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Carboxylterminal Receptor for Parathyroid Hormone and Circulating C-terminal PTH Ligands: Current Knowledge and Clinical Relevance

BY TIMOTHY M. MURRAY, MD, FRCPC

Parathyroid hormone (PTH) is the major regulator of serum calcium homeostasis. It is an 84 amino acid peptide hormone, secreted in response to hypocalcemia. It restores normocalcemia through a negative feedback loop mediated by the calcium-sensing receptor on parathyroid chief cells. Since the early 1970s, it has been recognized that the PTH molecule contains its major active region in its aminoterminal (N-terminal) part, and the remaining carboxylterminal (C-terminal) region of the hormone has been thought to be biologically inactive. The synthetic N-terminal fragment PTH(1-34) was found to be capable of producing all of the known classical actions of the hormone, both in vitro and in vivo. Further, the receptor for N-terminal PTH was cloned and recognized to be the receptor in skeletal and renal tissue that mediates the activities of both PTH and PTH-related peptide (PTHrP), referred to hereafter as PTH1R. However, later studies, of ligand binding in kidney and bone suggested the presence of discrete receptors for the carboxyl-terminal region. Recently, it has been demonstrated that C-terminal PTH fragments can inhibit both bone resorption and differentiation of osteoclast precursors in vitro and that they possess hypocalcemic activity in vivo. These findings, plus the well-established knowledge that C-terminal PTH fragments circulate in blood in relatively high concentrations, have given rise to the concept that receptors for the C-terminal portion of PTH, with distinct biological activities, are naturally liganded and are likely to play a regulatory role in calcium homeostasis and bone metabolism.

Evolution of PTH structure/function relationships and the classical aminoterminal receptor for PTH and PTHrP

Shortly after the amino acid sequence of this 84 amino acid peptide hormone was discovered in the early 1970s,¹ it was realized that the classical biological actions of the hormone could be subserved by amino terminal hormonal fragments, particularly PTH(1-34).² This concept was generally accepted and, for the next 3 decades, this synthetic hormonal fragment was used as a surrogate for the intact hormone molecule since supplies of the full-length hormone were limited. That this aminoterminal region was the major active site of the hormone molecule was solidified by the cloning of the PTH1R in 1991.³ This single receptor was shown to mediate the known biological actions of both PTH and PTHrP in both bone and kidney. The aminoterminal region of PTH is known to be both necessary and sufficient for full activity at PTH1Rs that mediate the classical biologic actions of the hormone. In addition, this region mediates the anabolic action of intermittently administered PTH. The human PTH (1-34) fragment, when administered intermittently, is now an approved, potent bone-forming treatment for osteoporosis.⁴

PTH secretion and metabolism

In 1968, it was recognized by Berson and Yalow that PTH circulated in multiple immunoreactive forms.⁵ In 1971, it was first demonstrated that fragments of PTH normally



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6121-61 Queen St. E.
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Fax: (416) 867-3696

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circulate in relatively large amounts in human blood.⁶ These circulating fragments are, for the most part, carboxylterminal fragments (C-fragments) that lack the aminoterminal determinants necessary to activate the PTH N-receptor. No PTH fragments that possess these aminoterminal determinants have been detected in the circulation under physiological conditions; the only circulating PTH moiety that possesses classical hormonal activity is full-length intact PTH(1-84).

C-fragments in blood may arise from parathyroid secretion or from hepatic metabolism of the hormone. It has been demonstrated that the major portion of circulating C-fragments is secreted by the parathyroids and regulated by the serum calcium level;^{7,8} their concentration increases in hypercalcemia. C-fragments are also generated by enzymatic cleavage in the Kupffer cells of the liver, but these form a smaller part of the circulating C-fragment pool. Their generation by hepatic metabolism is also regulated in the same manner by serum calcium levels. In hypocalcemia, the main product secreted by the parathyroids is PTH(1-84), but in hypercalcemia, the main secretory products are C-fragments. In hypercalcemia, the fraction of total PTH in the circulation may be as high as 85%.⁹

