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Antioxidant-Mediated Attenuation of the Induction of Cytochrome P450BM-3(CYP102) by Ibuprofen in *Bacillus megaterium* ATCC 14581

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ABSTRACT. *Bacillus megaterium* contains a soluble cytochrome P450 termed BM-3, which is highly inducible by barbiturates, peroxisome proliferators, and nonsteroidal antiinflammatory drugs. In rats and mice, the chronic administration of peroxisome proliferators induces a sustained oxidative stress in hepatic tissue and may be responsible for the nongenotoxic carcinogenesis observed with prolonged treatment. Here it is shown that ibuprofen induces a variety of enzymes associated with the oxidative stress response in *Bacillus*, including catalase, glucose-6-phosphate-dehydrogenase, and aldehyde reductase in a dose-related manner. Furthermore, evidence is presented to show that the expression of cytochrome P450 in *Bacillus* is associated with a marked depletion in cellular glutathione levels and that it renders these cells considerably more sensitive to oxidant insult. Finally, this work reports that a variety of structurally diverse antioxidants such as ascorbic acid, reduced glutathione, α-tocopherol acetate and the artificial antioxidant, butylated hydroxyanisole, all dramatically attenuate the expression of the cytochrome P450BM-3 gene and its repressor, Bm3R1, following ibuprofen treatment. These observations provide the first evidence that the expression of cytochrome P450 genes can lead to increased oxidant sensitivity but can be strongly modulated by dietary and artificial antioxidants, as well as antioxidant enzymes. The important implications of this phenomenon are also discussed. BIOCHEM PHARMACOL 54;4:443–450, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Cytochrome P450BM-3; induction; antioxidants; oxidative stress

Bacillus megaterium ATCC14581, contains a soluble cytosolic cytochrome P450, termed BM-3, active in the oxidation of fatty acids at the ω-1, ω-2 and ω-3 positions [1]. This enzyme is dramatically induced by a variety of structurally diverse chemicals including barbiturates [2], peroxisome proliferators [3], and nonsteroidal antiinflammatory drugs [4]. The mechanism for this induction involves the derepression of the cytochrome P450BM-3 operon through the interaction of these drugs with a transcriptional repressor, Bm3R1, to mediate the induction event [3, 4]. Furthermore, a study of the relative potency of the peroxisomal proliferators in *Bacillus* revealed a striking correlation with that obtained from studies in rodents [3]. This suggested that at least some of the effects of peroxisome proliferators appear to be conserved through evolution.

Peroxisome proliferator treatment produces very well-defined biochemical and morphological changes in the livers of rats and mice [5]. These include an increase in the size and the number of peroxisomes, the induction of genes encoding enzymes active in the peroxisomal oxidation of fatty acids, and the concomitant induction of members of the cytocrome P4504A† subfamily, which, like cytochrome P450BM-3, are also active in the hydroxylation of fatty acids [5]. Incidentally, cytochrome P450BM-3 is most highly related to members of the CYP4A gene subfamily. Moreover, the chronic administration of peroxisome proliferators to rats and mice induces a sustained oxidative stress thought to arise from the overstimulation of peroxisomal Acyl-CoA oxidase [6]. This is the first and rate limiting step in the oxidation of fatty-acyl-CoA species in peroxisomes. A product of this reaction is hydrogen peroxide, which is believed to provide a pro-oxidant environment intracellularly. In this capacity, it is hypothesised that the major carcinogenic effects of chronic peroxisome proliferator treatment may be attributed to the indirect ability of these compounds to produce reactive oxygen species, which modify biomolecules including nitrogenous bases in DNA.

A lesser studied question pertains to the relationship between cytochrome P450 expression and oxidative stress. It is known that cytochrome P450 can act as an NADPH oxidase, producing a number of reactive oxygen species [7,

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† *Abbreviations:* BHA, butylated hydroxy anisole; CFUs, colony forming units; CYP, cytochrome P450; DMSO, dimethyl sulphoxide; DNTB, 5,5′-Dithio-bis(2-nitrobenzoic acid); EMSA, electrophoretic mobility shift assay; GSSG, glutathione disulphide; HRP, horse radish peroxidase.

8], including superoxide anion ($O_2^{\cdot -}$) and singlet oxygen. $O_2^{\cdot -}$ could be converted to hydrogen peroxide through dismutation reactions and may also generate the highly reactive OH radical species through Fenton chemistry [9]. To address this possibility, the effects of cytochrome P450 expression on cellular resistance to oxidants as well as the effects of a number of natural and artificial antioxidants on the expression of cytochrome P450BM-3 were examined. These results demonstrate that cytochrome P450 expression significantly alters the redox status in *Bacillus* and that it is possible to modulate the expression profile of cytochrome P450 enzymes by the administration of dietary antioxidants.

MATERIALS AND METHODS

Reagents and Cells

B. megaterium ATCC 14581 was purchased from the American Type Culture Collection. The G39E mutant *B. megaterium* strain was a generous gift from Professor Armand J. Fulco, University of California at Los Angeles. All drugs and antioxidants, including catalase, used in this study were purchased from Sigma (Poole, Dorset) and were of the highest quality available. Polyclonal antisera to cytochrome P450BM-3 and the recombinant Bm3R1 were a generous gift from Professor Roland Wolf, ICRF, Dundee. Antirabbit IgG conjugated with horse radish peroxidase was purchased from Sigma.

