The vitamin D prodrugs 1α(OH)D2, 1α(OH)D3 and BCI-210 suppress PTH secretion by bovine parathyroid cells

Alex J. Brown1, Cynthia S. Ritter1, Joyce C. Knutson2 and Stephen A. Strugnell2

1Renal Division, Washington University School of Medicine, St Louis, MO and 2Preclinical Research, Bone Care International, Middleton, WI, USA

Abstract

Background. Active vitamin D compounds are widely used in the treatment of secondary hyperparathyroidism associated with renal failure. These compounds reduce PTH secretion through vitamin D receptor (VDR)-dependent repression of PTH gene transcription. In previous studies, 1α(OH)D3, a vitamin D prodrug, inhibited PTH secretion in cultured bovine parathyroid cells, but it was unclear whether 1α(OH)D3 itself or an active metabolite produced this inhibition.

Methods. We determined the effectiveness of the vitamin D prodrugs 1α(OH)D3, 1α(OH)D2 and 1α(OH)-24(R)-methyl-25-ene-D2 (BCI-210) at inhibiting PTH secretion in bovine parathyroid cell cultures, and examined the metabolism of [3H]1α(OH)D2 in these cells.

Results. All three prodrugs suppressed PTH secretion with approximately 10% of the activity of 1,25(OH)2D3; much higher activity than expected based on the VDR affinities of these prodrugs (0.25% of 1,25(OH)2D3). Parathyroid cells activated [3H]1α(OH)D2 to both 1,25(OH)2D2 and 1,24(OH)2D2. 1,24(OH)2D2 was detectable at 4 h, increased to a maximum at 8 h, and then decreased. In contrast, 1,25(OH)2D2 levels increased linearly with time, suggesting the presence of constitutively active vitamin D-25-hydroxylase not previously reported in parathyroid cells. The cytochrome P-450 inhibitor ketoconazole (50 μM) reduced 1α(OH)D2 metabolism to below detectable levels, but did not significantly affect suppression of PTH by 1α(OH)D2.

Conclusions. The vitamin D prodrugs 1α(OH)D3, 1α(OH)D2 and BCI-210 suppressed PTH production by cultured parathyroid cells. The ability of 1α(OH)D2 to reduce PTH despite inhibition of its metabolism suggests a direct action of this ‘prodrug’ on the parathyroid gland, but the mechanism underlying this activity is not yet known.

Keywords: BCI-210; 1α-hydroxyvitamin D2; 1α-hydroxyvitamin D3; ketoconazole; parathyroid hormone; vitamin D

Introduction

Vitamin D therapy is widely used for the treatment of the secondary hyperparathyroidism associated with chronic renal failure [1,2]. The active hormonal form of vitamin D, 1,25-dihydroxyvitamin D, and its analogues can reduce serum PTH levels by repressing PTH gene transcription [3,4] and blocking parathyroid gland proliferation [5]. These actions are mediated by the vitamin D receptor (VDR) present in the PTH-secreting chief cells of the parathyroid glands [6–8]. High-affinity binding to the VDR generally requires the presence of constitutively active vitamin D-25-hydroxylase not previously reported in parathyroid cells. The synthetic prohormones, 1α(OH)D3 (alfacalcidol) and 1α(OH)D2 (doxercalciferol) (Figure 1), are widely used for treatment of secondary hyperparathyroidism of kidney disease, but these compounds have relatively low intrinsic affinity for the VDR compared to active compounds such as calcitriol [9], and have been thought to require systemic activation to exert their effects on the parathyroid glands.

