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REVIEW

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Pathogenesis of parathyroid dysfunction in end-stage kidney disease

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Abstract Small decreases in serum calcium (Ca^{2+}) and more-prolonged increases in serum phosphate (Pi) stimulate the parathyroid (PT) to secrete parathyroid hormone (PTH). 1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2$ D_3] decreases PTH synthesis and secretion. The prolonged decrease in serum Ca^{2+} and $1,25(OH)_2$ D_3, or increase in serum Pi, observed in patients with chronic renal failure leads to a secondary increase in serum PTH. This secondary hyperparathyroidism involves increases in PTH gene expression, synthesis, and secretion and, if chronic, to proliferation of the PT cells. A low serum Ca^{2+} leads to an increase in PTH secretion, PTH mRNA stability, and PT cell proliferation. Pi also regulates the PT in a similar manner. The effect of Ca^{2+} on the PT is mediated by a membrane Ca^{2+} receptor. $1,25(OH)_2$ D_3 decreases PTH gene transcription. Ca^{2+} and Pi regulate the PTH gene post transcriptionally by regulating the binding of PT cytosolic proteins, *trans* factors, to a defined *cis* sequence in the PTH mRNA 3′-untranslated region, thereby determining the stability of the transcript. The PT *trans* factors and *cis* elements have been defined.

Keywords PTH gene expression · post transcriptional gene regulation · Calcium · Phosphate · Vitamin D · Chronic renal failure

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Introduction

In fish the predominant calcium-regulating hormone is calcitonin, which functions to protect the organism from the high concentration of Ca^{2+} in its exterior milieu. In amphibians and terrestrial organisms parathyroid hormone (PTH) is expressed and has an important role in preserving calcium (Ca^{2+}) in the face of a marginal supply. The parathyroid (PT) is unique in that it responds so avidly to a small decrease in extracellular Ca^{2+} by secreting PTH to rapidly correct serum Ca^{2+} by its action on the kidney and bone. In the kidney PTH also results in phosphaturia and an increase in the synthesis of 1,25-dihydroxyvitamin D3 [$1,25(OH)_2$ D_3]. There are negative feedback loops involving both these factors, as $1,25(OH)_2$ D_3 powerfully inhibits PTH gene transcription and hence the synthesis of PTH and its subsequent secretion [1, 2]. A chronically low serum phosphate (Pi) also acts on the PT to decrease PTH secretion. However, in the clinic the major problem is that of a high serum Pi, which stimulates the PT and results in increased PTH secretion. In patients with end-stage kidney disease there is a combination of a low serum $1,25(OH)_2$ D_3, a high serum Pi, and as a result a low serum Ca^{2+}. Together these result in secondary hyperparathyroidism, which leads to potentially disabling skeletal and systemic complications for the patient. It is important to understand how these factors act on the PT at a molecular level so that we may be able to devise rational treatment strategies.

Vitamin D and the PT

Nearly 20 years ago we showed in-vitro that $1,25(OH)_2$ D_3 decreases PTH gene expression and in the following year we showed in-vivo in rats that $1,25(OH)_2$ D_3 dramatically decreased PTH gene transcription [3, 4]. $1,25(OH)_2$ D_3 and its analogues have become the mainstays for the prevention and treatment of secondary hyperparathyroidism in patients with renal failure. Of interest, mice with knockouts of the $1,25(OH)_2$ D receptor have secondary

hyperparathyroidism, which can be corrected by a diet rich in Ca^{2+} [5]. This implies that the effect of vitamin D deficiency in causing secondary hyperparathyroidism is largely due to the secondary Ca^{2+} deficiency rather than a lack of effect of vitamin D on the PT itself. However, the effect of $1,25(OH)_2$ D_3 and its analogues on the PT are so potent that it is difficult to imagine that there is no physiological role for vitamin D in the PT.

