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ACCELERATED COMMUNICATION

Differentiated Induction of Cytochrome P450b/e and P450p mRNAs by Dose of Phenobarbital in Primary Cultures of Adult Rat Hepatocytes

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SUMMARY

Phenobarbital induces cytochromes P450 (P450s) of not only the class IIB gene subfamily (i.e., P450b and P450e) but also the class IIIA gene subfamily (P450p and P450pcn2). To determine whether coinduction of these structurally dissimilar gene products involves the same mechanism, we examined the dose dependency of phenobarbital induction of the mRNAs for these four P450s in a new responsive system for primary monolayer cultures of adult rat hepatocytes on Matrigel, a reconstituted basement membrane. Two-day treatments of the cultures with phenobarbital produced marked dose-dependent increases in the levels of P450b, P450e, and P450p mRNAs, which reached maximal inductions ranging from 11- to >193-fold. Although the dose-response relationships for the inductions of P450b and P450e mRNAs by phenobarbital were similar ($ED_{50} = 1.5 \times 10^{-5}$ and 5.7×10^{-6} M, respectively), the dose-response curve for the induction of P450p mRNA was positioned distinctly to the right ($ED_{50} = 3.0 \times 10^{-4}$ M). This difference reflects a potency ratio of 20-fold for P450p/P450b mRNA induction. Phenobarbital also produced a weak dose-dependent induction of P450pcn2 mRNA, with a potency ($ED_{50} = 3.4 \times 10^{-5}$ M) intermediate between those for P450b/e and P450p. In a similar experiment using two phenobarbital-like inducers, (*trans*)nonachlor and clotrimazole, the relative inductions of P450b, P450e, P450p, and P450pcn2 mRNAs proved to be similar to those produced by phenobarbital (P450p/P450b potency ratios = 14- and 16-fold, respectively). These findings provide strong further evidence in support of the newly emerging concept that "phenobarbital" induction of the responsive class IIB and class IIIA P450 isozymes likely reflects multiple mechanisms.

The hepatic P450s are members of a superfamily of microsomal enzymes that catalyze the oxidation of a vast array of endogenous substrates and foreign compounds. Some P450 forms are inducible by xenobiotics, an adaptive response that has also served as a basis for their categorization. By this nomenclature, P450s were first classified as those being inducible either by PB (i.e., P450b or P450e[1]) or by 3-methylcholanthrene (P450c or P450d) (1). This scheme was later appended to include a P450 form (later called P450p) that was inducible by the synthetic steroid PCN (2). Implicit in this classification scheme was the assumption that PB, 3-methylcholanthrene, and PCN each selectively induced its own unique P450 forms. However, Heuman *et al.* (3) reported that PB not only induced P450b and P450e in living rats but also induced P450p. Moreover, other compounds described as "PB-like" in their P450 induction profiles, including certain organochlorine pesticides, polychlorinated biphenyls, and imidazole antimycotic drugs, were found to induce not only P450b/e but also P450p (4–6). Following the isolation and sequencing of P450 genes, P450s were reclassified according to amino acid sequence similarity into 10 gene families (7). By this nomenclature, P450b/e and P450p fall into gene families II and III, respectively (7). Thus, PB and PB-like inducers induce P450s in different gene families.

The underlying molecular mechanisms through which PB and PB-like compounds induce P450b/e and P450p in mammalian cells are currently under investigation. Because P450b and P450e are reported to be induced by transcriptional activation (8–10) under coordinate regulatory control (11–13), it is generally assumed that the P450b and P450e genes share

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[1] By the nomenclature recommended by Nebert *et al.* (7), cytochromes P450b, P450e, P450c, P450d, P450p, and P450pcn2 correspond to P450s IIB1, IIB2, IA1, IA2, IIIA1, and IIIA2, respectively.

ABBREVIATIONS: P450, cytochrome P450; PB, phenobarbital; *t*-NC, (*trans*)-nonachlor; CTZ, clotrimazole; PCN, pregnenolone-16α-carbonitrile; SDS, sodium dodecyl sulfate; ED_{50}, 50% effective dose.

common receptors or gene-responsive elements. However, current evidence suggests that P450b/e and P450p are induced by PB and PB-like agents through different pathways. Thus, Hardwick *et al.* (14) previously reported that rats treated with a single dose of PB responded with a faster onset of P450b/e mRNA induction than occurred for P450p mRNA. Also, we have demonstrated striking structure-activity differences for the induction of P450b/e versus P450p by a series of polychlorinated biphenyls (4). Moreover, we have recently found that protein synthesis inhibition has opposite effects on the induction of P450b/e and P450p mRNAs by PB and PB-like inducers in primary cultures of rat hepatocytes (15).

