Parathyroid hormone potentiates the effect of insulin-like growth factor-I on bone formation

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Abstract. Insulin-like growth factor-I and parathyroid hormone are both known regulators of bone formation. In this study, human recombinant IGF-I and bovine PTH (1–34) and their combination were studied for their effects in vitro on the proliferation of embryonic chick osteoblast-like cells (osteoblasts) and in vivo on bone formation in normal rats. Osteoblasts from 17-day-old chick embryos were cultured in serum-free BGJb medium containing 0.1% bovine albumin. After 2 days, IGF-I and/or PTH were added. Twenty-four hours later [³H]thymidine incorporation into trichloroacetic acid precipitable material was quantified as an index of cell proliferation. This has previously been shown to reflect actual cell division. IGF-I at doses ranging from 0.85 to 13.6 nmol/l caused a dose-dependent increase in [³H]thymidine incorporation into osteoblasts. PTH alone (10 to 1000 pmol/l) had no significant effect. However, when combined with IGF-I, PTH potentiated the mitogenic effect of IGF-I and achieved statistical significance at 30 and 100 pmol/l (p < 0.05). This potentiation was also studied in vivo. The right hindlimbs of rats weighing 150 g were infused intra-arterially by an osmotic minipump with graded doses of IGF-I (0.1 to 0.4 nmol/day) and/or PTH (0.27 nmol/day) for 7 days. The rate of trabecular bone apposition (formation) was measured by double tetracycline labelling and compared with the contralateral uninjured limb which acted as the control. Histomorphometric data revealed that neither IGF-I nor PTH alone had a significant effect on trabecular bone apposition rate compared with control limbs. The co-infusion of IGF-I (0.4 nmol/day) and PTH (0.27 nmol/day) resulted in a marked increase in trabecular bone apposition rate. The results of 2 studies were significant at p < 0.01. These data suggest that PTH potentiates the effect of IGF-I on bone formation both in vivo and in vitro.

Insulin-like growth factors are anabolic polypeptides that are the major regulators of skeletal growth (1). There are two principal IGFs, IGF-I and IGF-II. They are growth hormone-dependent, have insulin-like activity, and are transported in plasma in an inactive form bound primarily to a large molecular weight carrier protein (2).

There is considerable evidence that IGF-I affects longitudinal bone growth. Clinically, IGF-I deficiency or cellular resistance to its actions results in decreased bone growth and sustained IGF-I excess in childhood results in acro-gigantism (1, 9). Experimentally, Russell & Spencer, Schlechter et al., and Isgaard et al. demonstrated that intra-epiphysial injection or unilateral perfusion of IGF-I into the rat hindlimb stimulated longitudinal bone growth in hypophysectomized rats (4–6). Schlechter et al. showed than antibodies to IGF-I blocked the increase in tibial epiphyseal width stimulated by growth hormone (7). Hizuka et al. reported an increase in tibial epiphyseal width of intact rats by sc injection of IGF-I (8). However, these are effects on enchondral bone function. The action of IGF-I on cortical and trabecular bone formation has not been described.

IGF-I has been shown in vitro to have anabolic effects on osteoblasts. Canalis first reported that high doses of IGF-I stimulated DNA, RNA, collagen, and non-collagen protein synthesis by 21-day-old fetal rat calvaria (9). Schmid et al. also described mitogenic effects of relatively high levels of
IGF-I on rat osteoblasts (10). Subsequently, Howard & Spencer used cultured embryonic chick calvarial osteoblasts to show that at physiologic doses IGF-I stimulated DNA and collagen synthesis (11). It was not clear if the in vitro data could be extrapolated to in vivo trabecular and cortical bone formation. The action of IGF-I on osteoclasts is of particular concern. Although we know that IGF-I slightly decreases osteoclast numbers, an effect on activity has not been explored (12).

Parathyroid hormone is known to stimulate cortical and trabecular bone formation and resorption (13–15). Selective stimulation of bone formation has been achieved under certain conditions, yet the precise mechanism of PTH’s anabolic effect remains unclear. In a preliminary in vitro study, Howard & Spencer suggested that PTH at a physiologic concentration was ineffective alone, but together with IGF-I, stimulated the proliferation of embryonic chick osteoblasts (11). Because only one PTH concentration was examined, this interaction was re-investigated to establish in vitro dose-response relationships.