Growth and Harvesting of Cells

B. megaterium cells were grown with aeration at 35° C in Glucose salts medium containing casamino acid hydrolysates as described previously [3, 4]. When cultures reached an optical density of 0.6 at 600 nm, ibuprofen was added to a final concentration of 1 mM using a stock ibuprofen solution in dimethyl sulphoxide (DMSO) together with various antioxidants. The water-soluble antioxidants, ascorbate and glutathione, were added directly to the cell culture, while the lipophilic species, α-tocopherol acetate and butylated hydroxy anisole (BHA) were dissolved in DMSO. Control cells were also treated with the maximum volume of DMSO (0.5% v/v). All cultures were incubated at this temperature for a further 8 hr. Cells were then pelleted by centrifugation (2,000 *g* for 5 min), the supernatant removed, and the pellets washed in ice-cold phosphate buffer (0.1 M), pH 7.4.

Cells were then resuspended in phosphate buffer before been disrupted by shearing using a minibeadbeater (Stratech Scientific, Luton, UK). Cell-free cytosolic extracts were obtained by centrifugation at 17,000 rpm. These 17,000 rpm supernatants were used in enzyme assays, Western Blot analysis, electrophoretic mobility shift assay (EMSA), and in the quantitation of cellular glutathione (GSH) levels.

Enzyme Assays

All assays were carried out on a Perkin–Elmer UV-visible recording spectrophotometer (Model Lambda 2). All reactions were carried out in a reaction volume of 1 mL. Catalase activities were monitored by following the disappearance of hydrogen peroxide at 240 nm in accordance with the method of Beers *et al.* [10]. Glucose-6-phosphate dehydrogenase activities were ascertained by following the reduction of $NADP^+$ at 340 nm as outlined previously [11]. Aldehyde reductase activities were measured by following the decrease in absorbance at 340 nm due to the oxidation of NADPH by *p*-nitrobenzaldehyde [12]. Cytochrome P450BM-3 activity was monitored as previously described by following the oleic acid-dependent oxidation of NADPH at 340 nm [3].

Determination of GSH Concentration

Bacterial GSH concentration was determined colourimetrically, based on the absorbance of the reaction product of GSH and 5,5′-Dithio-bis(2-nitrobenzoic acid)(DNTB) [13]. Although this technique is unable to differentiate between GSH and other low-molecular weight thiol containing compounds, GSH represents the overwhelming fraction of acid soluble thiols in many bacterial genera, including *Bacillus*.

The concentration of GSH was determined by measuring the reduction of DTNB at 412 nm using an absorbtivity coefficient of 13,600 M^{-1} cm^{-1}.

Protein Quantitation

All proteins were quantified by the method of Lowry *et al.* [14]. Bovine serum albumin was used as a protein standard.

Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) and Western Blot Analysis

SDS-PAGE was carried out using a 7.5 and 12% polyacrylamide gel for cytochrome P450BM-3 and the Bm3R1 [3] repressor, respectively. Cell-free supernatant extracts (30 μg), prepared as described previously [3, 4], were loaded onto into each lane. Resolved proteins were then transferred to nitrocellulose membranes and immunoblotting was carried out using polyclonal antisera to cytochrome P450BM-3 and Bm3R1. These polypeptides were visualised by incubation of these membranes with HRP-conjugated antirabbit antiserum and visualised by the addition of the HRP substrates, hydrogen peroxide and 4-chloro-2-naphthol, as outlined previously [3, 4].

Cell Viability Counts

B. megaterium 14581 cells and mutant G39E cells of the same strain were grown either in the absence or presence of 1.0 mM imidazole in glucose salts medium containing casamino acid hydrolysates to an optical density of 0.2 at 600 nm. Cells were then subjected to increasing concentrations of hydrogen peroxide (0, 2, 5, and 10 mM) for 20 min and diluted 10,000-fold with sterile medium before

TABLE 1. The effects of increasing concentrations of ibuprofen on the expression of cytochrome P450BM-3, catalase, aldehyde reductase (Ald-Reductase), and glucose-6-phosphate dehydrogenase (G-6-PDH)

Cell treatment Ibuprofen/mM	P450BM-3 (nmol/min/mg)*	Catalase μmol/min/mg†	Ald-Reductase nmol/min/mg‡	G-6-PDH nmol/min/mg§
0.0	13 ± 2	25 ± 7	6.5 ± 2	370 ± 15
0.2	25 ± 6	32 ± 8	8.9 ± 1	605 ± 22
0.4	97 ± 10	39 ± 4	9.7 ± 3	732 ± 17
0.8	194 ± 17	47 ± 8	10.4 ± 2	890 ± 16
1.0	255 ± 23	55 ± 5	14.4 ± 3	995 ± 24

Cells were treated with the indicated concentrations of ibuprofen and cell-free cytosolic extracts were obtained for the determination of cytochrome P450BM-3–associated oleic acid hydroxylase assays, catalase, aldehyde reductase, and glucose-6-phosphate dehydrogenase activities as outlined in the Materials and Methods section. The activities represent the means and standard deviations of three separate determinations.

