Biochemical Pharmacology, Vol. 43, No. 5, pp. 1067–1078, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

A PLEIOTROPIC RESPONSE TO PHENOBARBITAL-TYPE ENZYME INDUCERS IN THE F344/NCr RAT

EFFECTS OF CHEMICALS OF VARIED STRUCTURE

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(*Received* 25 *May* 1991; *accepted* 30 *October* 1991)

Abstract—The effects of a number of phenobarbital-type inducers on selected drug-metabolizing enzymes in male F344/NCr rats were determined by measuring specific catalytic activities and/or by measuring the levels of RNA which hybridize with specific probes for the corresponding genes. The effects on hepatic CYP2B1 were assessed by measuring the levels of CYP2B1-specific RNA and benzyloxyresorufin *O*-dealkylase and testosterone 16β-hydroxylase activities. Levels of CYP3A were monitored by measuring the rate of hydroxylation of testosterone at the 6β-position. Microsomal epoxide hydrolase activity was determined by measurement of cellular RNA specific for this form and by assaying the hydrolysis of benzo[*a*]pyrene-4,5-oxide. UDP-glucuronyltransferase activity was assayed by measuring the glucuronidation of 3-hydroxybenz[*a*]anthracene. Levels of glutathione *S*-transferase Ya/Yc were measured by quantifying total cellular RNA coding for the proteins. When male F344/NCr rats were administered various doses of phenobarbital or dichlorodiphenyltrichloroethane (DDT), strong correlations between the induction of CYP2B1 and the induction of epoxide hydrolase or UDP-glucuronyltransferase activities were observed. Treatment of rats with barbiturates, hydantoins, halogenated pesticides such as DDT or α-hexachlorocyclohexane, 2,4,5,2′,4′,5′-hexachlorobiphenyl, CYP2B1 inhibitors such as clotrimazole or clonazepam, or such structurally-diverse compounds as 2-hexanone or diallyl sulfide resulted in induction of CYP2B1-mediated enzyme activity and induction of certain other forms of cytochrome P450, microsomal epoxide hydrolase, at least one form of UDP-glucuronyltransferase, and multiple forms of glutathione *S*-transferase. This suggests that, as a class, compounds which induce CYP2B1 also induce a coordinate hepatic pleiotropic response which includes induction of these other phase I and phase II drug-metabolizing enzymes.

The long-acting sedative/anticonvulsant drug phenobarbital (PB‖) was initially synthesized over 80 years ago. Because of its clinical importance and various interesting pharmacologic side-effects, this compound and the barbiturates as a class have been investigated in great detail [1]. PB serves as the prototype for a class of xenobiotics which exhibit striking pharmacologic side-effects including liver enzyme induction, proliferation of hepatic smooth endoplasmic reticulum, liver hypertrophy, drug/drug interactions and liver tumor promotion in *N*-nitrosodiethylamine-initiated rodents [see, for example, Refs. 1–7].

Detailed studies of the induction of both cytochrome P450-mediated and non-P450-mediated drug-metabolizing activities by PB and related compounds have been limited previously by the fact that the assays employed to measure these activities have often not been highly isozyme specific. For example, induction of the cytochromes P450 was often determined by measuring total P450 or by measuring the dealkylation of aminopyrine or ethylmorphine. Neither of these assays appears to be highly specific for the individual forms of P450 which are inducible. Only more recently, with the development of monospecific and/or monoclonal antibodies [8, 9], recombinant DNA techniques [10, 11], and exquisitely specific substrates [12–14] has it become possible to examine the induction of individual isozymes of P450 by PB and related compounds. It is now apparent that PB induces various cytochromes P450, including CYP2B1, CYP2B2, CYP3A1 and CYP3A2 [15, 16]. A similar lack of specificity has been encountered in the

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‖ Abbreviations: α-HCH, α-hexachlorocyclohexane; ALB, allobarbital; APB, aprobarbital; BB, barbital; BZR, benzyloxyresorufin; CLT, clotrimazole; CZP, clonazepam; DAS, diallyl sulfide; DDT, dichlorodiphenyltrichloroethane; DMB, 5,5-dimethylbarbituric acid; DPH, 5,5-diphenylhydantoin; EEH, 5,5-diethylhydantoin; EPH, 5-ethyl-5-phenylhydantoin; ETR, ethoxyresorufin; HCB, 2,4,5,2′,4′,5′-hexachlorobiphenyl; HEX, 2-hexanone; MCA, 3-methylcholanthrene; PB, phenobarbital; PCN, pregnenolone-α-carbonitrile; PTB, pentobarbital; SCB, secobarbital; TCB, 3,3′,4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene.

determination of the induction of the glutathione *S*-transferases by means of catalytic assays with typical substrates [17].

A major impetus for conducting the present studies is that certain previous reports have implied that there is not a consistent response to barbiturates and certain related compounds in mice and certain strains of rats. Thus, Heubel *et al.* [18] suggested that the barbiturates as a class do not elicit a consistent pleiotropic response in mice. Similarly the work of Dunn *et al.* [19], Hassett *et al.* [20] and Hashimoto *et al.* [21] implied that there are striking differences in the isozymes of P450 induced by PB in various strains or substrains of rats. These latter studies were based upon their finding of a strain-dependent induction of CYP2B2 and aldehyde dehydrogenase (propionaldehyde, NAD^+).

Recent work has shown that a number of structurally-related compounds, including various barbiturates [5, 7], 5-ethyl-5-phenylhydantoin (EPH) [22] and 5-ethyl-5-phenylacetylurea [23], as well as such structurally-dissimilar xenobiotics as clotrimazole (CLT) [24], clonazepam (CZP) [25], α-hexachlorocyclohexane (α-HCH) [26] and certain other halogenated pesticides, 2-hexanone [27] and diallyl sulfide [28] are capable of inducing CYP2B1-mediated catalytic activities. However, the extent to which these diverse xenobiotics each induce other manifestations of the PB-type hepatic pleiotropic response has not previously been systematically examined. The importance of conducting such studies resides in the fact that, to date, the mechanism by which CYP2B1 induction occurs has not been resolved. In fact, the striking differences in chemical structure displayed by the above-mentioned inducers would appear to virtually preclude the mediacy of a specific high-affinity receptor, in contrast to the case of CYP1A-type induction which has been shown to occur via a cytosolic receptor protein [15, 29]. In addition, many of these CYP2B1 inducers cause liver tumor promotion in rodents, and the study of the pleiotropic response itself may help to elucidate the mechanism of their tumor-promoting action.

