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

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**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.**—Gloerich, J., 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, and S. Ferdinandusse. **A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARα-dependent and -independent pathways. *J. Lipid Res.* 2005.** 46: **716–726.**

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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

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Abbreviations: AMACR, α-methylacyl-CoA racemase; BB, γ-butyrobetaine; BBD, γ-butyrobetaine dioxygenase; BCOX, branched-chain acyl-CoA oxidase; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT2, carnitine palmitoyltransferase 2; CYP4A1, cytochrome P450 hydroxylase 4A1; DBP, D-bifunctional protein; Elovl, long-chain fatty acid elongase; ESI, electrospray ionization; LBP, L-bifunctional protein; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, mitochondrial trifunctional protein; PhyH, phytanoyl-CoA hydroxylase; PMP70, peroxisomal membrane protein 70; PPAR, peroxisome proliferator-activated 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; THIO, peroxisomal 3-ketoacyl-CoA thiolase; TMABADH, trimethylaminobutyraldehyde dehydrogenase; TML, trimethyllysine; VLCAD, very long-chain acyl-CoA dehydrogenase; VLCFA, very long-chain fatty acid.

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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).

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).

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.

### 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*-butyldimethylsilyl)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.

### 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′-CACCTTCCTTCATGTCTATCAC-3′; reverse, 5′-GAACAGGATGACCAGCGTCAT-3′. For Elovl3: forward, 5′-CAACAGTGATGTTTACAGTGGGC-3′; reverse, 5′-CATCTGCAGAATCTGCAGGCTG-3′. For Elovl4: forward, 5′-CAACCAAGTCTCCTTCCTTCAC-3′; reverse, 5′-GACAGTGCTGTGTGTCCGATG-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 will be described elsewhere (N. Van Vlies, R. J. A. Wanders, and F. M. Vaz, unpublished data).

### 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.

### 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 ac-

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[a] | ≤0.01 | 0.48 ± 0.13[a] |
| 2 weeks | ≤0.01 | 0.72 ± 0.24[a] | ≤0.01 | 0.89 ± 0.07[a] |
| 4 weeks | ≤0.01 | 0.75 ± 0.07[a] | ≤0.01 | 1.65 ± 0.28[a,b] |
| 8 weeks | ≤0.01 | 0.74 ± 0.41[a] | ≤0.01 | 1.71 ± 0.25[a] |
| Phytenic acid | | | | |
| 1 week | ≤0.001 | 1.1 ± 0.1[a] | ≤0.001 | 0.8 ± 0.1[a] |
| 2 weeks | ≤0.001 | 1.6 ± 0.1[a] | ≤0.001 | 0.7 ± 0.1[a,b] |
| 4 weeks | ≤0.001 | 2.4 ± 0.1[a] | ≤0.001 | 1.0 ± 0.04[a,b] |
| 8 weeks | ≤0.001 | 3.6 ± 0.4[a] | ≤0.001 | 1.3 ± 0.6[b] |
| Phytanic acid | | | | |
| 1 week | ≤0.1 | 2.3 ± 0.2[a] | ≤0.1 | 1.7 ± 0.1[a,b] |
| 2 weeks | ≤0.1 | 4.0 ± 0.4[a] | ≤0.1 | 1.6 ± 0.1[a,b] |
| 4 weeks | ≤0.1 | 8.4 ± 0.4[a] | ≤0.1 | 2.1 ± 0.3[a,b] |
| 8 weeks | ≤0.1 | 17.7 ± 1.4[a] | ≤0.1 | 2.7 ± 1.0[a,b] |
| Pristanic acid | | | | |
| 1 week | ≤0.01 | 0.4 ± 0.01[a] | ≤0.01 | 0.3 ± 0.02[a,b] |
| 2 weeks | ≤0.01 | 0.8 ± 0.1[a] | ≤0.01 | 0.3 ± 0.04[a,b] |
| 4 weeks | ≤0.01 | 1.8 ± 0.1[a] | ≤0.01 | 0.3 ± 0.02[a,b] |
| 8 weeks | ≤0.01 | 3.8 ± 0.2[a] | ≤0.01 | 0.5 ± 0.2[a,b] |

