Induction of cytochrome *P*-450_{BM-3} (CYP 102) by non-steroidal anti-inflammatory drugs in *Bacillus megaterium*

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Bacillus megaterium contains a cytochrome *P*-450 fatty acid mono-oxygenase which is inducible with barbiturate drugs. We have demonstrated that this enzyme system is inducible with peroxisome proliferators. In mammals, peroxisome proliferators also induce mono-oxygenases in the CYP4A gene family. In this paper we demonstrate that the non-steroidal anti-inflammatory drugs ibuprofen, ketoprofen and indomethacin are potent inducers of fatty acid mono-oxygenase activity as well as of *P*-450_{BM-3} protein in *B. megaterium*. The levels of induction of *P*-450 protein were 11.8-, 3.9- and 3.0-fold respectively. In addition,

we demonstrate that these inducing agents interact with a transcriptional repressor, Bm3R1, which leads to its dissociation from its operator sequence. This provides a rational mechanism for the induction process. This is the first report which demonstrates that non-steroidal anti-inflammatory drugs can interact directly with a transcription factor to initiate gene expression, and further substantiates the structure–activity relationships that identify inducers of cytochrome *P*-450_{BM-3} and compounds that have the potential to act as peroxisome proliferators and induce CYP4A expression in mammals.

INTRODUCTION

Cytochrome *P*-450s are a superfamily of mono-oxygenases involved in the oxidation of a wide range of compounds, including steroids, fatty acids and xenobiotics such as drugs and environmental chemicals. Hepatic levels of many mammalian cytochrome *P*-450s are increased by a wide range of compounds, and over the last decade much progress has been made in elucidating the molecular mechanisms mediating the regulation of these enzymes [1].

A range of structurally diverse chemicals, including fibrates, hypolipidaemic agents, herbicides and industrial plasticizers, can induce a well defined pleiotropic response in the livers of rodents and other mammals [2]. This involves hyperplasia, hypertrophy, peroxisomal proliferation and the co-ordinate induction of a number of genes involved in the oxidation of fatty acids [3]. Also associated with this phenomenon is the proliferation of the smooth endoplasmic reticulum and the induction of members of the cytochrome *P*-450 CYP4A subfamily, which are involved in the hydroxylation of fatty acids [4]. A member of the steroid hormone receptor superfamily, the peroxisome proliferator-activated receptor, has been implicated in the transcriptional activation of a variety of genes encoding peroxisomal enzymes involved in fatty acid metabolism [5]. This receptor has been shown to be activated by a variety of peroxisome proliferators and fatty acids, although it is not known whether these small molecules interact directly with the receptor [6].

The prokaryote *Bacillus megaterium* contains a barbiturate-inducible *P*-450, termed cytochrome *P*-450_{BM-3} [7]. This enzyme has a molecular mass of 117.5 kDa and possesses both cytochrome *P*-450 reductase and *P*-450 mono-oxygenase activities on the same polypeptide. It also has a similar molecular architecture to nitric oxide synthase, another fusion protein possessing both haem and reductase domains [8]. Cytochrome *P*-450_{BM-3} is the most catalytically efficient cytochrome *P*-450-dependent mono-oxygenase known. This enzyme, which is a fatty acid hydroxylase,

exhibits most sequence identity with members of the mammalian CYP4A subfamily [9]. The operon encoding the cytochrome *P*-450_{BM-3} gene is under negative regulation by a transcriptional repressor termed Bm3R1 [10]. It has been shown that a mutant with a single point mutation (Gly to Glu at amino acid 39) in the DNA binding domain of Bm3R1 inactivates the repression and allows cytochrome *P*-450_{BM-3} to be constitutively expressed [10]. Thus the inhibition of Bm3R1 binding to its DNA operator is tightly coupled to the induction of the cytochrome *P*-450_{BM-3} gene.

We have previously demonstrated that cytochrome *P*-450_{BM-3} is markedly induced by a variety of structurally diverse peroxisome proliferators [11]. Furthermore, we have shown that the effects of these agents are mediated by interaction with the Bm3R1 repressor to induce cytochrome *P*-450_{BM-3} gene expression. This repressor also interacts with fatty acids. We also observed that the relative potency of the peroxisome proliferator response in *B. megaterium* was closely correlated with the documented potency of the drugs in mammals.

