Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor α

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Abstract **Phytanic acid and pristanic acid are branched-chain fatty acids, present at micromolar concentrations in the plasma of healthy individuals. Here we show that both phytanic acid and pristanic acid activate the peroxisome proliferator-activated receptor α (PPARα) in a concentration-dependent manner. Activation is observed via the ligand-binding domain of PPARα as well as via a PPAR response element (PPRE). Via the PPRE significant induction is found with both phytanic acid and pristanic acid at concentrations of 3 and 1 μM, respectively. The *trans*-activation of PPARδ and PPARγ by these two ligands is negligible. Besides PPARα, phytanic acid also *trans*-activates all three retinoic X receptor subtypes in a concentration-dependent manner. In primary human fibroblasts, deficient in phytanic acid α-oxidation, *trans*-activation through PPARα by phytanic acid is observed. This clearly demonstrates that phytanic acid itself, and not only its metabolite, pristanic acid, is a true physiological ligand for PPARα. Because induction of PPARα occurs at ligand concentrations comparable to the levels found for phytanic acid and pristanic acid in human plasma, these fatty acids should be seen as naturally occurring ligands for PPARα. These results demonstrate that both pristanic acid and phytanic acid are naturally occurring ligands for PPARα, which are present at physiological concentrations.**—Zomer, A. W. M., B. van der Burg, G. A. Jansen, R. J. A. Wanders, B. T. Poll-The, and P. T. van der Saag. **Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor α.** ***J. Lipid Res.*** **2000.** 41: **1801–1807.**

Supplementary key words natural ligand • peroxisomal disorder • retinoic X receptor • phytol derivatives

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is an isoprenoid-derived 3-methyl fatty acid, which is derived solely from external sources. This branched-chain fatty acid originates from the phytol side chain of chlorophyll (**Fig. 1**). Microorganisms, which are present in the rumen of ruminants, are thought to release phytol from chlorophyll, after which phytol is converted into phytanic acid (1). Because humans are not capable of phytol release from chlorophyll (2), all phytanic acid enters the human body via the diet. In particular, ruminant fats, fish, and dairy products are rich sources of phytanic acid. Phytanic acid is metabolized into pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) in a process called α-oxidation. Pristanic acid, like phytanic acid, is present in the lipids from many sources (3). Peroxisomes play an indispensable role in both phytanic acid α-oxidation and pristanic acid β-oxidation (4, 5). Although the subcellular localization of the enzymes involved in phytanic acid degradation has not been resolved definitively, phytanoyl-CoA hydroxylase (PhyH), the first enzyme in the phytanic acid α-oxidation pathway, is strictly peroxisomal in both rats (6–8) and humans (9). With respect to pristanic acid β-oxidation, studies have shown that pristanic acid undergoes three cycles of β-oxidation in the peroxisome, followed by further β-oxidation in the mitochondrion (10).

Phytanic acid and pristanic acid accumulate in a variety of inherited disorders. First, in patients with a defect in peroxisome biogenesis, both metabolites are elevated in plasma and tissues because of a deficient oxidation of both phytanic acid and pristanic acid. Second, phytanic acid and pristanic acid accumulate in patients with D-bifunctional protein deficiency. In these patients pristanic acid β-oxidation is deficient (11). Despite the fact

Abbreviations: EC_{50}, median effective concentration; FCS, fetal calf serum; LBD, ligand-binding domain; PhyH, phytanoyl-CoA hydroxylase; PPAR, peroxisome proliferator-activated receptor; PTS2, peroxisome-targeting signal type 2; PPRE, PPAR response element; RXR, retinoid X receptor.

