Phosphodiesterase inhibitors stimulate osteoclast formation via TRANCE/RANKL expression in osteoblasts: possible involvement of ERK and p38 MAPK pathways

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Received 20 October 2004; revised 1 December 2004; accepted 14 December 2004

Available online 12 January 2005

Edited by Lukas Huber

Abstract Phosphodiesterases (PDEs) are enzymes that degrade intracellular cAMP. In the present study, 3-isobutyl-1-methylxanthine (IBMX) and pentoxifylline, PDE inhibitors, induced osteoclast formation in cocultures of mouse bone marrow cells and calvarial osteoblasts. These inhibitors induced the expression of the osteoclast differentiation factor, TNF-related activation induced cytokine (TRANCE, identical to RANKL, ODF, and OPGL), in calvarial osteoblasts. IBMX induced phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) in osteoblasts. Induction of TRANCE expression by IBMX was partially suppressed by the inhibitors of protein kinase A (PKA), ERK, and p38 MAPK, suggesting that activation of ERK and p38 MAPK, as well as PKA, is involved in TRANCE expression by IBMX. Osteoblasts expressed PDE4, a PDE subtype, and rolipram, a selective inhibitor of PDE4, induced TRANCE expression. These results suggest that PDE4 is a key regulator of TRANCE expression in osteoblasts, which in turn controls osteoclast formation.

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Keywords: Osteoblast; Osteoclast; Cyclic adenosine monophosphate; Phosphodiesterase; TNF-related activation-induced cytokine

1. Introduction

Osteoclasts are multinucleated giant cells responsible for bone resorption [1]. Osteoblasts, as well as stromal cells, are essentially required for osteoclastogenesis through cell–cell interactions with osteoclast precursors of monocyte/macrophage lineage [2,3]. In cocultures of mouse bone marrow cells and calvarial osteoblasts, osteoclasts are formed in response to several factors, such as 1,25-dihydroxyvitamin D\textsubscript{3} [1,25(OH)\textsubscript{2}D\textsubscript{3}], parathyroid hormone (PTH), interleukin (IL)-6 plus soluble IL-6 receptor, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), and calcium [2–4]. Those factors induce the expression of TNF-related activation-induced cytokine (TRANCE, also known as RANKL, ODF, or OPGL) in osteoblasts, which triggers osteoclast differentiation [5–8]. Osteoblasts also produce osteoprotegerin (OPG), a decoy receptor for TRANCE, to inhibit osteoclast formation [9].

Cyclic adenosine monophosphate (cAMP) is a secondary messenger in intracellular signaling cascades and elevation of intracellular cAMP levels activates protein kinase A (PKA) and controls gene expression and cell function [10,11]. This pathway is known to induce TRANCE expression in osteoblasts following stimulation with PTH and PGE\textsubscript{2} [12–14]. Intracellular cAMP is generated by adenylate cyclase from adenosine triphosphate (ATP) as a substrate, whereas cAMP-specific phosphodiesterases (PDEs) degrade cAMP by hydrolysis [10,11]. Thus, intracellular cAMP gradients are governed by a balance between its generation by adenylate cyclase and degradation by PDEs.

The PDE family consists of 11 isozymes, PDE1 to 11, and those involved in the degradation of cAMP are PDE1, 2, 3, 4, 7, 8, 10, and 11, with some PDE isozymes further classified into subtypes [11]. Various low molecular weight inhibitors of PDEs have been used as tools to study their biological roles in bone metabolism [15–17]. Pentoxifylline and 3-isobutyl-1-methylxanthine (IBMX) are PDE inhibitors that inhibit all members of the PDE family, and induce an elevation of intracellular cAMP levels by inhibiting cAMP hydrolysis [11]. It has been reported that pentoxifylline and rolipram, a selective PDE4 inhibitor, increase systemic bone-mass in mice [15]. Other PDE4 inhibitors have also been reported to show a therapeutic effect against bone loss in some animal osteopenia models [16,17]. Therefore, PDE inhibitors are considered to be promising candidates for anti-osteoporosis drug therapies, however, their effects on osteoclastogenesis have not been fully elucidated.

