Suppression of osteoclastogenesis via α2-adrenergic receptors

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Abstract. The sympathetic nervous system is known to regulate osteoclast development. However, the involvement of α2-adrenergic receptors (α2-ARs) in osteoclastogenesis is not well understood. In the present study, their potential role in osteoclastogenesis was investigated. Guanabenz, clonidine and xylazine were used as agonists of α2-ARs, while yohimbine and idazoxan were employed as antagonists. Using RAW264.7 pre-osteoclast and primary bone marrow cells, the mRNA expression of the osteoclast-related genes nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP) and cathepsin K was evaluated following induction with receptor activator of nuclear factor κB ligand (RANKL). TRAP staining was also conducted to assess effects on osteoclastogenesis in mouse bone marrow cells in vitro. Administration of 5-20 μM guanabenz (P<0.01, for RANKL-only treatment), 20 μM clonidine (P<0.05, for RANKL-only treatment) and 20 μM xylazine (P<0.05, for RANKL-only treatment) attenuated RANKL-induced upregulation of NFATc1, TRAP and cathepsin K mRNA. Furthermore, the reductions in these mRNAs by 10 μM guanabenz and 20 μM clonidine in the presence of RANKL were attenuated by 20 μM yohimbine or idazoxan (P<0.05). The administration of 5-20 μM guanabenz (P<0.01, for RANKL-only treatment) and 10-20 μM clonidine (P<0.05, for RANKL-only treatment) also decreased the number of TRAP-positive multi-nucleated osteoclasts. Collectively, the present study demonstrates that α2-ARs may be involved in the regulation of osteoclastogenesis.

Introduction

Numerous studies have reported that the central and peripheral sympathetic nervous systems serve important roles in bone remodeling and bone fracture healing (1-4). Particularly, bone-forming osteoblasts and bone-resorbing osteoclasts are established to express α- and β-adrenergic receptors (α- and β-ARs) (5-7). In osteoblastogenesis, it has been documented that α1-ARs promote cell proliferation through the suppression of potassium channels (8). Additionally, α1B-AR signaling may stimulate bone formation through the promotion of proliferation via upregulation of CCAAT/enhancer-binding protein δ in osteoblasts (9). Furthermore, it has been reported that leptin binding to hypothalamic receptors contributed to the regulation of bone homeostasis via β2-ARs (1), and that these β2-ARs inhibited cyclic-adenosine monophosphate (c-AMP)-responsive element-binding protein phosphorylation, leading to a decrease in osteoblast proliferation (10). α1-AR agonist but not β2-AR agonist may also induce fracture callus contraction via promotion of osteogenesis (11).

In osteoclastogenesis, it has been documented that an agonist to β-AR (isoprenaline) could promote bone-resorbing activity in human osteoclast-like cells (6). Furthermore, β2-ARs have been reported to stimulate osteoclastogenesis via reactive oxygen species generation (12). A key feature of ARs in bone remodeling is their ability to mediate interactions of osteoblasts with osteoclasts, since activation of α1- and β-ARs induces expression of receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) in osteoblasts, resulting in RANKL-driven promotion of osteoclastogenesis (13-15). To the best of our knowledge, however, little is known of the role of agonists to α-ARs in the development of osteoclast precursors.

α2-ARs, the prime focus in the present study, belong to the G-protein-coupled receptor (GPCR) family. There are three α2-AR subtypes (α2A, α2B and α2C), which are established to regulate various physiological functions via suppression of adenylyl cyclase and reduction of c-AMP (16-21). For instance, α2-ARs on presynaptic membranes may inhibit norepinephrine secretion from sympathetic nerves (16). It has also been reported that α2A-AR serves a principal role in the hypotensive...
response (17), and that it is a primary mediator of sedative, analgesic and anesthetic-sparing responses (18). Furthermore, α2A-AR on pancreatic β-cells has been documented to inhibit insulin secretion (19). Although α2-ARs have been established to serve various roles in homeostasis, little is understood of their direct involvement in osteoclastogenesis.

