Phenobarbital-induced Alterations in the Metabolism of [^{3}H]Vitamin D_3 by the Perfused Rachitic Rat Liver In Vitro

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ABSTRACT Anticonvulsant therapy of seizure disorders in man is associated with the development of complications involving bone and mineral metabolism including hypocalcemia, elevated serum immunoreactive parathyroid hormone levels, and increased amounts of unmineralized bone or osteoid. The latter has been attributed to a reduction in serum-25-hydroxycholecalciferol levels resulting from increased hepatic metabolism of vitamin D. Using an in vitro recycling hepatic perfusion system, we have demonstrated that 5 d of phenobarbital treatment increases the hepatic production of [^{3}H]25-hydroxyvitamin D_3 (4.3±0.3 vs. 3.3 ±0.2%/h, $P < 0.025$) without affecting the biliary excretion of radioactivity. Furthermore, rachitic livers perfused with blood obtained from animals treated with phenobarbital for 5 d also manifested an increase in [^{3}H]25-hydroxyvitamin D_3 production (4.6±0.5 vs. 3.3±0.2%/h, $P < 0.02$). Addition of phenobarbital or its major metabolite, *p*-hydroxyphenobarbital, directly to the perfusion apparatus had no effect on [^{3}H]25-hydroxyvitamin D_3 production. Phenobarbital treatment was also attended by a decrease in the intrahepatic content of [^{3}H]vitamin D_3 (11.7±0.4 vs. 17.5±0.7 dpm/mg liver protein, $P < 0.001$) without alterations in the content of [^{3}H]25-hydroxyvitamin D_3. The data collectively suggest that the increased hepatic conversion of [^{3}H]vitamin D_3 to [^{3}H]25-hydroxyvitamin D_3 attending phenobarbital treatment is secondary to stimulation of the hepatic 25-hydroxylation system(s) by a metabolite of phenobarbital other than *p*-hydroxyphenobarbital and/or by metabolic alterations resulting from phenobarbital therapy.

INTRODUCTION

Hypocalcemia, elevated serum immunoreactive parathyroid hormone levels, and increased amounts of unmineralized bone or osteoid are frequent complications attending anticonvulsant therapy of seizure disorders in man (1–7). This disordered bone and mineral metabolism resulting from chronic phenobarbital and dilantin treatment has been attributed to decreased circulating levels of 25-hydroxyvitamin D_3 (2–4) and increased metabolism of vitamin D_3 to more polar degradation products by the liver (8–10). However, normal to high circulating levels of 1,25-dihydroxyvitamin D_3, the most potent metabolite of the vitamin, have recently been reported in patients receiving anticonvulsant drugs (11), and phenobarbital has been demonstrated to increase the 1-hydroxylation of 25-hydroxyvitamin D_3 by the kidney in vitro (12). These observations, coupled with the finding that phenobarbital and dilantin blunt the bone rudiment response to parathyroid hormone and 1,25-dihydroxyvitamin D_3 (13), have led to the postulate that the disordered bone and mineral metabolism attending anticonvulsant therapy may be a consequence of a direct inhibitory effect of the drugs on bone, and not a result of the altered hepatic metabolism of vitamin D_3 (14).

The hepatic 25-hydroxylation of vitamin D_3 has been demonstrated to occur at two subcellular sites, the microsomes (15) and the mitochondria (16). Although the microsomal 25-hydroxylation of vitamin D_3 is not blunted by inhibitors of the cytochrome P450 system (17), it has been reported that administration of phenobarbital to humans and animals lead to induction of microsomal oxidase enzymes, presumably of the P450 system (8, 18), with resultant increased catabolism of vitamin D_3 and 25-hydroxyvitamin D_3, to polar, inactive products (8–10). In contrast, phenobarbital has been documented to increase the mitochondrial 25-hydroxylation of vitamin D (16).

