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Research Article

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25-Hydroxycholecalciferol-Enhanced Bone Maturation in the Parathyroprivic State

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ABSTRACT In vitro evidence presently favors a direct osteolytic effect of biologically active vitamin D metabolites. Studies were designed to evaluate the effect of 25-hydroxycholecalciferol ($25OHD_3$) on bone collagen and mineral maturation in vivo and its dependence on parathyroid hormone (PTH). After treatment of sham-operated control and parathyroidectomized (PTX) mature rats with either $25OHD_3$ or an oil vehicle for 2 wk, tibial bone mineral-collagen maturation was quantitated by bromoform-toluene density gradient fractionation techniques. Intestinal calcium absorption was measured by in vivo ^{45}Ca transport procedures. In contrast to the control group, the response to $25OHD_3$ of PTX rats was dramatic. Bone mineral and matrix maturation were both accelerated by $25OHD_3$ treatment without concomitant reduction in total bone mineral or collagen content or changes in the intestinal calcium absorption. These observations support the premise that biologically active vitamin D metabolites stimulate bone tissue maturation, and that PTH is not required in this regard.

INTRODUCTION

It has been well established that vitamin D must be metabolized to more polar hydroxylated compounds before exerting its physiological expression on bone and intestine (1–3). The initial site of this transformation is the liver wherein the parent vitamin is hydroxylated to 25-hydroxycholecalciferol ($25OHD_3$)[1] (1). Subsequent transformations by renal mitochondrial 24- and 1-hydroxylating systems have been documented (2, 3) and the 1,25-dihydroxycholecalciferol metabolite ($1,25(OH)_2D_3$) established as the most potent stimulus for the intestinal absorption of calcium (4) and the in vitro resorption of bone (5). The exact relationship between the in vitro potency of this dihydroxylated vitamin D metabolite in resorbing relatively immature skeletal tissue (5, 6) and its documented antirachitic in vivo effect is still ill defined and at best conjectural (7). Moreover, although the effect of parathyroid hormone (PTH) on bone mineral mobilization has been well established, its interaction with vitamin D and/or its metabolites in this regard is still controversial (8–10), as is the role it plays in the metabolic transformation of vitamin D to its hydroxylated renal metabolites (11, 12). This study was designed therefore to evaluate the in vivo effectiveness of $25OHD_3$ on bone metabolism and to determine the role of PTH in this regard.

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[1] *Abbreviations used in this paper:* $1,25(OH)_2D_3$, 1,25 dihydroxycholecalciferol; $25OHD_3$, 25-hydroxycholecalciferol; Pi, inorganic phosphorus; PTH, parathyroid hormone; PTX, parathyroidectomized.

METHODS

Female Holtzman rats, previously maintained on Purina laboratory chow and tap water, were utilized for all experiments. At 10 wk of age one group of animals was surgically parathyroidectomized under light ether anesthesia, the control group being sham operated. The morning after the operation and after an overnight fast, all rats were bled via the tail vein, and serum was analyzed for total calcium (13), inorganic phosphorus (Pi) (14), and ^{45}Ca concentrations (15). Only those animals with serum calcium levels below 8.0 mg/100 ml were considered to be parathyroidectomized (PTX). Thereafter, all PTX animals were maintained on a supplement of 2% calcium lactate in the drinking water. This additional calcium was sufficient to maintain the rat serum calcium concentration in the range of 8.0–9.0 mg/100 ml during the entire experimental period.

24 h after parathyroidectomy one-half of both the PTX and intact control groups were started on a regimen of $25OHD_3$ therapy (500 IU/day) administered orally in oil and continued for the subsequent 14 days. All "control" rats were given an identical volume of oil orally for the 14 days. The animals were bled via the tail vein, after an overnight fast, for calcium (Ca), inorganic phosphorus (Pi), and ^{45}Ca determinations on 20-μl portions of plasma, as described above on days 4, 7, and 14 of treatment. At time of sacrifice on day 14, serum samples from all treatment groups were additionally analyzed for levels of $25OHD_3$ by the method of Haddad and Chyu (16).

