Phytanic acid, a novel activator of uncoupling protein-1 gene transcription and brown adipocyte differentiation

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a phytol-derived branched-chain fatty acid present in dietary products. Phytanic acid increased uncoupling protein-1 (UCP1) mRNA expression in brown adipocytes differentiated in culture. Phytanic acid induced the expression of the *UCP1* gene promoter, which was enhanced by co-transfection with a retinoid X receptor (RXR) expression vector but not with other expression vectors driving peroxisome proliferator-activated receptor (PPAR) α , PPAR γ or a form of RXR devoid of ligand-dependent sensitivity. The effect of phytanic acid on the *UCP1* gene required the 5' enhancer region of the gene and the effects of phytanic acid were mediated in an additive manner by three binding sites for RXR. Moreover, phytanic acid activates brown adipocyte differentia-

tion: long-term exposure of brown preadipocytes to phytanic acid promoted the acquisition of the brown adipocyte morphology and caused a co-ordinate induction of the mRNAs for gene markers of brown adipocyte differentiation, such as UCP1, adipocyte lipid-binding protein aP2, lipoprotein lipase, the glucose transporter GLUT4 or subunit II of cytochrome *c* oxidase. In conclusion, phytanic acid is a natural product of phytol metabolism that activates brown adipocyte thermogenic function. It constitutes a potential nutritional signal linking dietary status to adaptive thermogenesis.

Key words: phytol, retinoic acid, retinoid X receptor.

INTRODUCTION

Brown adipose tissue (BAT) is a major site for non-shivering thermogenesis in rodents in response to low environmental temperatures and also for diet-induced thermogenesis. Thus transgenic mice with genetically ablated BAT are cold-intolerant and prone to develop obesity [1]. Most of the thermogenic capacity of BAT is due to the presence of the uncoupling protein-1 (UCP1) in brown adipocyte mitochondria, where it behaves as a natural uncoupler of oxidative phosphorylation [2]. In rodents, targeted disruption of the *UCP1* gene results in cold intolerance [3], whereas ectopic overexpression of *UCP1* in white fat confers resistance to genetic and diet-induced obesity [4,5].

The effect of noradrenaline ('norepinephrine') on *UCP1* gene transcription is considered to be the major mechanism for adaptive changes in the BAT thermogenic activity that occurs in response to sympathetic nervous system activity. Thus, noradrenaline activates cAMP-responsive elements in the *UCP1* gene and promotes *UCP1* gene transcription while it activates lipolysis, which provides fatty acids, the major metabolic fuel for thermogenesis in the tissue. Moreover, fatty acids are direct activators of the uncoupling activity of UCP1 in the mitochondria (for a review, see [6]). Retinoic acid (RA) has been identified as another activator of *UCP1* gene transcription that is as powerful as noradrenaline [7,8]. RA is a vitamin A derivative with key effects on gene expression in various cell types, including adipocytes. RA acts through two types of receptor, RA receptor (RAR) and the retinoid X receptor (RXR), members of the nuclear-hormone-receptor superfamily. RAR binds all-*trans* RA and 9-*cis* RA, and it activates the transcription of target genes when forming RAR–RXR heterodimers. 9-*cis* RA also binds RXR, and ligand activation of RXR induces the transcription of genes containing regulatory elements that bind RXR homodimers as well as heterodimers of RXR and other receptors of the thyroid/retinoid

receptor family [9]. For instance, heterodimers of RXR with peroxisome proliferator-activated receptors (PPARs) stimulate the transcription of target genes in response to ligand activation of either the RXR or the PPAR moiety [10].

The physiological significance of the high sensitivity of the *UCP1* gene to retinoids is not known. *UCP1* gene transcription is activated by all-*trans* RA and 9-*cis* RA and a complex array of binding sites for RAR and RXR in the enhancer region of the gene, a regulatory region approx. 3 kb upstream of the transcription start site, mediate this effect [7,8,11,12]. The positive action of RA on the *UCP1* gene contrasts with some of the effects of retinoids on adipogenesis. Thus all-*trans* RA usually inhibits adipocyte differentiation by mechanisms involving activation of RAR. In contrast, specific activation of RXR promotes adipogenesis, probably by enhancing the activation of PPAR γ –RXR heterodimers (for review, see [13]).

Phytanic acid is a branched-chain, isoprenoid-derived fatty acid constituent of the diet. The major sources of phytanic acid are dairy products, meat and fish. Phytanic acid can also result from the conversion of dietary phytol in the body. A deficiency in phytanoyl-CoA hydroxylase, the peroxisomal enzyme responsible for the first step of phytanic acid oxidation, leads to abnormal accumulation of phytanic acid in human serum and tissues, known as Refsum disease [14,15]. This disease causes retinal degeneration, peripheral neuropathy, cerebellar ataxia, cardiomyopathy and skeletal abnormalities. Animal models of abnormal phytanic acid accumulation, like sterol carrier protein-2-knockout mice, also show body-weight disturbances [16]. The molecular basis of the pathogenesis of phytanic acid accumulation in Refsum disease is unknown. In 1996, phytanic acid was reported to bind RXR [17,18]. The binding and *trans*-activation affinity of RXR by phytanic acid were much lower than by 9-*cis* RA, but in the range of the physiological levels of phytanic acid reported in normal humans. More recently, phytanic acid has

Abbreviations used: UCP1, uncoupling protein-1; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; COII, cytochrome *c* oxidase subunit II; BAT, brown adipose tissue; RA, retinoic acid; RAR, RA receptor; FCS, fetal calf serum; LPL, lipoprotein lipase; CAT, chloramphenicol acetyltransferase; TR, thyroid receptor.