An unpublished study by Olgaard and coworkers demonstrated a direct suppression of PTH secretion by 1α(OH)D3 in parathyroid tissue culture [10]. This initial study did not investigate whether suppression of PTH by 1α(OH)D3 required conversion to a more active metabolite. We have shown that parathyroid cells possess vitamin D hydroxylase (CYP24) activity [11,12], and may therefore be capable of activating...
Vitamin D prodrugs suppress PTH secretion

Methods

Preparation of dispersed bovine parathyroid cells

Dispersed bovine parathyroid cells were prepared as previously described [14]. Briefly, bovine parathyroid glands (obtained from MBH Enterprises, Tampa, FL, USA) were trimmed of extraneous fatty tissue, sliced to 0.5 mm thickness with a tissue slicer (Stadie Riggs; Thomas Scientific, Swedesboro, NJ) and placed in a mixture of DME:Ham’s F-12 medium (50:50) containing 0.5 mM calcium and collagenase (3000 U/ml of collagenase XI-S; Sigma, St Louis, MO, USA). The suspension (10 ml media per gram of tissue) was agitated in a shaking water bath at 37 °C for 90 min. Periodic passage of the mixture through the tip of a 10 ml pipette assisted in the disaggregation. The digested tissue was washed three times with serum-free culture medium containing DME:Ham’s F-12 (50:50), 1 mM CaCl2, 15 mM Hepes, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml holo-transferrin, 2 mM glutamine, 1% non-essential amino acids and 0.1% bovine serum albumin (fraction V).

Assessment of PTH suppression activity

Primary cultures of bovine parathyroid cells were grown to confluency in serum-free medium as previously described [15], and then treated for 72 h with 0.1, 1.0, 10 and 100 nM 1,25(OH)2D3, 1α(OH)D3, 1α(OH)D2, or BCI-210 with daily changes of medium. The cells were then changed into fresh medium and the amount of PTH secreted during a 4 h incubation was determined using an ELISA kit (Immutopics, San Capistrano, CA).

To determine the effects of ketoconazole, an inhibitor of metabolism, on PTH, confluent monolayers were treated with 1α(OH)D2 (100 nM) for 48 h with media changes every 12 h. RNA was harvested from the cells using RNAzol B (Cinna/Biotech), resolved on 1.2% agarose/formaldehyde gels and transferred to nylon membrane (Zeta-Probe, Bio-Rad). PTH mRNA and 18S rRNA were measured by hybridizing the membranes with riboprobes described previously [16]. The data are expressed as the ratio of PTH mRNA to 18S rRNA.

Metabolism

To determine if 1α(OH)D2 is metabolized to a more active compound in parathyroid cells, [3H]-1α(OH)D2 (100 nM, 1 μCi) was incubated with confluent monolayers of bovine parathyroid cells. After 0, 4, 8 or 24 h, the reactions were terminated by the addition of one volume of methanol (obtained from MBH Enterprises, Tampa, FL, USA) and placed in a mixture of DME:Ham’s F-12 medium (50:50) containing 0.5 nmol of 1,25(OH)2D2 and 1,24(OH)2D2. The cells plus medium were extracted by a modification of the method of Bligh and Dyer [17]. The metabolites of 1α(OH)D2 in the organic phase were resolved by normal phase HPLC in the mobile phase. To determine the efficiency of ketoconazole to inhibit the metabolism of 1α(OH)D2, confluent monolayers were treated with [3H]-1α(OH)D2 (100 nM, 1 μCi) for 48 h with media changes every 12 h. Medium from each plate was placed in a separate container after each media change; at the end of the incubation, pooled media from each individual plate was extracted and normal phase HPLC performed as above to detect the presence of 1,25(OH)2D2 and 1,24(OH)2D2. Radioactivity in the aqueous phase of the extraction was measured to assess the amount of conversion to side chain-cleaved metabolites.

Results

Suppression of PTH in vitro

The ability of the 1α-hydroxy analogues to suppress PTH was assessed in vitro in cultures of bovine parathyroid cells. The structures of the analogues

![Diagram of vitamin D prodrugs](https://academic.oup.com/ndt/article-abstract/21/3/644/1854458/1213644/184458)
tested are shown in Figure 1 and their effects on PTH secretion are shown in Figure 2. The 1α-hydroxy compounds, 1α(OH)D2, 1α(OH)D3 and BCI-210 were all effective in reducing PTH secretion, with potencies about 10% of 1,25(OH)2D3. The high activity of BCI-210, which was designed to be resistant to both 24- and 25-hydroxylation, suggests that the 1α-hydroxy compounds either do not require activation or that this compound is activated by an alternative pathway such as 26-hydroxylation.

