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Effects of β-adrenergic agonists on bone-resorbing activity in human osteoclast-like cells

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Abstract

In the present study, we demonstrate for the first time that β-adrenergic agonists stimulate bone-resorbing activity in human osteoclast-like multinucleated cells (MNCs). Osteoclast-like MNCs constitutively expressed mRNA for α1B-, α2B- and β2-adrenergic receptor (AR) in addition to characteristic markers of mature osteoclast, such as calcitonin receptor (CT-R), tartrate-resistant acid phosphatase (TRAP), αV-chain of integrin (Int αV), carbonic anhydrase II (CA-II) and cathepsin K (Cathe K). Epinephrine (1 μM; α,β-adrenergic agonist) up-regulated expression of Int αV, CA-II and Cathe K in the osteoclast-like MNCs. Osteoclastic resorbing activity was markedly increased by isoprenaline (1 μM; β-adrenergic agonist), moderately by epinephrine, but poorly by phenylephrine (1 μM; α1-adrenergic agonist). The actin ring, which was suggested to be correlated with bone-resorbing activity, was clearly observed in osteoclast-like MNCs treated with isoprenaline and epinephrine, but faintly in those treated with phenylephrine. These findings suggest that β-adrenergic agonists directly stimulate bone-resorbing activity in matured osteoclasts.

Keywords: Osteoclast; Bone-resorbing activity; Epinephrine; Isoprenaline; β-Adrenergic agonist

1. Introduction

Immunohistochemical studies showed that mammalian bones were widely innervated by sympathetic and sensory nerves [1,2]. We recently demonstrated the constitutive expression of axon guidance molecules, which are known to be chemoattractant or chemorepellent for growing nerve fibers, in osteoblastic and osteoclastic cells, suggesting the extension of axons of peripheral neurons to osteoblastic and osteoclastic cells and the possible neural regulation of bone metabolism in these osteogenic cells [3]. The possibility of adrenergic regulation in bone resorption has been documented in in vivo and in vitro studies. It was demonstrated that chemical sympathectomy by guanethidine-impaired bone resorption in rats [4], and that β-adrenergic agonists could stimulate bone resorption in intact mouse calvariae [5] and production of prostaglandin E_2 (PGE_2), a bone-resorption factor, in mouse osteoblastic cells [6].

Osteoclasts are multinucleated cells (MNCs) that are responsible for bone resorption [7]. The molecular basis for the regulation of osteoclast formation and function by cells of the osteoblastic lineage has been identified; osteoblastic cells induced osteoclastic differentiation and resorbing activity through expression of osteoclast differentiation factor (ODF, also known as RANKL/OPGL/TRANCE) [8]. In our recent study, we showed that isoprenaline and epinephrine increased ODF/RANKL mRNA expression in mouse osteoblastic cell line MC3T3-E1, and induced osteoclastogenesis in mouse bone marrow culture, suggesting β-adrenergic stimulation of bone resorption via osteoblastic cells [9]. However, there remains a possible mechanism for the stimulative effect of β-adrenergic agonist on osteoclasts. Frediani et al. [10] demonstrated that cathecolamines may act as inducers of osteoclast maturation in vitro and as stimulators of osteoclast activity via binding to β2-AR by

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use of a clonal cell line of human osteoclast precursors (FLG 29.1 cells).

In the present study, using human osteoclast-like MNCs, we examined if adrenergic stimulation could directly activate the osteoclastic function.

2. Materials and methods

2.1. Materials

Epinephrine bitartrate, phenylephrine hydrochloride, isoprenaline bitartrate and pronase E were purchased from Sigma (St. Louis, MO). Heat-inactivated horse serum and α-minimum essential medium (α-MEM) were purchased from Gibco BRL (Life Technologies, Grand Island, NY). All other chemicals used were of reagent grade.

