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Research Article

Thiamine pyrophosphate: An essential cofactor for the α-oxidation in mammals – implications for thiamine deficiencies?

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Abstract. The identification of 2-hydroxyphytanoyl-CoA lyase (2-HPCL), a thiamine pyrophosphate (TPP)-dependent peroxisomal enzyme involved in the α-oxidation of phytanic acid and of 2-hydroxy straight chain fatty acids, pointed towards a role of TPP in these processes. Until then, TPP had not been implicated in mammalian peroxisomal metabolism. The effect of thiamine deficiency on 2-HPCL and α-oxidation has not been studied, nor have possible adverse effects of deficient α-oxidation been considered in the pathogenesis of diseases associated with thiamine shortage, such as thiamine-responsive megaloblastic anemia (TRMA). Experiments with cultured cells and animal models showed that α-oxidation is controlled by the thiamine status of the cell/tissue/organism, and suggested that some pathological consequences of thiamine starvation could be related to impaired α-oxidation. Whereas accumulation of phytanic acid and/or 2-hydroxyfatty acids or their α-oxidation intermediates in TRMA patients given a normal supply of thiamine is unlikely, this may not be true when malnourished.

Keywords. Beriberi, 2-hydroxyfatty acids, peroxisomes, phytanic acid, thiamine-responsive megaloblastic anemia, vitamin B, Wernicke-Korsakoff syndrome.

Introduction

In mammals, long chain 3-methyl-branched fatty acids such as the naturally occurring phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), are shortened by one carbon atom (α-oxidation). This process, which occurs in peroxisomes, includes the following steps (Fig. 1): (1) activation to a CoA-ester, (2) hydroxylation of carbon 2 of the CoA-ester, and (3) cleavage between carbon 1 and 2, resulting in the generation of a 2-methyl-branched fatty aldehyde and formyl-CoA. The aldehyde is subsequently dehydrogenated (4) to a 2-methyl-branched fatty acid (pristanic acid in the case of phytanic acid breakdown), which will be further degraded by peroxisomal β-oxidation. Formyl-CoA is converted to formate, which will be oxidized to CO_2 (for review see [1, 2]). The most prototypical clinical picture of an isolated defect in α-oxidation is adult Refsum disease, which is characterized by retinitis pigmentosa, night blindness and anosmia; polyneuropathy, deafness, ataxia and ichthyosis are also quite

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common (for review see [3]). Increased phytanic acid levels, which are the biochemical hallmark of Refsum disease, usually result from mutations in the gene coding for the second enzyme of the α-oxidation pathway, phytanoyl-CoA hydroxylase [4, 5]. 2-Hydroxyphytanoyl-CoA lyase (2-HPCL), a tetrameric protein with homology to oxalyl-CoA decarboxylases and dependent on thiamine pyrophosphate (TPP), catalyzes the cleavage reaction [6]. Recently, it was shown that this enzyme also cleaves the CoA esters of straight chain 2-hydroxylated fatty acids (Fig. 1), and that peroxisomes play an important role in the α-oxidation of long chain 2-hydroxyfatty acids, constituents of certain sphingolipids [7].

2-HPCL provided the first example of a TPP-dependent enzyme in mammalian peroxisomes. TPP is the active form of thiamine, a water-soluble vitamin of the B-complex, and functions as a cofactor of various enzymes mainly involved in cellular glucose and energy metabolism: the pyruvate dehydrogenase complex, 2-ketoglutarate dehydrogenase, branched-chain keto-acid dehydrogenase and transketolase (Fig. 1). Like other micronutrients, thiamine is not synthesized by higher eukaryotes. Therefore, adequate nutritional levels of thiamine and subsequent absorption, via both a high-affinity saturable transporter and a low-affinity unsaturable transport mechanism, are essential for normal development and physiology. Upon entry into cells, most thiamine is converted by thiamine pyrophosphokinase to TPP, which is generally the predominant form of vitamin B (75–90% of total).

Severe thiamine deprivation as seen in gross malnutrition, chronic alcoholism and AIDS affects the nervous system by causing peripheral neuropathy and central nervous system lesions, referred to as the Wernicke-Korsakoff syndrome. Similar clinical manifestations and brain morphological changes have been described in animals given a thiamine-deficient diet and/or thiamine anti-metabolites such as oxythiamine (OT) or pyrithiamine (PT) [8–14]. The latter compounds block the coenzyme functions of thiamine. PT readily crosses the blood-brain barrier and PT-diphosphate competitively inhibits the activity of thiamine pyrophosphokinase [15]. OT-diphosphate competes with TPP for binding to apo-enzymes. Moreover, these cations cause a decrease in thiamine availability by competition for the saturable component of the thiamine/H^+ antiport [15–17].

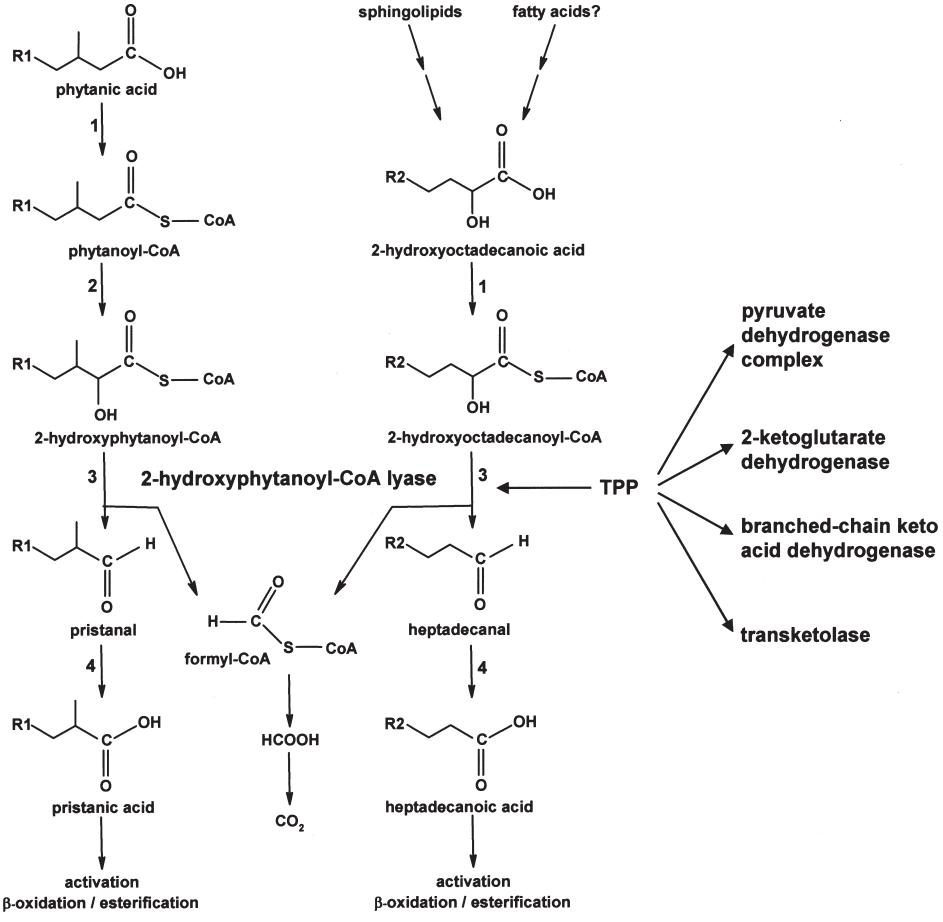

Figure 1. α-Oxidation of 3-methyl-branched and 2-hydroxy straight chain fatty acids and its relation with TPP. The scheme shows the involvement of TPP in the α-oxidation of 3-methyl-branched (left side, *e.g.* dietary phytanic acid) and 2-hydroxy straight chain fatty acids (right side, *e.g.* 2-hydroxyoctadecanoic acid, derived from sphingolipids or possibly other fatty acids) and in some enzymes involved in glucose and energy metabolism. R1 stands for 3,7,11-trimethyldodecyl and R2 for tetradecyl. See text for details.

