β-Adrenergic signaling stimulates osteoclastogenesis via reactive oxygen species

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Kondo H, Takeuchi S, Togari A. β-Adrenergic signaling stimulates osteoclastogenesis via reactive oxygen species. Am J Physiol Endocrinol Metab 304: E507–E515, 2013. First published November 20, 2012; doi:10.1152/ajpendo.00191.2012.—Sympathetic signaling regulates bone resorption through receptor activator of nuclear factor-κB ligand (RANKL) expression via the β-adrenergic receptor (β-AR) on osteoblasts. Reactive oxygen species (ROS) are known as one type of osteoclast regulatory molecule. Here we show that an antioxidant, α-lipoic acid (α-LA), treatment prevent the β-adrenergic signaling-induced bone loss by suppressing osteoclastogenesis, and sympathetic signaling directly regulates osteoclastogenesis through β2-AR expressed on osteoclasts via intracellular ROS generation. In an in vitro study, the β-AR agonist isoprenaline increased intracellular ROS generation in osteoclasts prepared from bone marrow macrophages (BMMs) and RAW 264.7 cells. Isoprenaline enhanced osteoclastogenesis through β2-AR expressed on BMMs and RAW 264.7 cells. The antioxidant α-LA inhibited isoprenaline-enhanced osteoclastogenesis. Isoprenaline increased the expression of osteoclast-related genes such as nuclear factor of activated T cells, cytoplasmic calcineurin-dependent 1, tartrate-resistant acid phosphatase, and cathepsin K on osteoclasts. α-LA also inhibited isoprenaline-induced increases of these gene expressions. These in vitro results led to the hypothesis that β-adrenergic signaling directly stimulates osteoclastogenesis via ROS generation. In an in vivo study, isoprenaline treatment alone caused oxidative damage in local bone and reduced bone mass because of an increase in bone resorption, and, in α-LA-treated mice, isoprenaline did not increase tibial osteoclast number even though the RANKL-to-osteoprotegerin ratio increased. These in vitro and in vivo results indicate that β-adrenergic signaling, at least in part, directly stimulates osteoclastogenesis through β2-AR on osteoclasts via ROS generation.

β-adrenergic receptor; bone metabolism; osteoclast

Osteoclast formation from its precursors requires two molecules. One is RANKL, which is produced by osteoblasts and stromal cells (27, 41). Recent studies have demonstrated that osteoclasts are the major source of the cytokine RANKL involved in osteoclast formation in cancellous bone (33). The other is macrophage colony-stimulating factor (M-CSF). RANKL acts by binding to its receptor, RANK, on the surface of osteoclast precursors, promoting osteoclast development from osteoclast precursors in vitro in the presence of M-CSF. β-AR is expressed on not only osteoblasts but also osteoclasts, and its stimulation induces osteoclast activity (3, 45). Furthermore, Suga et al. demonstrated direct nerve-osteoclast communication (38). These observations revealed that the sympathetic nervous system directly regulates bone resorption by osteoclasts.

Reactive oxygen species (ROS) are known as intracellular signaling molecules in stress responses in a variety of cell types (1). ROS are known to contribute to the etiology of various degenerative diseases such as atherosclerosis, arthritis, cancer, and the process of aging (13). ROS are also implicated, to some extent, in the bone loss due to aging, estrogen deficiency, and irradiation (2, 26, 28). ROS produced by osteoclasts or their precursors are important local factors involved in the activation and differentiation of osteoclasts (5, 16, 28). Treatment with a potent antioxidant, α-lipoic acid (α-LA), can prevent inflammation and irradiation-induced bone loss (17, 26). α-LA is a natural compound chemically named 1,2-dithiolane-3-pentanonic acid (C₆H₁₀O₅S₂), which is widely distributed in plants and animals in both cellular membranes and cytosol because α-LA is both water- and fat-soluble (47). α-LA acts as an essential cofactor of mitochondrial enzymes, has a powerful antioxidant capacity, and is used clinically for treating neuropathy (48). Here we demonstrate that α-LA treatment prevents the β-adrenergic signaling-induced bone loss by suppressing osteoclastogenesis and suggest that sympathetic signaling directly regulates osteoclastogenesis through β2-AR on osteoclasts via intracellular ROS generation.