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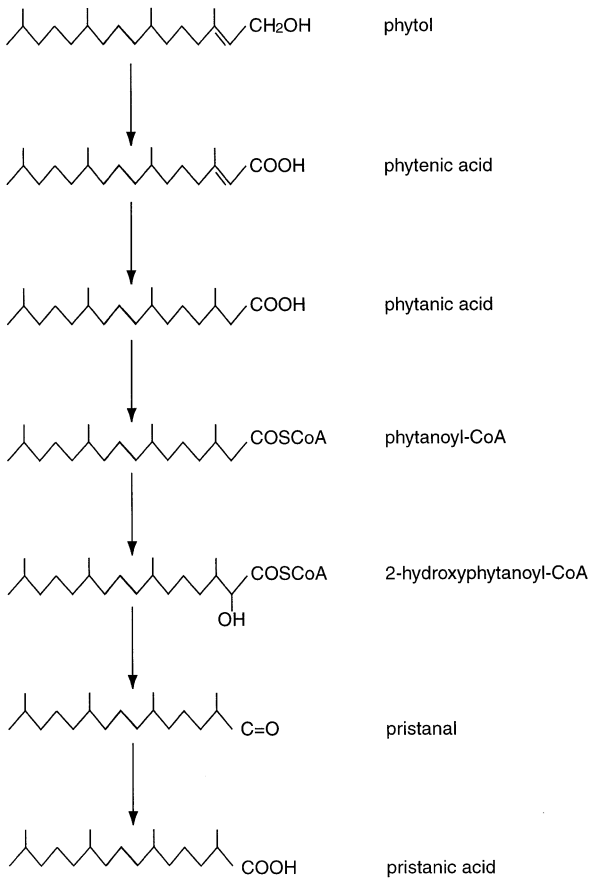

Fig. 1. Metabolic pathway from phytol to pristanic acid.

that α-oxidation can proceed normally, phytanic acid accumulates, probably as a result of feedback inhibition of the α-oxidation pathway by high levels of pristanic acid caused by deficient pristanic acid β-oxidation. In two other peroxisomal disorders phytanic acid accumulates whereas pristanic acid levels are normal. These two disorders are Refsum disease, in which PhyH is deficient because of mutations in the *PHYH* gene (12, 13), and rhizomelic chondrodysplasia punctata type 1. In the latter disease, the import of a distinct set of peroxisomal proteins equipped with a peroxisome-targeting signal type 2 (PTS2) is impaired because of mutations in the gene encoding the PTS2 receptor. As a consequence, all PTS2-containing proteins cannot be imported, including PhyH, resulting in a deficient α-oxidation (4). In the plasma of healthy individuals phytanic acid and pristanic acid are present at micromolar concentrations (<10 and <3 μM, respectively) (14). In patients the plasma concentration of phytanic acid and pristanic acid can rise, depending on the disorder, to 1,300 and 80 μM, respectively (5).

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors belonging to the family of nuclear hormone receptors. So far, three subtypes have been identified (α, δ/β, and γ) from humans, rodents, and *Xenopus* (15). The transcriptional regulation by PPARs is achieved through PPAR-retinoid X receptor (RXR) heterodimers, which bind to PPAR response elements (PPREs) in the promoter of target genes (16, 17). RXRs consist of RXRα, RXRβ, and RXRγ (18), which are activated by 9-*cis*-retinoic acid. Besides heterodimerization with various members of the nuclear hormone receptor family such as PPAR and retinoic acid receptor, RXRs can also homodimerize and regulate *trans*-activation of target genes.

In general, PPARs are activated by a variety of natural and synthetic ligands such as fatty acids (e.g., arachidonic acid), eicosanoids [e.g., 15-deoxy-prostaglandin J_2 and leukotriene B_4 (LTB_4)], plasticizers [e.g., di(2-ethylhexyl)phthalate], and hypolipidemic agents (e.g., clofibrate) (19–23). There are differences in the ability of the different compounds to activate the various receptor subtypes. In line with the fact that the major function of PPARα is the regulation of genes involved in lipid homeostasis (24, 25), fatty acids have been identified as PPARα-specific ligands. On the basis of this functional specificity we have searched for novel PPARα-specific ligands among naturally occurring fatty acids. We report the effect of different phytol-derived branched-chain fatty acids on transcriptional activation by RXRβ and PPARα. We then investigated the concentration-dependent *trans*-activation by two of these phytol derivatives, which were found to be ligands. Finally, we investigated the transcriptional activation by phytanic acid of PPARα in phytanic acid α-oxidation-deficient cell lines.

MATERIALS AND METHODS

Materials

Phytanic acid and pristanic acid were synthesized as described previously (26). Phytol and β-cyclodextrin were from Sigma (St. Louis, MO). 4,8,12-Trimethyltridecanoic acid, 4,8-dimethylnonanoic acid, and 2,6-dimethylheptanoic acid were a generous gift from G. Dacremont (Ghent, Belgium). SuperFect was from Qiagen (Hilden, Germany). The Luclite luciferase gene reporter assay kit and Topcount liquid scintillation counter were from Packard Instruments (Meriden, CT).