The non-steroidal anti-inflammatory drugs (NSAIDs) are a family of structurally diverse molecules which have the ability to inhibit cyclo-oxygenase. This enzyme family catalyses the production of the peroxide derivative of arachidonic acid, which is a precursor of a variety of prostanoid hormones [12]. Most of these agents are competitive inhibitors of either cyclo-oxygenase 1 or cyclo-oxygenase 2, or both [13]. A notable exception is aspirin, which has been shown to covalently modify Cox-1 through acetylation of a serine residue in the enzyme [14]. The metabolic products of the eicosanoid cascade include the prostaglandins, thromboxanes and leukotrienes [12]. The NSAIDs are widely used as effective analgesics, anti-inflammatory and antipyretic agents. Several of these compounds are converted intracellularly into CoA thioester derivatives [15], and in this manner are analogous to fatty acids in their metabolism. This suggests that cells respond to these agents as if they were unusual fatty acids. The structures of some of these compounds are shown in

Abbreviations used: NSAIDs, non-steroidal anti-inflammatory drugs; EMSA, electrophoretic mobility shift assay.

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[A] Non-Steroidal Anti-Inflammatory Drugs

[B] Peroxisome Proliferators

Figure 1 Structures of some NSAIDs and peroxisome proliferators

Figure 1. In general they have a long and safe clinical history, although one compound in this family, benoxaprofen, was removed from the market in 1984 following reports of associated liver toxicity in elderly patients [16].

The biochemical basis for the toxicity of benoxaprofen remains unknown, but it has been demonstrated that it can induce liver microsomal cytochrome *P*-450s in the rat, in particular CYP1A1 [16]. Recently Rekka et al. [17] reported that ibuprofen may elicit biochemical changes in rat liver typically induced by peroxisome proliferators. Study of the structural requirements for inducers of cytochrome *P*-450_{BM-3} led us to hypothesize that NSAIDs may also be inducers of this enzyme. We therefore investigated whether this was the case, as it would give important insights into the parallels between the mammalian and prokaryotic systems. The present work identifies important similarities in the properties of peroxisome proliferators and NSAIDs.

EXPERIMENTAL

Reagents

NSAIDs were purchased from Sigma, and were of the best quality available. Peroxisome proliferators were generously provided by Zeneca Central Toxicology Laboratories, Macclesfield, U.K. Antibodies to cytochrome *P*-450_{BM-3} and oligonucleotides were produced at the ICRF Laboratories, Clare Hall, Herts., U.K. Donkey anti-rabbit secondary antiserum was obtained from SAPU, Lanark, Lanarkshire, Scotland, U.K. *B. megaterium* A.T.C.C. 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 [11]. *B. megaterium* were grown at 37 °C with aeration in the 1 % (w/v) glucose salts medium described by

Grete [18] 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 absorbance of 0.6 at 600 nm. Drugs were added as required in DMSO; 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. The 30000 *g* supernatant fractions were used for enzyme assays and immunoblotting, and the 100000 *g* supernatant fractions were used for spectroscopic cytochrome *P*-450 quantification. All protein concentrations were quantified by the method of Lowry et al. [19].

Enzyme assays and total cytochrome *P*-450 quantification

Fatty acid hydroxylase activities associated with cytochrome *P*-450_{BM-3} were determined at room temperature by measuring the rate of NADPH oxidation using the substrate oleic acid, as described previously [20]. Each reaction was carried out in a volume of 1 ml containing 0.1 M potassium phosphate buffer, pH 8.0, between 200 and 800 μ g of 30000 *g* supernatant protein and NADPH (100 μ M). The reactions were initiated by the addition of sodium oleate (48 μ M) and the change in absorbance at 340 nm was monitored on a Shimadzu MPS 2.000 spectrophotometer. Initial reaction rates were measured for 30–60 s after the addition of oleate, when minimal uncoupling occurs [20]. The rate of oleic acid oxidation was calculated using the molar absorption coefficient for NADPH of 6.2 mM⁻¹·cm⁻¹. Total cytochrome *P*-450 content was calculated from the carbon monoxide reduced difference spectrum as described by Omura and Sato [21]. Absolute concentrations were calculated using a molar absorption coefficient of 91 mM⁻¹·cm⁻¹.

SDS/PAGE and Western blot analysis

SDS/PAGE and immunoblotting were performed as described previously [11,22–24].

Cloning of Bm3R1

Bm3R1 was cloned, expressed and purified as described previously [11].

Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out according to established protocols [25]. Drugs were dissolved in EMSA buffer [60 mM KCl, 12 mM Hepes, 12 % glycerol, 1 mM EDTA, 1 mM dithiothreitol and 2 μ g of poly(dI-dC)] in a final volume of 20 μ l. Drugs together with 1 mg of purified recombinant Bm3R1 [11] were added at the final concentrations indicated in the figure legends and left to incubate at room temperature for 15 min prior to probe addition. A 1 ng portion of radioactive double-stranded oligonucleotide encompassing the binding site for Bm3R1 (5'-CGGAATGAAC-GTTCATTCCG-3') was then added and allowed to incubate for a further 10 min. The entire reaction mixture was loaded on to a 4 % non-denaturing polyacrylamide gel and separated by electrophoresis. Gels were dried and bands visualized by autoradiography to reveal protein complexes with DNA. The specificity of the interaction between Bm3R1 and its operator was examined in previous work [11] and was shown to be highly specific for this oligonucleotide.

