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PTH receptors and apoptosis in osteocytes

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Abstract

Osteocytes comprise a heterogenous population of terminally differentiated osteoblasts that direct bone remodeling in response to applied mechanical loading of bone. Increased osteocyte density accompanies the anabolic effect of PTH *in vivo*, whereas accelerated osteocyte death may be precipitated by estrogen deficiency or excess glucocorticoid exposure (conditions benefitted by intermittent PTH therapy) and by renal failure (where circulating intact PTH and, especially, PTH carboxyl-fragments are elevated). Osteocytes express type-1 PTH/ PTHrP receptors (PTH1Rs), which are fully activated by amino-terminal PTH fragments and couple to multiple signal transducers, including adenylyl cyclase and phospholipase C. Activation of PTH1Rs in osteocytes promotes gap junction-mediated intercellular coupling, increases expression of MMP-9, potentiates calcium influx via stretch-activated cation channels, amplifies the osteogenic response to mechanical loading *in vivo*, and regulates apoptosis. Control of osteocyte apoptosis by PTH1Rs is complex, in that intermittent PTH(1-34) administration reduces the fraction of vertebral apoptotic osteocytes at 1 month in adult mice but increases femoral metaphyseal osteocyte apoptosis at 1-2 weeks in young rats. In MLO-Y4 cells, PTH(1-34) prevents apoptosis otherwise induced within 6 hr by dexamethasone. In older studies, large doses of intact PTH(1-84) caused rapid "degenerative" morphologic changes in osteocytes, similar to those described in renal osteodystrophy. We isolated clonal conditionally immortalized osteocytic (OC) cell lines from mice homozygous for targeted ablation of the PTH1R gene. OC cells express abundant (2-3 x 10^6 per cell) receptors specific for the carboxyl(C)-terminus of intact PTH(1-84) ("CPTHRs") but, as expected, do not express PTH1Rs or respond to PTH(1-34). CPTHRs are expressed at much lower levels by other skeletally-derived cell lines. Several highly conserved ligand determinants of CPTHR binding have been identified, including PTH(24-27), PTH(53-54) and the sequence PTH(55-84), loss of which reduces binding affinity by over 100-fold. Human PTH(53-84), like PTH(1-84), PTH(24-84), and PTH(39-84), increases OC cell apoptosis. Ala-scanning mutagenesis to define sequences within PTH(55-84) important for binding and bioactivity is underway. We conclude that osteocytes may be important targets for CPTH fragments that are secreted by the parathyroid glands or generated by peripheral metabolism of intact PTH and that accumulate in blood, especially in renal failure. Studies of functional interplay between responses to CPTHRs and (transfected) PTH1Rs, using receptor-specific ligands in OC cells, should provide new insight into PTH regulation of osteocyte function and survival.

Keywords: PTH, Receptors, Apoptosis, Osteoclasts, Osteocytes

Secretion and metabolism of parathyroid hormone

Native human parathyroid hormone (PTH), a single-chain polypeptide 84 amino acids in length, is secreted by the parathyroid glands in response to changes in serum ionized calcium concentration that are transduced by a cell-surface calcium-sensing receptor[1]. Secreted PTH consists of a mixture of intact hormone and various carboxyl (C) fragments, the ratio of which (C-fragments:intact PTH) increases at higher serum calcium concentrations[2-6]. Circulating intact PTH also undergoes rapid clearance and proteolytic cleavage in peripheral organs, most notably liver and kidney[7-12]. Hepatic proteolysis produces additional C-PTH fragments that are released back into blood, whereas the corresponding amino(N)-terminal fragment(s) are rapidly degraded *in situ* and are not normally detectable in the circulation[10,13,14]. The disappearance half-time of intact PTH is approximately two minutes[11,15,1], whereas C-PTH fragments, which are cleared more slowly via glomerular filtration, accumulate in blood and normally comprise 60-80% or more of circulating PTH immunoreactivity[17,18]. Renal clearance of intact PTH and intrarenally generated N-terminal fragments occurs mainly via glomerular filtration, followed by proximal tubular re-

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uptake by the multifunctional endocytic receptor, megalin[12,19-21]. The precise structures of secreted or circulating CPTH fragments are poorly understood. Cleavages within the region PTH (30-43) have been identified, and the size(s) of the major CPTH fragments isolated from blood or tissues are consistent with an intact C-terminus, although some immunoassay evidence suggests the presence of "mid-region" PTH fragments that lack both extreme N- and C-terminal epitopes[11,18,22-24]. Recently, application of a new PTH immunoradiometric assay with specificity for the extreme N-terminus of PTH has introduced the possibility that N-truncated PTH fragments much larger than those previously described may exist in blood and, like other "CPTH" fragments, may accumulate in renal insufficiency[25].