In the current study, RAW264.7 pre-osteoclast cells and primary bone marrow cells were used to evaluate the role of α2-ARs in osteoclastogenesis. In the presence and absence of α2-AR agonists (guanabenz, clonidine and xylazine) and α2-AR antagonists (yohimbine and idazoxan), these cells were cultured in an osteoclast differentiation medium. Real-time quantitative polymerase chain reaction (qPCR) and tartrate-resistant acid phosphatase (TRAP) staining, as well as western blot analysis, were then conducted to determine the effects of these agonists and antagonists in osteoclastogenesis.

Materials and methods

Animals. To harvest bone marrow cells, C57BL/6J mice were purchased from Chubu Kagaku Shizai Co., Ltd. (Nagoya, Japan). A total of 35 female mice (8-10 weeks old) were used in the current study. The mice were housed under a 12-h light/dark cycle, and water and food were provided ad libitum. The protocols for animal experiments were approved by the Aichi-Gakuin University Animal Research Committee (Nagoya, Japan).

Cell culture. After the female mice were sacrificed by cervical dislocation, mouse bone marrow cells were isolated from long bones (femur and tibia). For isolation of the bone marrow cells, the distal and proximal ends of the long bone were removed. Using a needle (25G), the bone marrow cavity was flushed out with phosphate-buffered saline. The buffer was then filtered through a cell strainer (100 µm; BD Falcon™; BD Biosciences, Hamburg, Germany) with a 45-min incubation at room temperature and stained with TRAP for 1 h at 37°C. The number of TRAP-positive cells containing three or more nuclei was determined. All positive cells in each well were counted using a light microscope (magnification, x100; Zeiss AG, Oberkochen, Germany).

RAW264.7 cells were plated at 1.0x10^5 cells into 60-mm dishes and cultured with 25 ng/ml RANKL in the presence or absence of 5-20 µM guanabenz, 10-20 µM clonidine or 10-20 µM xylazine with or without 10-20 µM yohimbine or 10-20 µM idazoxan (Sigma-Aldrich; Merck KGaA) at 37°C for 2-4 days for qPCR analysis.

Reverse transcription-qPCR. Mouse bone marrow cells and RAW264.7 cells were treated with RANKL and α2 agonists/antagonists at 37°C for 2-4 days prior to qPCR analysis. Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA). Reverse transcription was conducted with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and real-time qPCR was performed using a Takara Thermal Cycler Dice Real Time System III (Takara Bio, Inc., Otsu, Japan) with Thunderbird SYBR qPCR mix (Toyobo Life Science, Osaka, Japan). The PCR cycling conditions were 95°C for 10 min (pre-denaturation), 40 cycles at 95°C for 15 sec (denaturation) and 60°C for 1 min (extension). The mRNA levels of α2A-, α2B-, and α2C-ARs, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), TRAP and cathepsin K were evaluated with the PCR primers listed in Table I. The expression of GAPDH was used as the internal control. The PCR results were interpreted using the 2^-ΔΔCq method (23).

In vitro osteoclast formation and TRAP staining. Mouse bone marrow cells were plated at densities of 1.2x10^5 and 1.0x10^6 cells into 12-well and 60-mm dishes, respectively, and cultured with 10 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech, Inc., Rocky Hill, NJ, USA) at 37°C for 3 days. The surface-attached cells were used as osteoclast precursors (22). These cells were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech, Inc.). A total of 5-20 µM guanabenz (R&D Systems, Inc., Minneapolis, MN, USA) or 10-20 µM xylazine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was applied at the same time point as RANKL; 10-20 µM clonidine (Sigma-Aldrich; Merck KGaA) was administered with RANKL or 1 day after RANKL administration. After a 60-h treatment with RANKL at 37°C, cells were fixed in 10% formalin neutral buffer solution at room temperature and stained with TRAP for 1 h at 37°C. The number of TRAP-positive cells containing three or more nuclei was determined. All positive cells in each well were counted using a light microscope (magnification, x100; Zeiss AG, Oberkochen, Germany).
Statistical analysis. Statistical analyses were performed using Microsoft Excel for Mac 2011 (version 14.6.9; Microsoft Corporation, Redmond, WA, USA). Data were expressed as the mean ± standard deviation of three to five independent experiments. Statistical significance was evaluated using Student’s t-test at P<0.05.