In the present studies, therefore, we have undertaken to examine whether a number of structurally-diverse xenobiotics yield a qualitatively similar coordinate induction of selected P450 and non-P450 genes and gene products. The term coordinate is used, in this case, to imply that the various effects are induced by the compounds in a roughly proportionate manner, and is not intended to imply that the kinetics of induction of each effect are the same. In fact, the protocols we typically employed involved administration in feed for up to 2 weeks or multiple intraperitoneal or intragastric injections, and such exposures are expected to result in equilibrium (steady-state) conditions. We have examined the ability of a number of structurally-diverse chemicals (Fig. 1) or of various doses of PB or dichlorodiphenyltrichloroethane (DDT) to induce CYP2B1 and CYP3A, certain isozymes of UDP-glucuronyltransferase and glutathione *S*-transferase, and microsomal epoxide hydrolase in the F344/NCr rat. The results indicate that each of the compounds that substantially induce CYP2B1 also induce UDP-glucuronyltransferase, glutathione *S*-transferases, epoxide hydrolase and CYP3A1 activities. These results would appear to imply a coordinate induction of these enzymes, with the cumulative effect representing the so-called hepatic "pleiotropic" response to these PB-type inducers.

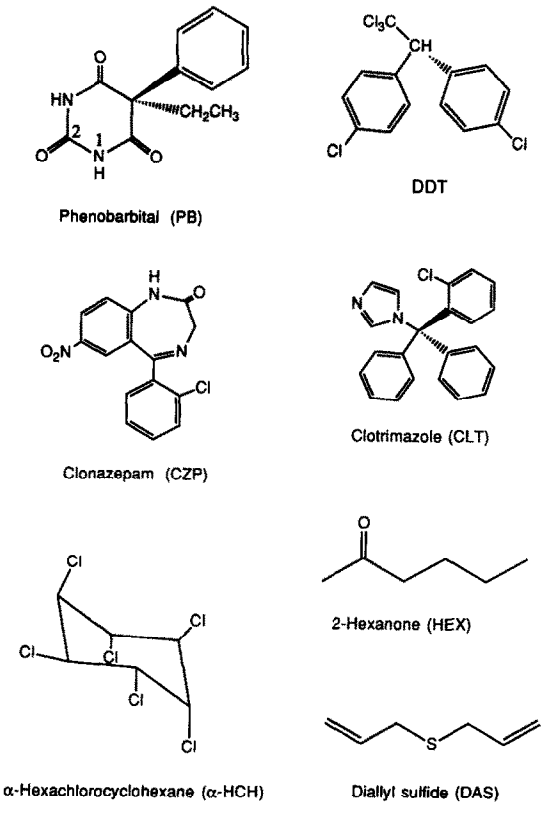

Fig. 1. Structural formulae of selected CYP2B1-type inducers, showing the high degree of structural diversity associated with inducers of this form of P450.

MATERIALS AND METHODS

Chemicals. PB, barbital (BB), allobarbital (ALB), aprobarbital (APB), secobarbital (SCB), 5,5-diphenylhydantoin (DPH), 5,5-dimethylbarbituric acid (DMB), pentobarbital (PTB), CLT, diallyl sulfide (DAS), NADPH, 3-methylcholanthrene (MCA) and dicumarol were obtained from the Sigma Chemical Co. (St. Louis, MO). Benzyloxyresorufin (BZR) and ethoxyresorufin (ETR) were obtained from Molecular Probes (Eugene, OR). Benzo[*a*]-pyrene-4,5-oxide, benzo[*a*]pyrene-4,5-dihydrodiol and 3-hydroxybenz[*a*]anthracene were obtained from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). DDT, 2-hexanone (HEX) and α-HCH were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 2,4,5,2′,4′,5′-hexachlorobiphenyl (HCB) and 3,3′,4,4′-tetrachlorobiphenyl (TCB) were provided by Dr. L. Robertson. Pregnenolone-α-carbonitrile (PCN) was obtained from the Upjohn Co. (Kalamazoo, MI) and CZP was supplied by

Hoffmann-La Roche (Nutley, NJ). EPH and 5,5-diethylhydantoin (EEH) were synthesized in our laboratory from the corresponding ketones via cyanohydrin intermediates following published procedures [30]. Chemical analysis of these materials has been published previously [22].

Treatment of animals. Male F344/NCr rats (Animal Production Area, FCRDC), 7–10 weeks of age, were administered control diet (Purina Lab Chow No. 5010) or diet containing PB (18–500 ppm), barbital (1500 ppm), allobarbital (500 ppm), aprobarbital (500 ppm), secobarbital (500 ppm), pentobarbital (500 ppm), DDT (4–500 ppm), α-hexachlorocyclohexane (320 ppm), ethylphenylhydantoin (500 ppm), diethylhydantoin (500 ppm), dimethylbarbituric acid (460 ppm), diphenylhydantoin (308 ppm) or clonazepam (1200 ppm) *ad lib.* for 14 days. For treatment with 2-hexanone, animals were administered 1500 mg/kg body wt i.g. on days 1, 2 and 3, and were killed 48 hr after the last exposure. Clotrimazole (75 mg/kg body wt, in corn oil) was given via intragastric intubation on days 1, 2 and 3 followed by termination 48 hr following the last dose (CLT 3-2), or alternatively, was administered for days 1–5 followed by termination 1 hr following the final dose (CLT 5). Diallyl sulfide was administered i.p. (200 mg/kg body wt) on days 1, 2 and 3 and animals were killed 48 hr following the last dose. Alternatively, rats were injected i.p. with 50 mg/kg MCA at 0 and 24 hr and were killed 24 hr later. 2,4,5,2′,4′,5′-Hexachlorobiphenyl and 3,3′,4,4′-tetrachlorobiphenyl were administered as i.p. injections in corn oil (30 mg/kg body wt) on days 1 and 4, with the rats killed on day 7. PCN was administered as an i.p. injection in corn oil (60 mg/kg body wt) on days 1 and 3, with the rats killed 48 hr later. Individual rats were killed by CO_2 asphyxiation, the livers were removed and weighed, and portions of the liver were taken for preparation of RNA. The remaining portions of the livers were homogenized in 0.15 M KCl/0.2 M sucrose (4 mL/g wet liver wt, 4°). Post-mitochondrial (S-9), microsomal and cytosolic subfractions were obtained by sequential 9000 *g* and 105,000 *g* centrifugations.

Alkoxyresorufin O-dealkylase assays. The *O*-dealkylation of 7-ethoxyresorufin or 7-benzyloxyresorufin was measured in hepatic S-9 fractions as described by Nims *et al.* [7]. Both substrates were used at a final concentration of 5 μM. S-9 protein concentrations were 25–100 μg/mL for highly induced samples and 250–750 μg/mL for control or weakly induced samples.

