4). Together, these data indicate that at

least the reaction catalyzed by FALDH is stereospecific for the conversion of the (*E*)-isomer. It remains unclear however, whether the alcohol dehydrogenase step is also stereospecific, because direct measurement of this reaction is not possible, due to instability of the reaction product, i.e. phytenal. Measurement of phytenoyl-CoA synthetase activity in multiple tissues showed that formation of (*E*)-phytenoyl-CoA was markedly higher than formation of the (*Z*)-isomer. Although both isomers of phytenoyl-CoA are reduced to phytanoyl-CoA in mouse liver homogenates, the reduction of (*E*)-phytenoyl-CoA was higher than that of the (*Z*)-isomer (chapter 4). In addition, studies with the recombinant TER showed that TER activity towards (*Z*)-phytenoyl-CoA was only 3% of its activity towards the (*E*)-isomer (unpublished data). Together, these data indicate that phytol degradation is stereospecific for the breakdown of (*E*)-phytol.

Degradation of phytanic acid

Phytanic acid is exclusively broken down in the peroxisome via a process called α-oxidation. β-oxidation of phytanic acid is not possible because of the presence of a methyl-group at the 3-position. During α-oxidation phytanic acid is shortened by one carbon atom (reviewed in [32]), yielding pristanic acid which can be further degraded by β-oxidation. A schematic overview of peroxisomal α-oxidation is shown in **figure 4**.

α-Oxidation

The exact mechanism of α-oxidation has remained unclear for a long time. An important step in the elucidation of the α-oxidation pathway was the finding that phytanic acid is activated to its CoA-ester prior to degradation.[33,34] At first, it was thought that activation of phytanic acid was catalyzed by a distinct phytanoyl-CoA ligase,[35] but later research showed that phytanic acid can be activated by the long-chain acyl-CoA synthetase, which also has affinity for straight-chain fatty acids.[29]

In early studies, 2-hydroxyphytanic acid formation was seen after injection of rats with radiolabelled phytanic acid, indicating that hydroxylation of phytanic acid at the 2-position was the first step of the α-oxidation pathway.[13] This was supported by the finding that radiolabelled 2-hydroxyphytanic acid was detected in plasma of human subjects who had received radiolabelled phytanic acid.[36,37] The enzyme responsible for the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA is phytanoyl-CoA hydroxylase (PhyH or PAHX).[38-40] PhyH is a non-haem iron (II) and 2-oxoglutarate-dependent hydroxylase that is targeted to the peroxisome by a peroxisomal targeting signal type 2 (PTS2).[41]

The second step in the α-oxidation of phytanic acid is the decarboxylation of 2-hydroxyphytanoyl-CoA to pristanal by 2-hydroxyphytanoyl-CoA lyase (HPCL). Pristanal was already hypothesized to be an intermediate of phytanic acid breakdown in the 1960s.[36] This hypothesis was supported by the observation that pristanic acid, and not pristanoyl-CoA was the end product of phytanic acid α-oxidation.[42] Final proof was obtained by the detection of pristanal after incubation of rat liver homogenates with 2-hydroxyphytanoyl-CoA.[43] Studies on the subcellular localization of HPCL showed activity in the peroxisomal fractions of rat liver, and the enzyme was purified from rat liver peroxisomes. In addition, the human cDNA of HPCL was cloned and a PTS1 sequence was identified at the carboxy-terminus.[44]

The third step in the α-oxidation of phytanic acid is the dehydrogenation of pristanal to pristanic acid. The enzyme responsible for this reaction remains unidentified up to date.

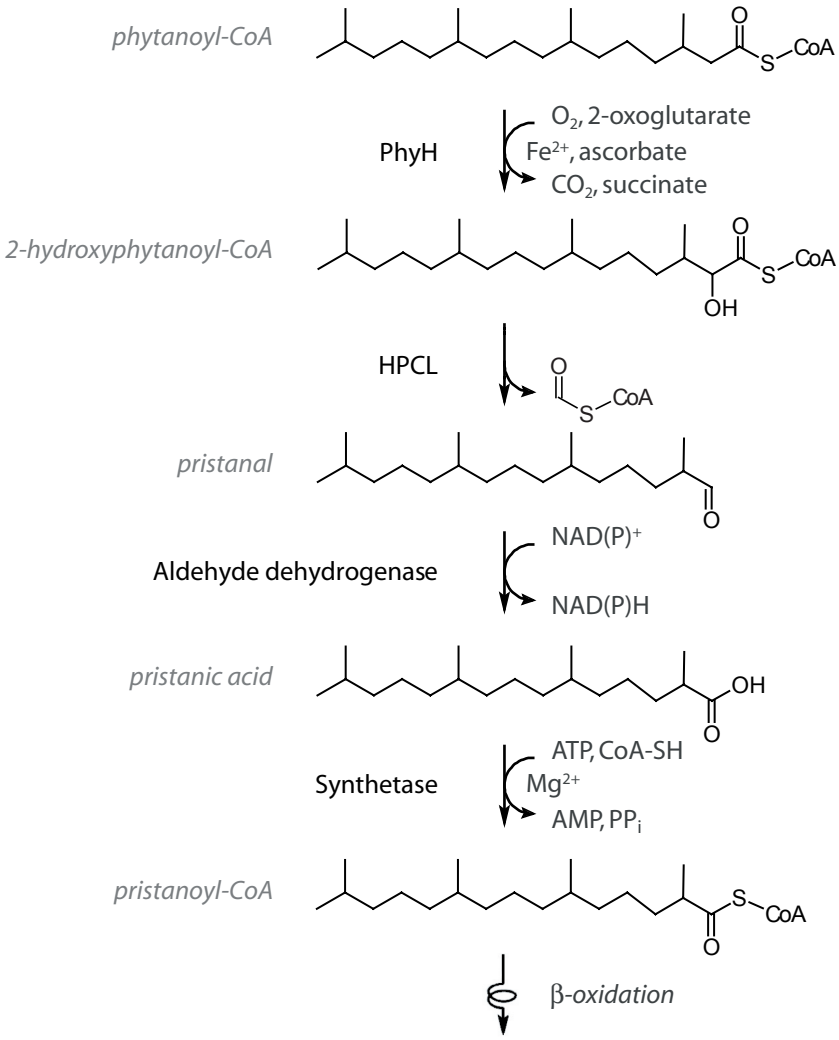


Figure 4: Schematic representation of phytanic acid α-oxidation. The first step in α-oxidation of phytanoyl-CoA is hydroxylation to 2-hydroxyphytanoyl-CoA by phytanoyl-CoA hydroxylase (PhyH). Subsequently, a formyl-CoA molecule is released from 2-hydroxyphytanoyl-CoA to form pristanal by the action of 2-hydroxyphytanoyl-CoA lyase (HPCL). Next, pristanal is converted to pristanic acid by the action of an aldehyde dehydrogenase and subsequently this acid is activated to pristanoyl-CoA, which can be further β-oxidized.

It was originally proposed that FALDH was the enzyme catalyzing this reaction, because in fibroblasts of SLS patients oxidation of [2,3-^{3}H]phytanic acid was reduced to 25% of normal values. In addition, a 4-fold increase in the accumulation of radioactivity in N-alkylphosphatidyl ethanolamine was detected after incubation with [2,3-^{3}H]phytanic acid.[45]

If correct, this hypothesis would imply that the pristanal produced in the peroxisome has to be transported to the ER-membrane for conversion to pristanic acid, followed by re-uptake of this product into the peroxisome for further degradation. Several observations, however, argue against the involvement of FALDH in this pathway. Firstly, phytanic acid levels have been found to be completely normal in plasma from SLS patients.[46] Secondly, a normal rate of α-oxidation of phytanic acid in SLS fibroblasts was observed when [1-^{14}C]phytanic acid was used as substrate.[39] Thirdly, subcellular localization studies have shown that pristanal dehydrogenase activity is not only localized in the microsomal fraction but can also be measured in the mitochondrial and peroxisomal fractions, even though the bulk of activity is microsomal (about 80%).[39] From these data it can be concluded that peroxisomes are able to produce pristanic acid from pristanal via a yet unidentified aldehyde dehydrogenase. However, involvement of FALDH in the α-oxidation of phytanic acid cannot be ruled out completely. Because of the large number of aldehyde dehydrogenases known in man (more than ten), it is no surprise that more than one of these can convert pristanal to pristanic acid. Further research will have to establish which peroxisomal aldehyde dehydrogenase is responsible for the conversion of pristanal into pristanic acid.

β-Oxidation

The product of phytanic acid α-oxidation, pristanic acid, is further degraded by peroxisomal β-oxidation. β-Oxidation requires activation of fatty acids to their CoA-ester by an acyl-CoA synthetase (reviewed in [47]). After activation, the carbon-chain of the CoA-ester is shortened by two carbon atoms per β-oxidation cycle. Each cycle consists of four sequential steps: 1) oxidation of the substrate forming a *trans*-2-enoyl-CoA, 2) hydration of the *trans*-2-enoyl-CoA to a 3-hydroxyacyl-CoA, 3) dehydrogenation forming a 3-ketoacyl-CoA, and 4) thiolytic cleavage yielding an acetyl-CoA unit (or propionyl-CoA unit in case of branched-chain substrates), plus a shortened fatty acyl-CoA that can reenter the β-oxidation cycle. A schematic model of the mechanism of β-oxidation is depicted in **figure 5A**. β-Oxidation occurs not only in peroxisomes, but also in mitochondria. In the peroxisome, very long-chain fatty acids and long branched-chain fatty acids are chain shortened by a couple of rounds of peroxisomal β-oxidation. The shortened products of peroxisomal β-oxidation are then transported to the mitochondria and degraded to completion by the mitochondrial β-oxidation.

Peroxisomal β-oxidation

For a long time it was thought that a single set of β-oxidation enzymes was involved in peroxisomal β-oxidation. However, at this moment it is well established that in man peroxisomes contain two sets of β-oxidation enzymes (reviewed in [48]).

The first step of peroxisomal β-oxidation, involving the desaturation of an acyl-CoA, is catalyzed by flavin adenine dinucleotide (FAD)-dependent acyl-CoA oxidases, which transfer electrons directly to molecular oxygen, resulting in the production of hydrogen peroxide. In man, two acyl-CoA oxidases are present in the peroxisome, whereas rat peroxisomes contain three distinct acyl-CoA oxidases,[49] which differ in substrate specificity. The first peroxisomal acyl-CoA oxidase isolated from rat liver is inducible by peroxisome proliferators and oxidizes the CoA-esters of very long-chain fatty acids, dicarboxylic acids, prostaglandins and glutaric acid.[50,51] The human homolog of this enzyme is the straight-chain acyl-CoA oxidase (SCOX). In rat, two additional acyl-CoA oxidases have been identified: pristanoyl-CoA oxidase and trihydroxycholestanoyl-CoA oxidase. Pristanoyl-CoA oxidase is not inducible by peroxisome

proliferators and catalyzes the oxidation of branched-chain fatty acyl-CoAs, such as pristanoyl-CoA, but can also handle straight-chain acyl-CoAs.[52,53] Trihydroxycholestanoyl-CoA oxidase is active with the CoA-esters of the bile acid intermediates, di- and trihydroxycholestanoic acid (DHCA and THCA, respectively).[51,54] Remarkably, humans have only one additional oxidase, called branched-chain acyl-CoA oxidase (BCOX), which is active with both pristanoyl-CoA and DHC-CoA and THC-CoA.[55]

In humans, rats and mice, two distinct bifunctional proteins have been identified. They both have enoyl-CoA hydratase and NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase activities, and catalyze the conversion of *trans*-2-enoyl-CoAs to 3-ketoacyl-CoA via the formation of a 3-hydroxyacyl-CoA. The first bifunctional protein identified was L-bifunctional protein (LBP), because it forms and dehydrogenates L-3-hydroxyacyl-CoAs, whereas D-3-hydroxyacyl-CoAs are formed as intermediates of the reaction catalyzed by the second bifunctional protein, D-bifunctional protein (DBP). Alternative names are multifunctional enzymes I and II (MFE I and II), multifunctional proteins 1 and 2 (MFP1 and 2) and L- and D-peroxisomal bifunctional enzyme (L-PBE and D-PBE). Despite the fact that DBP was identified many years after the first identification of LBP,[56-61] it is now well established that DBP is the enzyme involved in the breakdown of very long-chain fatty acids, pristanic acid and bile acid intermediates.[58-65] Identification of patients with a deficiency of DBP[66-69] and the generation of a DBP knockout mouse[19] have provided unequivocal evidence for the important role of DBP in peroxisomal β-oxidation. Only recently, a possible function for LBP was identified in the degradation of long-chain dicarboxylic acids.[70]

The last step in the peroxisomal β-oxidation process is catalyzed by a thiolase, which thiolytically cleaves 3-ketoacyl-CoAs into chain-shortened acyl-CoAs and acetyl-CoA or propionyl-CoA (in case of 2-methyl-branched-chain fatty acids). The first peroxisomal thiolase identified, was purified from rat livers treated with peroxisome proliferators.[71] It is synthesized as a 44 kDa precursor and undergoes proteolytic processing to a 41 kDa mature protein after import into the peroxisome. A second peroxisomal thiolase, designated peroxisomal thiolase 2 or sterol carrier protein X (SCPx) was identified years later.[72] Studies on the substrate specificities of these enzymes showed that straight-chain fatty acids are handled by both thiolases, whereas SCPx is the only thiolase that acts on ketoacyl-CoAs of pristanic acid and THCA.[73-76] In **figure 5B**, the peroxisomal β-oxidation enzymes are depicted.

Mitochondrial β-oxidation

After three rounds of peroxisomal β-oxidation, the chain-shortened product of pristanoyl-CoA, i.e. 4,8-dimethylnonanoyl-CoA, is not a substrate for the peroxisomal acyl-CoA oxidases anymore and is degraded further in the mitochondrion. There are several differences between the mitochondrial and peroxisomal β-oxidation system (reviewed in [77,78]). Firstly, β-oxidation in the two organelles is catalyzed by different enzymes, which are products of different genes. Secondly, in mitochondrial β-oxidation more energy is conserved, because the first oxidative step is directly coupled to oxidative phosphorylation, whereas the energy produced in the first step of peroxisomal β-oxidation is released as heat. Thirdly, in contrast to the mitochondrial β-oxidation system, peroxisomal β-oxidation does not go to completion; after a few cycles the chain shortened products are transported to the mitochondrion where they are further β-oxidized. Finally, the two systems have different substrate specificities. Mitochondria are able to β-oxidize short-, medium and long-chain fatty acids, whereas peroxisomal β-oxidation is responsible for the oxidation of very long-chain fatty acids, pristanic acid, long-

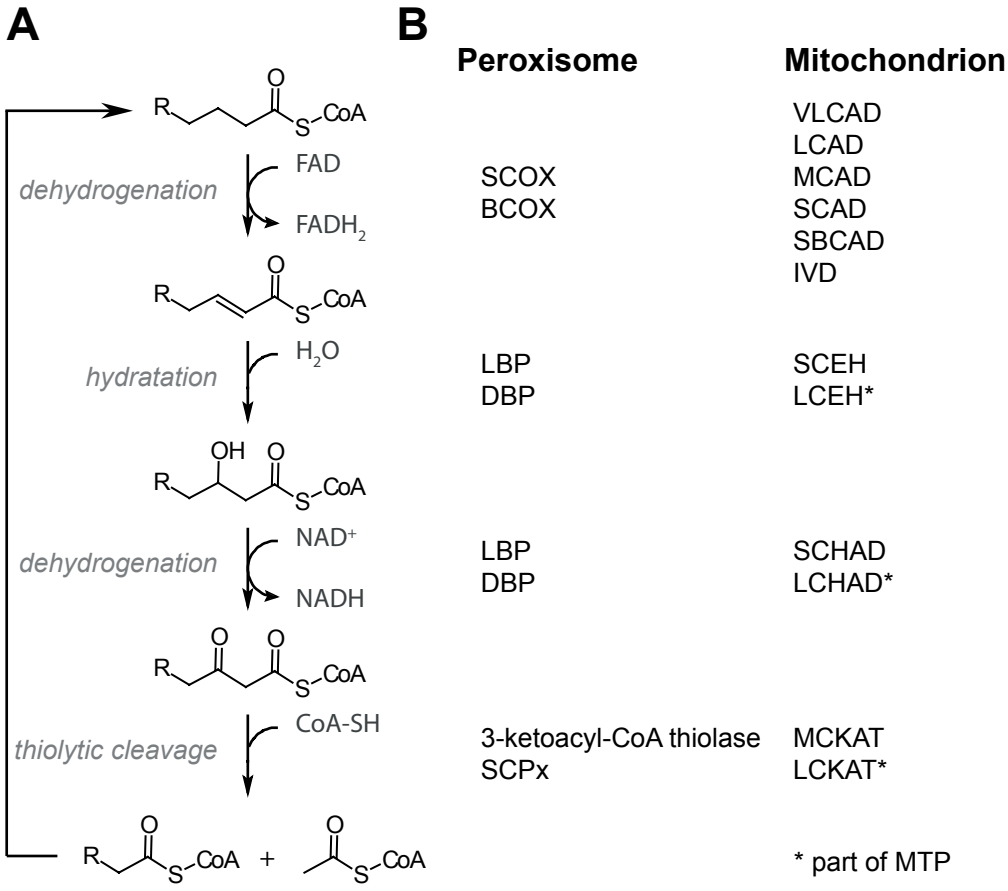


Figure 5: Schematic representation of the β-oxidation pathway. A: Each cycle of β-oxidation consists of a dehydrogenation, a hydration, a second dehydrogenation and a thiolytic cleavage. B: Enzymes involved in the different steps of peroxisomal and mitochondrial β-oxidation. Peroxisomal enzymes: straight-chain acyl-CoA oxidase (SCOX), branched-chain acyl-CoA oxidase (BCOX), L-bifunctional protein (LBP), D-bifunctional protein (DBP), 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx). Mitochondrial enzymes: very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD), short branched-chain acyl-CoA dehydrogenase (SBCAD), isovaleryl-CoA dehydrogenase (IVD), short-chain enoyl-CoA hydratase (SCEH), long-chain enoyl-CoA hydratase (LCEH), short-chain hydroxyacyl-CoA dehydrogenase (SCHAD), long-chain hydroxyacyl-CoA dehydrogenase (LCHAD), medium-chain ketoacyl-CoA thiolase (MCKAT) and long-chain ketoacyl-CoA thiolase (LCKAT). LCEH, LCHAD and LCKAT are part of the mitochondrial trifunctional protein complex (MTP).

chain dicarboxylic acids, bile acid intermediates and certain mono- and polyunsaturated fatty acids.

In figure 5B, the different enzymes catalyzing each step of mitochondrial β-oxidation are depicted (reviewed in [79,80]). For all steps of the mitochondrial β-oxidation process, two or more enzymes with different substrate specificities have been identified that carry out the reaction.

Transport of fatty acids from peroxisomes to mitochondria

Fatty acyl-CoAs that have been chain-shortened by a couple of cycles of peroxisomal β-oxidation, like 4,8-dimethylnonanoyl-CoA, are transported out of the peroxisome and into the mitochondrion, where they are oxidized to completion. For 4,8-dimethylnonanoyl-CoA, the breakdown product of pristanoyl-CoA, this transport from peroxisome to mitochondrion takes place as a carnitine ester. In the peroxisome, 4,8-dimethylnonanoyl-CoA is converted into 4,8-dimethylnonanoyl-carnitine by carnitine octanoyltransferase (COT).[81] This carnitine ester is exported from the peroxisome, transported across the inner mitochondrial membrane via a specific carrier, carnitine-acylcarnitine translocase (CACT). In the mitochondrial matrix, the acyl-group of 4,8-dimethylnonanoyl-carnitine is transferred back from carnitine to CoASH by carnitine palmitoyltransferase 2 (CPT2) and the resulting 4,8-dimethylnonanoyl-CoA can then undergo β-oxidation in the mitochondrion.[82]

For long-chain fatty acyl-CoAs that are directly oxidized by mitochondrial β-oxidation, without involvement of the peroxisomal pathway, import into the mitochondrion also occurs via the formation of a carnitine ester in a reaction catalyzed by carnitine palmitoyltransferase I (CPT1) at the outer mitochondrial membrane. The resulting acylcarnitines are also transported across the inner mitochondrial membrane by CACT, and reconverted to CoA-esters by CPT2 and can then undergo β-oxidation in the mitochondrion.[83] In contrast to long-chain fatty acids, medium- and short-chain fatty acids are able to cross the mitochondrial membranes without involvement of the carnitine cycle. These fatty acids are activated to their corresponding CoA-esters by mitochondrial acyl-CoA synthetases.

Stereospecificity of β-oxidation

In the breakdown of branched-chain substrates, such as pristanic acid and the bile acid intermediates, another enzyme is important, i.e. α-methylacyl-CoA racemase (AMACR). Both the peroxisomal and the mitochondrial β-oxidation system are stereospecific for the breakdown of (*2S*)-methylacyl-CoAs. This is in contrast to the α-oxidation system, which converts both the (*3R*)- and (*3S*)-stereoisomers of phytanic acid into (*2R,6R,10R*)- and (*2S,6R,10R*)-pristanic acid. To further degrade (*2R,6R,10R*)-pristanic acid, it first has to be converted to its (*2S*)-stereoisomer. This reaction is catalyzed by AMACR,[84,85] which is localized in both peroxisomes and mitochondria, because it has a mitochondrial targeting signal at the N-terminus and a PTS1 sequence at the C-terminus. In the mitochondrion, AMACR is responsible for the conversion of the degradation products of pristanic acid to their (*2S*)-isomers to enable complete β-oxidation of these compounds.

Branched-chain fatty acids are ligands for transcription factors

Studies on branched-chain metabolites have revealed that phytanic acid and pristanic acid play a role in the regulation of fatty acid degradation. At first, it was shown that phytol

metabolites, including phytanic and phytenic acid, are able to activate the nuclear retinoid-X receptor (RXR) *in vitro*.[86,87] RXR belongs to the family of nuclear hormone receptors, which are transcription factors regulating gene expression in response to lipophilic ligands such as steroid hormones and fatty acids.[88] The family of nuclear receptors includes the oestrogen receptors, thyroid hormone receptors, retinoic acid receptors, RXRs and the peroxisome proliferator-activated receptors (PPARs). Later studies revealed that phytanic acid and pristanic acid are also ligands for PPARα, but not for the other PPAR isoforms, PPARβ and PPARγ.[11,89] Whether phytol itself is able to activate PPARα remains unclear at the moment, since conflicting results on this topic have been published.[11,90]

PPARs are ligand-activated transcription factors: after ligand binding, PPARs heterodimerize with RXR and modulate the expression of target genes by binding to specific peroxisome proliferator response elements (PPREs) in the promotor region of regulated genes.[91] The three PPAR isoforms have different tissue distributions and functions. PPARα is mostly expressed in organs with a high rate of fatty acid catabolism, such as brown adipose tissue, liver, kidney, and heart, and it plays an important role in various aspects of lipid and glucose metabolism.[77,92]

PPARα has a broad range of both artificial and natural ligands also called peroxisome proliferators. The artificial ligands of PPARα consist of a variety of compounds, including hypolipidemic drugs (e.g., clofibrate and Wy-14,643), phthalate ester plasticizers, herbicides, and several chlorinated hydrocarbons. Besides the phytol metabolites, a broad array of unsaturated fatty acids and also long-chain fatty acids are natural ligands for PPARα.[10,11,93-95] Administration of peroxisome proliferators to rodents results in hepatomegaly and an increase in the number and size of peroxisomes. In addition, it changes the expression of a variety of genes involved in various aspects of lipid metabolism, ranging from fatty acid transport, mitochondrial and peroxisomal fatty acid β-oxidation to microsomal fatty acid ω-oxidation. Chronic treatment of rodents with peroxisome proliferators results in hepatocellular carcinomas.[96] These changes are all mediated by PPARα, as demonstrated by studies with PPARα-knockout (PPARα$^{-/-}$) mice.

Under normal conditions, PPARα$^{-/-}$ mice are indistinguishable from wild-type animals and have normal levels of hepatic peroxisomes. Upon fasting, however, PPARα$^{-/-}$ mice are unable to switch to fatty acid oxidation. As a consequence, they develop a fatty liver and become severely hypoglycemic.[92] Similarly, feeding these mice a high-fat diet results in massive accumulation of lipids in the liver, attributable to their inability to enhance fatty acid degradation.[97] PPARα$^{-/-}$ mice display an altered constitutive expression of several mitochondrial and peroxisomal enzymes involved in the oxidation of fatty acids.[98] Moreover, PPARα$^{-/-}$ mice are nonresponsive to treatment with peroxisome proliferators; hence, they do not show any physiological, toxicological, or carcinogenic responses to peroxisome proliferators.[99]

We have studied the *in vivo* effects of phytol and its metabolites on fatty acid metabolism and the role of PPARα in its regulation by feeding wild-type and PPARα$^{-/-}$ mice a diet enriched with phytol (chapter 3 and 4, [20]). The phytol-enriched diet resulted in increased plasma and liver levels of the phytol metabolites phytanic and pristanic acid. In wild-type mice, plasma fatty acid levels decreased after phytol feeding, whereas in PPARα$^{-/-}$ mice, the already elevated fatty acid levels increased. Furthermore, phytol feeding resulted in a PPARα-dependent induction of various peroxisomal and mitochondrial β-oxidation enzymes, but also of several enzymes involved in the degradation of phytol to phytanic acid.

Human disorders of branched-chain fatty acid degradation

Several genetic metabolic disorders have been identified, in which the degradation of branched-chain fatty acids is (partially) impaired. In patients with a peroxisome biogenesis disorder, who have a defect in peroxisome assembly and thereby a generalized loss of peroxisomal functions, branched-chain fatty acids can accumulate, because α- and β-oxidation of phytanic and pristanic acid are peroxisomal processes. In addition to peroxisome biogenesis disorders, elevated branched-chain fatty acids are observed in single enzyme deficiencies of enzymes involved in the breakdown of branched-chain fatty acids, such as Refsum disease, AMACR deficiency and DBP deficiency. Because branched-chain fatty acids are derived from exogenous origin only, the accumulation is diet- and age-dependent in these disorders. In SLS, which is caused by a deficiency of FALDH, catalyzing the second step of phytol degradation, no elevated branched-chain compounds have been detected in plasma.

Peroxisome biogenesis disorders

The peroxisome biogenesis disorders (PBDs; MIM #601539) are a group of disorders with different genetic causes. Despite the genetic heterogeneity, they have a defect in the biogenesis of peroxisomes in common, which leads to an impairment in one or more peroxisomal functions. The PBDs include Zellweger syndrome (ZS; MIM #214100), neonatal adrenoleukodystrophy (NALD; MIM #202370), infantile Refsum disease (IRD; MIM #266510) and rhizomelic chondrodysplasia punctata type 1 (RCDP1; MIM #215100) (reviewed in [100]).

ZS, NALD, and IRD are clearly distinct from RCDP1 and are usually referred to as the Zellweger spectrum with ZS being the most severe, and NALD and IRD the less severe disorders. Liver disease, variable neurodevelopmental delay, retinopathy and perceptive deafness are common to all the three disorders.[101] Patients with ZS are characterized by a typical craniofacial dysmorphism and are severely hypotonic from birth and die before 1 year of age. Patients with NALD experience neonatal onset of hypotonia and seizures, they suffer from progressive white matter disease and usually die in late infancy.[102] Patients with IRD may survive beyond infancy and some may even reach adulthood.[103] Clinical differentiation between these disease states is not very well defined and patients can have overlapping symptoms. The absence of functional peroxisomes leads to a number of biochemical abnormalities including impaired plasmalogen synthesis and peroxisomal α- and β-oxidation.[101,104-106] This leads to elevated plasma levels of very-long chain fatty acids, phytanic and pristanic acid and the bile acid intermediates THCA and DHCA.

RCDP1 is clinically quite different from ZS, NALD, and IRD. It is characterized by a disproportionally short stature primarily affecting the proximal parts of the extremities, a typical facial appearance, congenital contractures, characteristic ocular involvement, dwarfism, and severe mental retardation with spasticity. Most RCDP patients die in the first decade of life. In RCDP1, caused by mutations in the *PEX7* gene encoding the PTS2-receptor, plasmalogen biosynthesis and peroxisomal α-oxidation, but not peroxisomal β-oxidation, are impaired, resulting in a deficiency of plasmalogens and elevated levels of phytanic acid in plasma.

Refsum disease

Refsum disease (MIM #266500) is caused by a deficiency of PhyH, the first enzyme of the peroxisomal α-oxidation system.[107,108] As a result, these patients accumulate high amounts

of phytanic acid. In plasma, phytanic acid levels may reach the mM range (control range 0-9 μM). The main clinical symptoms include retinitis pigmentosa, peripheral neuropathy, anosmia and cerebellar ataxia. Additionally, deafness, ichthyosis and cardiac arrhythmias are also regularly present.[109] The onset of symptoms is usually relatively late in life, with most patients presenting in adolescence.

α-Methylacyl-CoA racemase deficiency
Only a few patients with a deficiency of AMACR (MIM #604489) have been identified.[110,111] The deficiency of AMACR causes an accumulation of the branched-chain fatty acids, phytanic and pristanic acid. The level of phytanic acid is only marginally elevated, whereas the pristanic acid level is strongly increased. In addition, these patients accumulate the bile acid intermediates DHCA and THCA, whereas the very long-chain fatty acid levels are normal. The main clinical symptom of AMACR deficiency is adult-onset sensory motor neuropathy, whereas eye problems, including retinitis pigmentosa and optic atrophy are sometimes observed. Additionally, tremor, cerebellar dysarthria, spastic paraparesis and epileptic seizures can be present.

D-Bifunctional protein deficiency
The clinical presentation of DBP deficiency (MIM #261515) is very severe. Symptoms include neurological abnormalities (seizures, hypotonia and craniofacial dysmorphia), severe developmental delay, and death in early infancy. DBP deficiency can be divided into three subgroups. In the first group, the patients have a complete DBP deficiency (type I),[69] in the second group there is an isolated DBP enoyl-CoA hydratase deficiency (type II)[68] and in the third group an isolated DBP 3-hydroxyacyl-CoA dehydrogenase deficiency (type III).[67] Although no distinction can be made between these subtypes based on clinical symptoms, it is possible to differentiate between the different types of DBP deficiency by measuring both total DBP activity[67] and the isolated DBP 3-hydroxyacyl-CoA dehydrogenase activity (chapter 2, [112]). In chapter 2 the development of a specific enzyme assay for the isolated 3-hydroxyacyl-CoA dehydrogenase activity is described. Plasma analysis in DBP-deficient patients reveals elevated levels of very long-chain fatty acids, pristanic acid and in most cases also elevated levels of DHCA and THCA.

Sterol carrier protein X deficiency
Only recently, the first patient with a deficiency of SCPx was described.[113] The patient presented with torticollis and dystonic head tremor, slight cerebellar signs with intention tremor, nystagmus, hyposmia and azoospermia. Magnetic resonance imaging showed a leukencephalopathy and involvement of the thalamus and pons. Metabolite analyses in plasma revealed an accumulation of pristanic acid. In urine, abnormal bile alcohol glucuronides were excreted, implicating a block in the metabolism of bile acid intermediates at the level of SCPx.

Sjögren-Larsson syndrome
The main clinical features of SLS (MIM #270200) include ichthyosis, mental retardation and spastic di- or tetraplegia.[114] SLS is caused by a deficiency of FALDH.[115] FALDH is part of the microsomal NAD^+-oxidoreductase complex that functions in the conversion of long-

chain fatty alcohols into fatty acids.[116] A deficiency in FALDH leads to the accumulation of long-chain fatty alcohols in plasma of patients.[117,118] Furthermore, FALDH plays a role in the degradation of leukotriene B_4 (LTB_4), leading to elevated urinary concentrations of LTB_4 and its metabolite 20-hydroxy-LTB_4.[119] Finally, FALDH is also involved in the degradation of phytol, catalyzing the conversion of phytenal to phytenic acid, as shown by studies in human fibroblasts of SLS patients.[25] So far, however, no elevation of phytol has been detected in plasma of SLS patients (D.M. van den Brink, personal communication).

A variety of mutations have been detected in the *ALDH3A2* gene of SLS patients, including deletions, insertions, splicing defects and missense mutations.[46,114,115,120-127] Most of these mutations result in a total loss of FALDH activity, but a few missense mutations result in a protein which still shows some residual activity, when expressed in Chinese hamster ovary cells.[46] In chapter 5, we have investigated whether FALDH expression and activity in human skin fibroblasts of control subjects and SLS patients can be upregulated in a PPAR-dependent manner.[128]

Outline of this thesis

The accumulation of branched-chain fatty acids in plasma and tissues of patients is thought to be involved in the pathogenesis of the diseases described above. The degradation of phytanic and pristanic acid has been extensively studied in the past and the mechanism of peroxisomal α- and β-oxidation has been resolved at the molecular level. Several enzymatic assays have been developed to aid in the diagnosis of patients with defects in these pathways. In chapter 2, a novel assay to differentiate between DBP deficiency type I and II is described.

Studies on branched-chain fatty acids have resulted in the identification of phytanic and pristanic acid as activators of PPARα and RXR *in vitro*. In chapter 3 and 4, we describe investigations on the effects of elevated branched-chain fatty acids *in vivo*. To this end, we fed wild-type and PPARα$^{-/-}$ mice a control diet or a diet enriched with phytol. We investigated the effects of elevated plasma and tissue levels of phytol metabolites on the regulation of peroxisomal and mitochondrial β-oxidation via PPARα and measured plasma fatty acid levels. Furthermore, we investigated the metabolism of phytol and the role of PPARα in its regulation. We show that several enzymes involved in this pathway, including FALDH, are regulated via PPARα.

In chapter 5, we investigated the hypothesis that also in humans FALDH is regulated via PPARs. To this end, we studied the effects of bezafibrate, a pan-agonist of all PPAR-isoforms on FALDH activity and mRNA levels in human fibroblasts of control subjects and SLS patients.

Since the degradation of phytol can also contribute to phytanic acid levels in patients, more recently studies have been performed to elucidate the mechanism of conversion of phytol to phytanic acid. Although the mechanism of this pathway has been resolved, not all the enzymes involved have been identified. In chapter 6 of this thesis, it is shown that the peroxisomal *trans*-2-enoyl-CoA reductase catalyzes the last step in the degradation of phytol to phytanic acid, namely the conversion of phytenoyl-CoA to phytanoyl-CoA.