There are a number of C-fragments of different lengths in the circulation but, for the most part, their precise chemical nature has not been definitively determined by direct sequence analysis. Until recently, circulating C-fragments were thought to consist mainly of fragments with N-termini at positions 34, 37, 41, and 43, although some smaller mid-fragments truncated at both ends and some smaller, much shorter, fragments have been detected. All PTH peptides are cleared from the circulation by the kidney, but their clearance is reduced in renal failure and C-fragments accumulate, particularly in the blood of renal failure patients. Of great interest is the recent discovery by the D'Amour laboratory of very long circulating C-fragments of PTH that are detected by the commonly used assay for intact PTH, but these lack the N-terminal determinants necessary to activate the PTH1R and are biologically inactive in the classical sense.¹⁰ D'Amour initially termed these fragments "non-(1-84) PTH." These fragments have an elution profile on high performance liquid chromatography (HPLC) similar to PTH(7-84), although their structure has not yet been rigorously determined by direct sequence analysis. These fragments account for as much as 50% of circulating immunoreactive PTH in renal failure patients (see below).

PTH carboxylterminal receptors in historical perspective

PTH receptors were initially studied by classic binding studies of radio-labeled hormone to cell mem-

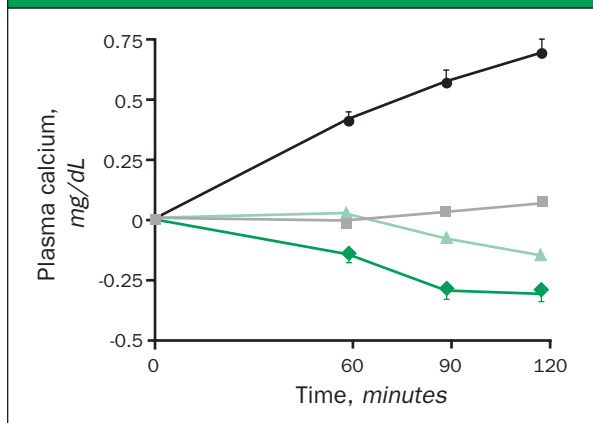
Table 1: Some biologic responses to C-terminal PTH

Biologic response	Experimental system
Stimulation of alkaline phosphatase (ALP) activity and gene expression	ROS 17/2.8 osteosarcoma cells ^{15,17,20}
Osteocalcin gene expression	Rat 17/2.8 osteosarcoma cells ¹⁷
Stimulation of IGFBP-5 expression	UMR106 osteosarcoma cells ³⁰
Tooth enamel formation and ALP activity	Embryonic tooth germ ³¹
Connexin-43 activity	PTH1R null osteocytes ²⁵
Apoptosis	PTH1R null osteocytes ²⁵
Hypocalcemia	TPTX rats ^{21,22}
Blocking effect of PTH on serum calcium and bone turnover	TPTX rats with renal failure ²⁷
⁴⁵ Ca release from bone	Mouse calvariae in vitro ²³
Inhibition of osteoclast formation	Whole mouse bone marrow ²³ purified osteoclast precursors ³²

branes or intact cells. The first studies used radioactive derivatives of PTH(1-34) and hormone binding, and correlated closely with measurements of biological activity as assessed by adenylate cyclase activity. However, when radioligands derived for intact PTH(1-84) were utilized, a different pattern was observed. Substantial amounts of bound intact hormone could not be displaced from binding sites by PTH(1-34).^{11,12} Kinetic analyses in renal tissue using the PTH(53-84) C-terminal probe indicated two distinct binding sites: a site with N-terminal specificity and a C-terminal site linked to the binding of the C-terminal PTH(53-84) fragment.¹³ Using the same approach, over 70% of intact PTH binding occurred via the C-terminal region in bone cells.¹⁴ While these findings suggested the presence of receptors for C-terminal PTH (CPTHs), little interest was generated in such a possibility until biological activity could be demonstrated for C-terminal agonists. In 1989, it was discovered that PTH(53-84) could stimulate alkaline phosphatase (ALP) activity in bone cells (an action opposite to that of PTH(1-34)^{15,16}) and that it could also regulate gene expression for ALP and osteocalcin.¹⁷ These Toronto contributions stimulated further research in the field.