Epoxide hydrolase assay. The continuous fluorimetric assay developed by Dansette *et al.* [31] was performed essentially as described. The formation of benzo[*a*]pyrene-4,5-dihydrodiol from the 4,5-oxide was monitored in a Perkin–Elmer spectrophotofluorimeter. Substrate (benzo[*a*]pyrene-4,5-oxide, 10 μM) was combined with 50–100 μg S-9 protein and 0.1 M Na_2HPO_4, pH 8.8, in a total volume of ~ 2 mL. Incubations were carried out at 28° for 10–20 min. Increases in relative fluorescence per unit time were compared to those resulting from known amounts of benzo[*a*]pyrene-4,5-dihydrodiol.

UDP-glucuronyltransferase assay. An assay with 3-hydroxybenz[*a*]anthracene used as the aglycone was carried out according to the method of Lilienblum *et al.* [32], with some modification. The assay was conducted under subdued light. Incubation mixtures (1.0 mL) contained 0.1 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, Lubrol-Px activated post-mitochondrial supernatants (0.1 to 0.5 mg protein/reaction), and 100 μM substrate. The reaction mixtures were preincubated at 37° for 5 min in a shaking water bath, and then were initiated by the addition of 3 mM UDP-glucuronic acid. After 5 min, the reactions were terminated by the addition of 1.75 mL chloroform:methanol:water (4:2:1). The tubes were then centrifuged at 2000 rpm for 10 min at 4°. The supernatants obtained (0.5 mL) were mixed with 0.6 mL of sodium hydroxide and then briefly centrifuged in a microcentrifuge. The supernatants were read in a Perkin–Elmer spectrophotofluorimeter (excitation, 292 nm; emission, 417 nm). The activity of the treated groups is presented relative to the activity of the control group.

Hydroxylation of testosterone. Cytochrome P450-mediated hydroxylation of testosterone was measured essentially as described by Sonderfan *et al.* [33]. Briefly, the incubations consisted of a 3-mL mixture containing 50 mM phosphate buffer, pH 7.4, 4 mM NADPH, 500 μM testosterone, 20 μM 11-hydroxytestosterone and 10 μM 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one. The latter chemical was added to the reaction mixtures to inhibit 5α-reductase activity [34]. The reaction mixtures were preincubated, while shaking, for 5 min at 30°. The reactions were initiated by the addition of microsomal protein (300–700 μg/mL) into the incubation mixtures. Aliquots (1 mL) were withdrawn from each incubation mixture at 2 and 4 min after the start of the reaction and pipetted directly into 6 mL of dichloromethane, followed immediately by mixing for 30 sec on a vortex mixer. The extraction of each reaction mixture was repeated using an additional 6 mL of dichloromethane and the pooled extracts were evaporated to dryness under a stream of nitrogen. The dried residues were redissolved in a final volume of 300 μL of solvent. A 50-μL aliquot was injected for HPLC analysis using the conditions described by Sonderfan *et al.* [33]. The hydroxylated metabolites of interest were identified and quantitated by comparison with authentic standards (Steraloids, Wilton, NH). The values of the 2- and 4-min time points for each treatment were averaged and reported in units of picomoles hydroxylated product per minute per milligram of microsomal protein.

Protein determination. Protein contents in S-9 and microsomal samples were measured using fluorescamine [35] with bovine serum albumin as standard.

Quinone oxidoreductase assay. This activity was measured by determining the reduction of resorufin as previously described [36]. In brief, the decrease in fluorescence due to the enzymatic reduction of resorufin (10 μM) was measured in the presence of 50 mM Tris/25 mM $MgCl_2$, pH 7.5, 670 μM NADPH, and 10–100 μg S-9 protein.

Aldehyde dehydrogenase. Induction of the MC-inducible forms of aldehyde dehydrogenase was

determined as described by Törrönen *et al.* [37]. Activities were determined in a 1.0 mL final assay volume containing 5 mM benzaldehyde, 1 mM $NADP^+$, and 15–30 μg of cytosolic protein in 80 mM pyrophosphate buffer, pH 8.9, to which 0.75 mM pyrazole had been added. Enzyme activity was measured at room temperature by monitoring the increase in absorbance at 340 nm for 3–5 min. An extinction coefficient of 6220 M^{-1} cm^{-1} for NADPH was used in the calculation of enzyme activity.

Isolation of total cellular RNA. Liver tissue from three individual rats per treatment was pooled and placed in 10 mL of cold 4 M guanidine isothiocyanate/0.1 M Tris–HCl (pH 7.5)/1% 2-mercaptoethanol solution prepared as previously described [38]. Tissues were homogenized by a 45-sec treatment in a Polytron homogenizer. Sodium sarkosyl (*N*-lauroylsarcosine, sodium salt) was added to a final concentration of 0.5% and the homogenates were layered over 6.1 M cesium chloride/25 mM sodium acetate (pH 5.2)/10 mM EDTA solution. RNA was collected by centrifugation for 20 hr at 20° in a Beckman SW41 rotor at 110,000 *g*. The RNA pellets were dissolved in glass-distilled diethylpyrocarbonate-treated water brought to 0.3 M sodium acetate and then precipitated with 2.5 vol. of ethanol. The RNA pellets were rinsed with 70% ethanol and air dried. RNA was then dissolved in 10 mM Tris/1 mM EDTA (pH 7.5) and the concentration determined by measurement of A_{260}.

RNA blot analysis. Total cellular RNA was denatured by treatment with formaldehyde (2.2 M) for 15 min at 65° and blotted directly onto Biodyne A nylon membrane filters (Pall Ultrafine Filtration Corp., Glen Cove, NY) using the Schleicher & Schuell Minifold II apparatus (Schleicher & Schuell, Inc., Keene, NH) [39]. Plasmids pGTB-38 (glutathione *S*-transferases Ya/Yc) [40], pEPH302 (microsomal epoxide hydrolase) [41], and the 0.6 kb cDNA β-actin fragment were labeled with ^{32}P by the random primer labeling techniques to 0.3 to 1.2×10^9 cpm/μg. Hybridization of the blots with plasmid probes was performed at 42° in 50% formamide. Following hybridization, the blots were washed two to five times at room temperature in 2 × SSC*/0.1% sodium dodecyl sulfate (SDS), followed by 3 washes with 0.1 × SSC/0.1% SDS for 15 min at 52°. An oligonucleotide (5′-GGTTGGTAGCCGGTGTGA-3′), was 5′-end-labeled with ^{32}P to 5×10^8 cpm/μg and used to detect rat CYP2B1 as recommended by Giachelli and Omiecinski [10]. Hybridization of the oligonucleotide was performed for 18 hr at 42°. The blots were washed five times with 4 × SSC/0.1% SDS at room temperature for 5 min followed by two washes with the same buffer at 52° for 15 min [10]. Following the washes, the blots were wrapped in Saran Wrap and autoradiographed for various time periods (up to 6 days) in the presence of an intensifying screen (Cronex Lightning Plus, DuPont, Wilmington, DE) with preflashed Kodak X-Omat XAR-5 film at −80°. After hybridization with one of the labeled probes and autoradiography, the probe was removed by treatment of the blot with 0.1 × SSC/0.5% SDS (95°, 20 min) prior to being rehybridized with another probe.