Expression and reporter plasmids

pSG5-RXRβ was a gift from P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France, Strasbourg, France). GAL4-mRXRα-LBD, GAL4-mRXRβ-LBD, and GAL4-mRXRγ-LBD have been described previously (27). pBK-CMV-Gal4-rPPARα-LBD, pBK-CMV-Gal4-rPPARδ-LBD, and pBK-CMV-Gal4-rPPARγ-LBD were gifts from E. Treuter (Biosciences at Novum, Karolinska Institute, Huddinge, Sweden). 3*PPRE-tk-Luc, pCMX-mPPARα, pCMX-mPPARδ, and pCMX-mPPARγ were gifts from R. M. Evans (Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA).

Cell culture

Monkey COS-1 cells and human HepG2 cells were purchased from the American Type Culture Collection (Rockville, MD). Human primary fibroblasts from a Refsum disease patient, a Zellweger syndrome patient, and a healthy individual (control) were from the Mutant Cell Repository (Laboratory for Genetic Metabolic Diseases, University of Amsterdam, The Netherlands). The diagnoses of Refsum disease and Zellweger syndrome, respectively, were based on clinical symptoms and confirmed by de-

tailed studies of fibroblasts showing an isolated deficiency of PhyH and a complete loss of peroxisomal functions, respectively (14). All cell lines were grown at 37°C in the presence of 7.5% CO_2. COS-1 cells were cultured in DF medium (1:1 mixture of Dulbecco's minimal essential medium and Ham's F12, buffered with 44 mM $NaHCO_3$) supplemented with 7.5% fetal calf serum (FCS). HepG2 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% FCS. The primary fibroblasts were cultured in Ham's F12 buffered with 44 mM $NaHCO_3$ and supplemented with 10% FCS.

Transient transfections

COS-1 and HepG2 cells were transiently transfected in 24-well plates by the calcium phosphate coprecipitation method, using 1.0 μg of reporter construct, 0.25 μg of β-galactosidase expression vector SV2LacZ, 0.125 μg of receptor construct, and pBluescript SK^- to a total of 1.5 μg of DNA per well (28). After 16 h medium was refreshed, and cells were exposed to various additions as indicated. All ligands were solubilized in β-cyclodextrin (10 mg/ml in 100 mM Tris-HCl, pH 8.0). Cells were harvested 24 h later. Primary fibroblasts were transiently transfected in 12-well plates with SuperFect according to the manufacturer instructions by using 0.7 μg of reporter construct, 0.2 μg of SV2LacZ, 0.1 μg of receptor construct, and 5 μl of SuperFect per well. Medium was refreshed after 3 h and after 16 h, when ligands were added as indicated. Cells were harvested 24 h later. Cell extracts were analyzed for luciferase activity by using the Luclite luciferase gene reporter assay kit and measured in a Topcount liquid scintillation counter. Values were corrected for β-galactosidase activity as a measure of transfection efficiency (29).

RESULTS

Phytol derivatives differentially stimulate RXR and/or PPARα

To examine whether different phytol-derived branched-chain fatty acids can mediate transcriptional effects through PPARα/RXR, we used a chimeric receptor system in which the respective ligand-binding domains (LBDs) were fused to the DNA-binding domain of the yeast tran-

