Estrogen modulates osteoblast proliferation and function regulated by parathyroid hormone in osteoblastic SaOS-2 cells: role of insulin-like growth factor (IGF)-I and IGF-binding protein-5

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Abstract

Although there is clinical evidence showing that combined therapy with parathyroid hormone (PTH) and estrogen is additively effective in increasing the bone mass of patients with osteoporosis, the mechanism of the interaction between these hormones remains unclear. The present study was performed to determine whether estrogen would affect osteoblast proliferation and function modulated by PTH in human osteoblastic SaOS-2 cells. Human PTH-(1–34) significantly inhibited [3H]thymidine (TdR) incorporation, which was attenuated by 24 h pretreatment with 10–10 to 10–7 M 17β-estradiol (17β-E2) in a concentration-dependent manner. PTH significantly stimulated alkaline phosphatase (ALP) activity, collagen synthesis and type-I procollagen mRNA expression after pretreatment with 17β-E2 in these cells. Tamoxifen, an anti-estrogen, antagonized these 17β-E2-induced effects. Pretreatment with insulin-like growth factor-I (IGF-I) mimicked estrogen action, and coincubation of 3 μg/ml anti-IGF-I antibody antagonized the effects of 17β-E2 as well as those of IGF-I. In the presence of 17β-E2 pretreatment, PTH strongly stimulated IGF-binding protein (IGFBP)-5 mRNA expression in these cells, and recombinant IGFBP-5 increased type-I procollagen mRNA expression and ALP activity. In conclusion, estrogen attenuates PTH-induced inhibition of osteoblast proliferation and PTH stimulates osteoblast function in the presence of estrogen pretreatment. IGF-I and/or IGFBP-5 seemed to be involved in the estrogen-induced modulation of PTH action on osteoblast proliferation and function.

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Introduction

There has been a recent improvement in the treatment of patients with osteoporosis. Although it is necessary to develop drugs that increase bone mass for severe osteoporosis, there is no good drug that considerably increases bone mass. The drugs for the treatment of osteoporosis include reagents that stimulate bone formation and reagents that inhibit bone resorption. Combining both types of drug is considered to be a reasonable approach in the treatment of patients with severe osteoporosis.

Parathyroid hormone (PTH) possesses bi-directional actions, i.e. it is bone-catabolic and bone-anabolic. The intermittent administration of PTH increases bone mass, whereas continuous infusion causes a decrease (Dempster et al. 1993). On the other hand, estrogen inhibits bone resorption by directly inhibiting osteoclast activity as well as by decreasing the production of cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α and IL-6 from stromal cells (Oursler et al. 1991b, Jilka et al. 1992, Pacifi ci 1996). Combined therapy with PTH and estrogen in ovariectomized rats and in osteoporotic patients results in better improvements in bone mass than is achieved using either agent alone (Bradbeer et al. 1992, Shen et al. 1993, 1995). We previously reported that estrogen inhibits PTH-stimulated osteoclast-like cell formation by selectively inhibiting the cAMP-dependent pathway (Kaji et al. 1996). Estrogen maintains bone volume in rats not only by suppressing bone resorption but also by stimulating bone formation (Chow et al. 1992). Several pieces of evidence (Ernst et al. 1989, Bellido et al. 1993, Ikegami et al. 1993) indicate that estrogen has a bone-anabolic function that directly affects osteoblasts. Although a few reports have indicated that there is an interaction between PTH and estrogen in osteoblasts (Rao et al. 1994, 1996, Kudo et al. 1995, 1996), the mechanism has not been clearly defined. The rate of bone formation is largely determined by the number of osteoblasts (Parfitt 1990) and it was widely believed that the anabolic effect of PTH was the result of increased osteoblast differentiation (Dempster et al. 1993). We therefore examined the effects of the interaction between PTH and estrogen on osteoblast proliferation, alkaline phosphatase (ALP) activity and type-I collagen synthesis, by using human osteoblastic SaOS-2 cells.
Insulin-like growth factor (IGF)-I regulates bone formation in autocrine and/or paracrine fashions (Raisz 1988). The production and/or secretion of IGF-I and transforming growth factor (TGF)-β have been shown to be modulated by estrogen and PTH (Ernst et al. 1989, Gray et al. 1989, Linkhart & Mohan 1989, McCarthy et al. 1989, Oursler et al. 1991a, Kudo et al. 1995). Oursler et al. (1991a), however, reported that coincubation with estrogen and PTH does not affect TGF-β1 mRNA expression or protein synthesis, whereas treatment with either agent alone significantly stimulates it in human osteoblast-like cells. On the other hand, estradiol stimulates IGF-I mRNA expression in RCT-3 cells and stimulates the secretion of IGF-I in UMR-106 cells (Ernst et al. 1989, Gray et al. 1989). PTH also stimulates the release of IGF-I in neonatal mouse calvariae and enhances the transcript and polypeptide levels of IGF-I in osteoblast-enriched cultures from fetal rat bone (Linkhart & Mohan 1989, McCarthy et al. 1989). Moreover, it seems likely that IGF-I partly mediates the anabolic action of PTH in bone (Canolis et al. 1989, Ishizuya et al. 1997). However, the role of IGF-I in the modulation of PTH action by estrogen remains unknown.