Receptors for parathyroid hormone in bone

The classical biologic actions of intact PTH in bone and other target tissues, such as kidney, are mediated by a G-protein coupled, seven-membrane -spanning receptor which also is activated by PTH-related peptide (PTHrP) and is designated herein the (type-1) PTH/PTHrP receptor ("PTH1R")[26]. This receptor is fully activated by the N-terminal one-third of the intact PTH sequence, as represented in the widely used synthetic peptide PTH(1-34), and also by the corresponding N-terminal domain of PTHrP, which is highly homologous to that of PTH. The PTH1R activates adenylyl cyclase, phospholipases C and D, and protein kinase C (possibly via mechanisms independent of PLC) and is known to be highly expressed by osteoblasts but not by osteoclasts[26-30].

Despite the fact that most of the C-terminal sequence of PTH(1-84) has been highly conserved among species and that the glandular secretion of CPTH fragments is strongly regulated by serum calcium, CPTH fragments traditionally have been regarded as biologically inactive degradation products of the active intact hormone. This view derived principally from observations that CPTH peptides can neither bind nor activate PTH1Rs and that PTH(1-84) and PTH(1-34) are essentially equipotent in classical PTH bioassays *in vivo* or *in vitro*. The presence of multiple forms of incompletely defined circulating CPTH fragments generated by both secretion and peripheral PTH metabolism seemed also to argue against an important or specific biological role for these peptides. Not surprisingly, perhaps, recognition of the potential physiologic importance of CPTH fragments in bone biology has lagged far behind that of the PTH1R. In fact, evidence for a distinct class of PTH receptors with specificity for C-terminal epitopes of the ligand ("CPTHRs") first emerged almost 20 years ago, when kinetic analysis showed that ^{125}I-PTH(1-84) binding to renal membranes fit much better with a two-site than a one-site model[31,32]. Subsequently, Demay et al. and Rao et al. found that ^{125}I-bPTH(1-84) binding to osteosarcoma cells and renal membranes was displaced only incompletely by bPTH(1-34), compared with bPTH(1-84), and that oxidation of N-terminal methionines, which eliminated PTH1R binding, only modestly reduced bPTH(1-84) binding[33,34]. A decade later, Inomata et al. showed that ^{125}I-[Tyr^{34}]hPTH(19-84), which cannot bind to recombinant PTH1Rs, did bind to both ROS 17/2.8 osteosarcoma and PT-r3 parathyroid cells with apparent affinity of approximately 20-30 nM and could be specifically crosslinked to a predominant 80 kDa protein[35]. Concurrently, several groups have observed functional responses of bone cells to CPTH peptides alone or effects of intact PTH not shared by PTH(1-34), including upregulation of alkaline phosphatase, collagen I and IGFBP-5 expression and stimulation of ^{45}Ca uptake in osteosarcoma cells[36,41], production of Ca^{2+} transients and upregulation of collagen II and X in chondrocytes[42,43], regulation of murine embryonic tooth germ development[44], stimulation of hepatic glucose release *in vivo*[45], activation of osteoclast formation *in vitro*[46] and stimulation of osteoblastic fibronectin secretion *in vivo* and *in vitro*[47]. Very recently, two groups independently reported that the acute calcemic response to hPTH(1-84) or hPTH(1-34) in TPTX rats could be strikingly inhibited by hPTH(7-84), which also lowered basal serum calcium in these animals[48,49]. Importantly, hPTH(7-84) does not bind to PTH1Rs or antagonize cAMP signaling via PTH1Rs[49] (our unpublished observations), which strongly implies that these *in vivo* effects are mediated by receptors distinct from PTH1Rs, likely CPTHRs.

