Fatty acid signals in *Bacillus megaterium* are attenuated by cytochrome P-450-mediated hydroxylation

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In previous publications [English, Hughes and Wolf (1994) J. Biol. Chem. **269**, 26836–26841; English, Hughes and Wolf (1996) Biochem. J. **316**, 279–283], we have demonstrated that peroxisome proliferators and non-steroidal anti-inflammatory drugs are inducers of the cytochrome *P*-450_{BM-3} gene in *Bacillus megaterium* ATCC14581. Their mechanism of action involves binding to and subsequent displacement of the transcriptional repressor, Bm3R1, from its operator site, which results in the activation of cytochrome *P*-450_{BM-3} gene transcription. We now present evidence that the branched-chain fatty acid, phytanic acid, is a potent inducer of cytochrome *P*-450_{BM-3}. We have also observed that phytanic acid and peroxisome proliferators are inducers of Bm3R1 protein accumulation and associated DNA-binding activity. In contrast, several barbiturates, although capable of inducing cytochrome *P*-450_{BM-3} and Bm3R1 gene

transcription, were unable to induce the Bm3R1 protein. We also demonstrate that cytochrome *P*-450_{BM-3} readily oxidizes phytanic acid, and provide evidence that, although the ω -1 hydroxy acid derivatives of phytanic acid can associate with Bm3R1, they do so with an affinity two orders of magnitude lower than the unmodified fatty acid. As a consequence, the ability of the hydroxylated product to induce cytochrome *P*-450_{BM-3} gene expression *in vivo* is markedly reduced. These data collectively suggest that metabolism of fatty acids by cytochrome *P*-450_{BM-3} leads to an attenuation of their ability to activate the transcription of the BM-3 operon. This work places the action of bacterial fatty acid hydroxylases in an autoregulatory loop where they may be responsible for the inactivation or clearance of the inducing fatty acid signal.

INTRODUCTION

Bacillus megaterium ATCC 14581 contains at least three soluble cytochrome *P*-450s termed *P*-450_{BM-1}, *P*-450_{BM-2} and *P*-450_{BM-3} [1]. Cytochromes *P*-450_{BM-1} and *P*-450_{BM-3} have been cloned, and their genes encode polypeptides of molecular mass 47 and 117.5 kDa respectively [2,3]. Both cytochrome *P*-450s are induced by barbiturates [4], and research in our laboratory has revealed that cytochrome *P*-450_{BM-3} is also profoundly induced by peroxisome proliferators and non-steroidal anti-inflammatory drugs [5,6]. Investigations into the mechanism of regulation of the cytochrome *P*-450_{BM-3} operon have identified an open reading frame located immediately 5' of cytochrome *P*-450_{BM-3} which encodes a transcriptional repressor, termed Bm3R1 [7]. This repressor binds to a perfect palindromic operator upstream of its own open reading frame. We have demonstrated that barbiturates, peroxisome proliferators and non-steroidal anti-inflammatory drugs have a capacity to interact with Bm3R1 and to abrogate its binding to operator DNA sequences [5,6]. The displacement of Bm3R1 from its operator by barbiturates results in the activation of transcription across both the coding regions of Bm3R1 and *P*-450_{BM-3} [7,8]. Furthermore the relative potency of these compounds as *P*-450_{BM-3} inducers *in vivo* is closely correlated with their ability to inhibit the formation of the Bm3R1–operator complex *in vitro* [5,6]. Further evidence illustrating the critical role of the Bm3R1 repressor in the regulation of this operon comes from the observation that a single point mutation in the DNA-binding helix–turn–helix motif of Bm3R1 leads to an inability to bind its operator and allows constitutive expression of cytochrome *P*-450_{BM-3} [7].

Cytochrome *P*-450_{BM-3} is a fatty acid hydroxylase which shares more primary sequence similarity with members of the mammalian CYP4A subfamily of fatty acid ω -hydroxylases than with any other prokaryotic mono-oxygenase [9]. The spectrum of compounds that induces cytochrome *P*-450_{BM-3} is very similar to that which induces CYP4A enzymes in mammals [10]. This led us to speculate that the capacity of specific chemicals to induce either CYP4A or cytochrome *P*-450_{BM-3} is due to their ability to mimic fatty acid signalling compounds. Indeed, we have shown that unsaturated fatty acids are the most effective inhibitors of Bm3R1 binding to its operator DNA sequence *in vitro* [5].

