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Estrogen Inhibition of PTH-Stimulated Osteoclast Formation and Attachment *in Vitro*: Involvement of Both PKA and PKC

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Estrogens modulate the catabolic effects of PTH on bone *in vivo* and *in vitro*. PTH-stimulated cAMP accumulation in osteoblasts is thought to be linked to increased osteoclastic activity, but the precise mechanism is still unknown. In cocultures of clonal marrow stromal cells (MS1) and normal mouse spleen cells, both 1,25-dihydroxyvitamin D_3 and rat PTH (rPTH)-(1–34) can induce the formation of tartrate-resistant acid phosphatase- and calcitonin receptor-positive multinucleated osteoclast-like cells, which can attach to dentine slices and produce resorption pits. In this system, osteoclastogenesis stimulated by PTH, but not by 1,25-dihydroxyvitamin D_3, was suppressed by 17β-E2 (10^{-10}–10^{-8} M), whereas 17α-E2 (10^{-8} M) had no effect. Exposure to 10^{-8} M 17β-E2, but not 17α-E2, also significantly decreased the PTH-induced attachment of osteoclast-like cells to dentine slices. 17β-E2 inhibited osteoclast-like cell formation induced by 8-bromo-cAMP (10^{-4} M), 12-*O*-tetradecanoylphorbol 13-acetate (10^{-8} M), or rat PTH-(1–34) (10^{-7} M) in combination with either rp-adenosine-3′,5′-cyclic monophosphorothioate (10^{-4} M) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (10^{-5} M). 17β-E2 suppressed the partial stimulation of tartrate-resistant acid phosphatase-positive multinucleated osteoclast-like cell formation induced by [Arg^2]human (h) PTH-(1–34) (10^{-7} M) or hPTH-(3–34) (10^{-7} M), but not that caused by 10^{-7} M hPTH-(53–84). We conclude that estrogens suppress PTH-stimulated osteoclast-like cell formation by blocking both the cAMP-dependent PKA pathway and the PLC-coupled calcium/PKC pathway. In addition to inhibiting formation of osteoclasts and promoting their apoptosis, estrogen may regulate bone resorption by blocking attachment of osteoclasts to bone. (*Endocrinology* 143: 627–635, 2002)

ESTROGEN DEFICIENCY, leading to accelerated bone resorption, is the primary cause of postmenopausal osteoporosis. This is prevented by estrogen replacement therapy (1, 2), which indicates that the hormone plays a central role in the regulation of bone resorption. The negative regulation of bone resorption by estrogens is complex and may not be fully understood. Estrogens are known to inhibit the proliferation and differentiation of osteoclast precursors, possibly via actions on both hemopoietic osteoclast progenitors and stromal cells of the osteoblastic lineage (3, 4), but they also reduce the survival of mature osteoclasts by promoting their apoptosis (5, 6).

PTH is a major osteotropic factor that plays a critical role in calcium homeostasis and in regulating the rate of bone turnover. PTH stimulates marrow stromal cells or osteoblasts to produce soluble and membrane-associated factors that act upon osteoclast precursors to increase their proliferation and/or differentiation and upon mature osteoclasts to increase osteoclastic resorptive activity. Among these factors are macrophage colony-stimulating factor (M-CSF) (7), IL-6 (8), IL-11 (9), and the TNF family ligand, RANKL (10, 11). Osteoclasts and their precursors express the M-CSF receptor c-*fms* and the RANKL receptor RANK (12, 13) and thereby respond to M-CSF and RANKL, respectively. These cytokines, delivered via direct cell to cell interactions with stromal cells or osteoblasts, then promote the differentiation and survival of mature osteoclasts.

Estrogen can influence bone resorption by down-regulating the production of bone-resorbing cytokines by stromal cells and osteoblasts, which then decrease osteoclastic activity by a paracrine mechanism (4, 14). In some systems estrogen also has been shown to exert direct effects on osteoclasts, including increased apoptosis (3, 15–18), although this remains controversial (19, 20). Recently, estrogen was reported to up-regulate the expression by stromal cells of OPG, a soluble decoy receptor for RANKL that blocks its access to, and activation of, RANK receptors on osteoclasts (21, 22). Moreover, estrogen also may enlist TGFβ, a known antiresorptive factor, in its proapoptotic action on osteoclasts (5). Thus, estrogen may regulate osteoclast formation, activity and survival both by inhibiting the production of osteoclast stimulatory factors and by stimulating the production of osteoclast inhibitory factors.

In experiments with mammalian tissue, estrogen suppresses osteoclastic resorption stimulated by PTH (23, 24). After binding to a single receptor, PTH exerts its biological effects via activation of dual signal transduction systems, *i.e.* the cAMP-dependent PKA pathway and the PLC-coupled

Abbreviations: Arg2, [Arg^2,Tyr^{34}]hPTH-(1–34); 8-Br, 8-bromo-cAMP; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; hcFBS, heated-inactivated and charcoal-stripped FBS; hPTH, human PTH; IFN-γ, interferon-γ; M-CSF, macrophage colony-stimulating factor; MNC, multinucleated osteoclast-like cells; mOPG, mouse OPG; PTHIR, type 1 PTH/PTHrP receptor; Rp-cAMPS, rp-adenosine-3′,5′-cyclic monophosphorothioate; rPTH, rat PTH; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRAP, tartrate-resistant acid phosphatase.

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calcium/PKC pathway (25, 26). Several studies (27–29) have implicated estrogen inhibition of PTH-stimulated accumulation of cAMP in osteoblasts as a mechanism for suppression of osteoclastic activity, but the precise effects of estrogen on PTH-dependent bone resorption, including possible modulation of cAMP-independent type 1 PTH/PTHrP receptor (PTHIR) signaling, are unknown.

Previously, we demonstrated that PTH could induce osteoclast-like cell formation when normal murine spleen cells were cocultured with the clonal murine marrow stromal cell line, MS1 (30). Moreover, the full effect of PTH could not be mimicked by pharmacological activation of PKA or PKC alone, but was simulated by concurrent activation of both pathways (30). The major goal of the present study was to use this coculture model to investigate the role of estrogen in the regulation of PTH-dependent osteoclast function and to characterize the participation of specific PTHIR second messenger systems in the inhibition by estrogen of PTH-stimulated osteoclast formation.