The purpose of these studies was to investigate the net effect of phenobarbital on the microsomal and mitochondrial 25-hydroxylation reactions, as well as

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the effect of the drug on vitamin D_3 catabolism, by examining the metabolism and biliary excretion of [^{3}H]-vitamin D_3 by the perfused rat liver in vitro. Our results indicate that phenobarbital increases the production of [^{3}H]25-hydroxyvitamin D_3 (^{3}H-25-OH D_3)[1] by the perfused rat liver, without altering the biliary excretion of radioactivity and that the enhanced 25-hydroxyvitamin D_3 production is secondary to hepatic stimulation by a metabolite of phenobarbital and/or metabolic changes resulting from phenobarbital treatment.

METHODS

40 3-wk-old male weanling Holtzman rats were fed a vitamin D-deficient diet containing 0.2% calcium and 0.4% phosphate for 7 wk. The animals were then divided into two groups. Experimental animals were injected intraperitoneally with phenobarbital, 65 mg/kg, for 5 d before sacrifice, and the blood phenobarbital levels determined by gas liquid chromatography (19). Control animals were injected with vehicle. Circulating 25-hydroxyvitamin D_3 (20) levels were determined in the two groups of animals.

Three protocols for the in vitro perfusion of the rachitic rat liver were undertaken. In the first protocol, 32 rachitic untreated rats were sacrificed by phlebotomy under light ether anesthesia and the blood used for the liver perfusate (*vide infra*). Four rats were injected with phenobarbital and four received the vehicle for 5 d. These latter animals served as the liver donors. In the second protocol, 18 rachitic rats were treated with phenobarbital for 5 d before sacrifice by phlebotomy and 18 rats were given the vehicle. The blood from each of these groups was used for the perfusion of a liver from an untreated rachitic rat. In the final protocol, either phenobarbital, 1.5 μg/ml, or its major derivative, *p*-hydroxyphenobarbital (21), 0.55 μg/ml, was directly added to the perfusate at the initiation of the liver perfusion. Experimental and control livers were simultaneously perfused in independent chambers at a rate of 15 ml/min. The perfusate was recycled during the course of the 4-h experiments. [^{3}H]vitamin D_3, 0.5 μCi, purchased from New England Nuclear (Boston, Mass.) was added to each perfusate at zero time. Humidity and temperature were maintained in the chambers at 80–90% and 37°C, respectively.

The surgical removal and perfusion of the livers were performed as described (22). The esophagus was ligated and cut and the bile ducts cannulated. The hepatic artery and inferior vena cava were ligated and the portal vein and superior vena cava cannulated. The liver was perfused through the portal vein with 3:1:1 Krebs-Ringer bicarbonate: erythrocytes: plasma at a pH of 7.4. The Po_2 was maintained at 100 mm Hg. Bile was collected during the entire perfusion period.

The viability of the perfused livers was evaluated by assessing hepatic histology, bile production (23), bromsulfophtalein (BSP) clearance (24), and lactate and pyruvate concentrations of the perfusate. Phenobarbital-treated and control livers were fixed in formalin and stained with hematoxylin and eosin before and after 4 h of perfusion. Bile was collected via the inserted cannula and its production expressed in milliliters per hour. BSP clearance by the perfused liver was determined by a modification of a described method (24). BSP, 10 mg/dl, was added to the experimental and control perfusates after either 2 or 3 h of perfusion. Portions of perfusate were removed at 15-min intervals over the ensuing hour and the BSP concentration of the perfusate determined (25). Clearance of the dye over the 60-min period was expressed as nanomolar per gram liver per minute. Lactate and pyruvate (26) concentrations were quantitated in the perfusate at zero time, and after 30, 60, 120, 180, and 240 min of perfusion. Portions of perfusate were removed at the designated times and centrifuged at 2,000 *g* for 10 min at 2°C. Lactate and pyruvate levels in the supernate were determined and expressed as the lactate:pyruvate ratio.