The role of cytochrome *P*-450_{BM-3} in the life cycle of soil-living bacillus is unclear. As a fatty acid mono-oxygenase, it may be involved in the biosynthesis of specific fatty acids required for normal homeostasis or provide a protection mechanism against the harmful accumulation of endogenously or exogenously produced fatty acids. In support of the latter possibility, we have found that many plant-derived unsaturated fatty acids are extremely toxic to *B. megaterium* ([5]; N. English, C. N. A. Palmer, W. L. Alworth, L. Kang, V. Hughes and C. R. Wolf, unpublished work). Unsaturated fatty acids have been reported to be very effective inhibitors of phospho-relay signalling in *Bacillus subtilis*, which may contribute to their observed toxicity [11]. The induction of enzymes involved in the metabolism and inactivation of fatty acids would provide a logical autoregulatory loop for cells to respond to fatty acid accumulation. In the present study we have demonstrated the induction of cytochrome *P*-450_{BM-3} by a branched-chain fatty acid, phytanic acid. We have also established that oxidation of phytanic acid by cytochrome *P*-450_{BM-3} results in the formation of ω -1 hydroxyphytanic acid.

Abbreviation used: EMSA, electrophoretic mobility-shift assay.

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The ω -1 hydroxyphytanic acid has a markedly reduced capacity to inhibit Bm3R1 binding to its operator DNA sequence *in vitro* and induce cytochrome *P*-450_{BM-3} *in vivo*.

EXPERIMENTAL

Reagents

Phytanic acid (> 96% pure) was purchased from Sigma. Peroxisome proliferators were a gift from Zeneca Central Toxicology Laboratories, Macclesfield, Cheshire, U.K. and from Dr. Brian Lake at Bibra, Carshalton, Surrey, U.K. Antibodies and oligonucleotides were produced at the Imperial Cancer Research Fund laboratories at Clare Hall, Potters Bar, Herts., U.K. Donkey anti-rabbit secondary antiserum was purchased from SAPU, Carlisle, Lanarkshire, Scotland, U.K. *B. megaterium* ATCC 14581 was purchased from the American Type Culture Collection.

Growth and harvesting of cells

Growth, harvesting and preparation of bacterial extracts was as described previously [5]. *B. megaterium* were grown at 37 °C with aeration in 1% (w/v) glucose salts medium described by Grelet [12] but with the additional salts 0.004% (w/v) ammonium ferric citrate and 0.008% (w/v) CaCl₂ and a supplement of 0.5% (w/v) casamino acids, to an A_{600} of 0.6. Phytanic acid and drugs were added as required in DMSO, or in the case of controls, vehicle alone was added. The final DMSO concentration did not exceed 0.5% (v/v) in any incubation. Cells were grown for approx. 8 h, harvested by centrifugation at 2000 *g*, washed in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, and lysed by sonication. Subcellular fractions were obtained by centrifugation. Supernatant fractions (30000 *g*) were used for immunoblotting and electrophoretic mobility-shift assay (EMSA). All protein concentrations were quantified by the method of Lowry *et al.* [13].

Protein immunoblotting

SDS/PAGE and immunoblotting were performed using standard protocols [14,15]. Briefly, proteins within the *B. megaterium* 30000 *g* supernatant extract (30 μ g) were resolved using SDS/PAGE. A 7.5% acrylamide gel was used to resolve cytochrome *P*-450_{BM-3}, and a 12.5% gel was used for the detection of the Bm3R1 protein. After separation by SDS/PAGE, the proteins were transferred to nitrocellulose. Both cytochrome *P*-450_{BM-3} and Bm3R1 protein were identified using a polyclonal rabbit antiserum (1:2000), raised to the reductase domain of BM-3 and the purified recombinant repressor respectively [5], followed by donkey anti-rabbit serum (1:2000) conjugated with horseradish peroxidase. 4-Chloro-1-naphthol was used as the peroxidase substrate. To increase the sensitivity of the Western blot, filters were further incubated for 60 min with Protein A labelled with ²⁵I followed by extensive washing [16]. Bands were then visualized by autoradiography.

Cloning of Bm3R1

Bm3R1 was cloned, expressed and purified as described previously [5].