If, in fact, nonidentical pathways are involved in the induction by PB of P450b/e and P450p, then differences in their dose-response relationships might be demonstrable. However, it is often difficult to detect modest differences in dose-response relationships *in vivo* because of interanimal variability and the difficulty of precisely administering the desired doses to the target organ. Furthermore, because expression of P450b/e and P450p genes is influenced by extrahepatic factors, such as growth hormone (16, 17), the direct effects of PB upon the hepatocytes may be obscured.

Use of liver cell cultures would circumvent all of the aforementioned shortcomings, but until recently it has been difficult to elicit appropriate responses to PB in any of the available systems of continuously replicating cell lines derived from liver or in nonproliferating primary cultures of adult rat hepatocytes. However, this obstacle has been overcome by culturing rat hepatocytes on a substratum of Matrigel (a reconstituted basement membrane extracted from the Engelbreth-Holm-Swarm sarcoma) instead of the traditional type I collagen matrix (18). These cultured hepatocytes, maintained in a chemically defined medium free of serum and pituitary hormones, respond to PB with inductions of P450b/e and P450p comparable to those reported in the liver *in vivo* (18). In the current study we have taken advantage of this new system to proximately compare the responses of the P450b/e and P450p genes to induction by PB and two PB-like inducers in cultured hepatocytes. Our results reveal a striking dissociation in responsiveness of P450p from P450b/e, as well as from P450pcn2, a class IIIA P450 gene subfamily member that shares 89% amino acid and 90% nucleotide similarity with P450p (19).

Experimental Procedures

Materials. Adult male Sprague Dawley rats (Dominion Laboratories, Dublin, VA) weighing 180–200 g were maintained in wire-bottom cages, with free access to animal chow and water, for 2 weeks before use. Collagenase type I was purchased from Cooper Biochemical Co. (Malvern, PA). Drugs were obtained from the following sources: sodium PB, J. T. Baker, Inc. (Phillipsburg, NJ); *t*-NC, Velsicol Corp. (Chicago, IL); and CTZ, Sigma Chemical Company (St. Louis, MO). Oligonucleotides to P450b, P450e, P450p, and P450pcn2 were synthesized, according to published sequences (13, 19), either in our laboratory (P450b and P450e) or by Dr. Bryan Johnson (P450p and P450pcn2) of the University of Alabama (Birmingham, AL).

Hepatocyte cultures and drug treatments. Rat hepatocytes were isolated and plated onto 60-mm plastic dishes coated with 120–150 μl of Matrigel, as previously described (18). Cultures were maintained in a humidified incubator at 35° under an atmosphere of 5% CO_2/95% air. The culture medium, a modification of Waymouth MB-752 containing insulin as the only peptide (18), was renewed daily. In each experiment, cells were incubated for the first 3 days with medium only and then were treated for 2 days with medium containing various doses of drug (three dishes/dose). (Inasmuch as the total volume of medium for each dish was kept constant throughout each experiment, we have elected to refer to "dose-response" rather than "concentration-response" in describing drug effects.) Cells from one rat received PB (10^{-6} to 3×10^{-3} M); cells from a second rat received CTZ (10^{-8} to 10^{-4} M) or *t*-NC (10^{-8} to 3×10^{-5} M). Drugs were added to the cultures as concentrated stock solutions dissolved in water (PB) or in dimethyl sulfoxide (*t*-NC and CTZ)(maximum 0.3% of total volume).

Northern blot analysis. Total RNA was isolated from the pooled cells of the three culture dishes used for each treatment. RNA samples (10 μg) were resolved on denaturing 1% agarose gels and capillary transferred onto reinforced nitrocellulose filters. Oligonucleotides to P450b, P450e, P450p, and P450pcn2 were radiolabeled to greater than 5×10^8 cpm/μg, using a commercial 5′-labeling kit (BRL, Gaithersburg, MD) and [γ-^{32}P]ATP (4500 Ci/mmol) as substrate. The filters were prehybridized for at least 1 hr, hybridized overnight with 2×10^6 cpm/ml oligonucleotide, and washed. Prehybridization and hybridization conditions were the following: 1) P450b and P450e oligonucleotides: 5× Denhardt's solution (1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 6× SSPE (3 M sodium chloride, 0.2 M sodium phosphate monobasic, 0.02 M ethylenediaminetetraacetic acid, pH 7.4), 0.1% SDS, 200 μg/ml transfer RNA, 52°; 2) P450p and P450pcn2 oligonucleotides: 5× Denhardt's solution, 5× SSPE, 0.1‰ SDS, 200 μg/ml transfer RNA, 15% formamide, 42°. Final washing conditions were the following: 1) P450b and P450e oligonucleotides: 30 min at 42°, 0.1× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.5), 0.1% SDS; 2) P450p and P450pcn2 oligonucleotides: 30 min at 42°, 0.1× SSC, 0.1% SDS. RNA bands were visualized by autoradiography and quantified by scanning densitometry.