The present study, therefore, was performed to examine how estrogen might modulate PTH action in osteoblasts and to investigate the possible role of IGF-I in the actions of estrogen.

Materials and Methods

Materials
SaOS-2 cells were a generous gift from Dr T J Martin (Melbourne, Australia). The 17β-estradiol (17β-E2), 17α-E2 and tamoxifen were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The human (h) PTH-(1–34) was obtained from the Peptide Institute, Inc. (Osaka, Japan), the rabbit anti-human IGF-I immunglobulin G fraction and the recombinant human IGF-binding protein (IGFBP)-5 were from Austral Biological (San Ramon, CA, USA), the recombinant human IGF-I was from Life Technologies, Inc. (Gaithersburg, MD, USA) and the bacterial collagenase was from the Advance Biofactures Co. (Lynbrook, NY, USA). All other chemicals were of analytical grade.

Cell culture
SaOS-2 cells were maintained in Dulbecco’s Modified Eagle’s Medium containing 10% fetal calf serum in a 5% CO₂:95% air atmosphere at 37 °C. Cells were passaged weekly using a 0.05% trypsin/0.02% EDTA solution.

The culture medium was changed to serum-free, phenol-red-free medium for 12 h before the start of an experiment, because of the existence of the intrinsic estrogenic activity in phenol red. Pretreatment with 17β-E2, 17α-E2 or IGF-I at the concentrations given in the Results was performed for 24 h. Although pretreatment with these reagents was performed for the same incubation time for each assay, different incubation times were used for PTH stimulation.

β²H/Thymidine incorporation (TdR)
After preincubation with or without 17β-E2 or 17α-E2, hPTH-(1–34) was added. Twenty-two hours after incubation with the indicated concentration of substances, cells were pulsed with methyl-[³H]thymidine (Amersham Japan, Tokyo, Japan) (1 μCi/ml). The reagents were removed before the addition of methyl-[³H]thymidine in this assay. Three hours later, the incubation was terminated by removal of the medium and the addition of 5% trichloroacetic acid (TCA). After removal of the TCA, the precipitated layer was washed with ethanol and the wells were desiccated at room temperature. The residue was dissolved in 20 mmol/l NaOH and 1% sodium dodecyl sulfate (SDS) and scintillation cocktail was added. Each sample was counted in a liquid scintillation counter.