Those animals with ^{45}Ca bone labels had been injected intraperitoneally 2 wk before the experimental period with

37.0 μCi $^{45}CaCl_2$ in saline (New England Nuclear, Boston, Mass.). Previous studies have demonstrated that after a 2-wk period of labeling most of the retained radioactive calcium can be considered to be in the "stable" as opposed to the "labile and exchangeable" bone calcium pools (17).

Before sacrifice on day 14 the animals were fasted and placed in metabolic cages for urine collections. Ca and Pi were analyzed as described above. In addition, determinations of urinary hydroxyproline (18) and creatinine (19) were made.

Those animals in which an in vivo measurement of duodenal calcium absorption was to be made according to a modification of Coates and Holdsworth (20) were similarly fasted overnight. On the following day each rat was injected intraduodenally, under light ether anesthesia, with 0.5 μCi of ^{45}Ca containing 2.0 mg of ^{40}Ca in 0.1-ml volume. The diluent was isotonic saline. 30 min after injection each animal was bled via the abdominal aorta and serum analyzed for Ca, Pi, and ^{45}Ca as outlined above.

At the time of sacrifice the tibiae were excised, freed of all connective tissue, and washed clean of marrow constituents with isotonic saline. Tibiae were further subjected to bromoform-toluene density gradient fractionation for analyses of the bone mineral-collagen maturational state, as previously described (21).

In those animals given [^{3}H]proline (100 μCi), the radioactive isotope was injected intraperitoneally 4 days before sacrifice. At the time of sacrifice the tibiae were cleaned and subjected to bromoform-toluene density gradient fractionation as described above. In addition, a 20-μl portion of each density fraction was counted on a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.), as previously described (21).

RESULTS

As seen in Table I, circulating 25OHD$_3$ levels were not altered by parathyroidectomy. After the 2-wk experimental period of 25OHD$_3$ therapy, no significant alterations in the circulating Ca or Pi concentrations were observed in the intact control group despite a 15-fold increase in plasma 25OHD$_3$. Additionally, 25OHD$_3$ administration to control animals resulted in a 200% increase in urinary Ca although no concomitant change was noted in the urinary output of either Pi or hydroxyproline (Table II). There was hypercalciuria despite the lack of significant change in the in vivo duodenal transport of Ca in the 25OHD$_3$-treated control animals (Table I).

In contrast to the results obtained in the intact animals, serum Ca rose and Pi fell in the PTX animals during 25OHD$_3$ therapy (Table I). The significant increment in serum Ca and diminution of serum Pi of 25OHD$_3$-treated PTX animals obtained despite insignificant changes in urinary Ca and Pi compared to the nontreated PTX animals (Table II).

For those rats which were given ^{45}Ca parenterally 2 wk before parathyroidectomy and 25OHD$_3$ therapy, the serum Ca, Pi, and ^{45}Ca values during the ensuing 2-wk experimental period are presented in Fig. 1. As early as 4 days after institution of 25OHD$_3$ therapy, a significantly higher serum Ca level was apparent, an elevation which was maintained throughout the 2-wk period. From 7 to 14 days a significantly higher level of ^{45}Ca existed in the 25OHD$_3$-treated PTX group (Fig. 1). Intact rats, similarly treated with 25OHD$_3$, evidenced no alteration in circulating Ca, Pi, or ^{45}Ca during the entire 2-wk experimental period.

In distinction to these changes in circulating Ca and Pi of the PTX rat, no significant change in bone Ca

TABLE I

*Rat Serum Values and Intestinal Calcium Absorption after 2 wk of 25OHD$_3$ Therapy**

Treatment	Serum Ca	Serum Pi	Intestinal Ca absorption (plasma ^{45}Ca)	Serum 25OHD$_3$ levels
	mg/100 ml		*dpm/0.10 ml*	*ng/ml*
Intact	9.96 ±0.15	6.85 ±0.19	488 ±25	10.8 ±0.37
Intact + 25OHD$_3$ therapy	9.89 ±0.10	6.43 ±0.16	506 ±30	159‡ ±22
PTX	8.28 ±0.13	10.76 ±0.41	316 ±25	12.3 ±1.19
PTX + 25OHD$_3$ therapy	9.46‡ ±0.11	9.26‡ ±0.24	285 ±30	112‡ ±13

* Values represent the mean of each group±SEM. The course of therapy was as outlined in the text.