References

1. Willstätter R, Mayer EW, and Huni E (1910) *Liebigs Ann* **378:** 73
2. Hansen RP and Shorland FB (1952) The branched-chain fatty acids of butterfat. 2. The isolation of a multi-branched C_{20} saturated fatty acid fraction. *Biochem J* **50:** 358-360.
3. Hansen RP and Shorland FB (1953) The branched-chain fatty acids of butterfat. 3. Further investigations on a multibranched C_{20} saturated fatty acid fraction. *Biochem J* **55:** 662-663.
4. Klenk E and Kahlke W (1963) Über das Vorkommen der 3,7,11,15-Tetramethylhexadecansaüre (Phytansäure) in den Cholesterinestern und anderen Lipidfraktionen der Organe bei einem Krankheitsfall unbekannter Genese (Verdacht auf Heredopathia Atactiva Polyneuritiformis [Refsum-Syndrome]). *Hoppe Seylers Z Physiol Chem* **333:** 133-139.
5. Wanders RJ, Van Roermund CW, Visser WF, Ferdinandusse S, Jansen GA, Van den Brink DM, Gloerich J, and Waterham HR (2003) Peroxisomal fatty acid alpha- and beta-oxidation in health and disease: new insights. *Adv Exp Med Biol* **544:** 293-302.
6. Idel S, Ellinghaus P, Wolfrum C, Nofer JR, Gloerich J, Assmann G, Spener F, and Seedorf U (2002) Branched chain fatty acids induce nitric oxide-dependent apoptosis in vascular smooth muscle cells. *J Biol Chem* **277:** 49319-49325.
7. Kahlert S, Schonfeld P, and Reiser G (2005) The Refsum disease marker phytanic acid, a branched chain fatty acid, affects Ca2+ homeostasis and mitochondria, and reduces cell viability in rat hippocampal astrocytes. *Neurobiol Dis* **18:** 110-118.
8. Schonfeld P, Kahlert S, and Reiser G (2004) In brain mitochondria the branched-chain fatty acid phytanic acid impairs energy transduction and sensitizes for permeability transition. *Biochem J* **383:** 121-128.
9. Monnig G, Wiekowski J, Kirchhof P, Stypmann J, Plenz G, Fabritz L, Bruns HJ, Eckardt L, Assmann G, Haverkamp W, Breithardt G, and Seedorf U (2004) Phytanic acid accumulation is associated with conduction delay and sudden cardiac death in sterol carrier protein-2/sterol carrier protein-x deficient mice. *J Cardiovasc Electrophysiol* **15:** 1310-1316.
10. Ellinghaus P, Wolfrum C, Assmann G, Spener F, and Seedorf U (1999) Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice. *J Biol Chem* **274:** 2766-2772.
11. Zomer AW, Van der Burg B, Jansen GA, Wanders RJ, Poll-The BT, and Van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. *J Lipid Res* **41:** 1801-1807.
12. Goldstein JL and Brown MS (1990) Regulation of the mevalonate pathway. *Nature* **343:** 425-430.
13. Mize CE, Avigan J, Baxter JH, Fales HM, and Steinberg D (1966) Metabolism of phytol-U-14C and phytanic acid-U-14C in the rat. *J Lipid Res* **7:** 692-697.
14. Steinberg D, Avigan J, Mize C, Eldjarn L, Try K, and Refsum S (1965) Conversion of U-C14-phytol to phytanic acid and its oxidation in heredopathia atactica polyneuritiformis. *Biochem Biophys Res Commun* **19:** 783-789.
15. Hansen RP (1980) Phytol: its metabolic products and their distribution. A review. *N Z J Sci* **23:** 259-275.
16. Baxter JH, Steinberg D, Mize CE, and Avigan J (1967) Absorption and metabolism of uniformly 14C-labeled phytol and phytanic acid by the intestine of the rat studied with thoracic duct cannulation. *Biochim Biophys Acta* **137:** 277-290.
17. Baxter JH (1968) Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease. *J Lipid Res* **9:** 636-641.
18. Steinberg D, Avigan J, Mize CE, Baxter JH, Cammermeyer J, Fales HM, and Highet PF (1966) Effects of dietary phytol and phytanic acid in animals. *J Lipid Res* **7:** 684-691.
19. Baes M, Huyghe S, Carmeliet P, Declercq PE, Collen D, Mannaerts GP, and Van Veldhoven PP (2000) Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids. *J Biol Chem* **275:** 16329-16336.
20. Gloerich J, Van Vlies N, Jansen GA, Denis S, Ruiter JP, Van Werkhoven MA, Duran M, Vaz FM, Wanders RJ, and Ferdinandusse S (2005) A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARalpha-dependent and -independent pathways. *J Lipid Res* **46:** 716-726.
21. Savolainen K, Kotti TJ, Schmitz W, Savolainen TI, Sormunen RT, Ilves M, Vainio SJ, Conzelmann E, and Hiltunen JK (2004) A mouse model for {alpha}-methylacyl-CoA racemase deficiency: adjustment of bile

acid synthesis and intolerance to dietary methyl-branched lipids. *Hum Mol Genet* **13:** 955-965.
22. Klenk E and Kremer GJ (1965) [Studies on the metabolism of phytol, dihydrophytol and phytanic acid]. *Hoppe Seylers Z Physiol Chem* **343:** 39-51.
23. Muralidharan FN and Muralidharan VB (1986) Characterization of phytol-phytanate conversion activity in rat liver. *Biochim Biophys Acta* **883:** 54-62.
24. Muralidharan FN and Muralidharan VB (1985) In vitro conversion of phytol to phytanic acid in rat liver: subcellular distribution of activity and chemical characterization of intermediates using a new bromination technique. *Biochim Biophys Acta* **835:** 36-40.
25. Van den Brink DM, Van Miert JN, Dacremont G, Rontani JF, Jansen GA, and Wanders RJ (2004) Identification of fatty aldehyde dehydrogenase in the breakdown of phytol to phytanic acid. *Mol Genet Metab* **82:** 33-37.
26. Van den Brink DM, Van Miert JM, and Wanders RJ (2005) Assay for Sjogren-Larsson syndrome based on a deficiency of phytol degradation. *Clin Chem* **51:** 240-242.
27. Lee T (1979) Characterization of fatty alcohol:NAD+ oxidoreductase from rat liver. *J Biol Chem* **254:** 2892-2896.
28. Ichihara K, Noda Y, Tanaka C, and Kusunose M (1986) Purification of aldehyde dehydrogenase reconstitutively active in fatty alcohol oxidation from rabbit intestinal microsomes. *Biochim Biophys Acta* **878:** 419-425.
29. Watkins PA, Howard AE, Gould SJ, Avigan J, and Mihalik SJ (1996) Phytanic acid activation in rat liver peroxisomes is catalyzed by long-chain acyl-CoA synthetase. *J Lipid Res* **37:** 2288-2295.
30. Van den Brink DM, Van Miert JN, Dacremont G, Rontani JF, and Wanders RJ (2005) Characterization of the final step in the conversion of phytol into phytanic acid. *J Biol Chem* **280:** 26838-26844.
31. Gloerich J, Ruiter JP, Van den Brink DM, Ofman R, Ferdinandusse S, and Wanders RJ (2006) Peroxisomal trans-2-enoyl-CoA reductase is involved in phytol degradation. *FEBS Lett* **580:** 2092-2096.
32. Wanders RJ, Jansen GA, and Lloyd MD (2003) Phytanic acid alpha-oxidation, new insights into an old problem: a review. *Biochim Biophys Acta* **1631:** 119-135.
33. Singh I, Lazo O, Kalipada P, and Singh AK (1992) Phytanic acid alpha-oxidation in human cultured skin fibroblasts. *Biochim Biophys Acta* **1180:** 221-224.
34. Watkins PA, Howard AE, and Mihalik SJ (1994) Phytanic acid must be activated to phytanoyl-CoA prior to its alpha-oxidation in rat liver peroxisomes. *Biochim Biophys Acta* **1214:** 288-294.
35. Pahan K and Singh I (1995) Phytanic acid oxidation: topographical localization of phytanoyl-CoA ligase and transport of phytanic acid into human peroxisomes. *J Lipid Res* **36:** 986-997.
36. Mize CE, Avigan J, Steinberg D, Pittman RC, Fales HM, and Milne GW (1969) A major pathway for the mammalian oxidative degradation of phytanic acid. *Biochim Biophys Acta* **176:** 720-739.
37. Ten Brink HJ, Schor DS, Kok RM, Poll-The BT, Wanders RJ, and Jakobs C (1992) Phytanic acid alpha-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxisomal disorders. *J Lipid Res* **33:** 1449-1457.
38. Croes K, Casteels M, De Hoffmann E, Mannaerts GP, and Van Veldhoven PP (1996) alpha-Oxidation of 3-methyl-substituted fatty acids in rat liver. Production of formic acid instead of CO2, cofactor requirements, subcellular localization and formation of a 2-hydroxy-3-methylacyl-CoA intermediate. *Eur J Biochem* **240:** 674-683.
39. Jansen GA, Mihalik SJ, Watkins PA, Moser HW, Jakobs C, Denis S, and Wanders RJ (1996) Phytanoyl-CoA hydroxylase is present in human liver, located in peroxisomes, and deficient in Zellweger syndrome: direct, unequivocal evidence for the new, revised pathway of phytanic acid alpha-oxidation in humans. *Biochem Biophys Res Commun* **229:** 205-210.
40. Mihalik SJ, Rainville AM, and Watkins PA (1995) Phytanic acid alpha-oxidation in rat liver peroxisomes. Production of alpha-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors. *Eur J Biochem* **232:** 545-551.
41. Jansen GA, Ofman R, Denis S, Ferdinandusse S, Hogenhout EM, Jakobs C, and Wanders RJ (1999) Phytanoyl-CoA hydroxylase from rat liver. Protein purification and cDNA cloning with implications for the subcellular localization of phytanic acid alpha-oxidation. *J Lipid Res* **40:** 2244-2254.
42. Verhoeven NM, Wanders RJ, Schor DS, Jansen GA, and Jakobs C (1997) Phytanic acid alpha-oxidation: decarboxylation of 2-hydroxyphytanoyl-CoA to pristanic acid in human liver. *J Lipid Res* **38:** 2062-2070.
43. Verhoeven NM, Schor DS, Ten Brink HJ, Wanders RJ, and Jakobs C (1997) Resolution of the phytanic acid alpha-oxidation pathway: identification of pristanal as product of the decarboxylation of 2-hydroxyphytanoyl-CoA. *Biochem Biophys Res Commun* **237:** 33-36.
44. Foulon V, Antonenkov VD, Croes K, Waelkens E, Mannaerts GP, Van Veldhoven PP, and Casteels M (1999)

Purification, molecular cloning, and expression of 2-hydroxyphytanoyl-CoA lyase, a peroxisomal thiamine pyrophosphate-dependent enzyme that catalyzes the carbon-carbon bond cleavage during alpha-oxidation of 3-methyl-branched fatty acids. *Proc Natl Acad Sci U S A* **96:** 10039-10044.

45. Verhoeven NM, Jakobs C, Carney G, Somers MP, Wanders RJ, and Rizzo WB (1998) Involvement of microsomal fatty aldehyde dehydrogenase in the alpha-oxidation of phytanic acid. *FEBS Lett* **429:** 225-228.
46. Rizzo WB, Carney G, and Lin Z (1999) The molecular basis of Sjogren-Larsson syndrome: mutation analysis of the fatty aldehyde dehydrogenase gene. *Am J Hum Genet* **65:** 1547-1560.
47. Watkins PA (1997) Fatty acid activation. *Prog Lipid Res* **36:** 55-83.
48. Wanders RJ, Vreken P, Ferdinandusse S, Jansen GA, Waterham HR, Van Roermund CW, and Van Grunsven EG (2001) Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem Soc Trans* **29:** 250-267.
49. Schepers L, Van Veldhoven PP, Casteels M, Eyssen HJ, and Mannaerts GP (1990) Presence of three acyl-CoA oxidases in rat liver peroxisomes. An inducible fatty acyl-CoA oxidase, a noninducible fatty acyl-CoA oxidase, and a noninducible trihydroxycoprostanoyl-CoA oxidase. *J Biol Chem* **265:** 5242-5246.
50. Osumi T, Hashimoto T, and Ui N (1980) Purification and properties of acyl-CoA oxidase from rat liver. *J Biochem (Tokyo)* **87:** 1735-1746.
51. Van Veldhoven PP, Vanhove G, Assselberghs S, Eyssen HJ, and Mannaerts GP (1992) Substrate specificities of rat liver peroxisomal acyl-CoA oxidases: palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase), and trihydroxycoprostanoyl-CoA oxidase. *J Biol Chem* **267:** 20065-20074.
52. Van Veldhoven PP, Vanhove G, Vanhoutte F, Dacremont G, Parmentier G, Eyssen HJ, and Mannaerts GP (1991) Identification and purification of a peroxisomal branched chain fatty acyl-CoA oxidase. *J Biol Chem* **266:** 24676-24683.
53. Wanders RJ, Denis S, Jakobs C, and ten Brink HJ (1992) Identification of pristanoyl-CoA oxidase as a distinct, clofibrate non-inducible enzyme in rat liver peroxisomes. *Biochim Biophys Acta* **1124:** 199-202.
54. Casteels M, Schepers L, Van Veldhoven PP, Eyssen HJ, and Mannaerts GP (1990) Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycoprostanoyl-CoA in human liver. *J Lipid Res* **31:** 1865-1872.
55. Vanhove GF, Van Veldhoven PP, Fransen M, Denis S, Eyssen HJ, Wanders RJ, and Mannaerts GP (1993) The CoA esters of 2-methyl-branched chain fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acids are oxidized by one single peroxisomal branched chain acyl-CoA oxidase in human liver and kidney. *J Biol Chem* **268:** 10335-10344.
56. Furuta S, Miyazawa S, Osumi T, Hashimoto T, and Ui N (1980) Properties of mitochondria and peroxisomal enoyl-CoA hydratases from rat liver. *J Biochem (Tokyo)* **88:** 1059-1070.
57. Leenders F, Tesdorpf JG, Markus M, Engel T, Seedorf U, and Adamski J (1996) Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. *J Biol Chem* **271:** 5438-5442.
58. Dieuaide-Noubhani M, Novikov D, Baumgart E, Vanhooren JC, Fransen M, Goethals M, Vandekerckhove J, Van Veldhoven PP, and Mannaerts GP (1996) Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C27 bile acids di- and tri-hydroxycoprostanic acids are metabolized by separate multifunctional proteins. *Eur J Biochem* **240:** 660-666.
59. Jiang LL, Miyazawa S, and Hashimoto T (1996) Purification and properties of rat D-3-hydroxyacyl-CoA dehydratase: D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J Biochem (Tokyo)* **120:** 633-641.
60. Jiang LL, Kurosawa T, Sato M, Suzuki Y, and Hashimoto T (1997) Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J Biochem (Tokyo)* **121:** 506-513.
61. Qin YM, Poutanen MH, Helander HM, Kvist AP, Siivari KM, Schmitz W, Conzelmann E, Hellman U, and Hiltunen JK (1997) Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization. *Biochem J* **321 (Pt 1):** 21-28.
62. Dieuaide-Noubhani M, Asselberghs S, Mannaerts GP, and Van Veldhoven PP (1997) Evidence that multifunctional protein 2, and not multifunctional protein 1, is involved in the peroxisomal beta-oxidation of pristanic acid. *Biochem J* **325 (Pt 2):** 367-373.
63. Dieuaide-Noubhani M, Novikov D, Vandekerckhove J, Van Veldhoven PP, and Mannaerts GP (1997)

Identification and characterization of the 2-enoyl-CoA hydratases involved in peroxisomal beta-oxidation in rat liver. *Biochem J* **321 (Pt 1):** 253-259.

64. Qin YM, Haapalainen AM, Conry D, Cuebas DA, Hiltunen JK, and Novikov DK (1997) Recombinant 2-enoyl-CoA hydratase derived from rat peroxisomal multifunctional enzyme 2: role of the hydratase reaction in bile acid synthesis. *Biochem J* **328 (Pt 2):** 377-382.
65. Xu R and Cuebas DA (1996) The reactions catalyzed by the inducible bifunctional enzyme of rat liver peroxisomes cannot lead to the formation of bile acids. *Biochem Biophys Res Commun* **221:** 271-278.
66. Suzuki Y, Jiang LL, Souri M, Miyazawa S, Fukuda S, Zhang Z, Une M, Shimozawa N, Kondo N, Orii T, and Hashimoto T (1997) D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein deficiency: a newly identified peroxisomal disorder. *Am J Hum Genet* **61:** 1153-1162.
67. Van Grunsven EG, Van Berkel E, IJlst L, Vreken P, de Klerk JB, Adamski J, Lemonde H, Clayton PT, Cuebas DA, and Wanders RJ (1998) Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc Natl Acad Sci U S A* **95:** 2128-2133.
68. Van Grunsven EG, Mooijer PA, Aubourg P, and Wanders RJ (1999) Enoyl-CoA hydratase deficiency: identification of a new type of D- bifunctional protein deficiency. *Hum Mol Genet* **8:** 1509-1516.
69. Van Grunsven EG, Van Berkel E, Mooijer PA, Watkins PA, Moser HW, Suzuki Y, Jiang LL, Hashimoto T, Hoefler G, Adamski J, and Wanders RJ (1999) Peroxisomal bifunctional protein deficiency revisited: resolution of its true enzymatic and molecular basis. *Am J Hum Genet* **64:** 99-107.
70. Ferdinandusse S, Denis S, Van Roermund CW, Wanders RJ, and Dacremont G (2004) Identification of the peroxisomal beta-oxidation enzymes involved in the degradation of long-chain dicarboxylic acids. *J Lipid Res* **45:** 1104-1111.
71. Miyazawa S, Osumi T, and Hashimoto T (1980) The presence of a new 3-oxoacyl-CoA thiolase in rat liver peroxisomes. *Eur J Biochem* **103:** 589-596.
72. Seedorf U, Brysch P, Engel T, Schrage K, and Assmann G (1994) Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. *J Biol Chem* **269:** 21277-21283.
73. Antonenkov VD, Van Veldhoven PP, Waelkens E, and Mannaerts GP (1997) Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates. *J Biol Chem* **272:** 26023-26031.
74. Bun-ya M, Maebuchi M, Kamiryo T, Kurosawa T, Sato M, Tohma M, Jiang LL, and Hashimoto T (1998) Thiolase involved in bile acid formation. *J Biochem (Tokyo)* **123:** 347-352.
75. Wanders RJ, Denis S, Wouters F, Wirtz KW, and Seedorf U (1997) Sterol carrier protein X (SCPx) is a peroxisomal branched-chain beta-ketothiolase specifically reacting with 3-oxo-pristanoyl-CoA: a new, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes. *Biochem Biophys Res Commun* **236:** 565-569.
76. Wanders RJ, Denis S, Van Berkel E, Wouters F, Wirtz KW, and Seedorf U (1998) Identification of the newly discovered 58 kDa peroxisomal thiolase SCPx as the main thiolase involved in both pristanic acid and trihydroxycholestanoic acid oxidation: implications for peroxisomal beta-oxidation disorders. *J Inherit Metab Dis* **21:** 302-305.
77. Reddy JK and Mannaerts GP (1994) Peroxisomal lipid metabolism. *Annu Rev Nutr* **14:** 343-370.
78. Wanders RJ and Tager JM (1998) Lipid metabolism in peroxisomes in relation to human disease. *Mol Aspects Med* **19:** 69-154.
79. Bartlett K and Eaton S (2004) Mitochondrial beta-oxidation. *Eur J Biochem* **271:** 462-469.
80. Eaton S, Bartlett K, and Pourfarzam M (1996) Mammalian mitochondrial beta-oxidation. *Biochem J* **320 (Pt 2):** 345-357.
81. Ferdinandusse S, Mulders J, IJlst L, Denis S, Dacremont G, Waterham HR, and Wanders RJ (1999) Molecular cloning and expression of human carnitine octanoyltransferase: evidence for its role in the peroxisomal beta-oxidation of branched-chain fatty acids. *Biochem Biophys Res Commun* **263:** 213-218.
82. Verhoeven NM, Roe DS, Kok RM, Wanders RJ, Jakobs C, and Roe CR (1998) Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J Lipid Res* **39:** 66-74.
83. Kerner J and Hoppel C (2000) Fatty acid import into mitochondria. *Biochim Biophys Acta* **1486:** 1-17.
84. Schmitz W, Fingerhut R, and Conzelmann E (1994) Purification and properties of an alpha-methylacyl-CoA

racemase from rat liver. *Eur J Biochem* **222:** 313-323.
85. Schmitz W, Albers C, Fingerhut R, and Conzelmann E (1995) Purification and characterization of an alpha-methylacyl-CoA racemase from human liver. *Eur J Biochem* **231:** 815-822.
86. Kitareewan S, Burka LT, Tomer KB, Parker CE, Deterding LJ, Stevens RD, Forman BM, Mais DE, Heyman RA, McMorris T, and Weinberger C (1996) Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Mol Biol Cell* **7:** 1153-1166.
87. Lemotte PK, Keidel S, and Apfel CM (1996) Phytanic acid is a retinoid X receptor ligand. *Eur J Biochem* **236:** 328-333.
88. Lemberger T, Desvergne B, and Wahli W (1996) Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol* **12:** 335-363.
89. Wolfrum C, Ellinghaus P, Fobker M, Seedorf U, Assmann G, Borchers T, and Spener F (1999) Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. *J Lipid Res* **40:** 708-714.
90. Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T, and Kawada T (2005) Phytol directly activates peroxisome proliferator-activated receptor alpha (PPARalpha) and regulates gene expression involved in lipid metabolism in PPARalpha-expressing HepG2 hepatocytes. *Biochem Biophys Res Commun* **337:** 440-445.
91. Berger J and Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53:** 409-435.
92. Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, and Rao MS (2000) Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem* **275:** 28918-28928.
93. Forman BM, Chen J, and Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* **94:** 4312-4317.
94. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A* **94:** 4318-4323.
95. Moya-Camarena SY, Van den Heuvel JP, Blanchard SG, Leesnitzer LA, and Belury MA (1999) Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARalpha. *J Lipid Res* **40:** 1426-1433.
96. Gonzalez FJ (2002) The peroxisome proliferator-activated receptor alpha (PPARalpha): role in hepatocarcinogenesis. *Mol Cell Endocrinol* **193:** 71-79.
97. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, and Wahli W (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* **103:** 1489-1498.
98. Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, and Gonzalez FJ (1998) Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). *J Biol Chem* **273:** 5678-5684.
99. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, and Gonzalez FJ (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* **15:** 3012-3022.
100. Wanders RJ and Waterham HR (2005) Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. *Clin Genet* **67:** 107-133.
101. Gould SJ, Raymond GV, Valle D. The peroxisome biogenesis disorders. In: Scriver CR, Beaudet AL, Valle D, Sly WS, eds. *The metabolic and molecular bases of inherited disease.* 8 ed. New York: McGraw-Hill, 2001:3181-3217
102. Kelley RI, Datta NS, Dobyns WB, Hajra AK, Moser AB, Noetzel MJ, Zackai EH, and Moser HW (1986) Neonatal adrenoleukodystrophy: new cases, biochemical studies, and differentiation from Zellweger and related peroxisomal polydystrophy syndromes. *Am J Med Genet* **23:** 869-901.
103. Poll-The BT, Saudubray JM, Ogier HA, Odievre M, Scotto JM, Monnens L, Govaerts LC, Roels F, Cornelis A, and Schutgens RB (1987) Infantile Refsum disease: an inherited peroxisomal disorder. Comparison with Zellweger syndrome and neonatal adrenoleukodystrophy. *Eur J Pediatr* **146:** 477-483.
104. Datta NS, Wilson GN, and Hajra AK (1984) Deficiency of enzymes catalyzing the biosynthesis of glycerol-ether lipids in Zellweger syndrome. A new category of metabolic disease involving the absence of peroxisomes. *N Engl J Med* **311:** 1080-1083.
105. Heymans HS, Schutgens RB, Tan R, Van den Bosch H, and Borst P (1983) Severe plasmalogen deficiency in

tissues of infants without peroxisomes (Zellweger syndrome). *Nature* **306:** 69-70.
106. Wanders RJ, Schutgens RB, and Barth PG (1995) Peroxisomal disorders: a review. *J Neuropathol Exp Neurol* **54:** 726-739.
107. Jansen GA, Ofman R, Ferdinandusse S, IJlst L, Muijsers AO, Skjeldal OH, Stokke O, Jakobs C, Besley GT, Wraith JE, and Wanders RJ (1997) Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. *Nat Genet* **17:** 190-193.
108. Mihalik SJ, Morrell JC, Kim D, Sacksteder KA, Watkins PA, and Gould SJ (1997) Identification of PAHX, a Refsum disease gene. *Nat Genet* **17:** 185-189.
109. Wanders RJA, Jacobs C, Skjeldal OH. Refsum Disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic & Molecular Bases of Inherited Disease.* 8 ed. New York: McGraw-Hill, 2001:3303-3321
110. Ferdinandusse S, Denis S, Clayton PT, Graham A, Rees JE, Allen JT, McLean BN, Brown AY, Vreken P, Waterham HR, and Wanders RJ (2000) Mutations in the gene encoding peroxisomal alpha-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy. *Nat Genet* **24:** 188-191.
111. Van Veldhoven PP, Meyhi E, Squires RH, Fransen M, Fournier B, Brys V, Bennett MJ, and Mannaerts GP (2001) Fibroblast studies documenting a case of peroxisomal 2-methylacyl-CoA racemase deficiency: possible link between racemase deficiency and malabsorption and vitamin K deficiency. *Eur J Clin Invest* **31:** 714-722.
112. Gloerich J, Denis S, Van Grunsven EG, Dacremont G, Wanders RJ, and Ferdinandusse S (2003) A novel HPLC-based method to diagnose peroxisomal D-bifunctional protein enoyl-CoA hydratase deficiency. *J Lipid Res* **44:** 640-644.
113. Ferdinandusse S, Kostopoulos P, Denis S, Rusch H, Overmars H, Dillmann U, Reith W, Haas D, Wanders RJA, Duran M, and Marziniak M (2006) Mutations in the gene encoding peroxisomal sterol carrier protein X (SCPx) cause leukencephalopathy with dystonia and and motor neuropathy. *Am J Hum Genet* **78:** 1046-1052.
114. Willemsen MA, IJlst L, Steijlen PM, Rotteveel JJ, de Jong JG, Van Domburg PH, Mayatepek E, Gabreels FJ, and Wanders RJ (2001) Clinical, biochemical and molecular genetic characteristics of 19 patients with the Sjogren-Larsson syndrome. *Brain* **124:** 1426-1437.
115. De Laurenzi V, Rogers GR, Hamrock DJ, Marekov LN, Steinert PM, Compton JG, Markova N, and Rizzo WB (1996) Sjogren-Larsson syndrome is caused by mutations in the fatty aldehyde dehydrogenase gene. *Nat Genet* **12:** 52-57.
116. Rizzo WB and Craft DA (1991) Sjogren-Larsson syndrome. Deficient activity of the fatty aldehyde dehydrogenase component of fatty alcohol:NAD+ oxidoreductase in cultured fibroblasts. *J Clin Invest* **88:** 1643-1648.
117. Rizzo WB, Dammann AL, Craft DA, Black SH, Tilton AH, Africk D, Chaves-Carballo E, Holmgren G, and Jagell S (1989) Sjogren-Larsson syndrome: inherited defect in the fatty alcohol cycle. *J Pediatr* **115:** 228-234.
118. Rizzo WB and Craft DA (2000) Sjogren-Larsson syndrome: accumulation of free fatty alcohols in cultured fibroblasts and plasma. *J Lipid Res* **41:** 1077-1081.
119. Willemsen MA, Rotteveel JJ, de Jong JG, Wanders RJ, IJlst L, Hoffmann GF, and Mayatepek E (2001) Defective metabolism of leukotriene B4 in the Sjogren-Larsson syndrome. *J Neurol Sci* **183:** 61-67.
120. Aoki N, Suzuki H, Ito K, and Ito M (2000) A novel point mutation of the FALDH gene in a Japanese family with Sjogren-Larsson syndrome. *J Invest Dermatol* **114:** 1065-1066.
121. De Laurenzi V, Rogers GR, Tarcsa E, Carney G, Marekov L, Bale SJ, Compton JG, Markova N, Steinert PM, and Rizzo WB (1997) Sjogren-Larsson syndrome is caused by a common mutation in northern European and Swedish patients. *J Invest Dermatol* **109:** 79-83.
122. Kraus C, Braun-Quentin C, Ballhausen WG, and Pfeiffer RA (2000) RNA-based mutation screening in German families with Sjogren-Larsson syndrome. *Eur J Hum Genet* **8:** 299-306.
123. Rizzo WB, Carney G, and De Laurenzi V (1997) A common deletion mutation in European patients with Sjogren-Larsson syndrome. *Biochem Mol Med* **62:** 178-181.
124. Sillen A, Jagell S, and Wadelius C (1997) A missense mutation in the FALDH gene identified in Sjogren-Larsson syndrome patients originating from the northern part of Sweden. *Hum Genet* **100:** 201-203.
125. Sillen A, Anton-Lamprecht I, Braun-Quentin C, Kraus CS, Sayli BS, Ayuso C, Jagell S, Kuster W, and Wadelius C (1998) Spectrum of mutations and sequence variants in the FALDH gene in patients with Sjogren-Larsson syndrome. *Hum Mutat* **12:** 377-384.
126. Tsukamoto N, Chang C, and Yoshida A (1997) Mutations associated with Sjogren-Larsson syndrome. *Ann*

Hum Genet **61 (Pt 3):** 235-242.

127. Willemsen MA, Steijlen PM, de Jong JG, Rotteveel JJ, IJlst L, Van Werkhoven MA, and Wanders RJ (1999) A novel 4 bp deletion mutation in the FALDH gene segregating in a Turkish family with Sjogren-Larsson syndrome. *J Invest Dermatol* **112:** 827-828.
128. Gloerich J, IJlst L, Wanders RJ, and Ferdinandusse S (2006) Bezafibrate induces FALDH in human fibroblasts; implications for Sjögren-Larsson syndrome. *Mol Genet Metab* **89:** 111-115.

2

A novel HPLC-based method to diagnose peroxisomal D-bifunctional protein enoyl-CoA hydratase deficiency

Jolein Gloerich, Simone Denis, Elisabeth G. van Grunsven, Georges Dacremont, Ronald J. A. Wanders and Sacha Ferdinandusse (2003) *J Lipid Res* **44**, 640-644

A novel HPLC-based method to diagnose peroxisomal D-bifunctional protein enoyl-CoA hydratase deficiency

J. Gloerich[*], S. Denis[*], E.G. van Grunsven[*], G. Dacremont[†], R.J.A. Wanders[*] and S. Ferdinandusse[*]

[*]*Laboratory for Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma's Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.* [†]*Department of Pediatrics, University of Ghent, 9000 Ghent, Belgium.*

Abstract

D-bifunctional protein (DBP) plays an indispensable role in peroxisomal β-oxidation, and its inherited deficiency in humans is associated with severe clinical abnormalities. Three different subtypes of DBP deficiency can be distinguished: *1*) a complete deficiency of DBP (type I), *2*) an isolated DBP enoyl-CoA hydratase deficiency (type II), and *3*) an isolated DBP 3-hydroxyacyl-CoA dehydrogenase deficiency (type III). In this study, we developed a method to measure DBP dehydrogenase activity independent of DBP hydratase (DBP HY) activity to distinguish between DBP deficiency type I and type II, which until now was only possible by mutation analysis. For this assay, the hydratase domain of DBP was expressed in the yeast *Saccharomyces cerevisiae*. After a coincubation of yeast homogenate expressing DBP HY with fibroblast homogenate of patients using the enoyl-CoA ester of the bile acid intermediate trihydroxycholestanoic acid as substrate, DBP dehydrogenase activity was measured. Fibroblasts of patients with a DBP deficiency type II displayed DBP dehydrogenase activity, whereas type I and type III patients did not. This newly developed assay to measure DBP dehydrogenase activity in fibroblast homogenates provides a quick and reliable method to assign patients with deficient DBP HY activity to the DBP deficiency subgroups type I or type II.

Introduction

In humans, both mitochondria and peroxisomes are able to β-oxidize fatty acids. Peroxisomal β-oxidation is essential for the oxidation of a distinct set of substrates that cannot be broken down by the mitochondrial β-oxidation system. Substrates of the peroxisomal β-oxidation system include *1*) very long-chain fatty acids, *2*) α-methyl-branched-chain fatty acids such as pristanic acid, and *3*) the bile acid intermediates dihydroxycholestanoic acid and trihydroxycholestanoic acid (DHCA and THCA, respectively) (as reviewed by Wanders et al.[1]). Peroxisomal β-oxidation is impaired in peroxisomal biogenesis disorders, as well as in patients with a single enzyme defect in the peroxisomal β-oxidation system.[2] One of the single enzyme defects in peroxisomal β-oxidation is a deficiency of D-bifunctional protein (DBP; also known as MFE-2, MFP-2, D-PBE). The enoyl-CoA hydratase (DBP HY) and 3-hydroxyacyl-CoA dehydrogenase (DBP DH) domains of DBP catalyze the second and third step of peroxisomal β-oxidation, respectively. DBP deficiency can be divided into three subgroups. In the first group, the patients have a deficiency in both the hydratase

and dehydrogenase component of DBP (type I).[3,4] In the second group, only the hydratase component of DBP (type II) is deficient,[5] and in the third group, only the dehydrogenase component of DBP is deficient (type III).[3]

In the assay that is currently used to diagnose DBP deficiency,[6] DBP activity is measured by incubating fibroblast homogenates with the enoyl-CoA ester of the bile acid intermediate THCA (THC:1-CoA). From this substrate, 24-hydroxy-THC-CoA is produced by the action of DBP HY, which is subsequently converted into 24-keto-THC-CoA via the dehydrogenase component of DBP. With this assay, however, no distinction can be made between DBP deficiency type I and II, since the substrate for DBP DH cannot be synthesized by the defective hydratase component of the patient's DBP.

