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Estrogen Blocks Parathyroid Hormone (PTH)-Stimulated Osteoclast-Like Cell Formation by Selectively Affecting PTH-Responsive Cyclic Adenosine Monophosphate Pathway*

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ABSTRACT

Several lines of evidence have previously indicated that estrogen inhibits PTH-induced bone resorption *in vivo* and *in vitro*. However, its mechanism remains unknown. Therefore, the present study was performed to investigate the effect of estrogen on PTH-stimulated osteoclast-like cell formation and clarify its mechanism. 17β-estradiol (17β-E_2) significantly antagonized osteoclast-like cell formation stimulated by 10^{-8} M human (h) PTH-(1–34) as well as 10^{-8} M hPTH-related peptide (PTHrP)-(1–34) in osteoblast-containing mouse bone cell cultures. The conditioned medium derived from osteoblastic SaOS-2 cells or MC3T3-E1 cells pretreated with both PTH-(1–34) (10^{-8} M) and 17β-E_2 (10^{-8} M) stimulated osteoclast-like cell formation from hemopoietic blast cells more weakly than conditioned medium from cells pretreated with PTH-(1–34) alone. Moreover, 10^{-8} M 17β-E_2 significantly blocked the formation of osteoclast-like cells stimulated by 10^{-8} M hPTH-(1–34) in spleen cell cultures derived from 5-fluorouracil-pretreated mice. On the other hand, 10^{-8} M 17β-E_2 significantly inhibited osteoclast-like cell formation stimulated by dbcAMP (10^{-4} M) and Sp-cAMPS (10^{-4} M), as well as forskolin (10^{-5} M) in mouse bone cell cultures. In contrast, 10^{-8} M 17β-E_2 did not affect PMA (10^{-7} M)-, A23187 (10^{-7} M)-, or BAYK-8644 (5×10^{-6} M)-stimulated osteoclast-like cell formation. In conclusion, the present study demonstrated that estrogen inhibits PTH-stimulated osteoclast-like cell formation by directly acting on hemopoietic blast cells as well as by indirectly acting on them via osteoblasts. The inhibitory effects of estrogen on PTH-stimulated osteoclast-like cell formation seemed to be mediated through blocking the cAMP-dependent protein kinase pathway but not by blocking calcium/protein kinase C. (*Endocrinology* **137:** 2217–2224, 1996)

PTH POSSESSES anabolic as well as catabolic actions in bone. A previous study (1) indicated that the intermittent administration of PTH to rats causes an increase in bone volume and stimulates bone formation, although the continuous administration of PTH increases bone resorption and causes osteopenia. Moreover, cortical bone thickness was reduced in patients with hyperparathyroidism, but cancellous bone volumes were often higher in patients with mild hyperparathyroidism than in control patients (2, 3). Thus, the action of PTH in bone seems to be complex, and its mechanism remains unknown.

Estrogen replacement therapy effectively prevents postmenopausal osteoporosis, and several reports have suggested that estrogen is effective in controlling serum calcium and osteopenia in postmenopausal women with primary hyperparathyroidism, possibly by inhibiting PTH-induced bone resorption (4, 5). Estrogen modulates the balance between bone formation and bone resorption by stimulating the bone-forming activity of osteoblasts (6, 7) as well as by reducing osteoclast-mediated bone resorption (8–10). Previous reports have shown that estrogen inhibits PTH-induced bone resorption in mouse organ cultures (11, 12). There has been accumulating evidence suggesting a direct effect of estrogen on mature osteoclasts (8, 10). These studies demonstrated the existence of functional estrogen receptors in human or avian mature osteoclasts. PTH is considered to stimulate bone resorption by accelerating the development of new osteoclast as well as the activation of quiescent osteoclasts (13, 14). However, the action of estrogen on PTH-induced osteoclast formation and its mechanism are still unknown.

We previously reported that the amino- and carboxyl-termini of PTH stimulate osteoclast-like cell formation by directly acting on hemopoietic blast cells as well as by indirectly acting on them via osteoblasts (13, 15, 16). On the other hand, our recent studies (15, 17) suggested that carboxyl-terminal PTH-related protein (PTHrP) and retinoic acid directly act on hemopoietic blast cells and that prostaglandin E_2 (PGE_2) indirectly acts on them via osteoblasts (18). However, whether estrogen would directly act on hemopoietic blast cells and/or indirectly act on them via osteoblasts in its effect on PTH-induced osteoclast formation also remains unknown.

PTH acts on bone and kidney through cAMP-dependent protein kinase (PKA) and phospholipase C-coupled calcium/protein kinase C (Ca^{2+}/PKC) pathways (19). Previous reports have demonstrated that estrogen significantly inhib-

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ited PTH-induced cAMP production in osteoblast-like cells (20, 21). These findings led us to postulate the hypothesis that estrogen selectively affects either the PKA or Ca^{2+}/PKC pathway in its action on PTH-induced osteoclast-like cell formation. The present study aims to clarify the mechanism by which estrogen affects PTH-stimulated osteoclast-like cell formation.