2.2. Isolation of human osteoclast-like MNCs

Human osteoclast-like MNCs were generated using bone marrow, which was obtained as surgical waste from elderly patients, who gave their informed consent before artificial hip replacement surgery. Briefly, the mononuclear cells were separated by density gradients centrifugation ($400 \times g$, 30 min) through Histopaque 1077 (Sigma) and were resuspended in α-MEM supplemented with 20% heat-inactivated horse serum and 10 nM $1\alpha,25(OH)_2D_3$ at a final concentration of 5×10^6 cells/ml; 2 ml of cell suspension was seeded in 35-mm plastic dishes and cultured at 37 °C in humidified air with 5% CO_2. When the numbers of osteoclast-like MNCs appeared in the culture about 2 weeks after the start of primary cultures, stromal cells and other cells that had adhered to the osteoclast-like MNCs were selectively removed by treatment with 0.002% pronase E and 0.02% EDTA for 5 min with agitation, as reported previously [11]. Greater than 95% of the remaining cells were osteoclast-like MNCs.

2.3. RNA extraction and RT-PCR procedures

RNA was extracted by the guanidium-thiocyanate method [12]. RT-PCR was performed using standard methods, as reported previously [13]. Briefly, cDNA was first synthesized using random primers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), followed by PCR amplification using specific primers. The oligonucleotides used as primers for PCR are shown in Table 1 [14–28]. PCR amplification was performed using the GeneAmp PCR System (Perkin Elmer/Cetus, Norwalk, CT) as follows: denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s for appropriate cycles. PCR products were electrophoresed on a 2% Nusieve agarose gel (FMC BioProducts, Rockland, ME) and stained with ethidium bromide and detected on a FluorImager 575 (Molecular Dynamics, Sunnyvale, CA).

2.4. Formation of resorption pits on calcium phosphate-coated disk

Bone-resorption activity of osteoclast-like MNCs was examined by the simple testing method using calcium phosphate-coated disks with culture wells (Osteologic; Millenium Biologix, Ontario, Canada) [29]. After osteoclast-like MNCs were dispersed with agitation in pronase E solution for 20 min, cells were reseeded onto calcium phosphate-coated disks and cultured in α-MEM with phenylephrine (1 μM), epinephrine (1 μM) and isoprenaline (1 μM) for 5 days. The culture medium containing each drug was replaced every 2 days. The culture was terminated on day 5 by the addition of 6% sodium hypochlorite to remove cells. Calcium phosphate-coated disks were stained with 5% silver nitrite for 2 h, fixed in 10% formalin containing 5% sodium carbonate, and

Table 1
Nucleotide of PCR primers

	Sense	Antisense	Size (bp)	Reference
α1A-AR	GTGCGCCTGCTCAAGTTCTCC	TCGGGCGTTCCTGGGGGTTCG	(434)	[14]
α1B-AR	TGGGCGCCTTCATCCTCTTTG	CGCACTCCTTGTCATCGTTGG	(432)	[15]
α1D-AR	TCTTTTCGGGGTGCTGGGTAA	TGGGTGACGATGGTTGGGTAG	(309)	[16]
α2A-AR	TCGTCATCATCGCCGTGTTCA	GCCGCCGCCGCCCTTCTTCTC	(380)	[17]
α2B-AR	GGGAGACCCCTGAAGATACTG	ACAAAAACGCCAATGACCACA	(367)	[18]
α2C-AR	GCGGCAGGCGAGGCGAGAACG	AACCAGCAGAGCACGAACACG	(386)	[19]
β1-AR	GCCATCGCCTCGTCCGTAGTC	CGTAGCCCAGCCAGTTGAAGA	(430)	[20]
β2-AR	TCTGATGGTGTGGATTGTGTC	ACGTCTTGAGGGCTTTGTGCT	(362)	[21]
β3-AR	CCCAATACCGCCAACACCAGT	CGACCCACACCAGGACCACAG	(427)	[22]
TRAP	CTTCAAGATCCCACAGACCAA	ATTCCCAGCCCCACTCAGCAC	(367)	[23]
CT-R	CTGCTGGCTGAGTGTGGAAAC	GATTCATTTCCAGGGCTTCTT	(294)	[24]
CAII	GAGGATCCTCAACAATGGTCA	GAAGAGGAGGGGTGGTCAGTG	(437)	[25]
Int αV	CTTCTTGGTGGTCCTGGTAGC	CACATCTGCATAATCATCTCC	(372)	[26]
Cathe K	CAAGAAGAAAACTGGCAAACT	TGTAAAACTGGAAGGAGGTCA	(323)	[27]
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	(452)	[28]