RESULTS

We demonstrated previously that a variety of peroxisome proliferators can induce cytochrome *P*-450_{BM-3} in *B. megaterium* A.T.C.C. 14581 [11]. Examination of the structures of the inducers indicated that an acidic functional group as well as a hydrophobic region were required for inducing activity. A variety of propionic acid and acetic acid derivative NSAIDs were observed to possess these chemical properties and were therefore tested as inducers. Treatment of cells with ibuprofen, ketoprofen and indomethacin caused marked increases in the total cytochrome *P*-450 content of 11.8-, 3.9- and 3.0-fold respectively over controls (Figure 2; Table 1). The induction observed using ibuprofen was more marked than with the previously described inducers nafenopin and secobarbital. In addition, we tested the ability of these compounds to induce cytochrome *P*-450_{BM-3}-mediated metabolism of oleic acid (Table 1). Ibuprofen, ketoprofen and indomethacin profoundly increased fatty acid hydroxylase activity to levels equivalent to or higher than those induced by the peroxisome proliferator nafenopin or the barbiturate secobarbital. There was a close agreement between the induction of activity and total cytochrome *P*-450 content (Table 1). These data support our hypothesis and demonstrate that NSAIDs are indeed inducers of cytochrome *P*-450_{BM-3} in *B. megaterium*. Two NSAIDs tested, aspirin and naproxen (results not shown), had no effect on activity or cytochrome *P*-450 levels.

In order to show that the induced *P*-450 was cytochrome *P*-450_{BM-3}, we carried out Western blot analysis. All three compounds tested induced cytochrome *P*-450_{BM-3} in a dose-dependent manner (Figure 3). In all cases the extent of induction was not saturable because of the toxicity of the compounds at the higher doses.

Peroxisome proliferators can interact with the Bm3R1 repressor, dissociating it from its DNA operator sequence to allow transcription to proceed [11]. In order to investigate whether this was the case for the NSAIDs, EMSAs were performed with the purified recombinant repressor using a synthetic oligonucleotide encompassing the binding site for Bm3R1. The recombinant

Figure 2 Effect of NSAIDs on cytochrome *P*-450 expression in *B. megaterium*

Ferrous cytochrome *P*-450-carbon monoxide spectra in *B. megaterium* 100 000 *g* supernatant fractions (15 mg/ml) are shown for cells treated with vehicle alone (left), ibuprofen (2 mM; middle) and ketoprofen (2 mM; right). Treatment details are given in the Experimental section.

Table 1 Effects of NSAIDs on cytochrome *P*-450 levels and associated oleic acid hydroxylase activity in *B. megaterium*

See the Experimental section for details. Cytochrome *P*-450 content is given in pmol/mg of supernatant protein, and activity is given in nmol of NADPH oxidized/min per mg of supernatant protein.

Treatment	<i>P</i> -450 content (pmol/mg)	<i>P</i> -450 _{BM-3} activity (nmol/min per mg)
Control	7.45	5.0
Aspirin (2 mM)	10.2	4.8
Ibuprofen (2 mM)	88.0	370
Ketoprofen (2 mM)	29.1	151
Secobarbital (2 mM)	27.2	119
Nafenopin (0.2 mM)	34.0	76
Indomethacin (1 mM)	22.4	52

Figure 3 Induction of cytochrome *P*-450_{BM-3} by NSAIDs

Cells were treated with different concentrations of NSAIDs and 30 000 *g* fractions prepared. Samples of 30 μ g of protein were then separated by SDS/PAGE, and Western blot analysis was carried out using a specific polyclonal antibody to cytochrome *P*-450_{BM-3}. The concentrations of each drug used in the culture media were, from left to right: ibuprofen (0, 10, 50, 200, 500 and 2000 μ M); ketoprofen (0, 50, 200, 500 and 2000 μ M) and indomethacin (0, 50, 200, 500 and 1000 μ M). Other details are given in the Experimental section.

protein formed a complex with the labelled oligonucleotide which could be dissociated by ibuprofen, ketoprofen and indomethacin, as well as by other cytochrome *P*-450_{BM-3} inducers such as nafenopin and secobarbital (Figure 4, upper panel). In contrast, incubation with aspirin or vehicle alone did not dissociate the Bm3R1-DNA complex, confirming the specificity of the interaction. Furthermore, ibuprofen dissociated Bm3R1 binding to DNA in a dose-dependent manner that closely followed its potency as a cytochrome *P*-450_{BM-3}-inducing agent (Figure 4, lower panel).