To examine the interaction in vivo a new model was used, the continuous intra-arterial perfusion of the right hindlimb in conscious, unrestrained rats. Unless the dose is excessive, this technique can significantly increase the local level of the infused agent, but not the systemic level. This is because there is a 15-fold dilution when the test agent enters the venous circulation and renal clearance of peptides is very rapid. Counterregulatory mechanisms do not complicate interpretations and the contralateral limb can serve as the control. Using this model we obtained in vivo data consistent with PTH potentiating the effect of IGF-I on trabecular bone formation, which are supported by in vitro studies on osteoblast proliferation.

Material and Methods

**Hormones and chemicals**

Bacterial-derived human recombinant IGF-I was obtained from Creative Biomolecules Inc, Hopkinton, MA. Bovine PTH (1–34), tetracycline, demeclocycline were purchased from Sigma Chemicals, St. Louis, MO. Porcine sodium heparin (1000 USP units/ml) was supplied by LyphoMed, Rosemont, IL.

**Tissue culture supplies**

Hanks Balanced Salt Solution, Fitton-Jackson Modified BGJb medium, Minimal Essential medium (MEM), magnesium and calcium free MEM, penicillin, streptomycin, and trypsin: EDTA were supplied by the Tissue Culture Facility of the University of California, San Francisco. Bacterial collagenase II was purchased from Worthington Biochemical Co, Freehold, NJ. [³H]thymidine (60 Ci/mm), and Ecolume were purchased from ICN Co, Irvine, CA. Fertilized chick eggs were supplied by Feather-hill Farm, Petaluma, CA at day 16 and kept in a humidified incubator at 37°C until used.

**Animals**

Female Sprague-Dawley rats, 150–170 g. 6–7 weeks old were supplied by Simonsen Laboratories, Inc, Gilroy, CA. They were housed in individual hanging cages with a 12-h light and dark cycle and were fed with Purina Rat Chow pellets and tap water ad libitum.

**Preparation of chick calvarial osteoblast-like cells**

Osteoblasts and osteoblast precursor cells were isolated from 17-day-old chick embryos according to our previously published procedure (11). The isolation and culture media contained no hormones or vitamins other than the experimental agents. Briefly, calvaria were aseptically dissected, rinsed, and incubated at 37°C with shaking in MEM containing penicillin (100 kU/l), streptomycin (69 μmol/l), bovine serum albumin (15 μmol/l), and collagenase (2 g/l). After 20 min. the incubation solution was discarded, which rinsed the calvaria of mainly fibroblast-like cells, and fresh solution was added. The incubation was continued for an additional 100 min. Cells were harvested, washed three times with MEM, and resuspended in serum-free Fitton-Jackson modified BGJb medium containing penicillin (100 kU/l), streptomycin (69 μmol/l), and bovine serum albumin (15 μmol/l). Cell number was determined by counting an aliquot with a hemocytometer. Cells harvested with this procedure were found to be predominantly osteoblasts which exhibit high alkaline phosphatase activity and increased cAMP production following PTH treatment (11).

**Determination of DNA synthesis**

The cells were plated at a density of 5 × 10⁴ cells per 16 mm well in Corning 24-well plates and incubated in an atmosphere of 5% CO₂ in air. After 48 h, the medium and unattached cells (mainly red blood cells) were removed, and replaced by fresh serum-free medium. Graded doses of test agents were then added to the culture. Twenty-four hours later [³H]thymidine (2 mCi/l) was added, and the culture incubated for 2 h. The 74-h residence in serum-free media and the 2-h [³H]thymidine pulse have previously been established to be the optimum times (11). After aspiration of the radioactive medium, the cells were washed twice with phosphate buffered saline, pH 7.4, and twice with 10% TCA. One ml of 0.1 mol/l HCl was

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added to each well to dissolve the TCA precipitable material. An aliquot from each well was quantified for radioactivity by liquid scintillation spectroscopy using Ecolorm as the scintillation fluid. Data were expressed as the percent radioactivity of the unstimulated culture.

**Hindlimb perfusion procedure**

**Hormone solution.** Test agents were dissolved in 10 mmol/l TRIS buffer, pH 8.0, containing 1.6% glycerol, 0.02% sodium azide, and 100 USP kU/l heparin to prevent absorption of hormones and clotting of the catheter.