After 4 h, the perfusion was terminated and the perfusate and bile extracted in a methanol:chloroform mixture (27, 28). ^{3}H-25-OH D_3 production was determined by chromatography of the lipid fraction of the perfusate with Sephadex LH-20-100 (Pharmacia Fine Chemicals, Piscataway, N. J.). Columns were packed to 35 cm and eluted with 50:50 hexane:chloroform. 2-ml fractions were collected. The percentage of total radioactivity appearing as ^{3}H-25-OH D_3 during the 4-h perfusion was determined and expressed as percent per hour ^{3}H-25-OH D_3 production. The ^{3}H-25-OH D_3 obtained from the Sephadex LH-20 columns was pooled and run on a high-pressure liquid chromatograph, 0.4 × 30 cm μm porisil column (Varian Instruments, Palo Alto, Calif.). The flow rate was 1 ml/min and 1 ml fractions were collected. A 95:5 and 97.5:2.5 hexane: isopopyl alcohol solvent system was used. In both instances, ^{3}H-25-OH D_3 obtained from the Amersham Corp. (Arlington Heights, Ill.) and purified by chromatography on a Sephadex LH-20 column was the standard. The ^{3}H-25-OH D_3 produced by the liver was mixed with cold 25-hydroxyvitamin D_3 and the UV absorbance at 263 nm determined along with the radioactivity of each fraction.

The liver was also homogenized in normal saline and extracted in the methanol:chloroform mixture. The liver was perfused with Krebs-Ringer bicarbonate buffer before extraction to remove trace isotope present in the vascular system. The amount of [^{3}H]vitamin D_3 and ^{3}H-25-OH D_3 in the lipid fraction of the hepatic extraction was quantitated by Sephadex LH-20 chromatography and expressed as disintegration per minute per milligram liver protein.

The amount of [^{3}H]vitamin D_3 metabolites conjugated to glucuronides in the aqueous fraction of the bile was determined by treating the aqueous fraction with 1,500 U of marine mollusk β-glucuronidase for 48 h at 37°C as described (29). This fraction was then extracted in the chloroform:methanol mixture and the radioactivity in the resultant aqueous and lipid fractions determined.

All data is expressed as the mean±SEM, and the probability of difference was determined with the paired *t* test.

RESULTS

Phenobarbital treatment (65 mg/kg body wt) for 5 d resulted in circulating levels of phenobarbital of 1.5 μg/ml, a concentration that is within the therapeutic range for human seizure disorders. Phenobarbital did not affect the in vivo concentration of 25-hydroxyvitamin D_3, the level in both treated and control rachitic rats being <1 ng/ml. The body weight of the rats whose livers were used for the perfusion were similar in the phenobarbital-treated and control groups (210±26 vs. 205±30 g). Likewise, the liver weights of the two groups were similar (8.95±3.12 vs. 8.25±1.81 g).

Hepatic viability. Hematoxylin- and eosin-stained sections of the phenobarbital-treated and controls

[1] *Abbreviations used in this paper*: BSP, Bromsulfophtalein; ^{3}H-25-OH D_3, [^{3}H]25-hydroxyvitamin D_3.

livers were similar before and after 4 h of perfusion. The routine light microscopy showed no evidence of hepatocyte damage. After the 4-h perfusion there were numerous polymorphonuclear leukocytes in the canniliculi of both livers.

Bile production was not significantly different in the two groups (0.31±0.04 vs. 0.26±0.03 ml/h). Similarly, phenobarbital treatment did not affect the BSP clearance of the perfused liver (19.5±2.9 vs. 20.5±4.4 nM/g liver per min).

The lactate:pyruvate ratios were determined in the perfusate throughout the 4-h experimental period. Initially, the ratios were elevated at zero time reflecting that period between the phlebotomy of the animals and the initiation of the perfusion. However, after only 30 min of perfusion the ratio closely approximated the normal of 10, where it remained during the first 3-h of the perfusion. After 4 h of perfusion, the ratio was slightly elevated in both phenobarbital-treated and control livers at 16.

