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INDUCTION OF A PLEIOTROPIC RESPONSE BY PHENOBARBITAL AND RELATED COMPOUNDS

RESPONSE IN VARIOUS INBRED STRAINS OF RATS, RESPONSE IN VARIOUS SPECIES AND THE INDUCTION OF ALDEHYDE DEHYDROGENASE IN COPENHAGEN RATS

COLLINS R. JONES* and RONALD A. LUBET†‡

*Biological Carcinogenesis and Development Program, Program Resources Inc./DynCorp, and †Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702–1201, U.S.A.

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Abstract—The ability of phenobarbital (PB) to induce a "pleiotropic response" which includes both cytochromes P450 (CYP) as well as other drug-metabolizing enzymes was investigated in mice, rabbits, hamsters, and various inbred strains of rats. PB induced similar drug-metabolizing enzymes (CYP2B, CYP3A, and epoxide hydrolase) in rats, mice, rabbits and hamsters. PB and two structural analogues (ethylphenylhydantoin and barbital) induced a variety of drug-metabolizing enzymes (CYP2B, CYP3A, CYP2A, epoxide hydrolase) in a series of inbred strains of rats. In contrast, levels of aldehyde dehydrogenase (ALDH) (propionaldehyde, NAD^+) which were expressed constitutively in all strains of rats were induced by PB in only two of the eight strains (ACI, Copenhagen). Further investigations of ALDH induction by structurally diverse compounds in Copenhagen rats demonstrated a strong correlation between the induction of ALDH and other elements of the pleiotropic response (CYP2B, CYP3A, epoxide hydrolase). These results imply that induction of ALDH (propionaldehyde, NAD^+) is associated with the PB pleiotropic response in Copenhagen rats.

Certain recent investigations, including our own, have strongly implied that there is a consistent "pleiotropic" drug-metabolizing enzyme response to phenobarbital (PB)§, certain structurally related compounds (barbiturates and hydantoins) and structurally diverse compounds, e.g. α-hexachlorocyclohexane, clonazepam, DDT, and 2,2′,4,4′,5,5′-hexachlorobiphenyl in male rats [1–6]. This pleiotropic response includes induction of specific forms of cytochrome P450 (CYP): CYP2B, CYP2C, and CYP3A; epoxide hydrolase; glutathione transferases Ya/Yc and Yb1/Yb2; and certain forms of glucuronyl transferases [1–9]. The various elements of this pleiotropic response were shown to be induced in female F344 and female DA rats as well [2]. Interestingly, we observed that the outbred line of Zucker rats showed a markedly diminished response to both PB and certain of its structural analogs (ethylphenylhydantoin, barbital), as well as to more structurally diverse PB-type inducers [2]. Diminished response is defined here as a more limited induction of various elements of the PB pleiotropic response (CYP2B, CYP3A, epoxide hydrolase, glutathione *S*-transferases Ya/Yc) than was observed following similar treatment of a variety of other rat strains. In fact, the results with the Zucker animals would appear to be most consistent with a dose–response curve which has been shifted substantially to the right [2]. This diminished effect was profound in the Zucker female obese animals, was still relatively strong in female Zucker lean animals and was least striking although still readily apparent in male Zucker lean animals. The work of May and coworkers [3] showed that inhibition of heme synthesis decreases all of the various elements of this phenobarbital-mediated pleiotropic response (CYP2B, CYP3A, GST Ya/Yc, epoxide hydrolase) while having minimal effects on Ah receptor-mediated induction by 5,6-benzoflavone. All of the above results would seem compatible with, although they are no proof of, a common biochemical mechanism for determining the response to these various PB-type inducers. However, no such receptor has been shown for PB-type compounds [10, 11] and, in fact, it would be hard to conceive of a high-affinity receptor which would be compatible with the structurally varied compounds which elicit the PB response (e.g. PB, pentobarbital, DDT, clotrimazole, and α-hexachlorocyclohexane).

In contrast to this more consistent pleiotropic response, certain authors have shown that at least two drug-metabolizing enzymes [CYP2B2; alde-

‡ Corresponding author: R. A. Lubet, Ph.D., Building 538, Room 205E, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702–1201. Tel. (301) 496-8563; FAX (301) 496-8567.

§ Abbreviations: ALDH, aldehyde dehydrogenase; BZROD, benzyloxyresorufin *O*-dealkylase; CYP, cytochrome P450; EH, epoxide hydrolase; EPH, ethylphenylhydantoin; PB, phenobarbital; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

hyde dehydrogenase (ALDH) (propionaldehyde, NAD^+)], which are induced in certain strains of rats following PB treatment, are not induced in other strains of rats [12–16]. Certain of these authors have used these results to hypothesize that there is no consistent pleiotropic response to PB and PB-type compounds [15]. The lack of response to CYP2B2 may be due to lack of expression of a functional structural gene, either constitutively or following PB induction [12–14]. In contrast, this does not appear to be the case for the ALDH gene where constitutive expression of the gene occurs in strains or substrains of rats that are either responsive or nonresponsive to PB-type induction [15].