* Cytochrome P450BM-3 activities expressed as nmols of NADPH oxidised/min/mg cytosolic protein.

† Catalase activities expressed as μmol of hydrogen peroxide degraded/min/mg cytosolic protein.

‡ Aldehyde reductase activities expressed as nmol NADPH oxidised/min/mg cytosolic protein.

§ G-6-PDH activities expressed as nmol $NADP^+$ reduce/min/mg cytosolic protein.

being plated out on nutrient agar plates. Petri dishes were left to incubate overnight at 37° C and the number of colony forming units (CFUs) enumerated. Imidazole was found to have essentially no effect on the viability of either the wild-type or the G39E cells (data not shown).

Electrophoretic Mobility Shift Assay (EMSA)

Assays were performed in accordance with established procedures [3, 4]. Briefly, 20 μg of cell-free cytosolic extracts from *B. megaterium* cells treated with various drugs as described above were incubated with EMSA buffer (60 mM KCl, 12 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol containing glycerol (10% v/v)]. Each incubation contained 2 μg of poly-DL-poly-dC as carrier DNA and 1 ng of a radiolabelled oligonucleotide encompassing the perfect palindromic binding site for Bm3R1. The final volume in each reaction mixture was 20 μL, and were left to incubate at room temperature for 20 min. The entire reaction mixture was then loaded onto a 4.5% nondenaturing polyacrylamide gel and the free DNA was resolved from protein-DNA complexes by electrophoresis in accordance with established protocols. The gel was dried and exposed to autoradiography. Supershift assays were essentially performed as above but with the addition of 1 μL of neat immune serum to Bm3R1 to the appropriate tubes as outlined in previous work [3].

The antioxidant EMSA experiment was performed in a similar manner to the EMSAs described above but with the addition of either α-tocopherol acetate (200 μM) or reduced glutathione (5 mM) to the EMSA buffer. Purified recombinant Bm3R1 (1 μg) [3, 4] was added to a series of reaction tubes, together with increasing concentrations of ibuprofen, 0, 100, 200, 400, 800, and 1,000 μM. The EMSA reaction contents were incubated with antioxidant, drug, and protein for 10 min at room temperature prior to the addition of radiolabelled probe. These reactions were then left for a further 10 min at room temperature before loading onto a 4.5% nondenaturing polyacrylamide gel and processed as described previously.

RESULTS

To establish whether the treatment of *B. megaterium* cells with peroxisome proliferators results in an oxidative stress, a number of enzyme activities associated with the oxidative stress response were examined. Treatment of *B. megaterium* cells with increasing concentrations of ibuprofen, (0, 200, 400, 800, and 1,000 μM) resulted in consistent dose-dependent inductions in cytochrome P450BM-3-associated activity, as well as, catalase, aldehyde reductase and glucose-6-phosphate dehydrogenase activities (Table 1). The level of induction varied from between 200 and 300% at the maximum dose of ibuprofen used (1 mM). This indicated that ibuprofen can induce a number of antioxidant enzyme defences in *Bacillus*, in concert with its ability to induce cytochrome P450BM-3–associated activity.

Because cytochrome P450 enzymes can catalyse a number of dismutation reactions which generate reactive oxygen intermediates [8], a study was performed on the oxidant tolerance of the wild-type *B. megaterium* strain compared to the G39E mutant, which has been characterised by its high, constitutive expression of cytochrome P450BM-3 [15]. The constitutive cytochrome P450BM-3 activity was reaffirmed and was found to be ~40-fold higher than wild-type *B. megaterium* (data not shown) (10 nmol/min/mg protein compared with 400 nmol/min/mg protein for the G39E mutant). Treatment of wild-type and G39E mutant cells with increasing concentrations of hydrogen peroxide revealed a notable difference in the degree of tolerance afforded by each strain. Wild-type cells displayed a fourfold increased resistance to hydrogen peroxide at a concentration of 2 mM compared to mutant cells (Fig. 1). Curiously, cotreatment of G39E cells with imidazole (2.0 mM), which acts as a general inhibitor of cytochrome P450 isoforms [16], resulted in a complete reversal of this sensitivity but