‡ $P < 0.001$, compared to respective nontreated group. Statistical comparison made according to Student's t test.

Serum mineral values represent nonfasting state. Intestinal absorption data and 25OHD$_3$ levels obtained after an overnight fast.

$n = 15$, for serum Ca and Pi; $n = 10$, for intestinal absorption data; $n = 5$, for serum 25OHD$_3$ levels.

TABLE II

*Urine Calcium, Phosphorus, and OH-Proline after 2 wk of 25OHD$_3$ Therapy**

Treatment	Ca	Pi	OH-proline
	mg/mg Cr	*mg/mg Cr*	*mg/mg Cr*
Intact (8)	0.082 ±0.025	2.21 ±0.21	0.059 ±0.004
Intact + 25OHD$_3$ therapy (8)	0.337‡ ±0.062	2.06 ±0.27	0.052 ±0.003
PTX (10)	0.367 ±0.071	1.28 ±0.28	0.057 ±0.005
PTX + 25OHD$_3$ therapy (11)	0.275 ±0.067	1.19 ±0.19	0.060 ±0.003

* Values represent the mean of each group±SEM. The course of therapy was as outlined in the text. Numbers of animals in each group are designated in parentheses.

‡ $P < 0.005$, compared to respective nontreated group. Statistical comparison made according to Student's t test.

TABLE III
*Tibial Mineral and Hydroxyproline Levels**

Treatment	Total Ca		Total Pi		Total OH-proline	
	mg/tibia	*mg/g bone*	*mg/tibia*	*mg/g bone*	*mg/tibia*	*mg/g bone*
Intact	58.92	143.6	40.04	97.2	4.13	10.00
(6)	±1.9	±5.5	±1.8	±3.0	±0.3	±0.5
Intact + 25OHD$_3$ therapy	58.55	146.9	36.27	91.2	4.08	10.02
(6)	±5.2	±7.6	±2.2	±1.2	±0.3	±1.2
PTX (10)	46.55	147.8	37.86	119.6	4.44	8.84
	±2.9	±6.4	±1.4	±3.0	±0.13	±0.46
PTX + 25OHD$_3$ therapy	50.57	155.7	40.22	123.7	6.94‡	13.39‡
(11)	±1.5	±5.2	±0.8	±2.2	±0.31	±0.49

* Values represent the mean of each group SEM. Numbers of animals are indicated in parenthesis. The course of therapy was as outlined in the text.
‡ P 0.001 compared to respective nontreated group. Statistical comparison made according to Student's t test.

or Pi content due to 25OHD$_3$ was demonstrated in the tibia of the PTX animals (Table III), expressed as total Ca and Pi per whole tibia or as mineral per milligram dry weight of bone. The Pi content of the bones from PTX rats, as compared to controls, was increased. Bone hydroxyproline (collagen) content was significantly elevated in the PTX group by 25OHD$_3$ therapy. No significant change in either ^{45}Ca content and/or ^{45}Ca sp act was observed in the tibia of 25OHD$_3$-treated PTX animals who had received parenteral injections of ^{45}Ca earlier for the purpose of labeling skeletal calcium pools (Table IV).

Density fractionation analysis of the tibiae from PTX rats (Tables V and VI) revealed a significant increment in the less dense or immature bone fractions and a corresponding decrease in the most mature bone fraction, as compared to control rat bones. Those bones from PTX animals treated with 25OHD$_3$ for 2 wk showed a statistically significant shift of mineral and matrix components from the less dense into the most mature fractions (Tables V and VI), compared to the untreated PTX group. Furthermore, the maturational profile of the PTX-25OHD$_3$ tibiae was indistinguishable from that of either the intact or the intact-25OHD$_3$ profiles.

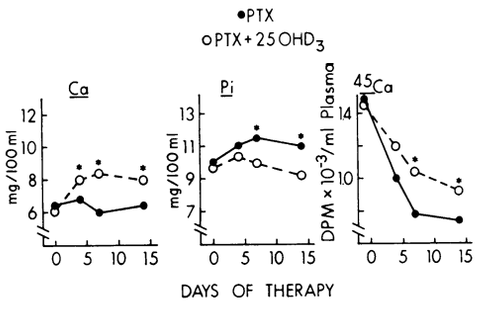

FIGURE 1 Response of PTX rats to 25OHD$_3$ therapy. All animals were fasted overnight $n \geq 13$ for total Ca and Pi curves, $n \geq 7$ for ^{45}Ca curves. *$P < 0.005$ compared to nontreated PTX animal. Statistical comparison according to Student's t test.

