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KINETIC ALTERATIONS IN RAT LIVER MICROSOMAL CHOLECALCIFEROL 25-HYDROXYLASE ASSOCIATED WITH PHENOBARBITAL ADMINISTRATION

EDGARD E. DELVIN,* ALICE ARABIAN and FRANCIS H. GLORIEUX
Genetics Unit, Montreal Shriners Hospital for Crippled Children, Departments of Surgery and Pediatrics, McGill University, Montreal, PQ, Canada H3G 1A6

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Abstract—The kinetics of cholecalciferol 25-hydroxylase in vitamin D-depleted rat liver microsomes, before and after phenobarbital induction, were studied. Three days of pretreatment with phenobarbital altered significantly both the apparent K_m and the V_{max} of the hydroxylase. Untreated vitamin D-repleted rats had lower cytochrome P-450 content and aminopyrine demethylase activity than the vitamin D-depleted animals. Phenobarbital administration reversed this nutritional effect on aminopyrine demethylase but not on cytochrome P-450 content. Furthermore, vitamin D deficiency potentiated the phenobarbital inductive effect upon microsomal protein. No inhibition of aminopyrine demethylase could be elicited in the presence of cholecalciferol or 25-hydroxycholecalciferol either prior to or after phenobarbital treatment, suggesting that these two oxidases are different entities.

Among the adverse effects of anticonvulsant drugs, when administered chronically, are low plasma levels of 25-hydroxyvitamin D,† often associated with osteomalacia [1–6]. To explain this metabolic disturbance, Hahn *et al.* [4] postulated that the apparent vitamin D deficiency observed in some anticonvulsant-treated patients was due to an induction of the vitamin D metabolism leading to more polar biologically inactive metabolites and to a depletion of the vitamin D pool. This hypothesis was logical, because the enzyme complex, cholecalciferol 25-hydroxylase, which converts cholecalciferol is mainly, although not exclusively [7], located in the microsomal fraction [8] and shares with other mixed function oxidases their requirements for an NADPH-generating system and molecular O_2 [8, 9]. However, the refractory response of some patients to vitamin D therapy which could be reversed by oral adminstration of small quantities (50 I.U) of 25-hydroxycholecalciferol speaks against this hypothesis [10]. Our demonstration [11] of a low plasma 25-hydroxycalciferol/calciferol ratio supports the clinical results and suggests an inhibition of the hepatic hydroxylation of vitamin D. To show whether or not the microsomal cholecalciferol hydroxylase is influenced by anticonvulsant treatment, we have studied, using rat liver as the source of enzyme, the effect of short-term phenobarbital treatment on the *in vitro* kinetics of cholecalciferol 25-hydroxylase and on aminopyrine demethylase activity as a marker for microsomal mixed function oxidases.

MATERIALS AND METHODS

Reagents. NADPH (sodium salt), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) type VII, cholecalciferol and semicarbazide HCl were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 25-Hydroxycholecalciferol was a gift of Roussel UCLAF (Paris, France). [1α, 2α-(*n*)-^{3}H]Cholecalciferol (12 Ci/mmole), purchased from Amersham–Searle (Oakville, Ontario, Canada), was periodically verified for its purity by high-pressure liquid chromatography by the method developed by Jones and DeLuca [12]. *N,N'*-diphenylphenylenediamine, a lipid peroxidation inhibitor [18], was from the Eastman–Kodak Co. (Rochester, NY, U.S.A.). Phenobarbital (sodium salt) was purchased from Winthrop Laboratories (Aurora, Ontario, Canada) and aminopyrine from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Cholecalciferol 25-hydroxylase assay. Male weanling Holtzman rats (Canadian Breeding Farms, St. Constant, Quebec, Canada) fed, *ad lib.*, a vitamin D-deficient diet (No. 170640, Teklad Test Diets, Madison, WI, U.S.A.) were kept in cages with maplewood shavings. After 4 weeks, they were injected intraperitoneally, on 3 consecutive days, with either phenobarbital sodium (100 mg/kg body wt) in 0.9% NaCl or the vehicle only. They were decapitated on day 4, after starvation overnight. The livers were excised at once, blotted and weighed, and then carefully perfused with cold 0.154 M KCl. They were homogenized with a Potter–Elvehjem homogenizer fitted with a Teflon pestel in ice-cold 0.25 M sucrose containing 1 mM NaH_2PO_4 buffer adjusted to pH 7.4 with 1 M NaOH. The combined microsomal–cytosol fraction, obtained as already described, was used for the cholecalciferol 25-hydroxylase assay [14].

