Direct suppression of *Pth* gene expression by the vitamin D prohormones doxercalciferol and calcidiol requires the vitamin D receptor

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Abstract

Vitamin D compounds regulate PTH at the transcriptional level, presumably via binding to the vitamin D receptor (VDR), but the exact mechanism is presently unclear. We recently reported that the several vitamin D prohormones with low VDR affinity suppressed PTH, even when their activation was inhibited, raising the possibility that their actions may be VDR independent. To test this hypothesis, we developed a novel organ culture that allowed the assessment of activities of the prohormones on PTH release from wild-type and VDR-null thyroparathyroid explants. The cultures remained viable with respect to PTH release for at least 2 weeks. Full suppression of PTH by the native vitamin D hormone, 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], required 2 days, consistent with a transcriptional mechanism, and was reversible, indicating that reduced PTH was not attributable to cell death. Inhibition of PTH release by 1α,25(OH)₂D₃ and two prohormones, 25-hydroxyvitamin D₃ and 1α-hydroxyvitamin D₂, was observed in explants from wild-type mice but not in those from VDR-null mice. These findings 1) are the first direct demonstration of the role of the VDR in regulation of PTH by 1α,25(OH)₂D₃, 2) confirm that the suppressive actions of the vitamin D prohormones are mediated by the VDR, and 3) introduce a novel organ culture model that allows the *ex vivo* study of the function of parathyroid glands from transgenic animals.

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Introduction

The parathyroid glands play a central role in calcium homeostasis. A drop in serum calcium triggers the release of PTH, which enhances renal calcium reabsorption and bone calcium mobilization. PTH also stimulates the renal synthesis and release of 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], which increases intestinal calcium absorption. Restoration of calcium levels reduces PTH release by inhibiting exocytosis, promoting PTH peptide degradation, and destabilizing *Pth* mRNA. PTH is also suppressed via a feedback loop in which 1α,25(OH)₂D₃ blocks *Pth* gene transcription.

The mechanism for transrepression of the *Pth* gene by 1α,25(OH)₂D₃ remains controversial. At least three distinct mechanisms have been proposed, but none have been tested directly in parathyroid cells. The first model proposes that 1α,25(OH)₂D₃-bound vitamin D receptor (VDR) binds to a negative vitamin D response element (VDRE) in the human PTH promoter, although the binding partner, if present, is not retinoid X receptor as is often seen with positive VDREs (Mackey *et al.* 1996). The second model proposes a more classical mechanism involving liganded VDR and RXR binding to a DR3-type element (two direct repeats of consensus 3-base elements separated by a 3-base spacer); this element is found in the rat and chick PTH promoters and was shown to act as a negative element in some, but not all, cell types, but has not been tested in parathyroid cells (Koszewski *et al.* 1999). More recently, a third model was proposed in which liganded VDR binds to and inactivates a positive effector (vitamin D interacting receptor or VDIR) of *PTH* gene transcription, with no binding of the VDR to DNA (Murayama *et al.* 2004). Furthermore, although all of the mechanisms assume that the repression by 1α,25(OH)₂D₃ is mediated by the VDR, this has not been demonstrated directly in parathyroid cells with the native gene.

We recently reported direct suppression of PTH by several vitamin D prohormones (or prodrugs) which lack key hydroxyl groups necessary for high affinity VDR binding (Brown *et al.* 2006, Ritter *et al.* 2006). The prodrugs 25(OH)D₃ (calcidiol) and 1α(OH)D₂ (doxercalciferol) retained full suppressive activity when their ‘activation’ by hydroxylases was blocked with cytochrome P450 inhibitors. The basis for the retention of PTH suppression is not clear, but several possibilities may be entertained. The prodrugs may accumulate in parathyroid cells to higher levels than 1α,25(OH)₂D₃, which could overcome their lower VDR affinities. It is also possible that the protocols for measuring VDR affinities *in vitro* may
not reflect the VDR affinities in the cells. At present, we cannot exclude the involvement of a unique receptor that mediates the direct actions of the prodrugs on Pth gene transcription. The existence of multiple receptors for vitamin D compounds was suggested by the studies of Panda et al. (2004), who found that the very high levels of 1α,25(OH)2D3 in Vdr knockout mice suppressed parathyroid growth; the receptor involved has not been identified.

In the present study, we test the hypothesis that the direct suppression of PTH by 1α(OH)D2 and 25(OH)D3 is independent of the VDR. In vivo studies would not be informative since the prohormones would be activated. Therefore, we established a novel organ culture model utilizing thyroparathyroid explants from wild-type and Vdr−/− mice to determine the role of the receptor in the inhibition of Pth gene expression by 1α(OH)D2 and 25(OH)D3.

Materials and methods

Animals

A Vdr+/+ breeding pair (B6.129S4-Vdr<sup>m1Mbd</sup>/J) was purchased from Jackson Laboratory (Bar Harbor, ME, USA; contributed by Dr Marie Demay (Li et al. 1997)). Offspring were genotyped using the recommended primers CAC GAG ACT AGT GAG ACG TG (mutant), CTC CAT CCC CAT GTG TCT TT (wild-type), and TTC TTC AGT GGC CAG CTC TT (common) to yield bands of 500 and 382 bp for the mutant and wild-type alleles, respectively. To prevent parathyroid hyperplasia, Vdr−/− mice were maintained on a diet containing 2.0% Ca, 1.25% P, and 20% lactose (Teklad TD96348). All animal protocols were approved the Animal Studies Committee of Washington University School of Medicine.