EMSA

EMSAs were carried out by established protocols [17]. A double-stranded oligonucleotide, encompassing the binding site of Bm3R1 (5'-CGGAATGAACGTTTCATTCG-3') [9], was incubated with *B. megaterium* 30000 *g* supernatant extracts (20

μ g). All assays were carried out in 20 μ l (final volume) EMSA buffer [60 mM KCl, 12 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol and glycerol (10%, v/v)] containing 2 μ g of carrier DNA poly(dI-dC) at room temperature for 15 min. Radioactive oligonucleotide (1 ng) was then added and the sample incubated for a further 15 min at room temperature. Drugs, diluted in EMSA buffer, were added to the incubations before the addition of the oligonucleotide. After incubation the entire sample was loaded on to a 4% non-denaturing polyacrylamide gel, electrophoresed at 16 mA constant current, dried and autoradiographed. A similar protocol was adopted when recombinant Bm3R1 protein was used. In this case, 1 μ g of Bm3R1 was used in each reaction. The binding specificity of Bm3R1 to its operator sequence has been demonstrated previously using mutant and non-specific competitor oligonucleotides [5,7]. Antibodies to Bm3R1 were used to establish the presence of this protein in the DNA-protein complex. Crude extract (30 μ g) obtained from nafenopin (100 μ M)-, Wy14643 (100 μ M)- and phytanic acid (400 μ M)-treated cells were incubated with 1 μ l of undiluted antiserum to Bm3R1 or preimmune serum for 15 min before probe addition and then for a further 10 min at room temperature. This antibody has been shown to exhibit a high specificity for Bm3R1 [5].

Assay of cytochrome *P*-450_{BM-3}-associated fatty acid oxidation

This was carried out by measuring substrate-induced NADPH oxidation, essentially as described previously [5] except that oleic acid was used as the fatty acid substrate.

Cytochrome *P*-450_{BM-3}-mediated oxidation of phytanic acid

Substrate-dependent NADPH oxidation activities were determined in 0.1 M potassium buffer, pH 7.4, at room temperature, as described by Black *et al.* [18]. The rate of NADPH oxidation was measured in the presence of purified recombinant cytochrome *P*-450_{BM-3} (expressed in *Escherichia coli*), phytanic acid and NADPH using a Cary 3E UV-visible spectrophotometer. The concentration of purified cytochrome *P*-450_{BM-3} was determined as described by Omura and Sato [19]. Buffer, enzyme and substrate were added to a 1 cm quartz cuvette to establish a reference absorbance, and 200 μ M NADPH was then added to initiate the reaction. The phytanic acid substrate was added as a solution in DMSO.

The Michaelis constant (K_m) and V_{max} of phytanic acid were derived from direct linear plots of the initial rate (V_o), of the cytochrome *P*-450_{BM-3}-dependent reaction versus substrate concentration [S], as described by Cornish-Bowden [20]. This method uses median estimates to reduce the relative error. The Sigmaplot Scientific Graph System Version 4.14 from Jandel corporation was used to determine the V_o of each reaction from the original recorded data and was used to plot the data.

Purification and analysis of the hydroxy acid derivatives of phytanic acid

Phytanic acid (5 mg; 16 μ mol) in 160 μ l of DMSO was mixed with 290 nM purified cytochrome *P*-450_{BM-3} in 30 ml of 0.1 M potassium phosphate buffer, pH 7.4, incubated at room temperature for 5 min, and then 10 ml of an NADPH-generating system added. The NADPH-generating system was produced by incubating 4.42 mM glucose 6-phosphate, 1.0 mM NADP⁺ and 3.02 mM MgCl₂ in 10 ml of 0.1 M potassium phosphate buffer, pH 7.4, at 37 °C for 5 min, then adding 0.5 unit of glucose-6-phosphate dehydrogenase, type XI (Sigma Chemical Co., St. Louis, MO, U.S.A.), and incubating the resulting solution at

37 °C for another 5 min. After 30 min at 37 °C the reaction was terminated by the addition of 4 ml of 1 M HCl, which reduced the pH to 2.0. Ethyl acetate (50 ml) was then added, the mixture mixed thoroughly with a vortex mixer and then centrifuged for 20 min. The resulting ethyl acetate phase was separated, and the extraction was repeated. The combined extracts were dried with Na_2SO_4 and the ethyl acetate evaporated. The residue was then resuspended in 100 μl of SIGMA-SIL-A reagent and analysed using a Shimadzu QP 5000 GC-MS. A DB5-MS column was used with helium as the carrier gas. The separation was performed at 60 °C for 4 min, and then the temperature was increased at 30 °C/min to a final value of 300 °C.