Thiamine-responsive megaloblastic anemia syndrome (TRMA, also known as Rogers syndrome) is a rare autosomal recessive disorder characterized by diabetes, megaloblastic anemia and deafness (OMIM 249270). In addition, optic atrophy, congenital heart defects and stroke-like episodes have been reported. Using positional cloning/candidacy approaches, three groups identified the disease gene as *SLC19A2*, which encodes a high-affinity thiamine transporter (THTR-1) that is present on the plasma membrane and on mitochondria [18–20]. THTR-1, a protein of 497 amino acid residues, is one of three members of the solute carrier family SLC19A [21]. The other members of this family include the reduced folate carrier (SLC19A1) [22, 23] and a recently identified second thiamine transporter without folate transport activity (SLC19A3) [24, 25].

Until now, thiamine deficiency has not been linked to perturbation in α-oxidation. One objective of the current study was to document the thiamine dependence of the α-oxidation pathway in cultured cells and in an animal model. Given the partial overlap in phenotype between Refsum disease and TRMA, the α-oxidation process was analyzed in cells from TRMA patients to see whether it was affected and could contribute to the pathophysiology of TRMA.

Materials and methods

Materials. Co-carboxylase (TPP), OT hydrochloride and PT were ordered from Sigma, thiamine monophosphate (TMP) chloride from Fluka, thiamine hydrochloride from Janssen Chimica. Phytol was obtained from Riedel-de Haen. [1-^{14}C]Palmitic acid and [1-^{14}C]pyruvate were from New England Nuclear. 3-Methyl-[1-^{14}C]hexadecanoic acid [26], 2-methyl-[1-^{14}C]hexadecanoic acid [26], 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoic acid [26], 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA [26] and 2-hydroxy-[1-^{14}C]octadecanoic acid [7] were synthesized as described previously.

Animals. Animal studies were approved by the University Ethics committee. Weaned Wistar rats, weighing 56–61 g, were kept in a constant light-dark cycle and on either a standard laboratory chow diet or a thiamine-depleted diet, which were obtained from Ssniff (Bio-Services). Subgroups were injected intraperitoneally for 8 consecutive days either with OT (4 mg/100 g in 0.5 ml 0.9% NaCl) or PT (50 µg/100 g in 0.5 ml 0.9% NaCl) as described by Okazaki et al. [27] and animals were weighed daily. Supplementation of chow with phytol (0.5%) was performed as described previously [28].

Cell isolation and cell culture. Primary mouse fibroblasts [28] and rat hepatocytes [29] were isolated essentially as described before. Fibroblasts from several TRMA patients were obtained from different pediatric centers and included two unrelated patients (A.R., M.M.; both homozygous for 239insA [18]), two siblings from one family (A. Z., K. Z.; both homozygous for 724delC [20]) and three siblings from a second family (I. Y., N. Y., Z. Y.; mutation(s) not yet identified; Van Maldergem L. and Gelb B., unpublished data). Control human fibroblasts were kindly provided by Dr. G. Matthys (Leuven, Belgium). Rat C6 glial cells were obtained from ATCC. Adherent cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% FCS (Invitrogen) or 2% Ultroser G (BioSepra), Glutamax (Invitrogen) and a mixture of antibiotics and antimycotics (Invitrogen). Thiamine-free DMEM-F12 was obtained from Life Technologies (Invitrogen), but its thiamine content, verified by standard lab techniques, was 9 nM (*versus* ~6400 nM for control medium). Hence, it is referred to as thiamine-depleted medium.

Fatty acid oxidation in cultured cells. Fibroblasts and rat C6 glial cells were grown to near confluence in 25-cm^2 flasks and incubated for 24 h with 4 µM substrate [30], either [1-^{14}C]palmitic acid (mitochondrial β-oxidation), 2-methyl-[1-^{14}C]hexadecanoic acid (peroxisomal β-oxidation of branched fatty acids), 3-methyl-[1-^{14}C]hexadecanoic acid or 2-hydroxy-[1-^{14}C]octadecanoic acid (α-oxidation). Depending on the substrate, the amounts of $^{14}CO_2$, [^{14}C]formate, labeled acid soluble β-oxidation products and label incorporated into cellular lipids were analyzed essentially as described before [30, 31].

Preparation of cell lysates and homogenates and enzyme measurements. Cells, grown to confluence in 175-cm^2 flasks, were harvested by trypsinization, washed twice with PBS and finally homogenized by sonication (Branson Sonifier; output 4) in 1 ml 0.25 M Tris-HCl pH 7.2 or 10 mM potassiun phosphate buffer pH 7.4. Liver pieces were freeze-clamped, stored at –80 °C until use and then homogenized in 7 volumes of 0.25 M sucrose, 5 mM Mops-NaOH pH 7.0 and 0.1% ethanol (2-HPCL activity) or in 9 volumes of 0.4 M $HClO_4$ (thiamine measurements, see below).

2-HPCL activity was quantified as described previously [6]. Incubations (37 °C) were started by the addition of 100 µl cell lysate (prepared in Tris-HCl buffer) or 50 µl liver homogenate to 150 or 200 µl reaction medium, respectively. Final concentrations were 50 mM Tris-HCl pH 7.5, 0.8 mM $MgCl_2$, 20 µM TPP, 6.6 µM BSA, and 40 µM 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA (2000 dpm/nmol), referred to as standard conditions. When using 2-hydroxy-[1-^{14}C]octadecanoic acid as a substrate (5000 dpm/nmol; 50 µM final concentration), the reaction mixture contained 4 mM ATP, 0.5 mM CoA,

2 mM NAD^+, 12.5 µM BSA, and 20 µM TPP in modified Krebs-Henseleit buffer (2.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 4.8 mM NaCl, 120 mM KCl, 1.2 mM KH_2PO_4, 20 mM HEPES-NaOH pH 7.5). Reactions were terminated after 10 min by addition of 125 µl $HClO_4$ (6%, w/v). After removal of protein, the acidified supernatant was used to measure ^{14}C-labeled formate [31]. During the incubation, the primary product, formyl-CoA, is (bio)chemically converted to [^{14}C]formate [31, 32]. Further oxidation of this metabolite to $^{14}CO_2$ is negligible in tissue homogenates and cell lysates in the absence of NAD^+ [32].

The decarboxylation of [1-^{14}C]pyruvate was measured essentially as described by Robinson et al. [33]. Briefly, 100 µl cell lysate (prepared in phosphate buffer) was added to 400 µl reaction medium containing 10 mM potassium phosphate pH 7.4, 1 mM EDTA, 1 mM DTT, 1% BSA, 2 mM $MgCl_2$, 0.15 mM CoA, 1.6 mM NAD^+, 0.1 mM TPP and 0.25 mM [1-^{14}C]pyruvate (2220 dpm/nmol). Incubations were performed for 45 min at 37 °C in closed glass vials and $^{14}CO_2$ was trapped, after acidification, on paper strips soaked in KOH.