Results

mRNA expression of α2-ARs. The mRNA levels of α2A-, α2B- and α2C-ARs were determined. All three of the α2-ARs were detectable (Fig. 1). However, the responses to RANKL differed between RAW264.7 and primary bone marrow cells. RANKL administration on days 0, 2 and 4 did not significantly alter the mRNA levels of α2-ARs in RAW264.7 cells (Fig. 1A); while it significantly altered their mRNA levels in primary bone marrow cells when detected after 2 days. Specifically, the mRNA expression of α2A- and α2C-ARs was significantly downregulated by RANKL administration (P<0.01 and P<0.05, respectively), while that of α2B-AR was upregulated (P<0.01; Fig. 1B).

α2-AR agonist-driven reduction in the expression of osteoclast genes. On day 2 following the administration of RANKL, the mRNA levels of NFATc1, TRAP and cathepsin K were significantly reduced by 5-20 µM guanabenz in RAW264.7 and primary bone marrow cells (P<0.01; Fig. 2). Administration of 20 µM clonidine consistently suppressed RANKL-induced
upregulation of NFATc1, TRAP and cathepsin K on days 2 and 4 in RAW264.7 cells (P<0.05; Fig. 3A and B). In primary bone marrow cells, administration of 10-20 µM clonidine suppressed the mRNA levels of the osteoclast genes when clonidine was applied alongside RANKL (P<0.01; Fig. 3C). However, when clonidine was administered 1 day after the administration of RANKL, it did not alter the mRNA levels of NFATc1, TRAP or cathepsin K (Fig. 3D). The mRNA levels of these osteoclast genes were also consistently downregulated by 20 µM xylazine in RAW264.7 and primary bone marrow cells on day 2 after administration of RANKL (P<0.05; Fig. 4).

**Suppression of α2-AR agonist-driven reduction of osteoclast gene expression by yohimbine or idazoxan.** The reduction in the mRNA levels of NFATc1, TRAP and cathepsin K in response to guanabenz was consistently suppressed by 20 µM yohimbine (P<0.05; Fig. 5A and B) or 20 µM idazoxan (P<0.05; Fig. 6). This result indicates that α2-AR antagonists inhibit the action of α2-AR agonists, and also supports the notion that α2-ARs may be involved in regulation of osteoclast gene expression. Of note, administration of yohimbine alone upregulated the expression of the osteoclast genes at concentrations of 10 (P<0.05) and 20 (P<0.01) µM guanabenz (P<0.01) and 10 and 20 µM clonidine (P<0.05 and P<0.01, respectively).

**Increase in eIF2α phosphorylation by guanabenz.** Guanabenz is established to suppress osteoclastogenesis by inhibiting dephosphorylation of eIF2α (24-26). To determine whether clonidine and xylazine also inhibit dephosphorylation of eIF2α, the level of p-eIF2α was assessed in RAW264.7 cells. Western blot analysis demonstrated that administration of 20 µM guanabenz increased the phosphorylation of eIF2α (P<0.05), while treatment with 20 µM clonidine or xylazine did not significantly affect the phosphorylation level (Fig. 8).

**Discussion**

The current study demonstrated that three chemical agents, guanabenz, clonidine and xylazine, which serve as α2-AR agonists, suppressed the mRNA expression of three osteoclast genes (NFATc1, TRAP and cathepsin K) in RAW264.7 and primary bone marrow cells, and reduced the number of TRAP-positive multi-nucleated osteoclasts in mouse bone marrow cells. Consistent with the observed involvement of α2-ARs in response to guanabenz and clonidine, administration of yohimbine and idazoxan, as α2-AR antagonists, suppressed the α2-AR agonist-induced reduction in the mRNA levels of the target genes. Compared with clonidine and xylazine, the results also indicated that the greater inhibitory effect of guanabenz in osteoclastogenesis may be associated with a guanabenz-driven elevation in p-eIF2α. Furthermore, the findings suggest that the responses to agents including clonidine...
may differ depending on the administration window during osteoclastogenesis.