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been reported to bind PPAR α with high affinity and to activate it [19]. Stimulation by phytanic acid of the transcriptional activity of both RXR and PPAR α has been confirmed [20]. In the present study we show that phytanic acid activates *UCP1* gene transcription and brown adipocyte differentiation.

EXPERIMENTAL

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and phytanic acid methyl ester were from Sigma. Phytol was from Merck. WY 14,643 was from Cayman. AGN19420 and BRL 49653 were gifts from Dr. R. Chandraratna (Allergan, Irvine, CA, U.S.A.) and Dr. L. Casteilla (UMR 5018, CHU Rangueil, Toulouse, France), respectively. Tissue-culture media, fetal calf serum (FCS) and supplements were from BioWhittaker and Sigma. DNA-modifying enzymes and poly(dI-dC) were purchased from Boehringer Mannheim or Promega. [α -³²P]dCTP and D-threo-[1,2-¹⁴C]chloramphenicol were from Amersham Bioscience. Oligonucleotides were from Roche Diagnostics.

Isolation and culture of primary brown preadipocytes were performed as described in [7]. Swiss mice (3 weeks old) were killed and interscapular, cervical and axillary deposits of BAT were removed. Precursor cells were isolated, plated on to 60 mm Petri dishes (7500 cells/cm²) and grown in 5 ml of Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) supplemented with 10% FCS, 20 nM insulin, 2 nM tri-iodothyronine and 1 μ M ascorbate (regular medium). The content of phytanic acid in this medium resulting from 10% FCS was 0.01 μ M (0.1 μ M in FCS), as determined by GLC after direct transmethylation [21]. Under these conditions cells were typically 85–90% differentiated (as determined by morphology) 7–8 days after plating. When indicated, cells were grown after day 4 of culture in a 'delipidated' medium containing 10% charcoal-stripped delipidated calf bovine serum (Sigma). Phytanic acid was undetectable in this serum.

The HIB-1B brown adipocyte cell line, a gift from Dr. B. Spiegelman (Dana-Farber Cancer Institute, Boston, MA, U.S.A.), was cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% heat-inactivated FCS. For treatment with phytanic acid and other phytol derivatives, the chemicals were dissolved in DMSO. The corresponding controls included equivalent amounts of solvent, always at a final proportion of less than 1:1000 in the culture medium. Cycloheximide was used at a dose of 5 μ g/ml, as reported elsewhere [7].

Total RNA from brown adipocytes in primary culture and from tissue was extracted following an affinity-column-based method (RNeasy; Qiagen, Hilden, Germany). For Northern-blot analysis, 10 μ g of total RNA was denatured, electrophoresed on 1.5% formaldehyde/agarose gels, and transferred to positively charged nylon membranes (Boehringer Mannheim). Ethidium bromide (0.2 μ g) was added to RNA samples in order to check equal loading of the gels and transfer efficiency. Prehybridization and hybridization were carried out at 55 °C using 0.25 M Na₂HPO₄ (pH 7.2), 1 mM EDTA, 20% SDS and 0.5% blocking reagent (Boehringer Mannheim) solution. Blots were hybridized using the following DNA probes: the rat cDNA for UCP1 [22], a 0.5 kb fragment of the murine cDNA for cytochrome *c* oxidase subunit II (COII) [23], the murine cDNA for adipocyte lipid-binding protein aP2 [24], the guinea pig cDNA for lipoprotein lipase (LPL) [25], the rat cDNA for the glucose transporter GLUT4 [26] and a genomic DNA probe for rat β -actin [27]. The DNA probes were labelled with [α -³²P]dCTP following the random oligonucleotide-primer method. Hybridization signals

were quantified using a Molecular Image System GS-525 (Bio-Rad).

The plasmid 4.5UCP1-CAT, in which the region –4551 to +110 of the rat *UCP1* gene drives the promoterless chloramphenicol acetyltransferase (CAT) gene, was a gift from Dr. D. Ricquier (CEREMOD, CNRS, Meudon, France). The derived plasmids containing 5'-deletion mutations, (–3628)UCP1-CAT, (–2494)UCP1-CAT and (–611)UCP1-CAT, internal deletion mutations, (Δ –3608/–2318)UCP1-CAT and (Δ –2469/–2283)UCP1-CAT, and the plasmids (–2494/–2318)UCP1-CAT and (–2469/–2318)UCP1-CAT, in which the indicated fragments were placed upstream of (–173)UCP1-CAT, have been described elsewhere [7,11,28]. The mutated versions of (–2494/–2318)UCP1-CAT and (–2469/–2318)UCP1-CAT, containing substitutions of 2, 3 or 4 bp (see Figure 4, below), were generated using the Quikchange site-directed mutagenesis kit (Stratagene) and sequenced to check for the introduction of the expected base-pair substitutions. Base-pair substitutions were designed to disrupt the GG pairs required for nuclear hormone receptor binding.