Figure 4 Effects of NSAIDs on the binding of the Bm3r1 repressor to its DNA operator

Upper panel: comparison of various compounds. Purified recombinant Bm3R1 (1 μ g) was incubated with the various NSAIDs, secobarbital or nafenopin. Incubations were carried out as described in the Experimental section. A sample of 1 ng of 32 P-labelled 20-mer oligonucleotide encompassing the palindromic operator site for Bm3R1 was used in each reaction. Lane 1, free DNA without protein; lane 2, Bm3R1 alone. Other assays were carried out by the addition of the specific drugs to the reaction mixture: lane 3, aspirin (2 mM); lane 4, ibuprofen (2 mM); lane 5, ketoprofen (2 mM); lane 6, nafenopin (0.2 mM); lane 7, secobarbital (2 mM); lane 8, indomethacin (1 mM). Lower panel: potency of ibuprofen in dissociating the Bm3R1–DNA complex. Reactions were carried out as described above. The concentrations of ibuprofen used, from left to right, were: lane 1, free DNA without Bm3R1; lanes 2–8, 0, 100, 250, 500, 800, 1000 and 2000 μ M ibuprofen respectively.

DISCUSSION

We have found that a variety of NSAIDs are inducers of cytochrome *P*-450_{Bm-3}. Rekka et al. [17] have shown that these drugs induce CYP4A1 in rats. These results suggest that NSAIDs have peroxisome proliferator-like properties. Furthermore, they link NSAID action with fatty acid metabolism. This is supported by our previous finding that fatty acids, particularly unsaturated species, can interact with the Bm3R1 repressor to induce cytochrome *P*-450_{Bm-3} gene expression [11]. The finding that the NSAIDs also appear to regulate gene expression in *B. megaterium* by directly interacting with Bm3R1 strongly suggests that these compounds are mimicking the effects of endogenous fatty acids.

The intriguing finding that NSAIDs can bind directly to the Bm3R1 repressor provides the first evidence that these compounds can associate with a transcription factor to alter patterns of gene expression. Furthermore, the observation that these compounds interact directly with the repressor *in vitro* provides evidence that it is not necessarily the acyl-CoA ester that is the active form. In support of this, barbiturate drugs that also have the capacity to induce cytochrome *P*-450_{Bm-3} do not form CoA esters. In addition, Hertz et al. [26] have shown that the co-

expression of acyl-CoA synthetase had a negative rather than a positive effect on peroxisome proliferator-activated gene expression. This finding favours the idea that peroxisome proliferators are more active in the non-esterified form. This is unlike the only other example of fatty acids acting as transcription factors, where in *Escherichia coli* only the CoA ester form associates with the FadR transcription factor [27]. If, like peroxisome proliferators, NSAIDs mimic fatty acids in the cell, then it is conceivable that they will associate with fatty acid binding proteins and directly modulate fatty acid homeostasis in mammals. This represents an alternative aspect to the mechanisms of action or side effects of these drugs. The strong similarity in the range of compounds that induce cytochrome *P*-450_{Bm-3} and CYP4A1 in mammals implies that the endogenous signalling pathways with which they interact have been highly conserved during evolution.

The ability to induce cytochrome *P*-450 CYP4A expression appears to be dependent on the presence of a carboxylic acid moiety and a hydrophobic domain. Certain inducers, such as the barbiturates, do not contain this carboxy group; however, this class of compounds still possesses considerable acidic character due to the tautomerization of the pyrimidine ring to the phenolic form. Thus the pyrimidine ring may act as a bioisostere of a carboxylic acid functional group. These structural requirements are intriguing, as they mirror the situation in mammals, where a variety of compounds not possessing a carboxy functional group, such as the tetrazole-substituted acetophenones, are potent inducers of peroxisomal proliferation and CYP4A1 [28]. Other members of the NSAID family, i.e. aspirin and naproxen, were found not to induce cytochrome *P*-450_{Bm-3}. Aspirin does not possess considerable hydrophobic character, while naproxen contains a polar methoxy group which may disrupt its hydrophobic interaction with Bm3R1. These data strongly indicate that the structure–activity relationships of NSAIDs and peroxisomal proliferators are strongly conserved, with a similar level of complexity observed in mammals and bacteria.

In summary, these data provide insights into common biochemical pathways linking NSAIDs and peroxisome proliferators. They also indicate that the use of *B. megaterium* may help to identify and characterize common features in these classes of compounds. The common denominator in the gene regulatory effects of these compounds appears to involve perturbations of fatty acid homeostasis.

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