Analysis of dose-response data. For each individual drug, the numerical data from the scanned autoradiographs were expressed as percentages of the maximum response observed for that drug and were plotted versus log dose. Least squares lines were fitted to the linear central portions of the plots, and responses corresponding to 50% of the observed maximum response (ED_{50} values) were calculated.

Results

Freshly isolated hepatocytes prepared from an adult male rat were incubated on Matrigel-coated plastic dishes in standard medium for 3 days and then were transferred to media containing various concentrations of PB for 2 days. Analysis of total cellular RNA on Northern blots confirmed our previous reports (17, 18), in that PB treatment induced mRNAs for P450b (>193-fold[2]) and for P450e (11-fold) (Fig. 1, Table 1). The dose-response relationship for induction of P450b mRNA (Fig. 2) was similar to that for P450e mRNA, with maximal effects occurring at 10^{-4} M PB and with calculated ED_{50} values of 1.5×10^{-5} and 5.7×10^{-6} M for P450b and P450e, respectively (Table 2). At higher doses of PB, the magnitude of induction of P450b/e mRNAs was progressively attenuated (Fig. 2).

In contrast, little induction of P450p mRNA occurred in cultures exposed to PB at 10^{-4} M, the maximally effective dose for P450b/e mRNA induction (Fig. 2). At higher doses of PB, dose-dependent induction of P450p mRNA ensued, which did not attain an obvious maximum by 3×10^{-3} M, the highest nontoxic dose of PB tolerated by our hepatocyte culture system. The level of P450p mRNA induction produced by 3×10^{-3} M PB (12.6-fold) was only 36% of that produced by 10^{-5} M dexamethasone, the most efficacious glucocorticoid P450p inducer (Fig. 1, Table 1). If 3×10^{-3} M PB is used as an estimate

[2] Percentages are maximal increases over untreated 120-hr control cultures. Because P450b mRNA was not detectable in untreated 120-hr cells, the percentage increase given in this case was calculated based on the lowest detected value, which was that from 72-hr control cells (see Table 1).

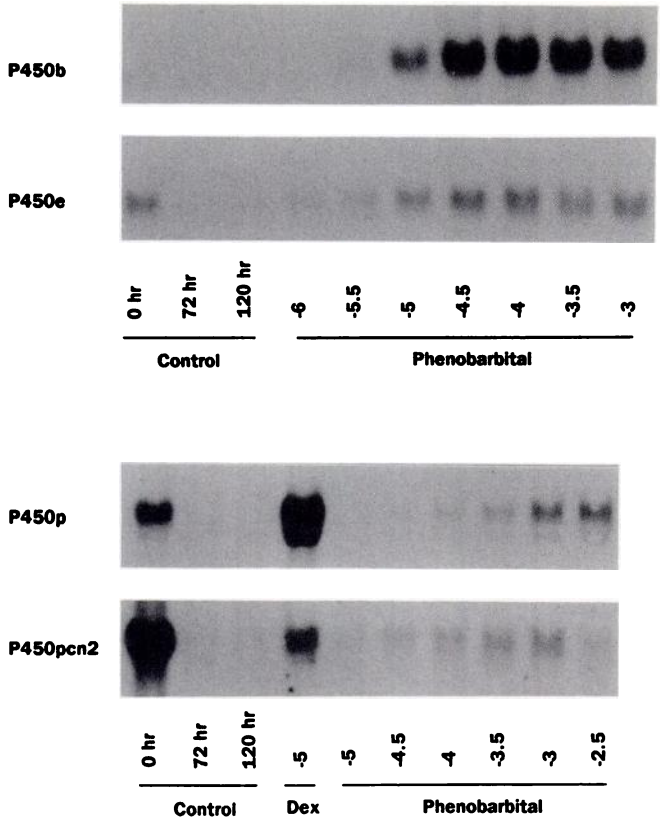

Fig. 1. Northern blot analysis of mRNAs for P450s in primary monolayer cultures of adult male rat hepatocytes treated with PB. Cells from a single liver were incubated for the first 3 days in standard, serum-free medium and then were treated for 2 days with medium containing PB (10^{-6} to 3×10^{-3} M; three dishes/dose). Total cellular RNA was extracted from the pooled dishes of each treatment group, resolved on denaturing 1% agarose gels, and transferred to reinforced nitrocellulose filters. The filters were hybridized with specific oligonucleotides to P450b, P450e, P450p, or P450pcn2, as described in Experimental Procedures. The autoradiographs displayed for P450b, P450e, P450p, and P450pcn2 mRNA hybridizations resulted from film exposures of 1, 1, 1, and 3 days, respectively. Drug doses are expressed as the logarithm of the molar medium concentration. Percentages of maximal PB induction were calculated from the densitometrically scanned autoradiographs and are given in Table 1. *DEX*, dexamethasone.