In the present study, the effects of PDE inhibitors on osteoclast formation were examined using a mouse coculture system. We found that IBMX, pentoxifylline, and the PDE4-selective inhibitor, rolipram, strongly induced osteoclast formation in the cocultures. Further, TRANCE mRNA and protein expression levels were elevated by IBMX and rolipram in calvarial osteoblasts. Our results also suggested that...
TRANCE expression by IBMX and rolipram is mediated not only by PKA, but also by extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Thus, PDE4 is considered to act as a key regulator of TRANCE expression in osteoblasts.

2. Materials and methods

2.1. Reagents

IBMX and toluidine blue were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). PTH (human, 1-34) was purchased from PEPTIDE Institute, Inc. (Osaka, Japan). PD98059 and SB203580 were obtained from Calbiochem (San Diego, CA, USA). Antibodies against ERK, phospho-ERK, p38 and phosphop38 were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cells and culture system

Primary calvarial osteoblasts were obtained from the calvariae of neonatal ddY mice (Japan SLC Inc., Shizuoka, Japan) using 0.1% collagenase and 0.2% dispase [18]. Bone marrow cells were obtained from the long bones of 4- to 6-week-old ddY male mice. UAMS-32 cells were kindly provided by Dr. Charles A. O’Brien (University of Arkansas for Medical Sciences, USA). To examine osteoclast formation, mouse bone marrow cells (1 × 10^5 cells) were cocultured with calvarial osteoblasts (5 × 10^3 cells), or UAMS-32 cells (5 × 10^3 cells) in the presence or absence of various concentrations of PDE inhibitors or 1,25(OH)2D3 (10 nM) in 96-well culture plates (CORNING, MA, USA). After 6 days of culture, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts, as described previously [18].

2.3. Pit formation assay

Mouse bone marrow cells (1 × 10^5 cells) and calvarial osteoblasts (5 × 10^3 cells) were cocultured in the presence or absence of IBMX (50 μM) on dentine slices (0.3 mm thick, 4 mm in diameter) placed in 48-well culture plates for 6 days, as described previously [18]. The slices were then recovered and stained with toluidine blue to visualize resorption pits.

2.4. Northern-blot analysis

Calvarial osteoblasts in 60-mm diameter dishes were cultured with agents for the indicated periods and then subjected to total RNA extraction using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer’s protocols. Total RNA (20 μg) was electrophoresed on 1.2% agarose-formaldehyde gels, transferred to nylon membrane filters (Hybond N+, Amersham Biosciences, Buckinghamshire, UK), and hybridized with 32P-labeled cDNA probes. After the final wash, the membranes were exposed to X-ray film (BioMax, Kodak, NY, USA) at −70 °C.

2.5. Flow cytometry analysis

Osteoblasts treated with or without IBMX (50 μM) for 24 h were stained with Fc-conjugated soluble TRANCE receptor (RANK) (kindly provided by Prof. Yongwon Choi, University of Pennsylvania, Fig. 1. Effects of PDE inhibitors on osteoclast formation. (A and B) Dose-dependent effects of IBMX and pentoxifylline on osteoclast formation in cocultures. Mouse bone marrow cells and calvarial osteoblasts were cocultured in the presence of the indicated concentrations of IBMX (A) or pentoxifylline (B) for 6 days. Cells were then fixed and stained for TRAP. TRAP-positive (+) multinucleated cells (MNCs) were counted. Data are expressed as means ± S.D. of triplicate cultures. (C) Photographs of cocultures (upper panels) and dentin slices (lower panels), on which cells were cocultured with (+) or without (−) 50 μM of IBMX for 6 days. Cells were fixed and stained for TRAP, which appear as dark-stained cells. Resorption pits were stained with toluidine blue after removing the cells and appear as dark dots. (D) Expression of calcitonin receptor (CTR) and cathepsin K mRNA in cocultured cells. Bone marrow cells and calvarial osteoblasts were cocultured for 6 days with (+) or without (−) 50 μM of IBMX. Total RNA was then isolated from the cells and cDNA templates prepared with (+) or without (−) reverse transcriptase (RT). mRNA expression was determined by RT-PCR using specific primers designed for each gene. Bar = 200 μm.
USA) followed by phycoerythrin-conjugated anti-IgG antibody (BD Pharmingen, San Diego, CA, USA). The stained cells were analyzed by flow cytometry.