[^3H]Vitamin D_3 metabolism. When phenobarbital was administered to rachitic rats and the liver of the treated animal perfused for 4 h, the production of ^{3}H-25-OH D_3 increased (4.3±0.3 vs. 3.3±0.2%/h, $P < 0.025$). A typical Sephadex LH-20 chromatograph is illustrated in Fig. 1, where the ^{3}H-25-OH D_3 represented 20.4% of the radioactivity after the 4-h perfusion of the

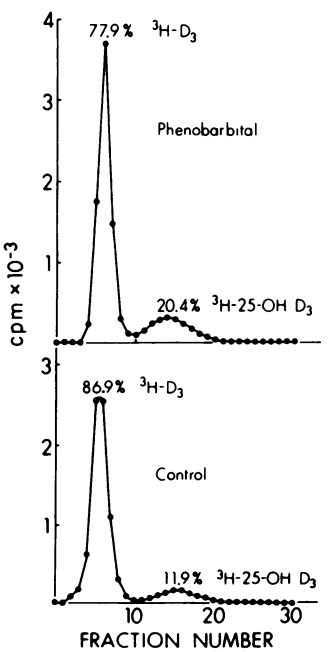

FIGURE 1 Sephadex LH-20 chromatograph of perfusate from a phenobarbital-treated (upper panel) and control (lower panel) rachitic liver after 4 h of perfusion. The ^{3}H-25-OH D_3 appears in fractions 10–20, and accounts for 20.4% of the total radioactivity of the phenobarbital-treated liver (upper panel) and 11.9% of the control liver (lower panel).

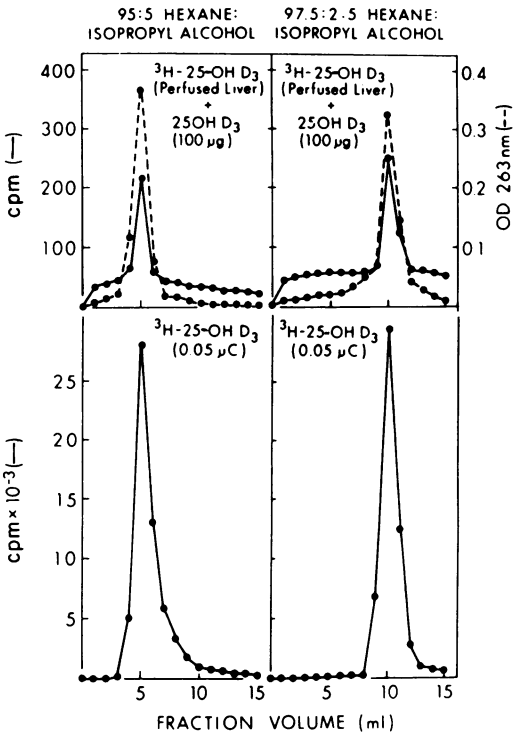

FIGURE 2 High-pressure liquid chromatographs of the ^{3}H-25-OH D_3 appearing in fractions 10–20 of Fig. 1 (upper panels solid lines), cold 25-hydroxyvitamin D_3 (25OH D_3) (upper panels dashed lines), and standard ^{3}H-25-OH D_3, 0.05 μCi (lower panels solid lines). The peaks comigrated in the 95:5 hexane:isopropyl (left panels) and the 97.5:2.5 hexane: isopropyl alcohol (right panels) solvent system indicating identity. Note the difference in scale.

phenobarbital-treated liver, and only 11.9% of the radioactivity of the control liver. To insure that the polar metabolite collected in fraction 10–20 of the Sephadex LH-20 column (Fig. 1) was ^{3}H-25-OH D_3, the fraction was pooled and run on a high-pressure liquid chromatograph with cold 25-hydroxyvitamin D_3 against a standard of ^{3}H-25-OH D_3. The high-pressure liquid chromatography was performed with a 95:5 and 97.5:2.5 hexane:isopropyl alcohol solvent system (Fig. 2). In the first instance, the ^{3}H-25-OH D_3 co-eluted in the 5th ml with cold 25-hydroxyvitamin D_3 and with the standard ^{3}H-25-OH D_3. When the solvent system was 97.5:2.5 hexane:isopropyl alcohol, the metabolites all co-eluted in the 10th ml. Thus, the polar metabolite produced by the liver during the 4-h perfusion was ^{3}H-25-OH D_3.