Materials and Methods

Materials

BDF_1 mice were obtained from the Shizuoka Experimental Animal Center (Shizuoka, Japan) and ICR mice from Japan Clea Co. (Tokyo, Japan). 17β-estradiol (17β-E_2), 17α-E_2, forskolin, dibutyryladenosine cAMP (dbcAMP), PGE_2, phorbol 12-myristate 13-acetate (PMA), and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO), hPTH-(1–34) from Peptide Institute (Osaka, Japan), hPTHrP-(1–34) from Peninsula Laboratories (Belmont, CA), hPTHrP-(107–111) from Bachem (Torrance, CA), Sp-cAMPS and Rp-cAMPS from Biolog Life Science Institute (Bremen, Germany), H-7 from Seikagaku Kogyo (Tokyo, Japan), A23187 from Hoechst Japan (Tokyo, Japan) and BayK-8644 from Biomolecular Research Laboratories (Plymouth Meeting, PA). 1, 25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$)], human recombinant interleukin (IL)-6 and rat antimouse IL-6 monoclonal antibody were provided by Chugai Pharmaceutical Co. Ltd. (Shizuoka, Japan). hPTH-(35–84) was a kind gift from Peptide Institute (Osaka, Japan). Five-fluorouracil (5-FU) was generously provided from Kyowa Hakko Co. (Osaka, Japan). Murine recombinant IL-3, human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF), and human recombinant interleukin 1β (IL-1β) were purchased from Genzyme Co. (Cambridge, MA). Tumor necrosis factor-α (TNF-α) was generously provided by Asahi Chem. Co. (Shiziuoka, Japan). All other chemicals used were of analytical grade.

Mouse bone cell culture and osteoclast-like cell formation

Osteoclast-like cell formation from mouse unfractionated bone cells was measured, as previously described (22, 23). Femora and tibiae of 10- to 15-day-old ICR mice were aseptically removed. The bones from ten mice were dissected free of soft tissues and mechanically minced with a scalpel blade in phenol red-free MEM containing 5% charcoal-treated FCS. After removal of bone fragments by sedimentation under normal gravity, unfractionated bone cell suspensions were collected from the supernatant. These unfractionated bone cells included tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs), alkaline phosphatase-positive mononuclear cells (probably osteoblasts), and other bone marrow cells. For cell spotting, the number of TRAP-positive cells containing three or more nuclei was counted as TRAP-positive MNCs in these bone cell suspensions. To examine the effect of each chemical on osteoclast-like cell formation using a population of preexistent osteoclast-free bone cells in the presence of osteoblasts, we spotted these mouse bone cells in 96-well plates at 160 TRAP-positive MNCs/well (1×10^5 cells/well). Unfractionated bone cells were cultured in MEM containing 5% FCS at 37 C in a humidified 10% CO_2-90% air atmosphere for 5 days, by which time preexistent osteoclasts (mature osteoclasts existing at the beginning of culture) had degenerated and few TRAP-positive MNCs were observed after 5 days of culture, as previously described in detail (22, 23). Thus, we could test the effects of osteotropic factors on the sequential process leading to osteoclast formation by adding those factors to the cultures after degeneration of preexistent osteoclasts. That is, this method allows us to analyze osteoclast-like cell formation in the presence of osteoblasts. Each chemical was added to these osteoclast-free bone cell cultures. After 7 days of culture, cells adherent to the plates were washed with PBS, dried, and promptly stained for TRAP. Cells were viewed under an inverted phase-contrast microscope, and the number of TRAP-positive MNCs was counted. These newly formed TRAP-positive MNCs had various characteristics of osteoclasts, including responsiveness to calcitonin and bone-resorbing activity, as previously described (24).

Formation of osteoclast-like cells from hemopoietic blast cells derived from mouse spleen cells

Osteoclast-like cell formation from hemopoietic blast cells was measured according to the method of Kurihara *et al.* (25). We previously showed that stromal cells and osteoblasts were not present in this culture system (26). Five-FU was administered to 6-week-old female BDF_1 mice at a dose of 150 mg/kg BW through the tail vein. Four days after injection, spleen cells were harvested from three to five mice, and cell suspensions were prepared by the filtration of the homogenate through nylon mesh. Aliquots of the suspension (2.6×10^6 spleen cells/ml) were plated into 35-mm culture dishes (Falcon, Oxnard, CA) containing 1 ml MEM supplemented with 1.2% methylcellulose, 50 U/ml IL-3, 10^{-8} M IL-6, 10 mg/ml BSA (Sigma Chemical Co., St. Louis, MO), and 30% FCS. The colonies of hemopoietic blast cells, which were TRAP-negative, appeared after approximately 7 days and were lifted from the dish with a 10-μl Eppendorf micropipette. For preparation of osteoclast precursors, aliquots of a suspension of hemopoietic blast cells (10^4 cells/ml) were cultured in 96-well microplates containing 100 μl MEM supplemented with 5% FCS and 200 U/ml GM-CSF for 7 days. Few TRAP-positive MNCs were viewed at this time. Each substance or conditioned medium was then added to this medium, followed by 4 more days of culture, and cells adherent to the plates were washed with PBS, dried, and promptly stained for TRAP. TRAP-positive cells containing three or more nuclei were counted as TRAP-positive MNCs. Stromal cells are not included in spleen cell preparations because spleen cells are supposed not to include stromal cells. Moreover, only hemopoietic cells and not stromal cells have the ability to form a colony. These MNCs had various characteristics of osteoclasts, including responsiveness to calcitonin, calcitonin binding, and osteoclastic bone resorption as evidenced by coculturing with bone rudiments (25). However, bone-resorbing activity of these cells could be demonstrated only in cultures containing stromal cells. Namely, our study revealed that TRAP-positive MNC formed by PTH in mouse spleen cell cultures did not form pits on dentine slice but that these cells could obtain the ability to form pits in coculture with MC3T3-G2/PA-6 stromal cells. These phenomena suggest that the terminal differentiation of osteoclasts requires the presence of stromal cells, as has been reported (27). Moreover, these TRAP-positive MNCs were observed in each colony derived from each replated blast cell, indicating that these hemopoietic blast cells would be precursors for osteoclasts. This method allowed us to analyze osteoclast-like cell formation in the absence of osteoblasts; that is, osteoclast-like cells in this culture system are formed without the help of nonhemopoietic cells including osteoblasts.