Calcitriol also potently decreases the transcription of the PTH gene. This action was first demonstrated in-vitro in bovine PT cells in primary culture, where calcitriol led to a marked decrease in PTH mRNA levels [3, 6] and a consequent decrease in PTH secretion [7, 8, 9]. The physiological relevance of these findings was established by in-vivo studies in rats [4]. The localization of the vitamin D receptor (VDR) mRNA to the PTs was demonstrated by in situ hybridization studies of the thyro-parathyroid and duodenum. VDR mRNA was localized to the PTs in the same concentration as in the duodenum, the classic target organ of calcitriol [10]. Rats injected with amounts of calcitriol that did not increase serum Ca^{2+} had marked decreases in PTH mRNA levels, reaching <4% of control at 48 h. There was also a decrease in calcitonin mRNA levels in these rats [11]. The effect of $1,25(OH)_2$ D_3 on the PTH and calcitonin genes was shown to be transcriptional both in in-vivo studies in rats [4] and in in-vitro studies with primary cultures of bovine PT cells [12]. Interestingly, in rats given large doses of vitamin D with a resultant hypercalcemia, there was still a decrease in calcitonin mRNA levels despite the elevated serum Ca^{2+}, which is a secretagogue for calcitonin [13].

Naveh-Many et al. [10] injected $1,25(OH)_2$ D_3 into rats and measured the levels of VDR mRNA and PTH mRNA in the parathyro-thyroid tissue. They showed that $1,25(OH)_2$ D_3 in physiologically relevant doses led to an increase in VDR mRNA levels in the PT glands, in contrast to the decrease in PTH mRNA levels. This increase in VDR mRNA occurred after a time lag of 6 h, and a dose response showed a peak at 25 pmol. Weanling rats fed a diet deficient in Ca^{2+} were markedly hypocalcemic at 3 weeks and had very high serum $1,25(OH)_2$ D_3 levels. Despite the chronically high serum $1,25(OH)_2$ D_3 levels, there was no increase in VDR mRNA levels. Furthermore, PTH mRNA levels did not fall and were markedly increased. The low Ca^{2+} may have prevented the increase in PT VDR levels and this may partially explain the PTH mRNA suppression. Whatever the mechanism, the lack of suppression of PTH synthesis in the setting of hypocalcemia and increased serum $1,25(OH)_2$ D_3 is crucial pathophysiologically, because it allows an increase in both PTH and $1,25(OH)_2$ D_3 at a time of chronic hypocalcemic stress. Vitamin D may also amplify its effect on the PT by increasing the activity of the Ca^{2+} receptor (CaR). Canaff and Hendy [14] showed that in fact there are vitamin D response elements (VDREs) in the human CaR promoter. The CaR, expressed in PT chief cells, thyroid C-cells, and cells of the kidney tubule, is essential for maintenance of Ca^{2+} homeostasis. Canaff and Hendy [14] showed that PT, thyroid, and kidney CaR mRNA levels increased twofold at 15 h after intraperitoneal injection of $1,25(OH)_2$ D_3 in rats. Functional VDREs have been identified in the CaR gene and provide the mechanism whereby $1,25(OH)_2$ D_3 upregulates PT, thyroid C-cell, and kidney CaR expression.

Calcium, phosphate, and the PT

Central to the understanding of the regulation of the PT by Ca^{2+} is the appreciation of the role of the PT CaR. This seven-transmembrane serpentine G-protein-coupled receptor has a large extracellular N-terminal region that recognizes changes in serum Ca^{2+} [15]. Inactivating mutations in the CaR in humans or mice result in an insensitivity of the CaR to increased concentrations of serum Ca^{2+} and a failure to inhibit PTH secretion [16]. Activating mutations lead to an inappropriate suppression of PTH secretion [17]. In patients with end-stage renal disease there is a down-regulation of the PT CaR and a shift in the set-point for the concentration of Ca^{2+} that decreases PTH secretion to the right, so that higher concentrations of extracellular Ca^{2+} are needed to decrease PTH secretion than in controls. Ca^{2+} also regulates the PT at the levels of PTH gene expression, PTH degradation, and PT cell hyperplasia.