Although phenobarbital treatment increased the production of ^{3}H-25-OH D_3, it had no effect on the distribution of [^{3}H]vitamin D_3 metabolites in either the aqueous (31,500±2,900 vs. 25,500±3,800 dpm) or lipid (5,200±1,100 vs. 4,500±900 dpm) fractions of bile produced by the perfused livers. Likewise, when the aqueous fraction of bile was treated with β-glucuronidase,

to determine if phenobarbital treatment increased the conjugation of [^{3}H]vitamin D_3 to water-soluble metabolites, the percentage of radioactivity appearing in the lipid fraction was similar for both phenobarbital-treated and control livers (37±3 vs. 35±6%).

The blood of animals treated with phenobarbital for 5 d was then used to perfuse an untreated rachitic liver. The production of ^{3}H-25-OH D_3 was increased after 4 h of perfusion (4.6±0.5 vs. 3.3±0.2%/h, $P < 0.02$) with blood obtained from animals treated with phenobarbital. However when phenobarbital, 1.5 μg/ml, or its major metabolite *p*-hydroxyphenobarbital, 0.55 μg/ml, were added directly to the perfusate at zero-time, neither stimulated the production of ^{3}H-25-OH D_3.

Phenobarbital treatment also altered the distribution of vitamin D in the liver. Whereas, the amount of ^{3}H-25-OH D_3 in hepatic tissue was not affected by phenobarbital (3.7±0.3 vs. 3.4±0.4 dpm/mg liver protein), treatment was associated with a significant decrease in the content of [^{3}H]vitamin D_3 (11.7±0.4 vs. 17.5±0.7 dpm/mg liver protein, $P < 0.001$).

DISCUSSION

The alterations in bone and mineral metabolism consequent to prolonged therapy with anticonvulsant medications are complex. The hypocalcemia, elevated levels of circulating immunoreactive parathyroid hormone, and increased amounts of unmineralized bone or osteoid attending anticonvulsant therapy (1–7) have been attributed to derangements in vitamin D metabolism resulting in decreased serum concentrations of 25-hydroxyvitamin D_3 (2–4). These decreased concentrations have been thought to be secondary to increased hepatic degradation of the 25-hydroxy metabolite to more polar by-products with resultant increased biliary excretion of the vitamin (8–10). However, using a recycling in vitro hepatic perfusion system, we have shown that phenobarbital treatment of rats for 5 d stimulates the hepatic conversion of [^{3}H]vitamin D_3 to its 25-hydroxy metabolite without altering biliary excretion. Similarly, other investigators have demonstrated that short-term phenobarbital treatment of chicks (30) and rats (31) results in increased blood levels of 25-hydroxyvitamin D_3.

We have also shown that acute phenobarbital treatment of rats results in increased hepatic production 25-hydroxyvitamin D_3 without alterations in total biliary radioactivity. When the aqueous fraction of methanol: chloroform-treated bile was incubated with β-gluconidase to determine the amount of radioactive metabolites of [^{3}H]vitamin D_3 being excreted as water-soluble glucuronide conjugates, there were no differences between phenobarbital-treated and control livers. These observations suggest that 5 d of phenobarbital treatment does not alter either glucuronide conjugation or biliary excretion of vitamin D_3 metabolites. Other investigators (9, 31) have reported that acute treatment with phenobarbital increases the bile flow and biliary excretion of [^{3}H]vitamin D_3 metabolites. However, it is important to note that in both previous studies, the livers of the phenobarbital-treated animals were significantly heavier than the control livers. Furthermore, the ratio of treated to control liver weight was similar to the ratios of treated to control bile flow rates and treated to control excretion of [^{3}H]vitamin D_3 metabolites, suggesting that the phenobarbital effects may have been nonspecific and related to hepatic weight. In contrast, we have found short-term phenobarbital treatment to have no significant effect on either bile flow rates or biliary excretion of [^{3}H]vitamin D_3 metabolites when hepatic weights are similar.