Materials and Methods

Chemicals

Rat (r) PTH-(1–34), [$Nle^{8,18}$,Tyr^{34}]human (h) PTH-(3–34) [PTH-(3–34)], and hPTH-(53–84) were obtained from Bachem (Torrance, CA), [Arg^{2},Tyr^{34}] hPTH-(1–34) (Arg2) was synthesized in the Core Laboratory of the Endocrine Unit of Massachusetts General Hospital (Boston, MA), 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA), and recombinant mouse interferon-γ (IFN-γ) was purchased from Genzyme (Boston, MA). Eagle's MEM, αMEM, FBS, penicillin, and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). ICI 182,780 was provided by Tocris Cookson (Bristol, UK). 17β-E2, 17α-E2, 8-bromo-cAMP (8-Br), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), dexamethasone, naphthol AS-BI phosphoric acid, and red violet LB salt were products of Sigma (St. Louis, MO). Stock solutions of 17β-E2 and 17α-E2 were prepared in ethanol, 8-Br and Rp-cAMPS in water, and TPA and ICI 182,780 in dimethylsulfoxide, stored at −20 C, and prepared as required. All other chemicals used were of analytical grade. All culture dishes and plates were purchased from Becton Dickson Labware (Franklin Lakes, NJ).

Cell culture

Clonal conditionally immortalized MS1 mouse marrow stromal cells (30) were maintained in Eagle's MEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5 U/ml mouse IFN-γ at 33 C in a humidified atmosphere of 5% CO_2 in air. The cells were immortalized and grew indefinitely at permissive conditions (33 C, in the presence of IFN-γ), but still were able to undergo differentiation at semipermissive conditions (37 C, in the absence of IFN-γ) (30).

Osteoclast-like cell formation in vitro

The coculture procedures required to generate osteoclasts *in vitro* were described in detail previously (30). C57BL/6 mice were obtained from the Laboratory Animal Center of Medical College of National Taiwan University (Taipei, Taiwan, Republic of China). Animal handling and treatment were performed according to protocols provided by the Center. All cocultures for generating osteoclast-like cells were performed in αMEM without phenol red at 37 C in a humidified atmosphere of 5% CO_2. FBS was heated-inactivated and charcoal-treated (hcFBS) to remove any steroids present and was added at a final concentration of 10%. In brief, the clonal stromal cells (4×10^4 cells/well) were plated in 24-well plates and cultured for 24 h before being overlaid with spleen cells (10^6 cells/well) from 8- to 11-wk-old C57BL/6 male mice. Cells were cultured in 0.5 ml phenol red-free αMEM supplemented with 10% hcFBS and 10^{-7} M dexamethasone with rPTH-(1–34) (10^{-7} M) or 1,25-$(OH)_2D_3$ (10^{-8} M) at 37 C for 3 wk.

To examine the effects of estrogen treatment on PTH- or 1,25-$(OH)_2D_3$-stimulated osteoclast formation, 17β-E2 (10^{-12}–10^{-8} M), 17α-E2 (10^{-8} M), 17β-E2 (10^{-8} M) combined with ICI 182,780 (10^{-10}–10^{-6} M), ICI 182,780 (10^{-6} M), or vehicle of ethanol or dimethylsulfoxide alone was added to the cultures from the first day of cocultures. The total concentrations of organic solvents in the medium were less than 0.1%, and the same volumes of the solvents were added to the control medium. For dissecting the signaling pathways affected by estrogen in PTH-stimulated osteoclast formation, cocultures were treated with various fragments of PTH (10^{-7} M), second messenger analogs (10^{-4} M 8-Br and 10^{-8} M TPA), or signaling inhibitors (10^{-4} M Rp-cAMPS and 10^{-5} M H-7) and combined with 17β-E2 (10^{-8} M) from the first day of cocultures. All cultures were refed by half-changes of fresh medium and treatment every 3 d for 3 wk, when tartrate-resistant acid phosphatase (TRAP) staining was performed.

TRAP-positive multinucleated cells quantitation

At the termination of the experiments, the cultures were fixed with ethanol-acetone (50:50, vol/vol), then stained for TRAP. TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were scored as osteoclasts as previously described (30). All experiments were performed in six replicates with n = 4 for each condition.

Immunohistochemistry for ER in MS1 cells

MS1 cells were seeded at $2 \times 10^4/cm^2$ into multichambered slides (Nunc, Naperville, IL) in phenol red-free αMEM supplemented with 10% hcFBS for 24 h under permissive conditions. The medium was changed, and the cells were treated with 17β-E2, ICI 182,780, or vehicle at 37 C for an additional 3 d and then processed for immunohistochemical studies. ER immunohistochemistry was performed using rabbit and goat polyclonal antibodies against the mouse ERα and ERβ, respectively, (sc-542 and sc-6821, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's instructions, with modification. Briefly, the cells were washed with cold calcium/magnesium-free PBS, fixed with methanol at −20 C, air-dried, and rinsed with PBS. The fixed cells then were blocked with 1.5% (vol/vol) normal goat or donkey serum in PBS (blocking serum-PBS) at room temperature for 30 min, rinsed with PBS containing 1% (wt/vol) BSA (PBS-BSA), and incubated for 30 min with 1 mg/ml rabbit antimouse ERα or goat antimouse ERβ antibody in PBS-BSA. A uterus from a 9-wk-old female C57BL/6 mouse was employed as a positive control. MS1 cells or uterus tissue sections were also incubated without primary antibody or with nonimmune rabbit or goat serum as a negative control. After washing three times with PBS, the cells were incubated with a biotin-conjugated goat antirabbit or donkey antigoat IgG for 30 min in 1.5% blocking serum-PBS, rinsed with PBS, and subsequently incubated for 30 min with avidin-biotin enzyme reagent (all from Santa Cruz Biotechnology, Inc.). The cells were further washed with PBS, followed by 0.5% Triton X-100 in PBS before incubation with 0.01% (wt/vol) 3,3-diaminobenzoidine and a few drops of 30% H_2O_2 in PBS for 2–5 min. Finally, the cells were washed extensively with distilled water, air-dried, and photographed using an Olympus Corp. BH2 microscope (New Hyde Park, NY).

Neutralizing antibody assays

Pan-specific polyclonal anti-TGFβ antibody (50 μg/ml, final concentration), polyclonal antimouse (m) OPG antibody (20 μg/ml), or an equal amount of preimmune host serum (all from R&D Systems, Minneapolis, MN) was added to the cocultures in the presence of PTH and 17β-E2. According to the manufacturer, 20 μg/ml anti-TGFβ antibody neutralizes approximately 80% of 0.25 ng/ml TGFβ1 in the HT-2 cell bioassay, whereas 2 μg/ml anti-mOPG antibody completely neutralizes 0.1 μg/ml soluble recombinant mOPG in the L929 cytolytic assay. The antibodies and nonimmune serum additives were maintained during the entire culture period until TRAP staining was performed.