Expression vectors containing murine cDNAs of PPAR isoforms [29], RAR α [30] and RXR α [31] driven by the simian virus-40 promoter were used. pRSV-RXR α is an expression vector for human RXR α [32]. pRSV-RXR- Δ AF2 is a vector driving the expression of a mutant-deleted form of RXR lacking the AF2 domain, obtained by a C-terminal deletion of human RXR α from residue 445 [33].

HIB-1B cells were transfected using the FuGENE 6 transfection reagent (Roche Diagnostics) for 16 h, exposed to phytanic acid or other phytol metabolites, and harvested 24 h later. Unless otherwise indicated, each transfection solution contained between 0.5 and 1 μ g of UCP1-CAT vector and 0.1 μ g of CMV- β -galactosidase (where CMV is cytomegalovirus), with or without 0.05 μ g of expression vector. CAT activity was analysed by TLC and quantified by radioactivity counting. The CAT activity was normalized for variation in transfection efficiency using the β -galactosidase activity measured for each sample as a standard.

Nuclear proteins were isolated from differentiated primary brown adipocytes as reported elsewhere [28]. For the gel-retardation assays, double-stranded oligonucleotides corresponding to the –2485/–2458, –2417/–2399 and –2357/–2330 regions of the rat *UCP1* gene were end-labelled using [α -³²P]dCTP and Klenow enzyme. The DNA probe (10000–20000 c.p.m.) was incubated for 30 min at 25 °C with 5 μ g of nuclear protein. Reactions were carried out in a final volume of 20 μ l containing 10 mM Tris/HCl (pH 8.0), 0.05% Nonidet P40, 1 mM dithiothreitol, 40 mM KCl, 6% glycerol and 2 μ g of poly(dI-dC). When indicated, 1 μ l of antiserum against RXR α (D-20) or ETS (negative control; C-275), from Santa Cruz Biotechnology, was added. Samples were analysed by electrophoresis at 4 °C for 60–80 min in non-denaturing 5% polyacrylamide gels in 0.5 $\times$ TBE (44.5 mM Tris/44.5 mM borate/1 mM EDTA). Gels were analysed by autoradiography.

RESULTS

Phytanic acid induces UCP1 mRNA expression in brown adipocytes

Exposure of brown adipocytes differentiated in primary culture to phytanic acid caused a significant induction of UCP1 mRNA levels (Table 1). This effect of phytanic acid in differentiated brown adipocytes was specific because, under these treatment conditions, COII mRNA was unaffected (Figure 1B). Dose-response analysis indicated that maximal induction was achieved with 10 μ M phytanic acid and remained high until at least 40 μ M (Figure 1A). Determination of the time course of the effects

Table 1 Effects of phytanic acid on UCP1 mRNA in differentiated brown adipocytes in primary culture

Brown adipocytes differentiated in primary culture (day 7) were treated with 10 μ M phytanic acid, 10 μ M phytol, 10 μ M phytanic acid methyl ester or treated with 5 μ g/ml cycloheximide and exposed or not to 10 μ M phytanic acid for 18 h. Total RNA (20 μ g) was analysed by Northern blotting as described in the Experimental section. Data are expressed relative to the untreated control (set to 1) and are means $\pm$ S.E.M. from at least three independent experiments.

Treatment	UCP-1 mRNA (arbitrary units)
—	1.0 $\pm$ 0.2
Phytol	1.3 $\pm$ 0.3
Phytanic acid	7.7 $\pm$ 1.4*
Cycloheximide	0.4 $\pm$ 0.1*
Phytanic acid + cycloheximide	3.2 $\pm$ 0.5*†
Phytanic acid methyl ester	0.8 $\pm$ 0.2

* Significantly different ($P < 0.05$) compared with the untreated control.

† Significantly different ($P < 0.05$) compared with cycloheximide treatment.

Figure 1 Dose-response effects of phytanic acid on UCP1 mRNA expression in differentiated brown adipocytes and representative Northern-blot analysis of the effects of phytol derivatives on UCP1 mRNA levels

(A) Brown adipocytes differentiated in primary culture (day 7) were treated with the indicated amounts of phytanic acid for 18 h. Total RNA was analysed by Northern blotting as described in the Experimental section. Points are means from at least two independent experiments and are expressed as the percentage of maximal induction. (B) Representative Northern-blot analysis of brown adipocytes treated with 10 μ M phytanic acid or 10 μ M phytol for 18 h. RNA (20 μ g) was analysed as described in the Experimental section. The filters were hybridized first with the UCP1 cDNA probe and thereafter with the COII cDNA probe.

Table 2 Effects of phytanic acid on the expression of the 4.5UCP1-CAT and effects of co-transfection of nuclear-receptor expression vectors

HIB-1B cells were transiently transfected with 0.5 μ g of the 4.5UCP1-CAT plasmid/plate and were exposed or not to 10 μ M phytanic acid, 10 μ M phytol or 10 μ M phytanic acid methyl ester. The expression vectors (0.05 μ g) for the nuclear-hormone receptors (PPAR α , PPAR γ , RAR α and RXR α) or the derived RXR- Δ AF2 mutant were co-transfected. Results are expressed as fold induction of CAT activity compared with non-cotransfected and non-phytanic acid-treated cells. Results are means $\pm$ S.E.M. from at least three independent experiments each performed in duplicate.