Effects of parathyroid hormone upon osteocytes

Actions of PTH upon osteocytes *in vivo* were first suggested by early experiments in which various adverse morphological changes (cellular retraction, mitochondrial swelling, cell "death") were observed in osteocytes (and osteoblasts) by light or electron microscopy within a few hours of administration of parathyroid extract (PTE)[50,51]. Others demonstrated evidence of increased proteolytic activity associated with expanded osteocyte lacunae in avian bone from animals given PTE daily for several days[52]. In retrospect, the purity and peptide composition of the PTE employed in these studies was not well defined, although these preparations likely contained intact bPTH(1-84) plus a variable admixture of PTH proteolytic fragments and unrelated proteins. Moreover, the administered doses of active hormone were huge (up to 10,000 U/kg, or 2-3 mg PTH/kg) and were recognized at the time as potentially "toxic".

When more modest doses of PTE were administered to thyroparathyroidectomized (TPTX) rats, however, changes also were observed in osteocyte morphology that were more suggestive of activation than injury, such as increased osteocyte size and enlargement of endoplasmic reticulum, cisternal dilation, irregularity of the surrounding lacunae and demineralization of subjacent matrix[53-55]. These morphologic features developed very rapidly - i.e. within 1 hour of PTE administration and were not dissimilar from those observed in biopsies from patients with chronic primary or secondary hyperparathyroidism[56].

More direct evidence of PTH1R expression by osteocytes derived from experiments showing immunohistochemically

detectable increases in cAMP within osteocytes within 20 minutes of PTE administration to young cats[57]. Later, binding of radiolabeled PTH(1-34) to osteocytes was observed, both *in vivo*[58] and *in vitro*[59]. Direct demonstration of PTH1R mRNA expression within osteocytes by *in situ* hybridization has not yet been accomplished[29,58], but osteocytic expression of PTH1R protein was shown by immunohistochemical staining of intact bone[60]. Purified osteocytes and clonal osteocytic cell lines also manifest increased cAMP generation and other responses to PTH(1-34) *in vitro*[59,61-64].

A physiologic role for PTH in osteocyte biology was strongly implicated by early *in vivo* experiments in which parathyroidectomy was found both to prevent the enlargement of osteocyte lacunae otherwise seen during immobilization[65,66] and to reduce immobilization-induced bone loss[67,68]. Subsequently, in an elegant *in vivo* model of selective vertebral mechanical loading, Chow et al. showed that the osteogenic response observed in rats 7-8 days after a brief session of cyclic compressive strain again was prevented by prior TPTX but was potentiated by a single injection of hPTH(1-34) (6 or 60 μg/kg) given 30-45 minutes before mechanical loading in either intact or TPTX animals[69]. Moreover, the combination of PTH(1-34) plus mechanical loading, but neither stimulus alone, led to increased osteocytic expression of *c-fos* mRNA, as detectable by *in situ* hybridization, at 1 hour after loading[69]. Ma et al. also showed in intact rats that intermittent administration of hPTH(1-38) (30 or 80 μg/kg once daily for 2-20 weeks) increased cortical bone mass and formation rates in chronically immobilized hind limbs and was significantly more effective when the limb was concurrently remobilized, pointing again to a functional interaction between mechanical loading and PTH1R activation[70]. A possible mechanism for these effects of PTH was suggested by the recent finding, in isolated normal rat calvarial osteocytes, that PTH(1-34) potentiates influx of extracellular calcium via stretch-activated channels, an effect that was mimicked by Sp-cAMPS, forskolin, dibutyryl cAMP and PTH(1-31) but not by PTH(3-34) or phorbol ester[64]. This effect of PTH was mirrored by a similar cAMP-dependent increase in IGF-1 mRNA in cyclically stretched, partially purified rat osteocytes that greatly exceeded the increased IGF-1 mRNA seen in unstretched cells. Collectively, these studies point to a powerful interactive effect of PTH1R receptor activation with concurrent mechanical loading of osteocytes, possibly mediated by the ability of PTH1R-activated adenylyl cyclase to augment stretch-activated calcium influx. The various rapid effects of PTE upon osteocyte morphology previously observed are less clearly due to PTH1R activation, although the rapid calcemic effect of PTH(1-34) seen within 1-2 hours in TPTX animals[49] could be due in part to PTH1R-triggered release of calcium via osteocytic osteolysis.

