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PDE4 inhibitor upregulates PTH-induced osteoclast formation via CRE-mediated *COX-2* expression in osteoblasts

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ABSTRACT

We investigated the interplay between parathyroid hormone (PTH) and phosphodiesterases (PDEs) in osteoblasts. PDE4 negatively regulated PTH-induced cAMP accumulation. PDE4 inhibitor enhanced PTH-induced osteoclast formation and *RANKL* mRNA expression, which is partially mediated by *COX-2* mRNA expression. Two CRE sites in the *COX-2* promoter were required for the increase in *COX-2* transcription by PDE4 inhibitor, and the expression of a dominant-negative form of CREB abolished *COX-2* mRNA expression in response to PDE4 inhibitor or PTH in osteoblasts. Taken together, our data indicate that PDE4 inhibitor promotes PTH-induced osteoclast formation partially via CRE-mediated *COX-2* mRNA expression.

1. Introduction

Osteoclasts are bone-resorbing, multinucleated cells that are derived from the monocyte-macrophage lineage under the tight regulation of stromal/osteoblastic cells [1–4]. In co-cultures of mouse bone marrow cells and calvarial osteoblasts, osteoclasts are formed in response to several osteotropic factors, including 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], interleukin (IL)-6 plus soluble IL-6 receptor, and prostaglandin E_2 (PGE_2) [2,3]. These factors induce the expression of receptor activator of NFκB ligand (RANKL, also known as TRANCE, ODF, or OPGL) in osteoblasts [4,5]. The binding of RANKL to its cognate receptor, RANK, which is expressed on the surface of osteoclast progenitor cells, induces osteoclastogenesis and activates osteoclasts, resulting in increased bone resorption.

Parathyroid hormone (PTH) is one of the main hormones regulating bone resorption. PTH stimulates osteoclast formation by binding to its receptor, PTH receptor 1 (PTHR1), on stromal/osteoblastic cells, leading to the increased production of RANKL [6]. The stimulation of RANKL by PTH requires cyclooxygenase-2 (COX-2) expression via cyclic AMP (cAMP) production in stromal/osteoblastic cells [7,8]. Notably, COX-2 expression is also required for the full induction of RANKL by IL-1, -6, and -17, basic fibroblast growth factor (bFGF), and 1,25$(OH)_2D_3$, indicating that COX-2 is a common component in various osteoclastogenic signaling pathways [9].

It was recently suggested that the properties of cAMP-induced signals are shaped by the presence of a large array of cyclic nucleotide phosphodiesterases (PDEs) [10]. PDEs are enzymes that degrade and inactivate cAMP/cGMP. The more than 20 genes encoding PDEs in the mammalian genome can be subdivided into 11 families based on sequence homology, substrate specificity, and inhibitor sensitivity. In addition, most PDE genes are expressed as multiple variants through the use of different promoters or alternative splicing, generating up to 100 individual PDE proteins, which implies a complex array of functions for these enzymes. PDEs involved in the degradation of cAMP include PDE1, 2, 3, 4, 7, 8, 10, and 11 [11].

Although PTH has been shown to stimulate osteoclast formation via a cAMP-dependent mechanism in osteoblasts, no data are available regarding the pattern of interaction between PTH and PDEs. Thus, in the present study, we used various PDE inhibitors to identify the PDEs involved in PTH signaling in osteoblasts, which in turn regulate osteoclast formation. Understanding the mode of interaction between PTH and PDEs has important pharmacological and clinical implications for bone-related diseases.

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2. Materials and methods

2.1. Reagents

PTH (human, 1–34) was purchased from Anygen Co., Ltd. (Gwang-ju, Korea). Antibodies against COX-2 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were from Sigma–Aldrich (St. Louis, MO).

2.2. Cells and culture system

Primary calvarial osteoblasts were obtained from the calvariae of neonatal ICR mice (Samtako Inc., Korea) as described before [12]. The murine osteoblastic cell line MC3T3-E1 subclone 4 cells (MC-4) [13] was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in α-MEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Bone marrow cells were obtained from the long bones of 4- to 6-week-old ICR male mice. To examine osteoclast formation, mouse bone marrow cells (1×10^5 cells) were co-cultured with osteoblasts (5×10^3 cells) in the presence or absence of PDE inhibitors and/or PTH (100 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 [12].