Until now, the only way to differentiate between these two types of DBP deficiency was by mutation analysis. To be able to distinguish between these two types of DBP deficiency in a less time-consuming and laborious way, we have developed a method to measure DBP DH activity in fibroblast homogenates of patients with a defective DBP HY based on the original DBP assay described above. In our newly developed assay, the inability to form 24-hydroxy-THC-CoA because of the patient's defective DBP HY is overcome by adding wild-type DBP HY expressed in yeast to the reaction mixture. The 24-hydroxy-THC-CoA formed is used as substrate for the patient's DBP DH. This way, formation of 24-keto-THC-CoA by DBP DH can be measured despite of DBP HY deficiency. In this study, we have assigned several patients with DBP deficiency to the DBP deficiency subgroups type I or type II with the help of our newly developed method.

Materials and Methods

Patient cell lines

All patient cell lines used in this study were taken from the cell repository of the laboratory for Genetic Metabolic Diseases (Academical Medical Center, University of Amsterdam, The Netherlands) and were derived from patients diagnosed in this Center. Informed consent was obtained from parents or guardians of the patients whose fibroblasts were studied in this article.

DBP enoyl-CoA hydratase expression in yeast

An expression vector containing the coding sequence of the human wild-type enoyl-CoA hydratase domain of DBP was used for the DBP HY expression in yeast. This plasmid (pHY-WT) was constructed as described.[5] The yeast strain used in this study, *Saccharomyces cerevisiae* (*MATα*, *leu2*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*), was transformed with pHY-WT using the lithium acetate procedure.[7] Transformed yeast cells were grown at 28°C on minimal essential medium (0.67% yeast nitrogen base without amino acids (YNB-WO, Difco Laboratories Inc., Detroit, MI), 0.3% glucose and amino acids (20–30 µg/ml) as required). To induce expression, cells were shifted to rich oleic acid medium (50 mM potassium phosphate, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.12% oleic acid, and 0.2% Tween-80).

Cells were harvested by centrifugation and washed twice with water. Yeast homogenates were made by resuspending cells in lysis buffer containing 40% (v/v) glycerol and 1 tablet-10 ml Complete mini protease inhibitor cocktail (Roche, Basel, Switzerland) in PBS, and subsequently disrupting cells by agitation at 4°C for 15 min on a vortex mixer in the presence of glass beads (Ø = 0.45 mm). The homogenate was centrifuged at 2,000 x *g* at 4°C for 2 min

and the supernatant was kept at -20°C until used for enzyme activity measurements.

DBP activity measurements

The activity of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of DBP were measured as described by Van Grunsven, with minor modifications.[6] Briefly, fibroblast homogenates were incubated for 30 min at 37°C in a reaction mixture (total volume 100 µl) containing 50 mM Bis-Tris Propane (BTP) pH 9.0, 1 mM NAD^+, 150 mM KCl, 0.1 mM 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA (THC:1-CoA, prepared as described by Xu and Cuebas[8]), 0.5 mM oxaloacetate, and 0.5 U/ml malate dehydrogenase. The final protein concentration was 0.3 mg/ml. Reactions were terminated by the addition of 2 M HCl to a final concentration of 0.18 M, followed by neutralization to a pH of ~5 by addition of 0.6 M MES plus 2 M KOH. After addition of acetonitril to the samples, resolution of the different CoA-esters was achieved by High Performance Liquid Chromatography (HPLC).

For measurement of 3-hydroxyacyl-CoA dehydrogenase activity in fibroblasts of DBP enoyl-CoA hydratase deficient patients, a DBP DH assay was developed based on the DBP assay described above. In the DBP DH assay, 0.3 mg/ml yeast homogenate expressing DBP HY was coincubated with fibroblast homogenate to synthesize the substrate for the dehydrogenase component of DBP, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oyl-CoA (24-hydroxy-THC-CoA). The assay was optimized for protein concentration and incubation time.

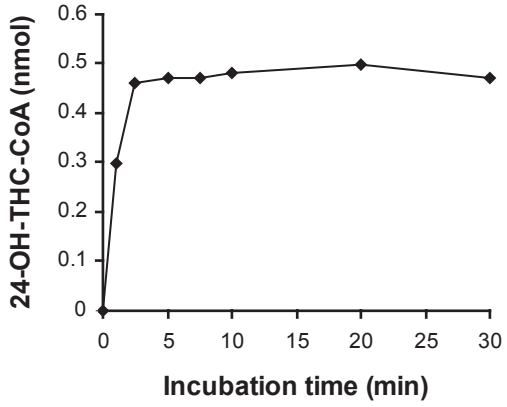


Figure 1 Activity of DBP enoyl-CoA hydratase (DBP HY) in homogenates of yeast cells transformed with the wild-type DBP HY domain. The total amount of 24-hydroxy-THC-CoA formed (nmol) is depicted against reaction time in minutes.

Immunoblot analysis

Patient fibroblast homogenates used for measuring DBP DH activity were also used for immunoblot analysis of DBP. Fifty micrograms of fibroblast homogenate was separated on a 10% (w/v) SDS-polyacrylamide gel, essentially as described by Laemmli,[9] and transferred to a nitrocellulose membrane (Schleicher and Shuell, Keene, NH). DBP was detected using an antiserum raised against DBP.[3] Goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase was used for detection, according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Densitometric analysis of the immunoblot was performed using NIH Image 1.61.

Results and Discussion

Hydratase activity in yeast homogenates

In order to measure the activity of DBP DH independent of DBP HY activity, the substrate for DBP DH, 24-hydroxy-THC-CoA, is needed. To synthesize this substrate, wild-type DBP HY was expressed in the yeast *S. cerevisiae*. Activity of DBP HY in yeast homogenate was measured in time, using the DBP activity assay (**Figure 1**) . Optimal levels of 24-hydroxy-THC-CoA were reached within 3 min of incubation. Increasing the amount of yeast homogenate in the reaction mixture from 0.3 mg/ml to 0.9 mg/ml did not lead to an increase in the amount of 24-hydroxy-THC-CoA formed. In homogenates of yeast cells transformed with the empty plasmid, no DBP HY activity could be detected. DBP HY yeast homogenates displayed no DBP DH activity.

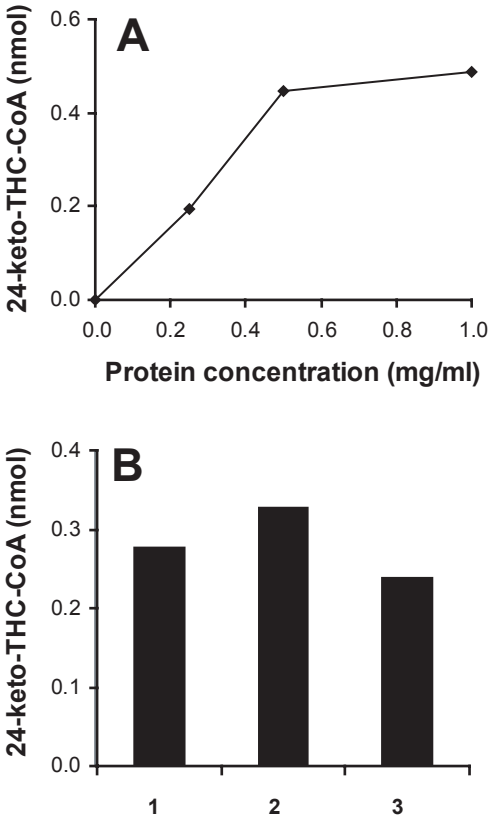


Figure 2. The formation of keto-THC-CoA by the action of DBP 3-hydroxyacyl-CoA dehydrogenase (DBP DH) in fibroblasts of a control subject (A) measured after a preincubation of 15 min with 0.3 mg/ml DBP HY yeast homogenate and an incubation of 30 min with different concentrations of fibroblast homogenate, and (B) measured after *1*) a coincubation of 30 min, *2*) a coincubation of 45 min with both 0.3 mg/ml yeast DBP HY and 0.5 mg/ml fibroblast homogenate of a control subject, and *3*) a preincubation of 15 min with 0.3 mg/ml DBP HY yeast homogenate and an incubation of 30 min with 0.5 mg/ml fibroblast homogenate of a control subject.

DBP DH assay

To be able to distinguish between patients with either DBP deficiency type I or DBP deficiency type II without having to sequence the gene, we developed a method to determine DBP DH activity in fibroblast homogenates. We used DBP HY expressed in yeast to generate 24-hydroxy-THC-CoA, the substrate for the DH component. To determine optimal conditions for this assay, fibroblast homogenates of control subjects were used.

First, the optimal protein concentration of fibroblast homogenate was determined by measuring the formation of 3α,7α,12α-trihydroxy-24-keto-5β-cholestanoyl-CoA (24-keto-THC-CoA) at different protein concentrations after a preincubation of 15 min with 0.3 mg/ml DBP HY yeast homogenate (**Figure 2A**) . Formation of 24-keto-THC-CoA was linear with protein concentration up to a concentration of 0.5 mg/ml. Based on these findings, a concentration of 0.5 mg/ml was used in future experiments.

Second, the optimal reaction time was determined. Two different assay set-ups were tested: *1*) a preincubation of 15 min with DBP yeast homogenate before addition of the fibroblast homogenate, and *2*) a coincubation of DBP HY yeast homogenate and fibroblast homogenate. Results are shown in **figure 2B**. Because sufficient 24-keto-THC-CoA was formed to be readily detectable, a coincubation of fibroblast and DBP HY yeast homogenate of 45 min was chosen for standard assay condition.

Table 1: Specific activity of DBP 3-hydroxyacyl-CoA dehydrogenase in fibroblast homogenates of controls and patients suffering from DBP deficiency type I and type II

Mutation	**Specific activity D-BP DH**	**References**
	pmol/min/mg	
Control (n=3)	125 ±30	
D-BP deficiency type I		
281-302 del (22 bp)	n.d.	10
281-622 del (342 bp)	n.d.	11
422-423 del (2 bp)	n.d.	5
869-881 del (13 bp)	n.d.	3
1211-1262 del (52 bp)	n.d.	4
D-BP deficiency type II		
N457Y	70	5
1439-1504 del (66 bp)	13	11
Patient 1	101	
Patient 2	90	
Patient 3	39	
Patient 4	39	
Patient 5	31	
Patient 6	30	
Patient 7	19	

DBP DH, DBP 3-hydroxyacyl-CoA dehydrogenase; n.d., not detectable

DBP DH activity in DBP deficient patients

To determine the specificity of our newly developed assay, DBP DH activities were measured in fibroblast homogenates of DBP deficient patients who were assigned to the three different subgroups by mutation analysis and expression studies (DBP deficiency type I, five patients; DBP deficiency type II, two patients; DBP deficiency type III, four patients).[3-6,10,11] None of the patients with DBP deficiency type I and type III showed any residual DBP DH activity in fibroblast homogenates. In contrast, all patients with DBP deficiency type II did display DBP DH activity in fibroblast homogenates.

With our newly developed assay, we subsequently measured DBP DH activity in 18 patients with deficient DBP HY who had not yet been assigned to subgroup DBP deficiency type I or type II. Based on these measurements we were able to assign seven of these patients

to subgroup II, and 11 patients to subgroup I. In **table 1**, the specific activities of DBP DH measured in fibroblast homogenates of these patients are shown. Mutations are listed for the patients used to validate the DBP DH activity assay.

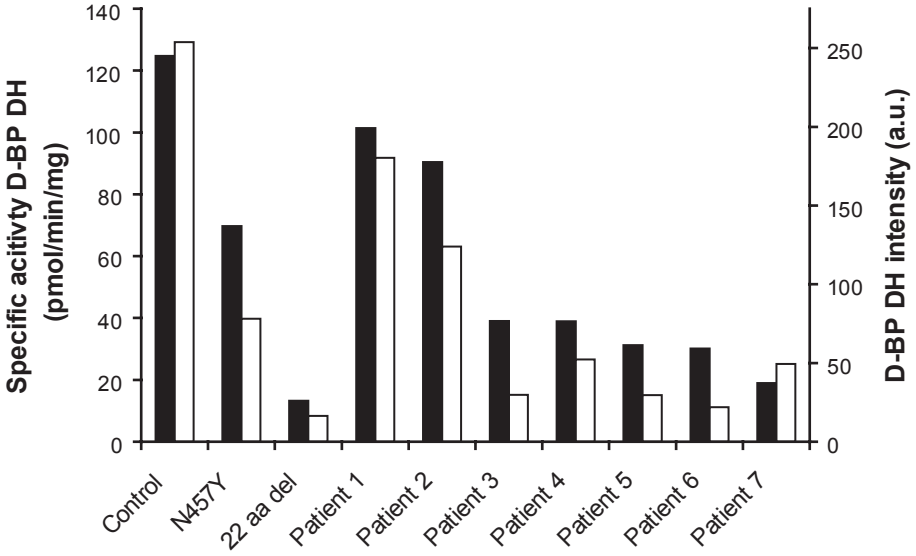


Figure 3: Specific activity of DBP DH measured in controls and DBP deficiency type II patients (two patients with described mutations, others determined by DBP DH assay) are depicted as black bars and the intensity of the DBP DH bands on immunoblot (the 79 kDa band of full length DBP plus the 35 kDa band of the DBP DH component) are depicted as white bars.

Immunoblot analysis of DBP in type II deficient patients

To study the relation between the measured DBP DH activity and the DBP protein level, immunoblot analysis was performed using antibodies against DBP. In control fibroblasts, three major bands could be detected: the full-length protein (79 kDa), the DBP HY component (45 kDa), and the DBP DH component (35 kDa). Full length DBP is proteolytically cleaved into two fragments. This process occurs after import of DBP into peroxisomes because patients with a defect in the peroxisomal targeting signal (PTS)1 receptor (Pex5p), who cannot import DBP into peroxisomes, only have full length DBP. Cleavage of DBP is not necessary for the activity of the protein, since DBP activity is found in fibroblast homogenates of patients with a defective PTS1 receptor.[5] In all fibroblast homogenates of DBP deficiency type II patients, both the 79 kDa and the 35 kDa band were reduced in abundancy compared with controls, but they were present, whereas the 45 kDa band of DBP HY was absent in all DBP deficiency type II patient fibroblasts, except for patient 1 (data not shown).

To compare DBP DH activity with the total amount of active DBP DH protein, the intensities of the 79 kDa and 35 kDa bands were determined by densitometric analysis. These two intensities together are a measure for the total amount of DBP DH present in the homogenate. In **figure 3**, both specific activities and blot intensities are plotted for all measured DBP deficiency type II patients. In all patients, the measured DBP DH activity was in good agreement with the amount of total DBP DH protein. These results indicate that mutations in

the DBP HY fragment might destabilize the full length DBP in a way that only little of the full-length protein reaches the peroxisome, where cleavage takes place. This destabilization of the DBP is probably dependent on the type of mutation in the hydratase domain of the protein, since both the DBP DH activity and the amount of protein was much higher in fibroblasts of a patient with a single amino acid substitution (N457Y) than in fibroblasts of a patient with a deletion of 22 amino acids (480–501 del). To substantiate this hypothesis, mutations of all assayed DBP deficiency type II patients will be determined.

Summarizing, the DBP DH assay described in this paper provides a quick and reliable method to differentiate between DBP deficiency type I and II. This assay can be performed to determine the DBP deficiency subgroup instead of time-absorbing methods like mutation analysis and expression studies of the protein in yeast. The DBP DH assay in combination with the normal DBP assay[6] make it possible to distinguish between all three types of DBP deficiency on the basis of DBP enzyme activities. This combination of assays is also very suitable for prenatal diagnosis of DBP deficiency.

Acknowledgments

This work was supported by the Netherlands Organization for Scientific Research (NWO, The Hague, Netherlands) grant number 901-03-097.

References

1. Wanders RJ, Vreken P, Ferdinandusse S, Jansen GA, Waterham HR, van Roermund CW, and Van Grunsven EG (2001) Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem Soc Trans* **29:** 250-267.
2. Wanders RJ, Schutgens RB, and Barth PG (1995) Peroxisomal disorders: a review. *J Neuropathol Exp Neurol* **54:** 726-739.
3. Van Grunsven EG, van Berkel E, Mooijer PA, Watkins PA, Moser HW, Suzuki Y, Jiang LL, Hashimoto T, Hoefler G, Adamski J, and Wanders RJ (1999) Peroxisomal bifunctional protein deficiency revisited: resolution of its true enzymatic and molecular basis. *Am J Hum Genet* **64:** 99-107.
4. Suzuki Y, Jiang LL, Souri M, Miyazawa S, Fukuda S, Zhang Z, Une M, Shimozawa N, Kondo N, Orii T, and Hashimoto T (1997) D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein deficiency: a newly identified peroxisomal disorder. *Am J Hum Genet* **61:** 1153-1162.
5. Van Grunsven EG, Mooijer PA, Aubourg P, and Wanders RJ (1999) Enoyl-CoA hydratase deficiency: identification of a new type of D- bifunctional protein deficiency. *Hum Mol Genet* **8:** 1509-1516.
6. Van Grunsven EG, van Berkel E, IJlst L, Vreken P, de Klerk JB, Adamski J, Lemonde H, Clayton PT, Cuebas DA, and Wanders RJ (1998) Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc Natl Acad Sci U S A* **95:** 2128-2133.
7. Becker DM and Guarente L (1991) High-efficiency transformation of yeast by electroporation. *Methods Enzymol* **194:** 182-187.
8. Xu R and Cuebas DA (1996) The reactions catalyzed by the inducible bifunctional enzyme of rat liver peroxisomes cannot lead to the formation of bile acids. *Biochem Biophys Res Commun* **221:** 271-278.
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
10. Paton BC and Pollard AN (2000) Molecular changes in the D-bifunctional protein cDNA sequence in Australasian patients belonging to the bifunctional protein complementation group. *Cell Biochem Biophys* **32 Spring:** 247-251.
11. Van Grunsven EG and Wanders RJ (1997) Genetic heterogeneity in patients with a disorder of peroxisomal beta- oxidation: a complementation study based on pristanic acid beta- oxidation suggesting different enzyme defects. *J Inherit Metab Dis* **20:** 437-440.

3

A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARα-dependent and -independent pathways

Jolein Gloerich, Naomi van Vlies, Gerbert A. Jansen, Simone Denis, Jos P.N. Ruiter, Michiel A. van Werkhoven, Marinus Duran, Frédéric M. Vaz, Ronald J.A. Wanders and Sacha Ferdinandusse (2004) *J Lipid Res* **46**, 716-726

A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARα-dependent and -independent pathways

J. Gloerich, N. van Vlies, G.A. Jansen, S. Denis, J.P.N. Ruiter, M.A. van Werkhoven, M. Duran, F.M. Vaz, R.J.A. Wanders, S. Ferdinandusse.

Laboratory for Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma's Children's Hospital, Academic Medical Center,University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Abstract

Branched-chain fatty acids (such as phytanic and pristanic acid) are ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα) *in vitro*. To investigate the effects of these physiological compounds *in vivo*, wild-type and PPARα-deficient (PPARα$^{-/-}$) mice were fed a phytol-enriched diet. This resulted in increased plasma and liver levels of the phytol metabolites phytanic and pristanic acid. In wild-type mice, plasma fatty acid levels decreased after phytol feeding, whereas in PPARα$^{-/-}$ mice, the already elevated fatty acid levels increased. In addition, PPARα$^{-/-}$ mice were found to be carnitine deficient in both plasma and liver. Dietary phytol increased liver free carnitine in wild-type animals but not in PPARα$^{-/-}$ mice. Investigation of carnitine biosynthesis revealed that PPARα is likely involved in the regulation of carnitine homeostasis. Furthermore, phytol feeding resulted in a PPARα-dependent induction of various peroxisomal and mitochondrial β-oxidation enzymes. In addition, a PPARα-independent induction of catalase, phytanoyl-CoA hydroxylase, carnitine octanoyltransferase, peroxisomal 3-ketoacyl-CoA thiolase, and straight-chain acyl-CoA oxidase was observed.

In conclusion, branched-chain fatty acids are physiologically relevant ligands of PPARα in mice. These findings are especially relevant for disorders in which branched-chain fatty acids accumulate, such as Refsum disease and peroxisome biogenesis disorders.

Introduction

Peroxisome proliferator-activated receptor α (PPARα) is one of the PPARs that form a subfamily of the nuclear hormone receptor superfamily. PPARs are ligand-activated transcription factors: after ligand binding, PPARs heterodimerize with the retinoic X receptor α and modulate the expression of target genes by binding to specific peroxisome proliferator response elements in the promotor region of regulated genes.[1] Three PPAR isoforms are known: PPARα, PPARβ, and PPARγ. The three isoforms have different tissue distributions and functions. PPARα is mostly expressed in organs with a high rate of fatty acid catabolism, such as brown adipose tissue, liver, kidney, and heart, and it plays an important role in various aspects of lipid and glucose metabolism.[2,3]

PPARα has a broad range of both artificial and natural ligands also called peroxisome proliferators. The artificial ligands of PPARα consist of a variety of compounds, including hypolipidemic drugs (e.g., clofibrate and Wy-14,643), phthalate ester plasticizers, herbicides,

and several chlorinated hydrocarbons. A broad array of unsaturated fatty acids, but also long-chain fatty acids and branched-chain fatty acids (e.g., phytanic acid), are natural ligands for PPARα.[4-8] Administration of peroxisome proliferators to rodents results in hepatomegaly and an increase in the number and size of peroxisomes. In addition, it changes the expression of a variety of genes involved in various aspects of lipid metabolism, ranging from fatty acid transport and mitochondrial and peroxisomal fatty acid β-oxidation to microsomal fatty acid ω-oxidation. Chronic treatment of rodents with peroxisome proliferators results in hepatocellular carcinomas.[9] These changes are all mediated by PPARα, as demonstrated by studies with PPARα-deficient (PPARα$^{-/-}$) mice. Under normal conditions, PPARα$^{-/-}$ mice are indistinguishable from wild-type animals and have normal levels of hepatic peroxisomes. Upon fasting, however, PPARα$^{-/-}$ mice are unable to switch to fatty acid oxidation; they develop a fatty liver and become severely hypoglycemic.[10] Similarly, feeding these mice a high-fat diet results in massive accumulation of lipids in the liver, attributable to their inability to enhance fatty acid degradation.[11] PPARα$^{-/-}$ mice do display an altered constitutive expression of several mitochondrial and peroxisomal enzymes involved in the oxidation of fatty acids.[12] However, PPARα$^{-/-}$ mice are nonresponsive to treatment with peroxisome proliferators; hence, they do not show any physiological, toxicological, or carcinogenic responses to peroxisome proliferators.[13]

Table 1: Hepatic levels of phytol and its branched-chain fatty acid metabolites from wild-type and PPARα$^{-/-}$ mice fed a control diet or a 0.5% phytol diet for 1, 2, 4, or 8 weeks

	Wild-Type Control (n = 3)	Wild-Type Phytol (n = 3)	PPARα$^{-/-}$ Control (n = 3)	PPARα$^{-/-}$ Phytol (n = 3)
Phytol				
1 week	≤ 0.01	0.38 ± 0.13*	≤ 0.01	0.48 ± 0.13*
2 weeks	≤ 0.01	0.72 ± 0.24*	≤ 0.01	0.89 ± 0.07*
4 weeks	≤ 0.01	0.75 ± 0.07*	≤ 0.01	1.65 ± 0.28$^{*,\#}$
8 weeks	≤ 0.01	0.74 ± 0.41*	≤ 0.01	1.71 ± 0.25*
Phytenic acid				
1 week	≤ 0.001	1.1 ± 0.1*	≤ 0.001	0.8 ± 0.1*
2 weeks	≤ 0.001	1.6 ± 0.1*	≤ 0.001	0.7 ± 0.1$^{*,\#}$
4 weeks	≤ 0.001	2.4 ± 0.1*	≤ 0.001	1.0 ± 0.04$^{*,\#}$
8 weeks	≤ 0.001	3.6 ± 0.4*	≤ 0.001	1.3 ± 0.6$^{\#}$
Phytanic acid				
1 week	≤ 0.1	2.3 ± 0.2*	≤ 0.1	1.7 ± 0.1$^{*,\#}$
2 weeks	≤ 0.1	4.0 ± 0.4*	≤ 0.1	1.6 ± 0.1$^{*,\#}$
4 weeks	≤ 0.1	8.4 ± 0.4*	≤ 0.1	2.1 ± 0.3$^{*,\#}$
8 weeks	≤ 0.1	17.7 ± 1.4*	≤ 0.1	2.7 ± 1.0$^{*,\#}$
Pristanic acid				
1 week	≤ 0.01	0.4 ± 0.01*	≤ 0.01	0.3 ± 0.02$^{*,\#}$
2 weeks	≤ 0.01	0.8 ± 0.1*	≤ 0.01	0.3 ± 0.04$^{*,\#}$
4 weeks	≤ 0.01	1.8 ± 0.1*	≤ 0.01	0.3 ± 0.02$^{*,\#}$
8 weeks	≤ 0.01	3.8 ± 0.2*	≤ 0.01	0.5 ± 0.2$^{*,\#}$

PPARα$^{-/-}$, peroxisome proliferator-activated receptor α-deficient. Concentrations of phytol and branched-chain fatty acids are expressed in nmol/mg protein. Values represent means ± SD.

n = number of animals.

* $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype.

$^{\#}$ $P < 0.01$, PPARα$^{-/-}$ versus wild-type mice on the same diet.

In the studies mentioned above, artificial ligands such as clofibrate and Wy-14,643 were used as PPARα ligands. Relatively little is known about the effects of natural PPARα ligands, such as the branched-chain fatty acids phytanic and pristanic acid, which have been shown to activate PPARα *in vitro*, in contrast to their precursor phytol.[6,14] Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is derived from the chlorophyll component phytol and undergoes α-oxidation in the peroxisome, which leads to shortening of the chain by one carbon atom, yielding pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and carbon dioxide. Pristanic acid is then further degraded in the peroxisome via β-oxidation.[15] In patients affected by different peroxisomal disorders, there is an accumulation of phytanic and/or pristanic acid. Patients suffering from Refsum disease have a deficiency of phytanoyl-CoA hydroxylase (PhyH), the first enzyme of the α-oxidation system, and as a consequence accumulate phytanic acid in tissues and plasma.[16] In α-methylacyl-CoA racemase (AMACR) or D-bifunctional protein (DBP) deficiency, two enzymes involved in the peroxisomal β-oxidation of branched-chain fatty acids, there is an accumulation of pristanic acid. Patients suffering from a peroxisome biogenesis disorder accumulate both phytanic and pristanic acid because they are deficient in both peroxisomal α- and β-oxidation (reviewed in [17]).

Table 2: Plasma values of very long-chain, long-chain, branched-chain, and polyunsaturated fatty acids from wild-type and PPARα$^{-/-}$ mice fed a control diet or a 0.5% phytol diet

	Wild-Type Control (n = 12)	Wild-Type Phytol (n = 12)	PPARα$^{-/-}$ Control (n = 12)	PPARα$^{-/-}$ Phytol (n = 12)
Phytenic acid	0.002 ± 0.0004	2.1 ± 0.6*	0.007 ± 0.007	1.4 ± 0.5$^{*,\#}$
Phytanic acid	0.8 ± 0.1	48.7 ± 5.8*	1.2 ± 0.2$^{\#}$	57.3 ± 7.7$^{*,\#}$
Pristanic acid	0.07 ± 0.02	3.7 ± 0.6*	0.08 ± 0.02	4.9 ± 1.1$^{*,\#}$
C14:0	20.8 ± 6.6	15.6 ± 3.3	25.3 ± 8.1	39.1 ± 7.8$^{*,\#}$
C16:0	1315 ± 209	1202 ± 196	1439 ± 273	1711 ± 279$^{\#}$
C18:0	663 ± 84	530 ± 49*	909 ± 144$^{\#}$	906 ± 140$^{\#}$
C20:0	19.1 ± 3.4	16.7 ± 1.9	30.2 ± 5.7$^{\#}$	33.6 ± 7.6$^{\#}$
C22:0	21.9 ± 3.0	16.9 ± 1.4*	27.0 ± 4.3$^{\#}$	24.3 ± 3.2$^{\#}$
C24:0	8.8 ± 1.1	8.3 ± 0.9	9.9 ± 1.2	11.5 ± 1.6$^{\#}$
C26:0	0.03 ± 0.04	0.20 ± 0.10*	0.07 ± 0.05	0.34 ± 0.18*
C18:3ω3	123 ± 33	93 ± 24	194 ± 57$^{\#}$	254 ± 65$^{\#}$
C22:6ω3	249 ± 40	177 ± 23*	255 ± 46	273 ± 46$^{\#}$
C18:2ω6	2146 ± 319	1751 ± 215*	2650 ± 478$^{\#}$	2871 ± 432$^{\#}$
C20:4ω6	631 ± 87	455 ± 50*	620 ± 102	542 ± 108
C22:5ω6	4.7 ± 1.2	4.8 ± 1.1	5.8 ± 1.5	8.6 ± 2.9$^{\#}$
C18:1ω9	742 ± 160	793 ± 175	775 ± 232	1073 ± 256$^{*,\#}$
C28:0†	1.0 ± 0.41	0.77 ± 0.38	1.35 ± 0.49	2.09 ± 0.65$^{*,\#}$
C30:0†	1.0 ± 0.42	0.82 ± 0.33	1.29 ± 0.45	2.48 ± 0.82$^{*,\#}$
C32:0†	1.0 ± 0.39	0.82 ± 0.38	1.41 ± 0.54	1.96 ± 0.48$^{\#}$

Concentrations of fatty acids are expressed in μmol/l. Values represent means ± SD. n = number of animals.

* P < 0.01, phytol-enriched diet versus control diet in mice of the same genotype.

# P < 0.01, PPARα$^{-/-}$ versus wild-type mice on the same diet.

† The amount of fatty acid present in plasma of wild-type mice on a control diet was set to 1.0.

In this study, we investigated the effects of the accumulation of these branched-chain fatty acids by feeding mice a diet enriched with phytol. Thus, we mimicked the situation in patients suffering from a peroxisomal disorder, because this results in an increase of phytol metabolites in tissues and plasma.[4,18] In several mouse models for peroxisomal β-oxidation disorders in which branched-chain fatty acids are increased, an altered expression of various fatty acid-metabolizing enzymes has been reported.[19-22]

We studied the expression of both peroxisomal and mitochondrial proteins involved in the metabolism of fatty acids in wild-type and PPARα$^{-/-}$ mice after phytol feeding. Furthermore, we investigated the effect of a phytol diet on the levels of various metabolites in plasma and liver, including very long-chain, branched-chain, and polyunsaturated fatty acids, acylcarnitines, and carnitine biosynthesis intermediates.

Materials and Methods

Animals

Male wild-type and PPARα$^{-/-}$ mice on a Sv/129 genetic background were used for this study.[13] Six week old wild-type and PPARα$^{-/-}$ mice were fed pelleted mouse chow (Hope Farms, Woerden, The Netherlands) containing no (control) or 0.5% (w/w) phytol for 1, 2, 4, or 8 weeks. Each group consisted of three animals. At the end of the experiment, mice were anesthetized using isoflurane, blood was collected by cardiac puncture, and tissues were harvested. The animals were always killed at the same time of day, and animals had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at –80°C until further analysis. A small piece of tissue was treated immediately with RNA*later* RNA stabilization reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions and stored at –80°C until RNA isolation. All animal experiments were approved by the University of Amsterdam Animals Experiments Committee.

Table 3: Values of acylcarnitines measured in plasma and liver of wild-type and PPARα$^{-/-}$ mice fed a control diet or a 0.5% phytol diet

	Wild-Type Control (n = 12)	Wild-Type Phytol (n = 12)	PPARα$^{-/-}$ Control (n = 12)	PPARα$^{-/-}$ Phytol (n = 12)
Plasma				
Free carnitine	36.3 ± 5.2	42.8 ± 7.5	19.2 ± 5.1$^{\#}$	19.4 ± 2.8$^{\#}$
Acetylcarnitine	12.3 ± 2.3	12.9 ± 2.5	8.1 ± 1.9$^{\#}$	7.6 ± 1.8$^{\#}$
Propionylcarnitine	0.53 ± 0.13	0.84 ± 0.14*	0.40 ± 0.11	0.57 ± 0.10$^{* \#}$
Palmitoylcarnitine	0.12 ± 0.04	0.12 ± 0.04	0.18 ± 0.06	0.19 ± 0.07$^{\#}$
Oleoylcarnitine	0.09 ± 0.03	0.07 ± 0.02	0.11 ± 0.03	0.10 ± 0.04
Liver^{c}				
Free carnitine	296 ± 41	376 ± 65*	175 ± 33$^{\#}$	185 ± 26$^{\#}$
Propionylcarnitine	2.2 ± 1.3	1.7 ± 0.9	1.3 ± 0.6	0.8 ± 0.5$^{\#}$

Concentration of acylcarnitines in plasma are expressed n μmol/l and those in liver are expressed in nmol/g wet weight. Values represent means ± SD. n = number of animals.
* P < 0.01, phytol-enriched diet versus control diet in mice of the same genotype.
# P < 0.01, PPARα$^{-/-}$ versus wild-type mice on the same diet.

Metabolite analysis in plasma and liver

Total very long-chain fatty acids (VLCFAs) up to 26 carbon atoms and branched-chain fatty acids in plasma and liver were analyzed using GC-MS.[23] Total fatty acids with more than 26 carbon atoms were analyzed using electrospray ionization (ESI)-tandem MS.[24] Because some of the necessary standards for precise quantitative analysis of these extremely long-chain fatty acids are not commercially available, only a comparative analysis between the different groups could be performed. For this reason, the amount of each fatty acid present in wild-type mice fed a control diet was set to 1. Total polyunsaturated fatty acids in plasma were measured by GC analysis.[25] Free carnitine and acylcarnitines in plasma and liver were analyzed as their propyl-esters using ESI-tandem MS as described previously.[26] The carnitine biosynthesis intermediates trimethyllysine (TML) and γ-butyrobetaine (BB) in plasma and liver were analyzed using ESI-tandem MS.[27]

Phytol levels were determined in freshly prepared liver homogenates in PBS. As an internal standard, 1 nmol of C19-OH dissolved in ethanol was added to samples containing 0.5 mg of liver protein. Subsequently, samples were subjected to alkaline hydrolysis by adding 2 ml of 1 M NaOH in methanol and incubated for 45 min at 110°C. After cooling to room temperature, the pH was decreased by adding 480 µl of 37% HCl. Phytol was then extracted with 2 ml of hexane. The organic layer was evaporated to dryness under nitrogen at 40°C. Samples were dissolved in 0.5 ml of heptane and purified on a silica gel column (J. T. Baker, Philipsburg, NJ) using 92:8 heptane-diethyl ether as eluent. Samples were evaporated to dryness under nitrogen at 40°C and derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsil yl)trifluoroacetamide (Pierce, Rockford, IL) and pyridine (50 µl each) at 80°C for 30 min. Samples were evaporated to dryness under nitrogen at 40°C, dissolved in 100 µl of hexane, and subjected to GC-MS analysis, essentially as described previously.[28] The [M-57]+ ions of phytol and C19-OH (corresponding to 353.3 and 341.3, respectively) were detected. The metabolites were quantified using a calibration curve of phytol.