The present series of experiments were intended to further explore this pleiotropic response and examine whether:

(1) this PB-mediated pleiotropic response is observed in various rodent and lagomorph species following treatment with PB.
(2) the pleiotropic response is observed in a wide variety of inbred strains of rats and whether any of these strains are relatively less responsive like the outbred Zucker rat.
(3) ALDH is associated with the pleiotropic response to PB-type inducers in those strains of rats in which it is induced.

MATERIALS AND METHODS

Chemicals. NAD^+, $NADP^+$, NADPH, dicumarol, testosterone, PB, and barbital were obtained from the Sigma Chemical Co. (St. Louis, MO). 5-Ethyl-5-phenylhydantoin and 5,5-diethylhydantoin were synthesized at FCRDC. Benzyloxy- and ethoxyresorufin (BZR and ETR, respectively) as well as resorufin were purchased from Molecular Probes (Eugene, OR). Benzo[*a*]pyrene-4,5-oxide and benzo[*a*]pyrene-4,5-dihydrodiol were obtained from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). Hydroxylated testosterone metabolites for use as HPLC standards were purchased from Steraloids (Wilton, NH). Rabbit polyclonal antisera to rat CYP2B and CYP3A were purchased from Oxygene (Dallas, TX). Alkaline phosphatase conjugated secondary antibody was purchased from Life Sciences Inc. (Gaithersburg, MD).

Treatment of animals. Male rats (6–9 weeks of age) were either obtained from Charles River (Kingston MA) (Brown Norway, Copenhagen, Noble) or from the FCRDC Animal Production Area (F344, Buffalo, Wistar Furth, Louvain, Lewis, ACI). $B6C3F_1$ mice and Syrian golden hamsters were obtained from the FCRDC animal production area. Animals were administered control diet (Purina Lab Chow No. 5010) or diet containing the indicated amount of test chemical *ad lib.* for 2 weeks. Individual rats were then killed by CO_2 asphyxiation, weighed, and the livers removed. The livers were weighed and homogenized in 0.15 M KCl/0.2 M sucrose (4 mL/g wet weight, 4°). Post-mitochondrial (S-9) subfractions were obtained by centrifugation of the homogenates at 9000 *g* for 15 min (4°) with the supernatant retained as the S-9 fraction. Microsomal and cytosolic fractions were prepared by ultracentrifugation (100,000 *g*, 75 min, 4°) of pooled S-9 fractions.

Alkoxyresorufin O-dealkylase assays. Benzyloxyresorufin *O*-dealkylase (BZROD) was measured using either S-9 or microsomal fractions as described in Ref. 17. S-9 protein levels were between 50 and 150 μg for induced animals and between 300 and 900 μg for control animals. Benzyloxyresorufin was employed at a final concentration of 5 μM in 2.0 mL of phosphate buffer, pH 7.5, containing 25 mM $MgCl_2$. The increases in relative fluorescence per unit time due to the formation of resorufin were measured in a Perkin–Elmer spectrophotofluorimeter (LS-5B) at 25° and were compared to the fluorescence of known amounts of resorufin. In all the studies employing rats, activities were determined on at least three individual animals.

Epoxide hydrolase (EH) assay. The continuous fluorometric assay developed by Dansette *et al.* [18] 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 (LS-5B). Substrate (benzo[*a*]pyrene-4,5-oxide, 10 μM) was combined with 50–100 μg of S-9 or microsomal protein and placed in 0.1 M Na_2HPO_4 buffer, pH 8.8, in a final volume of 2 mL. Incubations were carried out at 25° for 5–15 min. Increases in relative fluorescence per unit time were compared to the fluorescence of known amounts of benzo[*a*]pyrene-4,5-dihydrodiol.

Aldehyde dehydrogenase (ALDH) assay. Aldehyde dehydrogenase was determined as described by Lindahl and Evces [19]. Enzyme activities were determined in a 1.0-mL final assay volume containing 5 mM propionaldehyde, 1 mM NAD^+, 15–30 μg of cytosolic protein in 80 mM pyrophosphate buffer, pH 8.8, containing 0.75 mM pyrazole at 25°. The increase in absorbance at 340 nm due to the production of NADH was determined (Gilford UV-VIS spectrophotometer) and the rate of nanomoles of NADH produced per minute per milligram of protein was calculated using an extinction coefficient of 6220 $M^{-1}\,cm^{-1}$.