Organ culture

Eight-week-old wild-type and Vdr−/− mice were killed under CO<sub>2</sub>. The trachea was teased away from surrounding tissue, and then severed anteriorly and posteriorly to the thyroid glands. This thyroparathyroid complex (TPTG), consisting of thyroid–parathyroid–tracheal tissue, was excised and placed in 2 ml serum-free medium (DME:Ham’s F-12 (1:1, 1 mM Ca), 15 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml holo-transferrin, 2 mM glutamine, 1% nonessential amino acids, and 0.1% BSA) in a 24-well tissue culture plate. The medium was replaced daily and retained for analysis of PTH (Immutopics mouse intact PTH ELISA). The TPTG cultures were allowed to equilibrate for 3–6 days, with medium being replenished every 1–2 days. TPTGs were then washed one time and replenished with 2 ml of fresh medium, which was collected after 24 h for a baseline PTH level. TPTGs were then treated with vehicle (0.1% ethanol) or 100 nM 1α,25(OH)2D3, 25(OH)D3, or 1α(OH)D2, and daily collections of medium were continued. In initial studies, 1α,25(OH)2D3 was removed after 2 days of treatment to determine the reversibility of the suppression.

Results

Characterization of cultured mouse TPTGs

Organ culture of mouse TPTGs has not been reported previously. Therefore, we established the stability of the system with respect to PTH secretion. PTH secretion was maintained over a 2-week period, and the rate of PTH release consistently increased slightly during the cultures. As shown in Fig. 1, a 2-day treatment with 1α,25(OH)2D3 reduced PTH secretion by over 90%. The slow rate of suppression is consistent with a transcriptional repression and relatively long half-life of Pth mRNA (Ritter et al. 2008). Note that the reduction in PTH continued for 24 h after washout of the 1α,25(OH)2D3. PTH release returned to basal levels over several days, confirming that the reduction in PTH was not attributable to cell death. These findings illustrate the utility of the TPTG organ model to study parathyroid gland function.

The role of the VDR in the suppression of PTH by 1α,25(OH)2D3 and prohormones

The role of the VDR in the suppression of PTH by vitamin D compounds was investigated using
Transcriptional control of PTH expression by Vdr remains controversial (Mackey et al. 1999, Murayama et al. 2004). None of the transcriptional mechanisms have been confirmed in parathyroid cells, and direct involvement of the VDR has not been demonstrated. In the current study, we developed a novel organ culture model for the study of parathyroid gland function ex vivo. For the first time, we provide proof of the requirement of the VDR for repression of PTH by 1α,25(OH)2D3.

Previous studies from our group found that vitamin D prohormones can directly suppress PTH in bovine parathyroid cell culture. 1α(OH)D3, which has 600 times lower affinity for the VDR than 1α,25(OH)2D3 (Okamura & Zhu 1997), was shown to suppress PTH secretion and PTH mRNA in bovine parathyroid cultures, even when activation to 1α,25(OH)2D2 or 1α,24(OH)2D2 was blocked by the cytochrome P450 inhibitor ketoconazole (Brown et al. 2006). Similarly, 25(OH)D3, which also has 600 times lower affinity than 1α,25(OH)2D3 for the VDR (Norman et al. 2001), suppressed PTH in cultured bovine parathyroid cells when its activation to 1α,25(OH)2D3 was inhibited by clotrimazole (Ritter et al. 2006). The explanation for these surprising findings was unclear, but one possibility was that the control of PTH expression by 25(OH)D3 and 1α(OH)D3 is mediated by another receptor. This hypothesis was tested using cultured TPTGs from Vdr knockout and wild-type mice. The results revealed unequivocally that regulation of PTH by these two ‘precursors’ requires the VDR.

The mechanism for the direct effect of the two prohormones remains to be elucidated. It is possible that the equilibrium binding method used to measure VDR affinity may not accurately reflect the dynamic situation within the cell. For example, 25(OH)D3 and 1α(OH)D2 may dissociate from the VDR during the separation of bound and free ligand in the assay, causing an underestimation of the interaction that would occur in vivo or in cell culture where the concentrations of the compounds would be constant. Another possibility is that the two compounds, which are more hydrophobic than 1α,25(OH)2D3, may be sequestered at high enough concentrations within the cells to overcome the weaker VDR affinity. This hypothesis is consistent with preliminary findings that cellular uptake of 1α(OH)D2 is much higher than that of 1α,24(OH)2D2 (CS Ritter & AJ Brown 2006, unpublished data). The explanation for the higher cellular and nuclear content of 1α(OH)D2 is unclear, but the intracellular vitamin D-binding proteins, HSC70 (Adams et al. 2004) and BAG1 (Chun et al. 2007), have been shown to play a role in cellular distribution of vitamin D compounds and may enhance the activities of the prohormones relative to 1α,25(OH)2D3. Other explanations, such as differential recruitment of transcriptional co-regulators, cannot be excluded.

As illustrated by the current study, thyroparathyroid organ culture from transgenic mice provides a useful model for studying the effects of genetic modifications on parathyroid gland function ex vivo. In vivo approaches are limited in their interpretation because disruptions that affect PTH will be subject to feedback by calcium and vitamin D, making it difficult to identify
primary and secondary effects. Our findings demonstrate that PTH release is stable over several days and is regulated by vitamin D compounds. The potential utility of this model to study parathyroid hyperplasia is under investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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