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EFFECT OF LOW-DOSE PHENOBARBITAL ON HEPATIC MICROSOMAL UDP-GLUCURONYL TRANSFERASE ACTIVITY*

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Abstract—To determine whether hepatic microsomal enzyme induction occurs in rats following administration of phenobarbital at doses similar to those used in humans (0.5 to 7.5 mg/kg), UDP-glucuronyl transferase (UDPGT) and cytochrome P-450 activities were measured in liver homogenate and microsomal preparations from control rats and rats treated for 6 days with phenobarbital at 1 and 3 mg per kg per day. While no significant increases in liver weight and protein content of homogenate and microsomal preparations were observed with either dose of the drug, both UDPGT and P-450 activities were enhanced significantly following administration of phenobarbital at 3 mg per kg per day. The activity of P-450 was increased by approximately 30% and that of UDPGT by 15–24 and 45–66%, respectively, employing bilirubin and *p*-nitrophenol as the acceptor substrate. The extent of induction of bilirubin or *p*-nitrophenol UDPGT was similar when measured with "native" enzyme or with enzyme activated by UDP-*N*-acetyl glucosamine, digitonin or deoxycholate. These data suggest that the discordant effects of phenobarbital on UDPGT and cytochrome P-450 previously reported in humans and rats may not be attributable solely to differences in the drug doses employed.

Administration of phenobarbital to normal human volunteers, and to patients with congenital nonhemolytic jaundice type II and Gilbert's syndrome, is associated with a significant reduction of the plasma concentration of unconjugated bilirubin, which results from an increased hepatic bilirubin clearance [1–4]. In addition, drug administration increases the fraction of bilirubin excreted in bile as the diglucuronide, with a corresponding decrease in bilirubin monoglucuronide [5, 6]. Although the mechanisms of these drug effects are poorly understood, and those underlying the change in the diglucuronide : monoglucuronide ratio remain particularly controversial (reviewed in Ref. 7), induction of hepatic UDP-glucuronyl transferase (UDPGT)‡ is widely regarded as underlying the increased hepatic bilirubin clearance [1, 8, 9]. However, despite the abundant animal data demonstrating UDPGT induction by phenobarbital [10–15], direct evidence of an increased bilirubin conjugating capacity in man following administration of the drug is controversial, increases in UDPGT having been observed in a few studies [16, 17] but not in several others [2–4, 18]. One possible explanation for the failure to observe UDPGT induction in human studies is that phenobarbital was given in these studies [3, 18], for periods ranging from 7 days to years, at doses (0.5–7.5 mg per kg per day) which are far lower than those used experimentally (30–100 mg per kg per day [19]. To test the possibility that the different effects of phenobarbital on UDPGT reported in humans and rats reflect principally differences in the doses of drug employed, we have studied its effects when administered to rats at doses (1–3 mg per kg per day) comparable to those employed clinically.

METHODS

Animals and drug treatment. Male Sprague–Dawley rats (290–310 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). The animals were housed in a temperature-controlled room (22°) with alternating 12-hr light–dark cycles for at least 1 week prior to being used. They were fed Purina Lab Chow *ad lib.* with free access to water and were not fasted before being killed. Rats were randomly divided in three groups, and each group was treated with either saline or phenobarbital sodium at 1 or 3 mg per kg per day, i.p. Treatment was continued for 6 consecutive days before the day of sacrifice.

Chemicals and solutions. All chemicals used were reagent grade and, unless otherwise indicated, were purchased from the Sigma Chemical Co. (St. Louis, MO). The following solutions were prepared freshly just before use: UDP-glucuronic acid (UDPGA, 51.7 mM) in H_2O; UDP-*N*-acetyl glucosamine (UDPNAG, 30.7 mM) in H_2O; diazotized sulfanilic acid as described in Ref. 20; and digitonin (Fisher,

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‡ Abbreviations: UDPGT, uridine-5′-diphosphate glucuronyl transferase (EC 2.4.1.17); and UDPNAG, UDP-*N*-acetyl glucosamine.