Assay of ALP activity and DNA content
After preincubation with or without 17β-E2, 17α-E2, or IGF-I, hPTH-(1–34) was added. Forty-eight hours after incubation of nearly confluent cultures with the indicated concentrations of substances, the cells were rinsed three times with phosphate-buffered saline and then 600 μl distilled water was added to each well. The DNA-assay procedure of Labarca & Paigen (1980) was employed. This method is based upon the enhancement of fluorescence that occurs when bisbenzimidazole binds to DNA. Calf-thymus DNA was used as a standard. Our preliminary experiments revealed a linear correlation between the DNA contents of the cells and the cell numbers. ALP activity was assayed at 37 °C by a method modified from that of Lowry et al. (1954). The assay mixtures contained 0.1 mol/l 2-amino-2-methyl-1-propanol, 1 mmol/l MgCl₂, 8 mmol/l p-nitrophenyl phosphate disodium and cell homogenates. After 30 min incubation, the reaction was stopped with 0.1 N NaOH and the absorbance was read at 405 nm. Standard curves were prepared with p-nitrophenol.

Collagen synthesis
After preincubation with or without 17β-E2, 17α-E2 or IGF-I, hPTH-(1–34) was added. Twenty-four hours later, the cells were pulsed with [³H] proline (2 μCi/ml) in the presence of β-aminopropionitrile (0.5 mmol/l) and ascorbic acid (0.5 mmol/l). Three hours later, the incubation was terminated by removal of the medium and the addition of 10% TCA. The cells were scraped by

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a rubber policeman and transferred to tubes. Protein was extracted by the addition of 10% TCA and acetone. Desiccated protein was digested by collagenase at 37 °C for 90 min. The supernatant was removed and scintillation cocktail added. Samples were counted in a liquid scintillation counter. Collagen-digestible protein (CDP) and non-collagen protein (NCP) were measured. Collagen synthesis (%) was calculated after multiplying NCP by 5.4 to correct for the relative abundance of proline in collagen and NCP (Peterkofsky 1972).

**Northern hybridization**

Total RNA was extracted from cultured cells by the use of the acid guanidinium-thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). Polyadenylated (poly(A)+) mRNA was subsequently fractionated using oligo (dT)-Latex. Twenty micrograms total RNA or 8 µg poly(A)+ mRNA was denatured, run on a 1% agarose gel containing 2% formaldehyde, then transferred to a nitrocellulose membrane and hybridized to a 32P-labeled DNA probe overnight at 42 °C. The hybridization probe was the 2·8 kb EcoRI fragment of the α1 gene of type-1 procollagen (a gift from Dr T Kimura, Osaka University, Japan), the 6·0 kb Stul–SacII–HindIII fragment of the rat IGFBP-5 cDNA (kindly provided by Dr Shunichi Shimazaki, The Whitter Institute, La Jolla, CA, USA) or the 2·1 kb HindIII–BamHII fragment of human PTH/PTH-related protein (PTHrP) receptor cDNA (kindly provided by the Asahi Chem. Co., Shizuoka, Japan). A 1·2 kb β-actin cDNA was used as a reference. After hybridization, the filter was washed twice with 2× standard saline citrate (SSC) containing 0·5% SDS and subsequently washed twice with 0·1 × SSC containing 0·5% SDS at 58 °C for 1 h.

**Statistics**

Data were expressed as means ± s.e.m. The data used in the figures were representative of at least three separate cell preparations. Similar results were obtained from other cell preparations. A statistical analysis was performed using the Student’s t-test or Duncan’s multiple range test.

**Results**

We first examined whether PTH would affect osteoblast proliferation in SaOS-2 cells pretreated with estrogen. As shown in Fig. 1, PTH at 10⁻⁸ mol/l significantly inhibited TdR, but 24 h pretreatment with 17β-E₂ attenuated the inhibition of TdR by PTH in a concentration-dependent manner; the minimum effective concentration was 10⁻¹⁰ mol/l. Tamoxifen (an antagonist
of estrogen) at $10^{-6}$ mol/l, antagonized the $17\beta$-E$_2$-induced effect, and $17\alpha$-E$_2$ (a less-effective estrogen analog) at $10^{-8}$ mol/l did not affect it (Fig. 2).

