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Differential expression of CYP102 in *Bacillus megaterium* by 17-β-estradiol and 4-*sec*-butylphenol

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Abstract

Previously we have reported the induction of CYP102 in *Bacillus megaterium* by 17β-estradiol (E2) and 4-*sec*-butylphenol (4-sBP). Electrophoretic mobility shift assay analyses demonstrated that E2 and 4-sBP both cause a dose-dependent disassociation of the Bm3R1 repressor protein from its binding site on the operator sequence of the CYP102 gene. Equimolar combinations of E2 and 4-sBP demonstrated additive induction of CYP102 compared to equivalent samples of E2 and 4-sBP added alone. Two gene constructs were used in this investigation. One construct designated BMC143 contained the entire regulatory region of CYP102. The other gene construct, designated BMA45, had the "Barbie box" sequence deleted. While the induction of CYP102 by 4-sBP was much higher in the BMC 143 construct, E2 induced CYP102 in both constructs to the same extent. This difference in induction of CYP102 by these two inducers indicates that they act at different sites, either on the Bm3R1 repressor protein or on positive regulatory sites, or that they act, in part, through different mechanisms.

Keywords: Cytochrome P450; CYP102; *Bacillus megaterium*; 4-*sec*-Butylphenol; Estradiol

P450 BM-3 (CYP102) is a mono-oxygenase in *Bacillus megaterium* that requires only NADPH and O_2 for activity. CYP102 catalyzes the hydroxylation of long chain fatty acids at the ω-1, ω-2, and ω-3 positions, amides, alcohols, and the epoxidation and/or hydroxylation of monosaturated fatty acids [1–4]. The CYP102 gene is regulated by a derepression mechanism that acts at the transcriptional level. This gene has a high level of identity to mammalian CYP genes [1].

Fulco and co-workers [5,6] demonstrated that a variety of barbiturates induce CYP102 and characterized the CYP102 operon. Wolf and co-workers [1,7] have shown that CYP102 gene is also induced by nonsteroidal anti-inflammatory drugs (NSAIDs) and peroxisome proliferators. None of these substances are substrates of CYP102. Peroxisome proliferators, steroids, NSAIDs, and barbiturates may mimic fatty acids and other natural signaling molecules in both bacteria and mammals. This suggests that response to foreign chemical exposure is highly conserved and may involve the perturbation of fatty acid metabolism [9]. It also suggests possible alternative mechanisms or side effects of these drugs [7].

Fulco and co-worker [5] have characterized a secondary regulatory site in the CYP102 gene that has been designated as the Barbie box. The site was discovered during the investigation of barbiturate-mediated induction. The Barbie box sequence was first observed by using gel retardation assay with *B. megaterium* protein grown in either the presence or absence of barbiturates [8]. A Barbie box is found in all P450s induced by barbiturates, including animals [5]. This indicates that the Barbie box is an important control element in barbiturate-mediated induction [8]. Induction by barbiturates is not limited to prokaryotic cyp gene systems, but also occurs in many species, including rodents and humans. This suggests that elements of this gene regulation mechanism have been conserved throughout evolution

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[1]. The synthetic barbiturates seem to mimic certain aspects of the natural inducers of P450.

Alkylphenols have been found to mimic the female hormone estrogen 17-β-estradiol and can potentially disrupt endocrine-dependent activity [10–12]. Routledge and Sumpter, using an estrogen receptor assay, tested a variety of alkylphenols in order to identify the structural features responsible for estrogenic activity [13]. The alkylphenols were directly compared to the effects of E2. Their data indicated that maximal estrogenic activity was displayed by alkylphenols containing a para substituted branched alkyl side chain. The CYP102 gene in *B. megaterium* is induced by E2 and by the estrogenic alkylphenol 4-sBP [14]. EMSA analysis demonstrated that E2 and 4-sBP disassociated the Bm3R1 repressor in a dose-dependent manner [14]. The results of the EMSA analyses were directly proportional to the induction of CYP102. Induction by E2 and 4-sBP indicates that this CYP102 gene induction model is sensitive to certain characteristics of estrogen and could be used to screen compounds for those possessing estrogenic properties [14]. The observations that CYP102 is induced by E2 indicate that aspects of the gene regulation mechanism in the *B. megaterium* have been conserved throughout evolution. These observations reinforce similar findings involving CYP102 gene induction by barbiturates, NSAIDs, and peroxisome proliferators [9].

Materials and methods

17β-Estradiol (E2) was obtained from Sigma Chemical (St. Louis, MO). 4-*sec*-Butylphenol (4sBP) was purchased from Aldrich Chemical (Milwaukee, WI).

The *B. megaterium* clone, BMC143 used in this investigation, contains a plasmid with a truncated *cyp102* gene. This gene has been truncated a short distance past the start sequence encoding for the cytochrome P450. At the point of truncation, a bacterial luciferase reporter gene has been fused to the remaining portion of the *cyp102* gene.