Hypocalcemia dramatically increases PTH mRNA levels, secretion, and after prolonged stimulation, PT cell proliferation [18]. The increase in PTH mRNA levels is post transcriptional. Pi also regulates PTH secretion, gene expression, and PT cell proliferation [19, 20]. Dietary Pi depletion dramatically decreases PTH mRNA levels and this is also post transcriptional [19]. We have shown that this post transcriptional regulation is mediated by protein-RNA interactions involving protein binding to a specific element in the PTH mRNA 3′-UTR that determines PTH mRNA stability [18, 21]. Protein binding to the PTH mRNA 3′-UTR was increased by hypocalcemia and decreased by hypophosphatemia. The regulation of binding by Ca^{2+} and Pi was observed only in the PT and not in other tissues of the same rats. We have identified adenosine-uridine rich binding factor1 (AUF1) or heterologous ribonucleoprotein D (hnRNP D) as a protein *trans* -acting factor that stabilizes the PTH mRNA [22]. There is no PT cell line, therefore we have utilized a cell-free mRNA in-vitro degradation assay (IVDA) to demonstrate the functionality of the PT cytosolic proteins in determining the stability of the PTH transcript. This assay has been shown to authentically reproduce cellular decay processes. PT protein extracts from hypocalcemic rats stabilized and PT protein extracts from hypophosphatemic rats destabilized the full-length PTH transcript, correlating with mRNA levels in-vivo. Deletion of the protein-binding element of the PTH mRNA 3′-UTR resulted in stabilization of the transcript compared with the full-length PTH transcript. Moreover, Ca^{2+} and Pi did not regulate the stability of the truncated transcript. Therefore, the IVDA reproduces the in-vivo stabilizing effect of low Ca^{2+} and the destabilizing effects of low Pi on PTH mRNA levels and this regulation in the IVDA is dependent upon protein binding that protects an instability sequence in the PTH mRNA 3′-UTR

[18]. In rats with secondary hyperparathyroidism due to 5/6 nephrectomy, there are increased PTH mRNA levels, and we have studied the mechanism involved [23]. PT glands were microdissected from control and 5/6 nephrectomy rats and analyzed for PTH mRNA and control genes. The nuclei were used for nuclear run-on experiments. The cytosolic proteins of the PTs were used to study PTH mRNA protein binding by ultraviolet cross-linking and the degradation of the PTH transcript in-vitro. Nuclear run-ons showed that the increase in PTH mRNA levels was post transcriptional. Protein binding to the PTH mRNA 3′-UTR determines PTH mRNA stability and levels. PT proteins from uremic rats bound PTH mRNA similar to control rats by ultraviolet cross-linking. To determine the effect of uremia on PTH mRNA stability, the in-vitro RNA degradation assay was performed with PT proteins from uremic rats. When PT proteins from control rats were incubated with PTH mRNA, there was transcript degradation at 30 min, reaching 50% at 60 min and 90% at 180 min. With uremic PT proteins, the PTH mRNA was not degraded at all at 120 min and was moderately decreased at 180 min [23]. This decrease in degradation by uremic PT proteins suggests a decrease in PT cytosolic ribonuclease activity in uremia resulting in a more stable PTH transcript. The increased PTH mRNA levels would translate into increased PTH synthesis and serum PTH levels, and the subsequent metabolic bone disease present in many patients with chronic renal failure.

We have identified the minimal sequence for protein binding in the PTH mRNA 3′-UTR and determined its functionality. A minimal sequence of 26 nucleotides in the PTH mRNA 3′-UTR was sufficient for protein binding. To study the functionality of the protein binding element in the context of a different RNA, a 63-bp fragment encoding the 26 nucleotides and flanking nucleotides was fused to growth hormone (GH) reporter gene. RNAs were transcribed in-vitro and transcipts subjected to IVDA with PT proteins. The chimeric GH-PTH 63-nucleotide transcript, as the full-length PTH transcript, was stabilized by PT proteins from rats fed a low Ca^{2+} diet and destabilized by proteins of a low Pi diet, correlating with PTH mRNA levels in-vivo. The native GH transcript was more stable than PTH and the chimeric RNAs and was not affected by PT proteins from the different diet. These results demonstrate that the protein binding region of the PTH mRNA 3′-UTR is both necessary and sufficient to confer responsiveness to Ca^{2+} and Pi and determines PTH mRNA stability and levels [21].