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.
[a] $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype.
[b] $P < 0.01$, PPARα$^{-/-}$ versus wild-type mice on the same diet.

ids 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 lev-

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[a] | 0.007 ± 0.007 | 1.4 ± 0.5[a,b] |
| Phytanic acid | 0.8 ± 0.1 | 48.7 ± 5.8[a] | 1.2 ± 0.2[b] | 57.3 ± 7.7[a,b] |
| Pristanic acid | 0.07 ± 0.02 | 3.7 ± 0.6[a] | 0.08 ± 0.02 | 4.9 ± 1.1[a,b] |
| C14:0 | 20.8 ± 6.6 | 15.6 ± 3.3 | 25.3 ± 8.1 | 39.1 ± 7.8[a,b] |
| C16:0 | 1,315 ± 209 | 1,202 ± 196 | 1,439 ± 273 | 1,711 ± 279[b] |
| C18:0 | 663 ± 84 | 530 ± 49[a] | 909 ± 144[b] | 906 ± 140[b] |
| C20:0 | 19.1 ± 3.4 | 16.7 ± 1.9 | 30.2 ± 5.7[b] | 33.6 ± 7.6[b] |
| C22:0 | 21.9 ± 3.0 | 16.9 ± 1.4[a] | 27.0 ± 4.3[b] | 24.3 ± 3.2[b] |
| C24:0 | 8.8 ± 1.1 | 8.3 ± 0.9 | 9.9 ± 1.2 | 11.5 ± 1.6[b] |
| C26:0 | 0.03 ± 0.04 | 0.20 ± 0.10[a] | 0.07 ± 0.05 | 0.34 ± 0.18[a] |
| C18:3ω3 | 123 ± 33 | 93 ± 24 | 194 ± 57[b] | 254 ± 65[b] |
| C22:6ω3 | 249 ± 40 | 177 ± 23[a] | 255 ± 46 | 273 ± 46[b] |
| C18:2ω6 | 2,146 ± 319 | 1,751 ± 215[a] | 2,650 ± 478[b] | 2,871 ± 432[b] |
| C20:4ω6 | 631 ± 87 | 455 ± 50[a] | 620 ± 102 | 542 ± 108 |
| C22:5ω6 | 4.7 ± 1.2 | 4.8 ± 1.1 | 5.8 ± 1.5 | 8.6 ± 2.9[b] |
| C18:1ω9 | 742 ± 160 | 793 ± 175 | 775 ± 232 | 1,073 ± 256[a,b] |
| C28:0[c] | 1.0 ± 0.41 | 0.77 ± 0.38 | 1.35 ± 0.49 | 2.09 ± 0.65[a,b] |
| C30:0[c] | 1.0 ± 0.42 | 0.82 ± 0.33 | 1.29 ± 0.45 | 2.48 ± 0.82[a,b] |
| C32:0[c] | 1.0 ± 0.39 | 0.82 ± 0.38 | 1.41 ± 0.54 | 1.96 ± 0.48[b] |

Concentrations of fatty acids are expressed in μmol/l. Values represent means ± SD. n = number of animals.
[a] $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype.
[b] $P < 0.01$, PPARα$^{-/-}$ versus wild-type mice on the same diet.
[c] The amount of fatty acid present in plasma of wild-type mice on a control diet was set to 1.0.

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[a] | 19.4 ± 2.8[a] |
| Acetylcarnitine | 12.3 ± 2.3 | 12.9 ± 2.5 | 8.1 ± 1.9[a] | 7.6 ± 1.8[a] |
| Propionylcarnitine | 0.53 ± 0.13 | 0.84 ± 0.14[b] | 0.40 ± 0.11 | 0.57 ± 0.10[a,b] |
| Palmitoylcarnitine | 0.12 ± 0.04 | 0.12 ± 0.04 | 0.18 ± 0.06 | 0.19 ± 0.07[a] |
| Oleoylcarnitine | 0.09 ± 0.03 | 0.07 ± 0.02 | 0.11 ± 0.03 | 0.10 ± 0.04 |
| Liver | | | | |
| Free carnitine | 296 ± 41 | 376 ± 65[b] | 175 ± 33[a] | 185 ± 26[a] |
| Propionylcarnitine | 2.2 ± 1.3 | 1.7 ± 0.9 | 1.3 ± 0.6 | 0.8 ± 0.5[a] |

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

els 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.