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TGFβ1 production

MS1 cells were cultured on 24-well plates and grown to confluence with phenol red-free αMEM and 0.5% hcFBS under permissive conditions before transfer to phenol red-free αMEM with 0.1% BSA at 37 C. They were refed with the same medium 24 h later, and experiments were initiated by the addition of rPTH-(1–34) (10^{-7} M), 17β-E2 (10^{-8} M), and rPTH-(1–34) (10^{-7} M) combined with 17β-E2 (10^{-8} M) or with ethanol vehicle alone after an additional 24 h of culture. TGFβ1 concentrations were measured in cell-free supernatants collected 24 h after the stimulation. Active TGFβ1 and latent TGFβ (total TGFβ1 − active TGFβ1) in the conditioned media were analyzed by an ELISA kit (R&D Systems) according to the manufacturer's instructions. The sensitivity of this assay is less than 7 pg/ml.

Osteoclast attachment to dentine

Dentine slices were prepared as previously described, with some modification (30). Osteoclasts were obtained at the conclusion of cocultures by washing the cells with PBS and then incubating in 0.5% trypsin-EDTA for 10 min. The released cells were gently dispersed by passage through a 21-gauge needle. Equal aliquots of the dispersed cell suspensions were plated into wells containing human dentine slices in αMEM and then incubated for 2 h in 5% CO_2 at 37 C to facilitate attachment of the osteoclasts to the dentine slices. Nonadherent cells then were washed off the dentine slices by dipping them into media, and the dentine was placed into fresh phenol red-free αMEM containing 10% hcFBS and 1% penicillin-streptomycin with 17β-E2 or ethanol vehicle only. Six hours later, the dentine slices were washed three times with PBS to remove unattached cells before staining with TRAP to enable counting of the number of TRAP-positive cells as described above. Osteoclast attachment was expressed as the number of TRAP-positive mono- and multinucleated cells observed on dentine slices in four randomly selected areas per slice, with eight slices per experimental condition.

Statistical analysis

Data are expressed as the mean ± SEM. The data shown in the figures and the significance within each dataset were representative of at least six separate cell preparations. The significance of a difference between comparable groups was determined by *t* test.

Results

Demonstration of ER in MS1 cells

ERα and ERβ were readily detected by immunohistochemistry in sections of control uterus and in MS1 cells. Results for ERα are shown in Fig. 1. Within the epithelial compartment of the uterus, glandular epithelial cells showed the strongest signal, with virtually all cell nuclei staining strongly positively (Fig. 1A). ER-positive cells also were observed in most of the stromal cells and myometrial smooth muscle cells (Fig. 1A). Nuclear staining for ER also was observed in nearly all MS1 cells in the presence or absence of 17β-E2, whereas cytoplasmic staining was present in very few cells (Fig. 1, C and E). The pure antiestrogen ICI 182,780 has been reported to block estrogen action via multiple changes in ER function, including impaired receptor dimerization, increased turnover, and disrupted nuclear localization (31). When cells were treated with ICI 182,780 for 3 d, the subcellular distribution of ER was altered, in that there was a significant increase in the number of cells with cytoplasmic staining (Fig. 1F). In some cases, ER staining was even stronger in the cytoplasm than in the nucleus. ERβ also was expressed in control uterus and MS1 cells in a similar pattern (data not shown).

Effects of 17β-E2 on PTH-stimulated osteoclast-like cell formation

To determine the potential role of estrogen in the regulation of osteoclast formation, we initially assessed its ability to suppress the formation of TRAP-positive MNCs stimulated by PTH in cocultures of MS1 clonal marrow stromal cells with normal mouse spleen cells. The mean numbers of rPTH-(1–34) (10^{-7} M)- or 1,25-$(OH)_2D_3$ (10^{-8} M)-stimulated TRAP-positive MNCs found in 24 independent cocultures from 6 different experiments were 88.6 ± 10.6 and 96 ±

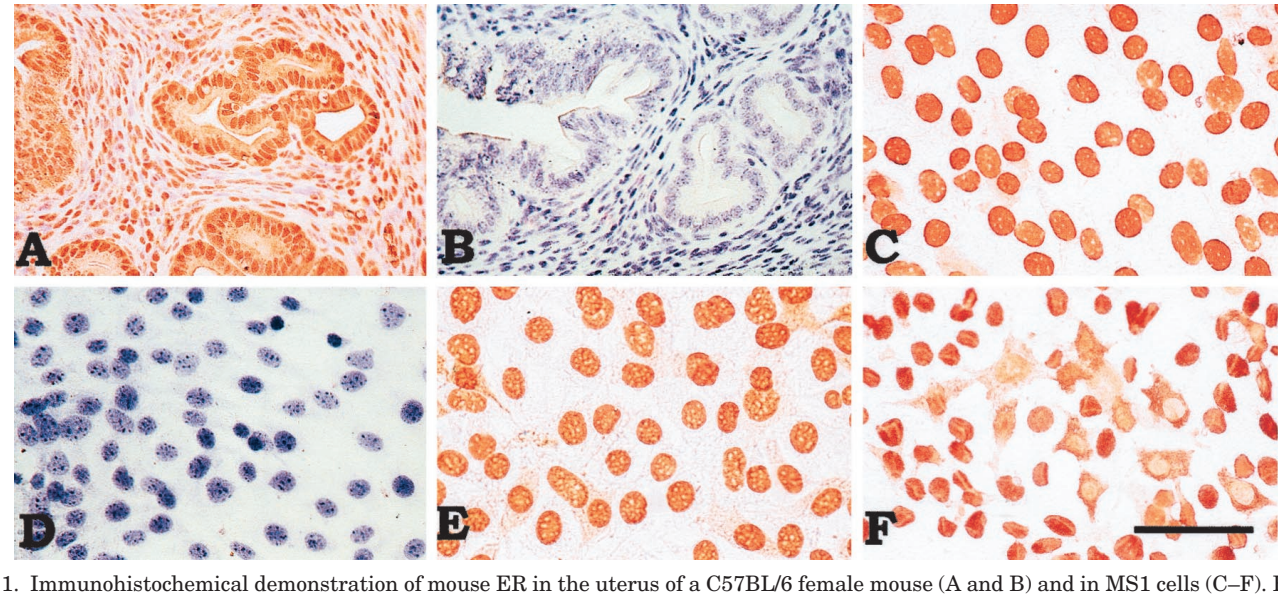

FIG. 1. Immunohistochemical demonstration of mouse ER in the uterus of a C57BL/6 female mouse (A and B) and in MS1 cells (C–F). For the effects of ligand on the alteration of ER, MS1 cells were treated with vehicle (C), 10^{-8} M 17β-E2 (E), or 10^{-6} M ICI 182780 (F) for 3 d. Receptors were detected by avidin-biotin immunohistochemistry using a polyclonal antibody to mouse ERα. Control studies stained with preimmune serum are shown in B and D. Original magnification: A–F, ×100. *Bar*, 50 μm.