**Catheterization.** The rats were weighed twice weekly. Sixteen hours before surgery, female rats were given 15 mg/kg tetracycline ip for fluorescent labelling of bone. The next day the rats were anesthetized with 0.1 ml/100 g of a solution containing ketamine HCl, xylazine, and acepromazine maleate (1:2:0.06) and a tributary of the right iliac artery was catheterized. The details of this technique were previously described (5). Briefly, a tapered polyethylene catheter connected to an Alzet osmotic minipump (Model #2001, Alza, Palo Alto, CA) filled with test agent(s) was inserted into the right superior vesical artery, and advanced until the tip just reached the junction of the iliac artery. It was ligated in place and additionally secured by a drop of cyanoacrylate glue (Krazy Glue, Itasca, IL). The minipump was left in the abdominal cavity which was closed with silk sutures. Six days later the second fluorescent label, demeclocycline (15 mg/kg), was given ip. The next day, rats were sacrificed by exsanguination. The continuity of the catheters was checked, the minipumps removed, and the residual fluid estimated.

**Measurements.** Both limb bones were carefully dissected free, the left serving as a control for the perfused right hindlimb. The tibias were kept in ice-cold phosphate buffered formalin for subsequent histomorphometric examination. In the last experiment (repetition of PTH study), femora were used for analysis because they could withstand more stress during bone processing. Blood samples, taken two days before the surgery and on the day of sacrifice, were assayed for PTH, calcium, and phosphorus.

**Bone histology**

After formaldehyde fixation, bones were processed in the following way (11). A segment of the proximal tibia (about 1.2 cm from the end) was cut with a Gillings-Hamco diamond saw and trimmed without demineralization to expose trabecular bone to facilitate tissue processing. The bone was then washed in 70% ethanol, dehydrated through 80%, 95%, and 100% ethanol, infiltrated under vacuum, and embedded in methyl methacrylate using a low-temperature embedding technique (18). Five μm longitudinal sections were obtained from the middle of the bone and mounted without staining for measurements of tetracycline labels. The new bone deposited on the trabecular bone surface, reflecting osteoblast activity, was measured under ×250 magnification from secondary spongiosa directly below primary spongiosa using Sigma Plot (Jandel Scientific, Sausalito, CA) and a digitizing tablet (Summagraphics, Fairfield, CT) interfaced with an IBM computer. The mean double-tetracycline label width calculated from 8–33 measurements on trabecular bone was then obtained and divided by 7 days to determine the rate of trabecular bone apposition.

**Serum PTH, calcium, and phosphorus measurements**

Serum immunoreactive PTH (iPTH) was measured by Nichols Institute, San Juan Capistrano, CA, employing an N-terminal specific radioimmunoassay. Serum calcium and phosphorus were determined by a Hitachi 705 multichannel analyzer.

**Statistical analysis**

For [3H]thymidine incorporation data, the mean ± SEM of 4 cultures was calculated and analysed using 1- or 2-way ANOVA followed by Student’s Newman-Keuls test. For the hindlimb perfusion studies, data were reported as means ± SEM. Statistical differences were evaluated with Student’s t-test for paired observations.

![Fig. 1](https://example.com/fig1.png)

Stimulation of DNA synthesis in chick osteoblast-like cells by IGF-I. Calvaria cells were incubated (see Materials and Methods) with various concentrations of bacterial-derived human recombinant IGF-I. [3H]thymidine (TdR) incorporation was quantified to determine DNA synthesis. Each bar is the mean of 4 cultures (±1 SEM) presented as percent of DNA synthesis in cultures without added IGF-I (control). The significance is represented as *p < 0.05 vs control and **p < 0.01 vs control.
Results

Cultured embryonic chicken osteoblasts

Fig. 1 shows that IGF-I caused a dose-related stimulation of the incorporation of [3H]thymidine into the chick osteoblast-like cells. Previous experiments have established that [3H]thymidine incorporation was positively correlated to cell number over the range of IGF-I concentrations used in this study (11, 16, 17). The effect of IGF-I was significant at concentrations as low as 1.7 pmol/l (p < 0.05), and was maximal at 13.6 pmol/l (p < 0.01). It should be noted that the actual IGF-I concentrations in the wells may be lower than those indicated above due to possible degradation and/or adherence to the walls of the peptide. In 4 experiments stimulation was always obtained.