Co-transfected receptor	Treatment	Relative CAT activity
—	—	1.0 $\pm$ 0.2
—	Phytanic acid	4.2 $\pm$ 0.7*
PPAR α	—	2.4 $\pm$ 0.4
—	Phytanic acid	5.9 $\pm$ 0.9*
PPAR γ	—	2.4 $\pm$ 0.3
—	Phytanic acid	4.9 $\pm$ 0.6*
RAR α	—	0.8 $\pm$ 0.2
—	Phytanic acid	3.9 $\pm$ 0.4*
RXR α	—	3.5 $\pm$ 0.6
—	Phytanic acid	25.7 $\pm$ 3.8*†
—	Phytol	4.6 $\pm$ 0.8
—	Phytanic acid methyl ester	3.1 $\pm$ 0.4
RXR α Δ AF2	—	3.0 $\pm$ 0.4
—	Phytanic acid	6.5 $\pm$ 0.8*
PPAR α + RXR α	—	19.1 $\pm$ 2.1
—	Phytanic acid	29.0 $\pm$ 4.3*†

* Significantly different ($P < 0.05$) between phytanic acid-treated and non-treated cells for each co-transfectant.

† Significantly different ($P < 0.05$) between phytanic acid treatments in the presence of a co-transfected receptor and in the non-cotransfected group.

revealed a maximum stimulus after 18 h of exposure (results not shown). The maximal effect of phytanic acid on UCP1 mRNA levels was approx. 40 % of that of 0.5 μ M noradrenaline or 1 μ M all-*trans* RA. Treatment with neither phytol nor the methyl ester derivative of phytanic acid altered UCP1 mRNA levels (Table 1). Although cycloheximide reduced the basal levels of UCP1 mRNA, as reported elsewhere [7], the extent of induction by phytanic acid, approx. 8-fold, was similar in the absence or presence of cycloheximide (Table 1), which shows that the effect of phytanic acid on the *UCP1* gene expression is independent of protein synthesis.

Phytanic acid activates *UCP1* gene promoter activity: RXR enhances the effects of phytanic acid

The 4.5UCP1-CAT plasmid construct, in which the 5' regulatory region of the gene drives the expression of the CAT reporter, was transiently transfected to the brown adipocyte HIB-1B cell line. Phytanic acid significantly induced 4.5UCP1-CAT activity, which reveals the presence of *cis*-acting elements in the *UCP1* gene promoter responsive to transcriptional activation by phytanic acid (Table 2). As with UCP1 mRNA levels, 4.5UCP1-CAT gene expression was not affected by phytol or phytanic acid methyl ester. Expression vectors for various members of the nuclear-receptor superfamily that are responsive to fatty acid derivatives and retinoids were co-transfected and their effect on the 4.5UCP1-CAT responsiveness to phytanic acid was determined. Co-transfection with PPAR α , PPAR γ or RAR α did not modify the responsiveness to phytanic acid. However, the *UCP1* gene promoter was responsive to these receptors in this cell system. Promoter activity was induced by co-transfection, probably due to the presence of ligand activity in the serum-containing medium or to ligand-independent activity of some

Figure 2 Dose–response effects of phytanic acid on 4.5UCP1-CAT expression: effects of co-transfected RXR α

HIB-1B cells were transiently transfected with 0.5 μ g of the 4.5UCP1-CAT plasmid/plate and were exposed or not to 10 μ M phytanic acid, 1 μ M or 5 μ M AGN19420. Cells were co-transfected or not with 0.05 μ g of pRSV-RXR α expression plasmid. Thereafter, cells were exposed to the indicated amounts of phytanic acid. Points are means from duplicate assays.

Table 3 Effects of phytanic acid plus AGN19420 on the expression of the 4.5UCP1-CAT

HIB-1B cells were transiently transfected with 0.5 μ g of the 4.5UCP1-CAT plasmid/plate and were exposed or not to 10 μ M phytanic acid, 1 μ M or 5 μ M AGN19420. Cells were co-transfected with the expression vector for RXR α (0.05 μ g). Results are expressed as fold induction of CAT activity compared with non-treated cells. Results are means $\pm$ S.E.M. from three independent experiments each performed in duplicate.

Treatment	Relative CAT activity
–	1.0 $\pm$ 0.2†
Phytanic acid	5.4 $\pm$ 0.6*†
AGN19420 (1 μ M)	13.0 $\pm$ 0.9*†
AGN19420 (5 μ M)	19.0 $\pm$ 1.1*
AGN19420 (1 μ M) + phytanic acid	16.7 $\pm$ 0.9*
AGN19420 (5 μ M) + phytanic acid	18.1 $\pm$ 0.8*

* Significantly different ($P < 0.05$) with respect to non-treated cells.