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, TMA-BADH 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 (**Fig. 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

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[a] | 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 | 1,471 ± 270 | 1,364 ± 206 | 1,153 ± 189[b] | 1,159 ± 183 |
| γ-Butyrobetaine dioxygenase | 134 ± 23 | 135 ± 15 | 112 ± 18 | 115 ± 17[b] |

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.
[a] $P < 0.01$, phytol-enriched diet versus control diet in mice of the same genotype.
[b] $P < 0.01$, PPARα$^{-/-}$ versus wild-type mice on the same diet.

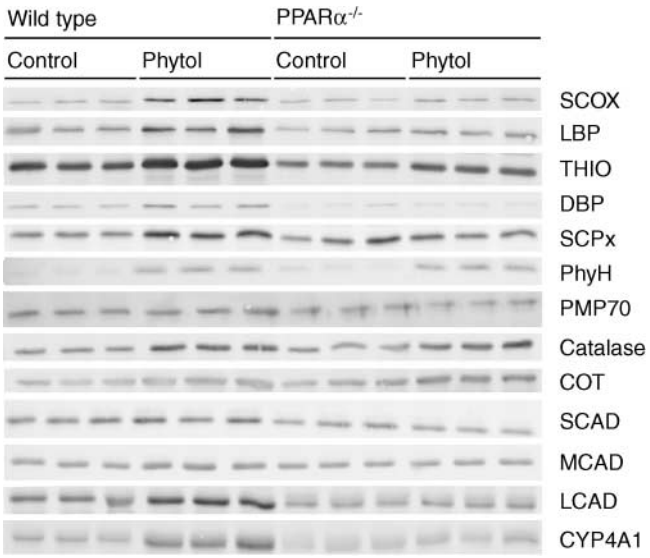


**Fig. 1.** Immunoblot analysis of different enzymes involved in fatty acid metabolism in liver of wild-type and peroxisome proliferator-activated receptor α-deficient (PPARα$^{-/-}$) mice on a control diet or a diet enriched with phytol. Results from three mice per group are shown. Antibodies against 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.

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 (**Fig. 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 (Fig. 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-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.

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[a] | 0.65 ± 0.34 | 1.73 ± 0.54[a,b] |
| D-Bifunctional protein | 1.0 ± 0.38 | 2.41 ± 0.57[a] | 0.42 ± 0.16 | 0.54 ± 0.20[b] |
| L-Bifunctional protein | 1.0 ± 0.25 | 2.45 ± 0.84[a] | 0.66 ± 0.25[b] | 0.86 ± 0.44[b] |
| Peroxisomal 3-ketoacyl-CoA thiolase | 1.0 ± 0.12 | 1.68 ± 0.22[a] | 0.55 ± 0.10[b] | 0.74 ± 0.11[a,b] |
| Sterol carrier protein x | 1.0 ± 0.34 | 1.91 ± 0.54[a] | 1.53 ± 0.59 | 1.41 ± 0.43[b] |
| Catalase | 1.0 ± 0.35 | 1.77 ± 0.62[a] | 0.94 ± 0.32 | 1.49 ± 0.43[a] |
| 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[a] | 1.22 ± 0.33 | 2.40 ± 0.77[a] |
| Carnitine octanoyltransferase | 1.0 ± 0.35 | 1.64 ± 0.41[a] | 1.12 ± 0.30 | 1.47 ± 0.25[a] |

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.