Parallel extracts were resuspended in DMSO and used in EMSA experiments to examine whether the phytanic acid metabolites could be used to displace the Bm3R1 repressor from its palindromic operator.

RESULTS

In previous work we demonstrated that peroxisome proliferators and non-steroidal anti-inflammatory drugs were effective inducers of cytochrome $P-450_{\text{BM-3}}$ in *B. megaterium* [5]. The rationale for these observations was that foreign compounds may mimic fatty acids or similar signalling molecules in the regulation of this gene. To gain further insight into the interactions with this system, we chose to study the effect of phytanic acid on events that are associated with the induction of the cytochrome $P-450_{\text{BM-3}}$ operon.

Phytanic acid has the advantage that, unlike many other fatty acids, particularly unsaturated fatty acids, it is relatively non-toxic to *B. megaterium* (N. English, C. N. A. Palmer, W. L. Alworth, L. Kang, V. Hughes and C. R. Wolf, unpublished work). On exposure of cells to this compound a potent dose-dependent induction of cytochrome $P-450_{\text{BM-3}}$ was observed (Figure 1).

Figure 1 Effects of phytanic acid on cytochrome $P-450_{\text{BM-3}}$ expression

Cytosolic proteins (20 μg) from cells treated with the increasing concentrations of phytanic acid were subjected to Western-blot analysis using an antibody to cytochrome $P-450_{\text{BM-3}}$. Lanes were, from left to right, DMSO-treated, 100, 200 and 400 μM phytanic acid-treated cells.

Figure 2 Effects of various compounds on Bm3R1 expression

Cells treated with phytanic acid, barbiturates or peroxisome proliferators and the level of Bm3R1 protein expression was determined by Western-blot analysis using a polyclonal antibody to Bm3R1. A 30 μg sample of protein was loaded per lane from extracts of cells treated with (from left to right): DMSO (lane 2), phenobarbital (4 mM) (lane 3), pentobarbital (2 mM) (lane 4), secobarbital (2 mM) (lane 5), phytanic acid (400 μM) (lane 6), nafenopin (100 μM) (lane 7) or Wy14643 (100 μM) (lane 8). Lane 1 contains recombinant Bm3R1 as a molecular-mass standard.

It has been reported that barbiturates induce the transcription of both the BM3 and the Bm3R1 repressor genes [7]. To establish that this was the case for the peroxisome proliferators and phytanic acid, Western blots using an antibody to Bm3R1 were carried out on supernatant fractions from cells treated with these compounds. Phytanic acid, as well as the peroxisome proliferators, Wy14643 and nafenopin, were effective inducers of Bm3R1 expression (Figure 2). Surprisingly, the three barbiturates tested, although capable of inducing cytochrome $P-450_{\text{BM-3}}$ (results not shown), did not induce Bm3R1 protein to a measurable extent (Figure 2). The presence or absence of Bm3R1 in the cell extracts was confirmed by EMSA using a synthetic oligonucleotide corresponding to the Bm3R1 operator site. Cell extracts from phytanic acid-treated cultures showed increased protein–DNA complex formation in a manner dependent on the dose of the inducing agent (Figure 3A). The binding of Bm3R1 to DNA is not affected by the inducing agent in these assays as the inducing agent is removed during the lysis procedure. Increased complex formation was also observed with cell extracts prepared from cultures that had been treated with peroxisome proliferators, nafenopin and Wy14643 (Figure 3B). In agreement with the Western-blot analysis, no detectable complex was observed when extracts obtained from cells grown in the presence of barbiturates were used (Figure 3C). To verify that Bm3R1 was a major component of this complex, two experiments were performed. In the first, extracts from the peroxisome proliferator- and phytanic acid-treated cells were incubated with a polyclonal antiserum to Bm3R1. This treatment completely inhibited complex formation (Figure 3B). Incubation with preimmune serum had essentially no effect on DNA-binding activity. This experiment alone does not preclude an indirect effect of Bm3R1 on the observed complex; however, in our second experiment we show that purified Bm3R1 binds directly to the operator sequence (Figure 3D). We also exploited the difference in molecular mass and charge of the endogenous and recombinant Bm3R1 that results from the presence of a histidine tag on the recombinant protein. In this experiment, recombinant Bm3R1 was added in increasing concentrations to the EMSA containing a constant amount of crude extract. The subsequent non-denaturing electrophoresis of this mixture resulted in an incremental loss of the endogenous complex with the faster mobility and the appearance of both a complex corresponding to the slower recombinant Bm3R1–DNA complex and a novel complex of intermediate mobility. This complex of intermediate mobility would suggest that the recombinant Bm3R1 is forming heterodimers with the endogenous binding activity. At high concentrations of recombinant Bm3R1, mainly homodimers of the recombinant Bm3R1 are observed. In addition to demonstrating that the complex formed in the cell extracts contains Bm3R1, these observations suggest that Bm3R1 binds to the operator as a homodimer. The above data, however, cannot exclude the possibility that, in the cell extracts, the Bm3R1 may also be present in heterodimeric complexes with unknown proteins of a similar mass.