† Significantly different ($P < 0.05$) with respect to cells treated with 5 μ M AGN19420.

of the receptors, especially RXR. When specific ligands were included, the activity of 4.5UCP1-CAT was induced by WY 14,643 in the presence of co-transfected PPAR α (2.6 $\pm$ 0.4-fold induction), by BRL49653 in the presence of co-transfected PPAR γ (6.4 $\pm$ 0.9-fold induction) and by all-*trans* RA in the presence of co-transfected RAR α (5.0 $\pm$ 0.8-fold induction). In contrast, co-transfection with RXR α strongly enhanced the effect of phytanic acid on 4.5UCP1-CAT activity. Co-transfection with a mutant form of RXR α lacking the ligand-dependent activation domain of the RXR receptor (Δ AF2) [33] increased basal activity but did not increase the sensitivity to phytanic acid. Co-transfection of RXR with PPAR α strongly promoted 4.5UCP1-CAT activity but did not enhance the responsiveness to phytanic acid.

The dose–response of phytanic acid effects on 4.5UCP1-CAT activity in the absence or presence of co-transfected RXR is shown in Figure 2. The doses were increasingly effective from 1 to 40 μ M. Higher doses were not tested for promoter activity due to toxic effects of phytanic acid at higher concentrations. A parallel dose–response analysis for responsiveness to AGN19420, a specific RXR activator, indicated that the maximal activation of 4.5UCP1-CAT activity was attained at 5 μ M (results not

shown), whereas 1 μ M AGN19420 resulted in 68% of the maximal activation (Table 3). Treatment with 10 μ M phytanic acid plus 1 μ M AGN19420 caused an additive effect and resulted in an activation similar to that elicited by the maximal dose of 5 μ M AGN19420. However, addition of 10 μ M phytanic acid to 5 μ M AGN19420 did not increase the maximal levels of induction caused by AGN19420 further, thus suggesting a common pathway of induction for AGN19420 and phytanic acid through the activation of RXR.

Effects of phytanic acid on *UCP1* gene transcription are mediated by three RXR-binding sites in the –2494/–2318 enhancer region

Figure 3 shows the deletion analysis of the effects of phytanic acid on the *UCP1* gene promoter. Progressive deletion of the 5' region of the *UCP1* gene only abolished the effects of phytanic acid when all but the –611 proximal region had been removed from the CAT constructs. An internal deletion between –2469 and –2283 sufficed to suppress the effects of phytanic acid. Moreover, the construct in which the region between –2494 and –2318 was linked to the proximal region of the *UCP1* gene was fully responsive to phytanic acid. It is concluded that the –2494/–2318 region, which comprises the regulatory enhancer of the *UCP1* gene [6], contains the elements mediating phytanic acid effects.

Figure 4(A) shows a schematic representation of the sites in the rat *UCP1* gene enhancer region to which RXR has been reported to bind as a partner of several members of the nuclear-receptor family [7,8,11,12,34]. Double point mutations disrupting the PPAR/RXR site reduced the extent of phytanic acid-dependent activation but the construct remained sensitive to phytanic acid (Figure 4B). The same was observed for the 5' deletion of the enhancer in which the PPAR/RXR-binding region is mutated. Further point mutations of the RA-responsive region of the *UCP1* gene enhancer were studied. This region is a complex site including an RAR/RXR-binding site at –2406/–2403 and an adjacent activator protein (AP) 1-like site required for RA effects [8]. Mutation of either of these two sites reduced the sensitivity to phytanic acid but did not abolish it completely. Mutation of the upstream thyroid receptor (TR)/RXR site did not affect phytanic acid responsiveness, whereas mutation of the downstream TR/RXR site reduced but did not completely suppress the effect of phytanic acid. Only when the PPAR/RXR element was absent and mutations were introduced into both the RA-response region and the downstream TR/RXR site was the resulting construct totally unresponsive to phytanic acid.

To check the capacity of these three sites to bind RXR in brown adipocytes, electrophoretic mobility-shift assays were performed. Double-stranded oligonucleotides for these sites were used as probes. They were incubated with nuclear extracts of primary brown adipocytes differentiated in culture and an RXR-specific antibody was added. As shown in Figure 5, the –2485/–2458 region (PPAR/RXR) and the –2357/–2330 region (TR/RXR) of the *UCP1* gene formed complexes with nuclear proteins from brown adipocytes that were supershifted in the presence of the RXR antibody. Addition of an unrelated antibody against ETS proteins had no effect (results not shown), thus revealing the specific binding of RXR to these two regions, as observed previously [7,8,34]. The addition of the RXR antibody to the –2417/–2399 DNA region in the presence of brown adipocyte nuclear extracts did not lead to a supershifted band but rather to a reduction in the intensity of two retarded bands (see Figure 5, arrows). These bands contained specific DNA–

Figure 3 Deletion analysis of the effects of phytanic acid on 4.5UCP1-CAT

HIB-1B cells were transiently transfected with 0.5 µg of 4.5UCP1-CAT/plate (top construct) or the deletion-mutant-derived constructs depicted on the left. Cells were co-transfected with 0.05 µg of pRSV-RXRα. Results are means ± S.E.M. from at least three independent experiments each performed in duplicate. Basal CAT activity is shown as arbitrary units with respect to 4.5UCP1-CAT activity (set to 1) and the fold induction due to phytanic acid treatment is also shown for each construct. Induction by phytanic acid was significant where indicated (**P* < 0.05).