Table 4: Concentration of the carnitine biosynthesis intermediates measured in plasma and liver, and carnitine biosynthesis enzyme activities in liver, of wild-type and PPARα$^{-/-}$ mice fed a control diet or a 0.5% phytol diet

	Wild-Type Control (n = 12)	Wild-Type Phytol (n = 12)	PPARα$^{-/-}$ Control (n = 12)	PPARα$^{-/-}$ Phytol (n = 12)
Plasma				
Trimethyllysine	0.20 ± 0.04	0.21 ± 0.02	0.20 ± 0.04	0.22 ± 0.04
γ-Butyrobetaine	0.61 ± 0.13	0.51 ± 0.09	0.62 ± 0.14	0.60 ± 0.13
Liver				
Trimethyllysine	1.15 ± 0.21	0.94 ± 0.25	0.94 ± 0.17	0.78 ± 0.11
γ-Butyrobetaine	8.37 ± 0.90	6.02 ± 0.64*	8.34 ± 1.84	6.82 ± 0.91
Trimethyllysine dioxygenase	25.4 ± 5.0	21.6 ± 4.0	26.9 ± 9.2	25.5 ± 8.3
Trimethylaminobutyraldehyde dehydrogenase	1471 ± 270	1364 ± 206	1153 ± 189#	1159 ± 183
γ-Butyrobetaine dioxygenase	134 ± 23	135 ± 15	112 ± 18	115 ± 17#

Concentrations of carnitine biosynthesis intermediates in plasma are expressed in µmol/l and those in liver are expressed in nmol/g wet weight. Activities of carnitine biosynthesis enzymes are expressed in pmol/mg/min. Values represent means ± SD. n = number of animals.

* $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype

# $P < 0.01$, PPARα$^{-/-}$ versus wild-type mice on the same diet

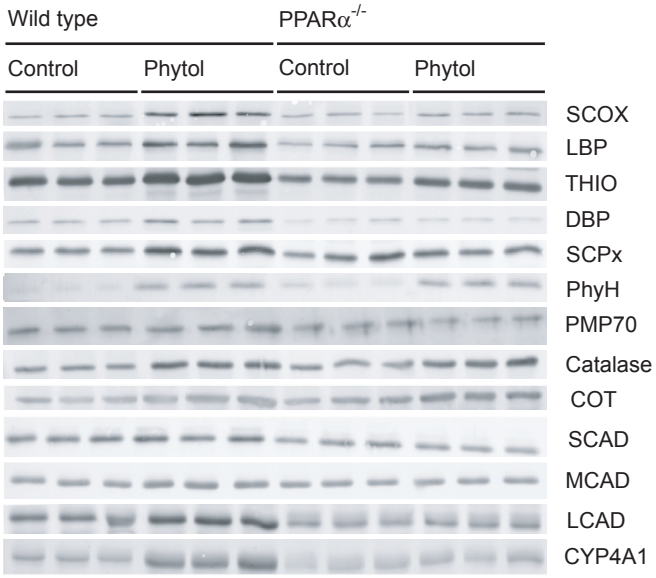


Figure 1: Immunoblot analysis of different enzymes involved in fatty acid metabolism in liver of wild-type and peroxisome proliferator-activated receptor α-deficient (PPARα$^{-/-}$) mice on a control diet or a diet enriched with phytol. Results from three mice per group are shown. Antibodies against straight-chain acyl-CoA oxidase (SCOX; the 70 kDa band is shown), L-bifunctional protein (LBP), peroxisomal 3-ketoacyl-CoA thiolase (THIO), D-bifunctional protein (DBP; the 79 kDa band is shown), sterol carrier protein x (SCPx; the 58 kDa band is shown), phytanoyl-CoA hydroxylase (PhyH), peroxisomal membrane protein 70 (PMP70), catalase, carnitine octanoyltransferase (COT), short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and cytochrome P450 hydroxylase 4A1 (CYP4A1) were used.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from RNA*later* -treated mouse liver and kidney samples using Trizol (Invitrogen, Carlsbad, CA) extraction, after which cDNA was prepared using a first-strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). Quantitative real-time PCR analysis of long-chain fatty acid elongases 2, 3, and 4 (Elovl2/3/4) and β-actin in liver and/or kidney was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche). The following primers were used. For Elovl2: forward, 5'-CACCTTCCTTCATGTCTAT CAC-3'; reverse, 5'-GAACAGGATGACCAGCGTCAT-3'. For Elovl3: forward, 5'-CAACA GTGATGTTTACAGTGGGC-3'; reverse, 5'-CATCTGCAGAATCTGCAGGCTG-3'. For Elovl4: forward, 5'-CAACCAAGTCTCCTTCCTTCAC-3'; reverse, 5'-GACAGTGCTGTG TGTCCGATG-3'. Primers for β-actin were used as described.[29] Melting curve analysis was carried out to confirm the generation of a single product. Amplification of a single product of the correct size was also confirmed by agarose gel electrophoresis. Duplicate analyses were performed for all samples. Data were analyzed using linear regression calculations as described by Ramakers et al..[30] To adjust for variations in the amount of input RNA, the values for Elovl2, Elovl3, and Elovl4 were normalized against the values for the housekeeping gene β-actin.

Immunoblot analysis

Small pieces of liver were homogenized in PBS containing a cocktail of protease inhibitors (Roche, Basel, Switzerland). Ten micrograms of liver homogenate was separated on a 10% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Shuell, Keene, NH).[31] After blocking of nonspecific binding sites with 30 g/l Protifar (Nutricia, Zoetermeer, The Netherlands) and 10 g/l BSA in 1 g/l Tween-20/PBS, the blot

was incubated with specific primary antibodies. Secondary antibodies conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA) were used for detection. Polyclonal antibodies directed against peroxisomal straight-chain acyl-CoA oxidase (SCOX), L-bifunctional protein (LBP), DBP, peroxisomal 3-ketoacyl-CoA thiolase (THIO), sterol carrier protein x (SCPx), PhyH, peroxisomal membrane protein 70 (PMP70) (Zymed, San Francisco, CA), carnitine octanoyltransferase (COT), catalase, short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), mitochondrial trifunctional protein α subunit (MTPα), short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD), short branched-chain 3-hydroxyacyl-CoA dehydrogenase (SBCHAD), and cytochrome P450 hydroxylase 4A1 (CYP4A1) (BD Gentest, Bedford, MA) were used according to the manufacturer's instructions or as described earlier.[12,32-36] Densitometric analysis of the immunoblots was performed using Scion Image software (version β3b).

Enzyme activity measurements

All enzyme activity measurements were done in freshly prepared liver homogenates.

Acyl-CoA oxidase activity measurements were performed spectrophotometrically, essentially as described previously.[37] Reactions were started with 50 μM palmitoyl-CoA and 50 μM pristanoyl-CoA for measurement of SCOX and branched-chain acyl-CoA oxidase (BCOX), respectively. DBP and SCPx activities were measured in a combined assay as described[38] with the exception that endogenous DBP was used to produce the substrate for SCPx. SCAD, MCAD, and very long-chain acyl-CoA dehydrogenase (VLCAD) activity measurements were performed using HPLC analysis.[39] Reactions were started using 25 μM butyryl-CoA, 200 μM 3-phenylpropionyl-CoA, and 250 μM palmitoyl-CoA, respectively. LCAD activity measurements were performed spectrophotometrically as described previously[40,41] using 200 μM 2,6-dimethylheptanoyl-CoA as substrate. Carnitine acetyltransferase (CAT) activity measurements were performed using radiolabeled substrate as described.[42] Carnitine palmitoyltransferase 2 (CPT2) activity measurements were performed using HPLC analysis, essentially as described by Slama et al..[43] Catalase activity was measured spectrophotometrically.[44] Trimethyllysine dioxygenase, trimethylaminobutyraldehyde dehydrogenase (TMABADH), and γ-butyrobetaine dioxygenase (BBD) activities were measured using ESI-tandem MS, as described by Van Vlies et al..[45]

Statistical analyses

Data are expressed as means ± SD. Statistical significance was evaluated using an unpaired Student's *t*-test. The results were considered significant at $P < 0.01$.

Results

Analysis of phytol and its metabolites phytenic, phytanic and pristanic acid in plasma and liver

Branched-chain fatty acids were measured in liver and plasma to establish the extent of the accumulation of these phytol metabolites after the phytol-enriched diet (**Table 1**). In liver from phytol-fed animals, there was a marked increase in hepatic levels of phytenic, phytanic, and pristanic acid. The levels increased with an increasing diet period, but strikingly, the increase was much stronger in wild-type animals than in PPARα$^{-/-}$ mice. These results led us to

investigate hepatic phytol levels. Interestingly, we found that there was a greater accumulation of phytol in PPARα$^{-/-}$ mice than in wild-type animals, suggesting a PPARα-dependent upregulation of the breakdown pathway of phytol to phytanic acid in wild-type animals upon phytol feeding. Remarkably, the plasma levels of phytenic, phytanic, and pristanic acid were increased after the phytol diet but did not increase with a longer duration of the diet. After 1 week of phytol feeding, the maximum plasma levels were reached. Therefore, the results in plasma are presented in **table 2** as a mean of all animals of the same genotype on the same diet (n = 12 per group), regardless of the length of the diet.

Although the hepatic levels of phytol and its metabolites increased with longer periods of the diet, no differences were observed for all other parameters that were investigated regarding the different diet periods. Based on these data, we conclude that already after 1 week of phytol feeding sufficient amounts of phytol metabolites have accumulated to induce the effects described in this study. Therefore, these effects are presented throughout this study as a mean of all animals of the same genotype on the same diet, regardless of the length of the diet.

Table 5: Immunoblot quantification of peroxisomal proteins in liver of wild-type and PPARα$^{-/-}$ mice fed a control diet or a 0.5% phytol diet

	Wild-Type Control (n = 12)	Wild-Type Phytol (n = 12)	PPARα$^{-/-}$ Control (n = 12)	PPARα$^{-/-}$ Phytol (n = 12)
Straight-chain acyl-CoA oxidase	1.0 ± 0.47	4.53 ± 0.92*	0.65 ± 0.34	1.73 ± 0.54$^{*\#}$
D-bifunctional protein	1.0 ± 0.38	2.41 ± 0.57*	0.42 ± 0.16	0.54 ± 0.20$^{\#}$
L-bifunctional protein	1.0 ± 0.25	2.45 ± 0.84*	0.66 ± 0.25$^{\#}$	0.86 ± 0.44$^{\#}$
Peroxisomal 3-ketoacyl-CoA thiolase	1.0 ± 0.12	1.68 ± 0.22*	0.55 ± 0.10$^{\#}$	0.74 ± 0.11$^{*\#}$
Sterol carrier protein x	1.0 ± 0.34	1.91 ± 0.54*	1.53 ± 0.59	1.41 ± 0.43$^{\#}$
Catalase	1.0 ± 0.35	1.77 ± 0.62*	0.94 ± 0.32	1.49 ± 0.43*
Peroxisomal membrane protein 70	1.0 ± 0.27	1.01 ± 0.28	0.86 ± 0.27	0.89 ± 0.44
Phytanoyl-CoA hydroxylase	1.0 ± 0.45	2.23 ± 0.66*	1.22 ± 0.33	2.40 ± 0.77*
Carnitine octanoyltransferase	1.0 ± 0.35	1.64 ± 0.41*	1.12 ± 0.30	1.47 ± 0.25*

The amount of protein present in liver from wild-type mice on a control diet was set to 1.0. Values represent means ± SD. n = number of animals.
* $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype.
# $P < 0.01$, PPARα$^{-/-}$ versus wild type mice on the same diet.

Plasma analysis of fatty acids

Plasma values of straight-chain and polyunsaturated fatty acids were measured to investigate the effect of a phytol-enriched diet on fatty acid metabolism (Table 2). In wild-type mice, the levels of virtually all fatty acids were decreased on the phytol-enriched diet compared with the control diet. In PPARα$^{-/-}$ mice, the levels of most fatty acids were increased, and this effect was even stronger after phytol feeding. In contrast to all other straight-chain fatty acids, the C26:0 levels were increased in both wild-type and PPARα$^{-/-}$ mice as a result of the phytol diet. Therefore, we investigated fatty acids longer than C26:0 using ESI-tandem MS. Interestingly, C28:0 and C30:0 levels (Table 2) and their monounsaturated counterparts (data not shown) were significantly higher after the phytol diet in PPARα$^{-/-}$ mice but not in wild-type mice.

An increase of VLCFAs in PPARα$^{-/-}$ mice on a phytol diet could be attributable to a diminished breakdown or an increased formation of these fatty acids by elongation. This led us to investigate the mRNA expression levels of Elovl2, Elovl3, and Elovl4 using quantitative

real-time PCR analysis. We found no significant differences in mRNA levels of Elovl2 (in liver and kidney), Elovl3 (in liver), and Elovl4 (in kidney) between the different experimental groups (data not shown), indicating that the increased levels of C26:0, C28:0, and C30:0 are not caused by increased elongation of long-chain fatty acids.

Acylcarnitines and carnitine biosynthesis intermediates

To further characterize the effects of a phytol diet in wild-type and PPARα$^{-/-}$ mice, we investigated the levels of acylcarnitines and free carnitine in plasma and liver (**Table 3**). In response to the phytol diet, the free carnitine level in liver increased significantly in wild-type animals. This trend was also observed in plasma, but the increase in plasma was not statistically significant. In PPARα$^{-/-}$ mice, however, no increase of free carnitine after the phytol diet was observed, and PPARα$^{-/-}$ mice on the control diet had markedly reduced levels of free carnitine in plasma (1.9-fold) and liver (1.7-fold) compared with wild-type animals.

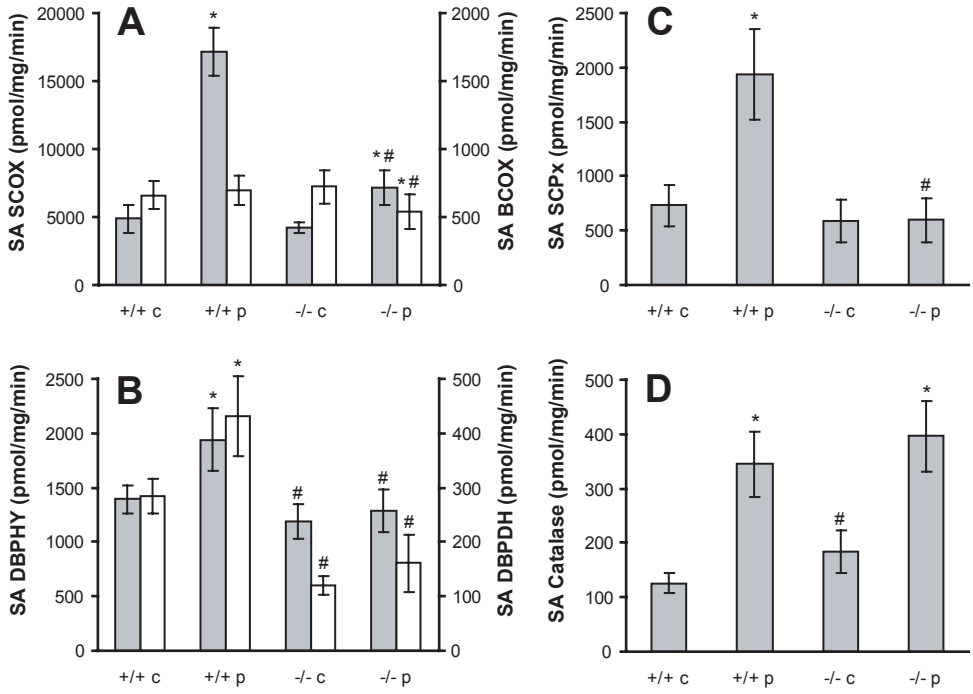


Figure 2: Enzyme activity measurements performed in liver homogenates of wild-type and PPARα$^{-/-}$ mice on a control diet (c) or a phytol-enriched diet (p). Specific activity (SA) of A: SCOX (gray bars) and branched-chain acyl-CoA oxidase (BCOX; white bars). B: DBP enoyl-CoA hydratase (DBP HY; gray bars) and DBP 3-ketoacyl-CoA dehydrogenase (DBP DH; white bars). C: SCPx. D: Catalase. Values represent means ± SD. * $P < 0.01$ between the phytol-enriched diet and the control diet in mice of the same genotype; # $P < 0.01$ between PPARα$^{-/-}$ mice and wild-type mice on the same diet.

Phytol feeding did not induce changes in acetylcarnitine levels in mice from either genotype, but PPARα$^{-/-}$ mice had significantly decreased plasma acetylcarnitine levels compared with wild-type mice. Liver acetylcarnitine levels varied considerably, probably because the animals

were not fasted before they were killed. Propionylcarnitine levels increased significantly in plasma of both wild-type and PPARα$^{-/-}$ mice on the phytol diet. Levels of palmitoylcarnitine were slightly increased in PPARα$^{-/-}$ mice compared with wild-type animals, but this was only significant on the control diet. No significant changes were observed for any of the other acylcarnitines that were analyzed.

In search of the cause of the decreased levels of free carnitine and acetylcarnitine in PPARα$^{-/-}$ mice and the increase of free carnitine in liver after phytol feeding, we measured the levels of the carnitine biosynthesis intermediates in plasma and liver and the activity of three enzymes involved in carnitine biosynthesis (**Table 4**). The only two intermediates that were detected in mouse plasma and liver were TML and the direct precursor of carnitine, BB. After phytol feeding, BB levels were decreased in liver from wild-type and PPARα$^{-/-}$ mice. Furthermore, TMABADH and BBD activities were decreased in PPARα$^{-/-}$ mice both on the control and the phytol-enriched diet.

The expression of peroxisomal β-oxidation enzymes

To determine the effect of the phytol diet on the expression of peroxisomal fatty acid-metabolizing enzymes and the role of PPARα therein, immunoblot analyses were performed followed by densitometric analysis of the immunoblots (**Figure 1**, **Table 5**). In PPARα$^{-/-}$ mice, the constitutive expression of several peroxisomal β-oxidation enzymes was lower, which is in agreement with previously reported data,[12] but this decrease was only significant for LBP and THIO. Phytol feeding of wild-type mice resulted in a significant increase in the expression of SCOX, LBP, DBP, THIO, SCPx, catalase, COT, and PhyH, with levels of induction ranging from 4.5-fold for SCOX to 1.6-fold for COT. In contrast, phytol feeding of PPARα$^{-/-}$ mice did not result in an induction of LBP, DBP, and SCPx, indicating that the effect of the phytol-enriched diet on the expression of these enzymes is PPARα-dependent. SCOX, THIO, and COT were induced by phytol treatment in PPARα$^{-/-}$ mice. However, this induction was less than in wild-type mice, indicating that there is both a PPARα-dependent and -independent effect on their expression. Interestingly, catalase and PhyH were induced to the same extent by the phytol diet in both wild-type and PPARα$^{-/-}$ mice. As a marker for peroxisome proliferation, the expression of the peroxisomal membrane protein PMP70 was studied, but no changes in the amount of protein were observed between the different experimental groups. In addition to the immunoblot analyses, enzyme activity measurements were performed for SCOX, BCOX, DBP, SCPx, and catalase (**Figure 2**) . Basal DBP activity was decreased in PPARα$^{-/-}$ mice compared with wild-type animals. Furthermore, in wild-type mice, the activity of SCOX, DBP (both the enoyl-CoA hydratase and the 3-hydroxyacyl-CoA dehydrogenase activity), SCPx, and catalase increased on the phytol diet. In PPARα$^{-/-}$ mice, catalase and SCOX activities increased as a result of the phytol treatment, although the increase in SCOX activity was lower than that observed in wild-type animals. In contrast, BCOX activity decreased in PPARα$^{-/-}$ mice after the phytol diet.

The expression of mitochondrial β-oxidation enzymes

Besides the peroxisomal fatty acid-metabolizing enzymes, PPARα also regulates the expression of several enzymes involved in the mitochondrial β-oxidation system. To investigate the influence of the branched-chain fatty acid metabolites of phytol on the expression of mitochondrial fatty acid-metabolizing enzymes, immunoblot analysis was performed using specific antibodies against several of these enzymes (Figure 1, **Table 6**). The constitutive

levels of SCAD and LCAD were significantly decreased in PPARα$^{-/-}$ mice. For SCHAD, an increased constitutive expression in PPARα$^{-/-}$ mice was reported previously,[12] but we could not confirm this. No differences in the constitutive expression of SCHAD, MCAD, and SBCHAD were found. The phytol diet did not induce significant changes in protein expression for the investigated mitochondrial β-oxidation enzymes in mice of either genotype. A significant increase was found for the expression of CYP4A1, a microsomal enzyme involved in ω-

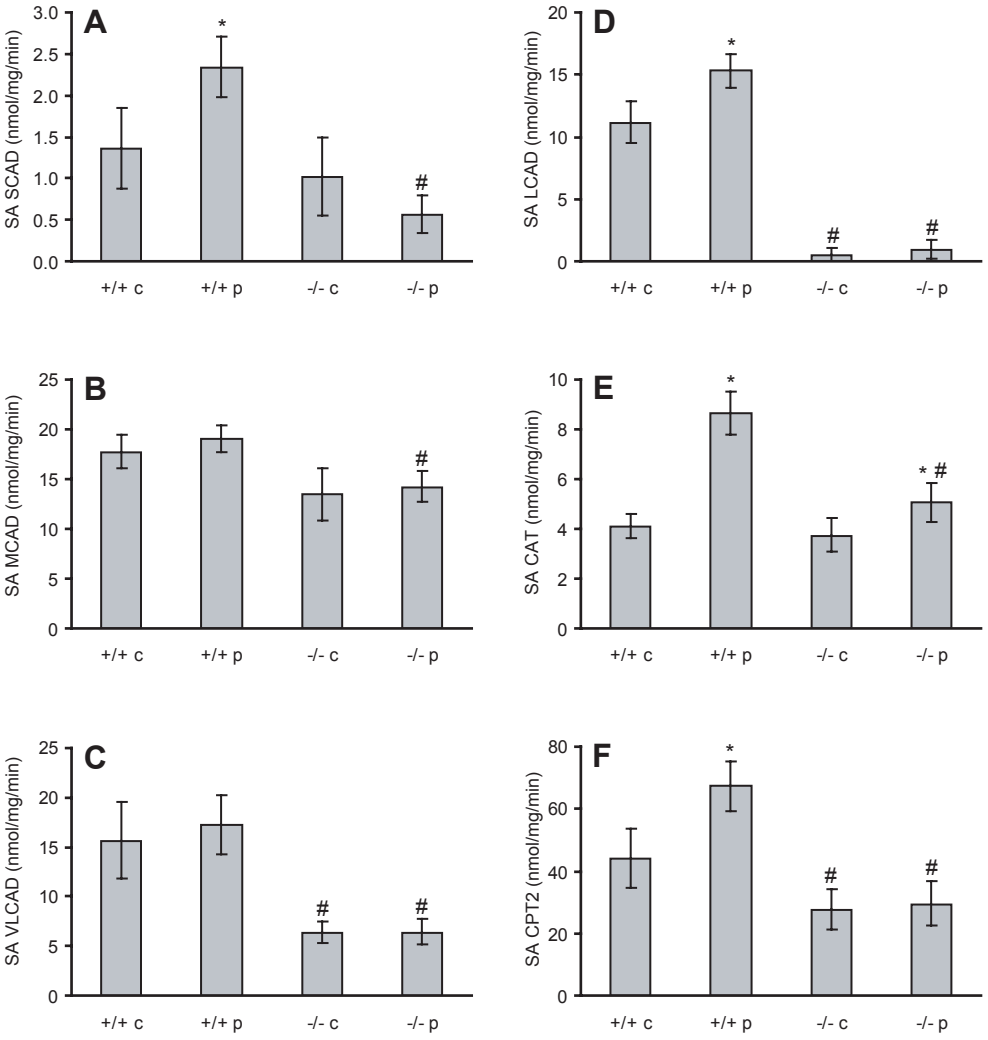


Figure 3: Enzyme activity measurements performed in liver homogenates of wild-type and PPARα$^{-/-}$ mice on a control diet (c) or a phytol-enriched diet (p). Specific activity (SA) of A: SCAD. B: MCAD. C: Very long-chain acyl-CoA dehydrogenase (VLCAD). D: LCAD. E: Carnitine acetyltransferase (CAT). F: Carnitine palmitoyltransferase 2 (CPT2). Values represent means ± SD. * $P < 0.01$ between the phytol-enriched diet and the control diet in mice of the same genotype; # $P < 0.01$ between PPARα$^{-/-}$ mice and wild-type mice on the same diet.

oxidation of fatty acids, in phytol-fed wild-type mice. This increase in expression of CYP4A1 was not found in PPARα$^{-/-}$ mice on a phytol diet, indicating that the induction of CYP4A1 is PPARα-dependent.

To investigate the effect of the phytol diet on the activity of the enzymes involved in mitochondrial fatty acid metabolism, enzyme activity measurements were performed for SCAD, MCAD, VLCAD, LCAD, CPT2, and CAT (**Figure 3**) . The basal activity levels of VLCAD, LCAD, and CPT2 were significantly decreased in PPARα$^{-/-}$ mice. On the phytol diet, a significant increase in enzyme activity was measured for SCAD, LCAD, CPT2, and CAT in wild-type animals. In PPARα$^{-/-}$ mice, no induction of enzyme activity was observed for these enzymes after phytol feeding, except for a slight but significant increase in CAT activity. However, this induction of CAT in PPARα$^{-/-}$ mice was less than in wild-type mice, indicating that there is both a PPARα-dependent and -independent effect of the phytol diet on CAT activity. The absence of induction of SCAD, LCAD, and CPT2 activity in PPARα$^{-/-}$ mice indicates that these enzymes are solely regulated via PPARα, at least with the branched-chain fatty acid metabolites of phytol as ligands.

Discussion

Many studies have been performed on the effect of artificial PPARα ligands on fatty acid metabolism and the expression of the enzymes involved. Much less is known about the effect of natural ligands such as the branched-chain fatty acids phytanic and pristanic acid. These fatty acids accumulate in several peroxisomal disorders, such as adult Refsum disease, AMACR deficiency, and peroxisome biogenesis disorders. In several mouse models for peroxisomal disorders in which these fatty acids accumulate, an altered pattern of gene expression is reported. For example, AMACR$^{-/-}$ mice have a normal phenotype on a control diet, but phytol feeding results in liver injury, peroxisome proliferation, and changes in the expression of fatty acid-metabolizing enzymes.[22] These changes are likely mediated via PPARα. Furthermore, the expression of the H_2O_2-producing SCOX is strongly increased in SCPx$^{-/-}$ and DBP$^{-/-}$ mice, which could lead to an overproduction of reactive oxygen species and therefore oxidative stress. This indeed has been shown to occur in patients with a deficiency of DBP.[46] Because of the accumulation of natural ligands of PPARα in patients with an inherited peroxisomal disorder, it is important to have full insight into the effect of these compounds. Therefore, we set out to study the effect of phytol metabolites on fatty acid metabolism and the role of PPARα therein.

Analysis of phytol and its branched-chain fatty acid metabolites revealed increased levels in plasma and liver of mice after the phytol-enriched diet. The increased levels of phytol and its metabolites in liver suggest that once phytol is taken up by the body it is transported to the liver, where it is broken down. Interestingly, we found that the level of hepatic phytol was higher in PPARα$^{-/-}$ mice, whereas the levels of the phytol metabolites were lower compared with wild-type animals, suggesting that the breakdown pathway of phytol to phytanic acid is under the control of PPARα. Further research is under way to investigate this. The observed effect of the phytol diet on all of the parameters studied was maximal after 1 week of diet, and increasing hepatic concentrations of branched-chain fatty acids and longer exposure to increased levels of these fatty acids did not influence the extent of the observed effects.

In this study, we found that the protein level and/or activity of all of the peroxisomal β-oxidation enzymes increased in wild-type mice after phytol feeding, except for the activity of

BCOX. Interestingly, the expression of BCOX is increased in DBP$^{-/-}$ mice,[19] but this apparently is not attributable to the accumulation of branched-chain fatty acids in this mouse model. COT and CAT are also induced by phytol treatment in wild-type animals. Both of these enzymes are involved in the (peroxisomal) conversion of shortened acyl-CoAs to carnitine esters, so that the latter can move to the mitochondrion for further breakdown.[42] Surprisingly, a response to the phytol diet was also observed in PPARα$^{-/-}$ mice for SCOX, THIO, COT, and CAT. However, the increase in expression in PPARα$^{-/-}$ mice was not as high as in wild-type animals; therefore, the enhanced expression of these genes in wild-type mice is probably regulated by PPARα as well as via another pathway that is still present in PPARα$^{-/-}$ mice. A PPARα-independent induction on the phytol diet was also observed for catalase and PhyH, the first enzyme of the peroxisomal α-oxidation pathway. Most likely, one of the phytol metabolites not only activates PPARα but also another, yet unknown, transcription factor, resulting in upregulation of a subset of peroxisomal enzymes. It is unlikely that phytol-mediated peroxisome proliferation, which has been described previously,[47] is involved in the increased protein levels/enzyme activity we observed, because the expression of the peroxisomal membrane protein PMP70 was not increased. This is supported by the observation that peroxisome proliferation can be achieved independently from induction of PPARα-regulated fatty acid β-oxidation genes, suggesting a separate regulation of these processes.[48]

The effect of the phytol diet on the expression of the enzymes involved in mitochondrial β-oxidation was less pronounced compared with that on the peroxisomal enzymes. CPT2, LCAD, and SCAD showed a slight PPARα-regulated induction on the phytol diet. This was not observed for MCAD, VLCAD, and MTP, which have been reported to be regulated via PPARα.[12]

Although there was a clear effect of dietary phytol on the expression of fatty acid-metabolizing enzymes, the highest induction level was 4.5-fold for SCOX. The reported induction of SCOX after treatment of mice with Wy-14,643 is much stronger (6.6-fold).[12] Thus, our results support the *in vitro* data that phytanic and pristanic acid are natural ligands for PPARα,[4] but we found that they are not as potent as artificial PPARα activators such as clofibrate or Wy-14,643. Besides the PPARα-regulated effect, we also found PPARα-independent regulation of the expression of peroxisomal enzymes involved in fatty acid metabolism, which has not been reported for the artificial PPARα ligand Wy-14,643.[12] Further research is needed to resolve which underlying mechanism is involved in this regulation.

Analysis of straight-chain fatty acids revealed a phytol diet-induced decrease of total plasma fatty acid levels in wild-type mice. In PPARα$^{-/-}$ mice on a phytol diet, the levels of the extremely long-chain fatty acids (with more than 26 carbon atoms) increased significantly. This was not attributable to enhanced elongation by the elongation enzymes; therefore, we suggest that these changes, like the changes in the other saturated and polyunsaturated fatty acids we have found, are attributable to differences in overall β-oxidation capacity (i.e., fatty acid breakdown). In PPARα$^{-/-}$ mice, the constitutive expression of many fatty acid-metabolizing enzymes is lower than in wild-type animals, resulting in a lower β-oxidation capacity and therefore higher levels of fatty acids in plasma. In wild-type animals, such an accumulation of monounsaturated and polyunsaturated fatty acids leads to an upregulation of the β-oxidation enzymes,[8] but this mechanism is abolished in PPARα$^{-/-}$ mice. Decreased levels of acetylcarnitine in plasma of the PPARα$^{-/-}$ mice likely reflect a lower production of acetyl-CoA units by fatty acid β-oxidation, which supports this hypothesis. However, no increase in acetylcarnitine was observed in wild-type mice after the phytol diet, despite the higher

β-oxidation capacity under this condition. The increased plasma levels of propionylcarnitine after phytol feeding in both wild-type and PPARα$^{-/-}$ mice could represent an increased production of propionyl-CoA during the β-oxidation of the phytol metabolite pristanic acid, because one pristanic acid molecule yields three propionyl-CoA units besides the acetyl-CoA units normally produced during β-oxidation of fatty acids.

Our results suggest that PPARα is involved in the changes in free carnitine and acetylcarnitine in liver and plasma. The PPARα$^{-/-}$ mice are carnitine and acetylcarnitine deficient in both liver and plasma and do not respond to the phytol diet, in contrast to wild-type animals, which display enhanced carnitine levels with this treatment. Previous studies have shown that under fasting conditions there is an increase in hepatic carnitine levels in wild-type mice that is not observed in PPARα$^{-/-}$ mice.[10] This is supported by the observation that in SCOX$^{-/-}$ mice, which have sustained PPARα activation, hepatic carnitine levels are higher than in wild-type mice.[10,49] The decreased carnitine levels in PPARα$^{-/-}$ mice could be attributable to decreased biosynthesis, decreased renal reabsorption of carnitine, or decreased hepatic uptake of carnitine from the circulation. The activities of the carnitine biosynthesis enzymes TMABADH and BBD were indeed lower in PPARα$^{-/-}$ mice, but this did not result in changes in the levels of the carnitine biosynthesis intermediates. Besides decreased production of hepatic carnitine as a result of lower enzymatic activity of the last two enzymes of carnitine biosynthesis, the lower levels of free carnitine and acetylcarnitine in PPARα$^{-/-}$ mice could also be the result of a lower flux through the carnitine biosynthesis pathway. This could be caused by decreased input of TML from protein degradation, which is known to be the rate-limiting step of the carnitine biosynthetic pathway.[50] In support of this, clofibrate indeed is known to induce protein breakdown in skeletal muscle.[51] Further research is needed to fully resolve the role of PPARα in carnitine metabolism.

In summary, our results show that increased levels of the phytol metabolites phytenic, phytanic, and pristanic acid result in the activation of PPARα but possibly also of another yet unknown transcription factor that induces several enzymes involved in peroxisomal fatty acid metabolism. This activation by phytol metabolites resulted in the increased expression of several peroxisomal and mitochondrial β-oxidation enzymes, leading to changes in fatty acid metabolism. These findings are especially relevant for disorders in which branched-chain fatty acids accumulate, such as Refsum disease and peroxisome biogenesis disorders.

