Understanding Parathyroid Hormone Action

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ABSTRACT: In addition to regulating serum calcium and stimulating bone resorption, parathyroid hormone (PTH) is known to stimulate bone formation under certain conditions. The mechanisms behind this counterintuitive anabolic action are largely unknown. We have set out to identify PTH-regulated genes that might be responsible for each of these contrasting effects of the hormone. This article describes our work on a select number of those genes.

KEYWORDS: parathyroid hormone; PTH; EGF; amphiregulin; bone resorption; bone formation; osteoblast; MCP-1

The classical regulation of serum calcium by parathyroid hormone (PTH) is well known.[1] In this situation low serum calcium levels cause the release of PTH from the four parathyroid glands behind the thyroid gland. The hormone has two sites of action; it acts on kidney to stimulate calcium re-absorption and also enhance the hydroxylation of 25 hydroxy vitamin D_3 to 1,25-dihydroxy vitamin D_3, which then has its subsequent actions on increasing calcium uptake by the small intestine. The other site of PTH action is bone, where it stimulates bone resorption. The actions on the kidney and bone then cause the restoration of serum calcium. This is our classical concept of PTH action, to have minute-to-minute responsibility for the regulation of serum calcium levels.

However, for some time now, we have known that PTH can also stimulate bone formation.[2] In other words, it has an anabolic action in contrast to its catabolic action to cause bone resorption. In fact, clinical trials where PTH (1-34) or (1-84) were given to postmenopausal women have shown significant increases in bone mineral density (BMD).[3] This then led to the approval by the FDA for the use of PTH 1-34 (teraparatide, Forteo™, Eli Lilly and Company) to treat osteoporosis. However, we still do not know why PTH has this anabolic effect in contrast to its catabolic effect.

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In our laboratory, we set out to identify PTH-regulated genes that might be responsible for each of these two effects of the hormone. Initially, we performed microarray analysis on UMR 106-01 cells, which is a rat osteoblastic osteosarcoma cell line. These cells had been treated with PTH (1-34) for 4, 12, or 24 hours. The RNA was isolated, converted to cDNA, then to biotinylated cRNA, and this was hybridized to Affymetrix rat chips containing 8,799 probes, which consisted of 7% known genes and 23% expressed sequence tags (ESTs). Our results showed that there are 125 known genes and 30 ESTs that were regulated by PTH.[4]

This article describes our work on a select number of those genes. The first genes investigated were members of the epidermal growth factor (EGF) family, which contains 10 ligands and four receptors. The ligands that we found stimulated were amphiregulin and transforming growth factor α (TGF-α). These together with EGF, heparin-binding EGF, β-cellulin, and epiregulin bind to the first-known EGF receptor, also known as ErbB1. This can heterodimerize with ErbB2 to form the most potent heterodimer. The four other ligands of this family, the neuregulins, bind to ErbB3 and ErbB4. We found that stimulation of amphiregulin was very rapid in the UMR cells with peak stimulation of the mRNA at 1 hour, which then declined by 12 hours. The stimulation was dose-dependent at 1 hour with a significant increase at 10^{-10} M rat PTH (1-34) and maximal effect at 10^{-8} M. The PTH regulation of amphiregulin was also observed in differentiating primary rat osteoblastic cultures. These cells are cultured so that they proceed through three stages, proliferation, differentiation, and mineralization. We found that rat PTH (1-34, 10^{-8} M) stimulated amphiregulin expression in all three stages; however, the greatest effect was seen in the mineralizing cells. In every case, there was maximal stimulation at 1 hour with a decline back to basal levels by 24 hours. We have also examined another osteoblastic cell line, the MC3T3 cells, and we also found rat PTH (1-34, 10^{-8} M) stimulated amphiregulin expression in these cells with a maximal regulation at 1 hour, which also declined by 6 hours; however, the stimulation was about fourfold in contrast to the 20- to 25-fold stimulations that we had seen in both the UMR and primary osteoblastic cells. Finally, we injected human PTH (1-38) into 4-week-old rats and then analyzed the metaphyseal region of the distal femur at various times after injection. We observed a very rapid and pronounced 13- to 14-fold increase in amphiregulin expression 1 hour after injection of PTH (1-38). This also declines very rapidly, reaching basal levels again 8 hours after the injection. Therefore, PTH stimulates the growth factor very rapidly, both in osteoblastic cultures *in vitro* as well as *in vivo*.[5]

The conclusion that we have reached from the work that we have performed on amphiregulin is that PTH acting on the mature osteoblast stimulates the expression of amphiregulin and this then acts on mesenchymal stem cells as well as committed preosteoblasts to stimulate their replication and also block their differentiation. This effect is likely to be transient since the injection of PTH is transient and, as we have seen, results in transient expression of amphiregulin.