Figure 4 Point-mutant analysis of the effects of phytanic acid on the activity of the *UCP1* gene enhancer

(A) Schematic representation of the enhancer of the rat *UCP1* gene showing the four regions for RXR binding, according to [7,8,11,12,34]. * indicates the sites where base-pair substitutions were introduced. (B) HIB-1B cells were transiently transfected with 0.5 µg of the constructs depicted on the left/plate; 0.05 µg of pRSV-RXRα was co-transfected. Results are means ± S.E.M. from at least three independent experiments, each performed in duplicate. Basal activity (arbitrary units with respect to -2494/-2318-UCP1-CAT activity, set to 1) and the fold induction due to phytanic acid treatment for each construct are shown. **P* < 0.05, significant induction by phytanic acid; #*P* < 0.05, significant differences between the phytanic acid effects in the point-mutated constructs and the corresponding parental non-mutated construct.

Figure 5 Electrophoretic mobility-shift analysis of the interaction of the $-2485/-2458$, $-2417/-2399$ and $-2357/-2330$ regions of the *UCP1* gene enhancer with nuclear proteins from brown adipocytes: effects of incubation with an RXR antibody

Double-stranded synthetic oligonucleotides corresponding to the regions mentioned were end-labelled, incubated with $5 \mu\text{g}$ of nuclear-protein extracts from murine brown adipocytes differentiated in culture including (+) or not (–) $1 \mu\text{l}$ of antiserum against RXR α , or a 100-fold molar excess of the unlabelled oligonucleotide (Competitor), and subjected to PAGE. Arrows indicate the retarded bands affected by the presence of the antibody.

protein complexes, as shown by their reduced intensity in the controls with excess of unlabelled probe. Although the binding of RXR to this site has been demonstrated to be involved in the formation of RAR–RXR dimers [8], the difference with respect to the other sites can be attributed to the nature of the DNA–protein complexes in this region. Unlike the more canonical structures of half-sites in the PPAR/RXR and the downstream TR/RXR regions, the RA-responsive region in the *UCP1* gene has an atypical architecture for an RA-response element [8]. We conclude that the effects of phytanic acid are mediated in an additive manner by the three sites in the *UCP1* gene enhancer that bind RXR as partner of several nuclear receptor complexes.

Phytanic acid induces brown adipocyte differentiation

In order to establish whether, together with direct effects on the *UCP1* gene, brown adipocyte differentiation is also affected by phytanic acid, primary cultures of brown preadipocytes were exposed to long-term treatment with phytanic acid.

Figure 6 shows the effects of various culture media on the differentiation of brown adipocyte precursor cells in primary culture. Culture of precursor cells in regular FCS-containing medium led to the acquisition of adipocyte morphology characterized by the accumulation of lipid droplets. On the basis of this morphology, around 90 % of cells were differentiated into brown adipocytes after 7 days of culture. Replacement of regular medium on day 4 of culture, before the start of differentiation, with a medium containing delipidated serum stopped the differ-

Figure 6 Effects of phytanic acid on brown adipocyte differentiation of precursor cells in primary culture

Microphotographs of primary brown adipocyte precursor cells grown in culture for 4 days and treated thereafter with regular differentiating medium (FCS), delipidated medium or delipidated medium plus $10 \mu\text{M}$ phytanic acid, $10 \mu\text{M}$ WY 14,643 or $1 \mu\text{M}$ AGN19420. Magnification, $\times 40$.

entiation process and, after 7 days of culture, cells remained with a fibroblastic morphology. When the delipidated medium was supplemented with phytanic acid, cells differentiated again into brown adipocytes and around 75 % acquired the brown adipocyte morphology by day 7. For comparison, parallel experiments were performed using other chemicals to supplement delipidated serum. Addition of AGN19420, the specific RXR agonist, also enhanced brown adipocyte differentiation. Addition of AGN19420 plus phytanic acid did not further increase the differentiation of brown adipocytes with respect to that obtained with AGN19420 alone, which was close to the maximal differentiation attained with regular differentiating medium (around 90 % differentiated cells; results not shown). In contrast, no major capacity to elicit differentiation into brown adipocytes was observed with the supplementation with $10 \mu\text{M}$ WY 14,643, a PPAR α -specific activator.

Table 4 shows how several gene markers of differentiation were affected as a result of the long-term effects of phytanic acid on brown adipocyte differentiation. Culture with the delipidated medium reduced the expression of all the gene markers of brown adipocyte differentiation, in agreement with the impairment in acquisition of the brown adipocyte morphology. Gene markers

Table 4 Effects of phytanic acid on the expression of the mRNAs for UCP1, aP2, LPL, GLUT4, COII and β -actin in differentiating brown adipocytes

Brown adipocyte precursor cells were grown in culture for 4 days and treated thereafter with regular differentiating medium (FCS), delipidated medium plus 10 μ M phytanic acid (delipidated + phytanic) or delipidated medium plus 2 μ l/plate DMSO, the vehicle of phytanic acid solution (delipidated). Cells were harvested on day 7 of culture and 10 μ g of RNA was used for Northern-blot assays. Results are means $\pm$ S.E.M. from densitometric analysis in three independent experiments and are expressed as percentages of mRNA abundance in differentiated brown adipocytes (FCS medium).