Acknowledgments

The authors thank L. van Lint, H. Overmars, J. van Miert, F. Valianpour, J. Komen, D. van den Brink, and J. Koster for technical assistance. This work was supported by Grant 901-03-097 from the Netherlands Organization for Scientific Research (The Hague, The Netherlands).

References

1. Berger J and Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53:** 409-435.
2. Reddy JK and Hashimoto T (2001) Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr* **21:** 193-230.
3. Braissant O, Foufelle F, Scotto C, Dauca M, and Wahli W (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* **137:** 354-366.
4. Ellinghaus P, Wolfrum C, Assmann G, Spener F, and Seedorf U (1999) Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient

mice. *J Biol Chem* **274:** 2766-2772.
5. Moya-Camarena SY, Van den Heuvel JP, Blanchard SG, Leesnitzer LA, and Belury MA (1999) Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARalpha. *J Lipid Res* **40:** 1426-1433.
6. Zomer AW, Van der Burg B, Jansen GA, Wanders RJ, Poll-The BT, and Van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. *J Lipid Res* **41:** 1801-1807.
7. Forman BM, Chen J, and Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* **94:** 4312-4317.
8. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, and Lehmann JM (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A* **94:** 4318-4323.
9. Gonzalez FJ (2002) The peroxisome proliferator-activated receptor alpha (PPARalpha): role in hepatocarcinogenesis. *Mol Cell Endocrinol* **193:** 71-79.
10. Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, and Rao MS (2000) Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem* **275:** 28918-28928.
11. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, and Wahli W (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* **103:** 1489-1498.
12. Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, and Gonzalez FJ (1998) Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). *J Biol Chem* **273:** 5678-5684.
13. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, and Gonzalez FJ (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* **15:** 3012-3022.
14. Heim M, Johnson J, Boess F, Bendik I, Weber P, Hunziker W, and Fluhmann B (2002) Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes. *FASEB J* **16:** 718-720.
15. Mukherji M, Schofield CJ, Wierzbicki AS, Jansen GA, Wanders RJ, and Lloyd MD (2003) The chemical biology of branched-chain lipid metabolism. *Prog Lipid Res* **42:** 359-376.
16. Wanders RJ, Jansen GA, and Skjeldal OH (2001) Refsum disease, peroxisomes and phytanic acid oxidation: a review. *J Neuropathol Exp Neurol* **60:** 1021-1031.
17. Wanders RJ, van Roermund CW, Visser WF, Ferdinandusse S, Jansen GA, van den Brink DM, Gloerich J, and Waterham HR (2003) Peroxisomal fatty acid alpha- and beta-oxidation in health and disease: new insights. *Adv Exp Med Biol* **544:** 293-302.
18. Atshaves BP, Payne HR, McIntosh AL, Tichy SE, Russell D, Kier AB, and Schroeder F (2004) Sexually dimorphic metabolism of branched-chain lipids in C57BL6/6J mice. *J Lipid Res* **45:** 812-830.
19. Baes M, Huyghe S, Carmeliet P, Declercq PE, Collen D, Mannaerts GP, and Van Veldhoven PP (2000) Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids. *J Biol Chem* **275:** 16329-16336.
20. Seedorf U, Raabe M, Ellinghaus P, Kannenberg F, Fobker M, Engel T, Denis S, Wouters F, Wirtz KW, Wanders RJ, Maeda N, and Assmann G (1998) Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function. *Genes Dev* **12:** 1189-1201.
21. Fan CY, Pan J, Chu R, Lee D, Kluckman KD, Usuda N, Singh I, Yeldandi AV, Rao MS, Maeda N, and Reddy JK (1996) Hepatocellular and hepatic peroxisomal alterations in mice with a disrupted peroxisomal fatty acyl-coenzyme A oxidase gene. *J Biol Chem* **271:** 24698-24710.
22. Savolainen K, Kotti TJ, Schmitz W, Savolainen TI, Sormunen RT, Ilves M, Vainio SJ, Conzelmann E, and Hiltunen JK (2004) A mouse model for {alpha}-methylacyl-CoA racemase deficiency: adjustment of bile acid synthesis and intolerance to dietary methyl-branched lipids. *Hum Mol Genet* **13:** 955-965.
23. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, and van Gennip AH (1998) Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas

chromatography-electron impact mass spectrometry. *J Chromatogr B Biomed Sci Appl* **713:** 281-287.
24. Valianpour F, Selhorst JJ, van Lint LE, van Gennip AH, Wanders RJ, and Kemp S (2003) Analysis of very long-chain fatty acids using electrospray ionization mass spectrometry. *Mol Genet Metab* **79:** 189-196.
25. Dacremont G and Vincent G (1995) Assay of plasmalogens and polyunsaturated fatty acids (PUFA) in erythrocytes and fibroblasts. *J Inherit Metab Dis* **18 Suppl 1:** 84-89.
26. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, and van Gennip AH (1999) Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. *J Inherit Metab Dis* **22:** 302-306.
27. van VN, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, and Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *Biochem J* **387:** 185-193.
28. van den Brink DM, van Miert JM, and Wanders RJ (2005) Assay for Sjogren-Larsson syndrome based on a deficiency of phytol degradation. *Clin Chem* **51:** 240-242.
29. Sousa M, Du Yan S, Fernandes R, Guimaraes A, Stern D, and Saraiva M (2001) Familial Amyloid Polyneuropathy: Receptor for Advanced Glycation End Products-Dependent Triggering of Neuronal Inflammatory and Apoptotic Pathways. *J Neurosci* **21:** 7576-7586.
30. Ramakers C, Ruijter JM, Deprez RH, and Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339:** 62-66.
31. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
32. Jansen GA, Ofman R, Denis S, Ferdinandusse S, Hogenhout EM, Jakobs C, and Wanders RJ (1999) Phytanoyl-CoA hydroxylase from rat liver. Protein purification and cDNA cloning with implications for the subcellular localization of phytanic acid alpha-oxidation. *J Lipid Res* **40:** 2244-2254.
33. Jiang LL, Miyazawa S, and Hashimoto T (1996) Purification and properties of rat D-3-hydroxyacyl-CoA dehydratase: D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J Biochem (Tokyo)* **120:** 633-641.
34. Tager JM, Van der Beek WA, Wanders RJ, Hashimoto T, Heymans HS, Van den Bosch H, Schutgens RB, and Schram AW (1985) Peroxisomal beta-oxidation enzyme proteins in the Zellweger syndrome. *Biochem Biophys Res Commun* **126:** 1269-1275.
35. Wanders RJ, Dekker C, Ofman R, Schutgens RB, and Mooijer P (1995) Immunoblot analysis of peroxisomal proteins in liver and fibroblasts from patients. *J Inherit Metab Dis* **18 Suppl 1:** 101-112.
36. Ossendorp BC, Voorhout WF, van Amerongen A, Brunink F, Batenburg JJ, and Wirtz KW (1996) Tissue-specific distribution of a peroxisomal 46-kDa protein related to the 58-kDa protein (sterol carrier protein x; sterol carrier protein 2/3-oxoacyl-CoA thiolase). *Arch Biochem Biophys* **334:** 251-260.
37. Wanders RJ, Denis S, and Dacremont G (1993) Studies on the substrate specificity of the inducible and non-inducible acyl-CoA oxidases from rat kidney peroxisomes. *J Biochem (Tokyo)* **113:** 577-582.
38. Ferdinandusse S, Denis S, van Berkel E, Dacremont G, and Wanders RJ (2000) Peroxisomal fatty acid oxidation disorders and 58 kDa sterol carrier protein X (SCPx). Activity measurements in liver and fibroblasts using a newly developed method. *J Lipid Res* **41:** 336-342.
39. Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, and IJlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. *J Inherit Metab Dis* **22:** 442-487.
40. Lehman TC, Hale DE, Bhala A, and Thorpe C (1990) An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. *Anal Biochem* **186:** 280-284.
41. Wanders RJ, Denis S, Ruiter JP, IJlst L, and Dacremont G (1998) 2,6-Dimethylheptanoyl-CoA is a specific substrate for long-chain acyl-CoA dehydrogenase (LCAD): evidence for a major role of LCAD in branched-chain fatty acid oxidation. *Biochim Biophys Acta* **1393:** 35-40.
42. Ferdinandusse S, Mulders J, IJlst L, Denis S, Dacremont G, Waterham HR, and Wanders RJ (1999) Molecular cloning and expression of human carnitine octanoyltransferase: evidence for its role in the peroxisomal beta-oxidation of branched-chain fatty acids. *Biochem Biophys Res Commun* **263:** 213-218.
43. Slama A, Brivet M, Boutron A, Legrand A, Saudubray JM, and Demaugre F (1996) Complementation analysis of carnitine palmitoyltransferase I and II defects. *Pediatr Res* **40:** 542-546.
44. Van Kuilenburg AB, Van Lenthe H, Wanders RJ, and van Gennip AH (1997) Subcellular localization of dihydropyrimidine dehydrogenase. *Biol Chem* **378:** 1047-1053.
45. Van Vlies N, Wanders RJ, and Vaz FM (2006) Measurement of carnitine biosynthesis enzyme activities by tandem mass spectrometry: Differences between the mouse and the rat. *Anal Biochem* **354:** 132-139.

46. Ferdinandusse S, Finckh B, de Hingh YC, Stroomer LE, Denis S, Kohlschutter A, and Wanders RJ (2003) Evidence for increased oxidative stress in peroxisomal D-bifunctional protein deficiency. *Mol Genet Metab* **79:** 281-287.
47. Van den Branden C, Vamecq J, Wybo I, and Roels F (1986) Phytol and peroxisome proliferation. *Pediatr Res* **20:** 411-415.
48. Baumgart E, Volkl A, Pill J, and Fahimi HD (1990) Proliferation of peroxisomes without simultaneous induction of the peroxisomal fatty acid beta-oxidation. *FEBS Lett* **264:** 5-9.
49. Fan CY, Pan J, Usuda N, Yeldandi AV, Rao MS, and Reddy JK (1998) Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J Biol Chem* **273:** 15639-15645.
50. Rebouche CJ, Lehman LJ, and Olson L (1986) epsilon-N-trimethyllysine availability regulates the rate of carnitine biosynthesis in the growing rat. *J Nutr* **116:** 751-759.
51. Paul HS and Adibi SA (1980) Leucine oxidation and protein turnover in clofibrate-induced muscle protein degradation in rats. *J Clin Invest* **65:** 1285-1293.

4

Metabolism of phytol to phytanic acid in the mouse and the role of PPARα in its regulation

Jolein Gloerich, Daan M. van den Brink, Jos P.N. Ruiter, Naomi van Vlies, Frédéric M. Vaz, Ronald J.A. Wanders, Sacha Ferdinandusse (2006) *J Lipid Res,* Accepted for publication

Metabolism of phytol to phytanic acid in the mouse and the role of PPARα in its regulation

J. Gloerich, D.M. van den Brink, J.P.N. Ruiter, N. van Vlies, F.M. Vaz, R.J.A. Wanders, S. Ferdinandusse

Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma's Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Abstract

Phytol, a branched-chain fatty alcohol, is the naturally occurring precursor of phytanic and pristanic acid, branched-chain fatty acids which are both ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα). To investigate the metabolism of phytol and the role of PPARα in its regulation, wild-type and PPARα knockout (PPARα$^{-/-}$) mice were fed a phytol-enriched diet or, for comparison, a diet enriched with Wy-14,643, a synthetic PPARα agonist. Following the phytol-enriched diet, phytol could only be detected in small intestine, the site of uptake, and liver. Upon longer duration of the diet, the level of the (*E*)-isomer of phytol increased significantly in the liver of PPARα$^{-/-}$ mice compared to wild-type mice. Activity measurements of the enzymes involved in phytol metabolism showed that phytol feeding or Wy-14,643 treatment resulted in a PPARα-dependent induction of the last three steps of phytol degradation in liver. Furthermore, the enzymes involved showed a higher activity towards the (*E*)-isomer than the (*Z*)-isomer of their respective substrates, indicating a stereospecificity towards the metabolism of (*E*)-phytol. In conclusion, the results described in this paper show that the conversion of phytol to phytanic acid is regulated via PPARα, and is specific for the breakdown of (*E*)-phytol.

Introduction

Phytol is a branched-chain fatty alcohol (3,7,11,15-tetramethylhexadec-2-en-1-ol) which is abundantly present in nature as part of the chlorophyll molecule. The release of phytol from chlorophyll occurs effectively in the digestive system of ruminant animals only, presumably by bacteria present in the gut.[1] As a result, a relatively high amount of free phytol is present in dairy products.[2] In mammals, free phytol is readily absorbed in the small intestine and is metabolized to phytanic acid, a fatty acid that accumulates in a number of metabolic disorders. Elevated levels of phytanic acid in the body are toxic and therefore this fatty acid needs to be broken down.[3-9] Because the methyl-group at the 3-position prevents β-oxidation, phytanic acid first has to undergo a round of α-oxidation. This results in formation of pristanic acid, which is one carbon atom shorter than phytanic acid and can be normally β-oxidized.[10] A deficiency in α-oxidation, such as in Refsum disease, leads to elevated levels of phytanic acid in plasma and tissues of patients, and this is thought to cause the main clinical symptoms in this disorder: retinitis pigmentosa, peripheral neuropathy and cerebellar ataxia.[3,4] Since the breakdown of phytol will contribute to the phytanic and pristanic acid levels in these patients, it is important to study its metabolism and regulation.

In many animal studies, phytol is used as a precursor of phytanic acid. Addition of phytol to the diet results in an increase of phytol metabolites in tissues and plasma.[6,11-13] This has been used as a model to study the effects of the accumulation of phytol metabolites on fatty acid metabolism, in particular via activation of the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα), which is an important transcription factor in the regulation of fatty acid metabolism. Both phytanic and pristanic acid have been shown to activate PPARα *in vitro*,[14,15] and more recently PPARα was also shown to be activated *in vivo* in mice fed a phytol-enriched diet.[13]

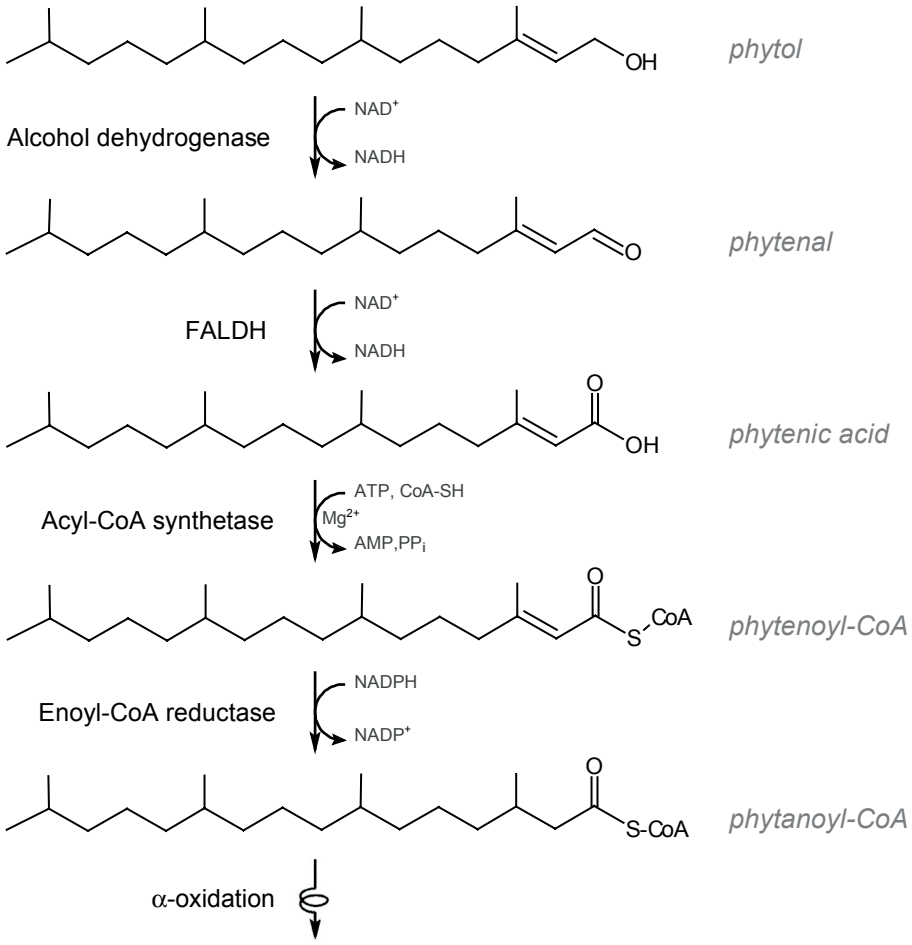


Figure 1: Pathway of the metabolism of phytol to phytanic acid. Phytol is first converted into (*E*)-phytenic acid by the subsequent action of an unknown alcohol dehydrogenase and fatty aldehyde dehydrogenase (FALDH). Phytenic acid is then activated by a synthetase to produce (*E*)-phytenoyl-CoA and finally converted by an enoyl-CoA reductase into phytanoyl-CoA. Phytanoyl-CoA is further broken down by α-oxidation.

Figure 1 shows a schematic representation of the metabolism of phytol to phytanic acid and the enzymes involved. First, phytol is converted into phytenal via a yet unknown alcohol dehydrogenase and subsequently, phytenal is converted into phytenic acid by the microsomal enzyme fatty aldehyde dehydrogenase (FALDH), which is encoded by the *ALDH3A2* gene.[16] Recently, it was shown that phytenic acid is activated to its corresponding CoA-ester, either at the ER or the peroxisome, which is then reduced by a reductase converting phytenoyl-CoA to phytanoyl-CoA either in the peroxisome or the mitochondrion.[17]

In this study, we investigated the metabolism of phytol to phytanic acid in the mouse and the role of PPARα in its regulation. To this end, phytol concentrations were determined and the activities of the enzymes involved in phytol metabolism were analyzed in different tissues of wild-type and PPARα$^{-/-}$ mice fed a phytol-enriched diet. To further investigate the role of PPARα, the activities of the enzymes involved in phytol metabolism were analyzed in different tissues of wild-type and PPARα$^{-/-}$ mice fed a diet enriched with Wy-14,643, a well-known synthetic PPARα agonist.

Materials and Methods

Materials

Pelleted mouse chow containing no or 0.5% (w/w) phytol (mixture of (*E*)- and (*Z*)-phytol) was purchased from Hope Farms (Woerden, The Netherlands). Phytol was from Merck (Darmstadt, Germany). Wy-14,643 was obtained from Tocris Bioscience (Ellisville, MO). $^{2}H_{3}$-phytanic acid was purchased from Dr. H.J. ten Brink (Free University Medical Center, Amsterdam, the Netherlands). C19-OH was obtained form Janssen Chimica (Beerse, Belgium). A mixture of (*E*)- and (*Z*)-phytenic acid was synthesized as described previously.[18] Phytenoyl-CoA isomers were chemically synthesized from a mixture of (*E*)- and (*Z*)-phytenic acid as described.[19] Methyl-β-cyclodextrin was purchased from Fluka (Buchs, Switzerland). NADPH, NAD^{+}, first-strand cDNA synthesis kit for RT-PCR, LightCycler FastStart DNA Master SYBR Green I kit and Complete^{mini} tablets, containing a cocktail of protease inhibitors, were obtained from Roche (Mannheim, Germany). *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL). Silica gel columns were purchased from J.T. Baker (Philipsburg, NJ). *E. coli* INVα cells and Trizol were obtained from Invitrogen (Carlsbad, CA). The pGEM-T vector was from Promega (Madison, WI). The pMAL-C2X vector and amylose-resin columns were purchased from New England Biolabs (Beverly, MA). Freund's complete and incomplete adjuvant were from Difco Laboratories (Detroit, MI). Goat anti-rabbit antibodies conjugated with alkaline phosphatase were obtained from Bio-Rad (Hercules, CA). Nitrocellulose membrane was from Schleicher and Shuell (Keene, NH). Protifar was obtained from Nutricia (Zoetermeer, The Netherlands). All other chemicals were of analytical grade.

Animals

Male wild-type and PPARα$^{-/-}$ mice on a Sv/129 genetic background were used for this study.[20] Six week old wild-type and PPARα$^{-/-}$ mice were fed pelleted mouse chow, containing no (control) or 0.5% (w/w) phytol for 1 or 8 weeks. Nine week old wild-type and PPARα$^{-/-}$ mice were fed pelleted mouse chow, containing no or 0.1% (w/w) Wy-14,643 for 2 weeks. Each group consisted of three animals. At the end of the experiments, mice were anesthetized using isoflurane and tissues were harvested. The animals were always killed at the same time of day,

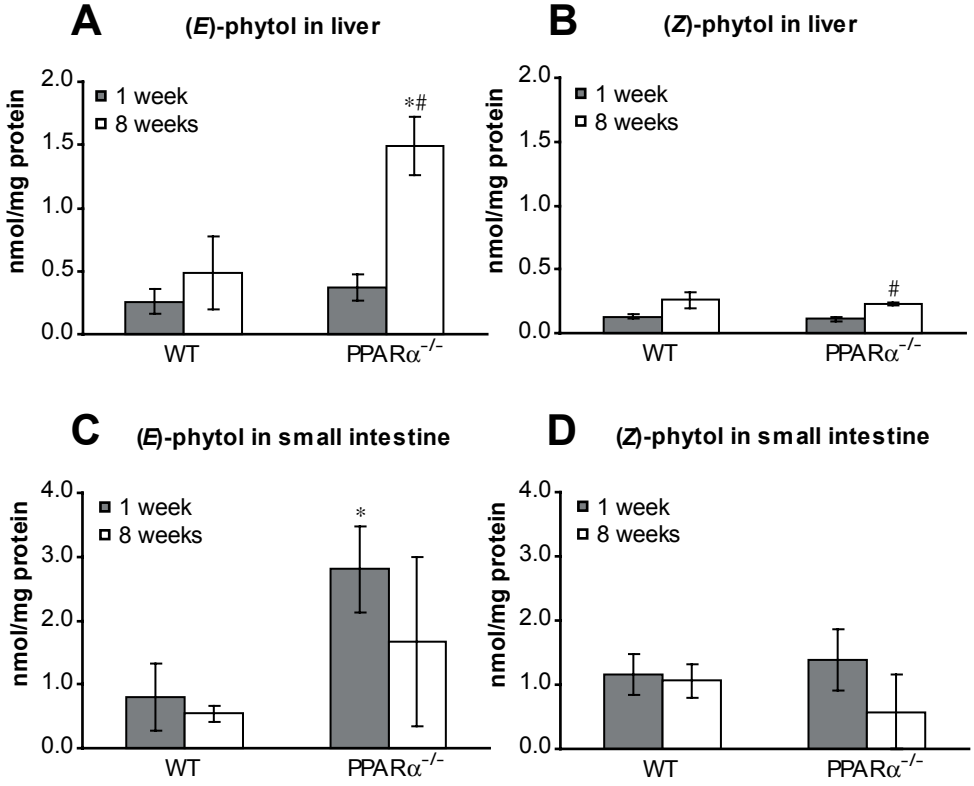


Figure 2: Phytol accumulation in wild-type (WT) and PPARα$^{-/-}$ mice fed a diet enriched with 0.5% phytol for 1 and 8 weeks. A: (*E*)-phytol levels in liver. B: (*Z*)-phytol levels in liver. C: (*E*)-phytol levels in small intestine. D: (*Z*)-phytol levels in small intestine. Values represent the mean of three animals per group ± SD. * $P < 0.01$ between wild-type mice and PPARα$^{-/-}$ mice after the same diet period. # $P < 0.01$ between 1 week and 8 weeks of phytol diet in mice of the same genotype.

and animals had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at –80°C until further analysis. All animal experiments were approved by the University of Amsterdam Animals Experiments Committee.

Synthesis of phytenal

Phytenal was synthesized from its precursor phytol essentially as described previously.[18] Briefly, phytol (a mixture of (*E*)- and (*Z*)-isomers) was oxidized to the corresponding aldehydes with CrO_3-pyridine in dry methylene chloride under a flow of nitrogen to exclude water and oxygen. The oxidant was prepared in 30 ml dichloromethane (dried over anhydrous Na_2SO_4) by addition of 24 mmol anhydrous pyridine, followed by 12 mmol CrO_3 in small batches under magnetic stirring. After clearing of the initial turbidity, 2 mmol phytol in 4 ml dichloromethane was added. The mixture was stirred for 1 hour. Subsequently, the solution was transferred into a separatory funnel and washed with aqueous 5% NaOH for elimination of the excess CrO_3, 5% HCl for the elimination of pyridine, 5% $NaHCO_3$ for neutralization, and finally washed with saturated NaCl. After drying over anhydrous Na_2SO_4, the organic

solvent was filtrated and evaporated under a flow of nitrogen. The residue was then purified over a silica gel column and eluted with chloroform, which was subsequently evaporated under a flow of nitrogen. Phytenal was stored in aliquots under argon at –80°C until use. Gas chromatography-mass spectrometry (GC-MS) analysis of the product showed two peaks corresponding to the (*E*)- and (*Z*)-isomers of phytenal with a molecular ion at *m/z* 294 and a fragmentation pattern specific for aldehydes.

Phytol analysis in tissue homogenates

Tissue phytol levels were determined as described.[13] Briefly, tissues were homogenized in PBS. C19-OH (10 µl of a 50 µM solution dissolved in ethanol) was added to 100 µl tissue homogenate (5 mg/ml) and the sample was subjected to alkaline hydrolysis by adding 2 ml of 1 M NaOH in methanol for 45 min at 110ºC. After cooling to room temperature, the pH was lowered by adding 480 µl 37% HCl. Subsequently, phytol was extracted with 2 ml hexane and the organic layer was evaporated to dryness under nitrogen at 40ºC. Samples were dissolved in 0.5 ml heptane and purified on a silica gel column using 92:8 (v:v) heptane:diethyl ether as eluent. The eluate was evaporated to dryness under nitrogen at 40ºC and derivatized with MTBSTFA and pyridine (50 µl each) at 80ºC for 30 min. Samples were evaporated to dryness under nitrogen at 40ºC, dissolved in 100 µl hexane and subjected to GC-MS analysis, essentially as described previously.[21] The metabolites were quantified using a calibration curve of phytol.

Enzyme activity measurements

All enzyme activity measurements were performed in freshly prepared tissue homogenates. FALDH activity measurements in liver were performed essentially as described previously.[21] Briefly, FALDH activity was measured with both phytol and phytenal as substrate. The reaction mixture constisted of liver protein (20 µg and 50 µg for phytol and phytenal incubations, respectively), 50 mM glycine buffer pH 9.2, 1 mM NAD^+, 0.1% sodium cholate and 1 mg/ml methyl-β-cyclodextrin in a total volume of 500 µl. Reactions were performed at 37ºC and initiated by the addition of substrate in a final concentration of 200 µM and 150 µM for phytol and phytenal, respectively. After 10 min, the incubations were terminated by the addition of 50 µl 2 M HCl. As internal standard 50 pmol of $^2H_3$-phytanic acid, dissolved in toluene, was added. Then 2 ml hexane was added, and after vigorous vortexing the samples were centrifuged, after which the organic layer was evaporated to dryness under a stream of nitrogen at 40ºC. After derivatization with MTBSTFA and pyridine (50 µl each) at 80ºC for 30 min, the samples were evaporated to dryness under nitrogen at 40ºC, dissolved in 200 µl hexane and subjected to GC-MS analysis as described previously.[21]

Enzymatic activation of phytenic acid to its CoA-ester was measured essentially as described.[17] Briefly, tissue homogenate (8 µg protein for liver homogenates, 10 µg protein for kidney and heart homogenates, and 25 µg protein for small intestine and brain homogenates) was incubated with a mixture containing 50 mM Tris pH 8.0, 10 mM ATP, 10 mM $MgCl_2$, 0.5 mM CoA, 1 mg/ml methyl-β-cyclodextrin and 100 µM phytenic acid (a mixture of (*E*)- and (*Z*)-phytenic acid) in a total volume of 100 µl. The incubation was allowed to proceed for 30 min when the activity was measured in liver, and for 60 min in all other tissues. Reactions were terminated by the addition of 100 µl acetonitrile and placed on ice. Samples were centrifuged for 10 min at 10,000 x *g* at 4ºC and supernatants were analyzed by HPLC.

Reduction of phytenoyl-CoA to phytanoyl-CoA was determined essentially as described

previously.[17] Briefly, the reaction mixture contained 50 mM Hepes pH 7.0, 1 mM NADPH, 40 µM phytenoyl-CoA (a mixture of (*E*)- an (*Z*)-phytenoyl-CoA) and tissue homogenate (25 µg for kidney homogenates, 100 µg for liver homogenates, and 200 µg for small intestine, heart and brain homogenates) in a total volume of 100 µl. Reactions were started by addition of substrate and incubated at 37ºC for 15 min for liver homogenates and 30 min for all other tissue homogenates. Reactions were terminated by the addition of 100 µl acetonitrile and placed on ice. Samples were centrifuged for 10 min at 10,000 x *g* at 4ºC and supernatants were analyzed by HPLC.

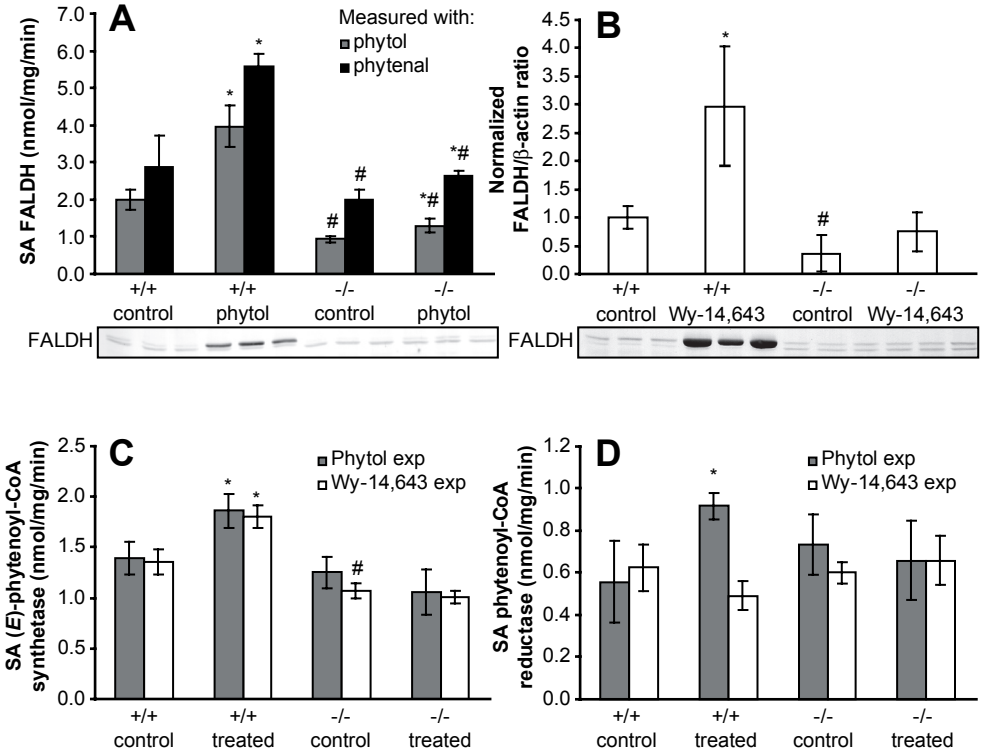


Figure 3: Enzyme activity measurements and immunoblot analysis performed in liver homogenates of wild-type (+/+) and PPARα$^{-/-}$ (-/-) mice (3 mice per group) on a control diet (control) or, either a phytol-enriched diet (phytol) for 8 weeks or a Wy-14,643-enriched diet for 2 weeks. A: Upper panel, rates of phytenic acid production with phytol as a substrate (grey bars) or specific activity (SA) of FALDH with phytenal as a substrate (black bars) in mice on a control diet or a phytol-enriched diet. Lower panel, immunoblot analysis of FALDH in liver from wild-type and PPARα$^{-/-}$ mice on a control diet or a phytol-enriched diet. B: Upper panel, quantitative RT-PCR analysis of FALDH in liver from mice of both genotypes on a control diet or a Wy-14,643-enriched diet. Presented are the normalized FALDH/β-actin ratios. The level of FALDH mRNA in wild-type mice on a control diet was set to 1.0. Lower panel, immunoblot analysis of FALDH in wild-type and PPARα$^{-/-}$ mice on a control diet or a Wy-14,643-enriched diet. C: Phytenoyl-CoA synthetase activity in mice from both genotypes on a control diet or, either a phytol-enriched diet or a Wy-14,643-enriched diet. D: Phytenoyl-CoA reductase activity in mice from both genotypes on a control diet or, either a phytol-enriched diet or a Wy-14,643-enriched diet. Values represent the mean of three animals per group ± SD. * $P < 0.01$ between the phytol-/Wy-14,643-enriched diet and the control diet in mice of the same genotype; # $P < 0.01$ between PPARα$^{-/-}$ mice and wild-type mice on the same diet.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from RNA*later*-treated mouse liver and kidney samples using Trizol extraction, after which cDNA was prepared using a first-strand cDNA synthesis kit for RT-PCR. Quantitative real-time PCR analysis of FALDH and β-actin in liver and kidney was performed using the LightCycler FastStart DNA Master SYBR Green I kit. The following primers were used. For FALDH: forward, 5'-CATACTTACAGATGTTGATCC-3'; reverse, 5'-CTCCAAAGGGCAGAGAATTA-3'. Primers for β-actin were used as described.[22] Melting curve analysis was carried out to confirm the generation of a single product. Amplification of a single product of the correct size was also confirmed by agarose gel electrophoresis. Duplicate analyses were performed for all samples. Data were analyzed using linear regression calculations as described by Ramakers et al..[23] To adjust for variations in the amount of input RNA, the values for FALDH were normalized against the values for the housekeeping gene β-actin.