Probes. The probes for the rat glutathione *S*-transferases Ya/Yc (pGTB-38) and microsomal epoxide hydrolase (pEPH302) were provided by Dr. C. Pickett. The 0.6 kb fragment of β-actin cDNA was obtained from Lofstrand Laboratories Ltd. (Gaithersburg, MD).

* 1 × SCC = 0.15 M sodium chloride and 0.015 M sodium citrate.

RESULTS

In the following studies we have examined the relationship between the ability of a variety of structurally-diverse xenobiotics (Fig. 1) or of differing doses of PB or DDT to induce CYP2B1 and to induce other enzymes of interest. The reason that induction of CYP2B1 has been used for comparison is that it has been shown previously that this gene and its associated enzymatic activities, such as pentoxy- and benzyloxyresorufin *O*-dealkylase and testosterone 16β-hydroxylase, are highly induced by PB [7, 8, 10, 12–14, 42], allowing one to readily distinguish differing levels of PB-type induction.

Induction of CYP2B1 and CYP3A. There was a significant degree of correlation ($r = 0.94$, $P < 0.01$) between the levels of BZR *O*-dealkylase activity and of testosterone 16β-hydroxylase activity (Fig. 2), both reactions being mediated preferentially by CYP2B1 [12, 14]. The induction of another major cytochrome P450 family, CYP3A (the forms which are highly induced by PCN), was monitored by measuring the hydroxylation of testosterone at the 6β position [14]. A significant correlation ($r = 0.87$, $P < 0.05$) between induction of activities mediated by these P450s was observed (Fig. 2).

Induction of epoxide hydrolase. There was a significant correlation between the induction of microsomal epoxide hydrolase and BZR *O*-dealkylase activities following administration of various doses of the barbiturate, PB, or of the halogenated pesticide, DDT ($r = 0.92$, $P < 0.01$), or following administration of various structurally-diverse xenobiotics ($r = 0.86$, $P < 0.01$, Fig. 3).

Induction of glucuronyltransferase. The induction of activities mediating the glucuronidation of 3-hydroxybenz[*a*]anthracene correlated significantly with the induction of BZR *O*-dealkylase activity by various doses of PB or DDT (Fig. 4; $r = 0.91$, $P < 0.01$) or by a variety of xenobiotics ($r = 0.90$, $P < 0.01$). We failed to observe induction of this phase II enzymatic activity following pretreatment of rats with either PCN or 3-methylcholanthrene (Table 1).

Induction of RNAs for CYP2B1, epoxide hydrolase and glutathione S-transferases (Ya/Yc). Quantitative RNA techniques were used to measure levels of RNA coding for CYP2B1, microsomal epoxide hydrolase, and for the glutathione *S*-transferase Ya/Yc gene products. As can be seen in Fig. 5, the levels of RNA for CYP2B1 were increased greatly (12- to 30-fold) in rats treated with strong inducers such as PB, ethylphenylhydantoin, pentobarbital, diallyl sulfide, or 2,4,5,2′,4′,5′-hexachlorobiphenyl. The magnitudes of the increases in levels of RNA coding for CYP2B1 correlated also with the

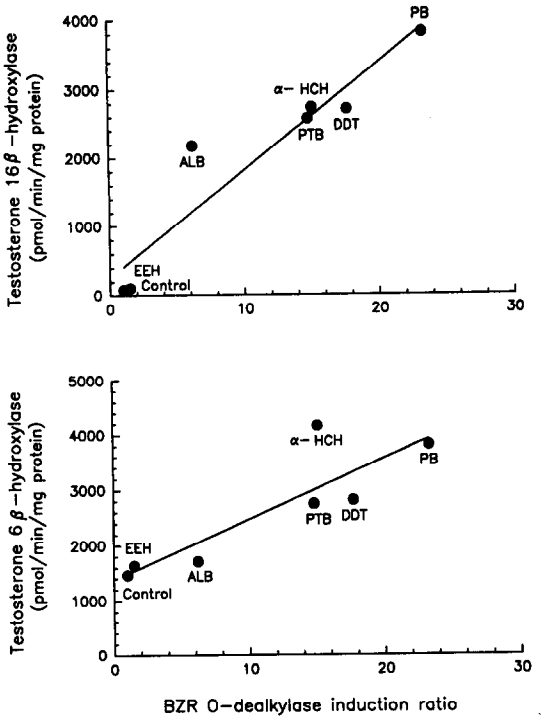

Fig. 2. Relationship between induction of BZR *O*-dealkylase (CYP2B1) and testosterone 16β-hydroxylase (top panel) or 6β-hydroxylase (bottom panel) activities. Three to five individual male F344/NCr rats were used per treatment group. BZR *O*-dealkylase induction is expressed as the ratio of activities measured in microsomes from treated rats to those measured in control rats (mean control value = 160 pmol/min/mg microsomal protein), while actual activities of testosterone hydroxylase were used in the correlation analyses. See Materials and Methods for abbreviations, and for dosing regimens employed.