The above experiments demonstrate that a fatty acid present in the natural environment has the capacity to induce cytochrome $P-450_{\text{BM-3}}$ by a mechanism probably involving binding to the repressor Bm3R1. To establish that such fatty acids are substrates of cytochrome $P-450_{\text{BM-3}}$, we studied whether cytochrome $P-450_{\text{BM-3}}$ could metabolize phytanic acid. Incubation of increasing concentrations of phytanic acid with cytochrome $P-450_{\text{BM-3}}$ caused a dose-dependent increase in the rate of NADPH oxidation (Figure 4). Cornish-Bowden analysis of the resulting data confirms that phytanic acid is a good substrate for cytochrome $P-450_{\text{BM-3}}$, with a calculated K_m of 20 μM . Indeed, under these

Figure 3 Bm3R1 operator DNA-binding activity in crude extracts from cells treated with phytanic acid, peroxisome proliferators or barbiturates

(A) Crude extracts (30 µg) derived from cells treated with the indicated concentrations of phytanic acid were subjected to EMSA using an oligonucleotide pair corresponding to the Bm3R1 operator as described in the Experimental section. (B) Crude extracts (20 µg) from cells treated with phytanic acid (Ph), pafenopin (Paf), or Wy14643 (Wy) were analysed for binding to the Bm3R1 operator oligonucleotide pair by EMSA. Anti-Bm3R1 polyclonal antiserum (1 µl) was added in lanes marked +. Preimmune serum (1 µl) was added in the lane marked (PI). (C) Bm3R1 operator DNA-binding activity of crude extracts (20 µg) from barbiturate-treated cells. Samples applied to lanes are (from left to right): oligonucleotide alone (Free probe), control extract (Control), extracts from cells treated with phenobarbital (Pb) (2 mM), pentobarbital (Pe) (2 mM), secobarbital (Se) (2 mM) and the peroxisome proliferator, Wy14643 (100 µM). (D) The effect of mixing crude extracts with the purified recombinant Bm3R1 protein on Bm3R1–operator DNA complex formation was analysed by EMSA. Reactions were performed as outlined in the Experimental section. Lanes contained, from left to right, 100 ng of recombinant Bm3R1 (Rec), 20 µg of cell-free extracts from cells treated with Wy14643 (100 µM) (Crude). The remaining lanes contained a constant amount (20 µg) of crude extract protein from Wy14643 (100 µM)-treated cells together with the indicated amounts of recombinant Bm3R1 protein.

assay conditions, phytanic acid proved to be a higher-affinity substrate for cytochrome *P*-450_{BM-3} than palmitic acid, which had an estimated K_m of 60.5 µM (results not shown).

To establish clearly that the phytanic acid was oxidized by cytochrome *P*-450_{BM-3}, the products of the incubation were analysed. TLC analysis of the reaction products clearly demonstrated the accumulation of a metabolite of phytanic acid (results not shown), and this metabolite was identified unambiguously by MS analysis as the ω -1 hydroxyphytanic acid (Figure 5).