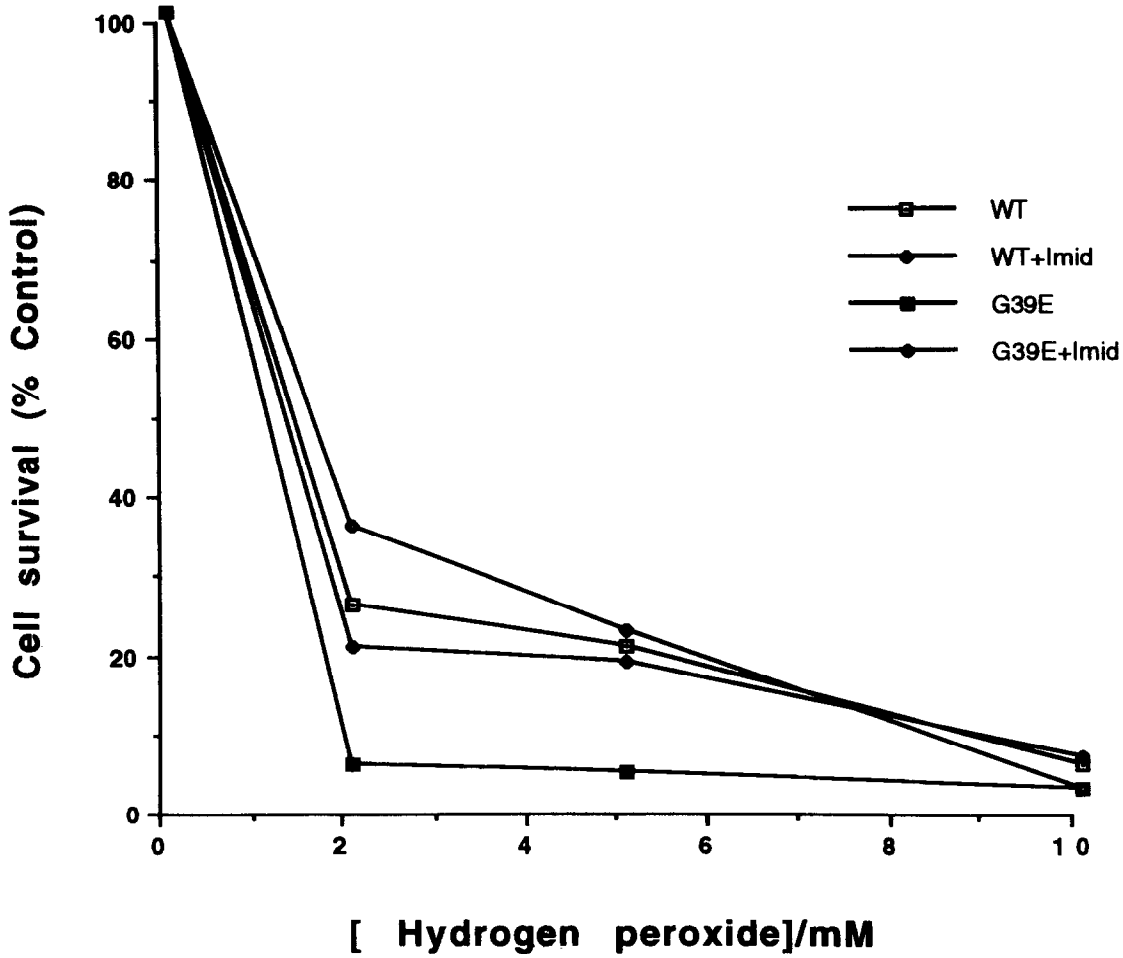

FIG. 1. The effects of oxidant insult and the P450 inhibitor, imidazole on the viability of wild-type and mutant *B. megaterium* cells. Wild-type and G39E mutant *B. megaterium* cells were grown in the absence or prescence of imidazole (1.0 mM) as outlined in Materials and Methods. Cells were then exposed to increasing doses of hydrogen peroxide (0–10 mM) and the number of colony forming units (CFUs) calculated after an appropriate dilution. The above shows the results of a typical experiment performed at least three times. Cell numbers not exposed to oxidant (0 mM) were taken as 100% viability in each case.

left the wild-type cells essentially unchanged (Fig. 1). These data suggested that enhanced sensitivity to oxidant insult occurs as a result of increased cytochrome P450 gene expression.

Next, the effects of a variety of antioxidants were examined for their ability to modulate the expression of the cytochrome P450BM-3 gene. Treatment of *B. megaterium* with ibuprofen (1 mM) alone produced a dramatic induction of cytochrome P450 activity relative to untreated cells, being induced over 20-fold relative to control activities (Table 2). Intriguingly, supplementation of the medium with increasing concentrations of ascorbic acid (3, 6, and 9 mM, respectively) with ibuprofen (1 mM) resulted in a dramatic attenuation of the induction of cytochrome P450BM-3–associated activity with complete inhibition of the induction phenomenon observed at 9 mM ascorbate (Table 2). In all cases, treatment of cells with antioxidants alone displayed P450 activities at or lower than controls (data not shown). These results suggested a possible role for reactive oxygen species in the induction event. To strengthen this hypothesis, cells were incubated with ibuprofen (1 mM) together with catalase (0.1 mg/mL) to degrade any hydrogen peroxide produced as a consequence of cytochrome P450 induction. Cytochrome P450BM-3–associated activity was consistently attenuated by approximately 50% relative to cells

TABLE 2. The effects of ascorbic acid on the ibuprofen-mediated expression of cytochrome P450BM-3–associated activity

Cell treatment	Cytochrome P450BM-3 activity (nmol/min/mg)*
Control (no addition)	17 ± 3
Ibuprofen (1 mM)	250 ± 47
Ibuprofen (1 mM) + ascorbate (3 mM)	94 ± 11
Ibuprofen (1 mM) + ascorbate (6 mM)	64 ± 9
Ibuprofen (1 mM) + ascorbate (9 mM)	14 ± 5

Cells were treated with the indicated drugs and cell-free cytosolic extracts were obtained for cytochrome P450BM-3–associated oleic acid hydroxylase assays as outlined in the Materials and Methods section. The activities represent the means and standard deviations of three separate determinations.