In those animals given [^{3}H]proline for 4 days (Table VII), there was an enhanced incorporation of ^{3}H into the most dense, mature collagen fractions in those bones from 25OHD$_3$-treated PTX animals. Concomitantly the total ^{3}H incorporated into the bone was also significantly increased by 4 days.

DISCUSSION

The response to 25OHD$_3$ of rats not suffering from a state of vitamin D deficiency and/or hypocalcemia depended on the PTH status of the animal (Table I). These observations that 25OHD$_3$ administration to intact rats produced no significant alterations in circulating Ca or Pi concentrations despite a marked calciuria (Table II) and unchanged duodenal Ca absorption (Table I) are markedly different from those obtained in the vitamin D-deficient state (4, 6, 7). However, the results of the present study are in agreement

TABLE IV
*Tibial ^{45}Ca Content**

Treatment	Metaphysis	Diaphysis	Metaphysis	Diaphysis
	dpm/tibia ($\times 10^{-3}$)		*dpm/mg bone Ca* ($\times 10^{-3}$)	
PTX (4)	579±60	958±55	33.6±2.8	25.4±1.9
PTX + 25OHD$_3$ therapy (5)	625±42	1,055±53	31.6±1.8	25.4±2.1

* Values represent the mean of each group±SEM. Numbers of animals are indicated in parenthesis. The course of therapy was as outlined in the text.

TABLE V

*Bone Calcium Density Distribution after 2-wk 25OHD$_3$ Therapy**

Treatment	Density gradient specific gravity						
	1.7	1.8	1.9	2.0	2.1	2.2	2.3
Intact (6)	0.38 ±0.08	0.35 ±0.08	0.69 ±0.21	2.34 ±0.62	9.34 ±1.67	48.34 ±2.19	38.53 ±1.30
Intact + 25OHD$_3$ therapy (6)	0.37 ±0.07	0.36 ±0.11	0.75 ±0.23	3.39 ±2.07	11.07 ±4.24	50.95 ±3.87	33.10 ±3.34
PTX (6)	0.33 ±0.05	0.11 ±0.07	1.03 ±0.31	4.30‡ ±0.41	15.31‡ ±1.05	52.70 ±3.88	26.31‡ ±2.43
PTX + 25OHD$_3$ therapy (7)	0.22 ±0.04	0.09 ±0.02	0.27§ ±0.10	1.04§ ±0.26	4.83§ ±0.21	55.71 ±3.32	37.83§ ±3.07

* Values represent the mean of each group±SEM. Methods and therapy as described in the text. Numbers of animals as indicated in parenthesis.
‡ $P < 0.02$, compared to intact group.
§ $P < 0.05$, compared to PTX, nontreated group.
Statistical comparisons made according to Student's t test.

with those of Younoszai and Schedl (22) which demonstrate that exogenous vitamin D$_3$ administration to normal animals had no stimulatory effect upon Ca absorption.

In contrast to these results obtained in intact animals, those animals subjected to PTX responded markedly to 25OHD$_3$ therapy with an elevation in serum Ca and fall in Pi despite any detectable increments in the intestinal absorption of calcium (Table I).

To substantiate whether the calcium mobilization seen in the PTX animals was related to duodenal absorption and/or to bone mineral, a parenteral dose of ^{45}Ca was given 2 wk before parathyroidectomy and 25OHD$_3$ therapy. This ^{45}Ca label was thereby incorporated into the "stable" as opposed to "exchangeable" pool of bone mineral (17), an area of bone known to be resorbed by PTH (23). As early as 4 days after institution of 25OHD$_3$ therapy a significantly higher serum calcium level was apparent (Fig. 1), an elevation which was maintained throughout the 2-wk period. From 7 through 14 days, a significantly higher level of serum ^{45}Ca existed in the 25OHD$_3$-treated group and was not accompanied by alterations in ^{45}Ca sp act. Accordingly the data were interpreted to support a