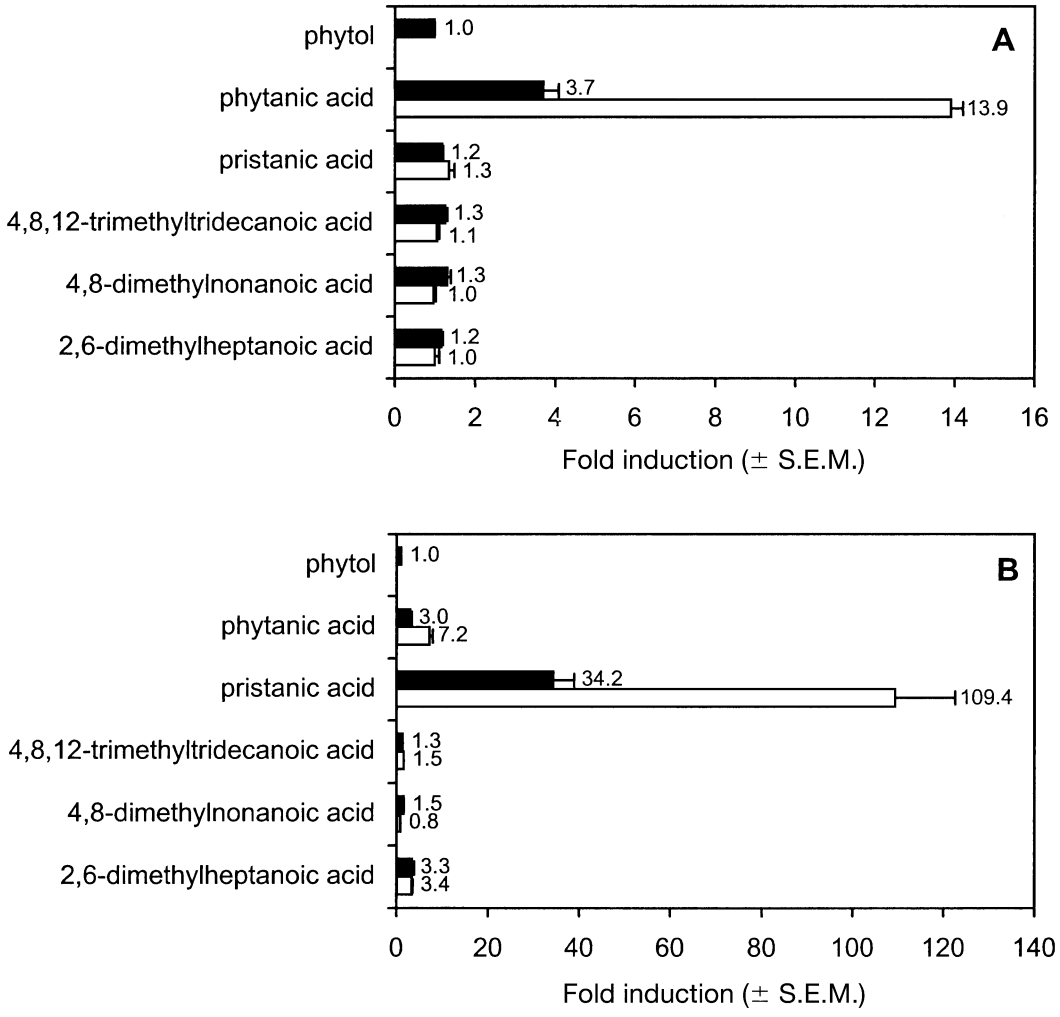

Fig. 2. *trans*-Activation of GAL4-RXRβ and GAL4-PPARα by phytol derivatives. HepG2 and COS-1 cells were transiently transfected with the reporter construct GAL4-tata-Luc, β-galactosidase expression vector SV2LacZ, and an expression vector (see Materials and Methods). The various phytol derivatives were added at a final concentration of 50 μM, from 2 mM stock solutions. (A) Expression vector GAL4-RXRβ. (B) Expression vector GAL4-PPARα. Data are presented as the mean (± SEM) fold induction. COS-1 (open columns); HepG2 (solid columns).

scription factor GAL4. By making use of transient transfections, the various branched-chain fatty acids were tested both in COS-1 and HepG2 cells. From the various phytol derivatives tested, only phytanic acid activated GAL4-RXRβ (**Fig. 2A**). No activation of GAL4-RXRβ was observed with pristanic acid. Previously, the transcriptional activation of full-length RXRα by phytanic acid has been described (30, 31). Two of the tested phytol derivatives were found to have a stimulatory effect on GAL4-PPARα: Phytanic acid and, with 10-fold higher induction levels, pristanic acid (Fig. 2B). The other derivatives, including 4,8,12-trimethyltridecanoic acid, 4,8-dimethylnonanoic acid, and 2,6-dimethylheptanoic acid, which are all intermediates of pristanic acid β-oxidation, did not show significant stimulation, either of GAL4-RXRβ or of GAL4-PPARα (Fig. 2A and B). The effects of phytanic acid and pristanic acid on the other two PPAR subtypes, determined via GAL4-PPARδ and GAL4-PPARγ, were negligible (**Fig. 3**). All three subtypes of RXR were stimulated by phytanic acid in a concentration-dependent manner (**Fig. 4**). The highest activation was seen with GAL4-RXRβ. However, this difference in activation was not specific for phytanic acid because the same difference in activation was seen with 9-*cis*-retinoic acid (data not shown). Moreover, the difference in activation, observed with the RXR subtypes, might be due to a difference in expression levels of these receptors. Further studies are necessary to elucidate the observed activation differences.

