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Diphenylhydantoin: direct inhibition of the vitamin D_3-mediated calcium absorptive mechanism in organ-cultured duodenum*

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There is now a substantial literature on the occurrence of disorders of bone and calcium metabolism in patients receiving anticonvulsant therapy. These disorders range from rickets in institutionalized, mentally retarded children [1], to osteomalacia in adult epileptics [2]. Hypocalcemia and raised serum alkaline phosphate are present in significant percentages of such cases. These findings suggested disturbances in vitamin D availability and/or metabolism.

Initial verification of disordered vitamin D availability came when it was shown that osteomalacia responded rapidly to vitamin D supplementation [2] and rickets was rapidly healed with smaller doses of 25-OH-D_3† [1]. This latter observation supported other work on the metabolism of vitamin D in phenobarbital-treated humans and rats. For example, in patients receiving phenobarbital, lower circulating levels of 25-OH-D_3 were found [3]. 25-Hydroxycholecalciferal is a vitamin D_3 metabolite formed mainly in the liver [4] and considered to be an intermediate in the biosynthesis of 1,25-$(OH)_2$-D_3 by the kidney [5], which latter metabolite has been considered to be the active form of vitamin D_3 [6]. In continuing studies with phenobarbital in man and animals, the view developed that the drug-related disorder stemmed from increased microsomal catabolism of vitamin D_3 and 25-OH-D_3 by the liver to more polar, inactive compounds and increased disappearance of vitamin D_3 and 25-OH-D_3 from the plasma [7, 8]. In direct contrast, others have shown an *increase* in circulating 25-OH-D_3 during anticonvulsant therapy [9, 10].

Other evidence of an anticonvulsant-vitamin D antagonism was the observation of reduced intestinal calcium absorption in DPH-treated rats [11, 12]. A concomitant decrease in the vitamin D_3-induced, intestinal calcium-binding protein (CaBP) was not observed. However, another study, using a more sensitive radial immunoassay technique [13], established that DPH-treated chicks exhibit both depressed intestinal calcium absorption and CaBP synthesis in parallel [14].

The present report provides support for the view that, while altered vitamin D metabolism may play a role in the DPH vitamin D antagonism and, consequently, inhibition of calcium transport and bone disorders, the possibility exists that at least part of the action of DPH on intestinal calcium transport is directly on the vitamin D-mediated, intestinal calcium absorptive mechanism. The relevant studies were done using a unique system for the maintenance of embryonic chick duodenum in organ culture [13].

The organ cultured duodenum, while completely isolated from systemic influences and maintained in a defined culture medium, responds to vitamin D_3 and its metabolites [15] by an early increase in cAMP production [16] followed by *de novo* synthesis of CaBP [17], and stimulation of calcium uptake and mucosal to serosal transport [13]. Thus, the system mimics what can be observed *in vivo* and allows distinction between direct and indirect effects of a specific treatment.

The organ culture procedure has been described in detail elsewhere [13]. Briefly, entire duodena from four 20-day chick embryos (300–400 mg tissue) were slit open and laid mucosal-side-up on a specially designed stainless steel grid inside a petri dish. The dish contained approximately 40 ml of McCoy's 5A medium (modified) without serum (Gibco, Grand Island, N.Y.) with 1.25 mM calcium, 0.625 mM phosphate and nystatin, 100 U/ml. The duodena on the grids, in bare contact at the serosal surface with the medium, were maintained at 37.5° for 48 hr in a humidified incubator continuously gassed with 5% CO_2:50% O_2: balance, air.

Addition of vitamin D_3 (Duphar, Amsterdam) to the culture medium (to a level of 26 μM) was made in ethanol such that the final ethanol concentration from this source was 0.1%. Diphenylhydantoin (Sigma, St. Louis) was added as an ethanolic solution such that the final ethanol concentration from this source was 0.4%. The ethanol concentrations of both control and treatment media were always identical.

After 48 hr incubation, some of the duodena from each treatment were homogenized prior to CaBP [13] and total protein [18] assays. Duodena for cAMP assay were liquid N_2-frozen, lyophilized, and extracted (homogenized) with 1 N-$HClO_4$ essentially as described by Cailla *et al.* [19]. Cyclic AMP assays on the neutralized extracts were then carried out by the radioimmunoassay technique of Steiner *et al.*, [20] using a commercially available kit (Schwartz Mann, Orangeburg, N.Y.). DNA analysis was performed on the pellet produced after centrifugation of the homogenate-extracts ($HClO_4$) by the method of Burton [21]. Radiocalcium uptake was measured on other duodena during a 30 min incubation at 37.5° in a ^{45}Ca-containing, low sodium buffer solution and calculations performed as detailed previously [13].