Testosterone hydroxylase. Cytochrome P450-mediated hydroxylation of testosterone was measured as described by Sonderfan *et al.* [20]. A 3-mL incubation mixture containing 50 mM phosphate buffer, pH 7.4, 4 mM NADPH, 250 μM testosterone (added in 20 μL methanol), 20 μM 11β-hydroxytestosterone (added as internal standard in 20 μL methanol) and 10 μM 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5-androstan-3-one [21] was preincubated at 30° for 5 min. The reaction was initiated by the addition of microsomal protein (300–600 μg) to yield a final concentration of 100–200 μg/mL. At 2 and 4 min, 1-mL aliquots were withdrawn and pipetted into 6 mL of methylene chloride to halt the reaction and extract the products. After a total of two extractions, pooled extracts were dried under nitrogen and redissolved in water/methanol/acetonitrile (60:39:1, by vol.). The redissolved residues were subjected to HPLC analysis (Waters System, Supelco C-18 column) exactly as described by Sonderfan *et al.* [20]. The values for the 2- and 4-min time points were averaged (SD ≤ 15% of mean).

Protein determination. Proteins were determined by the method of Bradford [22].

Detection of cytochrome P450 (CYP) proteins by immunoblotting. Immunochemical detection was performed using modifications of the method of Towbin *et al.* [23]. Briefly, microsomal samples were solubilized at a final concentration of 1 μg/μL by boiling for 2 min in buffer containing 1% sodium dodecyl sulfate and 10 mM β-mercaptoethanol. Samples were loaded (10 μg/lane) onto a 10% SDS–PAGE minigel (Schleicher & Schuell, Keene, NH). After separation by electrophoresis the resolved proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked overnight in 5% (w/v) nonfat powdered milk (Carnation, Inc., Los Angeles, CA). After washing, the CYP proteins bound to the membranes were detected using the appropriate antibody at a 1:200 dilution (2-hr incubation at room temperature). The membrane was washed again and incubated for 2 hr at room temperature with alkaline phosphatase conjugated secondary antibody at a 1:2500 dilution. Alkaline phosphatase activity was visualized using 5-bromo-4-chloro-3-indolylphosphate and *p*-nitro blue tetrazolium chloride according to the manufacturer's directions (Bio-Rad Inc., Richmond, CA).

RESULTS

We employed three different endpoints in determining the induction of various CYP proteins. First, we measured levels of specific alkoxyresorufin *O*-dealkylases: benzyloxy (Table 1) and pentoxy (data not shown). These results showed that BZROD and pentoxyresorufin *O*-dealkylase (PROD) were highly induced in rats, mice, and rabbits but were induced minimally in hamsters. Induced levels of pentoxyresorufin *O*-dealkylase were 2- to 5-fold lower than the rates of benzyloxyresorufin *O*-dealkylase in the various species [17].

Second we employed immunochemical detection techniques (Western blotting; Fig. 1), to further characterize CYP induction. In the mouse, rat, and rabbit we observed striking induction of material that cross-reacts with polyclonal antibody to the rat CYP2B family (Fig. 1, left panel). This CYP protein did not appear to be as strongly induced in the hamster. Similarly, when we examined the induction of the CYP3A family by PB (Fig. 1, right panel), employing polyclonal antibody directed against rat CYP3A, we observed fairly strong induction in mice, rats and hamsters but a relatively weak response in rabbit microsomes.

Finally, we examined the induction of the various CYPs by measuring specific metabolism of testosterone (Table 1). Hydroxylation of testosterone at the 16β position is closely associated with the CYP2B family in rats. This activity was highly induced in rats (>50-fold), moderately induced in mice (5-fold), weakly induced in rabbits (2-fold), and was not induced in hamsters. 6β-Hydroxylation of testosterone which is associated with the CYP3A family in various species was induced 2- and 3-fold in rats, mice, and rabbits, but not at all in hamsters.

Table 1. Induction of xenobiotic metabolizing enzymes in rodent species

Species/ Treatment*	BZROD† CYP2B	EH‡	ALDH§	Hydroxylated testosterone metabolite‖ 6β CYP3A	7α CYP2A	16β CYP2B
F344/NCr rat						
Control	60	1.6	4.9	1026	103	ND¶
Phenobarbital	3007	2.9	6.4	3759	357	3817
B6C3F$_1$ mouse						
Control	47	1.6	0.9	1871	341	84
Phenobarbital	1836	2.8	1.6	3763	532	340
Syrian hamster						
Control	93	2.2	4.1	3885	2866	ND
Phenobarbital	127	6.7	4.2	3785	1537	ND
New Zealand black rabbit						
Control	127	2.5		1157	ND	110
Phenobarbital	4792	5.0		2678	ND	221

* Animals were fed control diet or diet containing 500 ppm phenobarbital for 2 weeks *ad lib.*

† Benzyloxyresorufin-*O*-dealkylase (BZROD) activity is expressed as pmol resorufin formed/min/mg microsomal protein.

‡ Epoxide hydrolase (EH) activity is expressed as nmol benzo[*a*]pyrene-4,5-diol formed/min/mg microsomal protein.