Transcriptional activation of GAL4-PPARα with phytanic acid and pristanic acid

Concentration-dependent activation via the LBD of PPARα was seen with phytanic acid (**Fig. 5A**) and pristanic acid (Fig. 5B). The induction with pristanic acid is, depending on the cell line used, 6 to 10 times higher than the induction with phytanic acid, indicating a higher affinity of PPARα for pristanic acid than for phytanic acid. With phytanic acid at 10 μM no induction was detectable in either COS-1 or HepG2 cells, while the same concentration of pristanic acid elicited a 30-fold induction in COS-1 cells (Fig. 5B). In the case of pristanic acid, the median effective concentration (EC_{50}) value for induction was found to be approximately 40 μM. EC_{50} values for induction by phytanic acid could not be determined, because the phytanic acid curve had not reached a plateau at 100 μM. Higher phytanic acid concentrations could not be tested as these concentrations were found to be toxic in both cell lines.

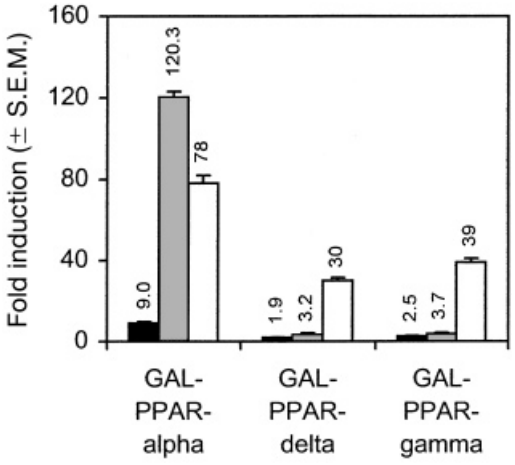

Fig. 3. Activation through the LBD of the PPAR subtypes with phytanic acid and pristanic acid. COS-1 cells were transiently transfected with the reporter construct GAL4-tata-Luc, β-galactosidase expression vector SV2LacZ, and the expression vector GAL4-PPARα, GAL4-PPARδ, or GAL4-PPARγ. Data are presented as means (± SEM) fold induction. Cells were stimulated with 50 μM ligand. Phytanic acid (solid columns); pristanic acid (shaded columns). As a control cells were also grown in the presence of 300 μM mono(2-ethylhexyl) phthalate (open columns).

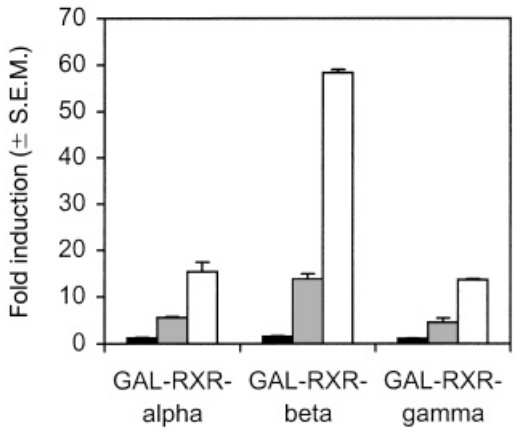

Fig. 4. Concentration-dependent activation through the LBD of RXR subtypes with phytanic acid. COS-1 cells were transiently transfected with the reporter construct GAL4-tata-Luc, β-galactosidase expression vector SV2-LacZ, and the expression vector GAL4-RXRα, GAL4-RXRβ, or GAL4-RXRγ by making use of the calcium phosphate coprecipitation method. Phytanic acid concentrations used: 10 μM (solid columns); 30 μM (shaded columns); 100 μM (open columns).