Determination of thiamine and its phosphate esters. The quantification of thiamine, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) was based on the formation of thiochrome derivatives by alkaline oxidation (adapted from [34, 35]). Briefly, perchloric cell or tissue extracts (100 µl; 0.4 M $HClO_4$ final) were derivatized with $K_3[Fe(CN)_6]$ (20 µl; 30.4 mM in 15% NaOH) after adding 10 µl methanol. After 60 s, 20 µl 1 M H_3PO_4 was added. Aliquots were injected on a C18-amide column (Alltima HP, 150 × 4.6 mm, 5 µm, Alltech) on a Waters 1525 HPLC, coupled to a Waters 2475 fluorescence detector (excitation 367 nm; emission 435 nm). The thiochrome derivatives were eluted with a gradient of solvent A/B (A: 140 mM potassiun phosphate buffer pH 7, 12% methanol; B: 70% methanol): linear from 100/0 to 50/50 in 10 min, followed by 50/50 to 0/100 in 5 min.

Results

To document the influence of thiamine/TPP on α-oxidation, we modulated the intracellular concentration of this vitamin, either by adding compounds interfering with thiamine uptake/metabolism or by growing cells in thiamine-depleted medium.

As rat C6 glial cells possess the highest basal α-oxidation rate for 3-methyl-branched fatty acids amongst different cultured cells analyzed (Foulon V., Casteels M., Van Veldhoven P.P., unpublished data), the first experiments were performed on these cells. When the cells were grown in the presence of increasing concentrations of OT for at least 2 days, α-oxidation of 3-methylhexadecanoic acid and 2-hydroxyoctadecanoic acid was depressed in a dose-dependent manner, up to 90–98% at 1 mM (Fig. 2). The OT effect was quite specific, as the treatment did not affect the β-oxidation of long 2-methyl-branched fatty acids, another peroxisomal pathway. Mitochondrial β-oxidation of long straight chain fatty acids was also unaffected by the addition of OT (Fig. 2). Furthermore, when 2-HPCL activity was measured in lysates of C6 cells, grown in OT-containing medium, a severe decrease was noticed, even when TPP was present in the assay (Fig. 3a, b). When the cells were grown in thiamine-depleted medium, however, α-oxidation rates (data not shown) and lyase activity (Fig. 3a, b) were only mildly affected. The activity of pyruvate decarboxylase, another TPP-dependent enzyme, was severalfold lower in thiamine-depleted cells when measured in the absence of TPP, but was restored to almost normal levels upon addition of the cofactor to the incubation mixture (Fig. 3c).

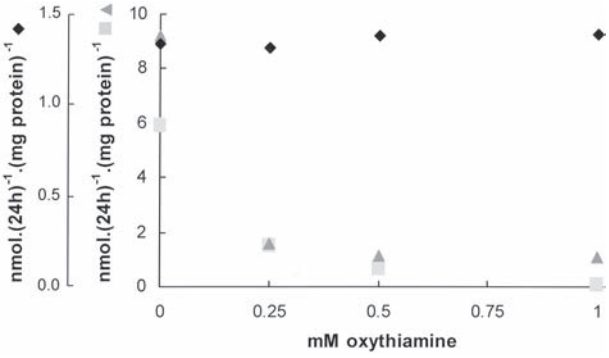

Figure 2. Effect of oxythiamine on α-oxidation in confluent C6 glial cells. Nearly confluent rat C6 glial cells, grown in the presence of increasing concentrations of OT for 48 h, were incubated with 4 µM 3-methyl-[1-^{14}C]hexadecanoic acid (■), 2-hydroxy-[1-^{14}C]octadecanoic acid (▲), or [1-^{14}C]palmitic acid (♦). Results represent the sum of $^{14}CO_2$ and [^{14}C]formate (α-oxidation) or sum of $^{14}CO_2$ and labeled acid soluble products (ASM) (β-oxidation), measured as described in the Materials and methods, and are mean values of two determinations. When grown in absence or presence of 1 mM OT for 48 h, oxidation rates for 2-methyl-[1-^{14}C]hexadecanoic acid were 7.25 *versus* 8.50 nmol/24 h/mg protein, those for 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoic acid 6.37 *versus* 0.59 nmol/24 h/mg protein.

As in C6 glial cells, α-oxidation in human skin fibroblasts cultured in thiamine-depleted medium was little affected. On the other hand, addition of 1 mM OT resulted in a 50% reduction in the oxidation rate of 3-methylhexadecanoic acid and 2-hydroxyoctadecanoic acid (Fig. 4). The decreased α-oxidation was not due to a nonspecific effect of OT on peroxisomes since β-oxidation of 2-methylhexadecanoic acid was normal (Fig. 4).

In mouse fibroblasts, similar findings were obtained: overall α-oxidation of 3-methylhexadecanoic acid was not affected by the use of thiamine-depleted medium, but was reduced after adding 1 mM OT (data not shown).

Together, these data indicate that 2-HPCL activity, and consequently the overall α-oxidation, is markedly af-

fected by the intracellular thiamine concentration or by thiamine anti-metabolites.

To study the *in vivo* effects of thiamine deprivation on α-oxidation, animal models for thiamine deficiency were tested. In a first set of experiments, young rats (about 50 g) were divided into four groups. One group received a thiamine-deficient diet; the other groups were fed a control diet and were daily injected intraperitoneally with OT, PT, or saline, respectively. On days 7, 11 and 13, hepatocytes were isolated from one rat of each group and the degradation of different fatty acids was determined. The oxidation capacity for 3-methylhexadecanoic acid as well as for 2-hydroxy-3-methylhexadecanoic acid, a synthetic 3-methyl-branched substrate that can be shortened by α-oxidation in the absence of phytanoyl-CoA hydroxylase (V. Foulon, M. Casteels and P.P. Van Veldhoven, unpublished results), and 2-hydroxyoctadecanoic acid was more depressed in hepatocytes from OT-injected animals and rats fed a thiamine-deficient diet than in hepatocytes from PT-injected rats (Fig. 5a). The effect of thiamine deprivation clearly increased with treatment duration. Although the α-oxidation rate dropped three- to fourfold in some conditions, no pathological symptoms were observed.

In a second set of experiments, weaned rats were fed either a control diet or a thiamine-deficient diet. The rats of the latter group were also injected with OT. Half of the animals on these diets received supplementary phytol. After 10 days, the OT-treated rats fed a thiamine-deficient diet had lost weight (final weight of 40–45 g) and were in very poor condition. Rather unexpectedly, addition of phytol to the food resulted in early death of all animals on a thiamine-deficient diet (and prevented assessment of their α-oxidation status in isolated hepatocytes). Animals on the control diet with and without phytol supplementation were