Preparation of conditioned medium (CM) derived from osteoblasts

For the preparation of CM from osteoblasts, human osteoblastic SaOS-2 cells or mouse osteoblastic MC3T3-E1 cells were cultured in 24-well plates, as previously described (15, 16). After treatment of SaOS-2 and MC3T3-E1 cells with each chemical for 24 h with or without 24 h pretreatment with 17β-E_2, these cells were washed several times with serum-free, phenol red-free DMEM or MEM, respectively. Then, 300 μl serum-free medium were added to each well. The resulting CM was collected 24 h later.

Statistical analysis

Statistical analysis was performed by Student's *t* test or Duncan's multiple range test. Results of figures are representative of at least three separate cell preparations. Similar results were obtained from other cell preparations.

Results

Effect of 17β-E_2 on PTH-stimulated osteoclast-like cell formation in mouse bone cell cultures

We examined the effects of 17β-E_2 on PTH-stimulated osteoclast-like cell formation by employing mouse bone cell

cultures. As shown in Fig. 1, 17β-E_2 significantly inhibited the formation of MNCs stimulated by 10^{-8} M hPTH-(1–34) at a minimal effective concentration of 10^{-9} M. 10^{-8} M 17β-E_2 similarly caused an inhibition of MNC formation stimulated by 10^{-8} M hPTHrP-(1–34), whereas it did not affect MNC formation stimulated by 10^{-8} M 1,25-$(OH)_2D_3$. On the other hand, 17α-E_2, a much less active stereoisomer of 17β-E_2, did not affect PTH-(1–34)-stimulated MNC formation.

Effect of 17β-E_2 on osteoclast-like cell formation stimulated by other bone-resorbing factors in mouse bone cell cultures

As shown in Fig. 2, 40 U/ml IL-1β, 10^{-8} M TNF-α, and 10^{-6} M PGE_2 significantly stimulated MNC formation in mouse bone cell cultures. Moreover, 10^{-8} M hPTH-(35–84) and 10^{-8} M hPTHrP-(107–111) also significantly stimulated MNC formation in these cultures, as we previously reported (13, 15). However, 10^{-8} M 17β-E_2 did not affect MNC formation stimulated by these bone-resorbing factors.

Effect of 17β-E_2 on PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells via osteoblastic cells

Our previous study indicated that PTH stimulates osteoclast-like cell formation through some soluble factors from osteoblasts (16). We therefore examined whether estrogen would affect the production of soluble factors from osteoblasts pretreated with PTH. As shown in Fig. 3, CM derived from SaOS-2 cells or MC3T3-E1 cells pretreated with hPTH-(1–34) (10^{-8} M) significantly increased the formation of TRAP-positive MNCs from hemopoietic blast cells. MNC formation by CM from cells pretreated with both PTH-(1–34) (10^{-8} M) and 17β-E_2 (10^{-8} M) was significantly reduced as compared with MNC formation by CM pretreated with PTH-(1–34) alone. These data suggested that estrogen blocks PTH-stimulated osteoclast-like cell formation partly through modulating the release of some soluble factors from osteoblasts.

Effect of 17β-E_2 on PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells in the absence of osteoblasts

As we previously reported (26), PTH directly acts on hemopoietic blast cells derived from spleen cells, resulting in the formation of TRAP-positive MNCs possessing calcitonin-binding sites. We next examined whether 17β-E_2 would directly act on hemopoietic blast cells, thereby causing an inhibition of PTH-stimulated osteoclast-like cell formation. As shown in Fig. 4, 10^{-8} M 17β-E_2 significantly inhibited the formation of MNCs stimulated by 10^{-8} M hPTH-(1–34) in mouse spleen cell cultures derived from 5-FU-pretreated mice. On the other hand, 10^{-8} M 17β-E_2 did not affect MNC formation stimulated by 10^{-8} M 1,25-$(OH)_2D_3$, and 10^{-8} M 17α-E_2 did not affect PTH-(1–34)-stimulated MNC formation.

Effect of 17β-E_2 on osteoclast-like cell formation stimulated by various kinds of second messenger analogues in mouse bone cell cultures

We examined whether 17β-E_2 would affect osteoclast-like cell formation stimulated by various second messenger analogues in osteoblast-containing mouse bone cell cultures. As shown in Fig. 5, 10^{-5} M forskolin, which affect the catalytic subunit of adenylate cyclase, and 10^{-4} M dbcAMP and Sp-cAMPS, both PKA activators, significantly caused a stimu-