Radioactivity was monitored in a Packard Tricarb 3385 liquid scintillation spectrometer with AQUA-

* To whom reprint requests should be addressed.

† Calciferol and 25-hydroxycalciferol are generic terms for vitamin D and 25-hydroxyvitamin D, respectively. Cholecalciferol and 25-hydroxycholecalciferol refer specifically to vitamin D_3 and 25-hydroxyvitamin D_3.

SOL (New England Nuclear Canada, Montreal, Quebec, Canada) as scintillation fluid.

Unless otherwise specified, the units of activity reported are pmoles of 25-hydroxycholecalciferol/min/g wet weight of tissue. The supernatant fraction from the same individual liver provided the enzyme for each set of experiments.

Aminopyrine demethylase assay. For these studies the animals were either fed, *ad lib.*, the vitamin D-deficient diet mentioned in the second paragraph or a normal rat chow (Raltston-Purina, Woodstock, Ontario, Canada) and injected intraperitoneally with either isotonic saline or phenobarbital sodium as described above. The perfused livers were homogenized as described, and the microsomes were isolated according to the method of Schenkman and Cinti [15]. They were resuspended in 0.154 M KCl buffered with 0.1 M NaH_2PO_4 adjusted to pH 7.4 with 0.1 M NaOH. Aminopyrine demethylase was assayed according to the method of La Du *et al.* [16] with the following modifications. To a 1.5-ml aliquot of the oxygenated cofactor (10 min with O_2) solution consisting of 1 mM NADPH, 16.7 mM glucose-6-phosphate and 15 mM semicarbazide in 0.5 M NaH_2PO_4, adjusted to pH 7.4 with 0.1 M NaOH, were added: 0.25 ml of glucose-6-phosphate dehydrogenase (3 units) in 25 mM $MgCl_2$, 0.25 ml of 1.2 mM aminopyrine, in 25 mM $MgCl_2$; and either 25 μl of 1,2-propanediol, 25 μl of 1 mM cholecalciferol or 25 μM 25-hydroxycholecalciferol in 1,2-propanediol. The mixtures were incubated for exactly 5 min at 37° after which time, at fixed intervals, 1 ml of the microsomal suspension, containing approximately 1.5 nmoles cytochrome P-450, was added to each flask. The incubations continued for 5 min, and the reaction was stopped by the addition of 1 ml of 0.52 M $ZnSO_4$ followed by 1 ml of saturated $Ba(OH)_2$. After centrifugation at 1000 *g* for 20 min at 4°, 2.5 ml of the clear supernatant fluid were used for the formaldehyde measurement by the method of Nash [17]. The units reported are, unless otherwise specified, nmoles formaldehyde produced/min/mg of microsomal protein.

Cytochrome and protein measurements. Cytochromes P-450 and b_5 were measured spectrophotometrically using the microsomal fraction resuspended in buffered 0.154 M KCl solution according to the method described by Mazel [18]. The proteins were measured by the method of Lowry *et al.* [19] using human serum albumin (Connaught Medical Research Laboratories, Toronto, Ontario, Canada) as standard.