2.6. RT-PCR analysis
Total RNA (1 μg) was reverse-transcribed using Superscript II (Invitrogen, CA, USA) according to the manufacturer’s protocols. Aliquots of the obtained cDNA pool were subjected to PCR amplification with Go Taq DNA polymerase (Promega Co., WI, USA). Primers for mouse PDE4s, TRANCE, and GAPDH used in this study are as follows: PDE4A, 5'-gggaatcacaactctgttgca-3' (forward), 5'-ggtgctgtcaagatggtec-3' (reverse); PDE4B, 5'-tggaatcactgtgctgccat-3' (forward), 5'-ttaagaagtggtgtc-3' (reverse); PDE4C, 5'-gtggatagagtaggtc-3' (forward), 5'-ctctgtgtaaaccttgctg-3' (reverse); PDE4D, 5'-taagatgctgtgctgacct-3' (forward), 5'-acagagagaggtggttac-3' (reverse); TRANCE, 5'-caagcttacaattgta-3' (forward), 5'-aagggagaattgac-3' (reverse); calcitonin receptor (CTR), 5'-tttcaaaacatgtcagctct-3' (forward), 5'-aaggtggaagggctgctg-3' (reverse); and GAPDH, 5'-aaggtggaagggctgctg-3' (forward), 5'-aaggtggaagggctgctg-3' (reverse). The PCR program was as follows: 32 (all mouse PDE4s, TRANCE, CTR, and calcinein K) or 28 (GAPDH) cycles, after an initial denaturation step at 94°C for 3 min, then denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min.

2.7. Immunoblot analysis
Total cell lysates were isolated, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat-milk in TBS-T (150 mM NaCl, 20 mM Tris, pH 7.4, and 0.1% Tween 20), and then immunostained with anti-phospho p38 (1:1000), anti-phospho ERK (1:1000), or with anti-ERK antibody (1:1000) followed by secondary horseradish peroxidase-conjugated antibody (1:1000). The membranes were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences).

3. Results

3.1. IBMX stimulates osteoclast formation in mouse coculture system
We first examined the effects of the PDE inhibitors, IBMX and pentoxifylline, on osteoclast formation using cocultures of mouse bone marrow cells and calvarial osteoblasts. Each inhibitor induced TRAP-positive multinucleated cell (MNC) formation in a dose-dependent manner (Fig. 1A–C). The mRNA expression levels of CTR and cathepsin K, osteoclast markers, were increased in the cultures treated with IBMX (Fig. 1D). When the cells were cocultured in the presence of IBMX (50 μM) on dentine slices, resorption pits were formed (Fig. 1C). These results indicate that both IBMX and pentoxifylline strongly induce osteoclasts in a mouse coculture system.

3.2. Induction of TRANCE expression in calvarial osteoblasts is involved in osteoclast formation
Osteoclast formation induced by IBMX was completely inhibited by addition of OPG, a decoy receptor for TRANCE (Fig. 2A). Furthermore, addition of soluble TRANCE receptor (RANK) also inhibited osteoclast formation induced by IBMX (data not shown). These data suggest that TRANCE is involved in the osteoclast formation induced by IBMX. To determine whether IBMX induces TRANCE expression, calvarial osteoblasts were treated with various concentrations of IBMX for 3 h. IBMX elevated intracellular cAMP levels in osteoblasts within 3 h (data not shown). IBMX dose-dependently stimulated TRANCE mRNA expression in calvarial osteoblasts, with the maximum effect seen at 100 μM (Fig. 2B). In agreement with this, analysis of osteoblastic cell surface by flow cytometry revealed that TRANCE expression is upregulated in protein level after IBMX treatment (Fig. 2C).