Two major breakthroughs were subsequently made in the Bringhurst laboratories in Boston. The first was the development of improved radioligands specific for C-receptors, eg, PTH(19-84), which detected the

Figure 1: Comparison of the calcemic effects of PTH isoforms.²¹



Parathyroidectomized rats fed a 0.02% calcium diet show a significant increase in plasma calcium after treatment with hPTH(1-84). In contrast, hPTH(7-84) produced a slight, but significant decrease in plasma calcium. When both peptides were given together in a 1:1 molar ratio, the calcemic response induced by hPTH was reduced by 94% ($p < 0.001$). Symbols are:

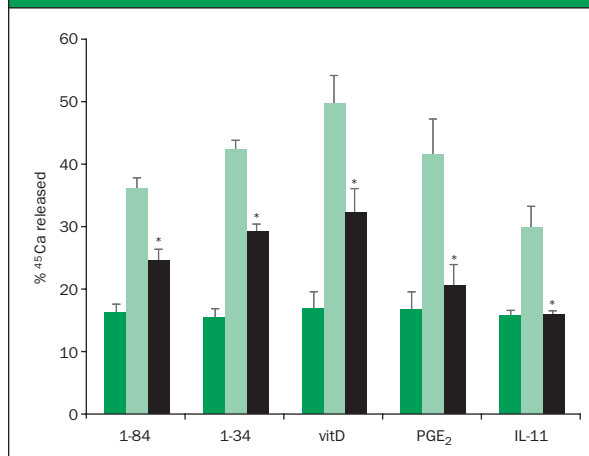
- hPTH(1-84), N = 9
 - hPTH(1-84) + hPTH(7-84), N = 6
 - ◆ control, N = 5
 - ▲ hPTH(7-84), N = 5.
- (Reprinted from Slatopolsky et al²¹)

C-receptors more efficiently and allowed for a more extensive analysis of their properties. C-fragments longer than PTH(53-84) have greater binding affinities for the C-receptors.¹⁸ These authors were also able to crosslink these longer C-terminal radioligands with a putative receptor protein having a molecular weight (MW) of 80 kDa.¹⁸ The second major breakthrough was the study of binding these C-terminal radioligands to cells from PTH1R^{-/-} mice.¹⁹ The study of CPTHs in cells lacking the PTH1R demonstrated that receptor binding and some biological activities of C-fragments could be observed independently of the N-terminal PTH/PTHrP receptor. Bone cell lines were generated that expressed high numbers of CPTHs. These cells had several properties of osteocytes, including a dendritic morphology and expression of connexin-43. Indeed, osteocytes are the bone cells with the highest number of CPTHs.

Biological activities of C-fragments in bone

The initial findings of Murray et al^{15,16} – that PTH(53-84) stimulated alkaline phosphatase activity – were subsequently confirmed by Nakamoto et al.²⁰ Since then, a much wider repertoire of activities with C-terminal PTH fragments have been described in different bone cell types, in vivo, as well as in vitro. These are summarized in

Figure 2: Inhibition of calcium release by hPTH (7-84)²³



Murine calvaria, prelabelled with ⁴⁵Ca, were cultured in the presence of doses of various stimulators of bone resorption sufficient to stimulate significant calcium release (pale green bars) compared to control (dark green bars). The addition of 100-200 nM hPTH(7-84) (black bars) inhibited the calcium release stimulated with each of the resorptive agents shown, hPTH(1-84), hPTH(1-34), 1,25(OH)₂D₃, PGE₂, and IL-11 (see text). (Reprinted from Divieti et al²³)