PTH1Rs and osteocyte apoptosis

A role for PTH1Rs in regulating osteocyte survival was highlighted by Jilka et al., who showed that increased bone formation induced in 4-5 month old SAMR1 mice by intermittently administered hPTH(1-34) (400 μg/kg once daily for 4 weeks) was accompanied by a striking decrease in the fraction of apoptotic vertebral osteocytes (and osteoblasts), as demonstrated by $CuSO_4$-enhanced TUNEL staining[63]. This anti-apoptotic effect of PTH(1-34) was also observed directly *in vitro* using various osteoblastic cells as well as the MLO-Y4 osteocytic cell line, which is immortalized by an SV40 large T antigen expressed under the control of the osteocalcin promoter[71]. In MLO-Y4 cells, apoptosis induced by etoposide or dexamethasone was blocked by bPTH(1-34) (10 nM and above) and by 0.1 mM dibutyryl cAMP or DEVD-CHO but not by 10 uM bPTH(3-34), which nevertheless did effectively antagonize the anti-apoptotic effect of a 100-fold lower concentration of bPTH(1-34)[63]. Coupled with the absence of increased marrow osteoblast progenitors in the PTH-treated animals, these findings suggested that the anabolic effect of intermittently administered PTH(1-34) proceeds mainly via enhanced osteoblast and osteocyte survival, rather than increased generation of new cells committed to the osteoblast lineage. On the other hand, Stanislaus et al. recently reported that hPTH(1-34), administered once daily at 80 ug/kg for up to 4 weeks to young (4-5 week old) male rats, induced a transient increase in apoptosis of both distal femoral osteoblasts (beneath the growth plate) and osteocytes (in terminal trabeculae adjacent to the marrow cavity)[60]. This effect, measured using a histochemical TUNEL stain, was maximal at 7 days and had dissipated completely by 28 days. Supportive data were obtained by flow-cytometric analysis of mixed metaphyseal bone cells, using annexin V and TUNEL staining, which showed an increase in apoptosis over the first 2-10 days of PTH treatment *in vivo*. Inexplicably, DEVD-specific caspase activity extracted from metaphyseal bone actually was reduced at 3 and 28 days, however, suggestive of an overall lower level of apoptosis in this tissue. Thus, while the idea that increased survival of osteoblasts and osteocytes drives the anabolic action of intermittently administered PTH1R agonists is appealing, further work is needed to clarify the temporal and possible site- or age-specific features of this response *in vivo*.

Osteocytes as targets for CPTH peptides

Previous studies suggestive of CPTHR expression in bone were performed using cells or tissues known to express PTH1Rs. Although binding of CPTH fragments to PTH1Rs never has been demonstrated and biologic effects of CPTH peptides clearly distinct from those of PTH(1-34) were observed, lingering uncertainty persisted as to the possible involvement of PTH1Rs in these "CPTH" effects. To eliminate this problem and to fully characterize CPTHR expression in different bone cell populations, we isolated clonal cell lines by enzymatic digestion of calvariae from murine embryos that were homozygous for targeted ablation of the PTH1R gene and that harbored also a transgene

encoding a temperature-sensitive SV40 large T antigen (tsA58) under the control of a ubiquitously expressed MHC promoter. Osteoblastic clones, isolated from such animals on the basis of high alkaline phosphatase expression, exhibited temperature-dependent proliferation and differentiation, generated no cAMP in response to rPTH(1-34) or hPTH(1-84) and expressed CPTHRs, as detected by specific binding of the ^{125}I-[Tyr^{34}]hPTH(19-84) CPTHR radioligand[72,73]. When clones were isolated blindly from the original calvarial digests exclusively on the basis of a high level of CPTHR expression, the resulting cell lines uniformly exhibited an osteocytic phenotype, including a dendritic morphology with extended cell processes; high expression of osteocalcin, connexin 43 and CD44 and low levels of alkaline phosphatase, Cbfa-1 and osteopontin[73]. Differentiation of these "OC" cells again was temperature-dependent, and, as expected, these cells expressed much higher levels of CPTHRs (2-3 x 10^6/cell) than did osteoblastic clones derived from the same calvarial bones (0.2 - 0.6 x 10^6/cell).