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11.4/0.5 cm^2, respectively (see *Materials and Methods*). Few TRAP-positive MNCs (10.3 ± 2) were observed in cocultures treated with vehicles alone. As we previously reported, these TRAP-positive MNCs exhibit various characteristics of osteoclasts, including responsiveness to calcitonin, calcitonin binding, and the ability to resorb bone, as evidenced by pit formation during coculture on dentine slices (30).

As shown in Fig. 2, 17β-E2 caused a concentration-dependent inhibition of TRAP-positive MNC formation stimulated by 10^{-7} M rPTH-(1–34). The minimally effective concentration of 17β-E2 was 10^{-10} M, and a 55% reduction of PTH-stimulated TRAP-positive MNC formation was seen with 10^{-8} M 17β-E2. In contrast, 10^{-8} M 17β-E2 did not affect TRAP-positive MNC formation stimulated by 1,25-$(OH)_2D_3$ (10^{-8} M). The inactive stereoisomer 17α-E2 (10^{-8} M) failed to inhibit TRAP-positive MNC formation induced by either PTH (Fig. 1) or 1,25-$(OH)_2D_3$ (not shown). Further evidence of specificity derived from observations that the pure ER antagonist ICI 182,780 caused a concentration-dependent reversal of the inhibitory effect of 17β-E2 on PTH-induced TRAP-positive MNC formation (Table 1).

Estrogen inhibition of osteoclast-like cell formation induced by activation of PKA and PKC

We previously reported that both the PKA activator 8-Br and the PKC activator TPA each stimulated TRAP-positive MNC formation in a dose-dependent manner in MS1/spleen cell cocultures (30). As shown in Table 1, 17β-E2 (10^{-8} M) significantly inhibited TRAP-positive MNC formation induced by either 8-Br (10^{-4} M) or TPA (10^{-8} M). The combination of 8-Br and TPA, at optimal concentrations of 10^{-5} and 10^{-8} M, respectively, unlike either drug alone, fully mimicked the action of rPTH-(1–34) on induction of osteoclastogenesis in the cocultures. Addition of 17β-E2 to these cocultures greatly reduced TRAP-positive MNC formation, and, as in the case of PTH-induced osteoclastogenesis, these actions of 17β-E2 were completely inhibited by 10^{-6} M ICI 182,780 (Table 1).

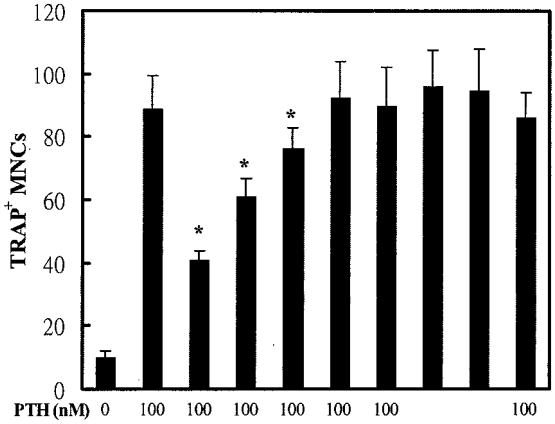

FIG. 2. Effects of 17β-E2 on rPTH-(1–34)- or 1,25-$(OH)_2D_3$-stimulated osteoclast formation in MS1/spleen cell cocultures. In the presence of rPTH-(1–34) (PTH), 1,25-$(OH)_2D_3$ (Vit D), or vehicle alone, cocultures were treated with or without the indicated concentrations of 17β-E2 (E2) or 10^{-8} M 17α-E2 (α-E2) for 21 d before staining for TRAP and counting of TRAP-positive MNCs, as described in *Materials and Methods*. Data are expressed as the mean ± SEM of six determinations. *, $P < 0.01$ compared with the rPTH-(1–34)-treated group.

To further pursue the possibility that both PKA and PKC activation by PTH might be independently involved in the osteoclastogenic response and subject to estrogen regulation, we first tested the effects of the respective kinase inhibitors, Rp-cAMPS and H-7. As shown in Fig. 3, Rp-cAMPS (10^{-4} M) and H-7 (10^{-5} M) each attenuated, but did not completely block, TRAP-positive MNC formation stimulated by rPTH-(1–34). Neither drug inhibited TRAP-positive MNC formation stimulated by 1,25-$(OH)_2D_3$ (data not shown). In each case, addition of 17β-E2 significantly further decreased the numbers of PTH-stimulated TRAP-positive MNC generated in the presence of the kinase inhibitor alone (Fig. 3). When these signaling inhibitors were used together, *i.e.* 10^{-4} M Rp-cAMPS combined with 10^{-5} M H-7 or 10^{-5} M Rp-cAMPS combined with 10^{-6} M H-7, cytotoxic effects were observed, with loss of substrate adhesion of cells (data not shown). Taken together, these data suggested that 17β-E2 inhibits PTH-stimulated osteoclast formation by affecting both the PKA and PKC signaling pathways.

Estrogen inhibition of osteoclast-like cell formation induced by PTH analogs

To further examine the effects of estrogen on the intracellular signaling responses that mediate PTH-induced osteoclast formation, cocultures were exposed to PTH analogs with selective signaling properties. The analog Arg2 previously was demonstrated to be a weak agonist for cAMP stimulation that did not retain any detectable PLC activation via rodent PTHIR (32). As shown in Fig. 4, Arg2 (10^{-7} M) did induce TRAP-positive MNC formation, although the response was much lower than that observed with the same concentration of rPTH-(1–34), as expected from the reduced intrinsic agonism of the analog (32). Moreover, 17β-E2 (10^{-8} M) completely blocked the formation of Arg2-induced TRAP-positive MNCs, as did Rp-cAMPS (10^{-4} M). The analog [$Nle^{8,18}$,Tyr^{34}]hPTH-(3–34) [PTH-(3–34)] is a partial agonist that activates PKC, but not adenylyl cyclase (33). As shown in Fig. 4, PTH-(3–34) (10^{-7} M) did modestly induce the formation of TRAP-positive MNCs, an effect that was completely blocked by 17β-E2 (10^{-8} M) or H-7 (10^{-5} M). We also observed a significant cooperative effect of coadministered Arg2 (10^{-7} M) and PTH-(3–34) (10^{-7} M) on TRAP-positive MNC formation compared with the effect of either analog alone. The stimulatory effect of the Arg2 and PTH-(3–34) combined treatment was fully inhibited by 17β-E2 (10^{-8} M; Fig. 4). By using signal-selective PTH analogs, these data suggested again that 17β-E2 inhibits PTH-stimulated osteoclast formation by affecting both the PKA and PKC signaling pathways.