* Specific activities were expressed as nmol of NADPH oxidised/min/mg of cytosolic protein.

TABLE 3. Measured glutathione levels in wild-type and mutant *B. megaterium* cells constitutively expressing cytochrome P450BM-3 with various drug additions

Cell treatment	[Glutathione] (nmol/mg cytosolic protein)
Wild type (no addition)	12.1 ± 4
Wild-type + Ibuprofen (1 mM)	3.0 ± 0.7
Wild-type + Ibuprofen (1 mM) + Imidazole (1 mM)	9.2 ± 1
G39E mutant (no drug addition)	2.4 ± 0.5
G39E mutant + Imidazole (1 mM)	11.5 ± 2

Wild-type cells and G39E mutant cells constitutively expressing cytochrome P450BM-3 were treated with the indicated drugs and cell-free cytosolic extracts were obtained for quantitation of reduced glutathione levels using DNTB as outlined in the Materials and Methods section. The measured glutathione levels represent the means and standard deviations of four separate determinations.

treated with the addition catalase but was essentially unaffected by the addition of bovine serum albumin (BSA) at a similar concentration, or by heat inactivating the enzyme prior to its addition to the medium (data not shown).

Glutathione is by far the most abundant small molecular weight, acid-soluble thiol in bacterial cells and provides a first line defence against the damaging effects of free radicals. Measurement of the concentration of the reduced form of intracellular GSH should, therefore, provide a sensitive indicator of the redox status of the cell. It was, therefore, hypothesised that the induction of cytochrome P450 in *Bacillus* would cause a depletion in the levels of reduced GSH relative to cells with little or no expression of this enzyme. A study was carried out to quantify the levels of reduced glutathione in wild type and the G39E mutant under different drug regimens (Table 3). In comparison to untreated wild-type cells, cells exposed to ibuprofen (1 mM) displayed a 75% reduction in the levels of this cellular antioxidant, whereas cotreatment of wild-type cells with ibuprofen (1 mM) as well as the cytochrome P450 inhibitor, imidazole (1 mM), restored the cellular levels of glutathione close to, but not quite as high as untreated controls (Table 3). In agreement with the cell viability studies, measured levels of glutathione in untreated G39E cells was markedly reduced to less than 20% that of wild-type cells. Moreover, treatment of these cells with imidazole (1 mM) maintained glutathione concentrations at comparable levels to wild-type cells. These data clearly indicate that the expression of cytochrome P450 in *B. megaterium* severely depletes the levels of the abundant antioxidant, glutathione, but can be prevented by incubating the cells with a cytochrome P450 inhibitor.

To verify that this attenuation of cytochrome P450BM-3 expression was not peculiar to ascorbic acid, experiments were carried out to establish the effects of a number of other natural and artificial antioxidants (Table 4). Incubation of cells with medium containing reduced glutathione (5 mM), α-tocopherol acetate (200 μM) and butylated hydroxyanisole (100 μM) all resulted in a consistent reduction in the expression of cytochrome P450BM-3–associated activity. The reductions were most pronounced in the case of glutathione. Western blot analysis of the effects of increasing ascorbic acid concentrations (Fig. 2A) (0–9 mM) and the associated effects of the other antioxidants (Fig. 2B) were also evaluated and found to correlate well with the enzymatic data obtained. Although much higher concentrations of the water-soluble antioxidants were required than the lipophilic species studied, the former attenuated the expression of cytochrome P450BM-3 much more thoroughly (Fig. 2A and B). In contrast, addition of the oxidised, disulphide form of glutathione, GSSG (5 mM), had essentially no effect on the ibuprofen-mediated induction of cytochrome P450BM-3. These results demonstrate that antioxidants can decrease the expression of cytochrome P450 apoprotein levels and explained the observed decrease in absolute activity.

The cytochrome P450BM-3 gene is under negative regulation by a transcriptional repressor, termed Bm3R1. In previous work it has been shown that a number of peroxisome proliferators can also induce the expression of Bm3R1 in a dose-dependent manner [3]. Therefore, the effects of antioxidants on the ibuprofen-mediated expression of Bm3R1 was also investigated (Fig. 3A and B). Cells treated with ibuprofen (1 mM) alone dramatically induced the expression of Bm3R1, as evidenced by immunoblot analysis using a specific polyclonal antiserum to the purified, recombinant Bm3R1 protein (Fig. 4A). Furthermore, coadministration of increasing amounts of ascorbate (3, 6, and 9 mM)

TABLE 4. The effects of a variety of antioxidants on the ibuprofen-mediated expression of cytochrome P450BM-3-associated activity

Cell treatment	Cytochrome P450BM-3 activity (nmol/min/mg)*
Control (no addition)	12 ± 4
Ibuprofen (1 mM)	260 ± 17
Ibuprofen (1 mM) + Vit E acetate (0.2 mM)†	125 ± 14
Ibuprofen (1 mM) + BHA (0.1 mM)	97 ± 11
Ibuprofen (1 mM) + Glutathione (5 mM)	15 ± 3
Ibuprofen (1 mM) + Catalase (0.1 mg/mL)	130 ± 12

Cells were treated with the indicated drugs and cell-free cytosolic extracts were obtained for cytochrome P450BM-3–associated oleic acid hydroxylase activity as outlined in the Materials and Methods section. The activities represent the mean and standard deviations of three separate determinations.