RESULTS

The effects of a rachitogenic diet and of phenobarbital treatment on rat body and liver weights, on liver microsomal protein and cytochrome P-450 contents, and finally on the microsomal aminopyrine demethylase activity are summarized in Table 1. As already reported by Jones [20], the vitamin D deficiency coupled to the phosphopenia markedly reduced the body weights. The nutritional status had no effect on either the liver weight or the microsomal

Table 1. Effects of vitamin D and phenobarbital treatment on body weight, liver weight, microsomal protein content, cytochrome P-450 content and aminopyrine demethylase activity in 4-week-old rats*

Treatment	Vitamin D status	Body wt (g)	Liver wt (g/100 g)	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Aminopyrine demethylase (nmole/mg protein/min)
Control	D^+	222 ± 25	4.4 ± 0.6	10.9 ± 1.3	1.0 ± 0.1	49.2 ± 4.5
	D^-	122 ± 12	3.6 ± 0.0	9.7 ± 3.6	1.4 ± 0.2	61.8 ± 2.9
P		< 0.001	NS	NS	< 0.05	< 0.01
Phenobarbital	D^+	216 ± 18	5.7 ± 0.4	17.6 ± 1.8	2.3 ± 0.1	125.6 ± 8.8
	D^-	110 ± 2	5.0 ± 0.3	20.9 ± 1.0	3.6 ± 0.4	111.4 ± 19.7
P		< 0.001	NS	< 0.05	< 0.01	NS

* Liver weights are expressed as g/100 g body wts. Microsomal proteins and cytochrome P-450 levels were measured as described in Materials and Methods. Aminopyrine demethylase was measured as described using 5 mM aminopyrine as substrate, and the activity is expressed as nmoles formaldehyde/mg of microsomal protein/min. The significance levels for difference in means (P values) were calculated with Student's *t*-test for independent observation (t_{ind}), d.f. = 4. Effect of PB treatment on: body weight (NS); liver weight ($P < 0.01$); microsomal proteins ($P < 0.01$); and cytochrome P-450 and aminopyrine demethylase ($P < 0.001$). All values are means ± S.D. Abbreviations: D^+; vitamin D-repleted rats; D^-; vitamin D-depleted rats; d.f., degrees of freedom; and NS, not significant.

protein levels in untreated animals. However, vitamin D deficiency potentiated the inductive effect of phenobarbital upon the microsomal protein content. Dietary vitamin D quantitatively decreased the cytochrome P-450 content and the aminopyrine demethylase activity in the control group. Phenobarbital treatment diminished this effect only on the demethylase activity. Phenobarbital administration had no effect on body weight but statistically increased the liver weight, the cytochrome P-450 levels and the aminopyrine demethylase activity. The cytochrome b_5 content was not enhanced by the anticonvulsant treatment (control, 0.44 ± 0.02 nmole/mg of protein; phenobarbital-treated, 0.60 ± 0.10 nmole/mg of protein).

The double-reciprocal plot of cholecalciferol 25-hydroxylase [21], shown in Fig. 1, reveals an apparent Michaelis constant (K_m) of 0.18 μM and a maximum velocty (V_{max}) of 32 pmoles/min/g of tissue for the control vitamin D-depleted animals. These data differ significantly from those observed in the phenobarbital-treated group (Fig. 1), which elicits a decrease in affinity for the substrate (higher K_m) with an increase in the capacity to hydroxylate cholecalciferol (higher V_{max}). Since Cinti *et al.* [22] have reported that, in Sprague–Dawley rats, ergocalciferol and 25-hydroxycholecalciferol induced a type I spectral change of cytochrome P-450, and since aminopyrine induces similar spectral changes [23], we investigated the possible effects of cholecalciferol and 25-hydroxycholecalciferol upon the aminopyrine demethylase activity in our animal model. 25-Hydroxycholecalciferol and cholecalciferol were dissolved in 1,2-propanediol, to obtain final concentrations in the incubation medium of 125 nmoles/l and 5 μmoles/l, respectively. These concentrations are higher than those reported by Cinti *et al.* [22] to obtain half-maximal spectral changes (K_s). We assumed that the K_s for cholecalciferol is of the same order of magnitude as that of ergocalciferol used in the former study [22]. From Fig. 2, it is clear that neither cholecalciferol nor 25-hydroxycholecalciferol interacts with aminopyrine demethylase both in control and phenobarbital-treated vitamin D-deficient rats. Similar results were obtained from the vitamin D-repleted animals.