Transcriptional activation of full-length PPARα with phytanic acid and pristanic acid

So far, the *trans*-activation by phytanic acid and pristanic acid was studied with GAL4-PPARα chimeric constructs on a GAL4 reporter. The next step was to use full-length PPARα in combination with RXRβ and a PPRE. Because no difference in activation was observed between the full-length RXR subtypes (not shown), RXRβ was used in all experiments. The maximal induction found with phytanic acid (**Fig. 6A**) was in the same range as the induction found for pristanic acid (Fig. 6B). This was not only true for PPARα alone, but also for the combination of PPARα and RXRβ. Under these conditions the EC_{50} value for induction by phytanic acid was approximately 20 μM, while for pristanic acid it was about 3 μM. Because the plateau level was not reached completely with phytanic acid, the EC_{50} value for phytanic acid is a minimal estimate. The EC_{50} value for pristanic acid (3 μM) is within the physiological concentration at which this branched-chain fatty acid is present in the plasma of healthy individuals. On the basis of these experiments it is clear that the affinity of

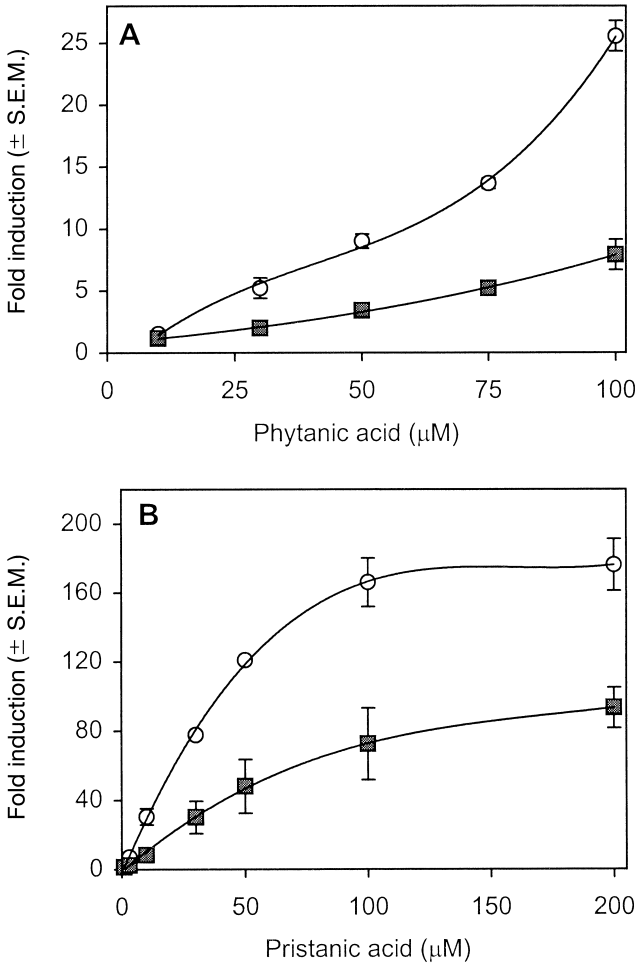

Fig. 5. Activation via the LBD of PPARα with concentration curves of phytanic acid and pristanic acid. COS-1 and HepG2 cells were transiently transfected with the reporter construct GAL4-tata-Luc, β-galactosidase expression vector SV2-LacZ, and the expression vector GAL4-PPARα. (A) Phytanic acid, added as indicated. (B) Pristanic acid, added as indicated. COS-1 (open circles); HepG2 (shaded squares).