Untreated livers perfused with blood obtained from animals given phenobarbital for 5 d also demonstrated increased production of ^{3}H-25-OH D_3. This finding cannot be accounted for by alterations in feedback control of 25-hydroxyvitamin D_3 production, since both phenobarbital-treated and control animals manifested similar serum 25-hydroxyvitamin D_3 concentrations of <1 ng/ml. Addition of phenobarbital or its major metabolite, *p*-hydroxyphenobarbital, directly to the perfusate had no effect on 25-hydroxyvitamin D_3 production. Hence, the data suggest that the increased hepatic conversion of [^{3}H]vitamin D_3 to ^{3}H-25-OH D_3 attending phenobarbital treatment is secondary to stimulation of the hepatic 25-hydroxylation system(s) by either a metabolite or phenobarbital other than *p*-hydroxyphenobarbital and/or by metabolic alterations resulting from phenobarbital therapy.

The intrahepatic concentrations of ^{3}H-25-OH D_3 after 4 h of perfusion were similar in both the phenobarbital-treated and control livers (3.7±0.3 vs. 3.4±0.4 dpm/mg liver protein), whereas concentrations of [^{3}H]vitamin D_3 were significantly less in the treated animals (11.7±0.4 vs. 17.5±0.7 dpm/mg liver protein, $P < 0.001$). Thus, it appears that phenobarbital does not stimulate the release of ^{3}H-25-OH D_3 from the liver, but that it enhances its production from [^{3}H]vitamin D_3.

These differences in the production of ^{3}H-25-OH D_3 induced by phenobarbital treatment cannot be accounted for by altered tissue viability. Although both preparations demonstrated increased intracannilicular numbers of polymorphonuclear leukocytes after 4 h of perfusion, the light microscopy showed no evidence of hepatic cell death. Likewise, bile production that has previously been used as an index of viability of the perfused liver (23), was similar in both groups (0.31±0.04 vs. 0.26±0.03 ml/h). Other investigators, using a different technique of a loading dose of BSP followed by a constant infusion of the dye, have reported a BSP clearance range of 24.4–43.2 nM/g liver per min in normal perfused livers (24). These results are similar to

our values of 19.5 and 20.5 nM/g liver per min in phenobarbital-treated and control rachitic perfused livers, where only a loading dose was administered. Finally, the lactate:pyruvate ratio of both preparations was similar and approximated the normal ratio of 10. The lactate: pyruvate ratio is an indicator of both ongoing aerobic glycolysis as well as a normal "coupling" of the anaerobic and aerobic pathways. Thus, both the phenobarbital and control preparations were viable, and the effects of phenobarbital on 25-hydroxyvitamin D_3 production cannot be attributed to altered tissue integrity.

Previous studies have demonstrated that the 25-hydroxylation of vitamin D_3 occurs at two subcellular sites, the microsomes (15) and the mitochondria (16), the latter responding to phenobarbital with an increase in the production of 25-hydroxyvitamin D_3 (16). In contrast, phenobarbital has been reported to inhibit the microsomal 25-hydroxylation of [^{3}H]vitamin D_3 (32). Although the whole liver perfusion preparation does not allow us to comment upon which subcellular 25-hydroxylation site(s) are affected by phenobarbital treatment, it does demonstrate that the net effect of acute therapy is to increase the production of 25-hydroxyvitamin D_3 by the liver without affecting biliary radioactivity. Furthermore, the data demonstrate that this effect is secondary to a metabolite of phenobarbital other than *p*-hydroxyphenobarbital and/or metabolic changes consequent to phenobarbital treatment. The results of this acute therapy regimen cannot be extrapolated to explain the metabolic effects of chronic phenobarbital treatment in humans, however the study indicates that the hepatic perfusion model may be used to further investigate the effects of long-term phenobarbital treatment on the hepatic metabolism of vitamin D.

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