Gene marker	Treatment	Relative mRNA abundance (%)
UCP1	FCS	100 $\pm$ 18
	Delipidated	12 $\pm$ 1*
	Delipidated + phytanic acid	39 $\pm$ 7*†
aP2	FCS	100 $\pm$ 21
	Delipidated	32 $\pm$ 8*
	Delipidated + phytanic acid	108 $\pm$ 19†
LPL	FCS	100 $\pm$ 14
	Delipidated	64 $\pm$ 9*
	Delipidated + phytanic acid	111 $\pm$ 17†
GLUT4	FCS	100 $\pm$ 21
	Delipidated	31 $\pm$ 5*
	Delipidated + phytanic acid	84 $\pm$ 17†
COII	FCS	100 $\pm$ 19
	Delipidated	22 $\pm$ 5*
	Delipidated + phytanic acid	66 $\pm$ 11*†
β -Actin	FCS	100 $\pm$ 18
	Delipidated	122 $\pm$ 14
	Delipidated + phytanic acid	114 $\pm$ 9

* Significant effect ($P < 0.05$) due to delipidated serum with respect to FCS.

† Significant effect ($P < 0.05$) of phytanic acid supplementation with respect to delipidated serum.

Table 5 Dose–response analysis of the effects of phytanic acid on aP2 and COII mRNA expression during brown adipocyte differentiation

Brown adipocyte precursor cells were grown in culture for 4 days and treated thereafter with delipidated medium plus the indicated amounts of phytanic acid until day 7 of culture. RNA (10 μ g) was used for Northern-blot assays. Data are means $\pm$ S.E.M. from densitometric analyses in three independent Northern-blot assays and are expressed as percentages of the maximal induction attained.

Phytanic acid (μ M)	Densitometric analysis (% of maximal induction)	
	aP2 mRNA	COII mRNA
0	28 $\pm$ 4	32 $\pm$ 5
0.1	55 $\pm$ 11	50 $\pm$ 8
1	83 $\pm$ 14	79 $\pm$ 13
10	100 $\pm$ 18	100 $\pm$ 16

of adipose differentiation shared by white and brown adipocytes, like aP2, LPL and GLUT4, were induced by the exposure to phytanic acid. Moreover, the mRNA for COII, a marker of the mitochondrial process occurring during differentiation of brown adipocytes, was also increased by phytanic acid treatment. UCP1 mRNA levels were also significantly higher in phytanic acid-treated cells than in delipidated controls. However, for some genes the mRNA levels attained by long-term exposure to phytanic acid were not different from those of cells fully differentiated by the regular medium (aP2 mRNA, LPL mRNA and GLUT4 mRNA), whereas others, like UCP1 mRNA and COII mRNA, remained lower.

In Table 5, a dose–response analysis shows the capacity of phytanic acid to affect brown adipocyte differentiation as shown

by the effects on the expression of aP2 mRNA, a marker of adipogenesis, and COII mRNA, a marker of mitochondrial biogenesis. The highest concentration of phytanic acid studied, 10 μ M, showed a maximal induction but 0.1 μ M sufficed to increase the expression of differentiation markers. This agrees with observations on cell morphology, which showed that the lower the dose of phytanic acid, the lower the percentage of cells acquiring brown adipocyte morphology (results not shown). Higher concentrations of phytanic acid, in the 100 μ M range, are not shown because they caused some toxicity to cells after 3 days of treatment.

DISCUSSION

The thermogenic activation of BAT is associated with two major events: an increase of *UCP1* gene transcription in mature brown adipocytes and activation of brown preadipocyte differentiation to form a larger mass of mature brown adipocytes. In the present study we identify phytanic acid, a phytol-derived dietary factor, as a novel activator of BAT thermogenic activity that enhances both processes.

Phytanic acid activates *UCP1* gene expression through a protein-synthesis-independent pathway that involves RXR-mediated activation of the regulatory enhancer of the *UCP1* gene. This is in agreement with previous findings showing that activation of RXR by synthetic ligands promotes *UCP1* gene expression [11] and that phytanic acid can bind and activate RXR [17,18]. Transfection assays on the *UCP1* gene constructs did not show any enhancement of responsiveness to phytanic acid by receptors other than RXR, including a mutant form of RXR in which ligand-dependent activation domain is suppressed. Transfection of PPAR α , the other nuclear receptor reported to mediate phytanic acid effects [19], was ineffective. However, it is difficult to distinguish between RXR- and PPAR α -dependent pathways in the mechanisms involved in the biological effects of phytanic acid, since PPAR α forms a heterodimer with RXR in which both receptors retain ligand-dependent sensitivity [10] and, for instance, RXR agonists activate PPAR α -inducible genes, both in transfection assays and *in vivo* [35]. The upstream RXR-binding site in the *UCP1* gene, which can bind the PPAR α –RXR heterodimer and is activated by PPAR α agonists [34], is involved in phytanic acid responsiveness but this could be due just to ligand activation of the RXR moiety. Moreover, other RXR-binding sites in the *UCP1* gene enhancer, unrelated to the PPAR/RXR-binding site, also contribute to phytanic acid effects, and therefore ligand-dependent activation of RXR appears as the major mechanism for the effects of phytanic acid on the *UCP1* gene. The binding affinity of phytanic acid has been reported to be higher for PPAR α than for RXR [19], but the biological effects of phytanic acid in the brown adipocyte may also involve molecules resulting from phytanic acid metabolism, such as pristanic acid or others [18,36]. These molecules may have different affinities for RXR than phytanic acid itself and they may be formed to different extents according to the cell type. In this sense, the high peroxisome content [37] as well as a high expression of the mRNA for phytanoyl-CoA hydroxylase (A. Schlüter and F. Villarroja, unpublished work) in BAT suggest an active pathway of phytanic acid metabolism in this tissue. Whereas the lack of effect of phytol is consistent with its failure to bind or activate RXR [18] and indicates a low capacity for phytol conversion into phytanic acid in brown adipocytes, the ineffectiveness of the phytanic acid methyl ester indicates a requirement for either the carboxylic end of phytanic acid for the biological effects or a further metabolism of phytanic acid to