Next, we examined whether PTH would affect type-I collagen and ALP activity in SaOS-2 cells with or without estrogen pretreatment. As shown in Fig. 3, PTH at $10^{-8}$ mol/l did not affect the mRNA expression of type-I procollagen without $17\beta$-E$_2$ pretreatment. In contrast, in SaOS-2 cells pretreated with $17\beta$-E$_2$ at $10^{-8}$ mol/l, PTH stimulated the expression. Tamoxifen at $10^{-6}$ mol/l antagonized this effect of $17\beta$-E$_2$; in these cells pretreated with $17\alpha$-E$_2$ at $10^{-8}$ mol/l, PTH slightly stimulated it, but much less so than $17\beta$-E$_2$. As shown in Figs 4 and 5, PTH did not affect ALP activity and collagen synthesis in SaOS-2 cells without $17\beta$-E$_2$ pretreatment, but in these cells pretreated with $17\beta$-E$_2$, PTH significantly stimulated them. Tamoxifen at $10^{-6}$ mol/l antagonized these effects of $17\beta$-E$_2$, but $17\alpha$-E$_2$ at $10^{-8}$ mol/l did not affect them.

Since $17\beta$-E$_2$ antagonized PTH-inhibited osteoblast proliferation and PTH stimulated osteoblast function only in the presence of $17\beta$-E$_2$ pretreatment, we examined the factors that might be involved in these effects. Kudo et al. (1995) showed, and we also confirmed (data not shown), that estrogen significantly increases the level of IGF-I in the conditioned medium from SaOS-2 cells. We therefore examined the role of IGF-I in the effects of $17\beta$-E$_2$ on the

![Figure 2](image1.png)

**Figure 2** Specificity of the effects of $17\beta$-E$_2$ on PTH-inhibited osteoblast proliferation. After 24 h preincubation with $10^{-8}$ mol/l $17\beta$-E$_2$, $10^{-8}$ mol/l $17\beta$-E$_2$ and $10^{-6}$ mol/l tamoxifen or $10^{-8}$ mol/l $17\alpha$-E$_2$, cells were treated with $10^{-8}$ mol/l PTH for 22 h. [$^3$H]thymidine incorporation was measured, as described in the Materials and Methods. Each bar represents the mean ± S.E.M. of six determinations. *P<0.01, compared with the control.

![Figure 3](image2.png)

**Figure 3** Effect of PTH on type-I procollagen mRNA expression in SaOS-2 cells pretreated with estrogen. After 24 h preincubation with vehicle (lanes 1 and 2), $10^{-8}$ mol/l $17\beta$-E$_2$ (lanes 3 and 4), $10^{-8}$ mol/l $17\beta$-E$_2$ and $10^{-6}$ mol/l tamoxifen (lanes 5 and 6), or $10^{-8}$ mol/l $17\alpha$-E$_2$ (lanes 7 and 8), cells were treated with vehicle (lanes 1, 3, 5 and 7), or $10^{-8}$ mol/l PTH (lanes 2, 4, 6 and 8) for 24 h, and total RNA (20 μg) was analyzed by Northern blotting using cDNA probes of procollagen and β-actin, as described in the Materials and Methods. The results of densitometric analyses (the ratios of procollagen:β-actin) are indicated below the Northern blot.
regulation of osteoblast proliferation and function by PTH in SaOS-2 cells. As shown in Fig. 6, IGF-I at 10 ng/ml mimicked the effect of 17β-E2 pretreatment on TdR, ALP activity and collagen synthesis. Anti-IGF-I antibody at 3 µg/ml antagonized the effects of 17β-E2 as well as antagonizing IGF-I.

IGFBP-5 and IGF-I are both produced by osteoblasts. Since IGFBP-5 potentiates IGF action (Andress 1995, Figure 4 Effect of PTH on collagen synthesis in SaOS-2 cells pretreated with estrogen. After 24 h preincubation with 10^{-8} mol/l 17β-E2, 10^{-8} mol/l 17β-E2 and 10^{-6} mol/l tamoxifen or 10^{-8} mol/l 17α-E2, cells were treated with 10^{-6} mol/l PTH for 24 h. CDP and NCP were measured and the percent collagen synthesis calculated, as described in the Materials and Methods. Each bar represents the mean ± S.E.M. of six determinations. *P<0.01, compared with the control.