MATERIALS AND METHODS

Osteoclast differentiation. Bone marrow cells were collected from male C57BL/6j mice by flushing out femora and tibiae with α-minimal essential medium (α-MEM; GIBCO-BRL, Grand Island, NY) containing penicillin and streptomycin. Bone marrow cells were cultured in α-MEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN) and then cultured for 16 h. Nonadherent cells were collected and counted using a flow cytometer (Guava EasyCyte Mini cell cytometer system) using Guava ViaCount fluorescent dye (Guava Technologies, Hayward, CA). These cells were replated at 1.5 × 10⁵ cells/well and incubated with 50 ng/ml M-CSF for 2 days in 48-well plates. The adherent cells were used as bone marrow-derived macrophages (BMMs). To differentiate into osteoclasts, the BMMs were

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cared for 3 days with 50 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech, London, UK) in the presence or absence of isoprenaline (Sigma-Aldrich, St. Louis, MO), hydrogen peroxide (Sigma-Aldrich), propranolol (Sigma-Aldrich), or α-LA (Sigma-Aldrich). After culturing for the specified periods, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Murine macrophage-like RAW 264.7 cells (46) were cultured in α-MEM containing 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. To differentiate osteoclasts, RAW 264.7 cells were plated at 2.5 × 10⁶ cells/cm² in a 96-well plate for TRAP staining and incubated with 50 ng/ml RANKL in the presence and absence of experimental reagents. For quantitative PCR analysis, these reagents were added after differentiation into osteoclasts from BMMs or RAW 264.7 cells plated in a sixwell plate.

Detection of intracellular ROS. For the detection of intracellular ROS, RAW 264.7 cells and BMMs were cultured for 3 days with 50 ng/ml RANKL for their differentiation into osteoclasts. On the last day of culture, cells were treated for 60 min at 37°C with 5 mM ROS-sensitive dye with the fluorogenic dye 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, 5 µM) (Invitrogen, Carlsbad, CA) with or without 1 µM isoprenaline and observed with a confocal laser scanning microscope (LSM-710; Carl Zeiss, Oberkothen, Germany); images were analyzed using Zen software (Carl Zeiss).

Quantitative real-time PCR analysis. Total RNA samples were prepared from cultured bone cells or the right distal femur using the SV total RNA isolation kit (Promega, Madison, WI) according to the manufacturer’s instructions, and cDNA samples were synthesized using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen). qRT-PCR experiments were performed using Step One Plus (Applied Biosystems, Foster City, CA), TaqMan MGP probe, and Eurogentec qPCR Master Mix Plus (Eurogentec, San Diego, CA). The primers used are listed in Table 1. The primers for β2-AR and β3-AR were purchased from Applied Biosystems, whose assay identifiers are Mm00431701_s1 for β2-AR and Mm00442669_s1 for β3-AR. Each experiment was performed in duplicate, and the results were standardized using Rodent GAPDH Control Reagents (Applied Biosystems). These experiments were performed at least three times.

Animals and experimental design. Male, 15 wk-old C57BL/6J mice (Japan SLC, Hamamatsu, Japan) were randomized by weight, and 5 or 6 mice of each group were housed together under automatic conditions of temperature (23 ± 1°C), humidity (50 ± 10%), and a 12:12-h light-dark cycle. To study the effects of the antioxidant α-LA (Sigma-Aldrich) on β-AR agonist, isoprenaline (Sigma-Aldrich) was used to induce bone loss. α-LA was dissolved in saline containing 5% dimethyl sulfoxide. Isoprenaline was dissolved in saline. For this, 60 mice were divided into five groups. The groups were subcutaneously injected with α-LA twice daily at 0, 0.25, 2.5, 25, and 50 mg/kg body wt⁻¹ day⁻¹. One-half of the mice in each group was intraperitoneally injected with isoprenaline (5 mg·kg body wt⁻¹ day⁻¹ ip), and the other half was injected with vehicle (0.9% saline). Body weight was measured every other day during the experimental period and recorded in grams (Sartorius BJ 600; Sartorius, Edgewood, NY).