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Pittsburgh, PA) in 0.25 M sucrose–1 mM EDTA (pH 7.4). Bilirubin was dissolved first in a few drops of NaOH (0.25 N) and subsequently added to a solution of crystallized and lyophilized bovine serum albumin. The final solution contained 0.5 mg/ml bilirubin in 2.5% albumin and was adjusted to a pH of 7.9 (22°). The bilirubin solution was clear for at least 15 days when stored in the dark at 4°. Tris buffers (Trizma: pH 7.9 and 7.4 at 37°) were dissolved in H_2O at 1 M concentrations.

Homogenate and microsomal preparation. Rats were killed by stunning, followed by decapitation and exsanguination. The liver was quickly removed, chilled on ice, weighed, minced with scissors, and homogenized in sucrose–EDTA. Tissue homogenization was performed in a Teflon–glass grinder, driven by an adjustable speed electric drill. All of the above procedures were conducted in a cold room at 4°. The homogenate was then diluted to a 25% suspension (w/v) with sucrose–EDTA and, after appropriate aliquots were removed for enzyme assays, was centrifuged for 20 min at 10,000 *g* in a refrigerated centrifuge (Sorvall, RC2-B). Thereafter, the supernatant fraction was cleared from the floating fat layer, aspirated, and centrifuged for 90 min at 105,000 *g* (average) in a Beckman L2-65 ultracentrifuge. The microsomal pellets were recovered and stored at −20° until used. Prior to use, the pellets were resuspended in an appropriate volume of sucrose–EDTA so that the microsomes isolated from 1 g liver were contained in a 4-ml suspension (25%, w/v).

Enzyme activation. The activity of UDPGT toward either bilirubin or *p*-nitrophenol as the acceptor substrate was measured in both homogenate and microsomal suspensions, in which the enzyme was either in its "native" form or activated. Bilirubin UDPGT was activated by UDPNAG or by the detergents digitonin or deoxycholate. The source of "native" enzyme was a 12.5% suspension of either homogenate or microsomes in sucrose–EDTA, which was obtained by diluting 1 vol. of the original (25%) homogenate or microsomal suspension with 1 vol. of sucrose–EDTA. Similarly, the detergent-activated enzyme was prepared by adding 1 vol. of the original homogenate or microsomal suspension to 1 vol. of sucrose–EDTA containing an appropriate concentration of the desired detergent. The optimal concentrations of deoxycholate in the final (12.5%) suspensions were 0.125% in homogenate and 0.1% in microsomes when bilirubin was the acceptor substrate and 0.2% in homogenate and 0.125% in microsomes when *p*-nitrophenol was employed. Digitonin produced maximal activation at 1.25% in homogenate and 0.65% in microsomes. The activity of the UDPNAG-activated enzyme was determined using untreated homogenate or microsomal suspensions, and by adding an appropriate amount of UDPNAG directly to the incubation mixture.

Analysis. The activity of UDPGT was measured aerobically at 37° using either bilirubin or *p*-nitrophenol as the acceptor substrate. When bilirubin was employed, the incubation mixture contained: Tris, pH 7.9, 250 mM; $MgCl_2$, 10 mM; UDP-glucuronic acid, 5.17 mM; bilirubin 0.214 mM with albumin, 6.25 mg/ml; homogenate or microsomal suspension (12.5%), 100 μl and UDPNAG, when applicable, 3.07 mM. These concentrations of all reagents were found to give optimal enzyme activity in a detailed study previously reported [21]. Similar incubation mixtures were prepared when *p*-nitrophenol was the acceptor, except that Tris, pH 7.4, was used instead of pH 7.9, albumin was not included, and *p*-nitrophenol (0.75 mM) was substituted for bilirubin. In both bilirubin and *p*-nitrophenol UDPGT assays, the final volume of the incubation mixture was 1 ml, and all components were added to 10-ml Erlenmyer flasks which were kept on ice until the incubation was initiated.

The activity of bilirubin UDPGT was determined in quadruplicate samples which contained "native" enzyme or enzyme activate by UDPNAG, digitonin or deoxycholate. Triplicate samples were prepared for the *p*-nitrophenol UDPGT assay, in which the enzyme activity was measured as "native" or activated by UDPNAG or deoxycholate. In the bilirubin UDPGT assay, incubations were done for 30 min, whereas in the *p*-nitrophenol UDPGT assay samples were incubated for 10 min. In both assays, however, selected samples were incubated for variable periods of time to examine the relationship between product formation and incubation time. A linear relationship was always obtained for incubation times in the range 0–50 and 0–15 min when bilirubin and *p*-nitrophenol were employed respectively. Similarly, in the bilirubin UDPGT assay, a linear relationship was observed between enzyme activity and protein concentration over the range 0–6 mg protein/ml when homogenate was used and 0–1 mg/ml when a microsomal suspension served as the source of the enzyme.