Preparation of antibodies

A polyclonal antiserum was raised against an N-terminal peptide corresponding to amino acids 1-96 of human FALDH, expressed as a fusion protein with maltose binding protein (MBP). The coding region for this peptide (base pairs 1 to 288) was amplified from human liver cDNA by PCR using the following primers: a *Bam*HI-tagged forward primer 5'-ggatccATGGAGCTCGAAGTCCGG-3' and a *Sal*I-tagged reverse primer 5'-gtcgacTTA GGCCTCATCCAGCATGGTGAG-3' (restriction sites are underlined). The resulting PCR product was ligated into the pGEM-T vector and sequence analysis was performed to exclude sequence errors introduced by Taq polymerase. The fragment was subsequently cloned into the pMAL-C2X vector to create pMAL-FALDH-Nterm, using the *Bam*HI and *Sal*I restriction sites downstream of the isopropyl-1-thio-β-D-galactopyranoside (IPTG) inducible promoter. The *E. coli* strain INVα was transformed with the expression plasmid pMAL-FALDH-Nterm. Transformed cells were grown at 37 °C in 100 ml LB medium supplemented with 0.2 % (w/v) glucose and 100 μg/ml ampicillin to an OD_{600} of approximately 0.6 and IPTG was added to a final concentration of 1 mM to induce expression of the fusion protein. Cells were lysed by sonication on ice (twice for 15 s at an output of 8 W, with an interval of 1 min). The MBP fusion protein was purified on amylose-resin columns according to the protocol of the manufacturer (New England Biolabs). The MBP-FALDH-Nterm was subjected to preparative SDS-PAGE, isolated from the gel and subsequently used to raise antibodies. To this end, a female New Zealand White rabbit was injected subcutaneously with 100 μg of the antigen mixed with an equal volume of Freund's complete adjuvant. After 1 month, the immunization was continued by booster injections (each containing 100 μg of antigen in Freund's incomplete adjuvant) until a satisfactory antibody titer was obtained.

Immunoblot analysis

Small pieces of tissue were homogenized in PBS containing a cocktail of protease inhibitors. Twenty micrograms of protein was separated on a 10% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose membrane.[24] After blocking of nonspecific binding sites with 30 g/l Protifar and 10 g/l BSA in 1 g/l Tween-20/PBS, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against FALDH diluted 1:1,500 in 4% BSA in PBS. Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used for detection.

Statistical analyses

Data are expressed as mean ± SD. Statistical significance was evaluated using an unpaired Student's *t*-test. The results were considered significant at $P < 0.01$.

Results

Phytol accumulation

Phytol levels were measured in several tissues to investigate the distribution of phytol in different organs of the mouse after a phytol-enriched diet. As expected, no phytol could be detected in any of the tissues studied of mice receiving a control diet. In the liver and small intestine of mice fed a phytol-enriched diet, there was an accumulation of phytol, whereas no phytol could be detected in brain, heart and kidney.

In **figure 2**, the amounts of (*Z*)- and (*E*)-phytol detected in liver and small intestine of wild-type and PPARα$^{-/-}$ mice are shown. The phytol levels in small intestine varied considerably

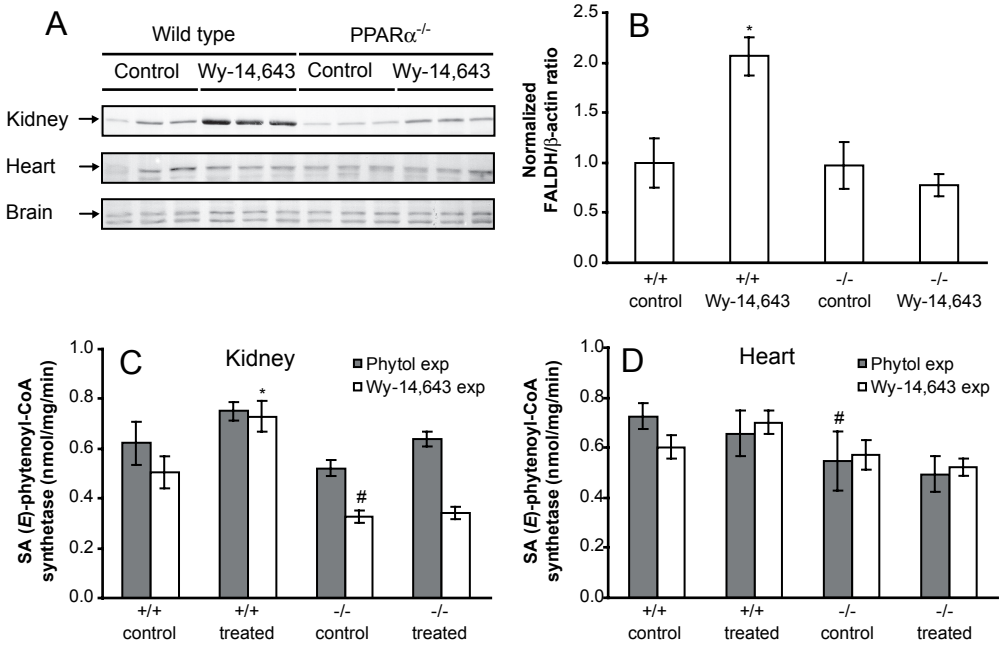


Figure 4: Enzyme activity measurements and immunoblot analysis performed in kidney, heart and brain homogenates of wild-type (+/+) and PPARα$^{-/-}$ (-/-) mice on a control diet (control) or, either a phytol-enriched diet (phytol) for 8 weeks or a Wy-14,643-enriched diet for 2 weeks. A: Immunoblot analysis of FALDH in kidney, heart and brain of wild-type and PPARα$^{-/-}$ mice on a control diet or a Wy-14,643-enriched diet (3 mice per group) B: Quantitative RT-PCR analysis of FALDH in kidney of mice from both genotypes on a control diet or a Wy-14,643-enriched diet. Presented are the normalized FALDH/β-actin ratios. The level of FALDH mRNA in wild-type mice on a control diet was set to 1.0. C: Phytenoyl-CoA synthetase activity in kidney of mice from both genotypes on a control diet or, either a phytol-enriched diet or a Wy-14,643-enriched diet D: Phytenoyl-CoA synthetase activity in heart of mice from both genotypes on a control diet or, either a phytol-enriched diet or a Wy-14,643-enriched diet. Values represent the mean of three animals per group ± SD. * $P < 0.01$ between the phytol-/ Wy-14,643-enriched diet and the control diet in mice of the same genotype; # $P < 0.01$ between PPARα$^{-/-}$ mice and wild-type mice on the same diet.

between individual mice, most likely due to the fact that the animals were fed ad libitum until sacrifice. Despite this high variance, the amount of (*E*)-phytol was higher in PPARα$^{-/-}$ mice than in wild-type animals (Figure 2C).

In a previous study, we determined the phytol levels in liver and showed that after 4 weeks of diet the accumulation of phytol in PPARα$^{-/-}$ mice was more substantial than in wild-type animals.[13] In this study, we determined the levels of both (*Z*)- and (*E*)-phytol in liver. After 1 week on a phytol-enriched diet there were no significant differences in phytol levels between wild-type and PPARα$^{-/-}$ mice (Figure 2). After 8 weeks on the phytol-enriched diet, however, the amount of (*E*)-phytol, in contrast to the (*Z*)-isomer, was increased significantly in PPARα$^{-/-}$ mice compared to wild-type animals. In wild-type mice, the phytol level did not increase with time, but it did increase in PPARα$^{-/-}$ mice.

Phytol degrading enzymes in the liver

Since FALDH only converts (*E*)-phytenal into (*E*)-phytenic acid, the predominant accumulation of (*E*)-phytol in liver of PPARα$^{-/-}$ mice in combination with lower hepatic levels of phytanic acid in PPARα$^{-/-}$ mice compared to wild-type animals[13] point to a decreased capacity of the phytol degradation pathway in PPARα$^{-/-}$ mice. To investigate this, we measured the activities of the enzymes of this pathway in liver (**Figure 3**).

Firstly, we measured the conversion of phytol and of phytenal into (*E*)-phytenic acid. When phytol is used as a substrate, the first and second step of the pathway are measured simultaneously (catalyzed by the alcohol dehydrogenase and FALDH, respectively). Using phytenal as a substrate, only the second step catalyzed by FALDH is measured. The specific activity of (*E*)-phytenic acid formation measured with both substrates was higher in wild-type than in PPARα$^{-/-}$ mice. Under influence of the phytol-enriched diet, both assays revealed a significantly increased (*E*)-phytenic acid formation in wild-type mice (Figure 3A). Because phytol metabolites are ligands for PPARα, this suggests that PPARα plays a role in the regulation of the FALDH expression and might also be involved in the regulation of the expression of the alcohol dehydrogenase. Because the fold induction of the activity measured with phytol and phytenal as substrate was the same, it is probable that the reaction catalyzed by FALDH is the rate-limiting step. Interestingly, a small but significant induction of (*E*)-phytenic acid formation also was observed with both substrates in PPARα$^{-/-}$ mice, although this was much less pronounced when compared to wild-type mice, indicating that there is both a PPARα-dependent and -independent effect. Immunoblot analysis of FALDH showed a clear induction of FALDH protein in wild-type mice fed a phytol diet compared to the control diet, whereas no induction was observed in PPARα$^{-/-}$ mice (Figure 3A). To substantiate that FALDH expression is under control of PPARα, FALDH expression was studied in wild-type and PPARα$^{-/-}$ mice treated with the PPARα ligand Wy-14,643. Figure 3B shows that induction of FALDH protein indeed occurred in wild-type mice, but not in PPARα$^{-/-}$ animals, fed a diet enriched with Wy-14,643. Investigation of liver mRNA levels using quantitative RT-PCR showed a 3.0-fold induction of FALDH mRNA after Wy-14,643 treatment in wild-type mice. The induction of FALDH mRNA is in agreement with the mode of action of Wy-14,643 which activates PPARα to induce transcription of its target genes.

Secondly, the activation of (*E*)-phytenic acid to its CoA-ester was significantly increased in liver from phytol-fed and Wy-14,643 treated wild-type animals when compared to animals on a control diet (Figure 3C). The specific activity for the formation of (*Z*)-phytenoyl-CoA was 17-fold lower than for (*E*)-phytenoyl-CoA (**table 1**) and, although it was slightly higher

in phytol-fed and Wy-14,643 treated wild-type mice, this difference was not statistically significant (data not shown). The increase in (*E*)-phytenoyl-CoA formation was not observed in PPARα$^{-/-}$ mice on a diet enriched with phytol or Wy-14,643, indicating that also the induction of phytenoyl-CoA synthetase is PPARα-dependent.

Thirdly, the specific activity of phytenoyl-CoA reductase, the last step of the pathway, was significantly increased in wild-type mice on a phytol-enriched diet, whereas no induction was observed in PPARα$^{-/-}$ mice. Interestingly, no induction of phytenoyl-CoA reductase activity was observed after feeding a Wy-14,643-enriched diet. These data suggest that, in addition to phytenoyl-CoA synthetase, phytenoyl-CoA reductase can be regulated in a PPARα-dependent way, although this regulation appears to be ligand specific. When studying the stereochemistry of the reductase reaction, the specific activity for the reduction of (*E*)-phytenoyl-CoA was found to be 2.8-fold higher than for the purified (*Z*)-isomer (data not shown).

Site of phytol degradation

In order to gain more insight into the site of phytol metabolism in the body, we investigated the activities of the enzymes involved in this pathway in different mouse tissues. The tissue distribution of FALDH was described previously.[25] Highest expression and activity was found in liver, followed by lung, kidney, small intestine and stomach. Low but detectable FALDH expression and activity was also present in brain, skin, heart and muscle. Immunoblot analysis of FALDH in kidney, heart and brain homogenates is depicted in **figure 4A**. In heart and brain, FALDH protein levels are detectable, but no induction of FALDH protein was observed after the Wy-14,643-enriched diet. In kidney, however, FALDH protein was significantly induced in wild-type mice after the Wy-14,643-enriched diet, indicating regulation via PPARα. Analysis of FALDH mRNA levels in kidney (Figure 4B) showed an induction of FALDH mRNA in wild-type mice after Wy-14,643 treatment, but not in PPARα$^{-/-}$ mice, which is in agreement with the immunoblot results.

Table 1: Specific activities for the activation of (*E*)- and (*Z*)-phytenic acid to their corresponding CoA-ester and for the reduction of phytenoyl-CoA in different tissues of wild-type mice on a control diet

	(*E*)-Phytenoyl-CoA synthetase (nmol/mg/min)	(*Z*)-Phytenoyl-CoA synthetase (nmol/mg/min)	Phytenoyl-CoA reductase (nmol/mg/min)
Liver	1.40 ± 0.02	0.08 ± 0.01	0.56 ± 0.04
Kidney	0.62 ± 0.09	0.10 ± 0.02	0.24 ± 0.12
Heart	0.72 ± 0.06	n.d.	0.03 ± 0.006
Small intestine	0.05 ± 0.03	n.d.	0.02 ± 0.006
Brain	0.12 ± 0.02	n.d.	0.04 ± 0.003

Values represent mean ± SD (n=3)
n.d., not detectable

In table 1, both (*E*)- and (*Z*)-phytenoyl-CoA synthetase activities are shown. In all the tissues tested, the specific activity for the formation of (*E*)-phytenoyl-CoA was markedly higher than for the (*Z*)-isomer. The highest activity of (*E*)-phytenoyl-CoA synthetase was measured in liver. In kidney and heart, the specific activity measured was approximately half of the value for liver. In small intestine and brain only very low specific activities of (*E*)-phytenoyl-CoA synthetase could be measured, whereas (*Z*)-phytenoyl-CoA synthetase activity was undetectable. In figure 4C and 4D, (*E*)-phytenoyl-CoA synthetase activities in

kidney and heart of mice in the different feeding experiments are depicted. After treatment of wild-type mice with Wy-14,643, a significant increase of (*E*)-phytenoyl-CoA synthetase activity was observed in kidney. A slight increase of (*E*)-phytenoyl-CoA synthetase activity also was observed in heart of wild-type mice after treatment with Wy-14,643, but this was not significant. The increase observed in kidney of wild-type animals fed a phytol diet was also not significant. In other tissues no change in (*E*)-phytenoyl-CoA synthetase activity was detected.

As for phytenoyl-CoA synthetase, the specific activity of phytenoyl-CoA reductase was highest in liver. In kidney the specific activity was approximately half of the value for liver. In all other tissues tested very low, but detectable, activities of phytenoyl-CoA reductase were measured (table 1). In the extrahepatic tissues, phytenoyl-CoA reductase activity was not induced by phytol or by Wy-14,643.

Discussion

Our results show that PPARα plays an important role in the regulation of the enzymes involved in phytol degradation. In livers from PPARα$^{-/-}$ mice, phytol was found to accumulate to much higher levels as compared to livers from wild-type animals upon longer duration of the phytol-enriched diet, suggesting a decreased capacity for phytol metabolism in PPARα$^{-/-}$ mice compared to wild-type animals. Further investigation showed that at least three of the four enzymes of this breakdown pathway (i.e. FALDH, phytenoyl-CoA synthetase and phytenoyl-CoA reductase) are under regulation of PPARα, because their activities increase after phytol or Wy-14,643 feeding in livers from wild-type mice but not from PPARα$^{-/-}$ animals. Only for FALDH, it was reported previously that expression can be induced by the action of PPARα, although no functional peroxisome proliferator response element has been identified yet.[26]

Both (*Z*)- and (*E*)-phytol are absorped in the small intestine of the mouse, as shown by the presence of both isomers. Upon longer duration of the phytol-enriched diet no increase in accumulation of phytol was observed in small intestine. In contrast, the accumulation of phytol in the liver increased with time, which was mainly due to an increase in (*E*)-phytol levels. The predominant accumulation of the (*E*)-isomer of phytol strongly suggests that the phytol degradation pathway is stereospecific. Indeed, FALDH, which catalyzes the second step in the breakdown of phytol, exclusively forms (*E*)-phytenic acid. The third and fourth step of the phytol degradation pathway, catalyzed by a phytenoyl-CoA synthetase and phytenoyl-CoA reductase, respectively, also show a strong preference for (*E*)-isomers as substrate. The peroxisomal *trans*-2-enoyl-CoA reductase identified by Das et al.[27] was suggested as a putative candidate for the reduction of phytenoyl-CoA to phytanoyl-CoA.[17] This reductase has been shown to be stereospecific towards the (*E*)-form of enoyl-CoA esters[17,27] and has now been shown to convert (*E*)-phytenoyl-CoA to (*E*)-phytanoyl-CoA.[28] It remains to be established, however, whether this enzyme is the sole reductase catalyzing the reduction of phytenoyl-CoA, especially since mitochondria, which lack the reductase identified by Das et al.,[27] are also able to reduce phytenoyl-CoA. Concluding, our results show that the phytol degradation pathway is stereospecific for the breakdown of (*E*)-phytol to phytanic acid.

All the enzymes involved in the breakdown of phytol to phytanic acid displayed high activity in mouse liver. The first step, catalyzed by the alcohol dehydrogenase, cannot be measured directly, since the product of this reaction, i.e. phytenal, is very unstable. At least in liver, this enzyme must be present since phytol was converted into (*E*)-phytenic acid in

liver homogenates. The enzyme activity measurements of the other enzymes involved in the breakdown of phytol, together with the measurement of phytol levels in different tissues of the mouse, strongly suggest that the main site of phytol degradation is the liver. Phytol itself only could be detected in liver and small intestine after the phytol-enriched diet. Most likely, the phytol levels in small intestine are a reflection of the phytol that has been absorbed from the diet just before sacrifice. As a consequence, no increase in small intestinal phytol levels was observed with time, in contrast to the hepatic phytol concentration, which did increase with time.

In summary, the results described in this paper show that the degradation of phytol to phytanic acid is regulated via PPARα. Furthermore, phytol accumulates almost exclusively in liver and the enzymes involved in phytol degradation display highest activities in liver, indicating that the liver is the main site of phytol metabolism in the body. Lastly, the pathway shows specificity for the breakdown of (*E*)-phytol, since this isomer predominantly accumulates in liver of PPARα$^{-/-}$ mice on a phytol-enriched diet and at least three of the enzymes of the pathway have been shown to be stereospecific towards this isomer.

Acknowledgments

The authors thank R. Ofman for technical assistance and Prof. Dr. J.F. Rontani for advice. This work was supported by grant 901-03-097 from the Netherlands Organization for Scientific Research (NWO, The Hague, The Netherlands) and grant QLG3-2002-00696 from the European Union.

References

1. Hansen RP (1966) Occurence of phytanic acid in rumen bacteria. *Nature* **210:** 841.
2. Brown PJ, Mei G, Gibberd FB, Burston D, Mayne PD, McClinchy JE, and Sidey M (1993) Diet and Refsum's disease. The determination of phytanic acid and phytol in certain foods and the application of this knowledge to the choice of suitable convenience foods for patients with Refsum's disease. *J Hum Nutr Diet* **6:** 295-305.
3. Wanders RJ, Jansen GA, and Skjeldal OH (2001) Refsum disease, peroxisomes and phytanic acid oxidation: a review. *J Neuropathol Exp Neurol* **60:** 1021-1031.
4. Wierzbicki AS, Lloyd MD, Schofield CJ, Feher MD, and Gibberd FB (2002) Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. *J Neurochem* **80:** 727-735.
5. Monnig G, Wiekowski J, Kirchhof P, Stypmann J, Plenz G, Fabritz L, Bruns HJ, Eckardt L, Assmann G, Haverkamp W, Breithardt G, and Seedorf U (2004) Phytanic acid accumulation is associated with conduction delay and sudden cardiac death in sterol carrier protein-2/sterol carrier protein-x deficient mice. *J Cardiovasc Electrophysiol* **15:** 1310-1316.
6. Savolainen K, Kotti TJ, Schmitz W, Savolainen TI, Sormunen RT, Ilves M, Vainio SJ, Conzelmann E, and Hiltunen JK (2004) A mouse model for {alpha}-methylacyl-CoA racemase deficiency: adjustment of bile acid synthesis and intolerance to dietary methyl-branched lipids. *Hum Mol Genet* **13:** 955-965.
7. Steinberg D, Avigan J, Mize CE, Baxter JH, Cammermeyer J, Fales HM, and Highet PF (1966) Effects of dietary phytol and phytanic acid in animals. *J Lipid Res* **7:** 684-691.
8. Atshaves BP, Payne HR, McIntosh AL, Tichy SE, Russell D, Kier AB, and Schroeder F (2004) Sexually dimorphic metabolism of branched-chain lipids in C57BL6/6J mice. *J Lipid Res* **45:** 812-830.
9. Atshaves BP, McIntosh AL, Payne HR, Mackie J, Kier AB, and Schroeder F (2005) Effect of branched-chain fatty acid on lipid dynamics in mice lacking liver fatty acid binding protein gene. *Am J Physiol Cell Physiol* **288:** C543-C558.
10. Verhoeven NM, Wanders RJ, Poll-The BT, Saudubray JM, and Jakobs C (1998) The metabolism of phytanic

acid and pristanic acid in man: a review. *J Inherit Metab Dis* **21:** 697-728.
11. Baes M, Huyghe S, Carmeliet P, Declercq PE, Collen D, Mannaerts GP, and Van Veldhoven PP (2000) Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids. *J Biol Chem* **275:** 16329-16336.
12. Ellinghaus P, Wolfrum C, Assmann G, Spener F, and Seedorf U (1999) Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice. *J Biol Chem* **274:** 2766-2772.
13. Gloerich J, Van Vlies N, Jansen GA, Denis S, Ruiter JP, van Werkhoven MA, Duran M, Vaz FM, Wanders RJ, and Ferdinandusse S (2005) A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARalpha-dependent and -independent pathways. *J Lipid Res* **46:** 716-726.
14. Heim M, Johnson J, Boess F, Bendik I, Weber P, Hunziker W, and Fluhmann B (2002) Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes. *FASEB J* **16:** 718-720.
15. Zomer AW, Van der Burg B, Jansen GA, Wanders RJ, Poll-The BT, and Van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. *J Lipid Res* **41:** 1801-1807.
16. van den Brink DM, van Miert JN, Dacremont G, Rontani JF, Jansen GA, and Wanders RJ (2004) Identification of fatty aldehyde dehydrogenase in the breakdown of phytol to phytanic acid. *Mol Genet Metab* **82:** 33-37.
17. van den Brink DM, van Miert JN, Dacremont G, Rontani JF, and Wanders RJ (2005) Characterization of the final step in the conversion of phytol into phytanic acid. *J Biol Chem* **280:** 26838-26844.
18. Rontani JF, Bonin PC, and Volkman JK (1999) Biodegradation of free phytol by bacterial communities isolated from marine sediments under aerobic and denitrifying conditions. *Appl Environ Microbiol* **65:** 5484-5492.
19. Rasmussen JT, Borchers T, and Knudsen J (1990) Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. *Biochem J* **265:** 849-855.
20. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, and Gonzalez FJ (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* **15:** 3012-3022.
21. van den Brink DM, van Miert JM, and Wanders RJ (2005) Assay for Sjogren-Larsson syndrome based on a deficiency of phytol degradation. *Clin Chem* **51:** 240-242.
22. Sousa M, Du Yan S, Fernandes R, Guimaraes A, Stern D, and Saraiva M (2001) Familial Amyloid Polyneuropathy: Receptor for Advanced Glycation End Products-Dependent Triggering of Neuronal Inflammatory and Apoptotic Pathways. *J Neurosci* **21:** 7576-7586.
23. Ramakers C, Ruijter JM, Deprez RH, and Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339:** 62-66.
24. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
25. Lin Z, Carney G, and Rizzo WB (2000) Genomic organization, expression, and alternate splicing of the mouse fatty aldehyde dehydrogenase gene. *Mol Genet Metab* **71:** 496-505.
26. Mandard S, Muller M, and Kersten S (2004) Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* **61:** 393-416.
27. Das AK, Uhler MD, and Hajra AK (2000) Molecular cloning and expression of mammalian peroxisomal trans-2-enoyl-coenzyme A reductase cDNAs. *J Biol Chem* **275:** 24333-24340.
28. Gloerich J, Ruiter JP, van den Brink DM, Ofman R, Ferdinandusse S, and Wanders RJ (2006) Peroxisomal trans-2-enoyl-CoA reductase is involved in phytol degradation. *FEBS Lett* **580:** 2092-2096.

5

Bezafibrate induces FALDH in human fibroblasts; implications for Sjögren-Larsson syndrome

Jolein Gloerich, Lodewijk IJlst, Ronald J.A. Wanders, Sacha Ferdinandusse
(2006) *Mol Genet Metab* **89**, 111-115

Bezafibrate induces FALDH in human fibroblasts; implications for Sjögren-Larsson syndrome

J. Gloerich, L. IJlst, R.J.A. Wanders, S. Ferdinandusse

Laboratory for Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma's Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Abstract

Sjögren-Larsson syndrome (SLS) is caused by a deficiency of fatty aldehyde dehydrogenase (FALDH), encoded by the *ALDH3A2* gene. In animal studies, the expression of the murine ortholog of FALDH, has been shown to be under the control of peroxisome proliferator-activated receptor α (PPARα). In the present study, we investigated whether the hypolipidemic drug bezafibrate, which is a pan-agonist of all PPAR isoforms, might induce FALDH activity in human fibroblasts of control subjects and SLS patients that still have some residual FALDH activity. Our results show that FALDH activity was induced 1.4-fold after a 3-day treatment with 800 μM bezafibrate in fibroblasts of control subjects. Interestingly, in fibroblasts of two SLS patients homozygous for the p.R228C substitution, FALDH activity could be induced to 37% of control values by bezafibrate treatment. mRNA analysis in fibroblasts of these patients also revealed a mean 1.8-fold induction of FALDH mRNA after bezafibrate treatment. No induction was observed in fibroblasts of patients with mutations that cause instability of FALDH mRNA or that result in a protein without any residual activity. These data suggest that bezafibrate treatment could be effective in patients with expression of FALDH protein and some residual enzyme activity. Further research is needed to resolve whether patients could benefit from treatment with bezafibrate.

Introduction

Sjögren-Larsson syndome (SLS) is an autosomal recessive disorder of fatty alcohol metabolism, characterized by ichthyosis, spastic di- or tetraplegia and mental retardation,[1] Fatty aldehyde dehydrogenase (FALDH), which is encoded by the *ALDH3A2* gene, is the enzyme that is deficient in SLS.[2] FALDH is part of the microsomal alcohol NAD^+-oxidoreductase complex that is responsible for the conversion of long-chain fatty alcohols into fatty acids. As a consequence, a deficiency of FALDH leads to elevated levels of fatty alcohols in plasma of patients.[3] FALDH is also involved in the breakdown of leukotriene B_4 and in the oxidation of fatty aldehydes derived from ether glycerolipids.[3,4] Recently, it was shown that, in addition, FALDH is involved in the degradation of phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol),[5] which is a branched-chain fatty alcohol abundantly present in nature as part of the chlorophyll molecule. In this pathway, FALDH is responsible for the conversion of phytenal into phytenic acid, which is further reduced to phytanic acid.

In a number of animal studies, phytol has been added to the diet so that phytol metabolites, most notably phytanic and pristanic acid, accumulate in tissues and plasma.[6-9] This model has been used to study the effects of the accumulation of phytol metabolites on fatty acid

metabolism, because phytanic and pristanic acid have been shown to activate peroxisome proliferator-activated receptor α (PPARα).[8-11] In one of these studies, FALDH protein and activity was induced in a PPARα-dependent manner after feeding a phytol-enriched diet (chapter 4). For murine FALDH, it was already reported that expression can be induced by the action of PPARα, although no functional peroxisome proliferator response element has been reported yet in the *ALDH3A2* promotor.[12]

PPARα is one of the PPARs that form a subfamily within the nuclear hormone receptor superfamily. PPARs are ligand-activated transcription factors. After ligand binding, PPARs heterodimerize with the retinoic X receptor α and modulate the expression of target genes by binding to specific peroxisome proliferator response elements in the promotor region of the regulated genes.[13] Three PPAR isoforms are known: PPARα, PPARβ, and PPARγ. The three isoforms have different tissue distributions and functions. PPARα is mostly expressed in organs with a high rate of fatty acid catabolism, such as brown adipose tissue, liver, kidney, and heart, and plays an important role in various aspects of lipid and glucose metabolism.[14,15] Given their roles in pivotal metabolic processes, PPARs are important targets in the modulation of several disorders, such as glucose intolerance, hyperinsulinemia, dyslipidemia, hypertension and atherosclerosis.[16,17] PPARs have attracted attention in terms of therapeutic potential, because many ligands or activators of these receptors have been identified, including common drugs like fibrates.[13]

In this study, we investigated whether FALDH expression and activity in human skin fibroblasts can be upregulated in a PPAR-dependent manner, just like in mice, as a potential therapeutic option for SLS patients. To do this, we measured FALDH activity and FALDH mRNA expression in human fibroblasts of control subjects and SLS patients after treatment with bezafibrate, a common hypolipidemic drug, which acts as an agonist of all PPAR-isoforms.

Materials and Methods

Materials

Cell culture reagents, media, fetal calf serum and Trizol were purchased from Invitrogen (Carlsbad, CA). Bezafibrate and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Phytol was purchased from Merck (Darmstadt, Germany). NAD^+, first-strand cDNA synthesis kit for RT-PCR and LightCycler FastStart DNA Master SYBR Green I kit were purchased from Roche (Mannheim, Germany). Methyl-β-cyclodextrin was from Fluka (Buchs, Switzerland). $^2H_3$-phytanic acid was obtained from Dr. H.J. ten Brink (Free University Medical Center, Amsterdam, the Netherlands). *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL). All other chemicals were of analytical grade.

Cultured Skin Fibroblasts

Human skin fibroblasts were cultured in HAM F-10 medium with L-glutamine and 25 mM HEPES, supplemented with 10 % fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37ºC with 5 % CO_2.

Patient cell lines

The FALDH-deficient fibroblasts were from established SLS patients as concluded from the

clinical history, deficient FALDH activity and distinct mutations in the *ALDH3A2* gene. These patients have been described in detail elsewhere.[1] All patient cell lines used in this study were taken from the cell repository of the laboratory for Genetic Metabolic Diseases (Academical Medical Center, University of Amsterdam, The Netherlands) and were derived from patients diagnosed in this Center.

Incubations of cultured fibroblasts with bezafibrate

Confluent fibroblast cultures were incubated with different concentrations of bezafibrate in the culture medium for 3 days. Bezafibrate was dissolved in DMSO. The final DMSO concentration in the medium was 1%. The untreated cells were incubated with medium containing 1% DMSO. Fibroblasts were harvested using trypsin and stored as cell pellets at –80ºC until use.

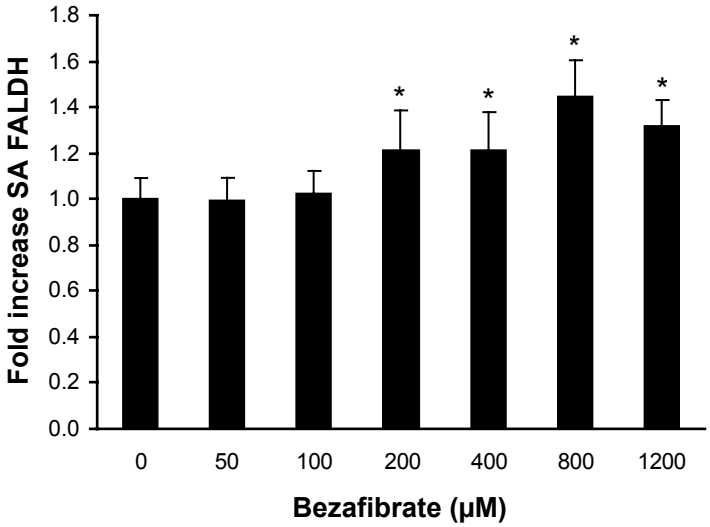


Figure 1: Increase in specific activity (SA) of fatty aldehyde dehydrogenase (FALDH) in human skin fibroblasts of control subjects after treatment with bezafibrate. Cells were cultured for 3 days in the presence of the indicated amounts of bezafibrate. The specific activity of cells on medium without bezafibrate was set to 1.0 in each experiment. Values represent the mean of measurements in four different control cell lines ± SD. * $P<0.001$ as calculated by Student's t-test compared with control fibroblasts without bezafibrate treatment.

FALDH activity measurements

All enzyme measurements were performed in freshly prepared homogenates of pelleted human fibroblasts. FALDH activity measurements were performed essentially as described previously.[18] Briefly, FALDH activity was measured with phytol as substrate. The reaction mixture constisted of 25 µg fibroblast protein, 50 mM glycine buffer pH 9.2, 1 mM NAD^+, 1 mg/ml sodium cholate and 1 mg/ml methyl-β-cyclodextrin in a total volume of 500 µl. Reactions were performed at 37ºC and initiated by the addition of substrate in a final concentration of 100 µM. After 60 min, the incubations were terminated by the addition of 50 µl 2 M HCl. As internal standard 50 pmol of $^2H_3$-phytanic acid dissolved in toluene, was added. Then 2 ml hexane was added, after which the organic layer was evaporated to dryness

under nitrogen at 40ºC. Samples were derivatized with MTBSTFA and pyridine (50 µl each) at 80ºC for 30 min. Subsequently, samples were evaporated to dryness under nitrogen at 40ºC, dissolved in 200 µl hexane and subjected to GC-MS analysis.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from human fibroblast pellets using Trizol, after which cDNA was prepared using a first-strand cDNA synthesis kit for RT-PCR. Quantitative real-time PCR analysis of FALDH and β-2-microglobulin was performed using the LightCycler FastStart DNA Master SYBR Green I kit. For FALDH the following primers were used: forward, 5'-GTACTTACCGATGTTGATCC-3' (located in exon 7); reverse, 5'-CCAAATGGGAAAGAGTTGAG-3' (located in exon 8) to amplify a fragment of 251 bp. Primers for β-2-microglobulin were used as described.[19] The PCR program comprised a 10 min initial denaturation step at 95ºC to activate the hot start polymerase, followed by 40 cycles of 95ºC for 10 s, 58ºC for 2 s and 72ºC for 10 s (9 s for β-2-microglobulin). Fluorescence was measured at 72ºC for FALDH and 80ºC for β-2-microglobulin. Melting curve analysis was carried out to confirm the generation of a single product. Amplification of a single product of the correct size was also confirmed by agarose gel electrophoresis. Duplicate analyses were performed for all samples. Data were analyzed using linear regression calculations as described by Ramakers et al..[20] To adjust for variations in the amount of input RNA, the values for FALDH were normalized against the values for the housekeeping gene β-2-microglobulin.

Statistical analyses

Data are expressed as mean ± SD. Statistical significance was evaluated using an unpaired Student's t-test. The results were considered significant at $P < 0.01$.