Another *B. megaterium* clone, BMA45 used in this investigation, contains a plasmid with a *cyp102* gene truncated to a point upstream from the Barbie box in the region encoding the Bm3R1 repressor protein. As a result, the Barbie box regulatory element is missing in this clone.

Two different constructs were made in order to better dissect the regulatory features of the *cyp102* gene. The BMC143 construct contains the full regulatory region, whereas the BMA45 construct has the Barbie Box region deleted. The coupled luciferase reporter gene permits determination of *CYP102* induction in *B. megaterium* spectrofluorometrically [14]. The regulatory mechanism was studied by comparing the induction data from these two gene constructs.

The *B. megaterium* clones BMC 143 and BMA 45 were grown under the conditions described for the wild-type bacteria [7,9] in media supplemented with 0.5% (w/v) glucose. The culture was incubated 14–18 h at 30 °C while being shaken at 150 rpm. Aliquots of 10 mL culture were transferred to T-25 flasks with vented caps. Test compounds were added in dimethyl sulfoxide (DMSO) and DMSO alone was added to the control flask. In no case did the concentration of DMSO exceed 0.25% (v/v). The cultures were incubated at 30 °C with aeration; aliquots were withdrawn at 2, 4, and 6 h intervals after the introduction of test compounds for the measurement of luciferase activity. Luciferase assays were carried out as reported earlier [14]. Assays were carried out in duplicate and each experiment was performed at least three times. Statistical analysis (ANOVA and Dunnet test) was carried out using Instat analysis software (GraphPad, San Diego, CA).

Results

Induction of CYP102 in *B. megaterium* by combinations of E2 and 4-sBP were compared to the induction by E2 and 4-sBP tested separately under identical conditions. E2 and 4-sBP were combined in equal amounts at concentrations of 100, 150, and 200 μM. Three samples contained only E2 and three samples contained only 4-sBP at the concentrations 100, 150, and 200 μM. The induction of CYP102 by both E2 and 4-sBP were measured with both the reporter gene constructs.

In BMC 143 (Fig. 1) and in BMA 45 (Fig. 2), 4-sBP demonstrated a dose-dependent response both alone

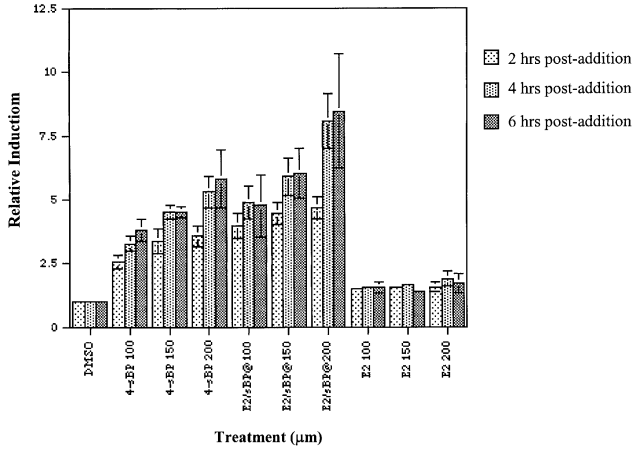

Fig. 1. Induction of CYP102 in BMC143 by 4-sBP and E2 at 2, 4, and 6 h post-addition to the cultures.

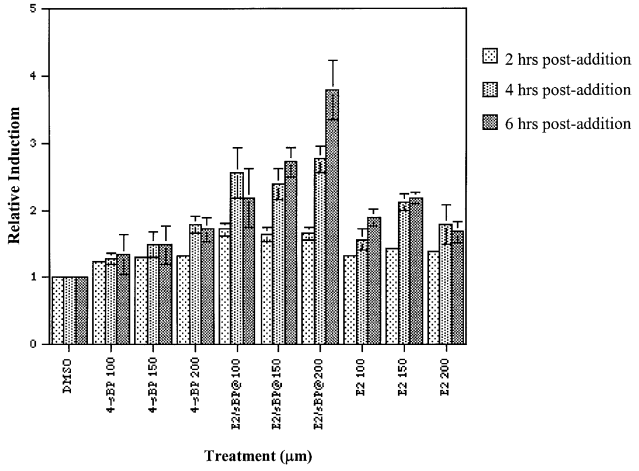

Fig. 2. Induction of CYP102 in BMA45 by 4-sBP and E2 at 2, 4, and 6 h post-addition to the cultures.

