

Phytanic acid, but not pristanic acid, mediates the positive effects of phytol derivatives on brown adipocyte differentiation

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Abstract The phytol derivatives phytanic acid and pristanic acid may activate nuclear hormone receptors and influence gene expression and cell differentiation. Phytanic acid induces brown adipocyte differentiation. It was determined that brown fat and brown adipocytes are sites of high gene expression of phytanoyl-CoA hydroxylase, the enzyme required for initiation of peroxisomal α -oxidation of phytanic acid. However, the effects of phytanic acid were not mediated by its α -oxidation product pristanic acid, which did not promote brown adipocyte differentiation or stimulate transcription of the uncoupling protein-1 gene. Moreover, acute cold exposure of mice caused a dramatic mobilization of the phytanic acid stores in brown adipose tissue thus suggesting that a high local exposure to phytanic acid in brown fat may contribute to signalling adaptive changes in the tissue in response to thermogenic activation. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Phytol; Phytanic acid; Brown adipose tissue; Cell differentiation

1. Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a methyl-branched fatty acid that originates from the phytol side of the chain of chlorophyll. Phytanic acid enters the human body in foods such as dairy products or other ruminant fats. Even though humans cannot release phytol from chlorophyll, they can convert it into phytanic acid [1]. The metabolism of phytanic acid begins with activation to phytanoyl CoA followed by peroxisomal α -oxidation. The first enzyme in the α -oxidation pathway of phytanic acid is phytanoyl-CoA hydroxylase (PhyH), deficiency of which leads to Refsum disease, an inherited disorder characterized by the accumulation of phytanic acid [2]. The product of phytanic acid α -oxidation is pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) which can also enter humans or rodents in the diet. Pristanic acid is oxidized by three cycles of peroxisomal β -oxidation, and the products are exported to mitochondria for further

β -oxidation in a transport pathway that involves peroxisomal carnitine octanoyl transferase (COT) [3].

Phytanic acid can bind and activate at least two types of nuclear hormone receptors, retinoid X receptors (RXR) [4,5] and peroxisome proliferator-activated receptor (PPAR) α [6], with affinities lower than other ligands but in the range of the physiological levels of this fatty acid in blood. Pristanic acid can also activate PPAR α [7]. We have recently reported that phytanic acid, when added to cell culture medium, is an activator of brown adipocyte differentiation and transcription of the uncoupling protein-1 (UCP1) gene, the specific marker of brown adipocytes [8]. Therefore, phytanic acid may affect adaptive thermogenesis and energy expenditure. As brown adipose tissue has a high peroxisome content, we hypothesized that phytanic acid metabolism in this cell type is highly regulated and that the action of phytanic acid is exerted, in whole or in part, through a product of its metabolism like pristanic acid. In this study we determined the gene expression of PhyH, the specific enzyme needed for phytanic acid metabolism, and we assessed the relative capacities of phytanic acid and pristanic acid to affect brown adipocyte differentiation. Measurement of phytanic acid in brown fat was performed to gain insight into the potential physiological significance of our findings.

2. Materials and methods

Phytanic acid and nonadecanoic acid were from Sigma (St. Louis, MO, USA). Pristanic acid was a kind gift of Dr. M. Girós (Institut de Bioquímica Clínica, Barcelona, Spain). Tissue culture media, fetal calf serum (FCS) and supplements were from BioWhittaker (Walkersville, MD, USA) and Sigma.

Swiss mice were fed a standard diet (B.K. Universal, Barcelona, Spain) with a phytanic acid content of 0.645 μ g/g. Adult mice (8 weeks old) were kept in standard conditions of light (12-h light/12-h dark) cycle and temperature ($21 \pm 1^\circ\text{C}$). One group of animals was exposed to 4°C for 4 h. Mice were killed by decapitation and interscapular brown adipose tissue, perigonadal white adipose tissue and the liver were dissected and frozen. Brown preadipocytes were isolated from 3-week-old mice and seeded for primary cultures as described [9]. When brown preadipocytes were grown in Dulbecco's modified Eagle's medium:Ham's F12 medium (DMEM/F12) (1:1) supplemented with 10% FCS, 20 nM insulin, 2 nM T3 and 1 μ M ascorbate (differentiating medium), 85–90% cells were differentiated to brown adipocytes 7–8 days after plating. When indicated, cells were grown after day 3 of culture in a medium containing 10% charcoal-stripped, delipidated calf bovine serum (Sigma), which blocked brown adipocyte differentiation (non-differentiating medium). The content of phytanic acid in 'differentiating medium' resulting from 10% FCS was 0.01 μ M but it was not detected in the 'non-differentiating' medium. The HIB-1B brown adipocyte cell line was cultured in DMEM/F12 containing 10% heat-inactivated FCS. For treatment, phytanic acid and pristanic