Since yohimbine and idazoxan are established to serve as α2-AR antagonists (27), it was determined whether yohimbine and idazoxan could suppress the effect of α2-AR agonists including guanabenz and clonidine. The results demonstrated that administration of yohimbine or idazoxan suppressed α2-AR agonist-driven reduction in the expression of osteoclast genes. This indicates that the selected antagonists may block binding of agonists to α2-ARs. Notably, administration of yohimbine alone increased mRNA expression of the osteoclast genes. It has been reported that certain ionotropic receptors, including the γ-aminobutyric acid A receptors, as well as GPCRs, including adrenergic, histamine and adenosine receptors, are constitutively activated even in the absence of agonists, and this constitutive activity may be inhibited by so-called inverse agonists (28-32). For instance, adenosine A1 receptor is constitutively activated in osteoclast precursors and rololofylline, a receptor antagonist, has been reported to inhibit osteoclast differentiation as an inverse agonist (32). Yohimbine may also serve as an inverse agonist of α2-ARs, resulting in increased expression of osteoclast genes.

Figure 3. Suppression of RANKL-induced osteoclast genes in response to clonidine in RAW264.7 and primary bone marrow cells. (A) Clonidine-induced inhibition of NFATc1, TRAP and cathepsin K on day 2 after administration of RANKL in RAW264.7 cells. (B) Clonidine-induced inhibition of NFATc1, TRAP and cathepsin K on day 4 after administration of RANKL in RAW264.7 cells. (C) Clonidine-induced inhibition of NFATc1, TRAP and cathepsin K on day 2 after administration of RANKL in primary bone marrow cells. (D) Undetectable alteration in the mRNA levels of NFATc1, TRAP and cathepsin K on day 2 by clonidine in primary bone marrow cells. Clonidine was applied at the same time point with RANKL in the experiment for (A-C) but was applied 1 day after administration of RANKL in the experiment for (D). *P<0.05 and **P<0.01 vs. RANKL-only treatment. NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor κB ligand.
Although guanabenz, clonidine and xylazine are $\alpha_2$-AR agonists, there are differences in specificity among these agents. Guanabenz is known to inhibit dephosphorylation of eIF2$\alpha$ and attenuate endoplasmic reticulum stress, leading to downregulation of osteoclast genes and attenuation of osteoclastogenesis (24-26). Consistent with the action of guanabenz, western blot analysis revealed that administration of guanabenz to RAW264.7 cells elevated the level of p-eIF2$\alpha$, while administration of clonidine or xylazine did not significantly alter the phosphorylation level. This result indicates that guanabenz serves as an inhibitor of eIF2$\alpha$ dephosphorylation, as well as an $\alpha_2$-AR agonist, and induces stronger suppression of osteoclast genes and attenuation of osteoclastogenesis compared with clonidine and xylazine.

The action of clonidine and guanabenz via $\alpha_2$-ARs is possibly mediated by c-AMP, since RANKL has been reported to increase the level of c-AMP in osteoclast precursors (33). Elevation of c-AMP may activate exchange protein directly activated by c-AMP (34,35), which has been documented to promote osteoclast differentiation via nuclear translocation of NF-κB (33). Furthermore, activation of an adenylyl cyclase followed by elevation of c-AMP upregulated c-Fos, which is established to promote osteoclast development (36,37). Since $\alpha_2$-ARs are known to suppress adenylyl cyclase and reduce c-AMP (21), and clonidine and guanabenz have been reported to reduce c-Fos expression (25,38), it is possible that the $\alpha_2$ agonists in the current study reduce RANKL-induced c-AMP, resulting in the suppression of osteoclastogenesis.