Table 1. Effects of phenobarbital (PB) treatment on weight, protein content and cytochrome P-450 activity of rat liver*

	Controls (N = 8)	PB (1 mg/kg) (N = 7)	PB (3 mg/kg) (N = 7)
Rat weight (g)	308 ± 12	306 ± 18	317 ± 11
Liver weight (g)	13.3 ± 1.1	13.9 ± 1.5	13.4 ± 0.9
Liver/rat weight (%)	4.31 ± 0.37	4.54 ± 0.34	4.32 ± 0.35
Homogenate protein (mg/g)	203.4 ± 12.1	198.9 ± 7.8	200.5 ± 9.7
Microsomal protein (mg/g)	25.6 ± 3.1	27.1 ± 2.9	26.5 ± 2.6
P-450†	0.083 ± 0.013	0.087 ± 0.010	0.108 ± 0.008‡

* Values are means ± S.D. (number of animals in parentheses).
† $\Delta O.D._{450-490}$/mg microsomal protein.
‡ Student's *t*-test: $P < 0.001$ compared to corresponding control group.

Table 2. Effect of phenobarbital (PB) on bilirubin UDPGT activity in rat liver microsomes*

Treatment	"Native"	Enzyme activation†		
		UDPNAG	Digitonin	Deoxycholate
Controls (N = 8)	3.64 ± 0.48	5.96 ± 0.75	13.33 ± 1.71	15.68 ± 1.67
PB, 1 mg/kg (N = 7)	3.85 ± 0.59	6.19 ± 0.64	14.94 ± 2.82	16.38 ± 3.00
PB, 3 mg/kg (N = 7)	4.50 ± 0.55‡	6.90 ± 1.06§	16.51 ± 1.35‖	18.33 ± 2.14¶

* Values (means ± S.D.) are expressed in nmoles per 10 min per mg microsomal protein (number of animals in parentheses).

† UDPNAG was added to the incubation mixture at 3.07 mM; digitonin (0.65%) and deoxycholate (0.1%) were added to the microsome suspension (12.5%) 30 min before use.

‡–¶ Levels of significance compared to the corresponding control group (determined by Student's *t*-test): ‡ $P < 0.005$, § $P < 0.05$, ‖ $P < 0.002$, and ¶ $P < 0.025$.

In the *p*-nitrophenol UDPGT assay, linearity was observed with protein concentrations ranging from 0 to 4 and 0 to 0.5 mg/ml when homogenate and microsomes were used respectively.

Incubations were terminated by transferring the samples onto ice. Thereafter, in the *p*-nitrophenol UDPGT assay, 2 ml of 0.5 N trichloroacetic acid was added and the tubes were centrifuged at 1000 *g* for 10 min. An appropriate volume of the supernatant fraction was then aspirated, diluted to a fixed volume with H_2O and, after the pH was adjusted to 11.5–12, read at 400 nm. In the bilirubin UDPGT assay, the conjugated bilirubin formed was extracted and coupled to diazotized sulfanilic acid, essentially as described by Strebel and Odell [20]. Conversion of the extinction values into units of enzyme activity was done using appropriate standards for the *p*-nitrophenol UDPGT assay and an extinction coefficient of $37.8\,M^{-1}\,cm^{-1}$ (570 nm) for the azo-pigment of conjugated bilirubin, as already reported from this laboratory [21].

Cytochrome P-450 was estimated in microsomal suspensions (3–4%) by its diothionite difference spectrum as described by Omura and Sato [22]. Protein concentrations in homogenate and microsomal suspensions were determined by the method of Lowry *et al.* [23] using bovine serum albumin as standard.

RESULTS

Phenobarbital treatment at 1 or 3 mg per kg per day × 6 days (i.p.) did not influence rat growth pattern, nor did it produce significant changes in liver weight, liver–body weight ratio, and protein content of liver homogenate and microsomes when compared to paired saline-treated controls (Table 1). Hepatic cytochrome P-450 activity was also unaffected by phenobarbital treatment at 1 mg/kg. However, when phenobarbital was injected at 3 mg/kg, a significant induction of microsomal P-450 was observed (Table 1).

