

25-Hydroxycholecalciferol-Enhanced Bone Maturation in the Parathyroprivic State

JEAN E. RUSSELL and LOUIS V. AVIOLI

From the Department of Medicine, Washington University School of Medicine, The Jewish Hospital of St. Louis, St. Louis, Missouri 63110

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 (25OHD₃) 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 25OHD₃ 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 ⁴⁵Ca transport procedures. In contrast to the control group, the response to 25OHD₃ of PTX rats was dramatic. Bone mineral and matrix maturation were both accelerated by 25OHD₃ 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 (25OHD₃)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)₂D₃) established as the most potent stimulus for the intestinal absorption of calcium (4)

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¹ Abbreviations used in this paper: 1,25(OH)₂D₃, 1,25 dihydroxycholecalciferol; 25OHD₃, 25-hydroxycholecalciferol; Pi, inorganic phosphorus; PTH, parathyroid hormone; PTX, parathyroidectomized.

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 25OHD₃ on bone metabolism and to determine the role of PTH in this regard.

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 ⁴⁵Ca 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 25OHD₃ 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 ⁴⁵Ca 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 25-OHD₃ by the method of Haddad and Chyu (16).

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

37.0 μCi $^{45}\text{CaCl}_2$ 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 ^{45}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 [^3H]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 25OHD₃ levels were not altered by parathyroidectomy. After the 2-wk experimental period of 25OHD₃ therapy, no significant altera-

TABLE I
Rat Serum Values and Intestinal Calcium Absorption
after 2 wk of 25OHD₃ Therapy*

Treatment	Serum Ca	Serum Pi	Intestinal Ca absorption (plasma ^{45}Ca)	Serum 25OHD ₃ levels
	<i>mg/100 ml</i>		<i>dpm/0.10 ml</i>	<i>ng/ml</i>
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 \ddagger ± 22
PTX	8.28 ± 0.13	10.76 ± 0.41	316 ± 25	12.3 ± 1.19
PTX + 25OHD ₃ therapy	9.46 \ddagger ± 0.11	9.26 \ddagger ± 0.24	285 ± 30	112 \ddagger ± 13

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

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

Serum mineral values represent nonfasting state. Intestinal absorption data and 25OHD₃ levels obtained after an overnight fast.

n = 15, for serum Ca and Pi; *n* = 10, for intestinal absorption data; *n* = 5, for serum 25OHD₃ levels.

TABLE II
Urine Calcium, Phosphorus, and OH-Proline after
2 wk of 25OHD₃ Therapy*

Treatment	Ca	Pi	OH-proline
	<i>mg/mg Cr</i>	<i>mg/mg Cr</i>	<i>mg/mg Cr</i>
Intact (8)	0.082 ± 0.025	2.21 ± 0.21	0.059 ± 0.004
Intact + 25OHD ₃ therapy (8)	0.337 \ddagger ± 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 \pm SEM. The course of therapy was as outlined in the text. Numbers of animals in each group are designated in parentheses.

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

tions in the circulating Ca or Pi concentrations were observed in the intact control group despite a 15-fold increase in plasma 25OHD₃. Additionally, 25OHD₃ 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 25OHD₃-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 25OHD₃ therapy (Table I). The significant increment in serum Ca and diminution of serum Pi of 25OHD₃-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 25OHD₃ 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 25OHD₃ 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 25OHD₃-treated PTX group (Fig. 1). Intact rats, similarly treated with 25OHD₃, 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 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 (6)	58.92 ±1.9	143.6 ±5.5	40.04 ±1.8	97.2 ±3.0	4.13 ±0.3	10.00 ±0.5
Intact + 25OHD ₃ therapy (6)	58.55 ±5.2	146.9 ±7.6	36.27 ±2.2	91.2 ±1.2	4.08 ±0.3	10.02 ±1.2
PTX (10)	46.55 ±2.9	147.8 ±6.4	37.86 ±1.4	119.6 ±3.0	4.44 ±0.13	8.84 ±0.46
PTX + 25OHD ₃ therapy (11)	50.57 ±1.5	155.7 ±5.2	40.22 ±0.8	123.7 ±2.2	6.94‡ ±0.31	13.39‡ ±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 25OHD₃ 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 25OHD₃ therapy. No significant change in either ⁴⁵Ca content and/or ⁴⁵Ca sp act was observed in the tibia of 25OHD₃-treated PTX animals who had received parenteral injections of ⁴⁵Ca 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 25OHD₃ 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-25OHD₃ tibiae was indistinguishable from that of either the intact or the intact-25OHD₃ profiles.