We previously reported that hPTH-(53–84), a carboxyl fragment of intact hPTH-(1–84) that cannot bind to or activate PTHIR, modestly stimulates osteoclast formation in MS1/spleen cell cocultures (30). This is believed to involve activation of novel receptors, distinct from PTHIR, with specificity for the carboxyl-terminal region of PTH (34). In the present studies, we observed no inhibition by 17β-E2 (10^{-8}

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TABLE 1. ICI 182,780 antagonizes 17β-E2 inhibition of TRAP-positive MNC formation

ICI 182,780	TRAP$^+$ MNCs							
	PTH	PTH/17β-E2	8-Br	8-Br/17β-E2	TPA	TPA/17β-E2	8-Br + TPA	8-Br + TPA/17β-E2
0	88.6 ± 10.6	40.7 ± 3.3	46.6 ± 5.4	23.8 ± 2.2	60.2 ± 6.6	30.8 ± 5.6	76.7 ± 7.4	39.2 ± 5.6
1	81.8 ± 9.2	82.4 ± 8.3[a]	44.2 ± 5.2	47.8 ± 5.4[a]	54.4 ± 7.9	53.6 ± 6.2[a]	77.6 ± 9.2	75.5 ± 8.6[a]
0.1	84.6 ± 10.5	84.1 ± 6.6[a]						
0.01	87.2 ± 9.7	66.2 ± 6.2[a]						
0.001	89.6 ± 9.7	50.5 ± 3.8[a]						
0.0001	87.0 ± 9.7	44.8 ± 3.5						

MS1 cells were cocultured with spleen cells and treated with rPTH-(1-34) (10^{-7} M), 8-Br (10^{-4} M), TPA (10^{-8} M), or a combination of 8-Br (10^{-5} M) and TPA (10^{-8} M) for 21 d in the presence or absence of 17β-E2 (10^{-8} M), ICI 182,780 (10^{-6} M), or 17β-E2 (10^{-8} M) combined with ICI 182,780 at the indicated concentrations ($\times 10^{-6}$ M) before enumeration of TRAP-positive MNC. Values shown are the mean ± SEM for six replicate wells (n = 4).

[a] Significantly different from corresponding control in the absence of ICI 182,780, $P < 0.05$.

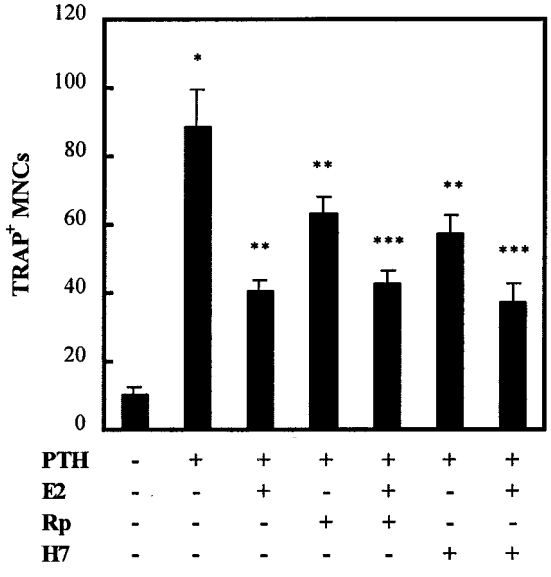

FIG. 3. Effects of signaling kinase inhibitors and 17β-E2 on PTH-stimulated osteoclast formation. In the presence of rPTH-(1–34) (10^{-7} M; PTH), PTH combined with Rp-cAMPS (10^{-4} M; Rp), PTH combined with H-7 (10^{-5} M), PTH combined with Rp-cAMPS and H-7, or vehicle, cocultures were treated with or without 10^{-8} M 17β-E2 (E2) for 21 d before staining for TRAP and counting of TRAP-positive MNCs, as described in *Materials and Methods*. Cytotoxic effects were observed with loss of substrate adhesion of cells when the cocultures were treated with either 10^{-4} M Rp-cAMPS and 10^{-5} M H-7 or 10^{-5} M Rp-cAMPS and 10^{-6} M H-7 in combination. Data are expressed as the mean ± SEM of six determinations. *, $P < 0.01$ compared with control; **, $P < 0.01$ compared with the PTH-only treated group; ***, $P < 0.01$ compared with the corresponding Rp-cAMPS- or H-7-treated, 17β-E2-untreated group.

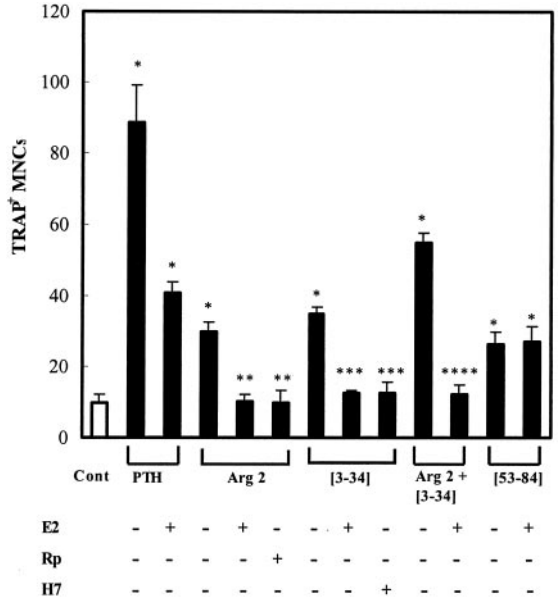

FIG. 4. Effects of signaling kinase inhibitors and 17β-E2 on osteoclast formation induced by signal-selective PTH analogs. Cocultures were incubated for 21 d with or without the following: 10^{-8} M 17β-E2 (E2), 10^{-7} M [Arg^2, Tyr^{34}]hPTH-(1–34) (Arg2), 10^{-4} M Rp-cAMPS (Rp), 10^{-7} M [Nle^{8,18}Tyr^{34}]hPTH-(3–34) ([3–34]), 10^{-5} M H-7, 10^{-6} M hPTH-(53–84) ([53–84]), or vehicle alone, after which they were stained for TRAP to enumerate TRAP-positive MNCs, as described in *Materials and Methods*. Data are expressed as the mean ± SEM of six determinations. *, $P < 0.01$ compared with control; **, $P < 0.01$ compared with Arg2-treated, 17β-E2-untreated group; ***, $P < 0.01$ compared with the [Nle^{8,18}Tyr^{34}[hPTH-(3–34)-treated, 17β-E2-untreated group; ****, $P < 0.01$ compared with the Arg2- plus [Nle^{8,18}Tyr^{34}]hPTH-(3–34)-treated, 17β-E2-untreated group.