Figure 5 Effect of PTH on ALP activity in SaOS-2 cells pretreated with estrogen. After 24 h preincubation with 10^{-8} mol/l 17β-E2, 10^{-8} mol/l 17β-E2 and 10^{-6} mol/l tamoxifen or 10^{-8} mol/l 17α-E2, cells were treated with 10^{-6} mol/l PTH for 48 h. ALP activity was measured, as described in the Materials and Methods. Each bar represents the mean ± S.E.M. of six determinations. *P<0.01, compared with the control.

Figure 6 The role of IGF-I in the effects of estrogen on PTH-modulated osteoblast proliferation and function. After 24 h preincubation with 10^{-8} mol/l 17β-E2, 10 ng/ml IGF-I, 10^{-6} mol/l 17β-E2 and 3 µg/ml anti-IGF-I antibody or 10 ng/ml IGF-I and 3 µg/ml anti-IGF-I antibody, cells were treated with 10^{-6} mol/l PTH. [3H]thymidine incorporation (A), collagen synthesis (B) and ALP activity (C) were measured, as described in the Materials and Methods. Each bar represents the mean ± S.E.M. of six determinations. *P<0.01, compared with the control.
Pretreatment with 17β-E2 inhibited PTH-induced cAMP production in SaOS-2 cells (Fukayama & Tashjian Jr, 1989), which raised the possibility that 17β-E2 down-regulates the level of PTH/PTHrP receptor expression in these cells. We therefore examined the effect of estrogen on PTH/PTHrP receptor mRNA expression in SaOS-2 cells. As shown in Fig. 8, 24 h treatment with 17β-E2 at 10^{-8} mol/l inhibited PTH/PTHrP receptor mRNA expression. Tamoxifen at 10^{-6} mol/l antagonized the 17β-E2-induced effect, and 17α-E2 at 10^{-8} mol/l slightly inhibited it, but much less so than 17β-E2. These findings indicate that estrogen specifically inhibits the level of PTH/PTHrP receptor expression in SaOS-2 cells.

**Discussion**

The present study revealed that PTH causes stimulation of osteoblast functions such as ALP activity and collagen synthesis in the presence of pretreatment with 17β-E2, although PTH did not affect them in the absence of estrogen pretreatment. A previous study (Rao et al. 1994) showed that simultaneous treatment with PTH and 17β-E2 stimulates ALP activity in SaOS-2 cells, whereas treatment with either one alone did not affect it. Our present results are compatible with those findings. In terms of osteoblast proliferation, pretreatment with 17β-E2 attenuated PTH-inhibited osteoblast proliferation. Both actions of 17β-E2 were unlikely to be non-specific, because 17α-E2 (a much less active estrogen analog) had no effect and tamoxifen (an anti-estrogenic agent) antagonized these 17β-E2-induced effects. How PTH affects osteoblasts remains unknown. PTH acts bidirectionally on osteoblasts. Since the inhibitory effects of PTH on osteoblast proliferation are considered to be bone-catabolic, blockade of the action of estrogen on the PTH effects will antagonize the catabolic action of PTH in SaOS-2 cells. On the other hand, PTH-induced ALP activity and type-1 collagen synthesis in the presence of estrogen pretreatment may be related to the bone-anabolic action of estrogen. The present findings may partly explain the mechanism by which PTH and estrogen have additive effects on bone formation.

The present study revealed that the effects of 17β-E2 on the regulation of proliferation, ALP activity and collagen synthesis by PTH were mimicked by IGF-I, and that anti-IGF-I antibody antagonized the effects of 17β-E2. These effects were not non-specific, because anti-IGF-I antibody antagonized the effects of IGF-I. The present findings, therefore, indicate that IGF-I is involved in the modulation of 17β-E2 effects on PTH action in osteoblast proliferation and function. Several IGFBPs modulate the action of IGF (Elgin et al. 1987, Feyen et al. 1991). Of these IGFBPs, IGFBP-5 is related to bone formation directly and/or indirectly via augmentation of IGF action.