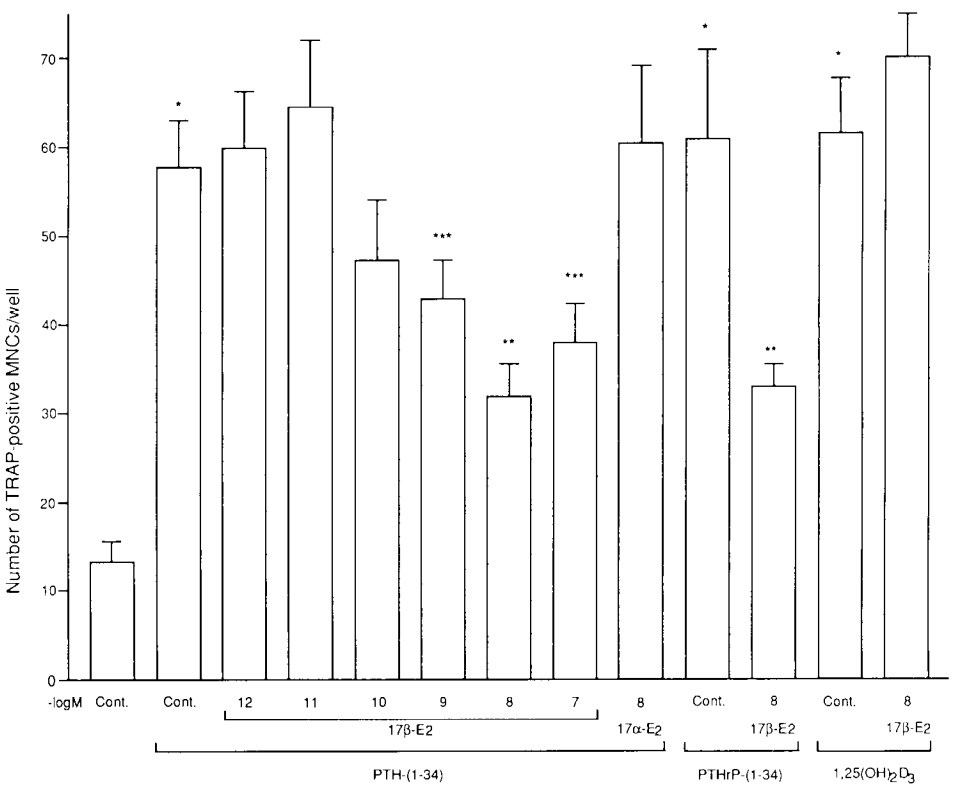

FIG. 1. Effect of 17β-E_2 on osteoclast-like cell formation stimulated by PTH, PTHrP, or 1,25-$(OH)_2D_3$ in mouse bone cell cultures. After degeneration of pre-existent osteoclasts, 10^{-8} M hPTH-(1–34), 10^{-8} M hPTHrP-(1–34), and 10^{-8} M 1,25-$(OH)_2D_3$ were added with or without the indicated concentrations of 17β-E_2 or 10^{-8} M 17α-E_2, followed by 7 days of culture. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Data are expressed as the mean ± SEM of four determinations. *, $P < 0.01$, compared with control. **, $P < 0.01$, compared with PTH- (1–34) or PTHrP-(1–34)-treated, 17β-E_2-untreated group; ***, $P < 0.05$, compared with PTH-(1–34)-treated, 17β-E_2-untreated group.

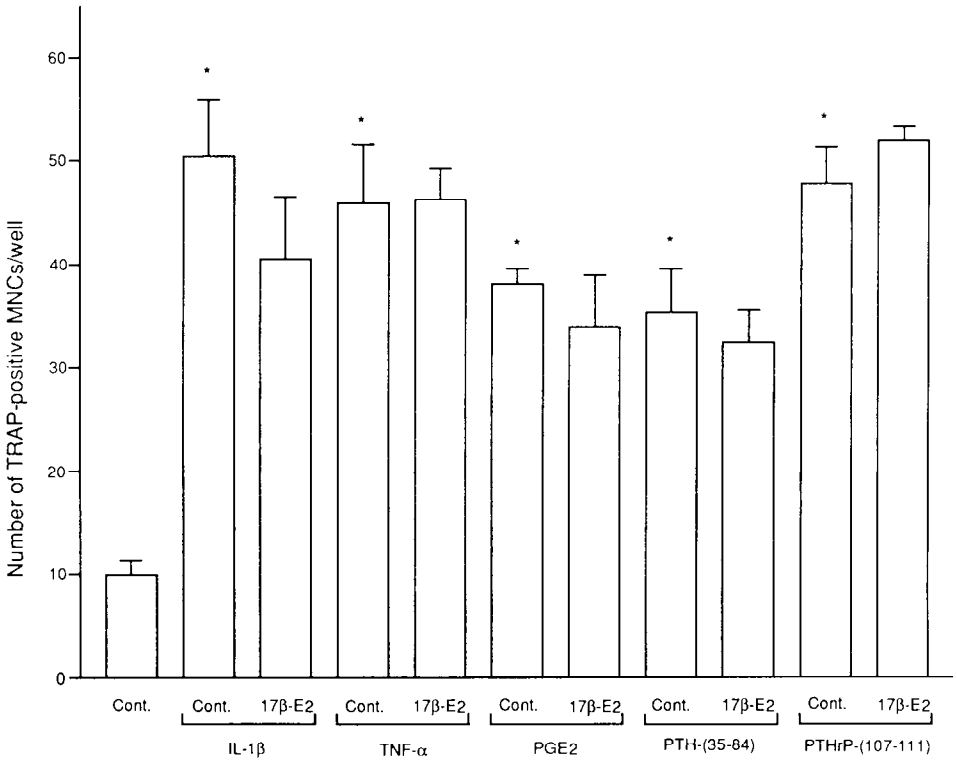

FIG. 2. Effect of 17β-E_2 on osteoclast-like cell formation stimulated by other bone-resorbing factors in mouse bone cell cultures After degeneration of preexistent osteoclasts, 40 U/l IL-1β, 10^{-8} M TNF-α, 10^{-6} M PGE_2, 10^{-8} M hPTH-(35–84), or 10^{-8} M hPTHrP-(107–111) were added with or without 10^{-8} M 17β-E_2, followed by 7 days of culture. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Data are expressed as the mean ± SEM of four determinations. *, $P < 0.01$, compared with control.