M) of TRAP-positive MNC formation induced by hPTH-(53–84) (Fig. 4).

Roles of TGFβ and OPG in PTH- and estrogen-regulated TRAP-positive MNC formation

As known inhibitors of osteoclast formation and survival, OPG and TGFβ were of particular interest as potential candidates to mediate the antagonistic action of estrogen on the osteoclastogenic response to PTH in the MS1/spleen cell coculture system (4, 21, 22, 35, 36). Moreover, TGFβ inhibits the expression of PTHIR (37) and induces that of OPG in osteoblastic cells (36).

As reported by others in osteoblastic cells (35, 38), rPTH-(1–34) (10^{-7} M) increased the production by MS1 cells of both active and latent TGFβ1, as measured in conditioned medium at 24 h (Table 2). Similar increases were observed after treatment with 17β-E2 (10^{-8} M; Table 2). Interestingly, the addition of 17β-E2 to rPTH-(1–34) did not further increase, but, rather, inhibited, TGFβ1 production, a response previously reported by others (35).

Consistent with the results of these TGFβ protein measurements, addition of a pan-specific anti-TGFβ neutralizing antibody did not increase the number of TRAP-positive MNCs produced in cocultures treated with PTH and 17β-E2.

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TABLE 2. TGFβ1 production in MS1 cells after rPTH-(1-34) and 17β-E2 treatment

TGFβ1 (pg/ml)	Treatment			
	Control	17β-E2	PTH	PTH + 17β-E2
Active	Undetectable	12.4 ± 0.8	15.8 ± 0.8	7.8 ± 0.7[a,b]
Latent	32.7 ± 2.4	76.2 ± 4.2[c]	97.8 ± 4.8[c]	70.8 ± 6.2[c,d]

MS1 cells were cultured in phenol red-free αMEM and 0.1% BSA for 24 h in the presence of rPTH-(1-34) (10^{-7} M), 17β-E2 (10^{-8} M), or rPTH-(1-34) (10^{-7} M) combined with 17β-E2 (10^{-8} M). Active and latent forms of TGFβ1 were measured using a commercial ELISA kit as described in *Materials and Methods*. Values shown are the mean ± SEM for three replicate wells (n = 3).

[a] Significantly different from 10^{-7} M 17β-E2 group ($P < 0.05$).
[b] Significantly different from 10^{-8} M rPTH-(1-34) group ($P < 0.01$).
[c] Significantly different from the control ($P < 0.01$).
[d] Significantly different from 10^{-7} M rPTH-(1-34) group ($P < 0.05$).

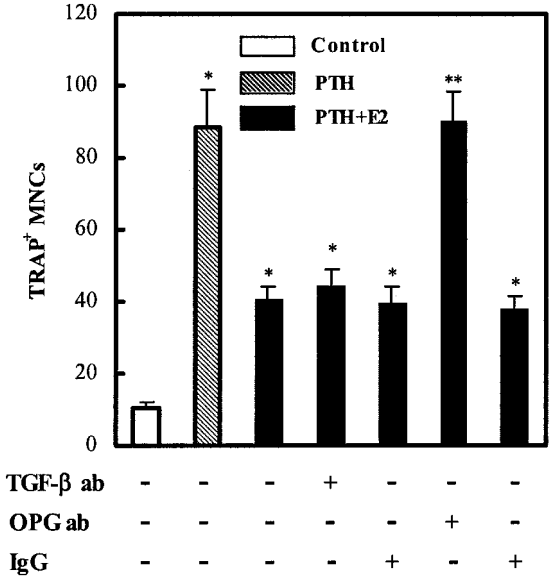

FIG. 5. Roles of TGFβ and OPG in estrogen antagonism of PTH-dependent osteoclast formation. Cocultures of MS1 and normal spleen cells were treated with vehicle alone (□), 10^{-7} M rPTH-(1–34) alone (▨), or both 10^{-7} M rPTH-(1–34) and 10^{-8} M 17β-E2 (■) in the absence or presence 50 μg/ml anti-TGFβ antibody (TGF-β ab), 20 μg/ml anti-mOPG antibody (OPG ab), or the appropriate preimmune serum (IgG), as indicated. The culture medium and treatment (with or without fresh antibodies) were changed every 3 d. After 21 d, the cocultures were stained with TRAP, and TRAP-positive MNCs were counted as described in *Materials and Methods*. Data are expressed as the mean ± SEM of six determinations. *, Significantly different from vehicle-treated control ($P < 0.01$). **, Significantly different from either vehicle-treated or PTH- plus 17β-E2-treated group ($P < 0.01$).

On the other hand, neutralizing antibody to mOPG completely reversed the inhibitory effect of 17β-E2 on PTH-induced osteoclast formation (Fig. 5).

Estrogen regulation of osteoclast attachment

Cellular adhesion to calcified matrix is an early event characteristic of functional osteoclasts that is subject to hormonal regulation. After sudden exposure to calcitonin, for example, osteoclasts rapidly exhibit cytoplasmic retraction, cessation of mobility, and detachment from bone surface (39, 40). To assess whether 17β-E2 acutely regulates osteoclastic attachment to bone, we first generated osteoclasts by treating cocultures with rPTH-(1–34) for 3 wk and then isolated them by trypsinization. We then counted the number of TRAP-positive cells attached to human dentine slices after replating the osteoclast-containing cell suspensions, removing nonadherent cells and incubating for 6 h in the presence *vs.* absence of added 17β-E2 (10^{-8} M). As shown in Table 3, 17β-E2 significantly inhibited dentine attachment of both mononuclear and multinucleated TRAP-positive cells (see also Fig. 6). Attachment could be blocked completely by calcitonin (Table 3). Interestingly, dentine attachment of TRAP-positive mono- and multinucleated cells generated by treatment of MS1/spleen cell cocultures with rPTH-(1–34) for 3 wk in the presence of 17β-E2 was not inhibited significantly by rechallenge with estrogen, whereas calcitonin still was effective (Table 3).