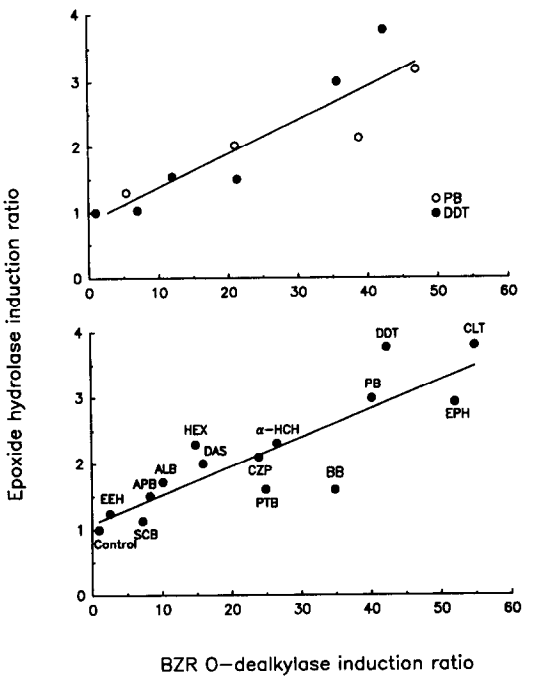

Fig. 3. Relationship between induction of BZR *O*-dealkylase (CYP2B1) and microsomal epoxide hydrolase activities. Three to five individual male F344/NCr rats were used per treatment group, and inductions are expressed relative to control activities (given in the footnotes to Table 1). Both activities were measured in hepatic S-9s. Top panel: Induction by various doses of PB (18.5, 67, 166 or 500 ppm) or DDT (16.4, 64, 256 or 500 ppm). Bottom panel: induction by structurally-diverse xenobiotics. See Materials and Methods for abbreviations, and for dosing regimens employed.

magnitudes of the increases in BZR *O*-dealkylase activities observed (data not shown). The pGTB-38 plasmid hybridizes to the Ya gene and to the Yc gene, both genes being members of the α subclass of glutathione *S*-transferase, and showing greater than 95% nucleotide homology within the regions of the cDNA incorporated into the plasmid. The results in Figs. 6 and 7 show that there was striking induction of RNAs hybridizing to the Ya/Yc family and to the epoxide hydrolase gene (plasmid pEPH302) by those compounds which are strong inducers of CYP2B1. The most striking discrepancy in these results is that obtained with diallyl sulfide, which appears to be relatively more efficient at inducing epoxide hydrolase or glutathione *S*-transferase than at inducing CYP2B1. When the relative levels of CYP2B1 RNA and RNAs for glutathione *S*-transferase Ya/Yc or epoxide hydrolase were compared (excluding DAS), rank correlation (r_s) values in excess of 0.685 ($P < 0.05$) were observed.

Pleiotropic responses caused by prototype inducers. The relative induction of selected catalytic activities by varying inducers is shown in Table 1. The major finding was that these xenobiotics yield a characteristic pattern which is similar among all PB-type inducers but which is quite distinct from patterns elicited by other prototype inducers, such as PCN, or the 3-methylcholanthrene/2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-type inducers, 3-methylcholanthrene and 3,3′,4,4′-tetrachlorobiphenyl. Interestingly, while the P450 induction patterns displayed a fairly high degree of specificity for the various prototype inducers, there was more overlap observed in the case of the non-P450 mediated enzymes. Thus, both PB- and 3-methylcholanthrene/TCDD-type inducers caused increases in levels of epoxide hydrolase and glutathione *S*-transferase activities. In contrast, PB caused increases only in that form of UDP-glucuronyltransferase which glucuronidates 3-hydroxybenz[*a*]anthracene. 3-Methylcholanthrene/TCDD-type inducers, on the other hand, specifically increased quinone oxidoreductase and aldehyde dehydrogenase (benzaldehyde, $NADP^+$) activities and induced a form of UDP-glucuronyltransferase that mediates the glucuronidation of 3-hydroxybenzo[*a*]pyrene [43].

DISCUSSION

The objective of this study was to determine

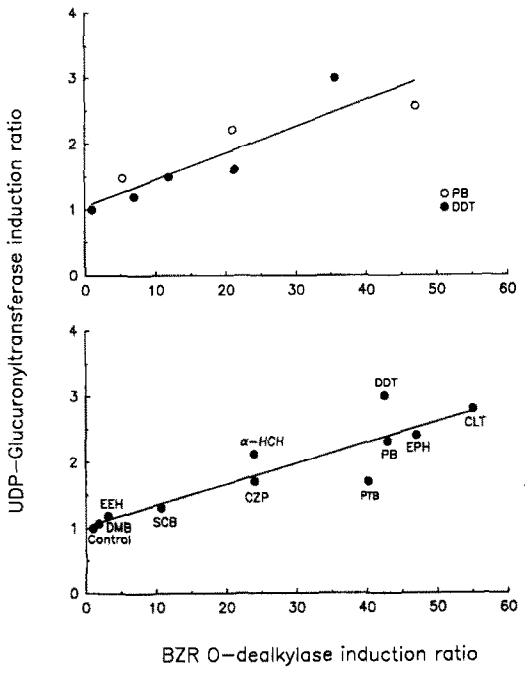

Fig. 4. Relationship between induction of BZR *O*-dealkylase (CYP2B1) and UDP glucuronyltransferase (glucuronidation of 3-hydroxybenz[*a*]anthracene). Three to five individual male F344/NCr rats were used per treatment group, and inductions are expressed relative to control activities (given in the footnotes to Table 1). Both activities were measured in hepatic S-9s. Top panel: induction by various doses of PB (18.5, 67, 166 or 500 ppm) or DDT (16.4, 64, 256 or 500 ppm). Bottom panel: induction by structurally-diverse xenobiotics. See Materials and Methods for abbreviations, and for dosing regimens employed.

whether a wide variety of xenobiotics, all of which have been demonstrated previously to induce CYP2B1 and/or its associated catalytic activities, each induce a similar set of P450 and non-P450 drug-metabolizing enzymes as a coordinate hepatic pleiotropic response. The endpoints which we have monitored were chosen based upon previous reports that the levels of each of these endpoints were increased by the prototype inducing agent PB. Interestingly, in those cases where gene expression has been studied, CYP2B, CYP3A, epoxide hydrolase and glutathione *S*-transferase induction appear to be mediated at some pretranslational level and increases in RNA coding for each of these proteins are observed quite rapidly following inducer treatment [10, 11, 19, 40, 44].

We have shown in the present study that various genes and their associated catalytic activities appear to be induced coordinately in rat liver following treatment with PB-type inducers possessing diverse structural attributes. Thus, when examining induction by various doses of PB or DDT, or by a structurally-diverse group of xenobiotics (Figs. 2–4), significant correlations were observed between the extent of induction of CYP2B1-mediated activities and activities mediated by CYP3A, microsomal epoxide hydrolase or UDP-glucuronyltransferase. In addition, the induction of RNAs coding for glutathione *S*-transferase Ya/Yc or epoxide hydrolase correlated significantly with the ability of a compound to induce RNA for CYP2B1. One compound which fit very poorly to the latter regression lines was diallyl sulfide. This compound has the chemical substructure —S—C=C— which is common to a variety of compounds which induce many phase II drug-metabolizing enzymes (including epoxide hydrolase, glutathione *S*-transferases, and quinone oxidoreductase) [45] through the mediation of a known DNA binding element which is independent of PB or TCDD [46, 47]. Since the induction of the phase II enzymes appears to be mediated via a separate mechanism, the larger than expected inductions of RNAs for epoxide hydrolase and glutathione *S*-transferase may represent the additive effects of the two induction mechanisms (—S—C=C— and PB-type pleiotropic responses). For this reason, the diallyl sulfide value was excluded from the correlation analysis.