AR, adrenergic receptor; TRAP, tartrate-resistant acid phosphatase; CT-R, calcitonin receptor; CAII, carbonic anhydrase II; Int αV, αV-chain of integrin; Cathe K, cathepsin K; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

examined by brightfield optics. Resorption activity was estimated from the pit area measured by counting the mesh number inside the pit on the microscopic photograph.

2.5. Formation of actin ring in osteoclast-like MNCs

Osteoclast-like MNCs were seeded onto the cover glass, which was placed on the bottom of the plastic dishes and cultured in α-MEM with phenylephrine (1 μM), epinephrine (1 μM) and isoprenaline (1 μM) for 24 h. At the end of the experiment, cells were fixed with 10% formalin in phosphate buffered saline (PBS) for 10 min at room temperature, and then permeabilized with 0.1% Triton X-100 for 5 min. After washing in PBS, F-actin in the osteoclast-like MNCs was stained with 0.3 μM rhodamine-conjugated phalloidine for 60 min at room temperature. The formation of ringed structure of F-actin was observed under a fluorescence microscope.

3. Results and discussion

3.1. Expression of mRNA for adrenergic receptor (AR) subtypes in osteoclast-like MNCs

The human osteoclast-like MNCs used in this study were formed from human bone marrow. The human osteoclast-like MNCs fulfilled the functional criteria of osteoclasts: expression of tartrate-resistant acid phosphatase (TRAP); binding of ^{125}I-labeled calcitonin; formation of resorption pits on calcium phosphate-coated materials and bovine bone; and calcitonin-induced inhibition of resorbing activity [29]. As shown in Fig. 1, the steady state of osteoclast-like MNCs expressed not only mRNA for characteristics of mature osteoclast, such as TRAP, calcitonin receptor (CT-R), carbonic anhydrase II (CA II), αV-chain of integrin (Int αV) and cathepsin K (Cathe K) (Fig. 1A) but also mRNA for AR subtypes, such as α1B-AR, α2B-AR and β2-AR (Fig. 1B). However, expressions of α1A-AR, α1D-AR, α2A-AR, α2C-AR, β1-AR and β3-AR were not observed. These findings suggested that osteoclast-like MNCs were regarded as mature osteoclastic cells and equipped with an ability to receive adrenergic stimulation.

3.2. Effect of epinephrine on mRNA levels for characteristic markers of bone-resorbing function in osteoclast-like MNCs

As characteristic markers for osteoclasts, CA II, Int αV and Cathe K were suggested to be essential for the bone-resorbing function [7]. CAII catalyzes protons from CO_2 and water to demineralize bone, αvβ3 integrin localized at the osteoclastic cell surface contributes to the osteoclast's attachment to the bone surface, and Cathe K, a lysosomal cysteine protease specifically produced in osteoclasts, is considered to degrade type I collagen of demineralized bone under acidic conditions. Therefore, the effects of epinephrine (α- and β-adrenergic agonist) at a concentration of 1 μM on the expression of mRNA for Int αV, CAII and Cathe K were examined in osteoclast-like MNCs. As shown in Fig. 2, mRNA levels of Int αV, CAII and Cathe K in osteoclast-like MNCs were up-regulated by treatment with epinephrine for 1–6 h, which suggested that the function of osteoclasts might be directly enhanced by adrenergic stimulation.