Fig. 2 shows that PTH alone had no effect on [3H]thymidine incorporation into chick osteoblast-like cells at concentrations ranging from 10 to 1000 pmol/l. However, PTH markedly potentiated the mitogenic effect of IGF-I. Statistical analysis revealed a significant interaction between the two hormones (ANOVA, p < 0.01). The potentiation was observed over a range of two log doses (10 to 1000 pmol/l), with 30 and 100 pmol/l PTH achieving statistical significance (p < 0.05). These results were also observed in two other experiments.

Hindlimb perfusion experiments

The unilateral arterial infusion of IGF-I into rat hindlimbs with doses ranging from 0.1 to 0.4 nmol/day failed to cause any effect on the tibial trabecular bone apposition rate determined histomorphometrically (Table 1). There was no change in the tibia length during the 7-day infusion period (data not shown).

The infusion of 0.27 nmol/day PTH raised the serum level of PTH from 0.35 to 0.56 pmol/l (normal range 0.27 to 0.81), but was ineffective in stimulating bone formation in the metaphysis of the proximal tibia (Fig. 3). Co-administration of 0.4 nmol/day of IGF-I and 0.27 nmol/day of PTH, however, caused a marked increase in the trabecular bone apposition rate. In a second experiment the femora were analysed instead of the tibia. Again,

Effect of IGF-I and bovine PTH (1–34) on chick osteoblast-like cells. Calvarial cells were incubated with bacterial-derived human recombinant IGF-I, bovine PTH (1–34), or both. [3H]thymidine (TdR) incorporation was determined to quantify DNA synthesis. Each bar is the mean of 4 cultures (± 1 s.e.m indicated) presented as percent of DNA synthesis in cultures without the addition of test substances (control). The concentration of bovine PTH (1–34) used is indicated in pmol/l as control ■, 10 □, 30 ▲, 100 △, and 1000 □. A significant difference from IGF-I only-treated cultures is indicated by *p < 0.05 or **p < 0.01 within each IGF-I concentration.
there was a significant increase in the trabecular bone apposition rate caused by the co-administration of the two hormones. The combined data from these two experiments is presented in Fig. 3. The increase was significant at p < 0.01.

The serum calcium and phosphorus levels were depressed after IGF-I and IGF-I plus PTH administrations (data not shown). Since sham-operated animals also have depressed levels, these changes are probably related to the effects of anesthesia and surgery.

Discussion

Physiologic, non-mitogenic concentrations of PTH significantly potentiated the mitogenic effect of IGF-I on cultured chick embryonic osteoblast-like cells. In vivo this potentiation between PTH and IGF-I was also demonstrated because co-arterial infusion of inactive doses of PTH and IGF-I into the rat hindlimbs caused a marked increase in the bone apposition rate. The synergism between IGF-I and PTH may explain the bone-stimulating effects of PTH in both intact and thyroparathyroidectomized rats (19–20). PTH could potentiate the circulating IGF-I and enhance the anabolic effect of IGF-I on bone tissues.

The inability of physiologic PTH concentrations to affect the proliferation of osteoblasts in vitro replicates our earlier finding (11). The discrepancy with previous studies that described stimulation may be due to their using: 1. pharmacologic concentrations (nanomolar) of PTH; 2. different model systems (rat osteoblasts or tumour cell lines); and 3. culture media with serum, which contains IGF-I and other growth factors (21–23). The maximum potentiation occurred at 30 pmol/l of PTH. The reason why higher concentrations were less effective is unknown. Stimulatory effects that have a maximum are commonly observed with growth factors, and presumably result from competing biochemical actions and/or down regulation of receptors. At higher concentrations PTH

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**Table 1.**

Effect of IGF-I infusion on bone formation of young, female rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tibia Trabecular bone apposition rate (μm/day ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I, 0.1 nmol/day × 7 days (N = 3)</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>IGF-I, 0.2 nmol/day × 7 days (N = 3)</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>IGF-I, 0.4 nmol/day × 7 days (N = 3)</td>
<td>0.96 ± 0.01</td>
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</table>

* The average of 8–33 measurements. b Infused limb.
may stimulate differentiated functions via cAMP which has been associated with inhibition of proliferation. It would be of interest to observe the effect of PTH and IGF-I on a differentiated function such as collagen synthesis.