§ Aldehyde dehydrogenase (ALDH) activity is expressed as nmol NADH produced/min/mg cytosolic protein using propionaldehyde as the substrate and NAD^+ as the cofactor.

‖ Activity is expressed as pmol hydroxylated testosterone metabolite formed/min/mg pooled microsomal protein (N = 3).

¶ ND = not detectable (<20 pmol hydroxylated testosterone metabolite produced/min/mg microsomal protein).

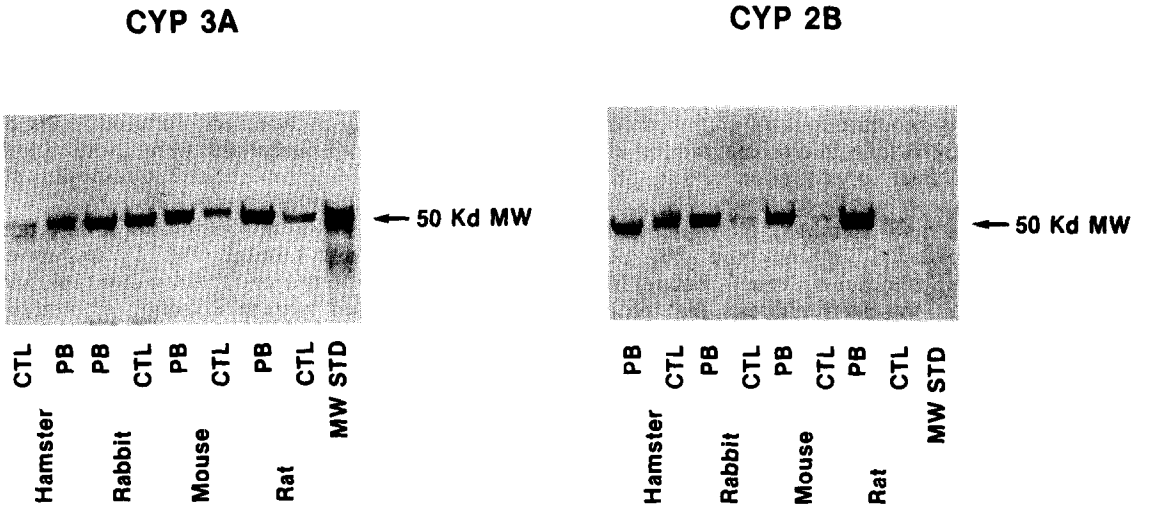

Fig. 1. Immunodetection of CYP2B and CYP3A related proteins in microsomes from control or phenobarbital-treated animals of various species. Ten micrograms of microsomal protein from control or phenobarbital-treated animals was loaded onto lanes of a 10% SDS–PAGE minigel. After electrophoresis the samples were electroblotted to a nitrocellulose membrane. CYB2B-related proteins (Fig. 1, left panel) were probed using polyclonal antisera directed against rat CYP2B. CYP3A related proteins (Fig. 1, right panel) were probed using polyclonal antisera directed against rat CYP3A.

Table 2. Induction of xenobiotic metabolizing enzyme activities by phenobarbital in male rates of different strains

Strain	Treatment*	BZROD†	EH‡	ALDH§
ACI	Control	20 ± 4	397 ± 8	8.4
	Phenobarbital	795 ± 32‖	1621 ± 61‖	34.4
Buffalo	Control	7 ± 1	176 ± 33	5.6
	Phenobarbital	494 ± 80‖	493 ± 21‖	3.4
Copenhagen	Control	35 ± 5	424 ± 49	5.1
	Phenobarbital	867 ± 98‖	1110 ± 153‖	23.7
Fisher 344	Control	22 ± 4	578 ± 27	5.9
	Phenobarbital	1007 ± 118‖	1639 ± 81‖	4.0
Lewis	Control	42 ± 5	437 ± 53	7.7
	Phenobarbital	1106 ± 63‖	1219 ± 121‖	6.6
Louvain	Control	33 ± 5	250 ± 18	6.5
	Phenobarbital	591 ± 109‖	546 ± 58‖	3.9
Brown Norway	Control	29 ± 2	513 ± 47	7.1
	Phenobarbital	821 ± 94‖	1237 ± 48	4.6
Noble	Control	39 ± 3	345 ± 33	4.3
	Phenobarbital	436 ± 63‖	786 ± 59‖	5.2
Wistar Furth	Control	38 ± 4	217 ± 16	5.1
	Phenobarbital	1173 ± 153‖	798 ± 51‖	4.5

* Animals were fed control diet or diet containing 500 ppm phenobarbital for 2 weeks *ad lib.*

† BZROD activity is expressed as pmol resorufin formed/min/mg S-9 protein produced by the O-dealkylation of benzyloxyresorufin. Values are means ±SD (N = 3).

‡ EH activity is expressed as pmol benzo[*a*]pyrene-4,5-diol formed/min/mg S-9 protein. Values are means ± SD (N = 3).