* Specific activities were expressed as nmols NADPH oxidised/min/mg cytosolic protein.

† Vit E acetate = α-tocopherol-acetate.

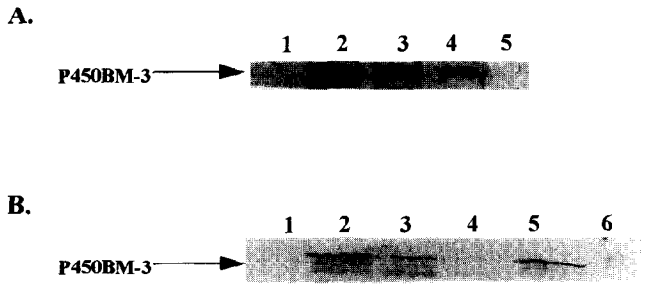

FIG. 2. The effects of antioxidants on the expression of ibuprofen-induced cytochrome P450BM-3 apoprotein. (A) Effects of ascorbic acid on P450BM-3 expression. Western blot analysis was carried out according to the procedure outlined in Materials and Methods. Lanes were from left to right, 1 = control (no addition), 2 = ibuprofen (1 mM), lanes 3, 4, and 5 were extracts obtained by growing cells in ascorbic acid at 3, 6, and 9 mM, respectively, together with ibuprofen (1 mM). (B) Effects of structurally diverse antioxidants on P450BM-3 expression. Cells were treated with the above antioxidants and ibuprofen as outlined in Materials and Methods. Lanes were 1 = control (no addition), 2 = ibuprofen (1 mM), all subsequent lanes contained ibuprofen (1 mM) together with, 3 = α-tocopherol acetate (200 μM), 4 = BHA (100 μM), and 5 = glutathione disulphide (GSSG) (5 mM), 6 = reduced glutathione (5 mM).

caused a marked dose-related decrease in the expression of this 19 kDa polypeptide (Fig. 4A). In accordance with that observed with cytochrome P450BM-3, administration of α-tocopherol acetate (200 μM) and glutathione (5 mM) also attenuated this response (Fig. 4B). Glutathione was again found to be the most effective in attenuating both cytochrome P450BM-3 and Bm3R1 gene expression.

To demonstrate that a decrease in the expression of Bm3R1 amounted to a concomitant decrease in associated DNA binding activity, electrophoretic mobility shift assays (EMSA) was carried out using cell free extracts obtained

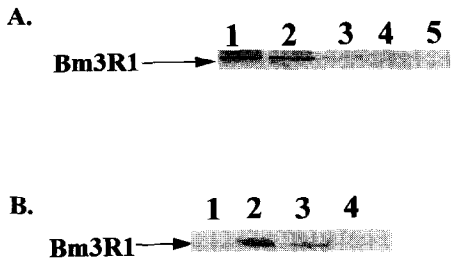

FIG. 3. Effects of antioxidants on the ibuprofen-mediated expression of the Bm3R1 repressor. (A) Effects of increasing concentrations of ascorbic acid on Bm3R1 expression. Cells were treated with the indicated concentrations of ascorbic acid as outlined in Materials and Methods. Lanes were from left to right, 1 = ibuprofen (1 mM), 2, 3, and 4 were extracts obtained from cells treated with increasing concentrations of ascorbic acid (3, 6, and 9 mM, respectively) together with ibuprofen (1 mM), 5 = control (no addition). (B) Effects of structurally diverse antioxidants on Bm3R1 expression. Cells were treated with the indicated concentrations of antioxidants together with ibuprofen (1 mM) as outlined in Materials and Methods. Lanes contained from left to right, 1 = control (no addition), 2 = ibuprofen (1 mM). Lanes 3 and 4 also contained ibuprofen (1 mM) together with cells treated with the following antioxidants: 3 = α-tocopherol acetate (200 μM), 4 = glutathione (5 mM).