Although IGF-I stimulated [3H]thymidine incorporation into cultured chick cells, it did not augment trabecular bone apposition rate in vivo in rats under our experimental conditions. This is because the bones of young, rapidly growing rats cannot easily be stimulated to grow faster, whereas the bones of older rats can (unpublished data). Yet young bones are continually responding to endogenous IGF-I, as evidence by decreased bone growth when IGF-I action is deficient or blocked (1, 3, 7). It requires the potentiating action of PTH to elicit an effect of additional IGF-I in young rats.

The mechanism of this interaction between IGF-I and PTH is currently being studied. PTH could potentiate the mitogenic effect of IGF-I by increasing the affinity and/or number of IGF receptors. IGF receptors have been reported to be modulated by various agents. Cortisol has been shown to increase IGF-I binding to cultured subconfluent rat osteoblasts and platelet-derived growth factor has been reported to up-regulate type 1 receptors on fibroblasts (24, 25). Insulin up-regulates type 2 receptors and down-regulates type 1 receptors (26–28). PTH could act by promoting the differentiation of osteoprogenitor cells into mature osteoblasts and thus increase the number of target cells for IGF-I. In this regard, PTH has been shown to promote the differentiation of rabbit costal chondrocytes, whereas IGFs stimulate the synthesis of proteoglycan, a major component of the cartilage matrix, in these differentiated cells (29). Theoretically, PTH could also influence IGF post-receptor events. For example, PTH has been shown to act via cAMP, CA++, and/or inositol triphosphate (30–32). These second messengers could in turn cause the phosphorylation of IGF-I receptors and augment the effect of IGF-I. Conversely, IGF-I may act on PTH receptor and post-receptor signaling. A common feature is these interactions could be the guanine nucleotide binding proteins (G proteins). The G proteins have come into prominence as possible major regulators of hormone and growth factor actions. The cAMP response to PTH has been reported to be dependent on G proteins (33). Glucocorticoids have been suggested to augment PTH-stimulated adenylate cy-

clase by facilitating transmembrane signalling in addition to increasing receptor number (34). Since IGF-I decreases PTH-simulated osteoblast cAMP, IGF-I may also act on G proteins (unpublished data). The effect of IGF-I on cAMP may steer cells into the proliferative pathway and away from differentiated functions. Conversely, pertussis toxin, which increases intracellular CAMP, probably by binding to G proteins, inhibits the mitogenic effect of IGF-I (35).

The potentiation between IGF-I and PTH probably does not extend to bone resorption, because IGF-I causes a slight decrease in osteoclast number (12). However, the effect of IGF-I on osteoclast activity has not been reported. Since PTH and IGF-I affect collagen synthesis in different directions, the net effect of the combination is unknown. The ability of IGF-I to inhibit PTH-stimulated adenylate suggests that increased synthesis of collagen would prevail.

Since the combination of IGF-I and PTH stimulate new bone formation, these two agents may be used clinically in treating or preventing the decrease in bone mass characteristic of osteoporosis. At present, treatment is unsatisfactory for the disease. Estrogen and calcitonin may be effective in halting the bone loss but are not effective in stimulating bone formation. Fluoride administration has been shown to stimulate new bone formation in vivo and possibly in vitro (36–38). However, when tested clinically, fluoride treatment did not reduce the hip fracture incidence of the patients (36). Furthermore, these treatments may have intolerable side effects, which frequently result in cessation of therapy. The positive anabolic interaction between IGF-I and PTH found in this study could be the basis of future therapy for osteoporosis.

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References


17. Farley JR, Tarbaux NM, Vermeiden JPW, Baylind DJ. In vitro evidence that local and systemic skeletal effectors can regulate [3H]-thymidine incorporation in chick calvarial cell cultures and modulate the stimulatory action(s) of embryonic chick bone extract. Calcif Tissue Int 1988; 42: 23–33.


30. Lowik CW, Van Leeuwen JP, Van der Meer JM, Van Zeeland ZK, Scheven BAA, Herrmann-Erlee MPM. A


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