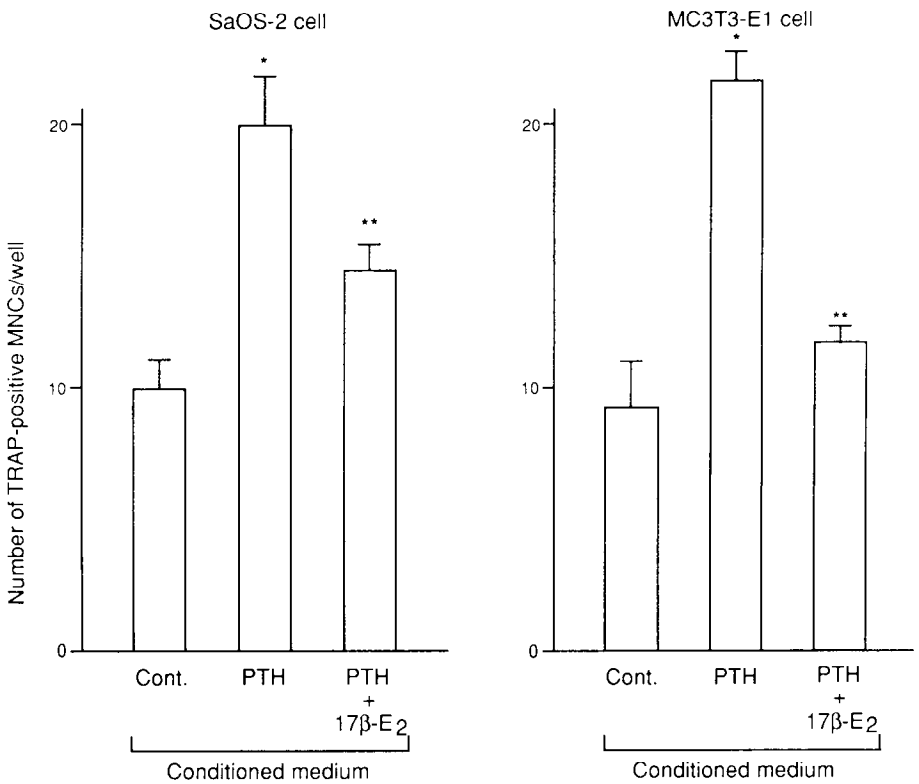

FIG. 3. Effect of 17β-E_2 on PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells via osteoblastic cells. Conditioned medium was obtained from SaOS-2 cells (*left panel*) or MC3T3-E1 cells (*right panel*) pretreated with 10^{-8} M hPTH-(1–34) for 24 h with or without 10^{-8} M 17β-E_2 24 h pretreatment. After treatment of mouse hemopoietic blast cells with 30% of each conditioned medium, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Each bar represents the mean ± SEM of four determinations. *, $P < 0.01$, compared with each control medium, **, $P < 0.01$, compared with each alone PTH-(1–34)-treated conditioned medium.

lation of MNC formation in mouse bone cell cultures. 10^{-8} M 17β-E_2 significantly inhibited MNC formation stimulated by dbcAMP and Sp-cAMPS as well as by forskolin. Moreover, 10^{-8} M 17β-E_2 significantly blocked MNC formation stimulated by 10^{-7} and 10^{-6} M forskolin as well as 10^{-5} M dbcAMP (number of MNC per well: 9.0 ± 0.5 for the control group, 18.9 ± 2.2 for 10^{-7} M forskolin, 10.0 ± 1.0 for 10^{-7} M forskolin, and 10^{-8} M 17β-E_2, 36.3 ± 4.2 for 10^{-6} M forskolin, 22.1 ± 2.7 for 10^{-6} M forskolin and 10^{-8} M 17β-E_2, 21.0 ± 3.6 for 10^{-5} M dbcAMP, 11.3 ± 1.0 for 10^{-5} M dbcAMP, and 10^{-8}

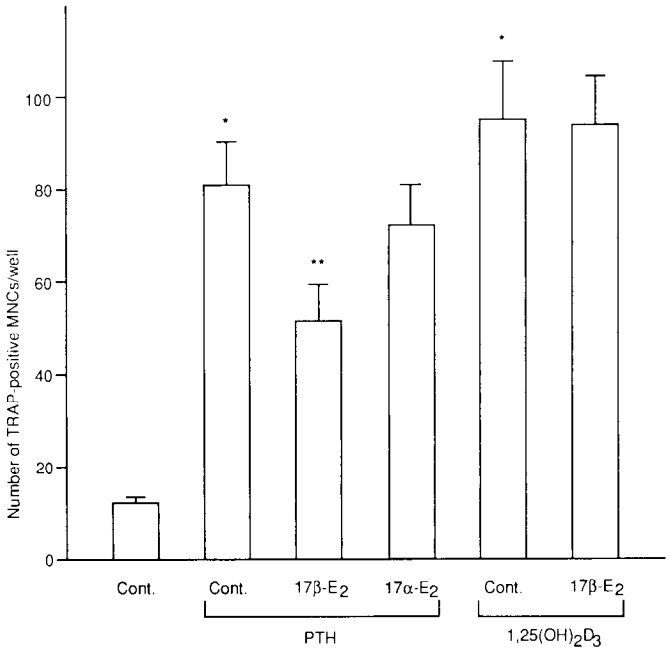

FIG. 4. Effect of 17β-E_2 on PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells in the absence of osteoblasts. Mouse hemopoietic blast cells supported by GM-CSF were treated with 10^{-8} M hPTH-(1–34) or 10^{-8} M 1,25-$(OH)_2D_3$ with or without 10^{-8} M 17β-E_2 or 10^{-8} M 17β-E_2 for 4 days. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Each *bar* represents the mean ± SEM of four determinations. *, $P < 0.01$, compared with control, **, $P < 0.05$, compared with alone PTH-(1–34)-treated group.