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Abbreviations: PhyH, phytanoyl-CoA hydroxylase; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; COT, carnitine octanoyl transferase; UCP1, uncoupling protein-1; CAT, chloramphenicol acetyltransferase

acid were dissolved in dimethylsulfoxide and the corresponding controls included equivalent amounts of the solvent, always at a final proportion of less than 1:1000 in the culture medium.

RNA from brown adipocytes in primary culture and from tissue was extracted following an affinity column-based method (RNeasy, Qiagen, Hilden, Germany). 10 µg RNA was used for Northern blot analysis, as reported [9]. For detection of PhyH mRNA, a probe was obtained after coupled reverse transcription and PCR amplification of mouse liver RNA using the primers 5'-GCAGTTTCGTCTGCTG-GCC-3' and 5'-GTACCTCACTCTTGAAGGGC-3', which correspond to positions 14–29 and 1080–1061 in the PhyH cDNA sequence (GenBank AF023463). Reactions were performed using 5 µg of RNA from mouse liver, 1 µM each primer, 2 µM each of the four nucleotide triphosphates, 3 mM MgSO₄, 0.1 U/µl *Thermus flavus* DNA polymerase and the AMV/*Tfl* as provided by the supplier (Promega, Madison, WI, USA). After 1 h incubation at 48°C, 40 cycles were performed, each at 94°C, 30 s, 55°C, 2 min, and 68°C, 1 min. The resulting DNA product, of approximately 1.8 kb, was purified, cloned into vector pGEM-T and sequenced. Other DNA probes used were the rat cDNA for COT [10], the rat cDNA for UCP1 [11] and the murine cDNA for aP2 [12]. The DNA probes were labeled with [α -³²P]dCTP following the random oligonucleotide primer method. Hybridization signals were quantified using a Molecular Image System GS-525 (Bio-Rad, Hercules, CA, USA).

The plasmid 4.5UCP1-CAT, in which the region –4551 to +110 of the rat *ucp-1* gene drives the chloramphenicol acetyltransferase (CAT) reporter gene, and expression vectors for human RXR α (pSG5–RXR) and mouse PPAR α (pSG5–PPAR α) were used. HIB-1B cells were transfected using the *FuGENE* Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) for 16 h, exposed to phytanic acid or pristanic acid and harvested 24 h later. Each transfection solution contained 0.5 µg of UCP1–CAT vector and 0.1 µg of cytomegalovirus- β -galactosidase, with or without 0.05 µg of expression vector. CAT activity was analyzed as reported [13] and it was normalized for variation in transfection efficiency using the β -galactosidase activity measured for each sample.

For phytanic acid quantification, total lipid extraction was performed on brown adipose tissue by the hexane/isopropanol method

[14]. Tissue extracts or serum were directly transmethyated [15] in the presence of nonadecanoic acid as internal standard. Phytanic acid methyl ester was analyzed by gas chromatography on a DB-7 1701 column (J&W Scientific) and the peak area was used for quantification followed by normalization using the internal standard. Retention times for the corresponding methyl esters and standards for phytanic acid quantification were established in parallel. To check the major lipid fraction containing phytanic acid, lipid extracts were subjected to thin-layer chromatography in silica gel plates using hexane/diethyl ether/acetic acid (80:20:1, v/v) as a mobile phase. The triacylglycerol fraction was separated from the other lipid fractions, extracted with chloroform/methanol (2:1, v/v), transesterified and analyzed by gas chromatography as reported above.