In toto, both the enzymatic and hydridization results seem to argue strongly for some overall coordinate induction of a pleiotropic response by all PB-type inducers, no matter how structurally diverse. We have also observed a coordinate induction of CYP2B1, CYP3A, epoxide hydrolase and glutathione *S*-transferases in F344/NCr, DA or Zucker female rats exposed to a variety of barbiturates and hydantoins [48]. This latter result confirms the general applicability of these observations to various strains of rats. In addition, our results in mice with 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) and various barbiturates (unpublished data) as well as our results with PB in different animal species (rat, mouse, rabbit, Patas monkey) [49, 50] appear to be compatible with the existence of such a pleiotropic response which includes CYP2B, CYP3A and epoxide hydrolase.

Our conclusion that CYP2B1-inducing agents in general cause a pleiotropic response appears to contradict the conclusions of certain other studies undertaken in mice or rats. The two studies in mice employing either a series of barbiturates [18] or TCPOBOP and PB [51] concluded, based upon examining multiple cytochrome P450-mediated catalytic endpoints, that the proportionality among the various catalytic endpoints was not absolutely maintained following treatment with the varying compounds, and therefore that these compounds were not necessarily inducing the same pleiotropic response. Alternatively, since the correlations by rank between induction of the various measured activities were highly significant, we would suggest that the failure to achieve absolutely equivalent proportionality must result from other factors. One of these may be that various constitutive forms of P450 metabolize to some extent many of the substrates examined by these investigators, and the contribution of these constitutive forms may be greater in the case of the weaker inducing agents. Additionally, profound induction of certain forms of P450 may be accompanied by reduction in the

Table 1. Relative hepatic induction of selected catalytic activities by diverse xenobiotics in the male F344/NCr rat

Xenobiotic*	Type of inducer	Fold induction of catalytic activities mediated by: CYP1A†	CYP2B1‡	CYP3A§	Epoxide hydrolase‖	Aldehyde dehydrogenase¶	Quinone oxidoreductase**	Glucuronyl-transferase††
Control		1.0	1	1	1	1	1	1
EEH		1.4	2	1	1	ND‡‡	<2	1
PB	PB	3.4	40	3	3	<2	<2	3
DDT	PB	2.5	35	3	4	<2	<2	4
α-HCH	PB	2.4	17	3	2	<2	<2	2
EPH	PB	3.0	35	4	3	<2	<2	3
ALB	PB	1.5	12	2	2	<2	<2	2
DAS	PB/?	ND	10	2	2	ND	3	ND
CLT	PB/PCN	2.7	42	6	4	<2	<2	3
PCN	PCN	1.5	2	5	1	<2	<2	1
TCB	MCA/TCDD	51	5	1	7	38	8	ND
MCA	MCA/TCDD	39	5	1	ND	30	ND	1

* Abbreviations, α-HCH, α-hexachlorocyclohexane; ALB, allobarbital; CLT, clotrimazole; DAS, diallyl sulfide; DDT, dichlorodiphenyltrichloroethane; EEH, 5,5-diethylhydantoin; EPH, 5-ethyl-5-phenylhydantoin; MCA, 3-methylcholanthrene; PB, phenobarbital; PCN, pregnenolone-α-carbonitrile; TCB, 3,3′,4,4′-tetrachlorobiphenyl; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

† CYP1A induction is based upon increases in hepatic S-9-mediated O-dealkylation of ethoxyresorufin; control activity: 25 ± 3 pmol resorufin formed/min/mg S-9 protein at 28°.

‡ CYP2B1 induction is based upon increases in hepatic S-9-mediated O-dealkylation of benzyloxyresorufin; control activity: 27 ± 5 pmol resorufin formed/min/mg S-9 protein at 28°.

§ CYP3A induction is based upon increases in the hepatic microsomal 6β-hydroxylation of testosterone; control activity: 1464 pmol 6β-hydroxytestosterone formed/min/mg pooled microsomal protein at 37°.

‖ Epoxide hydrolase induction is based upon increases in the hepatic S-9-mediated hydration of benzo[*a*]pyrene-4,5-oxide; control activity: 468 ± 43 pmol benzo[*a*]pyrene-4,5-diol formed/min/mg S-9 protein at 28°.

¶ Aldehyde dehydrogenase induction is based upon increases in the hepatic cytosolic dehydrogenation of benzaldehyde, using $NADP^+$ as cofactor; control activity: 2.8 nmol NADPH formed/min/mg pooled cytosolic protein.

** Quinone oxidoreductase induction is based upon increases in hepatic S-9-mediated reduction of resorufin; mean control activity: 1.8 ± 0.35 nmol resorufin reduced/min/mg S-9 protein at 28°.

†† Glucuronyltransferase induction is based upon increases in hepatic microsomal UDP-glucuronidation of 3-hydroxybenz[*a*]anthracene.

‡‡ Not determined.

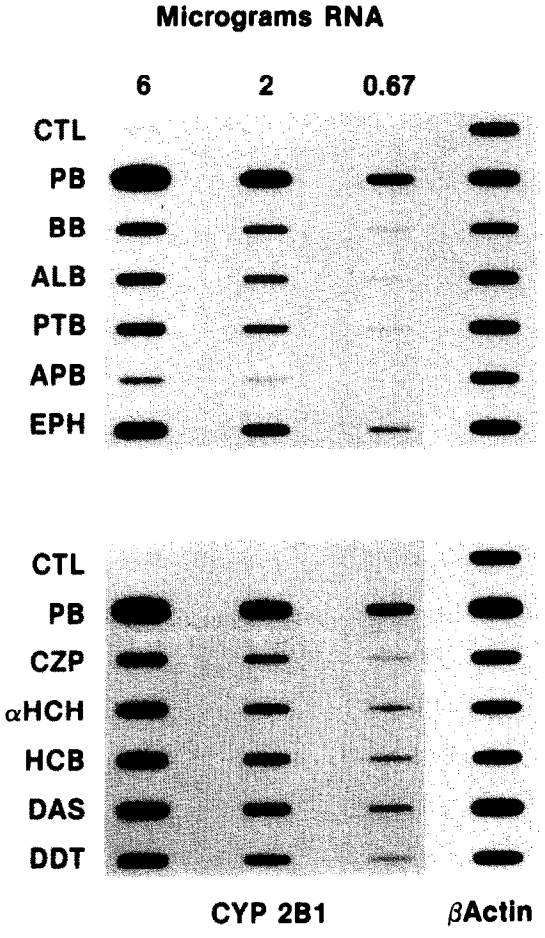

Fig. 5. Expression of the CYP2B1 gene in male F344/NCr rats exposed to various xenobiotics: (top panel) induction by various barbiturates; (bottom panel) induction by structurally-diverse xenobiotics. See Materials and Methods for abbreviations, and for dosing regimens employed. RNA was quantitated by hybridization to an oligonucleotide (5'-GGTTGGTAGCCGGTGTGA-3') [10].