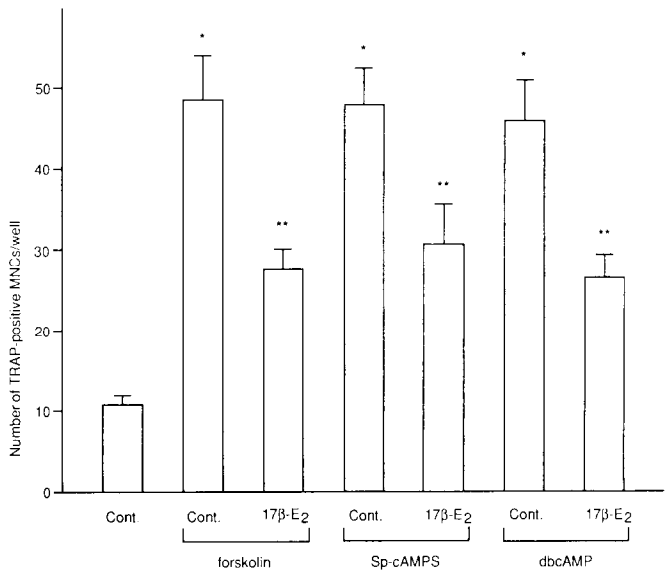

FIG. 5. Effect of 17β-E_2 on osteoclast-like cell formation stimulated by forskolin or PKA activators in mouse bone cell cultures. Unfractionated mouse bone cells after degeneration of preexistent osteoclasts were treated with 10^{-5} M forskolin, 10^{-4} M dbcAMP, or Sp-cAMPS with or without 10^{-8} M 17β-E_2 for 7 days. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Each *bar* represents the mean ± SEM of four determinations. *, $P < 0.01$, compared with control, **, $P < 0.01$, compared with 17β-E_2-untreated group.

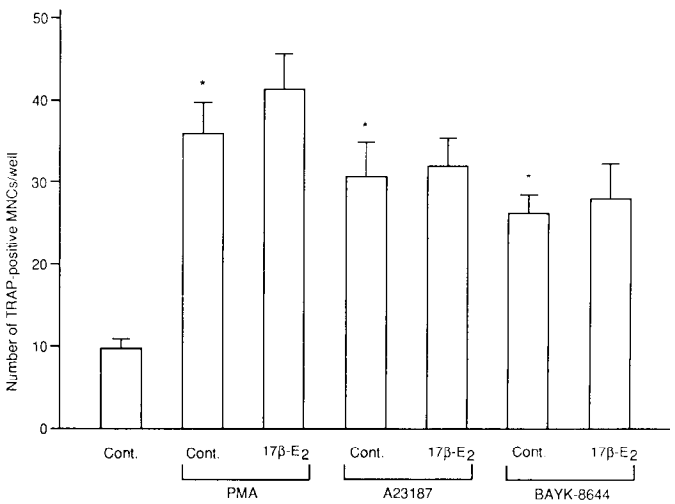

FIG. 6. Effect of 17β-E_2 on osteoclast-like cell formation stimulated by PMA, A23187, BAY-K-8644, in mouse bone cell cultures. Unfractionated mouse bone cells after degeneration of preexistent osteoclasts were treated with 10^{-7} M PMA, 10^{-7} M A23187 or 5×10^{-6} M BAYK-8644 with or without 10^{-8} M 17β-E_2 for 7 days. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Each *bar* represents the mean ± SEM of four determinations. *, $P < 0.01$, compared with control.

M 17β-E_2). 10^{-6} M tamoxifen, one of antiestrogens, almost completely blocked the inhibitory effects of 17β-E_2 on osteoclast-like cell formation stimulated by PTH-(1–34), forskolin, and PKA activators in unfractionated mouse bone cell cultures (data not shown). Therefore, the effects of 17β-E_2 seems to be specific. As shown in Fig. 6, 10^{-7} M PMA, a phorbol ester that activates PKC, 10^{-7} M A23187, a calcium ionophore, and 5×10^{-6} M BAYK-8644, an activator of voltage-dependent calcium channels, significantly stimulated MNC formation in these cultures. However, 10^{-8} M 17β-E_2 did not affect MNC formation stimulated by PMA, A23187, or BAYK-8644. As shown in Fig. 7, 17β-E_2 significantly inhibited MNC formation induced by the combined treatment with 10^{-8} M PTH-(1–34) and 10 μM H-7, a PKC inhibitor, whereas it did not affect MNC formation stimulated by the combined treatment with 10^{-8} M PTH-(1–34) and 10^{-4} M Rp-cAMPS, a direct PKA inhibitor. These data suggested that 17β-E_2 inhibits PTH-stimulated MNC formation by affecting the PKA pathway, and not by affecting Ca^{2+}/PKC.