TABLE VI

*Bone Hydroxyproline (Collagen) Density Distribution after 25OHD$_3$ Therapy**

Treatment	Density gradient specific gravity						
	1.7	1.8	1.9	2.0	2.1	2.2	2.3
Intact (6)	0.61 ±0.24	0.53 ±0.16	0.75 ±0.18	1.83 ±0.50	11.58 ±2.03	55.76 ±3.56	28.90 ±2.37
Intact + 25OHD$_3$ therapy (6)	1.50 ±0.38	1.16 ±0.46	1.75 ±0.63	3.93 ±1.77	10.22 ±2.83	57.28 ±4.88	24.15 ±3.45
PTX (6)	1.10 ±0.20	0.42 ±0.09	1.28 ±0.35	4.10‡ ±0.56	18.27‡ ±1.11	53.86 ±2.53	20.96‡ ±1.81
PTX + 25OHD$_3$ therapy (7)	0.45§ ±0.07	0.15§ ±0.02	0.22§ ±0.05	0.93§ ±0.19	9.24§ ±1.08	57.62 ±3.24	32.84§ ±3.19

* Values represent the mean of each group±SEM. Methods and therapy as described in the text. Numbers of animals as indicated in parentheses.
‡ $P < 0.02$, compared to intact group.
§ $P < 0.02$, compared to PTX, nontreated group.
Statistical comparisons made according to Student's t test.

TABLE VII

*Tibial Density Fractionation Profile of ^3H after a 4-day Pulse of [^3H]Proline**

Treatment	Density gradient specific gravity‡					dpm/g bone
	1.7–1.9	2.0	2.1	2.2	2.3	
PTX (4)	1.93 ±0.25	1.44 ±0.4	22.88 ±3.1	49.32 ±7.0	29.50 ±4.4	106.8 ±7.1
PTX + 25OHD$_3$ therapy (4)	1.70 ±0.15	3.68§ ±0.9	24.69 ±2.6	72.94§ ±6.4	46.20§ ±5.8	149.5§ ±7.1

* Values represent the mean of each group±SEM. Numbers of animals are indicated in parentheses. The course of therapy was as outlined in the text.

§ $P < 0.02$ compared to PTX group. Statistical comparison made according to Student's t test.

‡ dpm/g bone $\times 10^{-3}$.

stimulated resorption of bone, as opposed to enhanced intestinal absorption. These observations are in agreement with those of Harrison and Harrison (24) made in thyroparathyroidectomized rats and the recent observations of Liu et al. detailing vitamin D-enhanced osteoclastic bone resorption at vascular channels (25). They are also consistent with in vitro data defining a bone mobilizing effect of both 25OHD$_3$ and 1,25(OH)$_2$D$_3$ (6, 9, 10). The accumulated data would lead us to believe that biologically active vitamin D metabolites do possess bone resorbing potential in vivo and that this effect is independent of PTH.

However, the aforementioned changes in circulating Ca and Pi observed during 25OHD$_3$ therapy were not reflected by a change in the tibial Ca or Pi content of the PTX animals (Table III). The increased Pi concentration of the bones from PTX rats, as compared to values obtained from the controls, is consistent with the elevated serum Pi levels which characterize the parathyroprivic state in rats and with the known influence of the Ca and Pi bathing the mineral upon the composition of the nucleating hydroxyapatite crystals (26, 27).

Contrary to earlier reports of increased resorption of whole bone by vitamin D metabolites (5, 6, 9), bone hydroxyproline (collagen) content was significantly elevated in the 25OHD$_3$-treated PTX animals. The aforementioned serum and bone data interpreted collectively would therefore suggest that total skeletal turnover is increased during 25OHD$_3$ therapy with a preferential enhancement of collagen synthesis. The results are consistent with those of Paterson and Fourman (28) and others (29-32), wherein vitamin D and/or its active metabolites were reported to enhance bone collagen synthesis and to stimulate lysyl oxidase activity when administered to hypocalcemic and/or vitamin D-deficient animals. Unlike the vitamin D-deficient animal, however, the serum calciums of the PTX-calcium supplemented animals, although lower than normal, were never at the hypocalcemic levels which have been shown to decrease bone maturation (33). Rather, the 8.3 mg/100 ml serum calcium, which was maintained in this study (Table I), was comparable to the serum calcium concentration which Au and Raisz (34) found sufficient to maintain skeletal responsiveness in PTX rats. Thus the 25OHD$_3$ effect on bone in the present study cannot be solely a secondary reflection of the reversed hypocalcemia.