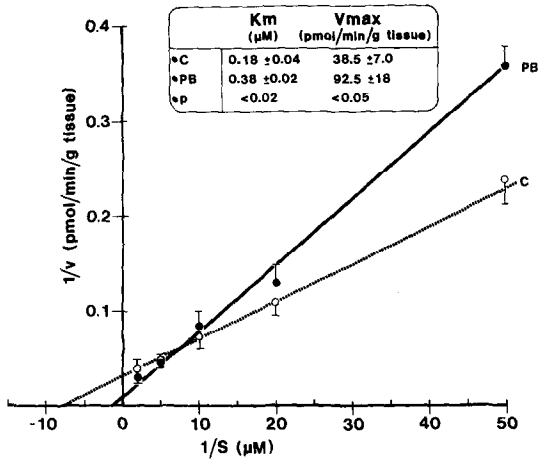

Fig. 1. Double-reciprocal plot of cholecalciferol 25-hydroxylase activity as a function of substrate concentration for vitamin D-depleted control and phenobarbital-treated rats. The 4 ml incubation medium contained: 2 ml of a cofactor solution consisting of 0.025 MK_2HPO_4 adjusted to pH 7.4 with 1 MNaOH, 0.25 mM NADPH, 1.25 mM $MgCl_2$, 0.025 MKCl, 6.0 mM glucose-6-phosphate, 6 units of glucose-6-phosphate dehydrogenase (where 1 unit of activity is defined as the amount of enzyme required to convert 1 μmole of substrate/min) and 0.01 mM *N,N'*-diphenyl-*p*-phenylenediamine in 10 μl ethanol. The amount of enzyme used was equivalent to 20 mg of liver and the cholecalciferol concentration varied from 0.02 to 0.5 μM. The regression lines were obtained from the pooled data of four control and four phenobarbital-treated animals, each assay at every substrate concentration being carried out in triplicate. The vertical bars represents ± S.D. from the mean. Insert: the K_m and V_{max} were derived from the regression line of the double reciprocal plot [21] for each separate experiment. The means (± S.D.) for the K_m and V_{max} were then calculated from each value obtained. The significant levels for the difference in means (P values) were calculated by Student's *t*-test for independent observations with 6 degrees of freedom. Abbreviations: C, control; PB, phenobarbital-treated.

DISCUSSION

The data represented in this report provide evidence that short-term phenobarbital adminstration to rachitic rats lowers the affinity of the enzyme for cholecalciferol (higher K_m) and increases its capacity of hydroxylation (higher V_{max}). This finding explains the apparent inhibition of the enzyme by phenobarbital reported by Sulimovici and Roginsky [24], since these workers measured the enzyme at a single substrate concentration well below the apparent Michaelis constant. On the other hand, it could explain, in part, the apparent phenobarbital induc-

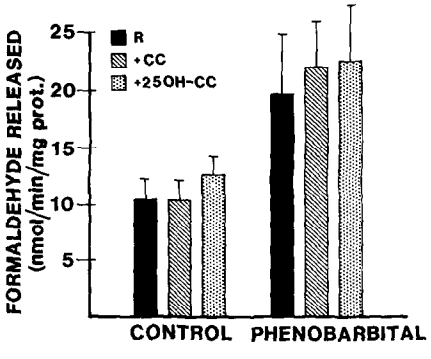

Fig. 2. Effects of cholecalciferol and 25-hydroxycholecalciferol on aminopyrine demethylase activity in control and phenobarbital-treated vitamin D-deficient rats. The incubations were performed as described in Materials and Methods in the presence of 0.2 mM aminopyrine. Activity is expressed as nmoles formaldehyde formed/min/mg of microsomal protein (± S.D.). The significance levels for the difference in means (N.S.) were calculated with Student's *t*-test for independent observations (t_{ind}), d.f. = 4. Abbreviations R, incubation done in the presence of 1,2,propanediol alone; + CC, incubation done in the presence of 5.0 μM cholecalciferol in 1,2-propanediol; + 25-OH-CC, incubation done in the presence of 125 nM 25-hydroxycholecalciferol in 1,2-propanediol; and d.f., degrees of freedom, N.S., not significant.