Discussion

The mechanisms by which estrogen prevents bone loss are complex and may include effects on commitment, survival, and differentiation of hemopoietic osteoclast progenitors and precursors, regulation of the number and activity of supporting osteoblasts or marrow stromal cells, and reduction of the survival of mature osteoclasts (3, 6, 17, 18, 21). Previous studies (24, 41) showed that estrogen suppresses PTH-stimulated osteoclastic bone resorption, but its interactions with the various intracellular signaling mechanisms triggered by the PTHIR remain incompletely understood. We used an *in vitro* mouse osteoclast development system (30) to study the mechanisms by which estrogen inhibits PTH-induced formation of osteoclasts. The TRAP-positive MNCs produced in these cocultures previously were shown to fulfill several criteria for osteoclasts, including multinuclearity, expression of TRAP and calcitonin receptors, and resorption of the calcified matrix of dentine slices (30). We also had shown that MS1 clonal marrow stromal cells express PTHIR and that this was sufficient, in the absence of PTHIR expression by cells of the osteoclast lineage, to support osteoclastogenesis (30).

The latter observation together with the failure of estrogen in the present studies to inhibit osteoclast formation induced by either 1,25-$(OH)_2D_3$ or hPTH-(53–84) in MS1/spleen cell cocultures strongly suggest that the estrogen antagonism of PTH-dependent osteoclastogenesis observed in this system is exerted via direct and specific effects on the MS1 stromal cells. Moreover, whereas an important aspect of the regulation of bone resorption by estrogen is to promote apoptosis of mature osteoclasts, either directly (6) or via TGFβ induction and subsequent stimulation of OPG expression by stromal cells (5, 36), these actions seem not to be evident under the conditions employed for our experiments, as 17β-E2 did not reduce the numbers of osteoclasts produced in response to 1,25-$(OH)_2D_3$ or hPTH-(53–84), nor did it augment TGFβ1 synthesis in the presence of rPTH-(1–34). This may be explained by our choice of a 3-wk culture period, which in this system corresponds to the time of peak appearance of new osteoclasts (30) and thus may reflect mainly the formation rate of new osteoclasts and not the duration of their survival.

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TABLE 3. Effects of 17β-E2 on osteoclast attachment to dentine slices

$TRAP^+$ cells	PTH, rechallenge			PTH + 17β-E2, rechallenge		
	Vehicle	sCT	17β-E2	Vehicle	sCT	17β-E2
Mononuclear	34.0 ± 3.7	0.7 ± 0.4[a]	15.9 ± 3.0[a]	18.5 ± 3.7	0.6 ± 0.5[a]	12.8 ± 3.3
Multinucleated	40.2 ± 4.7	0.8 ± 0.6[a]	25.6 ± 3.3[a]	22.0 ± 4.2	1.1 ± 1.2[a]	17.0 ± 4.4
Total	74.2 ± 8.5	1.5 ± 1.0[a]	41.5 ± 6.3[a]	40.5 ± 7.9	1.7 ± 1.7[a]	29.8 ± 7.6

Equal aliquots of trypsinized cell suspensions from cocultures pretreated for 21 d with rPTH-(1-34) (10^{-7} M) or rPTH-(1-34) (10^{-7} M) plus 17β-E2 (10^{-8} M) were settled onto dentine slices. After washing to remove nonadherent cells, the cultures were rechallenged with 17β-E2 (10^{-8} M), sCT (10^{-8} M), or vehicle alone for 6 h. Dentine slices were then washed extensively with PBS to remove unattached cells and stained for TRAP to enumerate attached TRAP-positive cells. Results are expressed as the mean ± SEM of the numbers of TRAP-positive mono- and multinucleated cell counted in four random light microscopy fields for eight slices per group from three different experiments.

[a] Significantly different from controls ($P < 0.05$; n = 32).