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

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

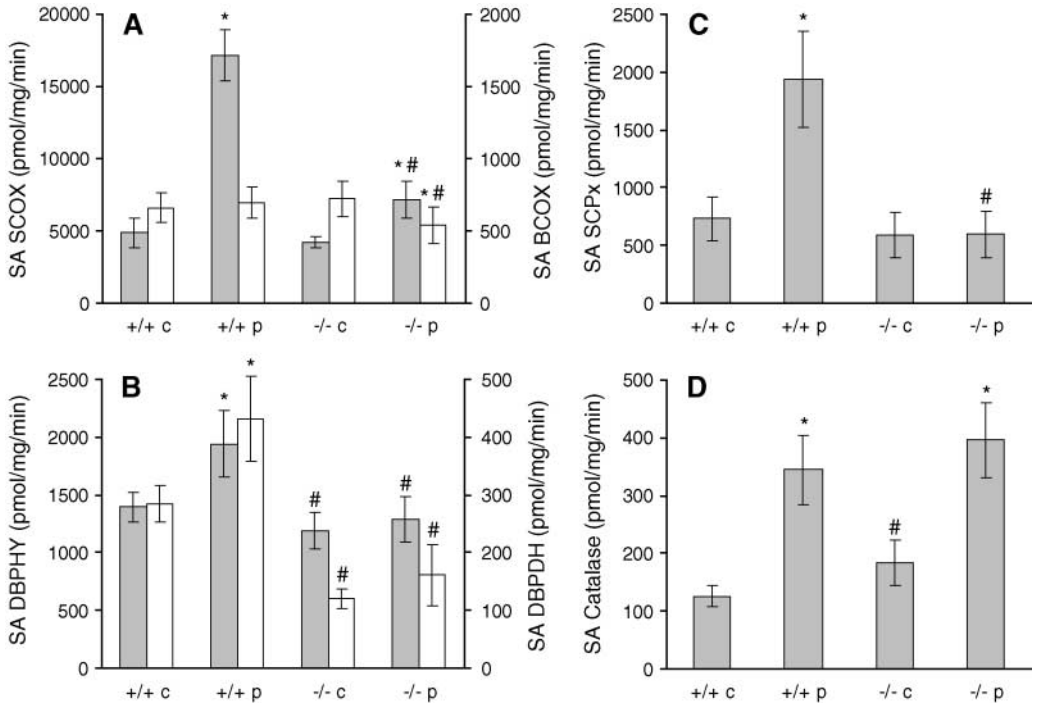


**Fig. 2.** Enzyme activity measurements performed in liver homogenates of wild-type and PPARα$^{-/-}$ mice on a control diet (c) or a phytol-enriched diet (p). Specific activity (SA) of A: SCOX (gray bars) and branched-chain acyl-CoA oxidase (BCOX; white bars). B: DBP enoyl-CoA hydratase (DBPHY; gray bars) and DBP 3-keto-acyl-CoA dehydrogenase (DBPDH; 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.

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 (**Fig. 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-

TABLE 6. Immunoblot quantification of mitochondrial β-oxidation enzymes and cytochrome P450 hydroxylase 4A1 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) |
|---|---|---|---|---|
| Short-chain acyl-CoA dehydrogenase | 1.0 ± 0.28 | 0.98 ± 0.15 | 0.75 ± 0.13[a] | 0.58 ± 0.14[a] |
| Medium-chain acyl-CoA dehydrogenase | 1.0 ± 0.18 | 1.31 ± 0.32 | 0.93 ± 0.27 | 0.83 ± 0.19[a] |
| Mitochondrial trifunctional protein α subunit | 1.0 ± 0.53 | 1.09 ± 0.60 | 0.55 ± 0.47 | 0.53 ± 0.53 |
| Short-chain 3-hydroxyacyl-CoA dehydrogenase | 1.0 ± 0.22 | 1.11 ± 0.27 | 1.17 ± 0.18 | 1.02 ± 0.22 |
| Short branched-chain 3-hydroxyacyl-CoA dehydrogenase | 1.0 ± 0.28 | 1.11 ± 0.28 | 0.99 ± 0.21 | 0.81 ± 0.14[a] |
| Long-chain acyl-CoA dehydrogenase | 1.0 ± 0.16 | 1.22 ± 0.24 | 0.69 ± 0.14[a] | 0.55 ± 0.14[a] |
| Cytochrome P450 hydroxylase 4A1 | 1.0 ± 0.21 | 1.88 ± 0.34[b] | 0.58 ± 0.24 | 0.88 ± 0.38[a] |

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.