We have studied the function of the PTH element in cells, using the heterologous cell line HEK293. Plasmids containing the native GH or GFP gene or chimeric genes containing the PTH 3′-UTR sequences at the 3′ end of the gene were transiently transfected into HEK293 cells and mRNA levels measured by Northern blot. There was a marked decrease in GH and GFP mRNA levels of the chimeric constructs compared with the wild type constructs or chimeric constructs that contained a truncated sequence of the PTH mRNA element. This effect was post transcriptional. These results are in agreement with the decreased stability of the chimeric transcript in the in-vitro degradation assay with PT proteins and they demonstrated the functional importance of the RNA protein binding region in the PTH 3′-UTR [21]. In order to understand the mechanism by which the specific sequences in the PTH mRNA 3′-UTR regulate mRNA half-life both in-vitro and in-vivo and PTH gene expression in response to changes in Ca^{2+} and Pi, we studied the structure of the PTH mRNA 3′-UTR. Our results showed that the 3′-UTR, and in particular the 100 nucleotides that include the 26-nucleotide protein binding core and flanking sequences, did not form a stable secondary structure. Mutation analysis confirmed the importance of specific sequence patterns for protein binding and for the destabilizing effect of the *cis* -acting element.

PT cell proliferation

Naveh-Many et al. [20] clearly demonstrated that hypocalcemia is a stimulus for PT cell proliferation. We studied PT cell proliferation by staining for proliferating cell nuclear antigen (PCNA) and found that a low Ca^{2+} diet led to increased levels of PTH mRNA and a tenfold increase in PT cell proliferation. The secondary hyperparathyroidism of 5/6 nephrectomized rats was characterized by an increase in both PTH mRNA levels and PCNA-positive PT cells. Therefore, both hypocalcemia and uremia induce PT cell proliferation in-vivo. The effect of $1,25(OH)_2$ D_3 on PT cell proliferation was also studied in this dietary model of secondary hyperparathyroidism. $1,25(OH)_2$ D_3 at a dose (25 pmol for 3 days), which lowered PTH mRNA levels, had no effect on the number of PCNA-positive cells. Higher doses (100 pmol for 7 days) dramatically decreased the number of proliferating cells (unpublished data). These findings emphasize the importance of a normal Ca^{2+} level in the prevention of PT cell hyperplasia. The importance of the CaR to PT cell proliferation is also evident in that the calcimimetic NPS R-568 largely prevents PT cell proliferation in rats with experimental uremia [24, 25]. Imanishi et al. [26] created transgenic mice with the cyclin D1 gene specifically targeted to the PT. In the PTs of these rats with hyperparathyroidism there was a downregulation of the CaR. These results indicate that the changes in the CaR may be secondary to the proliferative state and not causative. In patients with both primary and nodular secondary hyperparathyroidism due to chronic renal failure there is a decrease in VDR mRNA and protein levels [27, 28, 29]. In hyperparathyroidism there is a decrease in the cyclin kinase inhibitors p21 and p27, with an increase in transforming growth factor-α (TGFα) in the PTs [29, 30, 31]. Treatment with vitamin D metabolites increases p21 levels and prevents the increase in TGFα levels and PT cell proliferation.

Conclusion

In diseases such as chronic renal failure secondary hyperparathyroidism involves abnormalities in PTH secre-

tion, synthesis, and cellular proliferation. Progress has been made in understanding how Ca^{2+}, Pi, and vitamin D regulate the synthesis and secretion of PTH, as well as the proliferation of the PT cells. A more complete understanding of how the PT is regulated at each level will help to devise rational therapy for the management of such conditions.

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