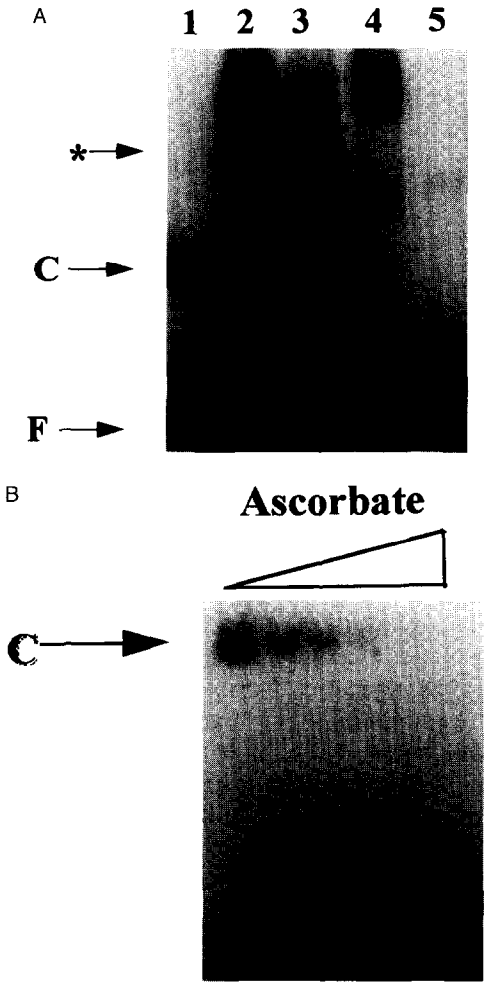

FIG. 4. The effects of antioxidants on the expression of Bm3R1-associated DNA binding activity. (A) Ibuprofen induces Bm3R1 binding to its palindromic operator. EMSA was carried out as outlined in Materials and Methods. Lanes illustrate additions. The supershift is indicated by the asterisk. C indicates the Bm3R1-DNA complex and F signifies the free radiolabelled oligonucleotide probe. Lanes 1, 2, 3, and 5 contained extracts of *B. megaterium* cells treated with ibuprofen (1 mM). Lane 1 = extract alone, lane 2 = extract with anti-Bm3R1 antisera, lane 3 = extract with preimmune serum, lane 4 = anti-Bm3R1antiserum alone, lane 5 = extract with 100-fold molar excess of unlabelled oligonucleotide probe. (B) The effects of ascorbate on the expression of Bm3R1-associated DNA binding activity. Cells were treated with ibuprofen (1 mM) alone or with increasing concentrations of ascorbic acid (from left to right 0, 1, 3, 6, and 9 mM, respectively) and subjected to EMSA analysis as described in Materials and Methods. The complex between Bm3R1 and its palindromic operator is indicated by the letter C.

from cells that had been treated with ibuprofen (1 mM) and increasing concentrations of ascorbic acid. Using a radiolabelled oligonucleotide probe encompassing the perfect palindromic binding site for Bm3R1 [3], an intense complex was obtained from extracts obtained from cells treated with ibuprofen (1 mM) alone (Fig. 4A). The specificity of this complex was verified by the addition of anti-Bm3R1 immune serum to the EMSA reaction and obtaining an intense supershift (Fig. 4A). In agreement with the immu-

noblot data, cells treated with ascorbic acid (1, 3, 6, and 9 mM), displayed a marked, dose-related reduction in the DNA binding activity (Fig. 4B). These data clearly indicate that antioxidants modulate the expression of Bm3R1 in a similar manner to cytochrome P450BM-3.

In an attempt to understand the nature of the inhibitory action of antioxidants on the expression of cytochrome P450BM-3, a series of EMSA reactions were performed to determine the *in vitro* effects of two antioxidants on the ability of ibuprofen to displace recombinant Bm3R1 from its DNA operator. In agreement with previous results, incubation of ibuprofen with a constant amount (1 μg) of recombinant Bm3R1, produced a dose-dependent displacement of the Bm3R1 repressor from its DNA binding site, with complete inhibition observed between 400 and 800 μM (Fig. 5A). This EMSA titration was repeated with the addition of a water-soluble antioxidant, glutathione (5 mM) (Fig. 5B), as well as the lipid soluble antioxidant, α-tocopherol acetate (200 μM) (Fig. 5C). Neither antioxidant additions had any significant effect on the ability of ibuprofen to displace the recombinant Bm3R1 protein from its DNA operator (Fig. 5). These results indicated that the inhibitory effects of antioxidants on cytochrome P450BM-3 expression cannot be attributed to a direct effect of antioxidants on the repressor.

