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Perspective Article

Hylonome

PTH and osteocytes

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Osteocytes are the most abundant cells in bone, yet their functions are still poorly understood. Osteocytes express several membrane receptors, including the parathyroid hormone (PTH) type 1 receptor (PTH1R)[1], estrogen receptors (both α and β[2-5]), and, as we recently have reported, a novel receptor that specifically recognizes the carboxyl-terminal region of PTH, the carboxyl-terminal PTH receptor (CPTHR)[6,7].

PTH is a single-chain polypeptide comprised of 84 amino acids and its main function is to maintain constant the serum calcium concentration. PTH accomplishes this via activation of a G-protein coupled receptor, the type 1 PTH/PTH-related peptide receptor (PTH1R), mainly expressed in kidney and bone[8].

The amino-acid sequence of PTH is highly conserved among species and this high degree of evolutionary conservation strongly suggests the possibility of additional, independent biological function(s) for the C-terminal region of the PTH molecule. Indeed, evidence of cellular receptors with specificity for the C-terminal portion of PTH(1-84) ("CPTHRs") has accumulated steadily over the past 25 years, as recently reviewed[9]. We reported abundant expression of CPTHRs (2-3 x 10^6/cell), detected using ^{125}I-[Tyr ^{34}]hPTH(19-84) as radioligand, on the surface of clonal osteocytic cells ("OC cells") isolated from calvarial bone of fetal PTH1R-null mice, thus providing the first conclusive evidence that CPTHRs exist independently of PTH1Rs[6,10,11]. Recently we have identified specific structural determinants of CPTHR binding[7] and we demonstrated that CPTHR activation in osteocytes leads to increased cell death, an effect opposite to that reported for the PTH1R, and to an increase in cell-to-cell communication.

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PTH1R and osteocytes

Direct actions of PTH on osteocytes "*in vivo*" were suggested by early experiments in which various adverse morphological changes (cellular retraction, mitochondrial swelling and cell death) were observed in osteocytes and osteoblasts by light electron microscopy within hours of administration of PTH extract (PTE)[12,13]. Other investigators demonstrated evidence of increased proteolytic activity associated with enlarged osteocytic lacunae in bones of animals given daily doses of PTE for several days. These findings initially suggested that the role of the osteocytes was osteolysis. The theory was quickly abandoned when it appeared evident that osteocytes in culture were unable to resorb bone (or mineralized matrix). Recently the concept of osteocytic osteolysis has found new support after the report of Tazawa et al., in which osteolysis was observed in lacunae of rats continuously infused with 80 μg/kg/day of PTH(1-34)[14].

Direct evidence of PTH1R expression on osteocytes derived from the work of Davidovitch et al.[15], who demonstrated an increase in cAMP (detected immunohistochemically) in osteocytes of cats treated with PTE. Specific binding of iodinated PTH(1-34) was then shown both *in vivo*[1] and *in vitro*[16].

The localization of PTH1Rs on osteocytes, as well as the synergistic effects of mechanical stress and PTH, indicated an important role of the hormone in regulating the signal transduction induced by loading in osteocytes. Early studies of Duncan et al. demonstrated stretch-activated cation channels and their activation by PTH in UMR106 osteoblast-like cells[17,18]. The subsequent work of Miyauchi et al. revealed the presence of stretch-activated Ca channels synergistically activated by PTH and mechanical stimulation (hypo-osmotic stress)[19]. Interestingly, another class of calcium channel, the L-type voltage-operated calcium channels (VOCCs), also was detected on MLOY4 osteocytic cells after stimulation with a very low dose (0.1 nM) of PTH(1-34). Similar effects were elicited by 17-β-estradiol and dexamethasone, suggesting that hormonal control might prime osteocytes to sense or respond to calcium. In line with this observation, *in vivo* studies in rats, showed that a single injection of PTH (80 μg/Kg) increased the effect of loading, measured as histo-

morphometrical parameters. This effect of PTH was specifically inhibited by verapamil, a VOCC blocker. Lastly, Jilka et al. reported that PTH exerts its anabolic effect on bone by suppressing osteoblast and osteocyte apoptosis[20]. They reported that the intermittent versus sustained effect of PTH is related to proteosomal degradation of Runx-2. Interestingly we have demonstrated that CPTHR activation on osteocytes exerts a pro-apoptotic effect, an action opposite that of PTH1R activation.