generate a biologically active product, which is impaired when the carboxylic end is methylated.

We found here that the effects of phytanic acid on cultured brown adipocytes started at micromolar concentrations. The phytanic acid levels in rodents and normal humans were approx. 8 μ M [38], and approx. 70% of circulating phytanic acid is present as esters [18]. Thus the estimated free phytanic acid would be equal to the threshold for the concentrations used to observe biological effects on *UCPI* gene expression and in the range of the effects observed on overall brown adipocyte differentiation (see below). However, BAT is not only a target for phytanic acid but it also contains substantial amounts of phytanic acid in its lipid stores and it is actively mobilized when lipolysis is activated (A. Schlüter and F. Villarroya, unpublished work). Regardless of whether phytanic acid affects the *UCPI* gene directly or through a derived metabolite, it may behave as a signal linking the mobilization of lipid stores and the thermogenic activation of brown adipocytes to promotion of *UCPI* gene expression. In this sense, identification of phytanic acid as an activator of the *UCPI* gene through the RXR-responsive sites may explain the functional significance of the sensitivity of the *UCPI* gene to retinoids in relation to thermogenic activation.

In addition to the stimulatory effects on *UCPI* gene transcription, phytanic acid also enhanced the brown adipocyte differentiation process. As for *UCPI* gene transcription, this action appears also to be mediated by the capacity of phytanic acid to activate RXR. Phytanic acid stimulates differentiation, as does the synthetic RXR ligand AGN19420, whereas WY 14,643, a PPAR α agonist, has no effect. This is consistent with previous observations on the positive role of RXR ligands on white fat adipogenesis [39] and the poor adipogenic action of PPAR α agonists [40]. As reported for white adipocytes, the major mechanism by which RXR activation by phytanic acid elicits brown adipocyte differentiation may be the activation of the heterodimer of RXR with PPAR γ , a master transcription factor for adipogenesis [40].

Whereas phytanic acid markedly induced the *UCPI* gene in brown adipocytes when these cells were already differentiated, the induction of *UCPI* gene expression associated with the brown adipocyte differentiation process elicited by long-term treatment with phytanic acid was not as high as with the differentiating medium. *UCPI* is a very late marker gene of brown adipocyte differentiation [41]. In this sense, *UCPI* mRNA is practically undetectable in cells not allowed to differentiate, whereas other brown adipocyte gene markers attain some degree of expression in the non-differentiated cultures. Most probably, the effects of phytanic acid are not strong enough to achieve the acquisition of the latter stages of brown fat cell differentiation. In any case, phytanic acid activates the programme of brown adipocyte differentiation not only for the characteristics shared by white adipocytes, as shown by the induction of the mRNAs of aP2, LPL and GLUT4, but also for the specific brown adipocyte phenotype, as shown by the enhanced expression of marker genes for mitochondrial biogenesis, such as COII, and of *UCPI*.

In summary, phytol metabolites may be nutritional signals linking an animal's dietary state with adaptive thermogenesis associated with BAT activity. Although the effects of phytanic acid on the body's energy balance have not been addressed directly, several observations link high phytanic acid levels to negative energy balance and a decrease in body weight. Mice bearing a targeted disruption of sterol carrier-2 protein [16] show high levels of circulating phytanic acid, and become hyperphagic but do not increase body weight, thus suggesting enhanced energy expenditure. Moreover, several reports in patients with

Refsum disease describe loss of body weight and reduction of white adipose tissue stores in the absence of intercurrent illness [15]. These observations suggest that phytanic acid activates energy expenditure and they are consistent with our present findings for BAT and *UCPI* gene activation.

The identification of natural inducers of *UCPI* gene expression and brown adipocyte differentiation is a major goal in the attempt to establish new pharmacological approaches to modulate adaptive thermogenesis and promote fat oxidation. The present identification of phytanic acid as a novel activator of *UCPI* gene expression and brown adipocyte differentiation opens a new research direction for the design of dietary and pharmacological agents affecting energy expenditure in obesity and other metabolic disturbances.

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