3. Results

3.1. PhyH mRNA is highly expressed in brown adipose tissue and differentiated brown adipocytes

Fig. 1 shows the mRNA expression for PhyH, the specific enzyme for initiation of phytanic acid oxidation, in brown adipose tissue compared with liver and white adipose tissue. The expression of PhyH mRNA in brown adipose tissue was even higher than in liver whereas expression in white fat was very low. Brown adipocytes differentiated in culture also showed high expression of PhyH mRNA, which was dramatically reduced when cells were prevented from differentiating by a delipidated culture medium. PhyH mRNA levels recovered partially following the treatment with phytanic acid. This pattern of regulation of expression was similar to marker genes for brown adipocyte differentiation such as UCP1 mRNA or aP2 mRNA. However, the positive effects of differentiation on PhyH mRNA were not common to gene expression for other peroxisomal proteins. Thus, the expression of

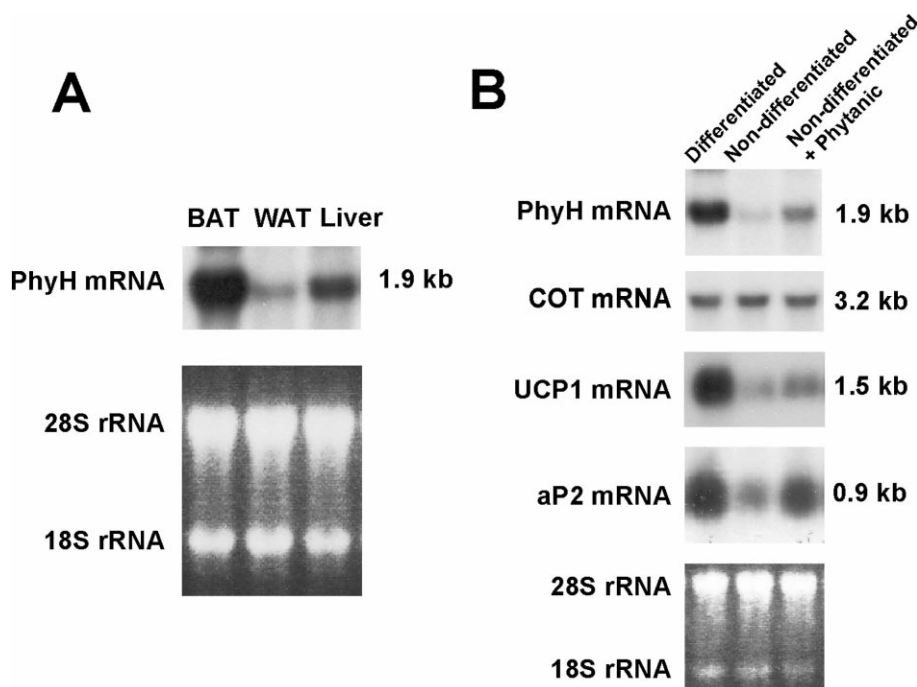


Fig. 1. Expression of PhyH mRNA in murine brown and white adipose tissues, liver and differentiating brown adipocytes in culture. Northern blot analysis of 10 µg RNA (A) from the interscapular brown adipose tissue (BAT), perigonadal white adipose tissue (WAT), liver from the adult mouse and (B) from brown adipocyte cells cultured in conditions leading to differentiation (Differentiated), to non-differentiation due to culture in delipidated medium (Non-differentiated) and conditions of supplementation of delipidated medium with 10 µM phytanic acid (Non-differentiated+Phytanic). The size of the detected transcripts is shown to the right.