Mohan et al. 1995), we examined whether IGFBP-5 could be involved in the effect of PTH on SaOS-2 cells pretreated with estrogen. As shown in Fig. 7A, PTH at 10^{-8} mol/l did not affect the gene expression of IGFBP-5 mRNA in SaOS-2 cells without 17β-E2 pretreatment. In contrast, in these cells pretreated with 17β-E2 at 10^{-8} mol/l, PTH stimulated the expression. In order to clarify whether IGFBP-5 could stimulate osteoblast function, we examined the effect of IGFBP-5 on the mRNA expression of type-1 procollagen: as shown in Fig. 7B, IGFBP-5 increased it. Moreover, IGFBP-5 caused a significant increase in ALP activity (control, 250·2 ± 5·3 pmol/min/µg DNA; IGFBP-5, 287·1 ± 4·8 pmol/min/µg DNA; P<0·01).
Conover et al. (1993) reported that PTH increases IGFBP-5 mRNA expression and its protein availability in UMR-106 cells. In addition, we recently reported that the transient treatment with PTH stimulates IGFBP-5 mRNA expression more effectively than the continuous treatment used in UMR-106 cells, and that IGFBP-5 would be partly involved in the anabolic action of PTH (Nasu et al. 1997). On the other hand, there has been some evidence that estrogen inhibits the PTH-induced increase in IGFBP-4 binding activity in SaOS-2 cells (Kudo et al. 1995). The present study revealed that PTH stimulates IGFBP-5 mRNA expression in SaOS-2 cells pretreated with 17β-E2 and that recombinant human IGFBP-5 increases both type-1 procollagen mRNA expression and ALP activity. These findings suggest that IGFBP-5 is partly involved in the 17β-E2-induced acceleration of PTH action on osteoblast function (although we did not have data about the protein level of IGFBP-5 and the modulation of IGF-I availability by IGFBP-5 in these cells).

The present findings indicate that estrogen specifically inhibits the level of PTH/PTHrP receptor expression in SaOS-2 cells. Goad & Tashjian Jr (1993) reported that IGF-I inhibits PTH-stimulated cAMP production by its action at the level of the PTH/PTHrP receptor in SaOS-2 cells. In addition, IGF-I suppressed PTH/PTHrP receptor expression via a mitogen-activated protein kinase pathway in UMR-106 osteoblast-like cells (Kawane & Horiuchi 1999). Taken together, the data indicate that the antagonism of 17β-E2 in PTH-induced inhibition of TdR is partly achieved by down-regulation of the PTH/PTHrP receptor by 17β-E2. We could not clarify why the PTH effects on proliferation were modified by 17β-E2 in a different manner to PTH effects on function. The previous study (Kudo et al. 1996) showed that 17β-E2 suppresses the inhibitory effect of PTH on osteoblastic activity by inhibiting the PTH-induced suppression of IGFBP-4 protease activity in SaOS-2 cells. Therefore, the different effects of 17β-E2 on osteoblast proliferation and function may be related to the different mediators, which may modify the effect of 17β-E2 and IGF-I.

We did not have the data on the interaction between 17β-E2 and PTH in osteoblastic cells other than SaOS-2 cells in the present study, although SaOS-2 cells were widely used to study the actions of PTH and/or estrogen in osteoblasts (Fukayama and Tashjian Jr 1989, Rao et al. 1994, Kudo et al. 1995, 1996). Therefore we cannot rule out the possibility that 17β-E2 modulates the effects of PTH differently in other osteoblast cell-lines. Further study is necessary to clarify this issue.

In conclusion, estrogen attenuated PTH-induced inhibition of osteoblast proliferation and PTH stimulated osteoblast function in the presence of estrogen pretreatment in osteoblastic SaOS-2 cells. IGF-I and/or IGFBP-5 seemed to be involved in the mechanism.

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