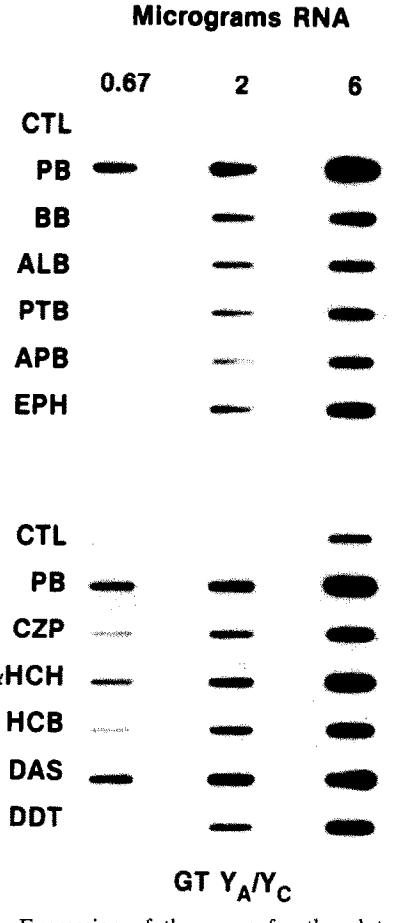

Fig. 6. Expression of the genes for the glutathione *S*-transferase family Ya/Yc in rats exposed to various xenobiotics: (top panel) induction by various barbiturates; (bottom panel) induction by structurally-diverse xenobiotics. See Materials and Methods for abbreviations, and for dosing regimens employed. RNA was quantitated by hybridization to plasmid pGTB-38 [40].

levels of the constitutive forms and also may cause deficiencies in the amounts of cytochrome P450 reductase available. Either of these factors may alter the relative catalytic activities towards specific substrates.

There are certain strains or substrains of rats in which CYP2B2 apparently is not expressed following administration of PB, while CYP2B1 induction does occur [20, 21]. That this is the case has been used as an argument against the existence of a coordinate pleiotropic PB response. In most of these animal models, however, there appears to be neither constitutive nor PB-inducible expression of the CYP2B2 gene, implying that for whatever reason the structural gene itself or its related 5'-regulatory elements have been altered substantially. If so, this finding should not be interpreted as a lack of coordination between the induction of CYP2B2 and CYP2B1. In contrast, the induction of aldehyde dehydrogenase (propionaldehyde, NAD^+), which has been found to be inducible by PB in some rat strains while not in others, does appear to reflect a true strain or substrain difference in responsiveness to PB. In this case the gene appears to be expressed constitutively in all of the rat strains/substrains examined. Irrespective of the actual mechanism(s) underlying these differences in inducibility for CYP2B2 and aldehyde dehydrogenase, such differences are not at variance with our finding that in those strains or species in which these activities are induced by PB, the activities also appear to be induced following administration of a variety of structurally-dissimilar PB-type inducers.

The findings of Guzelian and coworkers employing rat hepatocyte cultures [52] indicate that a higher concentration of PB is required for the half-maximal induction of CYP3A compared to the ED_{50} for CYP2B induction, a result which we have recently confirmed *in vivo* with the male F344/NCr rat (Nims RW and Lubet RA, unpublished). The *in vitro* hepatocyte studies also demonstrated that the

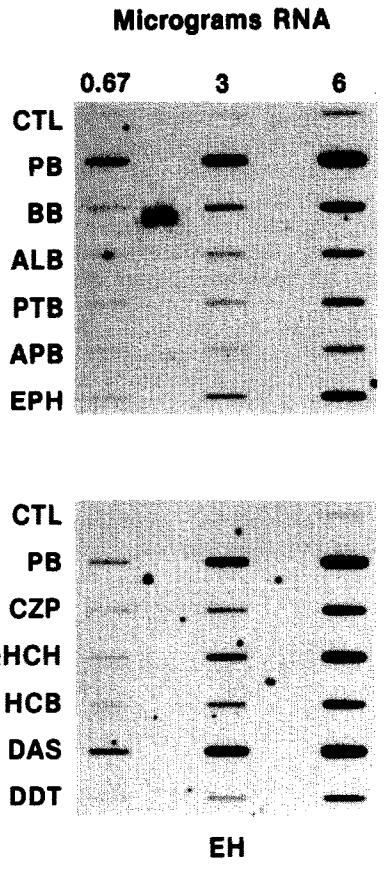

Fig. 7. Expression of the gene for microsomal epoxide hydrolase in rats exposed to various xenobiotics: (top panel) induction by various barbiturates; (bottom panel) induction by structurally-diverse xenobiotics. See Materials and Methods for abbreviations, and for dosing regimens employed. RNA was quantitated by hybridization to plasmid pEPH302 [41].

induction of CYP2B1 by PB is highly sensitive to inhibition by cycloheximide, while the induction of CYP3A is actually enhanced in the presence of the protein synthesis inhibitor [53]. These *in vitro* findings could be interpreted as evidence for mechanistic differences between the induction of the two families of P450 by PB and the absence therefore of coregulation of the P450s. However, the result obtained with cycloheximide is mitigated by the fact that treatment of female rats with cycloheximide in the absence of other inducing agents causes decreases in the constitutive levels of CYP2B RNA while simultaneously causing increases in the levels of CYP3A RNA [53]. Thus, the distinct responses obtained with these two P450 families following cycloheximide treatment may reflect differences in inducibility by cycloheximide itself, and may not relate specifically to the mechanism of action of PB on these two forms. Also, the observation of differences in the dose–response characteristics observed for the two families of P450 following PB administration may not reflect mechanistic differences in induction, and probably does not rule out coregulation of two forms. A shift to the right in dose response is also observed in the induction of aldehyde dehydrogenase (benzaldehyde, $NADP^+$) and quinone oxidoreductase, compared to CYP1A, following treatment of rats with TCDD-type inducers [54, 55], despite the general consensus that these non-P450 activities are an integral facet of the pleiotropic TCDD response.