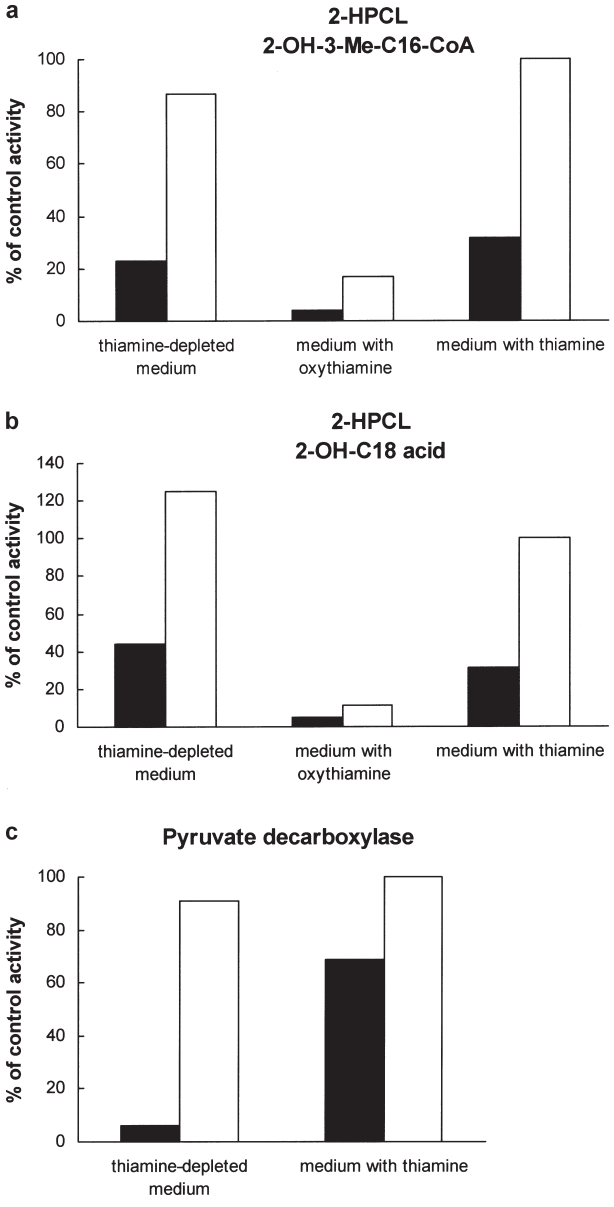

Figure 3. Effect of thiamine depletion on TPP-dependent enzymes in C6 glial cells. Rat C6 glial cells were grown in standard medium in the absence or presence of 1 mM OT or thiamine-depleted medium for 48 h. 2-HPCL activity {2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA (*a*) and 2-hydroxy-[1-^{14}C]octadecanoic acid (*b*) as substrate} and pyruvate decarboxylase activity (*c*) were measured on cell lysates as described in the Materials and methods, either without (black bars) or with (white bars) the addition of 20 µM (2-HPCL) or 0.1 mM TPP (pyruvate decarboxylase) to the assay mixtures. Control activity was defined as the activity measured in lysate prepared from C6 glial cells grown in standard medium, with TPP added to the incubation mixture (for pyruvate decarboxylase: 107 pmol/min/mg protein; for 2-HPCL: 64 ± 5.6 pmol/min/mg protein for *a*, mean ± SEM of three experiments, and 64 pmol/min/mg protein for *b*, mean of two experiments).

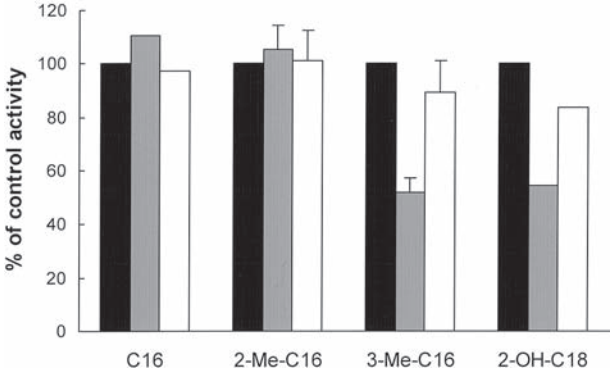

Figure 4. Effect of thiamine deprivation on fatty acid oxidation in human fibroblasts. Control fibroblasts, grown in standard medium (black bars), in the presence of 1 mM OT (gray bars) or in thiamine-depleted medium (open bars) for at least 5 days, were incubated with 4 µM 3-methyl-[1-^{14}C]hexadecanoic acid (3-Me-C16), 2-hydroxy-[1-^{14}C]octadecanoic acid (2-OH-C18), 2-methyl-[1-^{14}C]hexadecanoic acid (2-Me-C16) or [1-^{14}C]palmitic acid (C16). Labeled CO_2, formate and ASM were measured as described in the Materials and methods. Results were calculated as the sum of CO_2 and formate (α-oxidation) or CO_2 plus ASM (β-oxidation), and are shown as percentage of control activity (mean ± SEM of four cell lines, except for C16 and 2-OH-C18 where only two cell lines were used).

healthy and gained weight normally (120–125 g). Analysis of α-oxidation capacity in isolated hepatocytes of rats from the surviving three groups showed a significant decrease when the animal was made thiamine deficient (Fig. 5b). Moreover, 2-HPCL activity was not detectable in liver homogenates of the thiamine-deficient rats (Fig. 5c).

In view of the important influence of the thiamine status on the α-oxidation in cells and animals, fibroblasts from