Some duodena were fixed for 24 hr in Bouin's fluid and prepared for paraffin embedding. Sections, 6 μm thick, were routinely stained with H & E and examined microscopically.

The inhibitory effects of culturing embryonic chick duodena for 48 hr in the presence of DPH on the vitamin D-mediated calcium absorptive mechanism are shown in Fig. 1. Duodenal CaBP and cAMP concentrations declined and ^{45}Ca uptake by the tissue was diminished in a dose-dependent fashion. The effect was apparently not due to a generalized toxicity since, histologically, the DPH-treated tissues were indistinguishable from the control tissues. Also, total protein concentration of the duodena was unaffected by DPH-treatment whereas CaBP synthesis was specifically inhibited.

Previous studies with DPH *in vivo* were unable to distinguish indirect effects of the drug on bone and calcium metabolism via altered vitamin D metabolism, and a direct effect of the drug on the intestine. The hypothesis that

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† Abbreviations: CC = cholecalciferal (vitamin D_3); 25-OH-D_3 = 25-hydroxycholecalciferol; 1,25-$(OH)_2$-D_3 = 1α,25-dihydroxycholecalciferol; CaBP = vitamin D-induced, calcium-binding protein; DPH = diphenylhydantoin; cAMP = adenosine 3′,5′-cyclic monophosphoric acid.

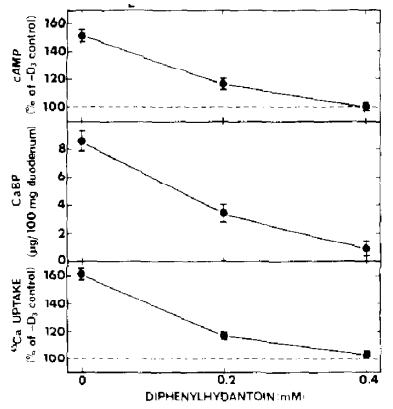

Fig. 1. Inhibitory effects of DPH on the vitamin D_3-mediated intestinal calcium absorptive mechanism. Values are the mean ± S.E.; 6–8 duodena per point. Vitamin D_3 concentration of the medium was 26 μM.

altered vitamin D metabolism was solely responsible for the disorders seen appears untenable on several grounds. First, DPH treatment is known to have a variety of effects on the intestine: including impairment of folic acid [22] and xylose absorption [23] and inhibition of ATPase activity [24]. Only the latter effect might possibly be related to vitamin D status, according to current information [25]. The interference with nutrient absorption by DPH, however, is not general since neither leucine nor galactose absorption was inhibited by DPH [11]. Second, the aforementioned discrepancies in the observations of lowered [7, 8] or raised [9, 10] serum 25-OH-D_3 levels in patients receiving anticonvulsants. Third, DPH interferes with the secretion or function of a variety of hormones: insulin [26], calcitonin [27], antidiuretic hormone [28], ACTH [29] and thyroid hormones [30]. Of these hormones, only calcitonin secretion might possibly be altered by vitamin D status according to current information. Fourth, DPH, but not phenobarbital, has been shown to have a direct inhibitory effect on PTH-induced bone resorption in organ-cultured mouse calvaria [31]. DPH was also an effective inhibitor of 25-OH-D_3-induced bone resorption [31]. The DPH-inhibition of bone resorption occurred without disturbing the well-known PTH-stimulation of cAMP concentration of bone [32]. This latter observation strengthens the contention that DPH has a direct, and very specific, effect on bone independent of its possible alteration of vitamin D metabolism. Finally, it is possible that DPH and phenobarbital have different effects, the former being by far the most potent in disrupting bone and calcium metabolism [12].