The ω -1 hydroxylation product was tested for its ability to interact with the Bm3R1–operator complex using EMSA. In-

Figure 4 Evidence that phytanic acid is a substrate for cytochrome *P*-450_{BM-3}

Phytanic acid metabolism was measured using substrate-induced NADPH oxidation as outlined in the Experimental section. A Cornish-Bowden plot of the resulting data is shown. The Michaelis constants, K_m and k_m , for phytanic acid metabolism derived from the above plot are 20 µM and 0.015 $\Delta A_{340}/s$ (3.11×10^3 nmol of NADPH/min per nmol of cytochrome *P*-450_{BM-3}) respectively for 46.7 nM cytochrome *P*-450_{BM-3}.

triguingly, the hydroxyphytanic acid could only displace the repressor from its operator at concentrations between 50 and 100 µM, whereas phytanic acid was shown to be far more potent, being able to inhibit Bm3R1–DNA binding at concentrations between 1 and 5 µM (Figure 6). Furthermore, on incubation of *B. megaterium* cells with 200 µM hydroxyphytanic acid, the cytochrome *P*-450_{BM-3}-associated fatty acid hydroxylase activity was increased from 11 to 18 nmol/min per mg. This is a very poor induction compared with the levels achieved by treatment with phytanic acid (89 nmol/min per mg). This difference in potency between phytanic acid and hydroxyphytanic acid is in agreement with the EMSA studies, which showed that the hydroxyphytanic acid only displaced the repressor from its operator at very high concentrations. It is also possible that differences in uptake or clearance may contribute to the lack of potency of the hydroxyphytanic acid in the induction of cytochrome *P*-450_{BM-3} activity. It is noteworthy, however, that considerably higher concentrations of phytanic acid were required to induce cytochrome *P*-450_{BM-3} gene expression *in vivo*, compared with the concentrations needed to abrogate the Bm3R1–DNA complex *in vitro*. The reason for this is unclear but could be explained by the metabolism of phytanic acid by cytochrome *P*-450_{BM-3} or other fatty acid-modifying enzymes. These data demonstrate that the metabolized form of phytanic acid has a greatly reduced capacity to induce cytochrome *P*-450_{BM-3} expression.

DISCUSSION

In previous reports we have shown that foreign compounds with the capacity to induce cytochrome *P*-450 fatty acid mono-oxygenases in mammals also have the ability to induce a fatty acid mono-oxygenase in *B. megaterium* [5,6]. The finding that in *B. megaterium*, peroxisome proliferators and non-steroidal anti-inflammatory drugs can dissociate the Bm3R1 repressor from its operator DNA sequence thus leading to the activation of transcription of the cytochrome *P*-450_{BM-3} gene has led us to

Scan : 630 B.G. Scan : (626-628)
 Mass Peak : 95 Ret. Time : 13.483
 Base Peak : 117.05 (72311)

Figure 5 GC/MS analysis of the product formed from cytochrome *P-450_{BM-3}* oxidation of phytanic acid

The chemical structure of the trimethylsilyl ester of the principle ω -1 hydroxy acid derivative of phytanic acid (molecular mass 400 Da) is shown below its corresponding mass spectrum. Further details are given in the Experimental section.

Figure 6 Ability of ω -1 hydroxylated phytanic acid to disrupt the Bm3R1-DNA complex

EMSAs were carried out as outlined in the Experimental section. Each lane contained 1 μ g of purified recombinant Bm3R1. For the experiments with the hydroxyacid derivative, additions were, from left to right, 0, 1, 10, 20, 50 and 100 μ M oxidized phytanic acid mixture. For the phytanic acid experiments, additions were, from left to right, 0, 1, 5, 10 and 20 μ M phytanic acid.

hypothesize that these compounds are mimicking the actions of fatty acids. The fact that many of them form CoA esters supports this hypothesis. It has long been proposed that non-steroidal anti-inflammatory drugs function by the competitive inhibition of arachidonic acid metabolism by cyclo-oxygenase-1 and -2. This concept has been reinforced by the co-crystallization of cyclo-oxygenase-2 and ibuprofen; ibuprofen was found to be bound to the arachidonic acid-binding site of the molecule [21]. This further strengthens the argument that specific drugs and

environmental chemicals can modulate patterns of gene expression by mimicking fatty acids.