Table 1. Most noteworthy are the recently demonstrated activities of lowering serum calcium in vivo, as shown in TPTX rats by two different laboratories (Figure 1),^{21,22} and of inhibiting bone resorption in vitro, as demonstrated by measuring ⁴⁵Ca release from mouse calvaria.²³ The inhibition of bone resorption appeared to be a general action, in that PTH(7-84) was shown to inhibit bone resorption stimulated by PTH(1-84), PTH(1-34), 1,25(OH)₂D₃, PGE₂, and IL-11 (Figure 2).²³ The action appears to involve inhibiting differentiation of hemopoietic osteoclast precursor cells in mouse bone marrow and was not mimicked by antagonists of the PTH1R.³² These investigators also demonstrated that this antiosteoclastogenic effect of hPTH(7-84) could be observed using purified hematopoietic osteoclast precursors found to exhibit specific binding of a CPTH(19-84) radioligand, but not of a PTH(1-34) PTH1R radioligand. It seems likely that the above action of C-fragments to lower serum calcium is mediated by bone. It was seen after 2 hours, and the antiresorptive action appears to be related specifically to the CPTHr. On the other hand, a very recent report indicates that N-truncated peptides, PTH(1-34) and PTH(7-84), can promote internalization of the PTH1R and thereby induce down-regulation of the PTH1R.²⁴ Thus, C-fragments may exert part of their hypocalcemic and antiresorptive actions via downregulation of the PTH1R.

A further skeletal action of C-fragments recently

demonstrated by the Boston group is the promotion of osteocyte apoptosis.²⁵

Physiological relevance of PTH receptors and fragments

The evidence is now convincing for the existence of receptors specific for the C-terminal region of PTH on various types of bone cells. Since there is also considerable evidence that natural ligands for these receptors exist in the circulation and that their concentrations are regulated by serum calcium concentration, it seems likely that a double feedback loop may exist for regulation of serum calcium and perhaps for skeletal calcium stores by the parathyroids, with the PTH1R dominant in hypocalcemia and the PTH C-receptors dominant in hypercalcemia. Not only does the parathyroid gland protect serum and bone calcium in times of calcium lack, but it may also protect against hypercalcemia and preserve bone calcium when dietary calcium is available or in pathological circumstances. However, present knowledge of this system is limited and much future research will be necessary to elucidate the mechanisms involved and the precise roles played by the C-receptor system in different cell types.

Clinical relevance of PTH C-receptors and C-fragments

The detection of substantial amounts of non-(1-84) PTH fragments in the blood means that commonly used assays for intact PTH also detect variable amounts of biologically inactive PTH. Assays that detect only full-length PTH(1-84) have been devised²⁶ and their clinical utility is being evaluated.

While the so-called “intact PTH” assays continue to be very useful for most purposes – particularly in the differential diagnosis of hypercalcemia and hypocalcemia – their usefulness in renal failure has been questioned since, in this condition, much of the immunoreactive hormone lacks bone resorptive and hypercalcemic activity. Furthermore, the concept that large, circulating amounts of C-fragments with activity at CPTHRS would likely have bone antiresorptive and hypocalcemic effects has important implications for the pathophysiology of renal failure. It has been recognized for some time that there is PTH resistance in renal failure and this has been recently attributed to the retention of PTH C-fragments.²¹ Indeed, it has also been shown that PTH(7-84) antagonizes the effects of PTH-(1-84) on bone turnover in nephrectomized rats.²⁷ The antiresorptive effects of long C-fragments may also be implicated in

low turnover osteodystrophy seen in dialysis patients.⁴ Thus, Moniere-Faugere et al demonstrated that the ratio of circulating PTH(1-84)/ long PTH C-fragments was a better predictor of bone turnover (as assessed histologically) in renal dialysis patients than serum levels of PTH(1-84), bone-specific alkaline phosphatase, intact PTH, or osteocalcin.²⁸ This suggests that the use of the new assays for full-length PTH may reduce the need for bone biopsy in the evaluation of renal bone disease and the diagnosis of adynamic renal bone disease. On the other hand, Coen et al did not find any relationship between the (1-84) to (7-84) ratio and bone turnover in such patients.²⁹

The relevance of the CPTHRS to osteoporosis therapy remains unknown. Current clinical use of PTH as a bone-forming therapy is based on a study by Neer et al that employed human PTH(1-34). This agent has been shown to have activity not only in increasing bone density and preventing fractures, but also in improving bone microarchitecture. However, intact PTH(1-84) is now under clinical investigation for osteoporosis therapy and the recent publication of a Phase II trial indicates its efficacy in increasing bone density and bone volume.³³ It could be hypothesized that a molecule opposing bone resorption (in addition to stimulating bone formation) might have advantages in improving the ultimate bone mass resulting from treatment and may also decrease the side effects of drugs associated with bone resorption (eg, hypercalcemia). Also, the proapoptotic effect of the CPTHRS might, by limiting the lifespan of cells in the osteoblast lineage, oppose any tendency for the development of osteosarcomas that has been observed in some rat studies of PTH(1-34) therapy. Whether any of these hypothetical differences actually exist with the pharmacological administration of these molecules may be explored in ongoing clinical trials.