tion of vitamin D_3 metabolism reported by Rising [25]. However, this author studied the *in vivo* metabolism vitamin D_3 in D-repleted animals. Therefore, this model may include not only the microsomal but also the mitochrondrial enzyme complex, as reported by Bjorkhem and Holmberg [7].

Several reports strongly suggest that there is a molecular heterogeneity of cytochromes P-450 depending upon the inducing subtance. Ingelman-Sundberg and Gustafsson [26] have resolved, from penobarbital-induced rabbit liver, four electrophoretically different forms of cytochrome P-450, of which only one had 6-β-androstenedione hydroxylase activity. Furthermore, Noshiro and Omura [27] have suggested the presence of multiple forms of cytochrome P-450 with different substrate specificities by selective inhibition of the hydroxylation of various xenobiotics with antibody raised against cytochrome P-450. Also by inhibition studies of arylhydrocarbon hydroxylase, Dent *et al.* [28] have shown qualitative changes of these enzymes after induction with polybrominated biphenyls. Finally, Alvares and Kappan [29] have demonstrated that Aroclor 1254, a polychlorinated biphenyl mixture, induced the synthesis of some proteins which had the combined catalytic properties of those induced by phenobarbital and those induced by 3-methylcholanthrene.

Since cholecalciferol 25-hydroxylase requires molecular O_2 and its activity depends upon the integrity of the cytochrome P-450 structure [9, 14], it belongs to the class of mixed function oxidases. The modifications of kinetics induced by phenobarbital treatment in the present study are, however, different from those observed for type I substrates [30]. The data presented may be another indication that phenobarbital induced the synthesis of a new cytochrome P-450 apoprotein.

The reduction in cytochrome P-450 content in vitamin D-repleted animals, concomitant with a decrease in aminopyrine demethylase, mimics the results obtained by Mackinnon *et al.* [31] for ethinyl estradiol-treated rats. However, in our hands only the aminopyrine demethylase activity is corrected after phenobarbital treatment. This, and the fact that vitamin D deficiency seemingly potentiates the inductive effect of phenobarbital on the microsomal protein synthesis, remain to be explained, but they also argue in favor of the heterogeneity of the proteins synthesized after induction by phenobarbital with a modulation of the functional properties of cytochrome P-450.

Contary to the report of Cinti *et al.* [22], we could not elicit any inhibition of aminopyrine demethylase by either cholecalciferol or 25-hydroxycholecalciferol, although incubations were done with secosteroid concentrations above the dissociation constants (K_s) reported. Our model, however, differs from the earlier report in two respects. First, Sprague–Dawley rats were used in the first instances, whereas we used Holtzman rats. Secondly, studies were conducted after long-term phenobarbital treatment (21 days) compared to a 3-day induction for the present report. The latter point warrants comment, since the ages of the animals at the time of the *in vitro* studies differ in the two studies and an age-dependent change in binding property has been reported in rats [32–34]. Therefore, it may be that vitamin D_3 and 25-hydroxycholecalciferol in our particular conditions did not act as type I substrates. This would then explain why they did not affect the aminopyrine demethylase activity and why aminopyrine or phenobarbital did not affect vitamin D_3 hydroxylation (data not shown).

We have thus shown that phenobarbital modifies the kinetics of the microsomal vitamin D_3 25-hydroxylase. These results corroborate those of Norman *et al.* [35] who showed marginal changes in vitamin D_3 metabolism in the phenobarbital-treated chicken. They also stress the importance of insuring an appropriate vitamin D intake in phenobarbital-treated individuals to prevent iatrogenic deleterious effects on bone and mineral homeostasis.

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