CPTHR and osteocytes

The high levels of CPTHR expression by OC cells enabled a reliable analysis, using OC59 cells and the CPTH radioligand ^{125}I-[Tyr^{34}]-hPTH(19-84) (which does not bind to the PTH1R), of the structural determinants for ligand binding. As recently reported[7], N-terminally truncated human PTH peptides hPTH(7-84), [Tyr^{34}]hPTH(11-84), [Tyr^{34}]hPTH(13-84), [Tyr^{34}]hPTH(19-84) and [Tyr^{34}]hPTH(24-84) displaced the radioligand as effectively as hPTH(1-84) (IC_{50}s: 10-40 nM), whereas a group of shorter peptides, including hPTH(28-84), hPTH(34-84), hPTH(37-84), [Asn^{76}]hPTH(39-84) and hPTH(53-84), bound with significantly lower apparent affinity (IC_{50}s: 200-600 nM). Further minimal N-terminal truncation beyond position 53, as in hPTH(55-84), hPTH(57-84) and hPTH(60-84), effectively abolished measurable binding affinity for CPTHRs (IC_{50}s >> 10,000 nM) highlighting the presence of at least two domains required for maximal binding affinity – one within the sequence hPTH(24-27), ("binding domain 1; BD1") and another represented by the dibasic sequence (Lys^{53}-Lys^{54}), termed "binding domain 2 ("BD2"). Further analysis of the intact hormone pointed to the presence of additional major determinants of binding affinity within the region hPTH(55-84), thereby defining a third "binding domain" ("BD3"). To identify key residues involved in the contribution of BD3 to overall CPTHR ligand binding affinity, clustered triple-alanine substitutions were introduced across the sequence of hPTH(53-84), to produce nine mutant hPTH(53-84) peptides. Three of these peptides, with substitutions at positions 71-74 ("M4"), 64-66 ("M6") and 55-57 ("M9"), respectively, showed dramatic (roughly 100-fold) reductions in apparent affinity. Further analysis of additional peptides harboring single-alanine substitutions within these regions identified three key residues - Asn^{57}, Lys^{65} and Lys^{72} - that appear to be critical for high affinity binding to CPTHRs.

As osteocytes are terminally differentiated osteoblasts, it was of interest to determine if CPTHR activation might play a role in regulating apoptosis in the OC cells. We found that incubation of OC cells, which lack functional PTH1R genes, for 6 hours with 100 nM hPTH(1-84) led to increased nuclear pyknosis and chromatin condensation, as revealed by DNA staining with Hoechst dye 33258. Increased apoptosis also was observed in response to the intact hormone or the short fragment hPTH(53-84) using a TUNEL immunocytochemical assay[7].

Our initial signaling studies demonstrated that CPTHR activation induces a rapid influx of calcium from the extracellular compartment, likely via opening of calcium channels. Since calcium is a major regulator of the cytoskeleton, we examined the effect of CPTHR-dependent calcium influx on cytoskeletal rearrangement. OC59 cells were treated with 100nM hPTH(53-84) for 2 and 10 minutes and then were examined by immuno-fluorescent staining of cytoskeletal components (vinculin and actin). OC59 cells treated with the CPTH fragment demonstrated a marked actin and vinculin condensation, suggestive of a rapid modification of the cytoskeleton. The specificity of this effect was examined by treating the cells with the mutant peptide [Ala$^{55-57}$]hPTH(53-84), which does not bind or activate calcium influx and, as expected, the mutant analog failed to induce any cytoskeletal changes in OC cells. The role of calcium influx was examined by blocking calcium influx with gadolinium chloride (GdCl) (1 and 10 μM). When OC59 cells were treated with GdCl, hPTH(53-84) failed to induce cytoskeletal changes, indicating that calcium influx might play an important role in the regulation of osteocyte cytoskeletal assembly and structure.

Thus, in summary, it seems possible that PTH might regulate osteocytic function via at least two receptor systems, the PTH1R and the CPTHR, although many questions still remain.

Frost had proposed that systemic hormones might alter bone remodeling by changing the thresholds at which mechanosensory cells in bone respond to differing intensities of mechanical stress or loading[21]. It therefore will be important, in the future, to further define the effects of PTH, acting both via the PTH1R and the CPTHR, on osteocytes undergoing mechanical stimulation.

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