It is still not clear whether the endogenous activator of cytochrome *P-450_{BM-3}* expression is a fatty acid or another endogenous signalling molecule. However, in this study we have clearly shown that a specific fatty acid can be an effective inducer of cytochrome *P-450_{BM-3}* and provide evidence that its mechanism of action involves interaction with the Bm3R1 repressor. This interaction results in the activation of transcription of both Bm3R1 and cytochrome *P-450_{BM-3}*. Close parallels between this mode of action and that observed with the peroxisome proliferators were observed. It is interesting to note, however, that, unlike these compounds, the barbiturates did not cause a significant induction of the Bm3R1 protein. It has been reported by Shaw and Fulco [7] that Bm3R1 mRNA is increased after the administration of barbiturates. This indicates that the lack of induction of Bm3R1 may be due to some post-transcriptional event. The mechanism of this differential regulation of Bm3R1 expression is currently under investigation. We have also demonstrated that phytanic acid is a substrate for cytochrome *P-450_{BM-3}*. As a consequence, an ω -1 hydroxylated product is formed with a markedly reduced affinity for the Bm3R1 repressor as well as a reduced capacity to induce cytochrome *P-450_{BM-3}*. This would provide an elegant regulatory loop for the regulation of cytochrome *P-450_{BM-3}* expression by fatty acids. Whether there are endogenous fatty acid-signalling pathways in *B. megaterium* is not known. However, it is possible that the regulatory pathway represents a response to exogenous environmental fatty acids. Phytanic acid represents an excellent example of this, as it is a major breakdown product of vegetative plant matter, and thus will be found in the soil, which is the natural habitat of bacillus species [22]. The regioselectivity of the metabolism of the ω -1 carbon of phytanic acid is in contrast with the multiple points of metabolism seen for simple saturated fatty

Figure 7 Structure of phytanic acid and retinoids

acids. Straight-chain saturated fatty acids such as palmitic and stearic acid are hydroxylated promiscuously at the ω -1, 2 or 3 positions. However, recent studies have also shown that cytochrome *P*-450_{BM-3} can metabolize the highly unsaturated arachidonic and eicosapentaenoic acids with exquisite regioselectivity [23].

We have previously remarked on the close parallels between the regulation of cytochrome *P*-450_{BM-3} and that of the mammalian CYP4A gene family [5,6]. There is growing evidence that the induction of cytochrome *P*-450 gene expression in mammals by foreign compounds can either be an adaptive response to toxic environmental challenge or through molecular mimicry of endogenous signalling involved in normal metabolic homeostasis [24]. The peroxisome proliferators activate the transcription of both cytochrome *P*-450_{BM-3} and the mammalian fatty acid hydroxylases. In the case of the mammalian enzymes, the fatty acids activate a nuclear receptor known as the peroxisome proliferator-activated receptor [25]. This receptor binds to the regulatory regions of the CYP4A genes as a heterodimer with the retinoid X receptor and mediates a peroxisome proliferator-dependent activation of transcription of these genes [26]. With this in mind it is particularly relevant to note that phytanic acid is a xenobiotic in mammals but is not an activator of the peroxisome proliferator-activated receptor/retinoid X receptor heterodimer [27]. The peroxisome proliferator-activated receptor has been shown to be a sensor for straight-chain unsaturated fatty acids [24,28]. In contrast, phytanic acid and its immediate precursor, phytyl acid, bind and activate homodimers of the nuclear retinoid X receptor [27,29]. This homodimeric complex may activate an overlapping subset of target genes that are also activated by peroxisome proliferator-activated receptor/retinoid X receptor, as the result of the similarity of their response elements [30]. We have not yet examined the potential for phytanic acid to regulate the CYP4A genes in rodents.

A human syndrome known as Refsum's disease is caused by a deficiency in phytanic acid metabolism [22]. Increased levels of phytanic acid are found in the tissues of afflicted individuals. These individuals suffer from neuropathies and retinal disorders which are reduced when a phytanic acid-free diet is implemented. It is not yet clear to what extent these disorders are mediated by inappropriate activation of the retinoid X receptor. These results do, however, suggest that the phytanic acid may act as a retinoid

mimic, and examination of the branched structure of phytanic acid reveals similarity to retinoids including all-*trans*-retinoic acid and 9-*cis*-retinoic acid (Figure 7). Studies are in progress to determine if retinoids are effective inducers of cytochrome *P*-450_{BM-3}.

It will be intriguing to establish whether there are further parallels between the *B. megaterium* system and the mammalian system, as this could provide further insight into how fatty acids and other lipophilic nutrient signals may be acting as transcriptional regulators of gene expression.

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