Discussion

Several lines of evidence have indicated that estrogen causes an inhibition of PTH-stimulated bone resorption in mouse organ cultures (11, 12). Moreover, the results of Orimo *et al.* (28) suggested that the sensitivity of bone to PTH is increased in the absence of endogenous estrogens. The present study demonstrated that 17β-E_2 blocked PTH-stimulated osteoclast-like cell formation in osteoblast-containing mouse bone cell cultures. Because 17α-E_2 did not affect PTH-stimulated osteoclast-like cell formation and 17β-E_2 did not affect 1,25-$(OH)_2D_3$-stimulated osteoclast-like cell formation, the inhibition by estrogen of PTH-stimulated osteoclast-like cell formation is unlikely to be nonspecific. The recent cloning of cDNA of the PTH/PTHrP receptor has demonstrated that both PTH-(1–34) and PTHrP-(1–36) are capable of bind-

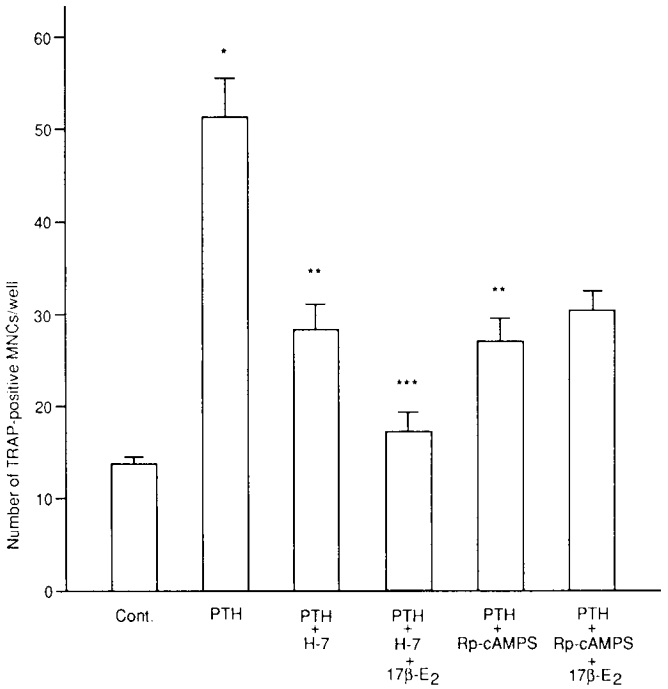

FIG. 7. Effect of 17β-E_2 on osteoclast-like cell formation stimulated by PTH-(1–34) and PKA inhibitor or PTH-(1–34) and PKC inhibitor in mouse bone cell cultures. Unfractionated mouse bone cells after degeneration of preexistent osteoclasts were treated with 10^{-8} M PTH-(1–34) and 10^{-4} M Rp-cAMPS or 10^{-8} M PTH-(1–34) and 10 μM H-7 with or without 10^{-8} M 17β-E_2 for 7 days. Cells were pretreated with Rp-cAMPS or H-7 for 30 min before PTH-(1–34) or 17β-E_2 addition. Then, the number of TRAP-positive MNCs was counted, as described in *Materials and Methods*. Each bar represents the mean ± SEM of four determinations. *, $P < 0.01$, compared with control; **, $P < 0.01$, compared with alone PTH-(1–34)-treated group; ***, $P < 0.05$, compared with PTH-(1–34)- and H-7-treated, 17β-E_2-untreated group.

ing to the same receptor (29). Moreover, our previous studies (26, 30) indicated that PTHrP-(1–34) stimulated osteoclast-like cell formation as well as bone-resorbing activity through the same receptor-mediated mechanisms as PTH-(1–34). The present study revealed that 17β-E_2 inhibited PTHrP-(1–34)- and PTH-(1–34)-stimulated osteoclast-like cell formation to a similar extent. These findings indicated that estrogen inhibits the PTH/PTHrP receptor-mediated stimulation of osteoclast-like cell formation in mouse bone cell cultures. IL-1β, TNF-α, and PGE_2 play important roles as the local bone-resorbing factors in various metabolic bone diseases. Our recent study demonstrated that carboxyl-terminal peptides from PTH and PTHrP stimulate osteoclast-like cell formation, possibly through a mechanism that is different from the mechanism of amino-terminal peptides in mouse bone cell cultures (13, 15). Therefore, we investigated whether estrogen would affect osteoclast-like cell formation stimulated by these osteotropic factors in mouse bone cell cultures. The present study revealed that 17β-E_2 did not affect osteoclast-like cell formation stimulated by these osteotropic factors in mouse bone cell cultures. The previous study (31) revealed that *ex vivo* bone marrow cultures from ovariectomized mice exhibit increased osteoclast formation in the presence of 1,25-$(OH)_2D_3$. This discrepancy may be partly due to the difference of culture systems. In that study, IL-6 mediates increased osteoclast development after estrogen loss. However, our previous study revealed that IL-6 did not stimulate osteoclast-like cell formation in unfractionated mouse bone cell cultures (24). Thus, there is the difference of response to IL-6 between these culture systems. Although it is well known that IL-6 plays an important role in the early stages of osteoclastgenesis and hematopoiesis, the role of IL-6 in the differentiation and fusion of osteoclast precursor cells still remains unclear. The culture system employed in the present study allow us to evaluate the effect of cytokines on the late stages of osteoclast differentiation. Therefore, we cannot rule out the possibility that estrogen inhibits osteoclast-like cell formation stimulated by the factors such as 1,25-$(OH)_2D_3$, when earlier stage of osteoclast precursors was employed. The recent study (32) revealed that IL-6 is downstream effector of PTH, resulting in the stimulation of bone resorption. However, our previous study revealed that IL-6 did not stimulate osteoclast-like cell formation in unfractionated mouse bone cell cultures (24), and our preliminary study revealed that antimouse IL-6 antibody did not affect PTH- and 1,25-$(OH)_2D_3$-stimulated osteoclast-like cell formation in these cultures (data not shown). Therefore, it seems unlikely that IL-6 is a mediator of PTH-stimulated osteoclast-like cell formation in these cultures.