Metabolism of 1α(C11)(OH)D2 in bovine parathyroid cells

To determine if the 1α-hydroxy compounds may be converted to more active metabolites, parathyroid cells were incubated with [3H]1α(OH)D2 for 0, 4, 8 and 24 h, and the cells plus medium were analysed for more polar metabolites. The HPLC profiles of a representative sample at each time point are shown in Figure 3A. There was a progressive loss of radioactive 1α(OH)D2 with the appearance of tritiated peaks co-eluting with 1,24(OH)2D2 and 1,25(OH)2D2 on normal phase HPLC. The amount of 1,24(OH)2D2 increased up to 8 h after substrate addition and then declined, while the amount of 1,25(OH)2D2 increased linearly for the entire 24 h incubation.

To confirm the identities of these metabolites, the putative 1,24(OH)2D2 and 1,25(OH)2D2 peaks were isolated and rechromatographed on reverse phase HPLC (data not shown). The radioactive peaks co-eluted with the internal standards, consistent with their identities as 1,24(OH)2D2 and 1,25(OH)2D2. A third metabolite peak in the normal phase HPLC profile was observed at fraction 38, in the position expected for 1,26(OH)2D2, but the lack of a standard prevented its further analysis. There was also a progressive increase in radioactivity in the methanol strip, indicating the presence of even more polar metabolites.

The amount of the metabolites present represented a very small proportion of the initial [3H]1α(OH)D2, as illustrated in Figure 3B, in which the scale of the y-axis of Figure 3A is expanded. The HPLC analysis indicates that about 1% of the input 10−7 M [3H]1α(OH)D2, equivalent to approximately 10−9 M 1,25(OH)2D2, was present at 24 h after substrate addition when 1,25(OH)2D2 levels were highest. In addition, we found these dihydroxylated metabolites predominantly in the medium rather than the cells (data not shown).

Effect of ketoconazole on 1α(OH)D2 metabolism and PTH suppression

The necessity of side chain hydroxylation in the suppression of PTH by 1α(OH)D3 was examined using the competitive cytochrome P450 inhibitor...
Vitamin D prodrugs suppress PTH secretion

Fig. 4. Effect of ketoconazole on 1α(OH)D2 metabolism. Confluent cultures of bovine parathyroid cells were incubated with [3H]1α(OH)D2 (100 nM, 1 μCi) for 48 h in the absence (solid bars) or presence (hatched bars) of 50 μM ketoconazole. Medium removed at the end of each 12 h period and the final cell monolayer extracted [17]. Radioactivity remaining in the aqueous phase after three extractions was quantified by scintillation counting. Data are expressed as mean ± SD (n = 3). *P < 0.05 vs no cell control. **P < 0.05 vs ketoconazole-treated cells.

We next examined the PTH mRNA levels in cells treated with 1α(OH)D2 (or vehicle) with or without ketoconazole under conditions identical to those above. As shown in Figure 6, 1α(OH)D2 alone decreased PTH mRNA levels by 50% comparable to the reduction in PTH secretion shown in Figure 2. Interestingly, ketoconazole alone also reduced PTH mRNA by an as yet unknown mechanism. In the presence of ketoconazole, 1α(OH)D2 further suppressed PTH mRNA by about 55%, as effectively as in the absence of ketoconazole. Similar results were obtained in two additional experiments in different cell preparations treated in the same manner.

