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, $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25$ (OH) $_2D_3$], 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 $1\alpha,25$ (OH) $_2D_3$ and two prohormones, 25-hydroxyvitamin D_3 and 1α -hydroxyvitamin D_2 , 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 $1\alpha,25$ (OH) $_2D_3$, 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 $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25$ (OH) $_2D_3$], 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 $1\alpha,25$ (OH) $_2D_3$ blocks *Pth* gene transcription.

The mechanism for transrepression of the *Pth* gene by $1\alpha,25$ (OH) $_2D_3$ remains controversial. At least three distinct mechanisms have been proposed, but none have been tested directly in parathyroid cells. The first model proposes that $1\alpha,25$ (OH) $_2D_3$ -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 $1\alpha,25$ (OH) $_2D_3$ 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) D_3 (calcidiol) and 1α (OH) D_2 (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 $1\alpha,25$ (OH) $_2D_3$, 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 $1\alpha,25(\text{OH})_2\text{D}_3$ 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 $1\alpha(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$ 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*-null mice to determine the role of the receptor in the inhibition of *Pth* gene expression by $1\alpha(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$.

Materials and methods

Animals

A *Vdr*^{-/+} breeding pair (B6.129S4-*Vdr*^{tm1Mbd}/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*-null mice were killed under CO₂. 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 $1\alpha,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, or $1\alpha(\text{OH})\text{D}_2$, and daily collections of medium were continued. In initial studies, $1\alpha,25(\text{OH})_2\text{D}_3$ 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 $1\alpha,25(\text{OH})_2\text{D}_3$ 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 $1\alpha,25(\text{OH})_2\text{D}_3$. 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 $1\alpha,25(\text{OH})_2\text{D}_3$ and prohormones

The role of the VDR in the suppression of PTH by vitamin D compounds was investigated using

Figure 1 Suppression of PTH by $1\alpha,25(\text{OH})_2\text{D}_3$. A representative wild-type mouse thyroparathyroid complex, cultured in serum-free medium, was treated at days 7 and 8 of culture with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$. Medium was collected daily for PTH measurements.

Figure 2 Effects of $1\alpha,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, and $1\alpha(\text{OH})\text{D}_2$ on PTH secretion by thyroparathyroid complexes from wild-type and *Vdr*-null mice. Thyroparathyroid complexes from *Vdr*-null mice and wild-type littermates were treated with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, and $1\alpha(\text{OH})\text{D}_2$ for 2 days, with daily measurement of PTH. Data are expressed as mean $\pm$ S.E.M., $n=3-5$.

cultured TPTGs from wild-type and *Vdr*-null mice. $1\alpha,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, and $1\alpha(\text{OH})\text{D}_2$ were all active in wild-type cultures but were completely inactive in cultures from the *Vdr*-null mice (Fig. 2).

Discussion

Transcriptional control of PTH expression by $1\alpha,25(\text{OH})_2\text{D}_3$ has been demonstrated in cell culture (Russell *et al.* 1986, Russell & Sherwood 1987) and *in vivo* (Silver *et al.* 1986), but the exact mechanism remains controversial (Mackey *et al.* 1996, Koszewski *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 $1\alpha,25(\text{OH})_2\text{D}_3$.

Previous studies from our group found that vitamin D prohormones can directly suppress PTH in bovine parathyroid cell culture. $1\alpha(\text{OH})\text{D}_2$, which has 600 times lower affinity for the VDR than $1\alpha,25(\text{OH})_2\text{D}_3$ (Okamura & Zhu 1997), was shown to suppress PTH secretion and *PTH* mRNA in bovine parathyroid cultures, even when activation to $1\alpha,25(\text{OH})_2\text{D}_2$ or $1\alpha,24(\text{OH})_2\text{D}_2$ was blocked by the cytochrome P450 inhibitor ketoconazole (Brown *et al.* 2006). Similarly, $25(\text{OH})\text{D}_3$, which also has 600 times lower affinity than $1\alpha,25(\text{OH})_2\text{D}_3$ for the VDR (Norman *et al.* 2001), suppressed PTH in cultured bovine parathyroid cells when its activation to $1\alpha,25(\text{OH})_2\text{D}_3$ 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 $25(\text{OH})\text{D}_3$ and $1\alpha(\text{OH})\text{D}_2$ 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, $25(\text{OH})\text{D}_3$ and $1\alpha(\text{OH})\text{D}_2$ 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 $1\alpha,25(\text{OH})_2\text{D}_3$, 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 $1\alpha(\text{OH})\text{D}_2$ is much higher than that of $1\alpha,24(\text{OH})_2\text{D}_2$ (CS Ritter & AJ Brown 2006, unpublished data). The explanation for the higher cellular and nuclear content of $1\alpha(\text{OH})\text{D}_2$ 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 $1\alpha,25(\text{OH})_2\text{D}_3$. 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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