Similarly, microsomal bilirubin UDPGT activity was increased only minimally following phenobarbital treatment at 1 mg/kg, but it was enhanced significantly when the drug was given at 3 mg/kg, irrespective of whether "native" enzyme or enzyme activated by UDPNAG, digitonin or deoxycholate was used (Table 2). However, when bilirubin UDPGT activity was determined in liver homogenate, and its activity expressed per g of liver, a significant induction over baseline could be demonstrated in the phenobarbital-treated rats (3 mg/kg) only when deoxycholate- [3.56 ± 0.26 (S.D.) vs control 2.72 ± 0.39 mg per hr per g liver; $P < 0.001$] or digitonin-activated enzyme (2.92 ± 0.17 vs 2.35 ± 0.32 mg per hr per g liver; $P < 0.001$) was used, but not with "native" (0.74 ± 0.14 vs 0.64 ± 0.12 mg per hr per g liver; $0.05 < P < 0.1$) or UDPNAG-activated enzyme (1.49 ± 0.22 vs 1.36 ± 0.19 mg per hr per g liver; $0.05 < P < 0.1$).

When UDPGT activity was measured using *p*-nitrophenol as the acceptor substrate, the *in vitro* activation of the enzyme by UDPNAG or detergent was far greater than that obtained when bilirubin was employed. In untreated rats, while UDPNAG

Table 3. Effect of phenobarbital (PB) treatment on *p*-nitrophenol UDPGT activity in rat liver microsomes*

Treatment	"Native"	Enzyme activation†	
		UDPNAG	Deoxycholate
Controls (N = 6)	3.54 ± 0.74	12.94 ± 2.65	39.31 ± 9.83
PB, 1 mg/kg (N = 5)	3.97 ± 0.81	14.52 ± 2.71	47.61 ± 8.07
PB, 3 mg/kg (N = 7)	5.33 ± 1.25‡	21.46 ± 3.32§	58.65 ± 7.93‡

* Values (means ± S.D.) are expressed in nmoles per min per mg microsomal protein (number of animals in parentheses).

† UDPNAG was added to the incubation mixture at 3.07 mM; deoxycholate (0.125%) was added to the microsome suspension (12.5%) 30 min before use.

‡,§ Levels of significance compared to the corresponding control group (determined by Student's *t*-test): ‡ $P < 0.01$ and § $P < 0.002$.

and deoxycholate increased microsomal bilirubin UDPGT activity by 55–65 and 400–440%, respectively, compared to "native" microsomes, they enhanced that of *p*-nitrophenol UDPGT by 350–410 and 1100–1250%. Phenobarbital administration produced a *p*-nitrophenol UDPGT induction pattern similar to that observed with P-450 and bilirubin UDPGT: no increase at 1 mg/kg, but a significant induction at 3 mg/kg (Table 3). However, in contrast to bilirubin UDPGT, phenobarbital at 3 mg/kg also produced a significant induction of *p*-nitrophenol UDPGT when liver homogenate was studied, regardless of whether "native" enzyme or enzyme activated by UDPNAG or by detergents was employed. Furthermore, the degree of induction of *p*-nitrophenol UDPGT by phenobarbital treatment at 3 mg/kg was more pronounced than that of bilirubin UDPGT, irrespective of enzyme activation status and of whether liver homogenate or microsomal suspensions were used. Thus, while UDPGT activity was induced by 15–24% when bilirubin was the acceptor substrate, a 45–66% increase was observed when *p*-nitrophenol was employed.