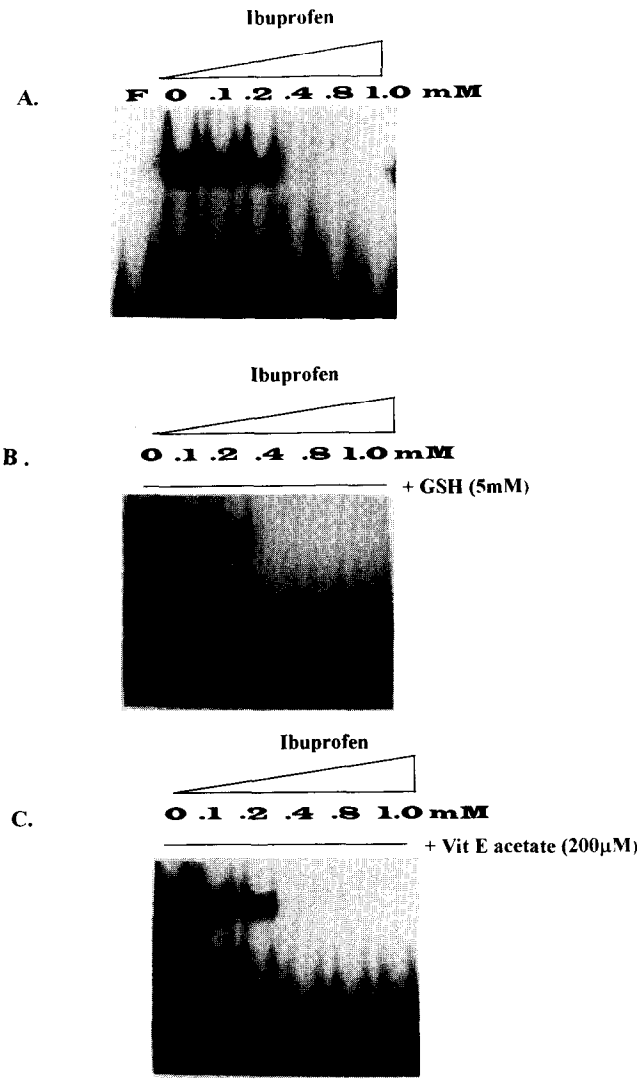

FIG. 5. The effects of antioxidants on the ability of ibuprofen to displace recombinant Bm3R1 from its DNA operator. (A) Effects of ibuprofen on Bm3R1-DNA complex formation. EMSA reactions were carried out as described in Materials and Methods. Briefly, 1 μg of purified recombinant Bm3R1 was added to each reaction mixture, together with increasing concentrations of ibuprofen as indicated. (B) Effects of ibuprofen together with glutathione on Bm3R1-DNA complex formation. Reactions were performed essentially as described in 5A, but with the prior addition of glutathione (5 mM) to the EMSA buffer. (C) Effects of ibuprofen together with α-tocopherol acetate on Bm3R1-DNA complex formation. Reactions were performed essentially as described in 5A, but with the prior addition of α-tocopherol acetate (200 μM) to the EMSA buffer.

DISCUSSION

Ibuprofen treatment of *B. megaterium* cells induced glucose-6-phosphate dehydrogenase, catalase, and aldehyde reductase activities, as well as cytochrome P450BM-3–associated activity in a dose-dependent manner. The former three enzymes are among a large number of proteins that are positively modulated by oxidants in both prokaryotic and eukaryotic species [17]. This suggested that *B. megaterium* was responding to an oxidative insult. This hypothesis was further substantiated by several lines of evidence. First, cells that expressed high levels of cytochrome P450 were rendered more sensitive to oxidant insult than cells expressing little or no cytochrome P450. Furthermore, this sensitivity could be ameliorated by incubating the cells with the general cytochrome P450 inhibitor, imidazole. Second, cells overexpressing cytochrome P450 were seen to have a marked decrease in the levels of the abundant cellular antioxidant, glutathione, whereas cells incubated with imidazole maintained relatively high levels of this antioxidant. Third, a number of structurally heterogeneous antioxidants, as well as the antioxidant enzyme, catalase, were capable of consistently attenuating the induction of the cytochrome P450BM-3 apoprotein and its associated activity.

Previous work has clearly demonstrated an important role for the Bm3R1 repressor in the activation of the cytochrome P450BM-3 gene. In particular, it has been shown that fatty acids, peroxisome proliferators, and a variety of nonsteroidal antiinflammatory drugs are ligands for the Bm3R1 and that their binding leads to a derepression of the operon [3, 4], allowing transcription to proceed. Studies with two antioxidants, including the hydrophilic molecule glutathione, as well as the lipid soluble antioxidant α-tocopherol acetate, had no effect on the ability of ibuprofen to inhibit Bm3R1–DNA complex formation. This suggests that the Bm3R1 repressor is not directly redox sensitive and that the effects of antioxidants are indirect. This is also supported by the observation that the induction of cytochrome P450BM-3 by ibuprofen can be partially attenuated by extracellular catalase (Table 4). One possi-

bility is that reactive oxygen intermediates are necessary, at least in part, to sustain the induction of the cytochrome P450BM-3 gene and that antioxidants help scavange these radicals, thereby attenuating the response. Because previous work has shown that the induction of this prokaryotic cytochrome P450 is intimately linked to perturbations in lipid homeostasis, a role for lipid peroxides cannot be excluded and, therefore, deserves closer attention.

An important implication of this work is that cytochrome P450 induction may contribute significantly to the generation of an oxidative stress in cells. Furthermore, because many classes of nongenotoxic carcinogens in mammals induce P450 expression in hepatic tissue, it raises the possibility that their propensity to act as tumour promoters may be attributed, at least in part, to increased expression of P450 isozymes.

In summary, this work demonstrates that cytochrome P450 enzymes may play an important role in generating a prooxidant environment in cells. It has been demonstrated that overexpression of cytochrome P450 genes can, at least in *Bacillus*, significantly compromise cell viability and resistance to oxidative insult. The work also demonstrates that dietary and artificial antioxidants can be used to effectively inhibit the expression of cytochrome P450BM-3. Finally, it highlights the increasing importance of using simple organisms to gain insights into complicated toxicological phenomena such as cytochrome P450 induction and peroxisome proliferation.

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