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

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

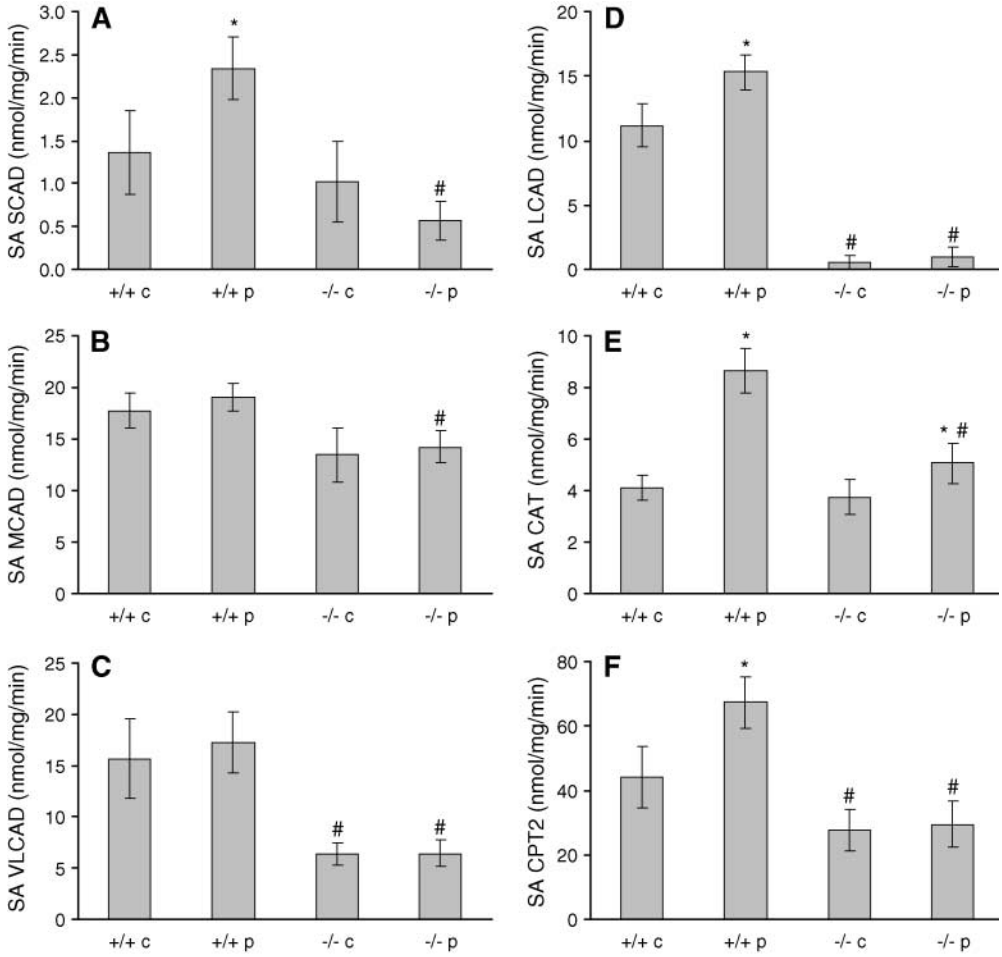


**Fig. 3.** Enzyme activity measurements performed in liver homogenates of wild-type and PPARα$^{-/-}$ mice on a control diet (c) or a phytol-enriched diet (p). Specific activity (SA) of A: SCAD. B: MCAD. C: Very long-chain acyl-CoA dehydrogenase (VLCAD). D: LCAD. E: Carnitine acetyltransferase (CAT). 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.

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 (45). 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 (46), 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 (47).

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, 48). 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 (49). In support of this, clofibrate indeed is known to induce protein breakdown in skeletal muscle (50). 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.

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