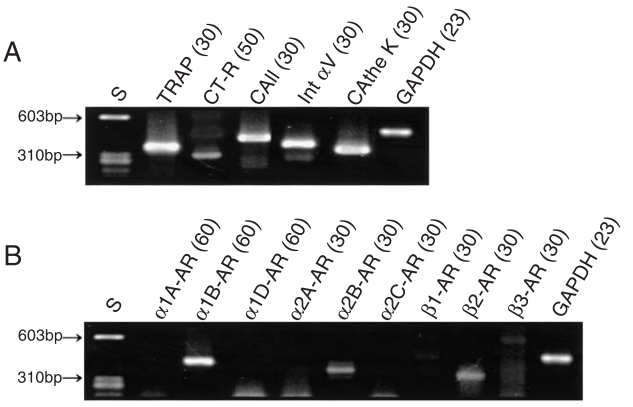

Fig. 1. Expression of mRNA for characteristic markers of mature osteoclasts (A) and adrenergic receptor phenotypes (B). (A) Total RNA extracted from human osteoclast-like MNCs was assayed by RT-PCR using specific primers for TRAP, CT-R, CAII, Int αV, Cathe K and GAPDH. (B) Total RNA from MNCs was assayed by RT-PCR using specific primers for α1A-AR, α1B-AR, α1D-AR, α2A-AR, α2B-A, α2C-AR, β1-AR, β2-AR, β3-AR and GAPDH. GAPDH is a housekeeping gene for internal standard. DNA size markers (φX174/*Hae*III digest) are shown on the left lane (S). Numbers in parentheses on the top of lane indicate cycles of PCR amplification.

3.3. Effects of phenylephrine, epinephrine and isoprenaline on bone-resorbing activity in osteoclast-like MNCs

Next, the effects of adrenergic agonists, phenylephrine (α1-adrenergic agonist), epinephrine and isoprenaline (β-adrenergic agonist) on bone-resorbing activity were examined in osteoclast-like MNCs by using the calcium phosphate-coated disks. Osteoclast-like MNCs were seeded on the calcium phosphate-coated disks and treated with phenylephrine, epinephrine and isoprenaline at a concentration of 1 μM for 5 days. As shown in Fig. 3, resorption pits were clearly larger in osteoclast-like MNCs treated with epinephrine and isoprenaline than in the cells treated with phenylephrine or the control cells (Fig. 3A). The total area of the resorption pits was significantly increased by treatment with epinephrine and isoprenaline, but not by the treatment with phenylephrine (Fig. 3B). As the potency of epinephrine for β-AR is lower than that of isoprenaline, epinephrine seems to enhance less the resorption activity compared with isoprenaline. In addition, treatment with clonidine (α2-adrenergic agonist) did not affect the resorbing activity (data not shown). These results suggested that β2-AR of mature osteoclasts was involved in resorbing activity, and were well consistent with the findings of Frediani et al. [10]. They also demonstrated that catecholamines may act as stimulators of