Therefore, we suggest that there should be a transient stimulation of replication and an increased pool of preosteoblasts. When the amphiregulin signal dissipates, the increased number of preosteoblasts will be able to progress into differentiated, mature osteoblasts.

The next set of genes we examined is related to growth of osteoblasts. The mitogen-activated protein kinase (MAPK) family is well known for being involved with the proliferation of cells. The major families are the ERKs, JNKs, and p38 families. All of these are activated by phosphorylation on both threonine and tyrosine. They must also be inactivated by dephosphorylation of those phosphates, and this is achieved by specific phosphatases. The phosphatases in question are the MAPK phosphatases. These are dual-specificity phosphatases in the overall protein tyrosine phosphatase superfamily. We found that one of these MAPK phosphatases, MKP1, was also another gene that was highly regulated by PTH (1-34) and was one of the genes shown to be increased in the microarray of the UMR cells. We examined its regulation in greater detail and found that this too was increased very rapidly, with the mRNA reaching maximal levels at 1 hour after PTH treatment in the UMR cells, dissipating back to lower levels by 7 hours. We found similar regulation in the normal differentiating osteoblasts, again with very rapid regulation. Maximum stimulation was observed at 1 hour and this again was reduced to basal within 4 hours. This regulation of MKP1 is protein kinase A dependent. We found that PTH (1-31), which does not activate the protein kinase C (PKC) pathway, was able to mimic the effect of PTH (1-34), whereas PTH (13-34), which activates the PKC pathway, did not stimulate MKP1 expression.

Eight bromo-cAMP could also stimulate MKP1 mRNA levels, as could the PKC stimulator, PMA. However, when we treated cells with PTH (1-34) in the presence of the protein kinase A inhibitor, H-89, we completely blocked the stimulation of MKP1 in contrast to when we used the protein kinase C inhibitor, GF109203X, there was no inhibition of the PTH stimulation of MKP1. These data would indicate that protein kinase A is necessary for PTH (1-34) stimulation of MKP1 whereas protein kinase C is not. This work was done on UMR cells but, as well, we injected PTH (1-34), (1-31), or (3-34) into 4-week-old rats, removed the metaphyseal region of the distal femur 1 hour after the injection, and found that PTH (1-34) and (1-31) had identical robust stimulation of MKP1 *in vivo* whereas PTH (3-34) had no effect at all. This would indicate that *in vivo* we have similar results to those seen *in vitro*; in other words, it is likely that the protein kinase A (PKA) pathway is responsible for the stimulation of MKP1 expression *in vivo*.

The activation of ERKs increases cyclin D1 mRNA expression while inhibition of ERKs (by MKP1) would block cyclin D1's induction. The cell cycle requires the transition from G1 to S and one of the first genes that is switched on is cyclin D1. Some of the inhibitors that regulate both cyclin D1 and cyclin E are p16, p15, p21, p27, and p57. We investigated the PTH regulation of some of these genes *in vivo*. Injection of PTH (1-38) into 4-week-old male rats