Discussion

The parathyroid glands were definitively identified as a vitamin D target tissue soon after characterization of the active form of vitamin D, 1,25(OH)2D3 [6–8]. The glands contain a high content of VDR, and 1,25(OH)2D3 has been shown to repress PTH gene transcription [3,4] and inhibit parathyroid cell proliferation [5] through a VDR-dependent mechanism. The secondary hyperparathyroidism that develops in patients with chronic renal failure is believed to be attributable in part to the reduction of renal synthesis of 1,25(OH)2D3 and loss of suppression of parathyroid...
gland function by 1,25(OH)_{2}D_{3}. Vitamin D therapy is widely used to treat hyperparathyroidism in these patients. Although 1,25(OH)_{2}D_{3} and its synthetic precursor, 1α(OH)D_{3} (alfacalcidol), have been successfully employed for this purpose for many years, the natural vitamin D hormone is a potent stimulator of intestinal calcium absorption and often produces hypercalcemia, especially in patients receiving calcium-based phosphate binders to control hyperphosphatemia. This has led to the development of vitamin D analogues that retain the suppressive actions of 1,25(OH)_{2}D_{3} on the parathyroid glands but have less calcemic activity. Several analogues are now available for treatment of secondary hyperparathyroidism in renal failure patients, including 19-nor-1,25(OH)_{2}D_{2} (paricalcitol), 22-oxa-1,25(OH)_{2}D_{3} (maxacalcitol), 1α(OH)D_{3} (alfacalcidol), 1α(OH)D_{2} (doxercalciferol) and 1,25(OH)_{2}26,27-F_{6}-D_{3} (falecalcitriol) [18,19]. Vitamin D prodrugs such as doxercalciferol owe their reduced calcemic activity at least in part to the pharmacokinetics of their activation, which leads to sustained serum levels of active compound at more physiological concentrations than can be produced by administration of active compounds. Recent studies indicate that 1α(OH)D_{3} produces a disproportionately large degree of PTH suppression relative to calcitriol, based on observed serum levels of active metabolites, suggesting either a direct suppressive effect of such prodrugs on the parathyroid, or activation in situ [20].

To further clarify the structural requirements for effective vitamin D analogues in controlling parathyroid gland function, we investigated the ability of synthetic vitamin D prodrugs to directly suppress PTH synthesis and secretion. As reported by Nielsen et al., 1α(OH)D_{3} was able to suppress PTH release from cultured bovine parathyroid cells. A similar suppression was observed in the present study with 1α(OH)D_{3} and BCI-210. BCI-210 is a novel prodrug with a 24 (R) methyl group and a 25–27 double bond that is designed to oxidize vitamin D analogues that retain the suppressive actions of 1,25(OH)_{2}D_{3} on the parathyroid glands but have less calcemic activity [11,12]. The 24-hydroxylase can oxidize carbons 24 and 23 of the vitamin D side chain, leading to more polar metabolites with limited VDR affinity and PTH suppressing activity [11], and eventually to side chain cleavage and inactivation [21].

Production of 1,25(OH)_{2}D_{3} from 1α(OH)D_{2} in parathyroid cells occurred in a linear fashion for the entire incubation period. The observation that parathyroid cells are capable of 25-hydroxylation of vitamin D compounds has not been previously reported. However, Correa et al. [13] recently reported the presence of transcripts for CYP27A1, a cytochrome P450 capable of 25-hydroxylating vitamin D, in human parathyroid glands. We confirmed the presence of these transcripts in human, rat and bovine parathyroid glands (data not shown), CYP27A1 may be responsible for the observed production of 1,25(OH)_{2}D_{3} in parathyroid cells. However, CYP27A1 does not 25-hydroxylate 1α(OH)D_{2} efficiently [22], suggesting that at least one other vitamin D 25-hydroxylase is also present in these cells.

The amount of 1,25(OH)_{2}D_{3} and 1α(OH)D_{2} in the parathyroid cells cultures is relatively small at all times examined (Figure 3B). Furthermore, when ketoconazole was added to the cells every 12 h over a 48 h period, no 1,24(OH)_{2}D_{2} or 1,25(OH)_{2}D_{2} were detected at the end of the final 12 h treatment period. In addition, the aqueous counts, a measure of side chain cleavage via the C24 oxidation pathway, in the ketoconazole-treated samples were the same as those found with substrate incubated under identical conditions without cells for a 12 h period, indicating virtually no metabolism of the 1α(OH)D_{3}. This complete block of metabolism did not prevent the reduction of PTH mRNA by 1α(OH)D_{3}.