§ ALDH activity is expressed as nmol NADH produced/min/mg cytosolic protein using propionaldehyde as the substrate and NAD^+ as the cofactor. Results are based on pooled cytosols from three animals.

‖ Significantly different from control ($P < 0.05$).

One should be aware that the constitutive levels of 6β testosterone hydroxylase are much higher (2- to 4-fold) in the hamster than in the other species. When we examined the induction of two other noncytochrome P450-mediated drug-metabolizing enzymes, we observed 2- to 3-fold induction of epoxide hydrolase in all three species, while we observed little or no induction of ALDH (propionaldehyde, NAD^+) in mice, F344 rats, or hamsters (Table 1). One should remember that in the species other than rats this activity was investigated in only a single strain. ALDH activity could not be measured in rabbits since we lacked cytosolic protein.

In an attempt to find a nonresponsive inbred strain of rats, similar to the outbred Zucker rats [2], we determined the induction of hepatic xenobiotic metabolizing enzymes by PB, ethylphenylhydantoin (EPH), and barbital in male animals of nine inbred strains of rats. Ethylphenylhydantoin and barbital were employed in addition to PB since Zucker rats proved to be particularly insensitive to induction by these compounds [2]. PB strongly induced CYP2B1 activity (15- to 90-fold), as measured by the O-dealkylation of benzyloxyresorufin in all nine strains tested (Table 2). The constitutive turnover of BZR in Buffalo rats was lower than that observed for the other strains (7 vs >20 pmol/min/mg S-9 protein). Two noncytochrome P450-mediated enzyme activities were investigated as well. Epoxide hydrolase activity was found to be induced 2.5- to 5-fold in all strains tested, while ALDH (NAD^+, propionaldehyde) was inducible (4-fold) by PB in only two strains: ACI and Copenhagen.

The induction of various CYPs in PB-treated strains of rats was measured indirectly by determining the regio- and stereospecific hydroxylation of testosterone as described by Sonderfan *et al.* [20] (Table 3). A number of specific hydroxylated metabolites have been shown to be products of specific CYP proteins or specific CYP families in the rat (16β-hydroxytestosterone/CYP2B; 6β-hydroxytestosterone and 2β-hydroxytestosterone/CYP3A; 7α-hydroxytestosterone/CYP2A) [17]. In each of the strains the hydroxylation of testosterone at the 16β position was highly induced (Table 3). Hydroxylation at the 6β and 2β positions was also inducible (1.5- to 4-fold) in each of the rat strains. Finally, hydroxylation at the 7α position was induced slightly (1.5- to 3-fold) in each of the strains examined.

Ethylphenylhydantoin and diethylbarbituric acid were also tested for their abilities to induce CYP2B1, CYP3A, and epoxide hydrolase. Prior studies showed that EPH (500 ppm) is a fairly strong inducer, similar to PB, in F344 male rats while barbital is somewhat weaker. In contrast, both are considerably weaker inducers than PB (500 ppm) in Zucker lean male or female rats [2]. In the present studies, parallel results were obtained in all strains of rats tested (data not shown). To wit, ethylphenylhydantoin treatment induced each of the enzyme activities (EH, CYP2B, CYP3A) comparably to PB, whereas barbital was a weaker inducer in all inbred strains tested.

Our studies of induction in various strains of rats (Table 2) demonstrated induction of ALDH in Copenhagen rats by PB. We therefore examined a variety of compounds for their abilities to induce both ALDH as well as other drug-metabolizing enzymes involved in the pleiotropic response to PB (Tables 4 and 5). The induction of each of the enzymes was compared to the induction of CYP2B (16β-hydroxytestosterone). Specifically the correlation coefficients for the various endpoints examined were: 16β-hydroxytestosterone: ALDH ($r = 0.93$, $P < 0.01$); 16β-hydroxytestosterone: BZROD ($r = 0.91$, $P < 0.01$); 16β-hydroxytestosterone: 6β-hydroxytestosterone ($r = 0.98$, $P < 0.01$); 16β-hydroxytestosterone: epoxide hydrolase ($r = 0.88$, $P < 0.01$). Thus, the ability of any of these chemicals to induce any of these enzymes in the pleiotropic response was closely related to its ability to induce CYP2B1.