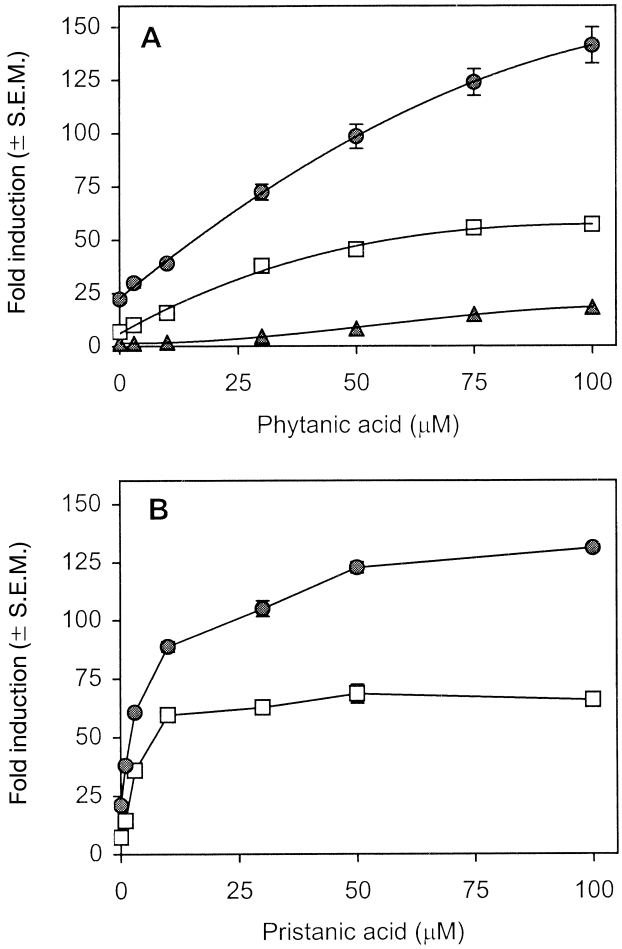

Fig. 6. Concentration-dependent activation of full-length PPARα with phytanic acid and pristanic acid. COS-1 cells were transiently transfected with the reporter construct 3*PPRE-tk-Luc, β-galactosidase expression vector SV2-LacZ, and the indicated expression vector pCMX-PPARα, pSG5-RXRβ, or both (see Materials and Methods). (A) Phytanic acid, added as indicated. (B) Transfections were done as described in Fig. 4A. However, pSG5-RXRβ alone was not tested because pristanic acid is not a ligand for RXRβ. Pristanic acid was added as indicated. RXRβ (shaded triangles); PPARα (open squares); PPARα plus RXRβ (shaded circles).

the full-length receptor is also significantly higher for pristanic acid than for phytanic acid.

Synergistic *trans*-activation with PPARα and RXRβ

The *trans*-activation by 100 μM phytanic acid of RXRβ was found to be 18-fold (Fig. 6), while with the same concentration of pristanic acid no stimulation of RXRβ was observed (Fig. 2A). The activation of PPARα caused by 100 μM phytanic acid or pristanic acid was in both cases approximately 60-fold, while the effect of these ligands on PPARα in combination with RXRβ was about 130-fold (Fig. 6). These results show, both with phytanic acid and pristanic acid, synergistic *trans*-activation of PPARα plus RXRβ when compared with the level caused by the summation of RXRβ and PPARα activities. Because phytanic acid can function as a ligand for RXRβ while pristanic acid cannot, these results also suggest that the synergistic process of *trans*-activation of RXRβ with PPARα is not RXR-ligand dependent.

Phytanic acid *trans*-activates PPARα in α-oxidation-deficient cell lines

Because pristanic acid is derived from phytanic acid after oxidative decarboxylation of phytanic acid, *trans*-activation by phytanic acid of PPARα might not be caused by phytanic acid itself, but by pristanic acid, derived from in vivo metabolized phytanic acid. Therefore human primary fibroblasts from patients with either Zellweger syndrome or Refsum disease, both deficient in phytanic acid α-oxidation, were used to study the *trans*-activation by phytanic acid of the GAL4-PPARα chimeric constructs. In both α-oxidation deficient cell lines a stimulatory effect of phytanic acid could be observed (**Fig. 7**) that was comparable to the effect found in primary fibroblasts from healthy individuals. In α-oxidation deficient cell lines a stimulatory effect of pristanic acid was also observed. These results clearly demonstrate that phytanic acid itself, like pristanic acid, is a ligand for PPARα.

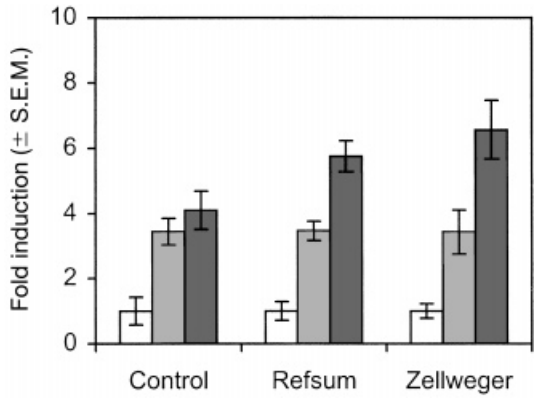

Fig. 7. *trans*-Activation of PPARα in primary human fibroblasts. The GAL-tk-Luc reporter construct was cotransfected with the GAL4-PPARα expression vector and the β-galactosidase expression vector SV2-LacZ by making use of SuperFect (see Materials and Methods). Cells were incubated for 16 h with a 100 μM concentration of the indicated ligand. Luciferase and β-galactosidase expression was measured from cell lysates of treated and untreated cells. Data are presented as the mean (± SEM) fold induction. β-Cyclodextrin (solvent; open columns); phytanic acid (grey columns); pristanic acid (dark columns).