Results and Discussion

Effects of bezafibrate on FALDH activity in human fibroblasts

To investigate whether FALDH activity can be induced in human cells via activation of one of the PPAR-isoforms, we measured FALDH activity in human skin fibroblasts of control subjects after treatment with bezafibrate. Cells of four different control subjects were incubated with different concentrations of bezafibrate (50-1200 µM) for three days. Because bezafibrate was dissolved in DMSO, all incubations were performed with a final concentration of 1% DMSO in the medium. In human control fibroblasts, bezafibrate induced FALDH activity in a dose-dependent manner (**Figure 1**). This increase in FALDH activity was statistically significant at a concentration of 200 µM bezafibrate or higher, and the maximal induction (1.4-fold) was observed after treatment with 800 µM bezafibrate. Bezafibrate was toxic to the fibroblasts at concentrations higher than 1200 µM. Therefore, 800 µM bezafibrate was chosen as standard condition for further studies.

Induction of FALDH activity in human fibroblasts of control subjects and SLS patients

A variety of mutations have been detected in the *ALDH3A2* gene of SLS patients, including deletions, insertions, splicing defects and missense mutations.[1,21-30] Most of these mutations result in a total loss of FALDH activity, but a few missense mutations result in a protein which still shows some residual activity when expressed in Chinese hamster ovary cells.[27]

Because FALDH activity could be induced in control fibroblasts by bezafibrate, we

investigated whether FALDH activity could also be induced in fibroblasts of SLS patients. To this end, we cultured patient fibroblasts for three days in the presence or absence of 800 μM bezafibrate and measured FALDH activity. Fibroblasts from four different patients were used in this study. Patient 1 and 2 had a homozygous missense mutation resulting in an amino acid substitution (p.R228C). Patient 3 was compound heterozygous for a missense mutation leading to the amino acid substitution p.K266N, and for a 2 basepair deletion causing a frameshift and a truncation (c.1297-1298del). The fourth patient was homozygous for this deletion. All four patient cell lines displayed low residual FALDH activity (**Figure 2**).

After treatment with 800 μM bezafibrate, a 2.5- and 2.8-fold induction of FALDH activity was observed in the fibroblasts of patient 1 and 2, respectively, both homozygous for the p.R228C substitution. This resulted in an FALDH activity in patient 1 and 2 of 34% and 39% of the activity in control subjects. In the cell line heterozygous for p.K266N and c.1297-1298del, and the cell line homozygous for this deletion, no induction of FALDH activity was observed. This could be explained by the fact that both mutations result in an unstable mRNA transcript that is rapidly degraded,[27,31] even though the p.K266N mutation displayed very high residual activity when expressed in Chinese hamster ovary cells. The recombinant FALDH protein containing p.R228C displayed 9% residual activity in these expression studies.[27]

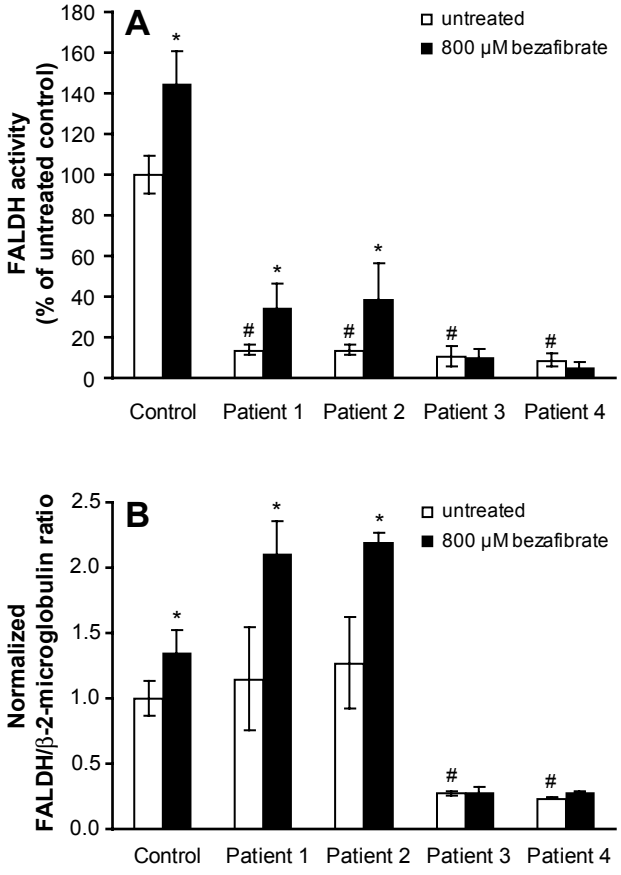


Figure 2: Measurement of fatty aldehyde dehydrogenase (FALDH) enzyme activity and mRNA levels in control and patient fibroblasts (patient 1 and 2: homozygous for p.R228C; patient 3: heterozygous for p.K266N and c.1297-1298del; patient 4: homozygous for c.1297-1298del) after 3-day treatment with or without bezafibrate. A: FALDH enzyme activity in control and patient fibroblasts, expressed as percentages of specific activity measured in control cells without bezafibrate. B: Quantitative real-time RT-PCR analysis of FALDH. Presented are the normalized FALDH/β-2-microglobulin ratios. The level of FALDH mRNA in control cells without bezafibrate treatment was set to 1.0. Control values represent the mean of the results obtained with two different control subjects. * $P < 0.01$ between untreated and bezafibrate treated cells of the same cell line. # $P < 0.01$ between control and patient cells.

Induction of FALDH mRNA expression in human fibroblasts of controls and SLS patients
Because FALDH activity was induced in fibroblasts from control subjects and patients homozygous for the amino acid substitution p.R228C, we investigated whether this induction was due to increased mRNA expression. To this end, mRNA levels of FALDH were investigated using quantitative RT-PCR after the 3-day treatment of the fibroblasts with or without 800 μM bezafibrate.

In figure 2B, mRNA expression of FALDH is depicted normalized to mRNA levels of the housekeeping gene β-2-microglobulin. In two control cell lines a 1.3-fold induction of FALDH mRNA expression was observed after bezafibrate treatment. In patient 1 and 2, FALDH mRNA levels were comparable to the levels in the control subjects, and after bezafibrate treatment a 1.8- and 1.7-fold induction of FALDH mRNA was observed in these cell lines, respectively. FALDH mRNA levels in fibroblasts from patient 3 and 4 were considerably lower than in control subjects, confirming the reported instability of the mRNA transcripts. No induction of mRNA levels was observed after bezafibrate treatment in these cell lines.

The increase in FALDH activity after bezafibrate treatment in cell lines of control subjects and patient 1 and 2 correspond well to the observed increase in mRNA level. The induction of FALDH mRNA levels after bezafibrate treatment is in agreement to the model of action of bezafibrate, which activates the PPARs, thereby inducing the transcription of target genes. The lack of induction in FALDH mRNA levels in patient 3 and 4 is most likely due to rapid degradation of the instabile mRNA transcripts. In contrast, the mRNA transcript containing the c.682C>T mutation leading to the p.R228C amino acid substitution is stable and leads to expression of FALDH protein with residual activity, that can be induced by treatment with bezafibrate.

Concluding remarks

Our results show that FALDH expression and activity can be induced via bezafibrate treatment in fibroblasts not only of control subjects but also of SLS patients. In cell lines of patients with a homozygous p.R228C substitution, FALDH activity could be induced to 37% of control values. No induction was observed in fibroblasts of patients with null mutations that cause instability of FALDH mRNA. This suggests that bezafibrate treatment could be effective in patients who have normal expression of a mutated FALDH protein with some residual activity. Based on our results, next to p.R228C, good candidates for bezafibrate therapy would be p.I45F, p.S365L and p.F419S, because recombinant FALDH with these amino acid changes display residual activity.[27] Of course, further research is needed to resolve whether patients could benefit from treatment with bezafibrate.

Acknowledgments

The authors thank Petra Mooijer, Patricia Veltman and Janet Koster for technical assistance. This work was supported by grant 901-03-097 from the Netherlands Organization for Scientific Research (NWO, The Hague, The Netherlands).

References

1. Willemsen MA, IJlst L, Steijlen PM, Rotteveel JJ, de Jong JG, Van Domburg PH, Mayatepek E, Gabreels FJ, and Wanders RJ (2001) Clinical, biochemical and molecular genetic characteristics of 19 patients with the Sjogren-Larsson syndrome. *Brain* **124:** 1426-1437.
2. Rizzo WB and Craft DA (1991) Sjogren-Larsson syndrome. Deficient activity of the fatty aldehyde dehydrogenase component of fatty alcohol:NAD+ oxidoreductase in cultured fibroblasts. *J Clin Invest* **88:** 1643-1648.
3. Rizzo WB and Craft DA (2000) Sjogren-Larsson syndrome: accumulation of free fatty alcohols in cultured fibroblasts and plasma. *J Lipid Res* **41:** 1077-1081.
4. Willemsen MA, Rotteveel JJ, de Jong JG, Wanders RJ, IJlst L, Hoffmann GF, and Mayatepek E (2001) Defective metabolism of leukotriene B4 in the Sjogren-Larsson syndrome. *J Neurol Sci* **183:** 61-67.
5. van den Brink DM, van Miert JN, Dacremont G, Rontani JF, Jansen GA, and Wanders RJ (2004) Identification of fatty aldehyde dehydrogenase in the breakdown of phytol to phytanic acid. *Mol Genet Metab* **82:** 33-37.
6. Savolainen K, Kotti TJ, Schmitz W, Savolainen TI, Sormunen RT, Ilves M, Vainio SJ, Conzelmann E, and Hiltunen JK (2004) A mouse model for {alpha}-methylacyl-CoA racemase deficiency: adjustment of bile acid synthesis and intolerance to dietary methyl-branched lipids. *Hum Mol Genet* **13:** 955-965.
7. Baes M, Huyghe S, Carmeliet P, Declercq PE, Collen D, Mannaerts GP, and Van Veldhoven PP (2000) Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids. *J Biol Chem* **275:** 16329-16336.
8. Gloerich J, Van Vlies N, Jansen GA, Denis S, Ruiter JP, van Werkhoven MA, Duran M, Vaz FM, Wanders RJ, and Ferdinandusse S (2005) A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARalpha-dependent and -independent pathways. *J Lipid Res* **46:** 716-726.
9. Ellinghaus P, Wolfrum C, Assmann G, Spener F, and Seedorf U (1999) Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice. *J Biol Chem* **274:** 2766-2772.
10. Heim M, Johnson J, Boess F, Bendik I, Weber P, Hunziker W, and Fluhmann B (2002) Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes. *FASEB J* **16:** 718-720.
11. Zomer AW, Van der Burg B, Jansen GA, Wanders RJ, Poll-The BT, and Van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. *J Lipid Res* **41:** 1801-1807.
12. Mandard S, Muller M, and Kersten S (2004) Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* **61:** 393-416.
13. Berger J and Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53:** 409-435.
14. Braissant O, Foufelle F, Scotto C, Dauca M, and Wahli W (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* **137:** 354-366.
15. Reddy JK and Hashimoto T (2001) Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr* **21:** 193-230.
16. Barbier O, Torra IP, Duguay Y, Blanquart C, Fruchart JC, Glineur C, and Staels B (2002) Pleiotropic actions of peroxisome proliferator-activated receptors in lipid metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* **22:** 717-726.
17. Vamecq J and Latruffe N (1999) Medical significance of peroxisome proliferator-activated receptors. *Lancet* **354:** 141-148.
18. van den Brink DM, van Miert JM, and Wanders RJ (2005) Assay for Sjogren-Larsson syndrome based on a deficiency of phytol degradation. *Clin Chem* **51:** 240-242.
19. Gootjes J, Schmohl F, Waterham HR, and Wanders RJ (2004) Novel mutations in the PEX12 gene of patients with a peroxisome biogenesis disorder. *Eur J Hum Genet* **12:** 115-120.
20. Ramakers C, Ruijter JM, Deprez RH, and Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339:** 62-66.
21. De Laurenzi V, Rogers GR, Hamrock DJ, Marekov LN, Steinert PM, Compton JG, Markova N, and Rizzo WB (1996) Sjogren-Larsson syndrome is caused by mutations in the fatty aldehyde dehydrogenase gene. *Nat Genet* **12:** 52-57.

22. De Laurenzi V, Rogers GR, Tarcsa E, Carney G, Marekov L, Bale SJ, Compton JG, Markova N, Steinert PM, and Rizzo WB (1997) Sjogren-Larsson syndrome is caused by a common mutation in northern European and Swedish patients. *J Invest Dermatol* **109:** 79-83.
23. Rizzo WB, Carney G, and De Laurenzi V (1997) A common deletion mutation in European patients with Sjogren-Larsson syndrome. *Biochem Mol Med* **62:** 178-181.
24. Sillen A, Anton-Lamprecht I, Braun-Quentin C, Kraus CS, Sayli BS, Ayuso C, Jagell S, Kuster W, and Wadelius C (1998) Spectrum of mutations and sequence variants in the FALDH gene in patients with Sjogren-Larsson syndrome. *Hum Mutat* **12:** 377-384.
25. Tsukamoto N, Chang C, and Yoshida A (1997) Mutations associated with Sjogren-Larsson syndrome. *Ann Hum Genet* **61 (Pt 3):** 235-242.
26. Sillen A, Jagell S, and Wadelius C (1997) A missense mutation in the FALDH gene identified in Sjogren-Larsson syndrome patients originating from the northern part of Sweden. *Hum Genet* **100:** 201-203.
27. Rizzo WB, Carney G, and Lin Z (1999) The molecular basis of Sjogren-Larsson syndrome: mutation analysis of the fatty aldehyde dehydrogenase gene. *Am J Hum Genet* **65:** 1547-1560.
28. Willemsen MA, Steijlen PM, de Jong JG, Rotteveel JJ, IJlst L, van Werkhoven MA, and Wanders RJ (1999) A novel 4 bp deletion mutation in the FALDH gene segregating in a Turkish family with Sjogren-Larsson syndrome. *J Invest Dermatol* **112:** 827-828.
29. Aoki N, Suzuki H, Ito K, and Ito M (2000) A novel point mutation of the FALDH gene in a Japanese family with Sjogren-Larsson syndrome. *J Invest Dermatol* **114:** 1065-1066.
30. Kraus C, Braun-Quentin C, Ballhausen WG, and Pfeiffer RA (2000) RNA-based mutation screening in German families with Sjogren-Larsson syndrome. *Eur J Hum Genet* **8:** 299-306.
31. Maquat LE (1996) Defects in RNA splicing and the consequence of shortened translational reading frames. *Am J Hum Genet* **59:** 279-286.

6

Peroxisomal *trans*-2-enoyl-CoA reductase is involved in phytol degradation

Jolein Gloerich, Jos P.N. Ruiter, Daan M. van den Brink, Rob Ofman, Sacha Ferdinandusse, Ronald J.A. Wanders (2006) *FEBS Lett.* **580**, 2092-2096

Peroxisomal *trans*-2-enoyl-CoA reductase is involved in phytol degradation

J. Gloerich, J.P.N. Ruiter, D.M. van den Brink, R. Ofman, S. Ferdinandusse, R.J.A. Wanders

Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma's Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Abstract

Phytol is a naturally occurring precursor of phytanic acid. The last step in the conversion of phytol to phytanoyl-CoA is the reduction of phytenoyl-CoA mediated by an, as yet, unidentified enzyme. A candidate for this reaction is a previously described peroxisomal *trans*-2-enoyl-CoA reductase (TER). To investigate this, human TER was expressed in *E. coli* as an MBP-fusion protein. The purified recombinant protein was shown to have high reductase activity towards *trans*-phytenoyl-CoA, but not towards the peroxisomal β-oxidation intermediates C24:1-CoA and pristenoyl-CoA. In conclusion, our results show that human TER is responsible for the reduction of phytenoyl-CoA to phytanoyl-CoA in peroxisomes.

Introduction

Peroxisomes are organelles, present in virtually each eukaryotic cell, that carry out a large variety of metabolic functions. One of these functions is the α-oxidation of 3-methyl-branched-chain fatty acids, like phytanic acid. In this process the carbon chain of phytanic acid is shortened by one carbon atom, leading to a 2-methyl-branched-chain fatty acid, i.e. pristanic acid.[1,2] In contrast to 3-methyl-branched-chain fatty acids, 2-methyl-branched-chain fatty acids can be degraded by β-oxidation, which is another metabolic process that takes place in the peroxisome. The first step in peroxisomal β-oxidation of pristanic acid is catalyzed by branched-chain acyl-CoA oxidase, which introduces a double bond at the 2,3-position, to form a *trans*-2-enoyl-CoA. After a dehydration and dehydrogenation reaction both catalyzed by the D-bifunctional protein localized in the peroxisome, the resulting 3-ketoacyl-CoA is thiolytically cleaved by one of the peroxisomal thiolases, resulting in a shortened fatty acyl-CoA. After three cycles of peroxisomal β-oxidation, the shortened acyl-CoA, i.e. 4,8-dimethylnonanoyl-CoA, is not a substrate for the peroxisomal acyl-CoA oxidases anymore. For further breakdown, the chain-shortened product is transported to the mitochondrion,[3] where it is totally degraded to acetyl-CoA and propionyl-CoA units.[4] Besides breakdown of pristanic acid, peroxisomal β-oxidation is also involved in the degradation of very long-chain fatty acids, long-chain dicarboxylic acids and bile acid intermediates.[4]

Another metabolic process that recently has been shown to occur, at least partly, in the peroxisome is the degradation of phytol. Phytol is a branched-chain fatty alcohol (3,7,11,15-tetramethylhexadec-2-en-1-ol) abundantly found in nature as part of the chlorophyll molecule. The release of phytol from chlorophyll occurs effectively in the digestive system of ruminant animals only, presumably by bacteria present in the gut.[5] As a result, a relatively high amount of free phytol is present in dairy products.[6] In mammals, free phytol is readily absorbed in

the small intestine and metabolized mainly in the liver (chapter 4). The product of phytol degradation is phytanic acid, which accumulates in a number of metabolic disorders. First, phytol is converted into phytenic acid at the endoplasmatic reticulum by the subsequent action of an alcohol dehydrogenase and an aldehyde dehydrogenase. Subsequently, phytenic acid is activated to its CoA-ester by an acyl-CoA synthetase. Phytenoyl-CoA synthetase activity has been detected in both microsomes and peroxisomes.[7] Finally, phytenoyl-CoA is reduced to phytanoyl-CoA by a reductase. Subcellular localization studies of rat liver homogenates have shown that phytenoyl-CoA reductase activity was present in both mitochondria and peroxisomes. Since phytanoyl-CoA is further broken down via the peroxisomal α-oxidation, it is tempting to speculate that *in vivo* the last steps of phytol degradation take place in the peroxisome. Interestingly, the degradation of phytol is stereospecific towards the breakdown of *trans*-phytol, also named (*E*)-phytol (chapter 4).

In a previous study, the cloning of a peroxisomal *trans*-2-enoyl-CoA reductase (TER), an enzyme with an unknown function but proposed to play a role in fatty acid chain elongation, was described.[8] This reductase has affinity for *trans*-2-enoyl-CoA esters with chain lengths up to 16 carbon atoms and appears to be ubiquitously expressed with high levels present in liver and kidney.[8]

In our study, we investigated whether this enzyme catalyzes the last step in the conversion of phytol to phytanic acid, i.e. the reduction of *trans*-phytenoyl-CoA into phytanoyl-CoA. The results described in this paper show that *trans*-phytenoyl-CoA is readily reduced by TER to yield phytanoyl-CoA. Furthermore, TER also showed activity towards straight-chain acyl-CoAs ranging in chain length from 4 to 16 carbon atoms. Interestingly, TER hardly reacted with pristenoyl-CoA and *trans*-2-tetracosenoyl-CoA (C24:1-CoA), two *trans*-2-enoyl-CoAs that are formed as intermediates during peroxisomal β-oxidation.

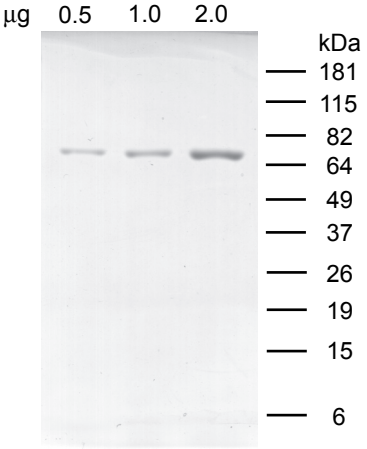


Figure 1: SDS-polyacrylamide gel analysis of purified recombinant human *trans*-2-enoyl-CoA reductase (TER). Human TER was expressed in E. coli as an MBP fusion protein and subsequently purified using amylose resin. Indicated amounts of the purified protein were applied on an SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue G-250.

Materials and Methods

Materials

E. coli strain INVα was purchased from Invitrogen (Carlsbad, CA). Yeast extract and tryptone were obtained from Difco Laboratories Inc. (Detroit, MI). The pGEM-T vector and T4-DNA ligase and ligation buffer were purchased from Promega (Madison, WI). The

pMAL-C2X vector and amylose resin columns were from New England Biolabs (Beverly, MA). Complete^{mini} tablets containing a cocktail of protease inhibitors were purchased from Roche (Basel, Switzerland). *Trans*-2-butenoyl-CoA (C4:1-CoA) was obtained from Sigma-Aldrich (St. Louis, MO). *Trans*-2-hexenoyl-CoA (C6:1-CoA), *trans*-2-octenoyl-CoA (C8:1-CoA), *trans*-2-decenoyl-CoA (C10:1-CoA), *trans*-2-dodecenoyl-CoA (C12:1-CoA), *trans*-2-tetradecenoyl-CoA (C14:1-CoA), *trans*-2-hexadecenoyl-CoA (C16:1-CoA), C24:1-CoA were enzymatically synthesized from the corresponding saturated CoA-esters using acyl-CoA oxidase from *Arthrobacter* (Sigma-Aldrich). A mixture of *cis*- and *trans*-phytenic acid was synthesized as described previously.[9] A mixture of *cis*- and *trans*-phytenoyl-CoA isomers was chemically synthesized from this mixture as described before.[10] All other chemicals were of analytical grade.

Cloning, expression and purification of human TER cDNA in E. coli

The complete open reading frame (ORF) of human TER was amplified from control human liver cDNA by PCR using the following primerset: a *Bam*HI-tagged forward primer 5'-tatag gatccATGGCCTCCTGGGCTAAGGG-3' and a *Sal*I-tagged reverse primer 5'-tatagtcgacAG GACACCTTGTTTCCTCAG-3' (restriction sites are underlined). The PCR product was first inserted into pGEM-T and sequenced to exclude PCR-introduced mutations. Subsequently, the ORF was cloned into the bacterial expression vector pMAL-C2X (New England Biolabs), using the *Bam*HI and *Sal*I sites downstream of the isopropyl-1-thio-β-D-galactopyranoside (IPTG) inducible promoter in frame with the ATG, in order to express TER as a fusion protein with maltose-binding protein (MBP). *E. coli* strain INVα was transformed with pMAL-TER. Transformed cells were grown at 37°C in 10 ml LB-medium supplemented with 0.2 % (w/v) glucose and 150 μg/ml ampicillin to an OD_{600} of approximately 0.6 and IPTG was added to a final concentration of 1 mM to induce expression of the fusion protein. After 4 h at 37°C, cells were collected by centrifugation for 5 min at 1000 x *g*. The pellet was dissolved in phosphate-buffered saline solution with 0.1 % (v/v) Triton X-100 and protease inhibitor cocktail. Cell lysates were prepared by sonication for 3 periods of 20 seconds at 8-9 Watt in an ice water bath followed by centrifugation at 12000 x *g* for 15 min at 4°C. The supernatant was used for purification of the MBP-fusion protein using amylose resin as described by the manufacturer (New England BioLabs). The purified protein was stored at –20°C in PBS containing 50% (v/v) glycerol.

SDS-polyacrylamide gel electrophoresis

0.5, 1 and 2 μg of the purified recombinant MBP-TER were applied on a 10% (w/v) SDS-polyacrylamide gel.[11] The gel was stained with Coomassie Brilliant Blue G-250 according to the method described by Neuhoff et al..[12]

2-Enoyl-CoA reductase activity measurements

Enoyl-CoA reductase activity with different *trans*-2-enoyl-CoA-esters as substrates, including phytenoyl-CoA, pristenoyl-CoA, C4:1-CoA, C6:1-CoA, C8:1-CoA, C10:1-CoA, C12:1-CoA, C14:1-CoA, C16:1-CoA and C24:1-CoA, was determined essentially as described previously.[7] Briefly, the reaction mixture contained 50 mM bis-Tris-propane pH 7.2, 1 mM NADPH, 50 μM of the 2-enoyl-CoA ester and 1 μg of the purified MBP-TER in a final volume of 100 μl. Reactions were started by addition of substrate and incubated at 37ºC for 15 min. Reactions with phytenoyl-CoA, pristenoyl-CoA and C24:1-CoA as a substrate were terminated by the

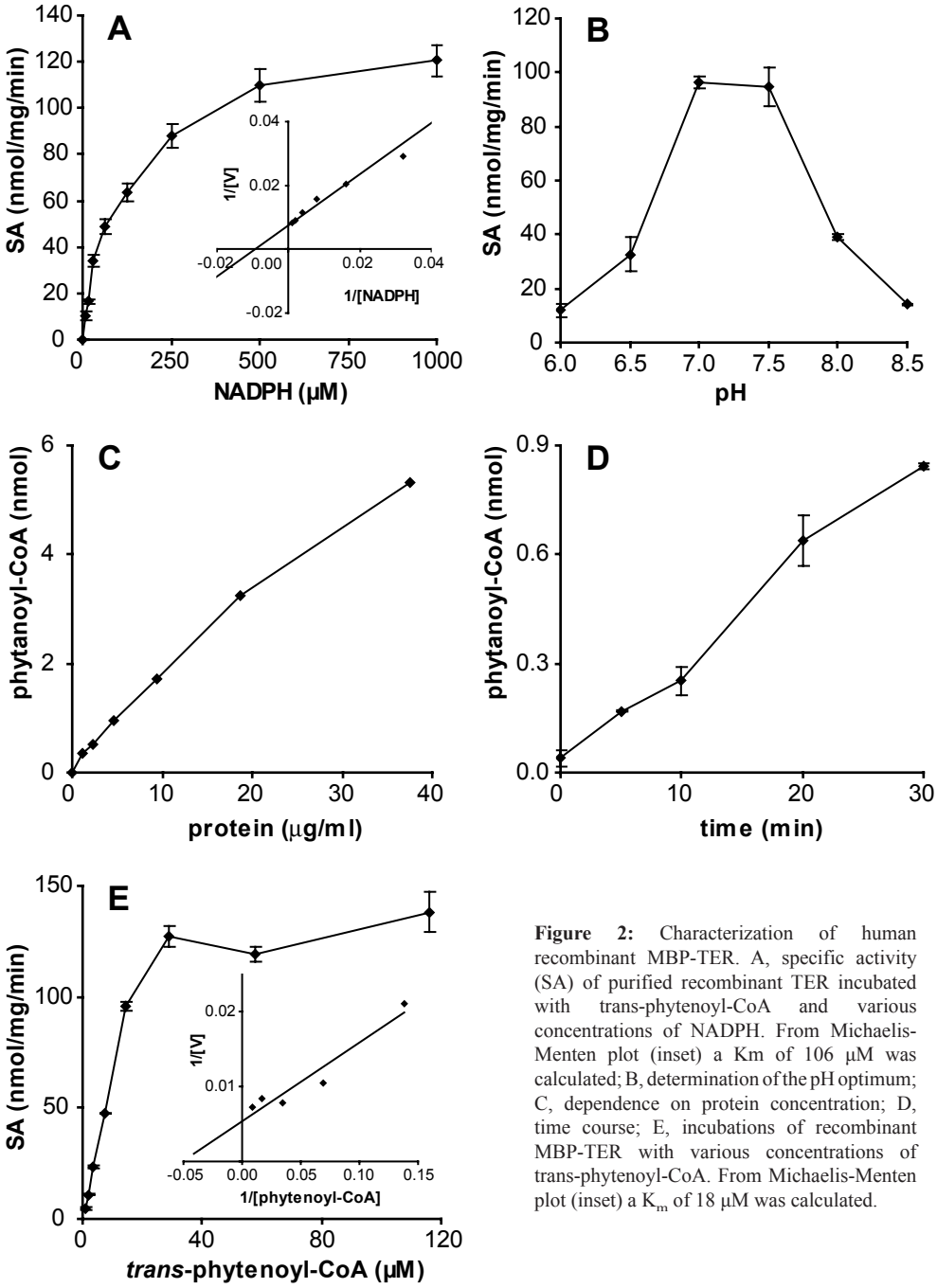


Figure 2: Characterization of human recombinant MBP-TER. A, specific activity (SA) of purified recombinant TER incubated with trans-phytenoyl-CoA and various concentrations of NADPH. From Michaelis-Menten plot (inset) a Km of 106 µM was calculated; B, determination of the pH optimum; C, dependence on protein concentration; D, time course; E, incubations of recombinant MBP-TER with various concentrations of trans-phytenoyl-CoA. From Michaelis-Menten plot (inset) a K_m of 18 µM was calculated.

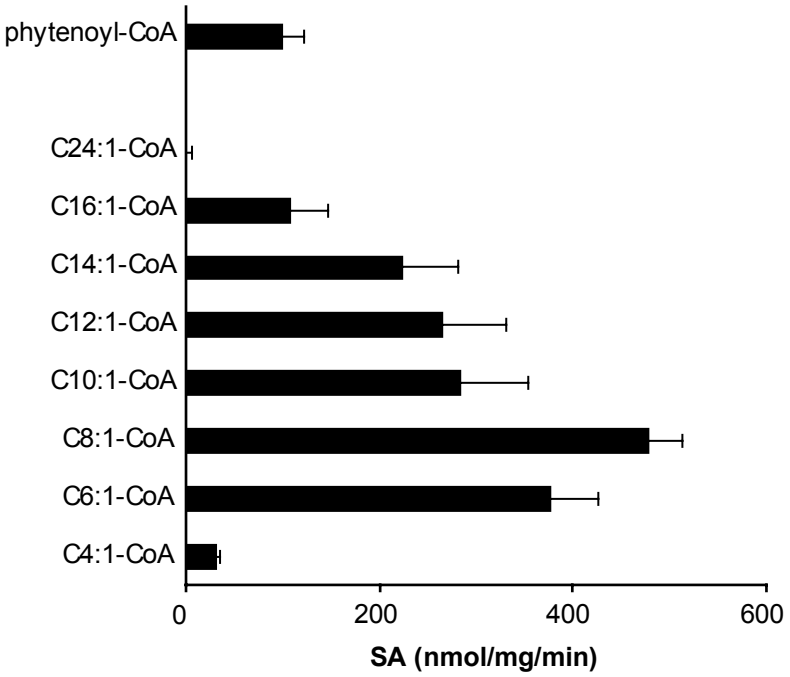


Figure 3: Specific activity (SA) of recombinant MBP-TER measured with different 2-enoyl-CoA esters. Enzyme activity measurements with different substrates were performed in a reaction mixture with a final concentration of 10 µg/ml recombinant MBP-TER, 1 mM NADPH, 50 mM Bis-Tris-Propane pH 7.2 and 50 µM of the substrate. Reactions were carried out for 15 min at 37ºC.

addition of 100 µl acetonitrile and placed on ice. Reactions with all other substrates were terminated by the addition of 2 M HCl to a final concentration of 0.18 M, followed by neutralization to a pH of ~6 by addition of 0.6 M MES plus 2 M KOH. Acetonitrile was added to a final concentration of ~20 %. All samples were spun for 10 min at 10000 x *g* at 4ºC. Supernatants were analyzed using high performance liquid chromatography and CoA-esters were quantified using UV-detection. To this end, 50 µl samples were injected on a C_{18} reversed-phase column (Supelcosil LC-18-DB, 250 mm x 4.6 mm internal diameter, particle size 5 µm, Supelco). Elution of the CoA-esters was performed using either a linear gradient of methanol in 50 mM potassium phosphate buffer (pH 5.3) for short-chain substrates (C4:1-CoA and C6:1-CoA), or a linear gradient of acetonitrile in 16.9 mM sodium phosphate buffer (pH 6.9) for all other substrates. Resolution between substrate and product using a methanol gradient was achieved using a linear gradient of 26% to 50% methanol over 20 min for C4:1-CoA incubations and of 14% to 26% methanol over 30 min for C6:1-CoA incubations. Resolution between CoA-esters using an acetonitrile gradient was achieved using a linear gradient of 16% to 34% acetonitrile over 30 min for C8:1-CoA incubations, of 40% to 54% acetonitrile over 23 min for incubations with substrates ranging in chain length from 10 to 16 carbon atoms, and of 49% to 51% in 24 min for incubations with C24:1-CoA and phytenoyl-CoA.

Results and Discussion

Expression and purification of recombinant MBP-TER

The full-length cDNA of human TER[8] was expressed as a fusion protein with MBP in *E. coli*. After induction, the cells expressing MBP-TER showed high reductase activity towards phytenoyl-CoA, whereas no phytenoyl-CoA reductase activity was observed in cells transformed with the empty pMAL vector (data not shown). The recombinant fusion protein, MBP-TER, bound tightly to an amylose resin column and could be eluted with maltose. The purity of the isolated fusion protein was established by SDS-PAGE (**Figure 1**). The expected molecular weight of the fusion protein is 75 kDa, which corresponds well with the apparent molecular weight of the protein observed on SDS-PAGE. The phytenoyl-CoA reductase activity of the purified enzyme was stable for at least a month, when stored at –20°C in PBS containing 50% (v/v) glycerol.

Characterization of the phytenoyl-CoA reductase activity of recombinant MBP-TER

Optimal reaction conditions were determined using a mixture of *cis*- and *trans*-phytenoyl-CoA as substrate. The reduction of phytenoyl-CoA by recombinant MBP-TER was stereospecific, since only the *trans*-isomer was reduced to phytanoyl-CoA. The reaction was dependent on NADPH as cofactor with a K_m of 106 μM (**Figure 2A**). Maximum activity was observed in incubation mixtures with a pH between 7 and 7.5 (Figure 2B). Based on these findings a pH of 7.2 was used in all other experiments. Phytanoyl-CoA production was linear with protein up to 20 μg/ml (Figure 2C) and linear over time up to at least 30 minutes (Figure 2D). In figure 2E, the dependence of the reductase reaction on the substrate *trans*-phytenoyl-CoA was studied. Calculation of the K_m and V_{max} resulted in 18 μM and 172 nmol/mg/min, respectively.