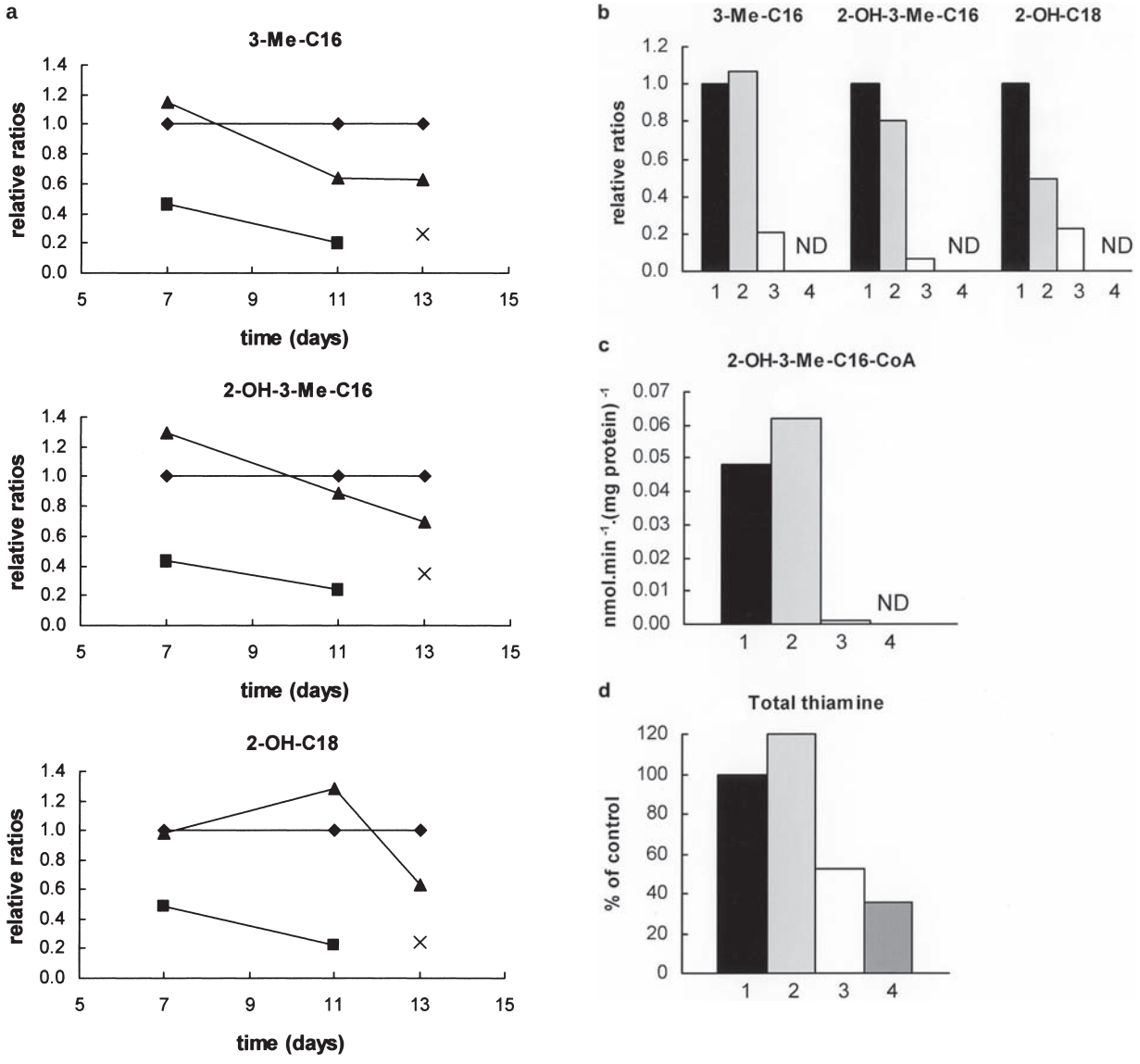

Figure 5. Hepatic fatty acid oxidation and 2-HPCL activity of thiamine-deficient rats. Hepatocytes, isolated from age-matched rats subjected to different treatments, were incubated with substrates for α-oxidation {3-methyl-[1-^{14}C]hexadecanoic acid (3-MeC16), 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoic acid (2-OH-3-Me-C16) or 2-hydroxy-[1-^{14}C]octadecanoic acid (2-OH-C18)}, or for β-oxidation ([1-^{14}C]palmitic acid). To compensate for possible effects of the animal's health on the yield/quality of the hepatocytes prepared on the different days, the ratio of the α-oxidation rates *versus* the β-oxidation rates of palmitic acid was determined for each condition and further related to this ratio obtained in the control animal. For the experiment shown in (*a*), rats were divided into four different groups: control group (♦), OT-treated group (■), PT-treated group (▲), and rats fed a thiamine-deficient diet (x). Hepatocytes were isolated on days 7, 11 and 13. Results presented are relative ratios based on single determinations. In (*b*), relative ratios calculated from single determinations on hepatocytes obtained from rats fed a control diet (1, black bars), a control diet supplemented with phytol (2, gray bars), or a thiamine-free diet and additional injections with OT (3 and 4; open bars) are shown. In (*c*), the lyase activity towards 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA (2-OH-3-Me-C16-CoA) measured in homogenates prepared from freeze-clamped pieces of livers of another set of animals, fed a control diet (1, black bar), a control diet supplemented with phytol (2, gray bar), a thiamine-free diet plus OT (3, open bar) and thiamine-free diet plus OT plus phytol (4, dark gray) is shown. (*d*) The thiamine content of freeze-clamped pieces of livers of the same animals as in (*c*), using the same symbols. Results represent the sum of thiamine, TMP and TPP and are expressed as % of control based on duplicate measurements of one (groups 1 and 3) or two animals (groups 2 and 4). Control value was 28.8 nmol total thiamine/g liver. TPP levels were between 42% to 65% of total thiamine content; due to storage, the actual TPP levels may be higher at time of sacrifice. ND, not detectable.

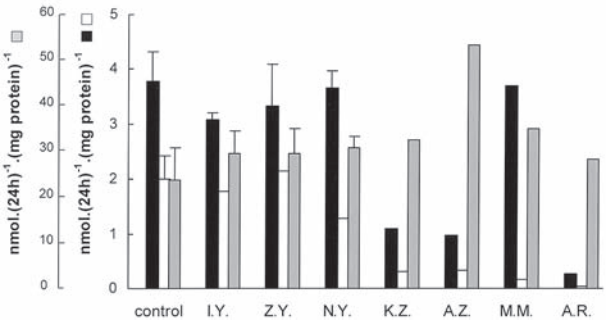

Figure 6. α-Oxidation in TRMA fibroblasts. α-Oxidation rates for 3-methyl-[1-^{14}C]hexadecanoic acid (3-Me-C16) in control and TRMA fibroblasts, grown in standard medium (black bars) or medium with 1 mM OT (open bars) for at least 5 days, are shown. Peroxisomal β-oxidation rates (2-methyl-[1-^{14}C]hexadecanoic acid, 2-Me-C16) (gray bars) are shown as comparison. Results were calculated as the sum of $^{14}CO_2$ plus [^{14}C]formate (α-oxidation) or $^{14}CO_2$ plus ^{14}C-labeled ASM (β-oxidation) and are mean values ± SEM of four control cell lines, and of two (M. M.; A. R.), three (I. Y.; Z. Y.; M. M.) or four (N. Y.) separate experiments for TRMA cells. Values for K. Z. and A. Z. and for conditions with OT are based on duplicate measurements in a single experiment.

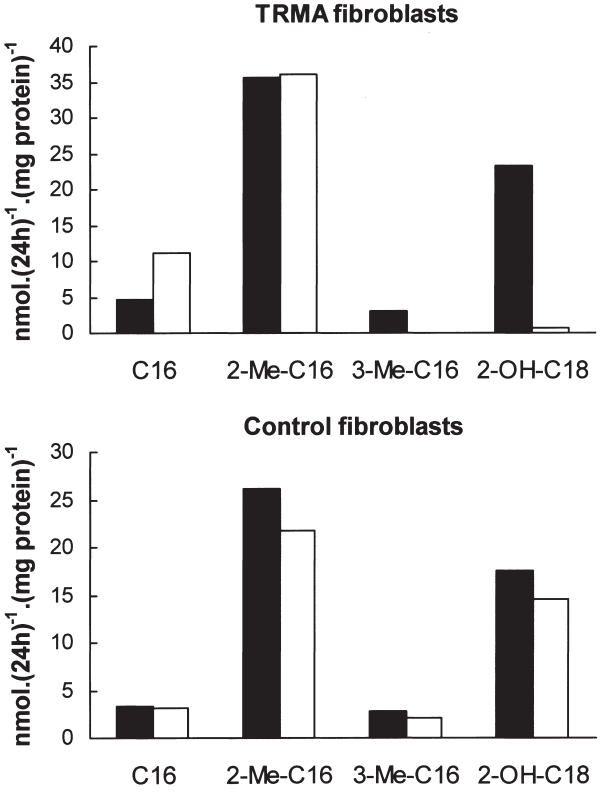

Figure 7. Effect of thiamine deprivation in TRMA fibroblasts. Two control and two TRMA fibroblast cell lines (K. Z. and M. M.), grown in normal (black bars) or thiamine-depleted medium (open bars) for at least 5 days, were incubated with 4 µM [1-^{14}C]hexadecanoic acid (C16), 2-methyl-[1-^{14}C]hexadecanoic acid (2-Me-C16), 3-methyl-[1-^{14}C]hexadecanoic acid (3-Me-C16) or 2-hydroxy-[1-^{14}C]octadecanoic acid (2-OH-C18), also in normal or thiamine-depleted medium. Results represent the sum of $^{14}CO_2$ plus ^{14}C-labeled ASM (β-oxidation) or $^{14}CO_2$ plus [^{14}C]formate (α-oxidation) measured as described in the Materials and methods, and are mean values of single determination on the different cell lines.

patients affected with TRMA, a disease model linked to thiamine transport, were analyzed. The α-oxidation capacity of these cells was somewhat variable, likely due to differences in passage number and growth conditions. In some cultures, however, α-oxidation rates were decreased compared with control cells. This becomes more obvious upon comparison with their peroxisomal β-oxidation capacity, measured under similar conditions (Fig. 6). In cells from some patients, the addition of 1 mM OT resulted in a substantial reduction of the overall α-oxidation rates, namely 71% (K.Z.), 82% (A.R.) and 96% (M.M.), being markedly higher than in control fibroblasts (48 ± 5.2%) (Fig. 6). The role of thiamine in the α-oxidation process was further analyzed in two well-growing TRMA fibroblast cultures (K.Z., M.M.). Due to poor growth, the other established TRMA cell lines could not be investigated in thiamine-depleted medium, whereas the fibroblasts of the TRMA patients in which the mutation has not been identified yet were not studied further. When the two TRMA cell lines were cultured in such medium, both 3-methylhexadecanoic acid and 2-hydroxyoctadecanoic acid breakdown was severely inhibited, but neither peroxisomal, nor mitochondrial β-oxidation was decreased (Fig. 7). The mitochondrial pathway was even stimulated (Fig. 7), but this might be due to an isotope effect as during thiamine deficiency acetyl-CoA levels are lowered [36].