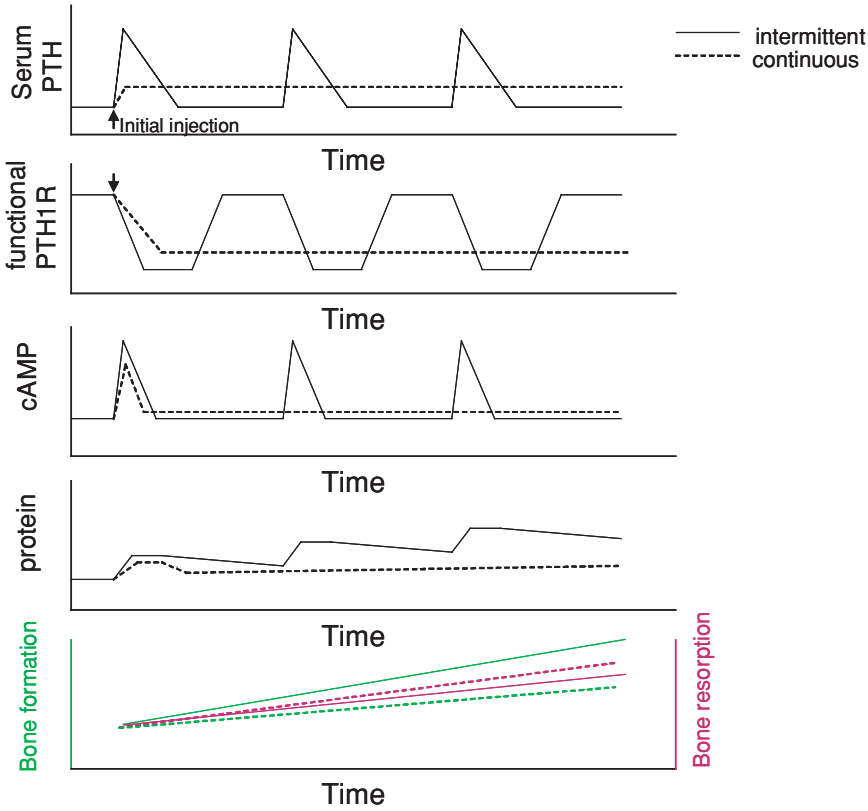

FIGURE 1. A theoretical time course of serum PTH (1-34), functional PTH receptor (PTH1R) level, cAMP amount, a putative PTH-regulated protein amount, and bone formation versus bone resorption after intermittent or continuous PTH (1-34) treatment. The arrow indicates the initial PTH administration. (Modified from Qin *et al.*[7] Reprinted with permission from Elsevier.)

showed a very rapid stimulation of MKP1; in fact, the maximal stimulation is at 30 minutes, which then declines back to basal by 4 hours. At the same time, we observe a decrease in cyclin D1 mRNA expression, but this actually rebounds to basal levels by 4 hours. PTH also regulates the inhibitor of the cyclins, p21. This is also very rapid with maximal effects at 30 minutes, which again declines to basal by 4 hours. It is noteworthy that we found that if 3-month-old female rats are given either one injection versus daily PTH (1-34) injections for 14 days we found that there were substantially greater increases in p21 mRNA with 14 days of injections compared with one injection.[6] This suggests that there is a ramping up in the sensitivity of cells to PTH. It should be noted this is not the case for all genes; for instance, MKP1 showed the same fold stimulation for one injection versus 14 daily injections.

Comparing the intermittent versus continuous effects of PTH we have proposed FIGURE 1. With daily intermittent injection the hormone levels are transient and return to baseline whereas with continuous infusion they rise, but not to the same levels as with the intermittent injection, but then stay constant throughout the whole period. We think that the PTH receptor will be transiently downregulated with the intermittent injections but that this then will rebound to normal within 24 hours prior to the next injection, whereas we think that with continuous treatment that the PTH receptor may decrease in abundance, both mRNA and protein, and may stay at lower levels throughout the time of infusion. With respect to cAMP, the intermittent injection would cause a transient increase after each injection whereas we think with the continuous injection that there may be a lesser increase in cAMP and that it may never return to basal but will stay slightly above basal throughout the infusion period. What we then think might happen in terms of particular proteins is that, with intermittent injections, there is a ramping up with each injection adding to the previous increase until there are much greater levels with each repeated injection. This is in contrast, we think, to the continuous infusion where you may observe an initial increase and this might slowly decline over the infusion period. We think these are the reasons that the result is greater bone formation versus resorption in the intermittent injections compared with the continuous infusion where there is more bone resorption than formation.[7]