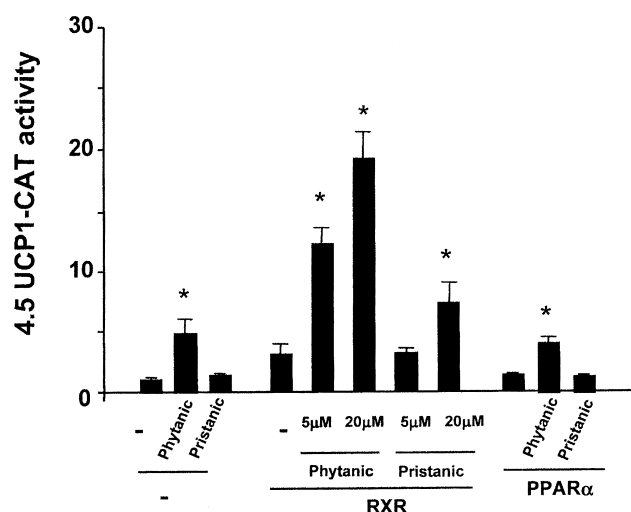


Fig. 2. Effects of pristanic acid and phytanic acid on UCP1 gene promoter activity. Influence of RXR or PPAR α co-transfection. HIB-1B cells were transiently transfected with 0.5 μ g/plate of the 4.5UCP1-CAT plasmid and were exposed to 20 μ M pristanic acid or 20 μ M phytanic acid unless otherwise indicated. When indicated 0.05 μ g of the RXR α or PPAR α expression vectors was co-transfected. Results are expressed as fold induction of CAT activity with respect to non-co-transfected non-treated control. Bars are means $\pm$ S.E.M. of three independent experiments. Significant differences ($P < 0.05$) between the fatty acid-treated cells with respect to non-treated cells for each set of co-transfection experiments is shown with an asterisk.

mRNA for COT, a peroxisomal enzyme involved in the export of products of fatty acid peroxisomal oxidation to mitochondria, was not affected by the differentiation status of the brown fat cell or by supplementation with phytanic acid.

3.2. Effects of pristanic acid and phytanic acid on brown adipocyte differentiation

Considering the positive effect of phytanic acid on brown adipocyte differentiation and the high expression of PhyH in these cells, the effects of pristanic acid were tested. For this purpose, brown adipocyte precursor cells were cultured in 'non-differentiating' medium and the capacity of pristanic acid to induce cells to differentiate was compared to that of phytanic acid supplementation. Pristanic acid was more toxic to primary brown preadipocyte cultures than phytanic acid. Thus, whereas phytanic acid could be used at concentrations of up to 40 μ M, 10 μ M exposure of preadipocytes to pristanic acid was deleterious as determined by detachment of cells from the culture plates. Therefore, a 1 μ M concentration was used to compare the capacities of the fatty acids to influence differentiation, which was monitored by examining the appearance of brown adipocyte morphology (accumulation of multivacuolar lipid droplets) and aP2 mRNA expression. 1 μ M phytanic acid had a positive effect on differentiation, characterized by the appearance of a low but significant percentage of cells with the adipocyte morphology (around 25%) and a 3.1 ± 0.4 -fold rise ($P < 0.05$) in aP2 mRNA with respect to non-treated cells, in agreement with previous findings [8]. No signs of adipocyte differentiation were observed in pristanic acid-treated cells and aP2 mRNA levels were indistinguishable from non-treated cells (0.9 ± 0.3 -fold induction).

3.3. Effects of pristanic acid and phytanic acid on UCP1 gene promoter activity

A second system was used to assess the potential of pristanic acid to mediate the biological actions of phytanic acid in the brown adipocytes. Previous work has shown that the gene promoter of UCP1, the specific molecular marker of differentiated brown adipocytes, was highly sensitive to phytanic acid [8]. To test the action of pristanic acid on the UCP1 gene promoter, the HIB-1B brown adipocyte-derived cell line was used. HIB-1B cells were transfected with 4.5UCP1-CAT, a plasmid in which the region -4551 to +110 of the rat *ucp-1* gene drives the CAT reporter, and exposed or not exposed to each fatty acid. HIB-1B cells were treated with up to 20 μ M pristanic acid with no signs of toxicity and therefore this concentration was used for the assays as well as 5 μ M. The effects of co-transfection of RXR or PPAR α receptors, potential mediators of the effects of phytanic acid or pristanic acid on gene transcription, were also determined. As shown in Fig. 2, in the absence of co-transfected receptors, only phytanic acid induced promoter activity. PPAR α co-transfection did not modify the effects observed in the absence of co-transfection. When RXR was co-transfected, either 5 μ M or 20 μ M phytanic acid caused a significant activation of promoter activity. In contrast, pristanic acid was ineffective at 5 μ M and only at 20 μ M a slight but significant induction was observed.

3.4. Phytanic acid levels in brown adipose tissue. Effects of cold-induced thermogenic activation

To contribute to the understanding of the potential significance of findings using cell cultures of brown adipocytes exposed to phytanic acid, the content of these fatty acids was determined in brown adipose tissue of adult mice. Phytanic acid in serum from adult mice maintained in standard conditions of ambient temperature and diet was 4.5 ± 0.9 μ M.