The ability of 1α(OH)D_{3} and probably the other vitamin D ‘prodrugs’, to suppress PTH without activation raises the question as to how this suppression is produced, given the relatively low VDR affinity of these compounds. One possibility is that these compounds accumulate in the parathyroid and reach intracellular concentrations sufficient to directly activate VDR and subsequently suppress PTH production. High intracellular levels of 1α(OH)D_{2} were, in fact, observed in the metabolism studies, supporting this possibility. Intraparathyroid accumulation may also explain the clinical observation that, although patients who received equal doses of intravenous or oral 1α(OH)D_{2} gave rise to equal serum levels of the active metabolite 1,25(OH)_{2}D_{2}, the dose of intravenous 1α(OH)D_{2} required for equivalent PTH suppression was 40% of the oral dose [23]. This clinical data is consistent with the possibility that the initial high serum levels of 1α(OH)D_{2} produced by intravenous administration deliver high levels of 1α(OH)D_{2} to the parathyroid glands, enhancing the effectiveness
of PTH suppression. Further studies will be necessary to confirm this possibility.

An alternative explanation for the observed PTH-lowering ability of 1z(OH)D2 is interaction with a novel receptor. Recent studies by Panda et al., using VDR and 1z-oxihydroxylase null mice have suggested the possibility that the high concentrations of 1z(OH)D3 may act independently of the VDR to block parathyroid hyperplasia [24]. VDR null mice did not develop parathyroid hyperplasia when fed a rescue diet containing 2.0% calcium, 1.25% phosphorus, 20% lactose and 2.2 U/g vitamin D3, whereas mice lacking both VDR and 1z-hydroxylase had enlarged parathyroid glands. The presence of a non-VDR receptor that is activated by high 1z(OH)D3 was proposed. This as yet hypothetical receptor may mediate the effects of vitamin D compound lacking side chain hydroxyl groups including those investigated in the present study. One or both of these two alternative mechanisms may explain the high potency of 20S-1z-oxihydroxy-2-methylene-19-nor-bishomopregnacalciferol (2M-bisP), an analogue lacking a side chain, to effectively lower PTH levels in both normal [25] and uremic rats [26].

The present study also revealed that ketoconazole itself can inhibit PTH, an observation that has not been previously reported. The addition of 1z(OH)D2 produced a similar percentage reduction in PTH regardless of presence of ketoconazole, indicating both that the two compounds act via distinct mechanisms and that the mechanism utilized by 1z(OH)D2 was not significantly impaired by ketoconazole. The mechanism for the suppression by ketoconazole is under investigation.

In summary, we have shown that PTH synthesis and secretion in cultured parathyroid cells is inhibited by exogenous vitamin D prodrugs lacking side chain hydroxyl groups. This suppression was not diminished when metabolism was blocked with the cytochrome P450 inhibitor ketoconazole indicating that side chain oxidation may not be required. This direct effect may be attributed to accumulation of 1z(OH)D2 in the parathyroid to high intracellular levels that activate the vitamin D receptor or, possibly, to interaction with a novel receptor. Further studies are necessary to define the mechanism(s) involved. These findings may have important implications for the design of vitamin D analogues for treatment of secondary hyperparathyroidism.

**Acknowledgements.** Supported by a grant from Bone Care International.

**Conflict of interest statement.** None declared.

**References**


4. Russell J, Sherwood LM. The effects of 1,25-dihydroxyvitamin D3 and high calcium on transcription of the pre-parathyroid hormone gene are direct. *Transact Assoc Am Physician* 1987; 100: 256–262


Received for publication: 17.6.05
Accepted in revised form: 7.9.05