Table 1
Comparison of the induction of CYP102 in BMC143 and BMA45 constructs by 4-sBP, E2 and their combination

	4-sBP	E2	Total	4-sBP/E2 combination
BMC143 2HR				
100 μM	2.58 ± 0.27	1.52 ± 0.09	4.10 ± 0.36	3.97 ± 0.50
150 μM	3.37 ± 0.49	1.55 ± 0.06	4.92 ± 0.55	4.47 ± 0.45
200 μM	3.58 ± 0.41	1.57 ± 0.20	5.15 ± 0.61	4.69 ± 0.44
BMC143 4HR				
100 μM	3.30 ± 0.29	4.53 ± 0.27	4.88 ± 0.40	4.91 ± 0.64
150 μM	1.58 ± 0.11	1.67 ± 0.09	6.20 ± 0.36	5.90 ± 0.73
200 μM	4.88 ± 0.40	1.90 ± 0.31	7.21 ± 0.94	8.10 ± 1.08
BMC143 6HR				
100 μM	3.81 ± 0.44	1.56 ± 0.21	5.37 ± 0.65	4.77 ± 1.23
150 μM	4.52 ± 0.20	1.38 ± 0.11	5.90 ± 0.31	6.02 ± 0.98
200 μM	5.83 ± 1.14	1.71 ± 0.36	7.54 ± 1.50	8.47 ± 2.23
BMA45 2HR				
100 μM	1.23 ± 0.06	1.32 ± 0.02	2.55 ± 0.08	1.71 ± 0.09
150 μM	1.29 ± 0.04	1.43 ± 0.04	2.72 ± 0.08	1.64 ± 0.11
200 μM	1.33 ± 0.05	1.38 ± 0.02	2.71 ± 0.07	1.65 ± 0.09
BMA45 4HR				
100 μM	1.28 ± 0.08	1.56 ± 0.15	2.84 ± 0.23	2.55 ± 0.38
150 μM	1.49 ± 0.19	2.12 ± 0.12	3.61 ± 0.31	2.39 ± 0.24
200 μM	1.78 ± 0.13	1.78 ± 0.30	3.56 ± 0.43	2.76 ± 0.20
BMA45 6HR				
100 μM	1.34 ± 0.30	1.89 ± 0.12	3.23 ± 0.42	2.18 ± 0.44
150 μM	1.48 ± 0.29	2.18 ± 0.08	3.66 ± 0.37	2.72 ± 0.22
200 μM	1.71 ± 0.17	1.67 ± 0.16	3.38 ± 0.33	3.79 ± 0.44

and in combination with E2. Additionally, 4-sBP demonstrated stronger relative induction in the BMC 143 gene construct. When added alone E2 is not as potent an inducer of CYP102 as 4-sBP and the relative induction levels by E2 are roughly equivalent in both gene constructs. When E2 and 4-sBP are added to *B. megaterium* cultures in combination, the observed induction response is much greater than the response produced by E2 or 4-sBP added alone. As shown in Table 1, for each given concentration the induction levels of E2 plus 4-sBP are nearly equivalent to the combined induction levels of the corresponding concentrations of E2 and 4-sBP, thus demonstrating the additive nature of the induction response of CYP102 by the two estrogenic chemicals. The BMC143 data demonstrate the best equivalence in relative induction levels. For the BMA45 data, the relative induction data for the combination, while higher than the induction response to either E2 or 4-sBP added alone, are lower than for the individual E2 and 4-sBP responses added together.

As shown in Fig. 3, 150 μM E2 produces the maximal E2-dependent response. To check that the induction response was indeed additive, varying concentrations of 4-sBP (25–200 μM) were added along with 150 μM E2 (Fig. 4). Additive induction that was dependent on the increasing concentrations of 4-sBP was observed. In a similar fashion, adding increasing concentrations of E2 to cultures containing saturating concentration of 4-sBP

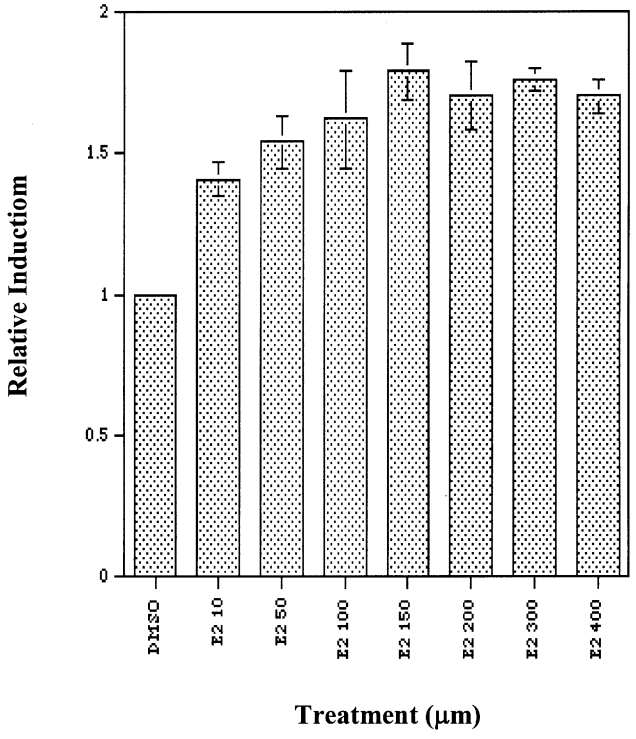

Fig. 3. Induction of CYP102 in BMC143 by E2 at 4 h post-addition to the cultures.