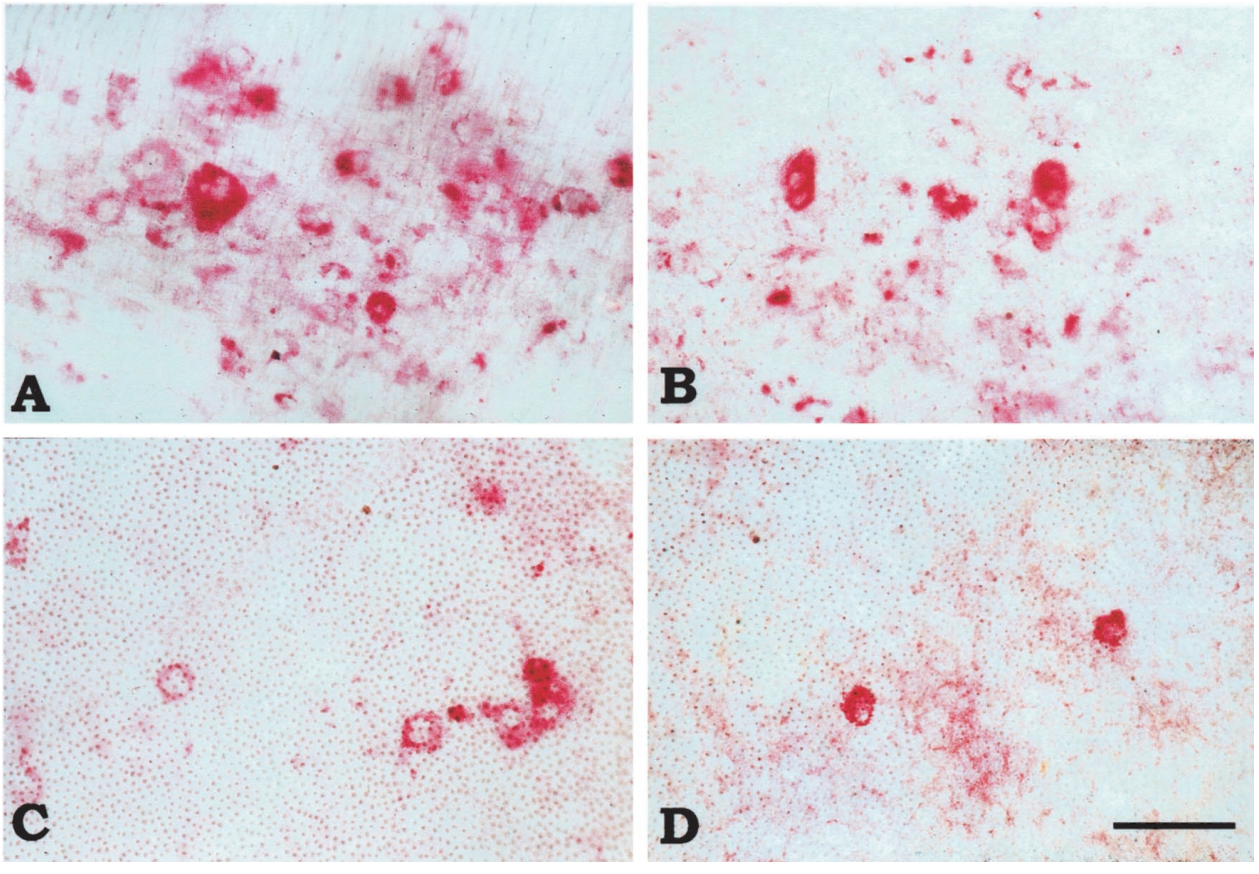

FIG. 6. Effect of estrogen on attachment of preformed osteoclasts. Equal aliquots of enzymatically dispersed, osteoclast-containing cell suspensions from cocultures pretreated with 10^{-7} M PTH with or without 10^{-8} M 17β-E2 for 21 d were settled onto dentine slices (see *Materials and Methods*). After washing away nonadherent cells, the cultures were rechallenged with 17β-E2 (10^{-8} M) or vehicle for 6 h. Dentine slices then were washed extensively with PBS to remove unattached cells and were stained for TRAP to enumerate the attached TRAP-positive cells. A, Cells precultured with PTH only, then challenged with vehicle. Numerous TRAP-positive cells are attached on the dentine slice. B, Cells precultured as described in A, but rechallenged with 17β-E2. The number of TRAP-positive cells attached on dentine slices is markedly decreased. C, Cells precultured with both rPTH-(1–34) and 17β-E2 for 3 wk, then rechallenged with vehicle. Few TRAP-positive cells are attached on the dentine slice. D, Cells precultured as described in C, then rechallenged with 17β-E2. Original magnification: A–D, ×100. *Bar*, 50 μm.

Our immunoneutralization studies did clearly indicate that OPG is essential for estrogen's antagonism of PTH-induced osteoclast formation in this coculture system. One interpretation of this finding, to be further addressed in future studies, is that estrogen may specifically prevent down-regulation of OPG expression by rPTH-(1–34), rather than directly induce OPG expression in a PTH-independent manner.

In the present study, we observed that the profound inhibition by estrogen of PTH-dependent osteoclast formation involves interference with both the PKA- and PKC-dependent pathways of PTHIR signaling. The contribution of both PKA and PKC to PTH-induced osteoclastogenesis was suggested by three types of evidence. First, direct pharmacological activators of PKA and PKC (*i.e.* 8-Br and TPA), when introduced separately at optimally effective concentrations in the coculture system, caused formation of osteoclasts in numbers lower than observed with PTH-(1–34), but produced a response equivalent to that of PTH-(1–34) when

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added in combination. Second, analogs of PTH known to selectively activate PKA or PKC via PTHIR (*i.e.* [Arg^2,Tyr^{34}]hPTH-(1–34) and [$Nle^{8,18}$,Tyr^{34}]hPTH-(3–34), respectively), each could induce osteoclast formation in the MS1/spleen cell cocultures, although, again, neither peptide alone could match the response to PTH-(1–34). Third, the formation of osteoclasts in response to PTH-(1–34) was only partially blocked by selective inhibitors of PKA or PKC (Rp-cAMPS and H-7, respectively). Repeated attempts to inhibit the PTH response completely by combined simultaneous treatment with both Rp-cAMPS and H-7 were thwarted because the cells detached from the culture plates under these conditions. This apparently was due to a toxic effect on the hemopoietic cells, as MS1 cells could survive the dual treatment for at least 3 wk. Our evidence that PTH-dependent osteoclastogenesis involves activation of both PKA and PKC is consistent with previous work using other systems (42–44).

With respect to the interaction of estrogen with these PTHIR signaling pathways, we observed that estrogen could inhibit PTH-dependent osteoclast formation in MS1/spleen cell cocultures in the presence of either Rp-cAMPS or H-7. Similarly, 17β-E2 inhibited osteoclast formation induced by either 8-Br or TPA (or both). This implies that both PKA- and PKC-dependent responses were susceptible to the inhibitory effects of estrogen. This conclusion is further supported by the ability of estrogen to inhibit osteoclast formation induced by [Arg^2,Tyr^{34}]hPTH-(1–34) and [$Nle^{8,18}$,Tyr^{34}]hPTH-(3–34), either alone or in combination. These effects of 17β-E2 appear to be specific, as they were not observed when osteoclast formation was induced by 1,25-$(OH)_2D_3$ or by hPTH-(53–84), which acts via a receptor distinct from the PTHIR; they were not mimicked by 17α-E2, and they were blocked by the ER antagonist ICI 182,780.

The precise mechanisms by which estrogen interrupts dual signaling by the PTHIR are unclear. Others have shown that estrogen strongly attenuates the cAMP response to PTH in osteoblastic cells (27), suggesting some uncoupling of occupied receptors from the adenylyl cyclase(s). On the other hand, more distal effects also must be important, as we observed inhibition of the effect of 8-Br, which bypasses adenylyl cyclase to directly activate PKA. Using different experimental models, others have reported also that estrogen can inhibit PTH-dependent osteoclast formation (45, 46), although in these studies the estrogen effect appeared to be specific for the PKA-dependent, rather than the PKC-dependent, component of the PTH response. This discrepancy cannot be readily explained, but probably relates to the use of different experimental systems and the focus upon actions of PTH in different cell types.

In this regard, it is of interest that others have provided evidence for direct effects of both PTH (42, 45, 46) and estrogen (5, 16, 17, 47, 48) on osteoclasts or osteoclast progenitors. Thus, although estrogen regulation of local cytokine production by stromal cells and osteoblasts undoubtedly is involved in its modulation of osteoclast formation induced by agents such as PTH (4, 19, 20, 49, 50), functional interactions within cells of the osteoclast lineage may play a role as well. We did establish that MS1 stromal cells express ER protein, but we cannot distinguish whether the effects of estrogen on PTH-induced osteoclast formation in our coculture system involve interactions primarily within the MS1 clonal stromal cells, within cells of the hemopoietic compartment, or both. Future studies with cells lacking ER, PTHIR, or both will be required to address this issue. We did observe a striking reduction in the ability of preformed osteoclasts to rapidly attach to dentine in the presence of added estrogen. Given the time frame involved, this effect is likely to reflect a direct action of estrogen on the mature osteoclasts or mononuclear osteoclast precursors in these preparations, although cells of greater purity would be required to establish this unequivocally. The inability of estrogen to produce this effect in cells previously exposed to the steroid is reminiscent of the escape phenomenon observed with calcitonin and is consistent with evidence that estrogen may functionally down-regulate its own receptor in osteoclasts (16).

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