Discussion

Previous research on the effects of thiamine deficiency in patients and animals has focused on the pentose phosphate shunt enzyme transketolase and on enzyme complexes involved in oxidative decarboxylation reactions (pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and branched-chain keto-acid dehydrogenase). Here, we show that 2-HPCL, a recently discovered peroxisomal TPP-dependent enzyme, has to be considered as well. Since this enzyme is essential for the α-oxidation of 3-methyl-branched fatty acids and of 2-hydroxyfatty acids, the breakdown of these substrates may also be influenced by the thiamine status of cells and animals. This has been proven experimentally in rat C6 glial cells and human and mouse fibroblasts by manipulation of the growth medium and in rat models by dietary treatments. It should be noted that thiamine deficiency does not affect other peroxisomal pathways, such as the degradation of 2-methyl-branched fatty acids (our data) and plasmalogen synthesis [37]. So far, defects in α-oxidation have only been linked to inherited disorders, including peroxisome biogenesis defects and Refsum disease. The problems in the latter, which are thought to be caused by increased phytanic acid levels, highlight the importance of a functional α-oxidation pathway. We suggest here that the α-oxidation may also be impaired in disorders with thiamine deficiency.

Whereas addition of OT resulted in reduced α-oxidation rates in all cells investigated, α-oxidation was minimally affected when the cells were grown in thiamine-depleted medium. One obvious explanation is that thiamine, although severalfold reduced, was still present in the medium used. The experimentally determined concentration of 9 nM is well below the K_m of the high-affinity thiamine transporter, but might be sufficient to maintain certain critical intracellular processes. Another possibility is that an OT metabolite, *e.g.* OT pyrophosphate, affects somehow 2-HPCL. At least this is consistent with a preliminary analysis of the thiamine status in OT-treated C6 glial cells. When treated with 1 mM OT for 2 days, their TPP content was still 82% of control value (377 *versus* 463 pmol/mg protein; TPP representing ~80% of total thiamine content in both conditions) (M. Sniekers, P. Fraccascia, M. Casteels and P.P. Van Veldhoven, unpublished results).

Two other observations are worth noting. The first is the susceptibility of 2-HPCL to thiamine depletion and OT treatment. The steady-state protein levels/activities of TPP-dependent enzymes upon withdrawal of thiamine or addition of anti-metabolites can be influenced by different factors: cell-type dependent differences [38], uptake of thiamine/anti-metabolite by the organ/cells, affinity of the enzyme for TPP or analogues, turnover and intracellular localization of the protein. Effects of thiamine concentration on mRNA levels have also been reported, both in mammals and yeast [39, 40]. For brain, thiamine levels are also controlled by the blood-brain barrier. In C6 glial and C-1300 neuroblastoma cells, pyruvate decarboxylase was more sensitive to thiamine deprivation than transketolase [41]. In brains of alcoholics, transketolase is more affected than the pyruvate dehydrogenase complex and 2-ketoglutarate dehydrogenase is unaffected [42]. In TRMA fibroblasts, transketolase activity was more susceptible to thiamine deprivation than control fibroblasts [38]. OT treatment reduced 2-HPCL activity in cultured cells, and, in liver from OT-treated rats fed a thiamine-deficient diet, 2-HPCL even became undetectable. Since addition of TPP is not able to restore 2-HPCL activity to normal levels, loss of apoenzyme can be inferred. Similar findings have been reported for transketolase in rat brain [43]. As in the OT-treated cells (see above) and liver of OT-treated rats (Fig. 5d), thiamine(PP) is still present, this loss seems to be caused by OT pyrophosphate, competing with TPP. Whether TPP plays a role in the tetramerization of 2-HPCL [6], and whether this process takes place in the cytosol or inside the peroxisome or is blocked by OT pyrophosphate, is presently not known. In addition, no experimental data are available with regard to the transport of thiamine or TPP across the peroxisomal membrane. Transport of thiamine followed by intra-peroxisomal conversion to TPP, however, seems less likely because the required enzymes do not possess an appropriate targeting signal (V. Foulon, M. Casteels and P.P. Van Veldhoven, unpublished data).

A second interesting observation relates to the toxicity of phytol under thiamine-deficient conditions. Phytol is a polyprenol found in chlorophyll, and is partially converted to phytanic acid, which is degraded via α-oxidation. In thiamine-depleted conditions, α-oxidation becomes likely defective, leading to the accumulation of a toxic compound, presumably 2-hydroxyphytanoyl-CoA or its precursor (phytanoyl-CoA or phytanic acid) or a metabolite (2-hydroxyphytanic acid). Not much is known about the bio-activity and toxicity of these compounds. One possible explanation is that these compounds, being lipophilic or detergent by nature, disturb membrane integrity and packing at high concentrations. For example, phytanic acid added to isolated adult rat brain mitochondria causes membrane depolarization [44]. Another, and probably more important, mechanism to be considered is the modulation of transcription. Phytanic acid has been shown to bind and activate the nuclear receptors RXR [45–47] and PPAR-α [47], to enhance transcription of uncoupling protein-1 [48] and of fatty acid binding protein [49], and to induce adipocyte differentiation [48, 50]. Other reported cellular effects of phytanic acid are nitric oxide-dependent apoptosis in vascular smooth muscle [51], morphological changes in retinal pigment epithelial cells, in particular generalized swelling, presence of lipid vacuoles and loss of apical microvilli, which are also seen in the retina of Refsum disease patients [52], and cytosolic calcium release, increase in reactive oxygen species and apoptosis due to mitochondrial impairment in rat hippocampal astrocytes [53, 54]. Based on the expression pattern of 2-HPCL [6], thiamine deficiency will lead to accumulation of toxic phytol derivatives mostly in liver, kidney, heart and muscle. It is worth noting that bovine serum, which is usually added to cultured cells, is a source of phytanic acid (10–230 μM in calf serum, 0.12 μM in fetal calf serum) [55–57], complicating the interpretation of thiamine deficiency studies in cultured cells. In our α- and β-oxidation studies on cultured cells, however, a synthetic supplement (Ultroser G) was chosen instead of serum [30]. In addition, Ultroser G, used at 0.2%, contributes only to 0.1 nM thiamine.

Given the established thiamine dependency of α-oxidation, it seemed logical to investigate this pathway under conditions whereby cellular thiamine uptake is affected. In fibroblasts from TRMA patients, when grown in standard medium, α-oxidation appeared to be affected to a variable extent. When grown in thiamine-depleted medium, however, α-oxidation was almost completely blocked in the investigated cell lines, although there is likely still some uptake of thiamine due to the presence of a low-affinity unsaturable thiamine uptake component. At the estimated thiamine concentration, ~9 nM under our conditions, the uptake would be 5–10% compared with control fibro-

blasts [58]. Remarkably, this concentration of thiamine has been reported to be sufficient to rescue TRMA fibroblasts from cell death, a process that is triggered when the cells are grown in complete absence of thiamine [55]. The apoptotic events have recently been linked to a defective synthesis of RNA ribose (pentose phosphate shunt pathway) [59]. However, our data indicate that 2-HPCL, and consequently α-oxidation, might be more sensitive to thiamine depletion than this process. Hence, abnormal phytanic acid and 2-hydroxyfatty acid metabolism might occur in TRMA patients, certainly under less well-nourished conditions. To prevent megaloblastic anemia, diabetes, and cardiac arrhythmia, TRMA patients are indeed dependent on the intake of large doses of lipophilic thiamine (benzoyloxymethyl-thiamine, benfothiamine).