TABLE 1

P450 induction by PB

Drug doses are expressed as the logarithm of the molar medium concentration. The autoradiographs in Fig. 1 were densitometrically scanned and percentages of maximal PB induction were calculated.

Treatment	Induction			
	P450b	P450e	P450p	P450pcn2
	% of maximum			
0 hr	4.47	64.6	123	428
72 hr	0.519	3.38	9.53	46.9
120 hr	<0.519	9.07	7.95	43.7
Dexamethasone−5			276	174
PB				
−6	0.778	17.4		
−5.5	3.18	29.3		
−5	42.2	70.6	6.47	29.3
−4.5	74.6	96.3	16.3	52.1
−4	100	100	23.4	61.8
−3.5	89.3	87.7	41.7	89.0
−3	77.6	95.9	86.8	100
−2.5			100	50.1

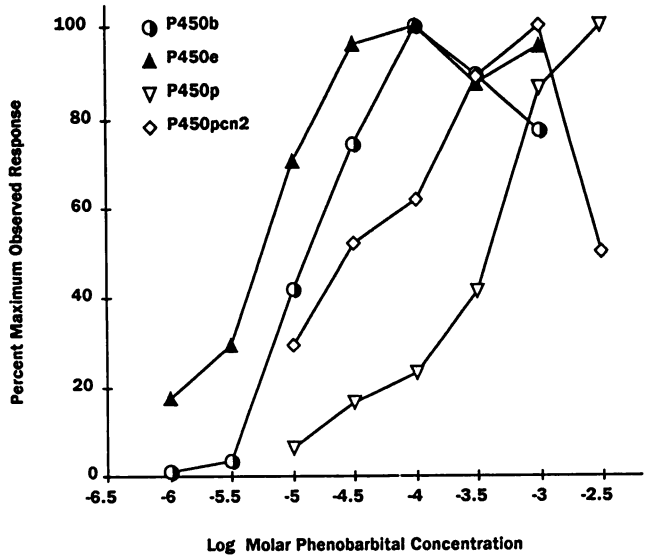

Fig. 2. Dose-response analysis of the induction of P450 mRNAs by PB in hepatocyte cultures. Percentage inductions of the P450 mRNAs are those given in Table 1.

TABLE 2

ED_{50} values and ratios for the induction of P450 mRNAs by PB, *t*-NC, and CTZ

ED_{50} values for PB, *t*-NC, and CTZ induction of P450b, P450e, P450p, and P450pcn2 mRNAs were calculated from the dose-response data, as described in Experimental Procedures.

	ED_{50}				ED_{50} ratios	
	P450b	P450e	P450p	P450pcn2	P450p/P450b	P450p/P450pcn2
	μM					
PB	15	5.7	300	34	20	8.8
t-NC	0.22	0.12	3.0	0.52	14	5.8
CTZ	0.16	0.11	2.5	0.23	16	11

(possibly low) of the maximally effective dose, the ED_{50} for induction of P450p mRNA is 3.0×10^{-4} M and is, therefore, at least 20-fold greater than the ED_{50} for induction of P450b mRNA (Table 2).

As an additional point of comparison, we examined induction by PB of the mRNA for P450pcn2, a class IIIA P450 that is highly similar to P450p (Fig. 1, Table 1). The dose-response curve for induction of P450pcn2 mRNA by PB was clearly positioned to the left of that for P450p (Fig. 2). The maximal response for P450pcn2 mRNA induction occurred in cells treated with 10^{-3} M PB, and the calculated ED_{50} was 3.4×10^{-5} M, a value 8.8-fold lower than that calculated for P450p mRNA (Table 2). A corresponding treatment of the same cultures with dexamethasone (10^{-5} M) produced an induction of P450pcn2 mRNA that was 1.7-fold greater than that produced by 10^{-3} M PB (Fig. 1, Table 1). We noted that the amount of P450pcn2 mRNA present in the cultures treated with 10^{-3} M PB represented only 23% of that found in freshly isolated hepatocytes (Fig. 1, Table 1).