Table 1 shows the content of phytanic acid in interscapular brown adipose tissue of adult mice. To assess the capacity of these stores to be metabolically mobilized, mice were exposed to 4°C for 4 h to activate the lipolysis associated with the thermogenic activation of the tissue. As shown in Table 1, the phytanic acid content of brown fat dropped to almost one-half of the control levels after 4 h of cold exposure. This drop paralleled that of total lipid content in the tissue that decreased due to cold exposure. More than 95% of the phytanic acid in brown adipose tissue was in the form of triacylglycerol esters in both the control and the cold-treated group (data not shown).

Table 1
Effects of acute cold exposure on content of phytanic acid in interscapular brown adipose tissue (IBAT) of mice

	Control (21°C)	Cold-exposed (4°C, 4 h)
Phytanic acid (ng/mg tissue)	69 $\pm$ 8	38 $\pm$ 4*
(μ g/IBAT)	1.9 $\pm$ 0.2	1.1 $\pm$ 0.1*
Total lipid (mg/100 mg tissue)	47 $\pm$ 7	25 $\pm$ 3*
(mg/IBAT)	12.8 $\pm$ 1.8	7.5 $\pm$ 0.9*

Controls were 8-week-old male mice kept at 21°C. Cold exposure was 4 h at 4°C.

Results are means $\pm$ S.E.M. of six mice. * $P \leq 0.05$, statistically significant with respect to controls.

4. Discussion

The present study shows that brown adipose tissue is a site for high expression of PhyH mRNA, the specific enzyme for peroxisomal phytanic acid oxidation. The expression was even higher than in the liver, the tissue considered to be a major site for phytanic acid metabolism. Therefore, a high capacity of phytanic acid oxidation may be expected in brown fat. A parallel observation came from brown adipocytes in culture: PhyH mRNA appeared as a marker of brown adipocyte differentiation and this was not the simple reflection of the high peroxisomal activity associated with the brown adipocyte phenotype as the mRNA of COT, another peroxisomal enzyme, which was unaltered during brown adipocyte differentiation. The induction of PhyH mRNA by phytanic acid treatment of preadipocytes is likely to be part of the overall promotion of brown fat differentiation, although a specific effect cannot be excluded on the basis of a recent report on induction of PhyH activity by phytanic acid in several cell types [16].

The potential capacity of brown adipocytes to metabolize phytanic acid led us to consider whether a product of its oxidation may be involved in its biological effects. Results showed that pristanic acid has almost no effect on the markers of brown adipocyte function and therefore neither pristanic acid itself nor a further product of downstream metabolism of this acid was involved in the action of phytanic acid. In fact, pristanic acid has been shown to act *in vitro* through PPAR α , and PPAR α activators are known to be poorly adipogenic in white or brown differentiation cell models [8,17]. Phytanic acid can activate either RXR or PPAR α and a previous study demonstrated that the RXR-mediated pathway mediated most of the phytanic acid effects on the brown fat cell, despite the presence of PPAR α in brown adipocytes [8]. Thus, the oxidation of phytanic acid to pristanic acid does not appear to provide active molecules mediating phytanic acid effects. In contrast, it is expected to act as a mechanism to reduce the phytanic acid-mediated stimulation of brown adipocytes.

Phytanic acid was present in mouse serum at a low micromolar range concentration, in agreement with previous findings in rodents or humans [18]. Moreover, brown fat appears as a site of phytanic acid storage, similarly to what has been described for white adipose tissue [18]. Short-time cold exposure rapidly reduces the lipid content of brown adipose tissue. This is due to the noradrenergic-mediated activation of lipolysis to provide fatty acids, which are rapidly oxidized in mitochondria to fuel thermogenesis [19]. In this situation, the phytanic acid content in the tissue dropped to an extent similar to that observed for total lipids. In fact, most phytanic acid depots in brown fat are esterified as triacylglycerols and, when lipolysis is activated, phytanic acid is released as a free fatty acid that is subsequently oxidized, although some may be re-released into circulation. As a consequence of this process, during thermogenic activation, phytanic acid may appear

transiently at a concentration higher than the serum level and it can activate UCP1 gene transcription and contribute to thermogenic activation in brown fat. Subsequent oxidation leading to pristanic acid and further downstream metabolites would down-regulate the phytanic acid-mediated effects.

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