OC cells manifested the characteristic ligand selectivity seen with previously-described CPTHRs. Thus, the binding affinities of hPTH(1-84), hPTH(7-84), [Tyr^{34}]hPTH(19-84) and hPTH(24-84) were similar (20-30 nM), whereas those of hPTH(28-84), hPTH(39-84), and hPTH(53-84) all were in the range of 500-700 nM and hPTH(60-84) could not displace the ^{125}I-[Tyr^{34}]hPTH(19-84) radioligand, even when introduced at 10 uM[74] (unpublished observations). In these PTH1R-null cells, hPTH(1-84), as well as hPTH(39-84), increased connexin-43 surface immunoreactivity within 2 hours. Most interestingly, PTH(1-84) strongly induced apoptosis, as shown by TUNEL immunoreactivity and Hoechst 33258 nuclear staining, when these cells were cultured in reduced serum under conditions nonpermissive for growth (39 C). This effect of PTH(1-84) was concentration-dependent (1-100 nM); mimicked by hPTH(24-84), hPTH(39-84) and hPTH(53-84); and blocked by the caspase inhibitor DEVD-CHO[73], (unpublished observations).

Summary

These recent data indicate that osteocytes, the most common cells in bone, may be preferential targets for the effects of CPTH fragments acting via CPTHRs that are especially abundant on these cells. Because osteocytes also express PTH1Rs, they may be uniquely poised to respond to changes in the relative circulating concentrations of intact vs. C-terminal PTH peptides through dynamic integration of signals simultaneously generated by both receptors. As the binding affinity of CPTHRs for various CPTH fragments is 10-100 fold lower than that of PTH1Rs for PTH(1-84), the relative sensitivities of these two receptors seem appropriately tuned to the correspondingly disparate ambient circulating levels of CPTH fragments vs. intact PTH normally present *in vivo*. Because of the large differences in their metabolic clearance rates, small shifts in the ratio of secreted CPTH fragments to intact PTH, known to occur with changes in serum calcium, could be amplified in blood and thus detectable by cells expressing receptors responsive to different domains within the PTH molecule. Moreover, in extreme pathologic states such as renal failure, where CPTH fragments accumulate disproportionately, one might anticipate severely unbalanced activation of CPTHRs vs. PTH1Rs in bone, with potentially deleterious consequences such as decreased osteoblast and osteocyte survival.

If, as suggested by our observations and those of Jilka et al., PTH1Rs and CPTHRs actually exert powerful but directionally opposite effects upon osteocyte apoptosis, how could such actions of CPTH fragments possibly have been overlooked for so long? One explanation may lie in the 10-100-fold disparity in binding affinities of PTH1Rs and CPTHRs for their respective ligands. In instances where PTH(1-34) and intact PTH were administered *in vivo* at equimolar doses, the duration of hormone exposure may have been too short or the dose too low to permit accumulation of generated CPTH fragments to levels sufficient for activation of CPTHRs. Moreover, because less than 20% of administered intact PTH(1-84) ultimately reappears as circulating CPTH fragments, it may be that ongoing secretion of CPTH fragments, and not peripheral proteolysis of secreted or administered intact hormone, is the major or exclusive source of biologically relevant CPTH. If so, bioactive concentrations of CPTH peptides may not be achievable via peripheral metabolism of intact PTH when administered in usual doses. In this regard, it is intriguing to speculate that "degenerative" changes observed in osteocytes many years ago following massive doses of PTE may have reflected a preponderant activation of CPTHRs by relatively long-lived CPTH fragments, produced in unusually large amounts by peripheral metabolism of the enormous quantities of administered but short-lived intact PTH.

It is clear that much remains to be learned about the function of CPTHRs in bone. The structure of the receptor(s) has not been defined, nor are its signaling mechanisms understood. The full spectrum of biologic responses to CPTHR activation remains unknown. The precise structures of circulating or locally generated CPTH ligands available to activate these receptors remain obscure. The possibility that, as for the PTH1R, CPTH fragments of differing length may elicit disparate responses via CPTHRs must be entertained. Whether CPTHRs recognize PTHrP, or peptides derived from it, has yet to be addressed. While pursuit of many of these questions will be facilitated by success in ongoing efforts to clone CPTHR cDNA(s), clonal osteocytes and related cell lines should prove invaluable in further defining their pharmacology.

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