μ-Computed tomography analysis. The distal region of the right femur was subjected to three-dimensional μ-computed tomography (μCT) analysis using an R-mCT μCT scanner (RIGAKU, Tokyo, Japan). The scanning was initiated 1.0 mm above the distal femoral growth plate, and a total of 75 consecutive 20-µm-thick sections were analyzed, encompassing a length of 1.5 mm of the secondary spongiosa. The measured volume of interest in the femur was obtained by selecting the cancellous bone (separate from the cortical shaft) using contour areas that were drawn semiautomatically. TR/3D-BON (Ratoc, Tokyo, Japan) software was used to analyze the cancellous parameters: bone volume/total volume (BV/TV), trabecular number (TB.N), trabecular separation (TB.Sp), and trabecular spacing (TB.Spac).

Bone histomorphometry. For dynamic histomorphometry analysis, all of the mice were injected intraperitoneally with calcine at 4 mg/kg at 4 and 2 days before death. At the end of the experiments, the right femora of each mouse were dissected and fixed in 70% ethanol. Next, 5-µm-thick sagittal sections were made as undecalcified sections. For mineral apposition rate (MAR), metaphyseal cancellous bone in the femora was used to obtain a bone fraction in a rectangular area of 0.34 mm² (0.5 mm × 0.67 mm) with its closest and furthest edges at 0.3 and 0.8 µm distal to the growth plate, respectively. For the decalcified sections, the left tibiae of the mice were dissected and fixed in 4% paraformaldehyde and then decalcified in 20% EDTA for 2 wk. Sagittal sections (4 µm thick) were made as decalcified sections and stained with TRAP for osteoclast analysis. Measurements were made within an area of 0.8 mm² (1.0 mm × 0.8 mm), with its closest and furthest edges being 2.0 and 3.0 mm distal to the growth plate of the proximal ends of the tibia, respectively. Osteoclast number/bone surface (Oc.N/BS) and osteoclast surface/bone surface (Oc.S/BS) were evaluated by scoring the TRAP-positive multinucleated cells attached to the bone surface as defined by Parfitt et al. (35).

Assessment of oxidative damage. Mineralized bones, left after flushing out the marrow, were stored at −80°C in 5 µM butyraldehyde until analysis. The plasma was taken from centrifuged blood samples and stored at −80°C. A commercially available kit that measures malondialdehyde (MDA) levels in tissue homogenates and plasma was used (MDA Assay Kit; Northwest Life Science Specialties). MDA levels were measured in duplicate and calibrated against a standard curve. Experimental procedures were performed in accordance with the manufacturer’s instructions.

Data analysis and statistics. All results shown are the means and SE. For in vivo study, results were analyzed statistically by ANOVA. Post hoc analysis, using the Tukey-Kramer test, was performed on values exhibiting an interaction effect. For in vitro study, results were analyzed by one-factor ANOVA with Fisher’s post hoc test. Stat View software was used (SAS Institute). A P value ≤0.05 was considered to be statistically significant.

RESULTS

β-AR agonist, isoprenaline, stimulates osteoclastogenesis through ROS generation. β-ARs are classified into three subtypes: β1-AR, β2-AR, and β3-AR. To identify the β-AR subtypes expressed by the osteoclasts, real-time PCR using primers specific for each receptor subtype was performed.