In a final study, we performed dose-response analyses for cultures treated with either of two PB-like inducers, *t*-NC and CTZ, representing the organochlorine pesticides and the imidazole antimycotics, respectively. As was observed with PB, *t*-NC and CTZ each produced dose-dependent inductions of the mRNAs for P450b, P450e, P450p, and P450pcn2 (data not shown). Furthermore, analysis of the *t*-NC and CTZ dose-

response curves revealed that the ED_{50} values for induction of P450p mRNA were 14- and 16-fold greater, respectively, than the ED_{50} values for P450b mRNA induction and were 5.8- and 11-fold greater, respectively, than the ED_{50} values for P450pcn2 mRNA induction (Table 2).

We emphasize that all points for each individual dose-response curve were generated in parallel from the RNA of the cells of a single rat liver, a design we have found to result in very low variability among identically treated dishes. Although we do observe some variability in the absolute levels of basal expression and responses to treatments among cell preparations derived from different animals, we have now observed the same relative pattern of induction of P450b/e and P450p mRNAs by PB and PB-like inducers in five independent cell preparations.

Discussion

We have used the rigorously controlled conditions of hepatocyte culture to demonstrate unequivocally that induction of P450p mRNA by PB and two PB-like inducers proceeds by a process having an apparent lower potency than that for induction of P450b/e. This result represents a striking departure from the general belief that PB induction of rat liver genes are jointly mediated events. The latter concept stems from the well known coordinate regulation by xenobiotics of two highly similar class IIB P450 genes, P450b and P450e, as is revealed in the present experiments in hepatocyte culture and is reported for living rats (11–13). Nevertheless, Ritter and Franklin (20) reported several years ago that CTZ produced a dose-differentiated induction of several mixed function oxidase activities in rat liver. The differences in the dose-response relationships for induction of the P450b/e and P450p mRNAs are consistent with the existing time course (14), structure-activity (4), and protein synthesis inhibition (15) data, and taken together these findings strongly suggest that the pathways by which PB and the PB-like xenobiotics induce the class IIB and IIIA P450s in liver are not identical.

Heterogeneity in the pathways of PB induction may extend to P450pcn2, a class IIIA P450 family member that is 90% similar to P450p at the nucleotide level (19). Each of the drugs we tested produced a dose-dependent (albeit weak) increase in the amount of P450pcn2 mRNA, with an apparent potency intermediate between those of P450b/e and P450p. It has been reported that P450p mRNA was not detected in untreated male rats and was induced by both dexamethasone and PB, whereas P450pcn2 mRNA was expressed in adult male rats and was most efficaciously induced by PB (19). Our results in cultured male hepatocytes are largely consistent with these findings, except that we readily detected P450p mRNA in freshly isolated cells (Fig. 1) and also found that dexamethasone was a more efficacious inducer of P450pcn2 than was PB.

It is now well established that inducers of class IA P450s act through the Ah receptor, a cytosolic protein that binds polycyclic aromatic hydrocarbons with high affinity and stereospecific selectivity (21). Likewise, there is evidence that glucocorticoids may induce the class IIIA P450s through a mechanism involving a steroid hormone receptor (22). In contrast, PB-like inducers represent a chemically and pharmacologically heterogeneous array of compounds whose only obvious shared characteristics appear to be lipophilicity and the ability to interact, either as substrates or inhibitors, with the hepatic P450 monooxygenase system (1, 23–25). Therefore, it is perhaps not surprising that, despite intensive efforts, no receptor protein for PB or the PB-like inducers has yet been identified (26). Nevertheless, it seems likely that one or more PB "receptors" or "recognition sites" do, in fact, exist that, as our results suggest, are linked to different mechanisms for induction of the class IIB and IIIA genes. One possibility is that the inductions of P450b/e and P450p proceed through separate receptors for which PB and the PB-like compounds have different affinities. It is also possible that there is a common PB receptor but that the regulatory regions of the P450b/e and P450p genes have separate response elements that differ in their affinities for drug-receptor interactions. Furthermore, the presence of different additional factors, such as negative regulatory elements, could serve to shift the dose-response curves. Finally, the "receptor" for PB and PB-like inducers need not be a cytosolic binding protein. There is a new growing recognition that P450s are regulated by endogenous substances. For example, physiological doses of growth hormone suppress induction by PB of P450b/e and P450p in cultured rat hepatocytes (17), presumably by affecting the levels of some intracellular messenger(s) within the hepatocyte. A recognition site could then be envisioned as an enzyme whose occupation by a PB-like compound alters the synthesis or degradation of such an endogenous regulatory substance.

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