2.3. cAMP measurement

Calvarial osteoblasts were treated with PTH (100 nM) in the presence or absence of PDE inhibitors for the indicated hours. Intracellular cAMP concentrations were measured using a commercially available cAMP enzyme-immunoassay kit (Amersham Biosciences, Bukinghamshire, UK) according to the manufacturer's protocol.

2.4. Northern blot analysis

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

2.5. Gene knock-down by oligonucleotide siRNA

The 22-nucleotide small interfering RNA (siRNA) for COX-2 and negative control (scrambled) was purchased from Dharmacon (Colorado, USA). For siRNA transfection, mouse osteoblastic MC-4 cells were seeded in 6-well plate at a density of 2×10^5 cells/well. Twenty-four hours later, cells were transfected with 100 nM siRNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The transfection took place in 2 ml serum-free media for 6 h after which the cells were cultured for 24 h in complete media. And then the cells were harvested for immunoblot, RT-PCR or osteoclast formation analysis.

2.6. RT-PCR analysis

Total RNA was prepared using easy-BLUE (iNtRON Biotechnology, Seoul, Korea) and cDNAs were synthesized from 1 μg of RNA using RevertAid First Strand cDNA Synthesis Kit (Fermantas, Hanover, MD). The PCR primer sequences used are as follows: RANKL, 5′-ccagccatttgcacacctc-3′ (forward), 5′-agcagggaagggttggaca-3′ (reverse); β-actin, 5′-tgtgatggtgggaatgggtcag-3′ (forward), 5′-tttgatgtcacgcacgatttcc-3′ (reverse). The PCR program was as follows: 32 (RANKL) or 22 (β-actin) 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 45 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, and then immunostained with anti-COX-2 (1:1000) or with β-actin (1:4000) antibody followed by secondary horseradish peroxidase-conjugated antibody (1:5000). The membranes were developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, Bukinghamshire, UK).

2.8. DNA mutation

Luciferase reporter pGL3 plasmid including 5′-flanking region (from −1302 to +123 bp) of the human COX-2 gene was kindly provided by Dr. S. Prescott (University of Utah, Salt Lake). DNA fragments of various lengths of the COX-2 promoter were prepared by PCR using the pGL3 plasmid as the template and subsequently subcloned into pGL3 plasmid. The mutant CRE1, CRE2 and CRE1/CRE2 plasmid clones were constructed by using Site-Directed Mutagenesis Kit (Stratagene, CA, USA). Primers for mutagenesis is as followings; CRE1, 5′-cagcctattaagcgt**cgtga**ctaaaacataaaac-3′; CRE2, 5′-gaaacagtcattt**cgtga**catgggcttgg-3′. Underlined regions indicate location of the specified response elements in the wild-type sequence; mutated bases are in bold. The correct clones were confirmed by sequencing.

2.9. Luciferase assay

For luciferase assay, calvarial osteoblasts were transfected with 1.5 μg pGL3-COX-2 promoter- or pCRE-luciferase reporter plasmid (Stratagene, CA, USA) and 0.5 μg pCMV-β-galactosidase plasmid. Cells were then incubated with PTH (100 nM) or rolipram (10 μM) for 6–8 h. Cells were harvested and lysed in a reporter lysis buffer, and the cleared lysates were transferred to each tube, and luciferase assay reagent (Promega, WI, USA) was added. The light intensity of the reaction was determined using a luminometer (Turner Biosystems Inc., Sunnyvale, CA) and the luciferase activity was normalized to β-galactosidase activity.

2.10. Overexpression of DN-CREB (CRE-binding protein)

Dominant-negative (DN)-CREB (pCMV-KCREB, that contains mutations in its DNA-binding domain) and wild-type (WT)-CREB (pCMV-CREB) were purchased from Clontech (Palo Alto, CA). Mouse osteoblastic MC-4 cells were co-transfected with pCRE-Luc, pCMV-β-galactosidase plasmid and (DN)-CREB, and transfected cells were selected with G418 (Sigma–Aldrich, USA). Transfected cells were treated with PTH (100 nM) or rolipram (10 μM) for indicated time, and then harvested with easy-BLUE for Northern blot analysis or reporter lysis buffer for luciferase assay.

2.11. Statistical analysis

Data are presented as the means + S.D. from at least three independent experiments.