In summary, thiamine depletion affects α-oxidation by lowering 2-HPCL levels and activity. Therefore, α-oxidation is likely impaired when dietary thiamine is restricted (beriberi, Wernicke-Korsakoff syndrome). In contrast to other TPP-dependent enzymes, which are linked to energy metabolism (pentose phosphate shunt, citric acid cycle), 2-HPCL is the first example of an enzyme directly involved in the breakdown of particular fatty acids, such as phytanic acid and 2-hydroxyfatty acids. Phytol-derived phytanic acid, present in ruminant fat and dietary products, and its breakdown intermediates such as 2-hydroxyphytanoyl-CoA, appear to be toxic if they accumulate. The same probably applies to 2-hydroxy straight chain fatty acids, originating from the turnover of sphingolipids, and their CoA esters. These observations may shed new light on some unexplained disparities seen in TRMA (affected tissues *versus* expression of SLC19A2), on the different clinical presentations of TRMA and dietary thiamine deficiencies, as well as on the mechanisms underlying the pathogenesis in these disorders.

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1 Casteels, M., Foulon, V., Mannaerts, G. P. and Van Veldhoven, P. P. (2003) Alpha-oxidation of 3-methyl-substituted fatty acids and its thiamine dependence. Eur. J. Biochem. 270, 1619–1627.
2 Wanders, R. J., Jansen, G. A. and Lloyd, M. D. (2003) Phytanic acid alpha-oxidation, new insights into an old problem: a review. Biochim. Biophys. Acta 1631, 119–135.
3 Wierzbicki, A. S., Lloyd, M. D., Schofield, C. J., Feher, M. D. and Gibberd, F. B. (2002) Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. J. Neurochem. 80, 727–735.
4 Jansen, G. A., Ofman, R., Ferdinandusse, S., Ijlst, L., Muijsers, A. O., Skjeldal, O. H., Stokke, O., Jakobs, C., Besley, G. T., Wraith, J. E. and Wanders, R. J. (1997) Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. Nat. Genet. 17, 190–193.
5 Mihalik, S. J., Morrell, J. C., Kim, D., Sacksteder, K. A., Watkins, P. A. and Gould, S. J. (1997) Identification of PAHX, a Refsum disease gene. Nat. Genet. 17, 185–189.
6 Foulon, V., Antonenkov, V. D., Croes, K., Waelkens, E., Mannaerts, G. P., Van, Veldhoven, P. P. 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. USA 96, 10039–10044.
7 Foulon, V., Sniekers, M., Huysmans, E., Asselberghs, S., Mahieu, V., Mannaerts, G. P., Van Veldhoven, P. P. and Casteels, M. (2005) Breakdown of 2-hydroxylated straight chain fatty acids via peroxisomal 2-hydroxyphytanoyl-CoA lyase: A revised pathway for the α-oxidation of straight chain fatty acids. J. Biol. Chem. 280, 9802–9812.
8 Dreyfus, P. M. (1976) Thiamine and the nervous system: an overview. J. Nutr. Sci. Vitaminol. 22, 13–16
9 Watanabe, L. (1978) Pyrithiamine-induced acute thiamine-deficient encephalopathy in the mouse. Exp. Mol. Pathol. 28, 381–394.
10 Hakim, A. M. (1984) The induction and reversibility of cerebral acidosis in thiamine deficiency. Ann. Neurol. 16, 673–679.
11 Hazell, A. S., Butterworth, R. F. and Hakim, A. M. (1993) Cerebral vulnerability is associated with selective increase in extracellular glutamate concentration in experimental thiamine deficiency. J. Neurochem. 61, 1155–1158.
12 Matsushima, K., MacManus, J. P. and Hakim, A. M. (1997) Apoptosis is restricted to the thalamus in thiamine-deficient rats. Neuroreport 8, 867–870.
13 Todd, K. G. and Butterworth, R. F. (1999) Early microglial response in experimental thiamine deficiency: an immunohistochemical analysis. Glia 25, 190–198.
14 Calingasan, N. Y., Huang, P. L., Chun, H. S., Fabian, A. and Gibson, G. E. (2000) Vascular factors are critical in selective neuronal loss in an animal model of impaired oxidative metabolism. J. Neuropathol. Exp. Neurol. 59, 207–217.
15 Wade, A. E., Wu, B. C., Holbrook, C. M. and Caster, W. O. (1973) Effects of thiamin antagonists on drug hydroxylation and properties of cytochrome P450 in the rat. Biochem. Pharmacol. 22, 1573–1580.
16 Rindi, G., De, Guiseppe, L. and Ventura, U. (1963) Distribution and phosphorylation of oxythiamine in rat tissues. J. Nutr. 81, 147–154.
17 Hobara, R., Okazaki, M., Yasuhara, H. and Sakomoto, K. (1981) Effect of oxythiamine and pyrithiamine on thiamine levels in the blood, liver and urine of rats. Folia Pharmacol. Japon. 78, 529–538.
18 Diaz, G. A., Banikazemi, M., Oishi, K., Desnick, R. J. and Gelb, B. D. (1999) Mutations in a new gene encoding a thiamine transporter cause thiamine-responsive megaloblastic anaemia syndrome. Nat. Genet. 22, 309–312.
19 Fleming, J. C., Tartaglini, E., Steinkamp, M. P., Schorderet, D. F., Cohen, N. and Neufeld, E. J. (1999) The gene mutated in thiamine-responsive anaemia with diabetes and deafness (TRMA) encodes a functional thiamine transporter. Nat. Genet. 22, 305–308.
20 Labay, V., Raz, T., Baron, D., Mandel, H., Williams, H., Barrett, T., Szargel, R., McDonald, L., Shalata, A., Nosaka, K., Gregory, S. and Cohen, N. (1999) Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. Nat. Genet. 22, 300–304.
21 Ganapathy, V., Smith, S. B. and Prasad, P. D. (2004) SLC19: the folate/thiamine transporter family. Pflügers, Arch. 447, 641–646.
22 Dixon, K. H., Lampher, B. C., Chiu, J., Kelley, K. and Cowan, K. H. (1994) A novel cDNA restores reduced folate carrier ac-