3.3. Inhibitors of PKA, ERK and p38 MAPK suppress TRANCE expression induced by IBMX
To identify the signaling pathways used by IBMX to induce TRANCE expression, the roles of the PKA, ERK, and p38 MAPK signaling cascades in TRANCE expression
were examined. Consistent with previous reports [12–14], pretreatment of calvarial osteoblasts with a PKA inhibitor, H89, decreased TRANCE mRNA expression levels induced by IBMX (Fig. 3A). Interestingly, a MAPK/ERK kinase (MEK) inhibitor, PD98059, and a p38 MAPK inhibitor, SB203580, also decreased TRANCE mRNA expression levels induced by IBMX (Fig. 3A). PD98059 and SB203580, but not H89 alone slightly decreased endogenous TRANCE mRNA expression levels (Fig. 3A). Immunoblot analysis using specific antibodies against phospho-ERK and phospho-p38 MAPK indicated that IBMX induced the activation of ERK and p38 MAPK pathways in osteoblasts (Fig. 3A). These results suggest that IBMX regulates TRANCE expression in osteoblasts via activation of the ERK and p38 MAPK signaling pathways, as well as PKA.

Since PTH is known to stimulate TRANCE mRNA expression via cAMP accumulation [12,14], we examined the effects of PD98059 and SB203580 on TRANCE mRNA expression induced by PTH. These inhibitors also decreased the expression levels of TRANCE mRNA induced by PTH (Fig. 3C), suggesting that ERK and p38 MAPK signaling pathways are involved in PTH-induced TRANCE expression.

3.4. PDE4 is a key regulator of TRANCE expression in calvarial osteoblasts

To determine the PDE isozymes responsible for TRANCE expression in calvarial osteoblasts, effects of various isozyme-selective PDE inhibitors on osteoclast formation and TRANCE mRNA expression in calvarial osteoblasts were examined. A PDE4 selective inhibitor, rolipram, dose-dependently induced osteoclast formation and TRANCE mRNA expression in calvarial osteoblasts (Fig. 4A–C). Vinpocetine, EHNA, milrinone, dipyridamole, and zaprinast (selective inhibitors of PDE1, PDE2, PDE3, PDE5 and 8, PDE5 and 9, respectively) did not induce osteoclast formation or TRANCE mRNA expression in calvarial osteoblasts as strongly as IBMX and rolipram (Fig. 4A and C). Similar to the results by IBMX, H89, PD98059, and SB203580 decreased the TRANCE mRNA expression levels induced by rolipram (Fig. 4D). These results suggest that TRANCE regulation in calvarial osteoblasts is attributable to PDE4.

3.5. PDEs regulate TRANCE expression in cells of osteoblastic lineage

Based on the results shown above, we compared the expression patterns of PDE4 subtypes in calvarial osteoblasts and the
mouse bone-marrow stromal cell line, UAMS-32. Calvarial osteoblasts, as well as UAMS-32 cells, expressed PDE4A, B, and D, however, not 4C (Fig. 5A). Both calvarial osteoblasts and UAMS-32 cells supported osteoclast formation, and induced TRANCE mRNA expression in response to IBMX and 1,25(OH)_{2}D_{3}, an activator of vitamin D receptor-mediated pathway independently of PKA and MAPK (Fig. 5B and C). These results suggest that not only vitamin D receptor but also PDEs regulate TRANCE expression in cells of osteoblastic lineage.