Table 3. Induction of testosterone hydroxylation in male rats of various strains

Strain*	Hydroxylated testosterone metabolite†				
	15β [3A]‡	6β [3A]	2β [3A]	7α [2A]	16β [2B]
Brown Norway					
Control	27	1080	167	130	46
Phenobarbital	52	2512	283	178	1690
Buffalo					
Control	20	323	44	86	49
Phenobarbital	37	1238	134	141	1598
Copenhagen					
Control	89	1200	173	118	69
Phenobarbital	235	2560	527	212	2313
F344/NCr					
Control	73	1026	155	103	ND§
Phenobarbital	252	3759	870	357	3817
Lewis					
Control	29	1016	139	221	52
Phenobarbital	41	2086	386	740	1816
Louvain					
Control	16	1588	125	95	79
Phenobarbital	109	3085	265	286	2452
Noble					
Control	16	1620	93	50	98
Phenobarbital	37	2924	176	139	1840
Wistar Furth					
Control	22	940	43	27	ND
Phenobarbital	47	1386	82	45	1500

* Animals were fed either control diet or diet containing 500 ppm phenobarbital for 2 weeks *ad lib.*

† Pooled (N = 3) microsomes were used. Values are reported as the average of activities (pmol/min/mg microsomal protein) determined at 2 and 4 min of incubation of microsomal protein with testosterone as described in Materials and Methods. The standard deviation was less than 15% of the mean for all values.

‡ The forms of cytochrome P450 corresponding to the specific hydroxylated testosterone metabolite are shown in brackets.

§ ND = not detectable (<20 pmol hydroxylated testosterone metabolite produced/min/mg microsomal protein).

Table 4. Induction of xenobiotic metabolizing activities by different chemicals in male Copenhagen rats

Chemical*	Dietary†	Enzyme activity			
		BZROD‡	16β§	EH‖	ALDH¶
Control	0	35 ± 5	69	424 ± 49	5.1 ± 1.3
PB	375	906 ± 158**	2313	1166 ± 262**	16.2 ± 4.0**
PB	750	867 ± 98**	2608	1110 ± 153**	23.7 ± 4.0**
BB	500	242 ± 32**	1020	552 ± 162	9.0 ± 2.0**
BB	2500	815 ± 219**	2528	1433 ± 103**	13.0 ± 3.3**
EPH	750	1001 ± 195**	2350	1070 ± 44**	14.0 ± 3.3**
EEH	500	28 ± 6	151	308 ± 29**	4.1 ± 0.7
DDT	350	615 ± 86**	2136	961 ± 72**	10.0 ± 2.0**

Values for BZROD, EH and ALDH are expressed as mean ± SD (N = 6).

* Abbreviations: PB, phenobarbital; BB, barbital; EPH, 5-ethyl-5-phenylhydantoin; and EEH, 5,5-diethylhydantoin.

† Animals were fed control diet or diet containing the indicated amount of chemical for 2 weeks *ad lib.*

‡ BZROD activity is expressed as pmol resorufin formed/min/mg S-9 protein resulting from the cytochrome P450-mediated O-dealkylation of benzyloxyresorufin.

§ Activity is expressed as pmol hydroxylated testosterone metabolite formed/min/mg pooled (N = 6) microsomal protein.

‖ EH activity is expressed as pmol benzo[*a*]pyrene-4,5-diol formed/min/mg S-9 protein.

¶ ALDH activity is expressed as nmol NADH produced/min/mg cytosolic protein using propionaldehyde as the substrate and NAD^+ as the cofactor.

** Significantly different from control ($P < 0.05$).

DISCUSSION

In a previous report, we demonstrated the presence of a "pleiotropic response" in male F344 rats exposed to PB [1] and a variety of structurally diverse compounds which induce CYP2B (e.g. DDT, α-hexachlorocyclohexane, clonazepam, and 2,2′,4,4′,5,5′-hexachlorobiphenyl). This response was characterized by a coordinate induction of hepatic xenobiotic metabolizing enzymes, including members of the cytochrome P450 superfamily, epoxide hydrolase, glucuronyl transferase and glutathione transferase, an increase in liver to body weight ratios, and a variety of other biologic effects. The specific endpoints employed to examine for this pleiotropic response were based primarily on the observation that phenobarbital, the prototype chemical, induces the specific endpoint of interest [1–9] although more varied structure activity studies have been performed with specific endpoints, e.g. CYP proteins or epoxide hydrolase [5, 6, 24, 25]. To determine the general applicability of this response, we examined for the induction of this pleiotropic response in four different species following PB administration, the results of which are summarized in Table 1. These findings imply a similar pleiotropic response in all four species. Thus, one observes moderate (hamster) to high (mouse, rat, rabbit) induction of CYP2B, as determined immunochemically; moderate levels of induction of CYP3A in three of the four species (not rabbit); moderate levels of induction of epoxide hydrolase in all three species; and finally no induction of ALDH in any of the species. We have observed a similar pleiotropic response (induction of CYP2B, CYP2C, CYP3A, EH and ALDH) in two species of Old World monkeys (*Erythrocebus patas, Macaca fascicularis*) which were exposed to PB [26]. One should be aware of certain potential limitations of this work. First, although a reasonably high dose of PB was used in all of the species, the doses were not optimized and are not necessarily maximally inducing. In fact, the serum levels of PB achieved by 500 ppm in the diet of F344 male rats (~50 μg/mL) are approximately twice the levels achieved in