To address this, we have undertaken microarrays in animals receiving PTH peptides by intermittent injections. We have also used different PTH peptides to try to determine which signal transduction pathway is responsible for the hormone's anabolic effect. We found that PTH (1-34) and (1-31) had the same stimulation of bone formation, in contrast to PTH (3-34), which does not. The histomorphometry shows a significant increase in the number of osteoblasts for both PTH (1-34) and (1-31) as well as an increase in the number of osteoclasts. This does not happen with PTH (3-34) although there is a trend toward an increase in both these parameters compared with the controls. When we examine the microarrays for the animals that have been injected for 14 days with these peptides we find that the set of genes that were regulated with PTH (1-34) and (1-31) overlap extensively, while there are much fewer numbers of genes regulated by PTH (3-34). Most of these are common to PTH (1-31) and (1-34). Thus, it appears that the gene expression profile is regulated by the PKA pathway rather than the intracellular calcium or PKC pathway. We have now done some preliminary work where we have given continuous injections of PTH (1-34) to 3-month-old female rats and undertaken microarrays. This is work in progress at present. We find that there are large numbers of genes regulated by the intermittent injections as well as to the continuous infusion. This is in significant contrast to the work published recently by Onyia *et al*.[8] where they found very few genes regulated by the intermittent injection. However, it should be noted that in that latter work they removed the bones of the rats 24 hours after the last PTH (1-34) injection and, it seems, immediately after

TABLE 1. Unique Genes Regulated by Intermittent PTH(1-34)

Amphiregulin	Jagged1	Syndecan 4
MKP1	CREM	Cox-2
p21	CEB/Pβ	MCP-1

the cessation of the PTH (1-34) infusion. In contrast, we removed the bones immediately after the end of the infusion and 1 hour after the last intermittent injection of PTH peptides. This is because of the observations we already had from the microarray of the osteoblastic cells as well as the data studying specific genes *in vivo*. We found significant stimulation of many genes 1 hour after the last PTH injection.

There are many genes also commonly regulated by both intermittent and continuous PTH treatment. Generally, we find that intermittent injection stimulates the expression of these genes to a greater level than that seen by the continuous injection. It is notable that RANK ligand is stimulated about sevenfold 1 hour after the last injection with PTH whereas with continuous infusion we see about a twofold stimulation.

Intermittent injection of PTH (1-34) also regulates many unique genes, and some of these are shown in TABLE 1. It should be noted that there are a number of genes that we have observed in the microarrays of the UMR cells, for instance, amphiregulin, MKP1, p21, Jagged1, and others. A notable gene, monocyte chemoattractant protein-1 (MCP-1), is regulated in the microarrays from the intermittent injections.

MCP-1 is a member of the CC chemokine family and has a characteristic –C-C- structure. We examined its expression in rats injected with PTH daily for 14 days. We found, again, that this is a gene that is regulated extremely rapidly with maximal expression 1 hour after the injection. As the injections increase we found a ramping-up of MCP-1 expression. After 14 days we observe almost a 250-fold stimulation of expression of MCP-1 mRNA in the distal femur. We think PTH acts on the mature osteoblast and stimulates the production of MCP-1 but this then has a number of roles acting on monocytes, preosteoclasts, and mature osteoclasts.

In summary, we think that PTH acts on the differentiating or mature osteoblast through the PKA pathway, causing expression of genes such as MKP1 or p21 that cause the differentiating or mature osteoblast to become terminally differentiated and to cease further proliferation. The hormone causes this post-proliferative osteoblast to express a number of growth factors and chemokines, amphiregulin, for instance, which stimulates the proliferation of bone marrow stem cells and preosteoblasts to then increase their numbers and at a later stage to become larger numbers of osteoblasts. We also think that PTH stimulates the expression of Jagged1, which has already been shown to stimulate the proliferation of hematopoietic stem cells and we think this is also involved with stimulating the proliferation of mesenchymal stem cells and preosteoblasts.

Finally, we think PTH stimulates the production of the chemokine MCP-1, which we think is involved with recruiting preosteoclasts, fusion of mononuclear osteoclasts, and possibly activating resident osteoclasts. This latter effect may be part of the requirement for osteoclast action in PTH's anabolic effect since it has been implicated in the clinical trials where bisphosphonates attenuated the anabolic effect of PTH.[9,10]

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