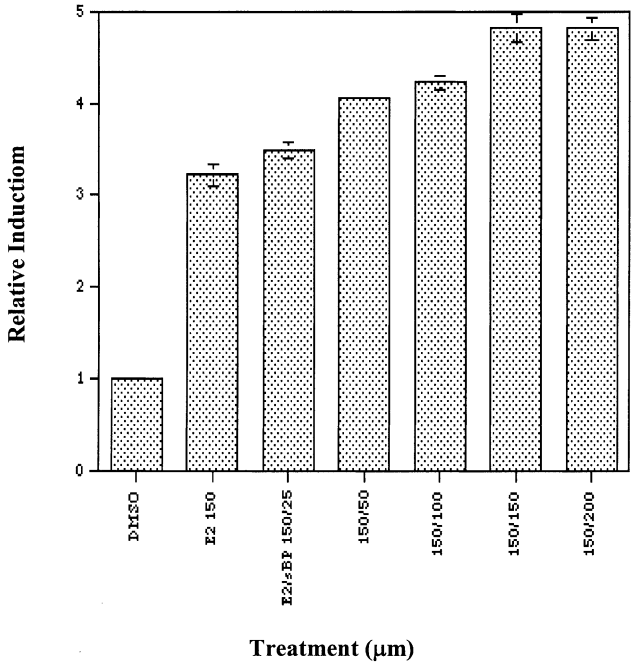

Fig. 4. Induction of CYP102 in BMC143 construct by varied 4-sBP concentrations plus 150 μM E2.

also demonstrated an additive induction response (data not shown).

Discussion

Combinations of E2 and 4-sBP produce additive induction of CYP102 in *B. megaterium*. The combined response due to E2 + 4-sBP was found to be nearly equivalent to the mathematical summation of individual responses produced by E2 and 4-sBP tested alone. For example, the induction of 100 μM 4-sBP and 100 μM E2 in the BMC143 clone added together are nearly equal to the induction produced by the combination of 100 μM of E2 plus 100 μM 4-sBP. This is the case at all three-time points measured (2, 4, and 6 h post-addition) and with statistical variation taken into account. This indicates that E2 and 4-sBP are acting at different sites or through different regulatory control element(s). If the different inducers acted on the same control element, for example, if they both acted only on the Bm3R1 protein and caused its dissociation from its operator site, the inducer with the highest affinity for Bm3R1 would simply displace the other potential ligand and the induction response would not be additive. The induction by E2 and 4-sBP is additive, even when one of the inducers is present at a level that produces its maximum response. This suggests that the two inducers must act at different control sites.

The results from the BMA 45 construct further reinforce the observation that two different control elements are being affected by E2 and 4-sBP. E2 produces similar induction in both gene constructs. E2 induces between 1.5- and 2-fold over the DMSO control at the same concentrations in both gene constructs. 4-sBP, however, produced dramatic difference in the induction responses in the two gene constructs. 4-sBP is a very potent inducer of CYP102 in *B. megaterium*. In the BMC 143 gene construct it demonstrated an induction level that is 4–10-fold higher than the DMSO control. In BMA 45, however, 200 μM 4-sBP by itself induced only about 2-fold over the DMSO control at 4 h post-addition (Table 1).

The difference between the two gene constructs is that the BMA 45 gene construct lacks the Barbie box sequence. According to Fulco's regulatory model for the CYP102 gene, the Barbie Box is a control element where the Bm3R1 repressor protein binds, prohibiting gene transcription [6,15]. Other positive regulatory elements also may act on the Bm3R1, causing it to be displaced from the Barbie box [6,15]. At the operator site of the CYP102 gene, however, the Bm3R1 is displaced directly by the inducer.

To explain the observed difference in induction response between E2 and 4-sBP, we suggest that 4-sBP interacts with both the Bm3R1 repressor protein and at other positive regulatory element(s) such as the Barbie box, thus demonstrating potent relative inductions observed with the BMC 143 gene construct. Since the BMA 45 gene construct lacks the Barbie box and thus one of the positive regulatory elements, the response due to 4-sBP is decreased in this construct. Since E2 demonstrates similar and low induction levels in both constructs, we suggest that E2 interacts only with the repressor protein Bm3R1 to cause its dissociation from the operator site. E2, unlike 4-sBP, does not appear to interact with positive regulatory elements such as the Barbie box.

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