Conclusion

The current knowledge reviewed above leaves no doubt about the existence of distinct receptors for the C-terminal region of PTH. However, further work is required to fully define the nature of circulating PTH forms and the full relevance of CPTHRS. Research will be facilitated by cloning the receptor, an endeavour that has been attempted unsuccessfully in the past. The use of expression libraries from osteocytic cells shown to have a high density of C-receptors may offer an advantage in pursuing this goal. Determining the structure of the receptors

may offer clues to the signaling mechanism(s) utilized by these receptors that, at present, remain unknown. In addition, further comprehensive studies are required to define the precise chemical structure of PTH C-fragments circulating in blood under different circumstances. These basic aspects then need to be applied to clinical situations to further elucidate the importance of PTHR and their ligands in disease states.

While the door has been opened in understanding a new aspect of calcium and bone regulation, many gaps in our knowledge remain. This new knowledge is likely to be clinically relevant, particularly with regard to renal bone disease, but it may also have pharmacological importance.

Dr. Timothy Murray is a Professor of Medicine, University of Toronto, Centre for Diabetes and Osteoporosis, St Michael's Hospital, Toronto, Ontario.

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Abstract of Interest

PTH(7-34) Downregulates the PTH1R Receptor by a Ubiquitin-Sensitive Pathway.

MAGYAR CE, SNEDDON WB, BISELLO A, FRIEDMAN PA. PITTSBURGH, PA, USA.

Renal failure and secondary hyperparathyroidism are accompanied by PTH resistance and downregulation of the type I PTH receptor (PTH1R). Increased circulating levels of PTH(7-84) have been suggested to competitively inhibit the action of PTH(1-84) and account for the PTH resistance of end-stage renal failure. However, PTH1R downregulation has been described in the same pathological setting. This would be inconsistent with a competitive inhibitory effect of PTH(7-84) on receptor endocytosis and degradation. We recently found that both activating ligands, PTH(1-34) and (1-84), and non-activating ligands, PTH(7-34) and (7-84) internalized the PTH1R in mouse distal convoluted tubule (DCT) cells and rat osteosarcoma cells. We hypothesized that whereas PTH1R internalization following activation is accompanied by receptor recycling, activation-independent internalization targets the PTH1R for degradation and downregulation. To determine the fate of the PTH1R after internalization by non-activating ligands, we analyzed receptor recycling in DCT cells after a 30-min exposure to PTH(1-34) or PTH(7-34). Receptor recycling was assessed by recovery of [¹²⁵I]PTH(1-34) binding. Over a 2-hr period, the PTH1R recycled faster after challenge with PTH(1-34) (>50% recovery within 30 min) than with PTH(7-34) (>50% recovery after 1h). Chronic exposure (30 min – 22h) to PTH(7-34) significantly decreased receptor abundance as determined by semi-quantitative immunoblotting. To test the hypothesis that proteasomal degradation mediates PTH1R downregulation, we examined PTH1R ubiquitination. PTH(7-34) but not PTH(1-34) was accompanied by marked PTH1R polyubiquitination in the presence of proteasome inhibitors, MG-132 and lactacystin. These data suggest that in response to PTH(7-34), the PTH1R is internalized, tagged by ubiquitin, and targeted to proteasomes for degradation. These findings support the view that in physiological settings when PTH(1-84) is prevalent, most of the receptor is rapidly recycled. In contrast, in pathological settings where PTH(7-84) is elevated, the PTH1R is internalized without activation and is targeted for degradation by a ubiquitin-

dependent process leading to receptor downregulation. In conclusion, PTH(7-84) internalizes the receptor without concurrent activation resulting in fewer membrane-bound PTH1Rs that are available for stimulation by PTH(1-84). We proposed that this effect contributes to hormone resistance and PTH1R downregulation in renal failure.

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