The present evidence strongly suggests a direct action of DPH on the vitamin D-mediated, intestinal calcium absorptive mechanism. Previous studies have shown that vitamin D_3 need not be, and is not, metabolized to 25-OH-D_3 and 1,25-$(OH)_2$-D_3 in the organ-cultured duodenum [15]. It seems unlikely, therefore, that the DPH effects observed in the present report were due to altered vitamin D_3 metabolism. A reasonable hypothesis would be that anticonvulsants, such as DPH, have a variety of actions in the animal but only part, if any, of these actions may be due solely to a drug enhanced catabolism of vitamin D.

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REFERENCES

1. N. Maclaren and F. Lifshitz, *Pediat. Res.* **7**, 914 (1973).
2. C. E. Dent, A. Richens, D. J. F. Rowe and T. C. B. Stamp, *Br. med. J.* **4**, 69 (1970).
3. T. J. Hahn, B. A. Hendin, C. R. Scharp and J. G. Haddad, *N. Engl. J. Med.* **287**, 900 (1972).
4. G. Ponchon and H. F. DeLuca, *J. clin. Invest.* **48**, 1273 (1969).
5. D. E. M. Lawson, D. R. Fraser, E. Kodicek, H. R. Morris and D. H. Williams, *Nature* **230**, 228 (1971).
6. M. F. Holick and H. F. DeLuca, *A. Rev. Med.* **25**, 349 (1974).
7. T. J. Hahn, S. J. Birge, C. R. Scharp and L. V. Avioli, *J. clin. Invest.* **51**, 741 (1972).
8. T. J. Hahn, C. R. Sharp and L. V. Avioli, *Endocrinology* **94**, 1489 (1974).
9. K. Schaefer, D. Kraft, D. von Herrath and A. Opitz, *Epilepsia* **13**, 509 (1972).
10. J. Silver, G. Neale and G. R. Thompson, *Clin. Sci. molec. Med.* **46**, 433 (1974).
11. W. F. Caspary, *Naunyn-Schmiedeberg's Arch. Pharmac.* **274**, 146 (1972).
12. H.-U. Koch, D. Kraft, D. von Herrath and K. Schaefer, *Epilepsia* **13**, 829 (1972).
13. R. A. Corradino, *J. Cell Biol.* **58**, 64 (1973).
14. M. Villarreale, L. V. Gould, R. H. Wasserman, A. Bar, R. T. Chiroff and W. H. Bergstrom, *Science* **183**, 671 (1974).
15. R. A. Corradino, *Science* **179**, 402 (1973).
16. R. A. Corradino, *Endocrinology* **94**, 1607 (1973).
17. R. A. Corradino, *Nature* **243**, 41 (1973).
18. O. H. Lowry, M. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. H. L. Cailla, M. S. Racine-Weisbuch and M. A. DeLaage, *Analyt. Biochem.* **56**, 394 (1973).
20. A. L. Steiner, C. W. Parker and D. M. Kipnis, *J. biol. Chem.* **247**, 1106 (1972).
21. K. Burton, *Biochem. J.* **62**, 315 (1956).
22. C. D. Gershon, G. W. Hepner and N. Brown, *J. clin. Invest.* **49**, 33a (1970).
23. E. H. Reynolds, J. F. Hallpike, B. M. Philips and D. M. Matthews, *J. clin. Path.* **18**, 593 (1965).
24. G. W. Hepner, L. M. Aledort, C. D. Gershom, N. Cohen, V. Herbert and H. D. Janowitz, *Clin. Res.* **18**, 382 (1970).
25. J. Melancon and H. F. DeLuca, *Biochemistry* **9**, 1658 (1970).
26. C. Malherbe, K. C. Burrill, S. R. Levin, J. H. Karam and P. H. Forsham, *N. Engl. J. Med.* **286**, 339 (1972).
27. J. T. Pento, S. M. Glick and A. Kagan, *Endocrinology* **92**, 330 (1973).
28. M. P. Fichman, C. R. Kleeman and J. E. Bethune, *Archs Neurol.* **22**, 45 (1970).
29. U. K. Rinnie, *Confin. Neurol., Basel* **27**, 431 (1966).
30. J. M. Hansen, L. Skovsted, U. B. Lauridsen, C. Kirkegaard and K. Siersbaek-Neilsen, *J. clin. endocrin. Metab.* **39**, 785 (1974).
31. M. V. Jenkins, M. Harris and M. R. Wills, *Calc. Tiss. Res.* **16**, 163 (1974).
32. L. R. Chase and G. D. Aurbach, *J. biol. Chem.* **245**, 1520 (1970).