### Table 1. Primers used for quantitative real-time PCR

<table>
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<th>Gene</th>
<th>Accession No.</th>
<th>Primer (Forward/Reverse)</th>
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<tr>
<td>β2-AR</td>
<td>NM_007420</td>
<td>(F) 5′-CAAGATTGCTCTTTCAAAGAGCTT-3′ (R) 5′-GTCTTGAAGTTGACGACTCT-3′</td>
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<tr>
<td>NFAT</td>
<td>NM_198249</td>
<td>(F) 5′-CCCAAGTCTCCCTCCCCACATC-3′ (R) 5′-CCTATGACCTGATAGCGCCAC-3′</td>
</tr>
<tr>
<td>TRAP</td>
<td>NM_007388</td>
<td>(F) 5′-GCGAATAATTCTACATCCCTGAGC-3′ (R) 5′-TCAGAGAAAAGAATCTGCAAGG-3′</td>
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<tr>
<td>Ctsk</td>
<td>NM_007802</td>
<td>(F) 5′-ATTCAGGCTGCAATTAGTGGAGAC-3′ (R) 5′-ATCCAGTGGCTGCCCTGCTGCT-3′</td>
</tr>
<tr>
<td>RANKL</td>
<td>NM_011613</td>
<td>(F) 5′-CCGAGATCTGAGGAAAGAAATAGC-3′ (R) 5′-GGCAAGACCTGACCCACCAAGAC-3′</td>
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<tr>
<td>OPG</td>
<td>NM_008764</td>
<td>(F) 5′-ACTCAGGAGGACTCTGGTGATT-3′ (R) 5′-TCAAGTCTTCTGCTGGTGCT-3′</td>
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Forward (F) and reverse (R) primers are listed. AR, adrenergic receptor; NFAT, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1; TRAP, tartrate-resistant acid phosphatase; Ctsk, cathepsin K; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin.
β1-AR, β2-AR, and β3-AR transcripts were detected in whole bone tissue (Fig. 1A). Mature osteoclasts (OC) derived from bone marrow cells contained mRNA encoding β2-AR but not β1-AR and β3-AR (Fig. 1A). The murine macrophage RAW 264.7 cells could differentiate into osteoclasts that also expressed only β2-AR (Fig. 1A).

Next we investigated whether β-AR agonist stimulates intracellular ROS generation via β2-AR on osteoclasts. Cells were loaded with the ROS-sensitive dye CF-H2DCFDA. In RAW 264.7 cells, 1 μM isoprenaline increased intracellular generation of ROS, causing a high level of fluorescence, as measured by confocal microscopy (Fig. 1B). In mature OCs derived from BMMs, 1 μM isoprenaline was sufficient to increase ROS levels at 1 h after isoprenaline addition (Fig. 1B). Hydrogen peroxide, one of the ROS, is a potent stimulator of osteoclastic bone resorption (5, 16). Hydrogen peroxide (1 and 10 μM) stimulated osteoclastogenesis from BMMs (Fig. 1, C and D) and increased expression levels of osteoclast-related genes (Fig. 1E). This concentration of hydrogen peroxide also increased osteoclastogenesis in the RANKL-treated RAW 264.7 cells (Fig. 1, C and D).

To examine the effects of β-AR agonist on osteoclastogenesis, RAW 264.7 cells were treated with the indicated dose of isoprenaline. Isoprenaline (1 and 10 μM) stimulated the formation of TRAP-positive multinucleated cells among RAW 264.7 cells in the presence of RANKL (Fig. 2, A top, and B). However, isoprenaline by itself did not stimulate the osteoclastogenesis of RAW 264.7 cells in the absence of RANKL (Fig. 2A, bottom). Treatment with propranolol inhibited isoprenaline-induced osteoclastogenesis (Fig. 2, A and B). α-LA treatment also inhibited isoprenaline-induced osteoclastogenesis (Fig. 2B). To investigate the molecular basis behind this finding, expression levels of osteoclast-related mRNAs were quantified by real-time PCR. Isoprenaline increased nuclear...