In a previous study, the reduction of phytenoyl-CoA in rat liver homogenate was investigated.[7] The pH optimum for this reaction was also ~ pH 7 and the reaction was also dependent on NADPH, although the K_m was much lower. This difference could be due to the MBP-moiety of the purified protein that was used in this study. Another explanation for the difference is the fact that phytenoyl-CoA reductase activity was observed in both mitochondria and peroxisome enriched fractions of rat liver density gradient centrifugation experiments. Since TER is a peroxisomal enzyme, the mitochondrial activity probably originates from another reductase with reactivity towards phytenoyl-CoA that has other enzymatic properties.

The reverse reaction, from phytanoyl-CoA to phytenoyl-CoA, did not take place in incubations with recombinant MBP-TER, $NADP^+$ and an NADP-regenerating system.

Characterization of the reductase activity of recombinant MBP-TER towards other substrates

Several straight-chain *trans*-2-enoyl-CoAs were tested as substrate for the recombinant MBP-TER and reductase activity was measured with substrates having a carbon chain ranging in length from 4 to 16 carbon atoms (**Figure 3**). Highest activity was observed for C8:1-CoA with a K_m and V_{max} of 17 μM and 651 nmol/mg/min, respectively. Although the V_{max} is higher for the reduction of C8:1-CoA than of phytenoyl-CoA, the K_m values for the reduction of C8:1-CoA and phytenoyl-CoA are quite similar.

Intermediates of peroxisomal β-oxidation, C24:1-CoA and pristenoyl-CoA (2,6,10,14-tetramethylpentadec-2-enoyl-CoA), were also tested as substrates for recombinant MBP-

TER. Interestingly, the specific activity for C24:1-CoA was only 1% of that of phytenoyl-CoA, whereas no reduction of the pristenoyl-CoA could be detected at all. This suggests that only CoA-esters with a methyl-group at the 3-position as in phytenoyl-CoA, and not at the 2-position as in pristenoyl-CoA, are substrates for TER.

TER has been shown to possess a peroxisomal targeting signal 1, that directs the protein to the peroxisome. Of the *trans*-2-enoyl-CoA esters that were tested as substrate for the recombinant MBP-TER, C24:1-CoA, pristenoyl-CoA and phytenoyl-CoA are formed in the peroxisome and not the mitochondrion, whereas medium-chain 2-enoyl-CoA esters are formed in both mitochondria and peroxisomes and short-chain 2-enoyl-CoA esters are only formed by β-oxidation in the mitochondria. Interestingly, the intermediates of peroxisomal β-oxidation, C24:1-CoA and pristenoyl-CoA, are not readily reduced by recombinant MBP-TER, leaving phytenoyl-CoA as the only true peroxisomal substrate with high affinity for TER.

Although the data presented suggest that TER has a function in phytol metabolism *in vivo*, further investigations are required. First, development of an antiserum against TER is needed to establish its exclusive location in the peroxisome. Also, immunoprecipitation experiments could be performed to investigate the percentage of phytenoyl-CoA reductase activity in liver homogenates that is attributable to TER. Finally, to elucidate the role of mitochondria in the reduction of phytenoyl-CoA *in vivo*, studies on phytenoyl-CoA import into the mitochondrion have to be done. For the import of phytenoyl-CoA into the mitochondrion, phytenoyl-CoA has to be converted to phytenoyl-carnitine by carnitine palmitoyl transferase 1 (CPT1). This carnitine ester then can be imported into the mitochondrion via the carnitine-acylcarnitine translocase and reconverted into the corresponding CoA-ester again in the mitochondrion by carnitine palmitoyl transferase 2. Preliminary studies revealed that CPT1 activity towards phytenoyl-CoA is less than 5% of its activity towards palmitoyl-CoA (data not shown). This suggests that phytenoyl-CoA is not readily imported into the mitochondrion for further degradation.

In conclusion, our results suggest that TER has a function in phytol metabolism, where it is responsible for the reduction of phytenoyl-CoA to phytanoyl-CoA. Since relatively low affinities towards peroxisomal β-oxidation intermediates were observed, its putative role in peroxisomal chain elongation as suggested by Das et al.[8] remains controversial.

Acknowledgments

The authors thank J. Koster for technical assistance. This work was supported by grant 901-03-097 from the Netherlands Organization for Scientific Research (NWO, The Hague, The Netherlands).

References

1. Verhoeven NM and Jakobs C (2001) Human metabolism of phytanic acid and pristanic acid. *Prog Lipid Res* **40:** 453-466.
2. Wanders RJ, Jansen GA, and Lloyd MD (2003) Phytanic acid alpha-oxidation, new insights into an old problem: a review. *Biochim Biophys Acta* **1631:** 119-135.
3. Verhoeven NM, Roe DS, Kok RM, Wanders RJ, Jakobs C, and Roe CR (1998) Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J Lipid Res*

39: 66-74.

4. Wanders RJ, Vreken P, Ferdinandusse S, Jansen GA, Waterham HR, Van Roermund CW, and Van Grunsven EG (2001) Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem Soc Trans* **29:** 250-267.
5. Hansen RP (1966) Occurence of phytanic acid in rumen bacteria. *Nature* **210:** 841.
6. Brown PJ, Mei G, Gibberd FB, Burston D, Mayne PD, McClinchy JE, and Sidey M (1993) Diet and Refsum's disease. The determination of phytanic acid and phytol in certain foods and the application of this knowledge to the choice of suitable convenience foods for patients with Refsum's disease. *J Hum Nutr Diet* **6:** 295-305.
7. Van den Brink DM, Van Miert JN, Dacremont G, Rontani JF, and Wanders RJ (2005) Characterization of the final step in the conversion of phytol into phytanic acid. *J Biol Chem* **280:** 26838-26844.
8. Das AK, Uhler MD, and Hajra AK (2000) Molecular cloning and expression of mammalian peroxisomal trans-2-enoyl-coenzyme A reductase cDNAs. *J Biol Chem* **275:** 24333-24340.
9. Rontani JF, Bonin PC, and Volkman JK (1999) Biodegradation of free phytol by bacterial communities isolated from marine sediments under aerobic and denitrifying conditions. *Appl Environ Microbiol* **65:** 5484-5492.
10. Rasmussen JT, Borchers T, and Knudsen J (1990) Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. *Biochem J* **265:** 849-855.
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
12. Neuhoff V, Arold N, Taube D, and Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9:** 255-262.

Summary & Samenvatting

Summary

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid which cannot be synthesized endogenously in mammals. It is abundantly present in a variety of food products, mainly those derived from grazing animals and fish. Besides direct intake of phytanic acid via the diet, degradation of free phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) to phytanic acid also contributes to phytanic acid levels.

Recent studies have elucidated the pathway of conversion of phytol to phytanic acid (see figure 3, chapter 1). Firstly, phytol is converted into phytenal by an unknown alcohol dehydrogenase. Secondly, phytenal is converted into phytenic acid by the microsomal enzyme fatty aldehyde dehydrogenase (FALDH). Thirdly, phytenic acid is activated to its CoA-ester by an unknown synthetase. This activity is detected in microsomal and peroxisomal fractions in studies using rat liver gradients. Finally, phytenoyl-CoA is reduced to phytanoyl-CoA by a reductase and this activity is observed in mitochondrial and peroxisomal fractions of rat liver gradients. In chapter 6 of this thesis, we describe a study on the peroxisomal *trans*-2-enoyl-CoA reductase (TER). Human TER was expressed as an MBP-fusion protein in *E. coli* and we showed that the purified recombinant protein had high reductase activity with *trans*-phytenoyl-CoA as substrate, but not with the peroxisomal β-oxidation intermediates C24:1-CoA and pristenoyl-CoA, indicating that TER is most likely responsible for the last step in the degradation of phytol to phytanic acid in the peroxisome.

The breakdown of phytanic acid in the body differs from the breakdown of straight-chain fatty acids. This is due to the methyl-group at the 3-position of the carbon-chain, which prevents normal β-oxidation. Therefore, phytanic acid is first shortened by one carbon atom via a process called α-oxidation, to yield pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can be further degraded by β-oxidation (for a schematic overview of both processes, see figure 4 and 5, chapter 1). Both α-oxidation of phytanic acid and β-oxidation of pristanic acid take place in the peroxisome.

In healthy persons, phytanic acid and pristanic acid are rapidly degraded. In a number of inherited disorders, however, branched-chain fatty acids accumulate as a result of a block in the degradation of these fatty acids. In the absence of functional peroxisomes, as is the case in cells from patients suffering from peroxisome biogenesis disorders (e.g. Zellweger syndrome), both α- and β-oxidation are deficient, and as a consequence phytanic and pristanic acid accumulate. Besides these generalized peroxisome biogenesis disorders, there are a number of single enzyme deficiency disorders in α-oxidation (Refsum disease) and β-oxidation (AMACR deficiency, DBP deficiency and SCPx deficiency) that result in the accumulation of branched-chain fatty acids in plasma and tissues of patients.

Besides analysis of very long-chain and branched-chain fatty acids in plasma, specific enzymatic assays have been developed to aid in the diagnosis of these patients. In chapter 2, we describe a newly developed method to measure DBP dehydrogenase activity independent of DBP hydratase activity to distinguish between DBP deficiency type I, in which both the DBP enoyl-CoA hydratase (DBP HY) and DBP 3-ketoacyl-CoA dehydrogenase (DBP DH) activities are deficient, and DBP deficiency type II, with an isolated DBP HY deficiency. Until now, differentiation between these subgroups was only possible by mutation analysis. For this assay, the hydratase domain of DBP was expressed in the yeast *Saccharomyces cerevisiae*. After a coincubation of yeast homogenate expressing DBP HY with fibroblast homogenate of patients using trihydroxycholestanoyl-CoA as substrate, DBP dehydrogenase activity could

be measured. This assay in combination with the normal DBP assay makes it possible to distinguish between all three types of DBP deficiency on the basis of DBP enzyme activities.

Studies on branched-chain metabolites have revealed that phytanic and pristanic acid are ligands of several transcription factors, including the three isoforms of the retinoid-X receptor (RXR) and the peroxisome proliferator-activated receptor α (PPARα). In chapter 3 and 4 of this thesis, we have studied the *in vivo* effects of phytol and its metabolites on fatty acid breakdown and phytol metabolism in mice and the role of PPARα in its regulation. To this end, we fed wild-type and PPARα$^{-/-}$ mice a diet enriched with phytol. In chapter 3, we describe that the phytol-enriched diet resulted in increased plasma and liver levels of the phytol metabolites phytanic and pristanic acid. In wild-type mice, plasma fatty acid levels decreased after phytol feeding, whereas in PPARα$^{-/-}$ mice, the already elevated fatty acid levels increased. Furthermore, PPARα$^{-/-}$ mice were found to be carnitine deficient in both plasma and liver. Dietary phytol increased liver free carnitine in wild-type animals but not in PPARα$^{-/-}$ mice. Investigation of carnitine biosynthesis revealed that PPARα is likely involved in the regulation of carnitine homeostasis, although further research is needed to fully resolve the role of PPARα in carnitine metabolism. In addition, phytol feeding resulted in a PPARα-dependent induction of various peroxisomal and mitochondrial β-oxidation enzymes. In conclusion, branched-chain fatty acids are physiologically relevant ligands of PPARα in mice.

In chapter 4, we describe the effects of phytol feeding and treatment with Wy-14,643, a synthetic PPARα agonist, on the degradation of phytol to phytanic acid. After the phytol-enriched diet, phytol could only be detected in small intestine, the site of uptake, and liver. Upon longer duration of the diet, the level of the (*E*)-isomer of phytol increased significantly in the liver of PPARα$^{-/-}$ mice compared to wild-type mice. Activity measurements of the enzymes involved in phytol metabolism showed that phytol feeding or Wy-14,643 treatment resulted in a PPARα-dependent induction of the last three steps of phytol degradation in the liver. Furthermore, the enzymes involved showed a higher activity towards the (*E*)-isomer than the (*Z*)-isomer of their respective substrates, indicating a stereospecificity towards the metabolism of (*E*)-phytol. Summarizing, the conversion of phytol to phytanic acid is regulated via PPARα, is specific for the breakdown of (*E*)-phytol, and the main site of phytol metabolism is the liver.

Since FALDH has been shown to be under the control of PPARα in mice, we investigated whether the hypolipidemic drug bezafibrate, which is a pan-agonist of all PPAR isoforms, might induce FALDH activity in human fibroblasts. In chapter 5, we show that FALDH activity was induced 1.4-fold after a 3-day treatment with 800 μM bezafibrate in fibroblasts of control subjects. We also investigated the effects of bezafibrate treatment in fibroblasts of patients suffering from Sjögren-Larsson syndrome (SLS), which is caused by a deficiency of FALDH. Interestingly, in fibroblasts of two SLS patients homozygous for a p.R228C substitution, FALDH activity could be induced to 37% of control values by bezafibrate treatment. mRNA analysis in fibroblasts of these patients also revealed a mean 1.8-fold induction of FALDH mRNA after bezafibrate treatment. No induction was observed in fibroblasts of patients with mutations that cause instability of FALDH mRNA or that result in a protein without any residual activity. These data suggest that bezafibrate treatment could be effective in patients with expression of FALDH protein and some residual enzyme activity. Further research is needed to resolve whether patients could benefit from treatment with bezafibrate.

Samenvatting

Het lichaam is opgebouwd uit cellen. De meeste cellen bevatten een aantal kleinere compartimenten, organellen genaamd, die elk één of meerdere specifieke taken vervullen in het functioneren van de cel. In twee organellen, het mitochondrion en het peroxisoom, vindt afbraak van vetzuren plaats. Dit gebeurt om energie te verkrijgen uit vetzuren, zodat deze kan worden gebruikt voor andere processen in de cel. Vetzuren zijn bestanddelen van het vet dat in allerlei voedingsmiddelen zit. Ze bestaan uit een keten van koolstof (C)-atomen met aan het uiteinde een zuurgroep. Er zijn verschillende soorten vetzuren, zo kan de lengte van de keten variëren van twee tot meer dan dertig C-atomen. Daarnaast kan een vetzuur onverzadigd zijn, wat betekent dat er een dubbele binding tussen twee C-atomen van de keten zit. Een vetzuur met een dubbele binding kan in twee verschillende ruimtelijke vormen voorkomen, namelijk de (*E*)- en de (*Z*)-isomeer. Ook zijn er vetzuren die vertakkingen van de hoofdketen hebben, zoals fytaanzuur en pristaanzuur (zie hoofdstuk 1, figuur 1). Zuivelproducten, vlees van herkauwers en vis bevatten van deze vertakt-keten vetzuren. Zowel fytaanzuur als pristaanzuur zijn afbraakproducten van fytol, een vertakt-keten alcohol. Fytol wordt via vier stappen omgezet in fytaanzuur (zie hoofdstuk 1, figuur 3). In de eerste twee stappen wordt de alcoholgroep, via de vorming van een aldehyde omgezet naar een zuurgroep, waarbij fyteenzuur ontstaat. Voordat de dubbele binding van fyteenzuur verbroken kan worden, wordt fyteenzuur eerst gemodificeerd door er een extra groep aan vast te maken. Deze groep heet co-enzym A (CoA) en heeft een essentiële functie in de afbraak van vetzuren. Het fytenoyl-CoA dat wordt gevormd, wordt in de laatste stap omgezet naar fytanoyl-CoA door de dubbele binding te verbreken. Deze laatste stap kan plaatsvinden in zowel het peroxisoom als het mitochondrion. In hoofdstuk zes wordt de identificatie van een enzym beschreven dat verantwoordelijk is voor de omzetting van fytenoyl-CoA naar fytanoyl-CoA. Dit enzym heet *trans*-2-enoyl-CoA reductase, en stond beschreven in de literatuur als een peroxisomaal reductase (een enzym dat dubbele bindingen verbreekt) met een nog onduidelijke functie. Door het enzym op te zuiveren was het mogelijk om verschillende substraten te testen en zo werd aangetoond dat dit enzym een hoge activiteit heeft voor de omzetting van de (*E*)-isomeer van fytenoyl-CoA naar fytanoyl-CoA.

Om fytaanzuur verder af te breken, moet het eerst worden omgezet in pristaanzuur (2,6,10,14-tetramethylpentadecaanzuur), dat één C-atoom korter is. Dit gebeurt via een proces dat α-oxidatie genoemd wordt en plaatsvindt in het peroxisoom (zie hoofdstuk 1, figuur 4). De verdere afbraak van pristaanzuur wordt β-oxidatie genoemd (zie hoofdstuk 1, figuur 5). Dit proces vindt ook plaats in het peroxisoom. Tijdens β-oxidatie wordt van de CoA-vorm van een vetzuur steeds een stukje van 2 C-atomen van de keten afgeknipt, totdat het vetzuur helemaal is afgebroken. Echter, in het peroxisoom kan het vetzuur alleen maar tot een bepaalde lengte worden afgebroken. Daarna moet het vetzuur worden getransporteerd naar het mitochondrion waar het helemaal wordt afgebroken, ook door middel van β-oxidatie.

In gezonde mensen worden fytaanzuur en pristaanzuur snel afgebroken en zijn de niveaus van deze vetzuren in lichaamsvloeistoffen en weefsels heel laag. Echter, hoge niveaus van vertakt-keten vetzuren worden gevonden in patiënten die lijden aan bepaalde erfelijke aandoeningen die het gevolg zijn van een blokkade in de afbraak van fytaanzuur en pristaanzuur. Deze aandoeningen kunnen worden onderverdeeld in twee categorieën. Bij peroxisoom biogenese defecten (bijvoorbeeld Zellweger syndroom) hebben patiënten geen

of lege peroxisomen, doordat de aanmaak van peroxisomen is verstoord. Hierdoor zijn alle functies van het peroxisoom, dus ook α- en β-oxidatie van vertakt-keten vetzuren, verstoord. Daarnaast zijn er patiënten die een specifiek defect hebben in één van de enzymen van de α-oxidatie (ziekte van Refsum) of β-oxidatie (AMACR deficiëntie, DBP deficiëntie en SCPx deficiëntie). Als gevolg van deze aandoeningen hopen de vetzuren die niet kunnen worden afgebroken (niet alleen vertakt-keten vetzuren maar ook zeer lange rechte-keten vetzuren) zich op in weefsels en lichaamsvloeistoffen van deze patiënten.

Naast de analyse van vertakt-keten en zeer lange rechte-keten vetzuren in plasma van patiënten, zijn er ook specifieke enzymatische tests ontwikkeld om te helpen bij het diagnosticeren van deze patiënten. In hoofdstuk twee wordt een nieuwe test beschreven om DBP dehydrogenase activiteit te meten onafhankelijk van DBP hydratase activiteit. Hiermee kan onderscheid gemaakt worden tussen twee verschillende types van DBP deficiëntie, namelijk DBP deficiëntie type I, waarbij zowel de hydratase als de dehydrogenase activiteit van DBP deficiënt is, en DBP deficiëntie type II, met een geïsoleerd defect in het hydratase gedeelte van DBP. Tot nu toe kon alleen onderscheid tussen deze groepen worden gemaakt door middel van mutatie analyse, een methode die erg tijdrovend is. Voor deze nieuwe test werd het hydratase gedeelte van DBP tot expressie gebracht in de gist *Saccharomyces cerevisiae*. De DBP dehydrogenase activiteit van humane fibroblasten (huidcellen) kon worden gemeten na co-incubatie van fibroblasten homogenaat met een gisthomogenaat dat DBP hydratase tot expressie brengt. Samen met de gebruikelijke enzymatische test om DBP deficiëntie te bepalen, kan deze methode onderscheid maken tussen alle drie de types van DBP deficiëntie.

Fytaanzuur en pristaanzuur zijn liganden van verschillende transcriptiefactoren, waaronder de drie isovormen van de retinoid-X-receptor (RXR) en peroxisome proliferator-activated receptor α (PPARα). Transcriptiefactoren zijn eiwitten die de expressie van andere eiwitten kunnen reguleren. Om expressie van een target eiwit te kunnen induceren, moet de transcriptiefactor eerst worden geactiveerd door binding van een ligand. De geactiveerde transcriptiefactor zorgt dan voor een verhoogde (of soms ook verlaagde) expressie van zijn target eiwitten. PPARα speelt een grote rol bij de regulatie van de vetzuurafbraak.

In hoofdstuk drie en vier van dit proefschrift, worden de effecten van fytol en zijn afbraakproducten in de muis beschreven en de rol die PPARα hierin speelt. Om dit te onderzoeken, werd aan normale (wildtype) muizen en PPARα-deficiënte (PPARα$^{-/-}$) muizen een dieet verrijkt met fytol gegeven. In hoofdstuk drie is beschreven dat dit dieet leidt tot verhoogde concentraties van fytaanzuur en pristaanzuur in plasma en lever van deze muizen. In wildtype muizen leidde het fytol-verrijkte dieet tot een verlaging van de plasma vetzuurconcentraties, terwijl in de PPARα$^{-/-}$ muizen de reeds verhoogde vetzuur concentraties nog verder stegen. Daarnaast, was een inductie in de expressie en activiteit van zowel peroxisomale als mitochondriale β-oxidatie enzymen zichtbaar in wildtype muizen op fytol dieet, maar niet in PPARα$^{-/-}$ muizen. Dit betekent dat deze inductie afhankelijk is van PPARα. Concluderend, de fytol metabolieten fytaanzuur en pristaanzuur zijn fysiologisch relevante liganden van PPARα en leiden tot inductie van verschillende enzymen in de β-oxidatie van vetzuren.

In hoofdstuk vier worden de effecten van het fytol dieet en van de behandeling met Wy-14,643, een synthetische ligand van PPARα, op de afbraak van fytol naar fytaanzuur beschreven. Na het fytol-verrijkte dieet kon alleen in dunne darm, waar fytol wordt opgenomen uit het voedsel, en in de lever fytol worden gedetecteerd. Naarmate het dieet langer duurde,

steeg de concentratie van fytol, met name de (*E*)-isomeer, in de lever van PPARα$^{-/-}$ muizen ten opzichte van wildtype muizen. Meting van de activiteiten van de enzymen die betrokken zijn bij de afbraak van fytol toonde aan dat een fytol-verrijkt dieet of behandeling met Wy-14,643 resulteerde in een PPARα-afhankelijke inductie van de laatste drie stappen in de afbraak van fytol in de lever. Daarnaast hadden de betrokken enzymen een hogere activiteit voor de afbraak van de (*E*)-isomeer dan de (*Z*)-isomeer van hun respectievelijke substraten, wat wijst op een specifieke afbraak van (*E*)-fytol. Samenvattend, de afbraak van fytol naar fytaanzuur is gereguleerd door PPARα, is specifiek voor de afbraak van (*E*)-fytol en vindt voornamelijk plaats in de lever.

De tweede stap in de afbraak van fytol wordt gekatalyseerd door het enzym fatty aldehyde dehydrogenase (FALDH). Patiënten met een deficiëntie van FALDH leiden aan Sjögren-Larsson syndroom (SLS). Omdat in muizen FALDH gereguleerd wordt door PPARα, werd onderzocht of in humane fibroblasten (huidcellen) FALDH ook geïnduceerd kon worden door activatie van één van de PPAR-isovormen. In hoofdstuk vijf wordt beschreven dat behandeling van humane fibroblasten van gezonde controles met bezafibraat, een ligand van alle PPAR-isovormen, leidde tot een inductie van FALDH. Daarnaast werd onderzocht of deze inductie van FALDH ook in SLS patiënten plaatsvond. In twee patiënten met een mutatie die leidde tot de aminozuur verandering R228C, werd de activiteit van FALDH door behandeling met bezafibraat geïnduceerd tot 37% van de waarde in controles. In patiënten die geen FALDH eiwit tot expressie brengen of een FALDH eiwit hebben zonder enige activiteit, werd geen inductie van FALDH activiteit gemeten. Hieruit kan worden geconcludeerd dat behandeling met bezafibraat effectief zou kunnen zijn in SLS patiënten die een FALDH eiwit tot expressie brengen dat nog enige restactiviteit heeft. Verder onderzoek is nodig om op te helderen of patiënten inderdaad gebaat zouden kunnen zijn bij behandeling met bezafibraat.

Dankwoord

Dankwoord

Alle proeven zijn gedaan, de artikelen zijn geschreven, de kaft is ontworpen en de lay-out is af. Alleen het meest gelezen hoofdstuk van het boek moet nog geschreven worden: het dankwoord ☺. Ik hoop dat ik niet al te veel in clichés verval (ik heb er hierboven al een aantal genoemd en er komen er ongetwijfeld nog een paar…). Dit kleine hoofdstukje is misschien wel het moeilijkst om te schrijven, omdat ik graag iedereen wil bedanken die direct of indirect een bijdrage geleverd heeft aan dit proefschrift. Ik pretendeer niet volledig te zijn, want dat zou personen die ik niet bij naam noem tekort doen, dus wil ik een aantal mensen in het bijzonder bedanken. Maar voordat ik hieraan begin, wil ik eerst zeggen dat ik een hele leuke tijd heb gehad op lab GMZ. Ik heb ontzettend veel geleerd, maar vooral de sfeer op het lab zal me bijblijven. Iedereen stond altijd meteen voor je klaar en de gezelligheid tijdens de koffie- en lunchpauzes en natuurlijk de onovertroffen borrels zal ik missen.

Beste Karel, jij hebt me geholpen bij mijn allereerste stapjes in de wetenschap. Toen ik zocht naar een mogelijkheid om mijn afstudeerstage gedeeltelijk in het buitenland te doen, was je meteen bereid om mee te denken. Al snel was alles geregeld en kon ik vier maanden naar Münster, waar ik een fantastische tijd heb gehad. Toen ik op zoek was naar een promotieplek, zei je meteen dat ik eens in Amsterdam moest gaan praten met Ronald Wanders. Dit boekje is het resultaat van die goede raad. De samenwerking tijdens mijn promotietijd was ook prettig, je was altijd zeer geïnteresseerd in mijn onderzoek. Jammer dat er uiteindelijk geen artikel is gekomen over al die Bodipy-proeven die ik op jouw lab in Utrecht gedaan heb, maar ik heb er wel ontzettend veel van geleerd.

Beste Ronald, tijdens onze eerste kennismaking vertelde je met zoveel vuur over peroxisomale ziekten en de fantastische mensen die er op het lab werkten, dat ik pas na drie kwartier kon uitleggen dat ik niet voor een afstudeerstage naar Amsterdam wilde komen, zoals jij had aangenomen, maar voor een promotieonderzoek. Aangestoken door je enthousiasme, was ik er snel van overtuigd dat dit inderdaad de plek was waar ik wilde promoveren. Je zorgde er meteen al voor dat ik goed begeleid zou worden tijdens mijn AiO-periode door me onder te brengen bij Sacha. Je enorme metabole kennis en kritische blik op mijn experimenten hebben me erg geholpen.

Beste Sacha, jij bent de motor achter dit proefschrift geweest. Vanaf het begin heb je gezorgd dat ik de grote lijn niet uit het oog verloor, wanneer ik geneigd was om in details te verdwalen, ook al was je toen zelf nog niet eens gepromoveerd. Ik heb er enorm veel bewondering voor hoe je mijn begeleiding hebt aangepakt naast je eigen promotie en ook daarna. Ook toen bleek dat er een drastische koerswijziging nodig was, had je meteen een back-up plan klaar. Jij hebt me de beginselen van het schrijven van artikelen bijgebracht en je stond altijd meteen voor me klaar als ik bij je binnenliep. Dank je wel!

Beste Dmitri, bedankt voor al je hulp met de CLSM, het trouble-shooten en het meedenken bij mijn experimenten. Ping, it was nice working with you and I hope you're enjoying your position as a PhD student in Maastricht. Ook alle andere mensen van Biochemie van Lipiden in Utrecht wil ik bedanken, ik voelde me altijd meteen weer thuis als ik naar Utrecht kwam.

Gelukkig hoef je niet alles alleen te doen, er zijn bij lab GMZ een heleboel mensen die meteen voor je klaarstaan om te helpen. Simone, jij was de eerste die ik een beetje leerde kennen, omdat je me meteen onder je hoede nam toen ik begon met mijn promotie. Ik denk met veel plezier terug aan de werkbesprekingen op woensdagochtend. Ik heb heel veel geleerd van

je kritische kijk op experimenten en de manier waarop je steeds een oplossing weet te vinden. Bedankt voor je betrokkenheid en alle hulp, ik zal je missen als buurvrouw! Lodewijk, ook jij was er altijd bij op woensdagochtend. Je was altijd heel geïnteresseerd in mijn onderzoek en ook heel nieuwsgierig naar de resultaten van de proeven die we tijdens de werkbespreking bedachten. Mirjam, bedankt voor alle gezelligheid en voor het feit dat je altijd klaarstond om te helpen of als ik een luisterend oor nodig had, je bent een fijne vriendin. Petra en Patricia, ontzettend bedankt voor het kweken van alle cellijnen die ik altijd gisteren al nodig had! Conny, bedankt dat je me ingewijd hebt in de wondere wereld van de immunoblot. Michiel, bedankt voor de hulp, zijn er nog leuke bandjes in Australië? Janet H en Wendy, bedankt dat ik altijd kon aankloppen met alle vragen over RNA en DNA, sequentieanalyse en wat al niet meer. Janet K, bedankt voor je hulp bij het cloneren, de RNA isolaties en nog een heleboel andere dingen. Fredoen en Henk van L., bedankt voor alle hulp met de isoprostanen. Arno, bedankt voor de MDA bepalingen. Iedereen die mij geholpen heeft met GC-MS bepalingen, GC bepalingen, het zoeken naar monsters in de –80 °C vriezer, bedankt dat jullie altijd klaarstonden voor al mijn kleine en grote vragen.

Dan wil ik natuurlijk alle postdocs, AiO's en ex-AiO's bedanken. Fred (voor al je ideeën en natuurlijk alle hulp met die verdomde computers), Gerbert (voor de planning van de PPARα-Phytol proef), Sander (ik was blij dat je weer terugkwam als mede-Limbo (biertje?)), Carlo (voor je altijd goede humeur en enthousiasme), Daan, bedankt voor al je hulp met de phytol proeven, Sietske (ik heb je toch wel gemist als moeder-AiO), Jeannette (is er vandaag geen peroxisoombespreking?), Pedro (wanneer is jouw boekje nou klaar?), Marit (we begonnen samen met één bureau), Saskia (waar ga je nu weer naartoe op vakantie?), Jasper, mijn redder in nood als de GC-MS weer eens raar deed, natuurlijk altijd pas na vijven... (misschien volgend jaar schuurfeest?), Naomi (gedeelde frustraties #$%@&$), Wouter (ik zal nooit je rode labjas vergeten…), Ference (ik ben lekker toch eerder!!!), Stef, Annemieke de R, Jörgen, Nadia, Hidde, Riekelt, Annemieke B, Mark, Robert-Jan, Linda. Ik zal de AiO-etentjes missen, misschien moeten we maar ex-AiO etentjes gaan organiseren…

Dan natuurlijk nog Romana, mijn eerste en enige echte student. Bedankt voor al je blotjes, ik vond het heel leuk en leerzaam om je te begeleiden. Succes met je studie, misschien kom je nog wel eens terug bij lab GMZ?

Dan een aantal mensen die het lab draaiende houden, Annelies, Moniek, Sharon, Rally, Gerrit-Jan, Jan, Maddy (jij was mijn grote hoop als ik Ronald weer eens niet kon vinden, bedankt voor alle hulp!), Iet, Susan, Jolanda, bedankt! Annie, ook jij bedankt, soms wel een beetje druk, maar zonder jou zou het veel saaier zijn op het lab.

Mijn paranimfen, Loesje en Jos. Lieve Loesje, ook al zien we elkaar niet zo vaak, en bellen we veel te weinig, ik ben blij dat je er altijd bent als ik een vriendin nodig heb. Op de een of andere manier begrijp je altijd precies waar ik het over heb. Ik zal onze vakantie in Californië nooit vergeten, misschien kunnen we dat nog eens doen als jij gaat postdoccen, zoek maar een mooi plekje uit ☺! Lieve Jos, bedankt voor al je hulp aan dit proefschrift en, nog veel belangrijker, voor je vriendschap. Onze mountainbike-tochten en squash-veldslagen vormden een goede afleiding op alle promotieperikelen, ik hoop dat we dat in de toekomst ook gewoon blijven doen! Dat zal alleen maar makkelijker worden nu je in Amersfoort woont, dat is toch weer dichterbij. Fantastisch dat jij en Mirjam elkaar gevonden hebben, ik wens jullie alle goeds! Lieve Loesje en Jos, ik ben er trots op dat jullie mijn paranimfen willen zijn!

Naast je promotie heb je natuurlijk ook de broodnodige afleiding nodig. Martha, bedankt voor de gezellige etentjes en verjaardagen. Nannette en Tobias, super dat Loesje en ik bij jullie

mochten logeren in San Francisco, dat was een fantastische vakantie! Mijn teamgenoten, ex-teamgenoten en tennismaatjes bij STP Rozendaal, fijn dat ik jullie altijd kon bellen als ik me af moest reageren op een tennisbal! Roberto, bedankt voor je hulp met de kaft, uiteindelijk best mooi toch? Thomas en Anouk, niets werkt beter als ontspanning dan een weekje skiën, bedankt voor de gezelligheid! Diane en Dennis, onze trip naar Londen was kort maar krachtig, dat moeten we beslist nog een keer doen!

Lieve pap en mam, dank je wel dat jullie er altijd voor me zijn. Jullie hebben me altijd vrij gelaten in al mijn keuzes, dat waardeer ik heel erg. Dank je wel voor alles! Lieve Carien, tussen ons botst het nog wel eens, maar dat komt omdat we even koppig zijn, je bent een lieve zus. Je zegt altijd dat mijn werk heel ingewikkeld is, maar ik zou nooit kunnen wat jij met je "kids" doet!

Lieve Marcel, jij mag natuurlijk niet ontbreken in dit dankwoord. Ik ben er zeker van dat het me zonder jouw steun nooit gelukt was. Het is fijn om thuis iemand te hebben die onvoorwaardelijk achter je staat. Als ik in een dalletje zit, weet jij me altijd weer die berg op te krijgen. Ik hou van je.