Table 5. The induction of testosterone metabolizing activities by different chemicals in male Copenhagen rats

Chemical* (ppm)	Hydroxylated testosterone metabolite†				
	15β	6β	7α	16β	2β
Control	92	1200	118	69	173
PB (375)	181	2353	212	2313	467
PB (750)	246	2560	315	2608	527
BB (500)	82	1483	205	1020	244
BB (2500)	220	2416	466	2528	461
EPH (750)	213	2200	261	2350	433
EEH (500)	101	1223	185	151	172
DDT (350)	195	2755	243	2136	460

* Animals were fed either control diet or diet containing the indicated chemical (in ppm) for 2 weeks *ad lib.* Abbreviations: PB, phenobarbital; BB, barbital; EPH, 5-ethyl-5-phenylhydantoin; and EEH, 5,5-diethylhydantoin.

† Pooled (N = 6) microsomes were used. Values are reported as the average of activities (pmol/min/mg protein) determined at 2 and 4 min of incubation of microsomal protein with testosterone as described in Materials and Methods. The standard deviation was less than 15% of the mean for all values.

human epileptics on long-term therapy [27]. Second, the ALDH activity was determined purely by enzymatic methods. Thus, if there are different substrate specificities for gene homologs of the different species, as is the case with CYP2B (see below), then we might get a false negative result. Third, the use of polyclonal antisera which have been characterized for specificity in the rat may not show exactly the same specificity or affinity in other species. Nevertheless, the results imply that in a wide variety of species, from rodents to primates, PB elicits a similar pleiotropic response. In fact, members of another CYP subfamily (2C) appear to be induced by phenobarbital in a variety of species (mouse [11, 28], rat [11, 28], rabbit [29]). Despite this similarity of a pleiotropic response to PB the structural requirements for chemicals to elicit this response can vary greatly even for closely related species (mice vs rats). Thus, when examining the effects of two halogenated pesticides, it was found that 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) [30] is 100–1000 times more effective as an inducer of CYP2B in mice than in rats, while DDT is vastly more effective as an inducer in the rat than in the mouse (Lubet RA, unpublished observations).

The second major finding is that structurally similar proteins, as assessed by polyclonal antibodies, may show greatly different substrate specificities. Thus, while all four species showed induction of CYP2B as assessed by reactivity with a polyclonal antibody directed against rat CYP2B, the hamster showed no induction as monitored by specific hydroxylation of testosterone or dealkylation of alkoxyresorufins. The PB-induced rabbit showed high levels of the BZROD and PROD activities but low levels of testosterone 16β-hydroxylase, while the mouse showed moderate levels of BZROD activity, but much lower (albeit inducible) levels of testosterone 16β-hydroxylase. The fact that testosterone 16β-hydroxylase is not as well induced in the mouse as in the rat was documented previously by Kelly and coworkers [31]. These results emphasize that although PB may induce CYP proteins with high degrees of amino acid homology, which would therefore cross-react when using polyclonal antisera or hybridization with a nucleic acid probe, the induced proteins may have greatly different substrate specificities. This result is not surprising since the naturally occurring CYP2B1 and CYP2B2, which have 98% amino acid homology, have vastly different turnover numbers for a variety of substrates. In addition, the work of Lindberg and Negishi [32] and Gonzalez and coworkers [33] have both shown that limited numbers of mutations in the proper regions of certain CYP proteins including CYP2B can greatly alter the substrate specificity of the resulting molecules.

In a previous study, we found that the outbred Zucker lean rat demonstrated a diminished response to PB and certain structural analogs (ethylphenylhydantoin, barbital), as well as a variety of structurally diverse PB-type inducers [2]. To determine whether inbred strains of rats which we could readily employ in tumor promotion studies might exhibit a diminished response to PB, eight strains of inbred rats were screened for induction of various hepatic xenobiotic metabolizing enzymes. To screen for less responsive strains, similar to the Zucker rat, animals were treated with barbital and ethylphenylhydantoin in addition to PB. These PB analogs were examined because the Zucker rat displayed a particularly weak response to induction with these compounds [2]. In all eight rat strains, a coordinate response (CYP2B, CYP3A, CYP2A, EH) was demonstrated, which paralleled that observed in the F344/NCr rat. Ethylphenylhydantoin induced CYP2B1, CYP3A1, CYP2A and epoxide hydrolase to approximately the same extent as PB. Barbital (1500 ppm) induced these enzymes to ~50% of the maximum induction observed for PB at a 500 ppm dietary dose, as in the F344/NCr rat. Thus, the coordinate induction of these enzymes by PB-type inducers appears to be a ubiquitous phenomenon among the various strains of rats tested. However, we failed to identify an inbred strain of rats which exhibited a lack of responsiveness similar to that of the Zucker outbred line.