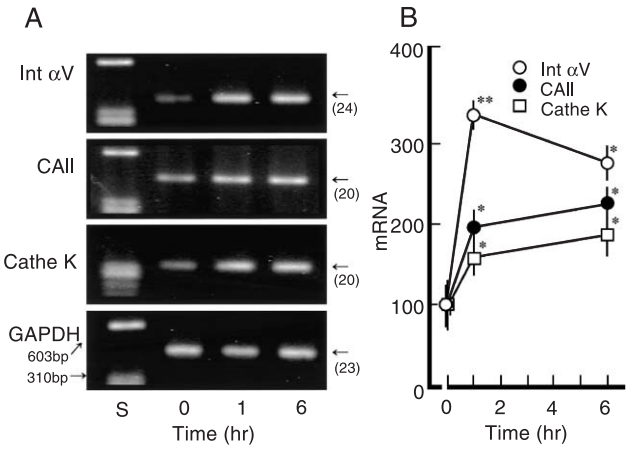

Fig. 2. Effects of epinephrine on expression of Int αV, CAII and Cathe K. (A) RT-PCR analysis for mRNA obtained from human osteoclast-like MNCs treated with epinephrine (1 μM) for 0, 1 and 6 h, and assayed by RT-PCR using specific primers. DNA size markers (ϕX174/*Hae*III digest) are shown on the left lane (S). Numbers in parentheses on the right indicate cycles of PCR amplification. Arrow indicates the predicted sizes of PCR products. GAPDH is a housekeeping gene for internal standard. (B) The mRNA level of Int αV, CAII and Cathe K was calculated by dividing the intensity of the Int αV, CAII and Cathe K bands by the intensity of the GAPDH band as determined by a fluorescent image analyzer. Pooled data from four independent experiments are expressed as means ± S.E. (*$P<0.05$ and **$P<0.01$, statistical difference from control, by Student's *t*-test).

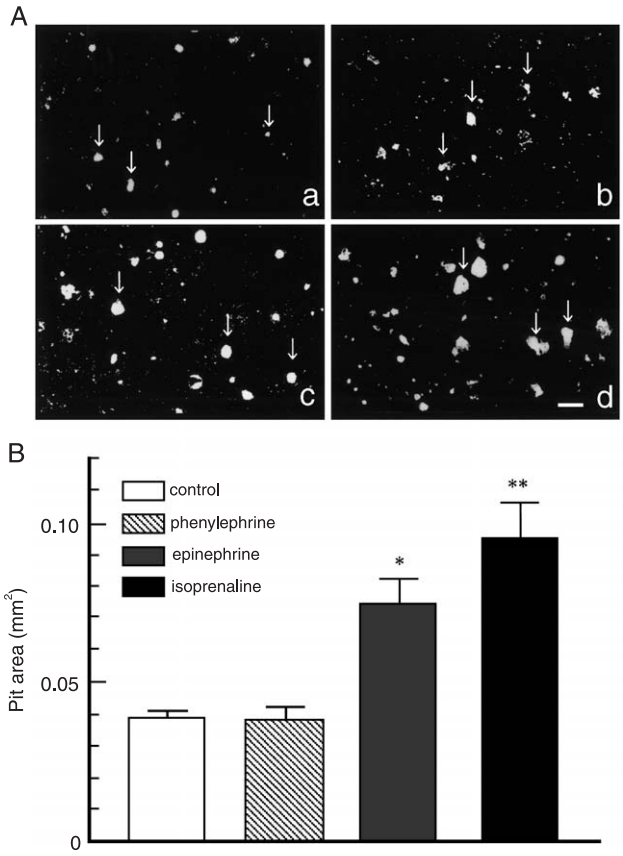

Fig. 3. Effects of adrenergic agonists on resorbing activity of human osteoclast-like MNCs. (A) Microscopic photograph of von Kossa stained calcium phosphate-coated disk on which osteoclast-like MNCs were cultured in the absence (a) or presence of 1 μM phenylephrine (b), epinephrine (c) and isoprenaline (d) for 5 days. Arrow indicates resorbed pit by hMNCs. Bar = 0.2 mm. (B) Total pit area was calculated at each microscopic photograph by counting mesh number inside the pit. Column and bars are expressed as means ± S.E. ($n=4$). (*$P<0.01$ and **$P<0.005$, statistical difference from control, by Student's *t*-test).

osteoclast activity via binding to β2-AR in FLG 29.1 cells, a clonal cell line of human osteoclast precursors. On the other hand, the physiological roles of α-ARs expressed in osteoclast-like MNCs were not clarified.