While clonidine served as an inhibitor of osteoclastogenesis in the current study, its effect during the course of osteoclastogenesis is not completely understood. A previous report identified that clonidine increased the number of TRAP-positive osteoclasts in mouse bone marrow cells, and that its administration did not alter the number of TRAP-positive osteoclasts in $\alpha_2A$ and $\alpha_2C$ double knockout mice (7). It has also been reported that the number of TRAP-positive osteoclasts was not affected by clonidine in cluster of differentiation 14$^+$ osteoclast precursors (39). A major difference among these studies appears to be the timing of clonidine administration. In the current study, clonidine was applied on day 0 together with RANKL or 1 day after administration of RANKL, while it was administered on days 2 and 11 in the previous reports (7,39). The present study revealed that when clonidine was applied alongside RANKL in primary bone marrow cells, it suppressed the mRNA levels of NFATc1, TRAP and cathepsin K; however, when clonidine was administered 1 day after the administration of RANKL, it did not alter mRNA levels. Therefore, as the mRNA levels of $\alpha_2A$- and $\alpha_2C$-ARs in primary bone marrow cells were downregulated on day 2, it is possible that the efficacy of clonidine as an inhibitor may depend on the timing of its administration as well as the expression profiles of $\alpha_2A$ and $\alpha_2C$ receptors.

While it was demonstrated in the current study that $\alpha_2$-ARs on osteoclast precursors suppressed osteoclastogenesis, pre-clinical studies in laboratory animals are necessary prior to clinical studies and application in patients. As the current study was an in vitro analysis, animal studies using conditional knockout mice or other appropriate models are recommended to verify the present findings. Nonetheless, the results indicated that $\alpha_2$-ARs may be involved in the regulation of osteoclastogenesis in RAW264.7 and primary bone marrow cells in vitro.
Figure 5. Reduction in guanabenz and clonidine-induced suppression of osteoclast genes by yohimbine in RAW264.7 cells. (A) Reduction in guanabenz-induced suppression of NFATc1, TRAP and cathepsin K by yohimbine on day 2 after administration of RANKL in RAW264.7 cells. Note that the asterisks denote the comparison of RANKL with guanabenz. (B) Reduction in clonidine-induced suppression of NFATc1, TRAP and cathepsin K by yohimbine on day 2 after administration of RANKL in RAW264.7 cells. Note that the asterisks are to compare RANKL with clonidine. (C) Upregulation of NFATc1, TRAP and cathepsin K by yohimbine on day 2 after administration of RANKL in RAW264.7 cells. *P<0.05 and **P<0.01 vs. RANKL with guanabenz treatment; #P<0.05 and ##P<0.01 vs. RANKL with clonidine treatment; $P<0.05 and $$P<0.01 vs. RANKL-only treatment. NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor κB ligand.

Figure 6. Reduction in guanabenz-induced suppression of osteoclast genes by idazoxan in RAW264.7 cells. Reduction in guanabenz-induced suppression of NFATc1, TRAP and cathepsin K by idazoxan on day 2 after administration of RANKL in RAW264.7 cells. *P<0.05 and **P<0.01 vs. RANKL with guanabenz treatment. NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor κB ligand.
Figure 7. Inhibitory effects of α2-adrenergic receptor agonist on the development of osteoclasts in the presence of 50 ng/ml RANKL. (A) Dose-dependent suppression of TRAP-positive multi-nucleated osteoclasts by guanabenz in bone marrow cells. (B) Dose-dependent suppression of TRAP-positive multi-nucleated osteoclasts by clonidine in primary bone marrow cells. *P<0.05 and **P<0.01 vs. RANKL-only treatment. RANKL, receptor activator of nuclear factor κB ligand; CN control.

Figure 8. Inhibition of eIF2α dephosphorylation by guanabenz. (A) Elevated level of p-eIF2α in RAW264.7 cells following treatment with 20 μM guanabenz for 24 h. Notably, 20 μM clonidine and xylazine did not significantly alter phosphorylation level. (B) Relative intensities of (p‑eIF2α)/(total eIF2α). *P<0.05 vs. RANKL-only treatment. eIF2α, eukaryotic translation initiation factor 2α; RANKL, receptor activator of nuclear factor κB ligand; p-, phosphorylated.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

KoH, KaH, HY, HK, KT, KI, DK, TH, KM, SG and AT designed the research. KoH, KaH, AC, HM and SY performed the experiments and analyzed the data. KaH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols for animal experiments were approved by the Aichi-Gakuin University Animal Research Committee (Nagoya, Japan).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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