The one exception to this coordinate induction in various strains of rats was ALDH (NAD^+, propionaldehyde) which was reported previously to be inducible in some rat strains but not in others. Cytosolic ALDH (NAD^+, propionaldehyde) was induced in two of eight strains of inbred rats: ACI and Copenhagen. To further examine the relationship of aldehyde dehydrogenase induction to the pleiotropic response to PB-type compounds, additional agents were examined. PB and a number of structurally diverse chemicals, known to be inducers of CYP2B1, were tested in male Copenhagen rats for their abilities to induce ALDH activity. If the induction of this particular aldehyde dehydrogenase isozyme (NAD^+, propionaldehyde) is a part of this pleiotropic response, then its induction would be expected to correlate to the induction of CYP2B1 for each treatment. In fact, correlation analyses of 16β-hydroxytestosterone (CYP2B1) vs ALDH or 16β-hydroxytestosterone vs 6β hydroxytestosterone (CYP3A) or 16β-hydroxytestosterone vs epoxide hydrolase all showed high correlates ($r > 0.88$, $P < 0.01$). Therefore, it is likely that in those strains of rat in which PB is able to induce cytosolic aldehyde dehydrogenase (NAD^+, propionaldehyde), the induction of this enzyme is a part of the pleiotropic response. This result was not totally unexpected since the results of Deitrich *et al.* [34] using an inbred strain of Long–Evans rats had similarly shown strong induction of ALDH by structurally diverse PB-type inducers (e.g. PB, DDT, and Mirex) although these previous authors had not investigated these chemicals for induction of the other portions of the PB-mediated pleiotropic effect.

The present studies are particularly interesting since they would appear to be a unique example where a specific element (ALDH) of an overall pleiotropic response has been gained or lost. In contrast, mutant strains of rodents exist which confer greatly diminished responsiveness to certain classes of cytochrome P450 inducers. Thus, various strains of mice (DBA, AKN, SWR) exhibit a diminished response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds [35, 36]. This

particular result appears to be mediated by an altered Ah receptor [37] and alters induction of various genes including CYP1A1, CYP1A2 and quinone reductase. More recently, a number of groups, including our own, have characterized an outbred strain of rats (Zucker), which have a diminished response to structurally diverse PB-type inducers [2, 38]. Similar to the mouse TCDD receptor mutants, these animals show a diminished response to all of the elements of the PB-type pleiotropic effect (i.e. CYP2B1, CYP3A, epoxide hydrolase, glutathione transferase Ya/Yc). Although non-responsiveness of particular genes has been observed phenotypically, e.g. specific UDP glucuronyl transferases in Gunn rats [39, 40], this typically results from an altered or nonfunctional gene resulting in decreased activity in both constitutive and induced states. Similarly, lack of expression of the CYP2B2 gene in certain strains of rats affects both constitutive and induced levels of these genes [12–14]. In contrast, the results with ALDH would appear to be a case of a loss or gain of a responsiveness element in only one gene of a pleiotropic response since the gene still appears to be expressed constitutively in various strains of rats.

As mentioned at the beginning of the paper and as discussed briefly above, there are various aspects of the pleiotropic response to PB-type inducers which might argue for a common biochemical mechanism. Waxman and Azaroff [11] recently reviewed the data dealing with the induction of certain of the CYP subfamilies (2B, 2C, 3A) by PB and possible mechanisms for such an induction. Interestingly the work of He and Fulco [41] which has been confirmed recently by Padmanhaban and coworkers [42] points to a potential mechanism for inducing such a pleiotropic response. He and Fulco have found a consensus DNA sequence in phenobarbital-inducible CYP genes in both *Bacillus megaterium* and rats [41]. This DNA sequence, which exhibits 11 conserved base pairs in a stretch of 17, is apparently bound by a PB-activated nuclear binding protein. Interestingly, regions with high nucleotide identity to this He and Fulco derived consensus sequence (7–9 base pairs out of 11) are found in a variety of PB-inducible genes including rodent glutathione transferase [43], rabbit CYP2C1 [29], rat epoxide hydrolase [44], and most interestingly rat ALDH [15]. Interestingly, the human homologs of EH [45] and ALDH 1 [46] similarly exhibit these regions of high identity. Recent studies of our own have shown that non-human primates exposed to PB show induction of genes similar to those observed in rodents including the CYP proteins [26] as well as EH and ALDH 1 [47]. Whether this particular DNA sequence and its associated binding protein are a major determinant of the pleiotropic response to PB and related compounds warrants careful examination.

In summary, we found that (1) PB induced a pleiotropic response (CYP2B, CYP3A, EH) in a variety of rodent species and lagomorphs; (2) unlike the outbred Zucker lean rats [2] a variety of inbred rat strains exhibited a similar response to PB, EPH and barbital, and (3) ALDH induction appeared to be a portion of the pleiotropic response to PB in those rat strains in which it was induced but was unusual insofar as this response appeared to have been lost or gained in certain strains of rats.

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