tivity and methotrexate sensitivity to transport deficient cells. J. Biol. Chem. 269, 17–20.
23 Moscow, J. A., Gong, M., He, R., Sgagias, M. K., Dixon, K. H., Anzick, S. L., Meltzer, P. S. and Cowan, K. H. (1995) Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. Cancer Res. 55, 3790–3794.
24 Eudy, J. D., Spiegelstein, O., Barber, R. C., Wlodarczyk, B. J., Talbot, J. and Finnell, R. H. (2000) Identification and characterization of the human and mouse SLC19A3 gene: a novel member of the reduced folate family of micronutrient transporter genes. Mol. Genet. Metab. 71, 581–590.
25 Rajgopal, A., Edmondnson, A., Goldman, I. D. and Zhao, R. (2001) SLC19A3 encodes a second thiamine transporter ThTr2. Biochim. Biophys. Acta 1537, 175–178.
26 Croes, K., Casteels, M., Dieuaide-Noubhani, M., Mannaerts, G. P. and Van Veldhoven, P. P. (1999) Stereochemistry of the alpha-oxidation of 3-methyl-branched fatty acids in rat liver. J. Lipid Res. 40, 601–609.
27 Okazaki, M., Sakamoto, H., Ohtsuki, A. and Oguchi, K. (1990) Changes in brain lipid composition in thiamine deficient rats. Jpn. J. Pharmacol. 54, 171–178.
28 Baes, M., Huyghe, S., Carmeliet, P., Declercq, P. E., Collen, D., Mannaerts, G. P. and Van Veldhoven, P. P. (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.
29 Mannaerts, G. P., Debeer, L. J., Thomas, J. and De, Schepper, P. J. (1979) Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. J. Biol. Chem. 254, 4585–4595.
30 Van Veldhoven, P. P., Huang, S., Eyssen, H. J. and Mannaerts, G. P. (1993) The deficient degradation of synthetic 2- and 3-methyl-branched fatty acids in fibroblasts from patients with peroxisomal disorders. J. Inherit. Metab. Dis. 16, 381–391.
31 Casteels, M., Croes, K., Van Veldhoven, P. P. and Mannaerts, G. P. (1994) Aminotrioazole is a potent inhibitor of alpha-oxidation of 3-methyl-substituted fatty acids in rat liver. Biochem. Pharmacol. 48, 1973–1975.
32 Croes, K., Van Veldhoven, P. P., Mannaerts, G. P. and Casteels, M. (1997) Production of formyl-CoA during peroxisomal alpha-oxidation of 3-methyl-branched fatty acids. FEBS Lett. 407, 197–200.
33 Robinson, B. H., Taylor, J. and Sherwood, W. G. (1980) The genetic heterogeneity of lactic acidosis: occurrence of recognizable inborn errors of metabolism in pediatric population with lactic acidosis. Pediatr. Res. 14, 956–962.
34 Batifoulier, F., Verny, M. A., Besson, C., Demigne, C. and Remesy, C. (2005) Determination of thiamine and its phosphate esters in rat tissues analyzed as thiochromes on a RP-amide C16 column. J. Chromatogr. B 816, 67–72.
35 Bontemps, J., Philippe, P., Bettendorff, L., Lombet, J., Dandrifosse, G., Schoffeniels, E. and Crommen, J. (1984) Determination of thiamine and thiamine phosphates in excitable tissues as thiochrome derivatives by reversed-phase high-performance liquid chromatography on octadecyl silica. J. Chromatogr. 307, 283–294.
36 Szutowics, A., Tomaszewicz, M., Bielarczyk, H. and Jankowska, A. (1998) Putative significance of shifts in acetyl-CoA compartimentalization in nerve terminals for disturbances of cholinergic transmission in brain. Dev. Neurosci. 20, 485–492.
37 Miller, B., Anderson, C. E., Vennart, G. P., Williams, J. N. Jr. and Piantadosi, C. (1965) Effect of thiamine deficiency and thiamine repletion on neutral glyceride, total and free cholesterol, phospholipids and plasmalogens in rat liver. J. Nutr. 85, 21–28.
38 Pekovich, S. R., Poggi, V., Martin, P. R. and Singleton, C. K. (1998) Sensitivity to thiamine deficiency in cultured human cells is dependent on cell type and is enhanced in cells from thiamine-responsive megaloblastic anemia patients, J. Nutr. Biochem. 9, 215–222.
39 Pekovich, S. R., Martin, P. R. and Singleton, C. K. (1998) Thiamine deficiency decreases steady-state transketolase and pyruvate dehydrogenase but not alpha-ketoglutarate dehydrogenase mRNA levels in three human cell types. J. Nutr. 128, 683–687.
40 Hohmann, S. and Meacock, P. A. (1998) Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. Biochim. Biophys. Acta 1385, 201–219.
41 Schwartz, J. P. and McCandless, D. W. (1976) Glycolytic metabolism in cultured cells of the nervous system. IV. The effects of thiamine deficiency on thiamine levels, metabolites and thiamine-dependent enzymes on the C-6 glioma and C-1300 neuroblastoma cell lines. Mol. Cell. Biochem. 13, 49–53.
42 Lavoie, J. and Butterworth, R. F. (1995) Reduced activities of thiamine-dependent enzymes in brains of alcoholics in the absence of Wernicke's encephalopathy. Alcohol. Clin. Exp. Res. 19, 1073–1077.
43 Giguere, J. F. and Butterworth, R. F. (1987) Activities of thiamine-dependent enzymes in two experimental models of thiamine deficiency encephalopathy: 3. Transketolase. Neurochem. Res. 12, 305–310.
44 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.
45 Kitareewan, S., Burka, L. T., Tomer, K. B., Parker, C. E., Deterding, L. J., Stevens, R. D., Forman, B. M., Mais, D. E., Heyman, R. A., McMorris, T. and Weinberger, C. (1996) Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. Mol. Biol. Cell 7, 1153–1166.
46 Lemotte, P. K., Keidel, S. and Apfel, C. M. (1996) Phytanic acid is a retinoid X receptor ligand. Eur. J. Biochem. 236, 328–333.
47 Zomer, A. W., van Der Burg, B., Jansen, G. A., Wanders, R. J., Poll-The B. T. and van Der Saag, P. T. (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. J. Lipid, Res. 41, 1801–1807.
48 Schluter, A., Barbera, M. J., Iglesias, R., Giralt, M. and Villarroya, F. (2002) Phytanic acid, a novel activator of uncoupling protein-1 gene transcription and brown adipocyte differentiation. Biochem. J. 362, 61–69.
49 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.
50 Schluter, A., Yubero, P., Iglesias, R., Giralt, M. and Villarroya, F. (2002) The chlorophyll-derived metabolite phytanic acid induces white adipocyte differentiation. Int. J. Obes. Relat. Metab. Disord. 26, 1277–1280.
51 Idel, S., Ellinghaus, P., Wolfrum, C., Nofer, J. R., 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.
52 Bernstein, P. S., Lloyd, M. B., O'Bay, W. T. and Bok, D. (1992) Effect of phytanic acid on cultured retinal pigment epithelium: an *in vitro* model for Refsum's disease. Exp. Eye Res. 55, 869–878.
53 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.
54 Reiser, G., Schonfeld, P. and Kahlert, S. (2006) Mechanism of toxicity of the branched-chain fatty acid phytanic acid, a marker of Refsum disease, in astrocytes involves mitochondrial impairment. Int. J. Dev. Neurosci. 24, 113–122.

55 Avigan, J. (1966) The presence of phytanic acid in normal human and animal plasma. Biochim. Biophys. Acta 116, 391–394.
56 Stead, D. and Welch, V. A. (1975) Lipid composition of bovine serum lipoproteins. J. Dairy Sci. 58, 122–127.
57 Zomer, A. W. M., Jansen, G. A., van der, Burg, B., Verhoeven, N. M., Jakobs, C., van der Saag, P. T., Wanders, R. J. and Poll-The B. T. (2000) Phytanoyl-CoA hydroxylase activity is induced by phytanic acid. Eur. J. Biochem. 267, 4063–4067.
58 Stagg, A. R., Fleming, J. C., Baker, M. A., Sakamoto, M., Cohen, N. and Neufeld, E. J. (1999) Defective high-affinity thiamine transporter leads to cell death in thiamine-responsive megaloblastic anemia syndrome fibroblasts. J. Clin. Invest. 103, 723–729.
59 Boros, L. G., Steinkamp, M. P., Fleming, J. C., Lee, W. N., Cascante, M. and Neufeld, E. J. (2003) Defective RNA ribose synthesis in fibroblasts from patients with thiamine-responsive megaloblastic anemia (TRMA). Blood 102, 3556–3561.