As is presented in summary form in Table 1, the pleiotropic responses elicited by different classes of inducers (PB-type, 3-methylcholanthrene/TCDD-type, PCN-type) appear to differ clearly. Thus, the varied chemicals that induced CYP2B1 activity also induced CYP3A as well as non-P450-mediated drug-metabolizing enzymes such as microsomal epoxide hydrolase and certain isozymes of UDP-glucuronyltransferase. In contrast, 3-methylcholanthrene and 3,3′,4,4′-tetrachlorobiphenyl induced a different, albeit partly overlapping, spectrum of activities (CYP1A1, CYP1A2, epoxide hydrolase, quinone oxidoreductase and certain isozymes of UDP-glucuronyltransferase and aldehyde dehydrogenase). A third type of inducer, exemplified by PCN, induced primarily CYP3A among the more limited types of enzymes which we examined. In contrast to the clearly defined receptors involved in 3-methylcholanthrene/TCDD or clofibrate-type inductions [15, 29, 56], such a receptor has not been demonstrated in the case of PB-type induction, despite repeated attempts [57]. It has been hypothesized by Poland *et al.* [57] that this may reflect primarily the absence of a sufficiently potent ligand with which to study receptor binding. In addition, there are other unusual aspects of PB-type induction, one of which is the fact that there are great variations in the structures of PB-type inducers, as exemplified in the present study (Fig. 1).

The possible lack of a receptor for PB-type induction raises questions relating to the mechanism of the coordinate pleiotropic response which we observe following treatment with structurally-diverse chemicals (Figs. 2, 3, and 7). Since most of the compounds which are strong PB-type inducers are relatively lipophilic, one might propose that the pleiotropic response relates to some fairly nonspecific membrane perturbation, not necessarily toxicity, caused by these chemicals. Arguing against such a proposal are the findings that TCPOBOP, a long-lived and potent inducer in mice, is two to three orders of magnitude less potent in rats [57], and that other lipophilic compounds, such as polycyclic hydrocarbons or clofibrate, some of which are potent inducers of other cytochrome(s) P450, do not induce the PB-type pleiotropic response even at relatively high doses. A second major hypothesis is that compounds which bind to and interact with specific forms of cytochrome P450 result in the induction of those forms of cytochrome P450 [58]. Although this might apparently explain the inducing properties of such inhibitors as clonazepam and clotrimazole, it seems less likely to explain the induction caused by other compounds, such as barbital, which only weakly interact with the specific form of cytochrome P450 which is induced [59].

Nonetheless there are a variety of findings that would give indirect support for either a receptor or

some common biochemical mechanism for induction by these varied chemicals. The first is the fact that coordinate induction of a number of different enzymes was observed (Figs. 2–4 and 7). In addition, other investigators have shown [10, 40, 44] that many of these genes (e.g. P450s, glutathione *S*-transferases and epoxide hydrolase) are induced, apparently at some pretranslational level, quite rapidly following PB administration. These results would appear to argue, at least indirectly, for some common biochemical determinant for altered expression of these various genes or proteins. Additional evidence, reported in the accompanying paper [48], is provided by the existence of a line of rats which displays a markedly diminished response in all aspects of the pleiotropic response (CYP2B1, CYP3A, microsomal epoxide hydrolase, glutathione *S*-transferases) following treatment with structurally-diverse PB-type inducers.

Recent work by He and Fulco [60] appears to provide a promising area for investigation of the PB-type pleiotropic response. These authors have identified a 17 bp sequence which is found in the 5′ portion of the PB-inducible CYP genes occurring in both bacteria (*Bacillus megaterium*, P450 BM-1, P450 BM-3) and the rat (CYP2B1 and CYP2B2) in which 10 bp are identical in all four genes and 11 are identical in three of the four genes. This sequence interacts with and binds with a PB-inducible/activated protein in the nuclei of liver cells. Interestingly, certain PB-inducible genes show nucleotide sequences with a high degree of homology to this 17 bp sequence. These include glutathione *S*-transferases Ya/Yc (displaying identity in 9/11 of the bases within the highly conserved bases in the region from −166 to −180 base pairs from the start site) [61]; aldehyde dehydrogenase, which shows identity in 8/11 of the highly conserved bases in the region from −150 to −164 base pairs from the start site) [19]; epoxide hydrolase, displaying identity in 7/11 of the highly conserved bases in the region from −84 to −98 bp from the start site [62]; and rabbit CYP2C1, which shows identity in 7/11 bases in the region from −228 to −243 bp from the start site [63]. Such a binding motif and its associated protein would obviously be of great interest as a potential mechanism for the induction of a PB-type pleiotropic response.

Although our primary focus has been upon the coordinate induction of various drug-metabolizing enzymes, the induction of this pleiotropic response is also associated with hepatocytomegaly and liver tumor promotion in *N*-nitrosodiethylamine-initiated animals. Thus, various barbiturates [7, 49, 64], certain hydantoins [22, 49], DDT [65], 2,4,5,2′,4′,5′-hexabromobiphenyl [66], and α-hexachlorocyclohexane [67], all of which are strong inducers of CYP2B1, are liver tumor promoters in rats. This has led us to hypothesize that compounds which are strong inducers of CYP2B1 may be predicted to display liver tumor promoting activity in rats [7, 22, 49] and mice. The liver tumor-promoting activity presumably reflects some aspect(s) of the hepatic pleiotropic response to these inducers.

In summary, we have shown that in the rat, diverse compounds which induce CYP2B1 also induce a variety of other drug-metabolizing enzymes, presumably as some portion of a coordinate pleiotropic response to these inducers. In the accompanying paper [48], we describe the decreased responsiveness of the outbred Zucker rat line to structurally-diverse PB-type inducers. This rat strain demonstrates a diminished response to the various elements (CYP2B1, CYP3A, microsomal epoxide hydrolase, glutathione *S*-transferases) of the entire pleiotropic response which we describe here, further supporting the coordinate nature of the response.

Acknowledgements—The authors would like to acknowledge the skilled technical assistance of Dan Logsdon and Craig Driver. This project has been funded at least in part with Federal funds from the Department of Health and Human Services under Contract No. N01-CO-74102 with Program Resources, Inc. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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