It is necessary, however, to reconcile the observed increments in circulating ^{45}Ca and total Ca (Fig. 1) with the unchanged bone Ca, Pi, and ^{45}Ca content (Tables III and IV). One might anticipate that in the face of an increased skeletal resorption and an elevated serum calcium, the bone ^{45}Ca and total Ca would be diminished. Indeed this would be the case if a significant percentage of the total bone mineral was being resorbed. In fact, the effective 1.2 mg/100 ml increase in the serum Ca of the PTX-25OHD$_3$ group compared to the untreated PTX group represents a calculated loss of 0.24 mg of calcium from the entire skeletal tissue or 1.2% of the total bone calcium in the tibiae alone. Thus, the significant elevation observed in the circulating Ca pool as a result of 25OHD$_3$ therapy appears to reflect the preferential loss of the available skeletal calcium pool which is beyond chemical quantitation.

To more fully evaluate the bone mineral-matrix composition in the PTX animals and the apparent increase in collagen synthesis observed in the 25OHD$_3$-treated animals, density fractionation analysis of the tibiae (Tables V and VI) was performed. The significant increment in the immature bone fractions of the tibiae from PTX rats and the corresponding decrease in the most mature fractions, as compared to control rat bones, reflects the influence of PTH on bone mineral-collagen maturation. In agreement with these data are observations by Burnell et al. (35), wherein a reduction in the total bone density and a decreased maturation of bone

were observed in PTX animals with a significant decrease in hydroxyproline/volume ratio of the bone.

From the profile of the tibiae from the 25OHD$_3$-treated PTX animals, additional corroboration was obtained that 25OHD$_3$ did not cause a net resorption of bone. In fact, a statistically significant shift of mineral and matrix components occurred from the less dense into the most mature fractions (Table V and VI). If resorptive processes alone had been operative, a decrease in the most dense, mature mineral and matrix components should have been observed, as noted earlier by Richelle and Bronner (36) in bones from rats subjected to PTH treatment. [^{3}H]proline administration to animals succeeded in further substantiating the enhanced bone maturation and collagen synthesis in response to 25OHD$_3$. In those bones from 25OHD$_3$-treated PTX animals, with a 4-day time of labeling, there was an enhanced incorporation of ^{3}H into the total bone as well as into the most dense, mature collagen fractions (Table VII).

The results of this study do not differentiate between the action of 25OHD$_3$ or its 1,25(OH)$_2$D$_3$ metabolite on bone tissue. Although the ionic control of 1,25(OH)$_2$D$_3$ production has proven to be quite complex (37-40), one would anticipate that 25OHD$_3$ is the primary circulating metabolite in PTX states since both acute (3, 8) and chronic (41) studies of PTH absence have demonstrated a diminution of 1,25(OH)$_2$D$_3$ synthesis. The accumulation of either 24,25(OH)$_2$D$_3$ or its 1,24,-25(OH)$_3$D$_3$ metabolite in the PTX animal (3, 8) would be of little consequence to the skeletal tissue since these metabolites have not been shown to have a significant effect on bone (42).

The observations obtained in this study of PTX animals, however, are consistent with a 25OHD$_3$- enhancement of bone maturation and with reported salutory effects of 25OHD$_3$ in reversing both the hypocalcemia of hypoparathyroidism (16, 43) and the osteodystrophy of anephric patients (44). They are also consistent with reported observations that 25OHD$_3$ accumulates in the skeleton in abundance (45). Suffice it to say that these experiments demonstrate that either 25OHD$_3$ or other skeletal biologically active metabolites thereof are capable of effecting skeletal turnover in the experimental parathyroprivic, nonrachitic state; and as such the metabolite(s) lead(s) to a restoration of abnormal mineral-collagen profiles to normal.

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