Osteoclasts are multinuclear bone-resorbing cells that display structural and functional changes between a resorbing and a nonresorbing/migrating state [7]. In the resorbing state, osteoclasts recognize bone matrix proteins, adhere to the bone surface through integrin receptors, and exhibit a highly polarized cytoplasmic organization with ruffled borders and clear zones. It is well known that the adhesive site of osteoclast shows actin rings (a ring formation of F-actin filaments), which are closely related to bone-resorbing activity [7,30,31]. Therefore, the effects of adrenergic agonists on actin ring formation were examined in osteoclast-like MNCs. As shown in Fig. 4, actin rings were faintly observed in control osteoclast-like MNCs similar to osteoclast-like MNCs treated with phenylephrine, which suggested that resorbing activity of those osteoclast-like MNCs was markedly lowered within 24 h. This finding was consistent with the evidence that most isolated mature osteoclasts placed on culture dishes decreased their bone-resorbing activity within 24 h in the absence of supporting cells, such as osteoblasts/stromal cells [31]. On the other hand, actin rings were clearly shown at the peripheral of osteoclast-like MNCs treated with isoprenaline and epinephrine, which suggested that β-adrenergic agonists act on osteoclast-like MNCs directly to maintain their bone-resorbing activity.

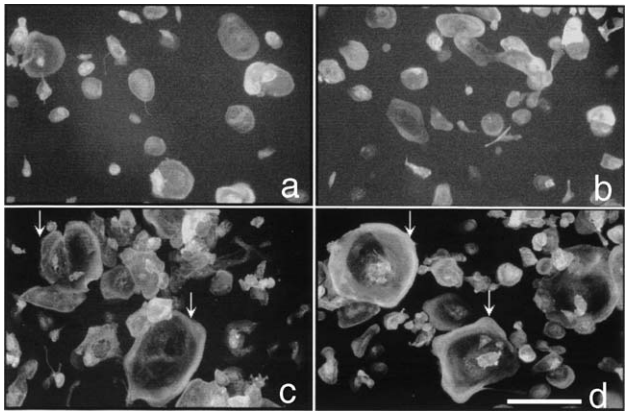

Fig. 4. Effects of adrenergic agonists on actin ring formation in human osteoclast-like MNCs. Osteoclast-like MNCs placed on cover glass were cultured in the absence (a) or presence of 1 μM phenylephrine (b), epinephrine (c) and isoprenaline (d) for 24 h. After culture, osteoclast-like MNCs were fixed and stained for F-actin with rhodamine-conjugated phalloidin. Bar = 0.1 mm.

3.4. β-Adrenergic stimulation of bone-resorbing activity

Bone resorption by β-adrenergic stimulation was demonstrated in in vivo and in vitro experiments. In neonatal mouse calvariae, adrenergic agonists increased cAMP production, and in the presence of a phosphodiesterase inhibitor and an antioxidant, they stimulated bone resorption [5]. Osteoclastic bone resorption consists of multiple steps such as the differentiation of osteoclast precursors into mononuclear prefusion osteoclasts, the fusion of prefusion osteoclasts to form multinucleate osteoclasts, and the activation of these osteoclasts to resorb bone [7]. Bone-resorbing factors, such as $1\alpha,25(OH)_2D_3$, parathyroid hormone, PGE_2, interleukin-1α and tumor necrosis factor-α, appear to act on osteoblasts/stromal cells to induce osteoclastogenesis in the mouse bone marrow culture system or the coculture system of mouse spleen cells and osteoblasts through expression of ODF/RANKL [8]. We also demonstrated that β-adrenergic stimulation induced expression of mRNA for ODF/RANKL in the mouse osteoblastic cell line, MC3T3-E1, and induced osteoclastogenesis in the mouse bone marrow culture system [9]. In the present study, we showed that both epinephrine and isoprenaline enhanced bone-resorbing activity of human osteoclast-like MNCs in the absence of osteoblasts/stromal cells, suggesting that β-adrenergic agonists not only indirectly stimulate osteoclastogenesis via osteoblastic cells but also directly stimulate bone-resorbing activity of osteoclasts.

In conclusion, we demonstrated that β-adrenergic agonist could directly stimulate bone-resorbing activity of mature human osteoclasts.

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