DISCUSSION

PPARα is activated by a variety of natural and synthetic ligands such as various long-chain fatty acids (linoleic acid and arachidonic acid), eicosanoids (leukotriene B_4 and 8(*S*)-hydroxyeicosatetraenoic acid), and hypolipidemic drugs (clofibric acid and Wy14,643). The structural similarities between the different peroxisome proliferators are not stringent. With the exception of an acidic group observed in all of them, their structures vary widely. Because of the involvement of PPARα in lipid homeostasis, we focused our research for two main reasons on the phytol pathway. First, two phytol derivatives, phytanic acid and pristanic acid, are known to accumulate in a variety of inherited disorders and second, various phytol derivatives are fatty acids and thus might be potential ligands for PPARα.

The results described in this article show that the naturally occurring fatty acids phytanic acid and pristanic acid can act as ligands for PPARα. Previous studies by Seedorf et al. (32, 33) have focused on phytanic acid as a ligand for PPARα. These studies were done in mice in which the gene encoding sterol carrier protein 2 was disrupted (33). Interestingly, this gene produces two mRNA transcripts, which correspond to a 58-kDa protein with both thiolase and sterol carrier protein 2 activity and a 13-kDa protein with sterol carrier protein activity only. The 58-kDa thiolase/sterol carrier protein plays a crucial role in the peroxisomal oxidation of 2-methyl fatty acids such as pristanic acid, which explains the deficient oxidation of pristanic acid in these mice (33). Under normal feeding conditions there is no accumulation of pristanic acid and phytanic acid because the standard laboratory diet is low in phytanic acid. Feeding phytol to the mice, however, caused a strong increase in serum levels of phytanic acid and pristanic acid. Furthermore, a variety of PPARα-regulated genes was found to be upregulated, which led to the identification of phytanic acid as a natural ligand for PPARα. However, as we have shown in this article, pristanic acid is a much more powerful ligand for PPARα than phytanic acid, which suggests that the effects observed in the sterol carrier protein-deficient mice on feeding phytol may well be due to pristanic acid rather than phytanic acid. Our findings may also be of importance for other inherited diseases in which the peroxisomal oxidation of pristanic acid and phytanic acid is deficient. In patients with D-bifunctional protein deficiency for instance, oxidation of pristanic acid is blocked at the level of the second and/or third step of β-oxidation (11, 34, 35). However, in such patients the oxidation of other fatty acids, such as $C_{26:0}$, is also deficient, leading to their accumulation and possibly disturbances of additional pathways.

A noticeable difference between the two ligands is that phytanic acid is also a ligand for RXR, albeit at supraphysiological concentrations. These supraphysiological concentrations, however, are readily reached in patients with Refsum disease, in whom phytanic acid plasma concentrations can rise to 1,300 μM (5). Aberrant activation of RXRs could, therefore, also contribute to the pathology of Refsum disease. Because RXRs are the natural partners of PPARα, accumulation of both PPARα/RXR ligands would lead to overactivation of pathways under the control of these nuclear receptors, which could lead to ectopic (over) expression of target genes. However, because RXRs also dimerize with several other different nuclear hormone receptors, other pathways may be stimulated as well.

We show in this article that pristanic acid is a PPARα ligand at physiological concentrations. Because of a deficient α-oxidation of phytanic acid, the plasma concentration of pristanic acid in patients with Refsum disease is lower compared when healthy individuals (5). It could therefore be reasoned that the pristanic acid concentration in patients with Refsum disease becomes too low to be able to *trans*-activate PPARα, which also might contribute to the pathology of this peroxisomal disorder. The identification of pristanic acid as a physiological ligand for PPARα gives us new leads, which may well have important implications for our understanding of the pathogenesis of peroxisomal disorders.

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