In those animals given [³H]proline for 4 days (Table VII), there was an enhanced incorporation of ³H into the most dense, mature collagen fractions in those bones from 25OHD₃-treated PTX animals. Concomitantly the total ³H incorporated into the bone was also significantly increased by 4 days.

DISCUSSION

The response to 25OHD₃ 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 25OHD₃ 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

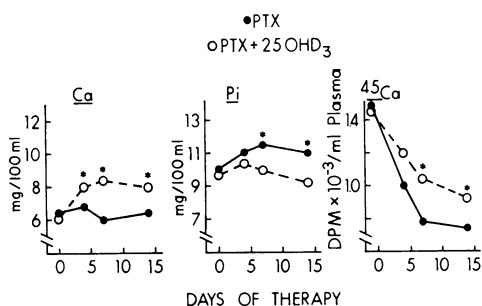


FIGURE 1 Response of PTX rats to 25OHD₃ therapy. All animals were fasted overnight $n \geq 13$ for total Ca and Pi curves, $n \geq 7$ for ⁴⁵Ca curves. * $P < 0.005$ compared to nontreated PTX animal. Statistical comparison according to Student's t test.

TABLE IV
Tibial ⁴⁵Ca Content*

Treatment	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 25OHD₃ 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 D₃ 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 25OHD₃ 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

⁴⁵Ca was given 2 wk before parathyroidectomy and 25OHD₃ therapy. This ⁴⁵Ca 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 25OHD₃ 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 ⁴⁵Ca existed in the 25OHD₃-treated group and was not accompanied by alterations in ⁴⁵Ca sp act. Accordingly the data were interpreted to support a

TABLE VI
Bone Hydroxyproline (Collagen) Density Distribution after 25OHD₃ 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 $[^3\text{H}]\text{Proline}^*$

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 \pm 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 25OHD₃ and 1,25(OH)₂D₃ (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 25OHD₃ 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 25OHD₃-treated PTX animals. The aforementioned serum and bone data interpreted collectively would therefore suggest that total skeletal turnover is increased during 25OHD₃ 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 nor-

mal, 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 25OHD₃ 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-25OHD₃ 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 25OHD₃ 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 25OHD₃-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 25OHD₃-treated PTX animals, additional corroboration was obtained that 25OHD₃ 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. [³H]proline administration to animals succeeded in further substantiating the enhanced bone maturation and collagen synthesis in response to 25OHD₃. In those bones from 25OHD₃-treated PTX animals, with a 4-day time of labeling, there was an enhanced incorporation of ³H 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 25OHD₃ or its 1,25(OH)₂D₃ metabolite on bone tissue. Although the ionic control of 1,25(OH)₂D₃ production has proven to be quite complex (37-40), one would anticipate that 25OHD₃ 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)₂D₃ synthesis. The accumulation of either 24,25(OH)₂D₃ or its 1,24,25(OH)₃D₃ 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 25OHD₃-enhancement of bone maturation and with reported salutary effects of 25OHD₃ in reversing both the hypocalcemia of hypoparathyroidism (16, 43) and the osteodystrophy of anephric patients (44). They are also consistent with reported observations that 25OHD₃ accumulates in the skeleton in abundance (45). Suffice it to say that these experiments demonstrate that either 25OHD₃ 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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