4. Discussion

In the present study, we demonstrated that PDE inhibitors induce osteoclast formation by inducing TRANCE expression in calvarial osteoblasts and UAMS-32 cells, suggesting that PDEs are the key molecules that negatively regulate TRANCE expression by degrading cAMP. Our results also suggested that PDE4 mainly regulates cAMP levels and TRANCE expression in osteoblasts.

In contrast to our results, previous reports have shown the anabolic effects of PDE inhibitors in vivo [15–17]. Kinoshita et al. [15] have reported that pentoxifylline and rolipram increased bone mass in animals that were given those drugs in daily injections. Interestingly, PTH as well as PDE inhibitors induce osteoclast formation in a mouse coculture system. However, injections of PTH to animals once daily produce a net anabolic effect in vivo [14,19]. Furthermore, continuous exposure to PTH leads to a coupled increase in bone formation and bone resorption, with a net loss of bone mass [19]. Therefore, PDE inhibitors may induce bone loss if the animals are administrated continuously. Additional analysis of PDE inhibitor-administrated animals under various conditions is necessary to understand the effects of PDEs on bone metabolism.

Elevated intracellular cAMP mainly activates PKA and this pathway has been implicated in TRANCE-mediated osteoclast formation [12–14]. In agreement with this, our experiments showed that the PKA inhibitor reduced the TRANCE mRNA expression induced by IBMX. Recently, Rawadi et al. [20] reported that ERK1/2 and p38 MAPK are activated by pentoxifylline to promote the differentiation of osteoblasts. Indeed, our results showed that IBMX activated ERK and p38 MAPK pathways, and the ERK inhibitor, PD98059, and p38 MAPK inhibitor, SB203580, reduced TRANCE mRNA expression induced by IBMX and rolipram. Furthermore, these MAPK inhibitors partially suppressed PTH-induced elevation of TRANCE expression as well. These results suggest that an alternative signaling pathway contributes to TRANCE expression via ERK and p38 MAPK activation following the cAMP elevation in osteoblasts. Further study will elucidate the precise relationships between PKA and MAPK.

In the present coculture system, only the PDE4 inhibitor rolipram, and not other inhibitors selective for PDE isozymes,
GAPDH mRNA were determined by RT-PCR. Extracted from the cells, and the expressions of TRANCE and 1,25(OH)2D3 (10 nM) or IBMX (50 μM) were measured. (A) Calvarial osteoblasts or UAMS-32 cells were treated with (+) or without (−) 1,25(OH)2D3 (10 nM) or IBMX (50 μM) for 6 days. Cells were then fixed and stained for TRAP. Data are expressed as means ± S.D. of triplicate cultures. (B) Mouse bone marrow cells were cocultured with calvarial osteoblasts or UAMS-32 cells in the absence (−) or presence (+) of 1,25(OH)2D3 (10 nM) or IBMX (50 μM) for 6 days. Cells were then fixed and stained for TRAP. Data are expressed as means ± S.D. of triplicate cultures. (C) Calvarial osteoblasts or UAMS-32 cells were treated with (+) or without (−) 1,25(OH)2D3 (10 nM) or IBMX (50 μM) for 3 h. Total RNA was then extracted from the cells, and the expressions of TRANCE and GAPDH mRNA were determined by RT-PCR.

Acknowledgments: We thank Dr. Charles A. O’Brien (University of Arkansas for Medical Sciences, USA) for providing the UAMS-32 cells. We also thank Dr. Yongwon Choi (University of Pennsylvania, USA), Dr. Nacksung Kim (Chonnam National University, Korea), Dr. Dong-Seok Lee (Korea Research Institute of Bioscience and Biotechnology, Korea), and Dr. Kunhong Kim (Yonsei University, Korea) for technical support.

References


In conclusion, PDE inhibitors promote osteoclast formation via TRANCE expression in osteoblasts. TRANCE expression induced by PDE inhibitors is possibly mediated by ERK and p38 MAPK, as well as PKA activation. Further analysis of animals given PDE inhibitors will contribute to the development of anti-osteoporosis drugs based on PDE activities.