DISCUSSION

The results of the present studies have demonstrated that phenobarbital, injected i.p. into rats for 6 days at a dose as low as 3 mg per kg per day, produces a significant increase of both hepatic UDPGT and P-450 activities. UDPGT induction could be demonstrated irrespective of whether "native" enzyme or enzyme activated by UDPNAG or by detergents was employed. Additionally, a significant increase in enzyme activity was obtained regardless of whether bilirubin or *p*-nitrophenol was the aglycone used. When *p*-nitrophenol was employed, however, UDPGT induction was more than twice that obtained with bilirubin. This finding is consistent with previous studies demonstrating different acceptor selectivities of UDPGT [13, 24–27] and provides additional evidence to support its functional heterogeneity. Our results, however, are in contrast with previous work by Bock *et al.* [13] who found that UDPGT induction by phenobarbital was far more pronounced when bilirubin was the acceptor substrate than when *p*-nitrophenol was used. At present we cannot offer a satisfactory explanation for this discrepancy, although differences in the dose and time of administration of phenobarbital, as well as in the technical details of the enzyme assays, exist between the two studies.

Studies reported previously have demonstrated unquestionably that phenobarbital administration to both normal human volunteers and patients with Gilbert's syndrome and congenital non-hemolytic jaundice type II is associated with a significant decline of plasma levels of bilirubin [1–4] and an increase in hepatic bilirubin clearance [4]. However, those studies in which liver biopsies were obtained for enzyme assay have failed to provide convincing evidence for an increased bilirubin conjugating capacity [2–4, 16–18]. To conciliate this clinical observation with abundant animal data supporting UDPGT induction by phenobarbital, several factors have been implicated, including large differences in the drug dose employed in experimental animals as opposed to humans.

Phenobarbital has, in fact, been given to man in these studies at doses ranging from 0.5 to 7.5 mg per kg per day [18], an amount which, on a body-weight basis, is only 0.5 to 7.5% of that usually administered to rats [19]. Our studies now indicate that this dose difference may not provide an explanation for the divergent effects on bilirubin UDPGT observed in man and rat. Thus, in the rat, a daily dose of the drug as low as 3 mg/kg produces a significant increase of both UDPGT and P-450 activities. Even at 1 mg per kg per day, slightly but not statistically significantly higher enzyme activities were obtained. Even though extrapolation of these animal findings to man must be done with caution, pharmacokinetic studies have shown that the drug is metabolized in man [28, 29] at a much slower rate than it is in the rat [30, 31]. This suggests that the 3 mg/kg dose which produced enzyme induction in the rat is pharmacologically equivalent to an even lower dose in man. Thus, the doses of phenobarbital which have failed to produce UDPGT induction in man [2–4, 18] are well within the range now shown to induce the enzyme in the rat.

Other factors, therefore, may underlie the failure of phenobarbital to produce measurable UDPGT induction in man. These may include technical deficiencies in measuring enzyme activity *in vitro*, lack of correlation between *in vitro* UDPGT activity and its functional levels *in vivo*, and unresponsiveness of human UDPGT to phenobarbital therapy. The possibility that bilirubin UDPGT may not be responsive to phenobarbital treatment warrants special consideration as most of the human studies of UDPGT have been carried out in patients with Gilbert's syndrome and patients with congenital non-hemolytic jaundice type II. These conditions are believed to be associated with a partial and severe deficiency, respectively, of bilirubin UDPGT [18], and a failure to respond to inducers unique to such patients is, therefore, not inconceivable. If UDPGT is not, in fact, induced, the mechanism responsible for the phenobarbital-associated acceleration of hepatic bilirubin clearance in man [1–4] requires further investigation and elucidation.

The present studies have also demonstrated that administration of phenobarbital to rats at a dose as low as 3 mg per kg per day is associated with a significant induction of hepatic cytochrome P-450 activity. P-450 is a member of the mixed function oxidase system and, as a hemoprotein, its turnover provides a significant source of the so-called "early labeled" bilirubin [32–34]. Studies in rats have shown that P-450 induction by phenobarbital treatment is associated with an enhanced incorporation of [2-^{14}C]glycine into "early labeled" bile bilirubin, and with a significant increase of biliary bilirubin output indicating increased heme turnover [34]. However, human studies in both normal volunteers and patients with Gilbert's syndrome, at a phenobarbital dose of 2.5 mg per kg per day, have failed to show any increase in early labeling of bile pigment or in total heme turnover [35].

Previous data, therefore, suggest that the response of hepatic microsomes to phenobarbital differs in

respect to both UDPGT and P-450 induction between man and rat. While there have been wide differences in the drug doses employed in the two species in these earlier reports, the present studies suggest that the discordant effects of phenobarbital in man and in the rat may not be related simply to drug dose differences.

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