PTH stimulates bone resorption through some soluble factors released from osteoblasts (33). In our recent study (16), conditioned medium from osteoblasts pretreated with PTH-(1–34) stimulated osteoclast-like cell formation from hemopoietic blast cells derived from mouse spleen cells, indicating that PTH stimulates osteoclast-like cell formation via some soluble factors released from osteoblasts. Because osteoblasts possess functional estrogen receptors and estrogen directly stimulates osteoblast proliferation and differentiation (6, 7), the inhibition by estrogen of PTH-stimulated osteoclast-like cell formation might be mediated through osteoblasts. In the present study, the CM derived from SaOS-2 and MC3T3-E1 cells pretreated with both PTH-(1–34) and 17β-E_2 was a less potent stimulator of osteoclast-like cell formation than the CM from cells pretreated with PTH-(1–34) alone. Thus, estrogen appears to inhibit PTH-stimulated osteoclast-like cell formation partly by modulating the release of some soluble factors from osteoblasts through directly acting on these cells.

Hakeda *et al.* (14) and we (26) previously reported that hPTH-(1–34) stimulated osteoclast-like cell formation from mouse hemopoietic blast cells that possess binding sites for this peptide. Fiorelli *et al.* (34) recently reported that functional estrogen receptors are present in the human preosteoclastic cell line FLG29.1, and that estrogen significantly reduced cell proliferation (34). Moreover, Hong *et al.* (9) revealed that mouse osteoclast precursors possess estrogen-binding sites, and that estrogen inhibits the proliferation and maturation of osteoclast precursors (31). The present study revealed that 17β-E_2 significantly inhibited PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells derived from mouse spleen cells pretreated with 5-FU. Because 17α-E_2 did not affect PTH-stimulated osteoclast-like cell formation and 17β-E_2 did not affect 1,25-$(OH)_2D_3$-stimulated osteoclast-like cell formation, the inhibition by estro-

gen of PTH-stimulated osteoclast-like cell formation is likely to be specific. The present findings, therefore, suggested that estrogen inhibits PTH-stimulated osteoclast-like cell formation by directly acting on hemopoietic blast cells.

The cloning of complementary DNA of the PTH/PTHrP receptor revealed that a single cDNA clone, expressing either the rat bone or opossum kidney PTH receptor, mediates the stimulation by PTH of both adenylate cyclase and phospholipase C, when expressed in COS cells (19). It has been reported that osteoblasts possess PTH-responsive dual signal transduction systems (PKA and Ca^{2+}/PKC systems) (19, 35). Moreover, we reported that the activations of PKA and PKC are directly linked to PTH-stimulated osteoclast-like cell formation from hemopoietic blast cells (26). That report (26) and our earlier study (15) indicated that an increase in $[Ca^{2+}]_i$ might be involved in the stimulation of osteoclast-like cell formation by PTH. The present study revealed that 17β-E_2 significantly inhibited osteoclast-like cell formation stimulated by PKA activators and forskolin in mouse bone cell cultures, whereas it did not affect osteoclast like cell formation stimulated by a PKC activator, a calcium ionophore, and a calcium channel activator. Moreover, 17β-E_2 significantly blocked osteoclast-like cell formation stimulated by the combined treatment with PTH and a PKC inhibitor, but not by the combined treatment with PTH and a PKA inhibitor. These findings suggest that estrogen inhibits PTH-stimulated osteoclast-like cell formation through blocking the PKA pathway but not by blocking Ca^{2+}/PKC.

PTH accelerates both bone formation and bone resorption, and its potent bone-forming activity is the reason why PTH is employed as a drug to increase bone volume in osteoporotic patients (36). On the other hand, PTH causes osteopenia by stimulating bone resorption. Thus, PTH possesses complex dual actions in bone, although these mechanisms are unclear. The present study demonstrated that estrogen specifically inhibited PTH-stimulated osteoclast-like cell formation in mouse bone cell cultures. Moreover, Shen *et al.* (37) reported that a combination of estrogen and PTH was significantly better than either agent alone, both in terms of bone mass and trabecular structure in ovariectomized rats. These findings led us to speculate on the possibility that estrogen is effective in preventing the adverse effects that occur in the administration of PTH to the osteoporotic patients by inhibiting the bone-resorbing action induced by PTH as well as by its own effects on bone. The effective coadministration of estrogen and PTH may clinically result in more dramatic effects than either agent alone in the treatment of osteoporosis.

In conclusion, the present study demonstrated that estrogen inhibits PTH-stimulated osteoclast-like cell formation by directly acting on hemopoietic blast cells as well as by indirectly acting on them via osteoblasts. The inhibitory effects of estrogen on PTH-stimulated osteoclast-like cell formation seemed to be mediated through blocking PKA pathway but not by blocking Ca^{2+}/PKC.

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