**Fig. 1.** Isoprenaline (ISO) stimulates reactive oxygen species (ROS) generation in osteoclasts through β2-adrenergic receptor (AR). A: to determine the expression levels of β1-, β2-, and β3-AR mRNA, real-time RT-PCR was performed in bone marrow macrophages (BMMs) and RAW 264.7 cells during osteoclast differentiation. β2-AR, but not β1 or β3, was expressed in mature osteoclasts and in undifferentiated cells. β1-, β2-, and β3-ARs were expressed in whole bone as a positive control for the primers. ND, not detected. B: RAW 264.7 cells and BMMs were cultured with receptor activator of nuclear factor-κB ligand (RANKL) for 3 days to differentiate into mature osteoclasts (OC) and analyzed for ROS generation after incubation with 5- (and 6-) chloromethyl-2′,7′-dichloroflourescin diacetate (CM-H2DCFDA) using a confocal microscope. ISO increased ROS generation in RANKL-treated RAW 264.7 cells and BMMs compared with that in the control group. C and D: hydrogen peroxide (HP) stimulated the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells among RANKL-treated RAW 264.7 cells and BMMs. Oc.N, osteoclast number. E: hydrogen peroxide increased the expression levels of osteoclast-related genes such as nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), TRAP, and cathepsin K in RANKL-treated RAW 264.7 cells. Data are shown as means ± SE (n ≥ 3) and were analyzed by ANOVA. *P < 0.05 relative to the control (ctrl). Experiments were performed at least three times with similar results.
factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), TRAP, and cathepsin K mRNA levels in RANKL-treated RAW 264.7 cells, and α-LA inhibited ISO increase in the expression of osteoclast-related genes. D and E: ISO stimulated the formation of TRAP-positive multinucleated cells among RANKL-treated RAW 264.7 cells, and α-LA inhibited ISO increase in osteoclastogenesis. Bar = 0.1 mm. F: ISO increased the expression of osteoclast-related genes in RANKL-treated BMMs. Data are shown as means ± SE (n = 3) and were analyzed by ANOVA. *P < 0.05 relative to the control. Experiments were performed at least three times with similar results.

Antioxidative α-LA inhibits β-AR agonist-induced osteoporosis by protecting against oxidative damage. To test if treatment with the antioxidant α-LA prevents β-AR agonist-induced osteoporosis, mice were treated with α-LA (0, 0.25, 2.5, 25, and 50 mg·kg body wt⁻¹·day⁻¹ sc) during β-AR agonist (isoprenaline; 5 mg·kg body wt⁻¹·day⁻¹ ip) vehicle treatment for 2 wk. Isoprenaline treatment alone significantly decreased BV/TV and Tb.N and increased Tb.Sp and Tb.Spac, as reported previously (11, 24, 25, 39). In contrast, 0.25 and 2.5 mg·kg body wt⁻¹·day⁻¹ α-LA treatments partially suppressed isoprenaline-induced bone loss. The 2.5 mg·kg body wt⁻¹·day⁻¹...
α-LA treatment did not suppress isoprenaline-reduced BV/TV, but suppressed isoprenaline-reduced Tb.N and increased Tb.Sp and Tb.Spac (Fig. 3, C–E). The 25 and 50 mg·kg body wt⁻¹·day⁻¹ α-LA treatments blocked isoprenaline-induced alteration of bone microarchitecture. On the basis of these experiments, we focused on 25 mg·kg body wt⁻¹·day⁻¹, which is the minimum effective dose. Next, we performed assessment of oxidative damage to lipids of bone and plasma by measuring the levels of MDA, which is commonly measured as an index of lipid peroxidation. Isoprenaline treatment alone significantly increased MDA levels in bone. In contrast, treatment with 25 mg·kg body wt⁻¹·day⁻¹ α-LA treatment suppressed isoprenaline-induced MDA levels in bone (Fig. 4A). On the other hand, isoprenaline treatment did not increase MDA levels in plasma in either α-LA-treated or untreated groups (Fig. 4A). To assess general health, body weight was measured during the experimental period and was not altered significantly by the drug treatment (Fig. 4B). Because chronic isoprenaline treatment is known to increase heart weight through cardiac hypertrophy, we also measured heart weight (6, 12, 25). This dose of isoprenaline was not sufficient to increase the ratio of heart weight/body weight (Fig. 4B).

α-LA prevents β-AR agonist-enhanced osteoclast formation independently of RANKL expression in vivo. To examine the cellular mechanisms behind the effect of α-LA on β-AR agonist-induced bone loss, histomorphometric analysis was conducted. Isoprenaline treatment alone significantly increased bone resorption parameters such as bone surface covered with TRAP-positive osteoclasts, Oc.S/BS (Fig. 5A), and Oc.N/BS (Fig. 5B). In contrast, treatment with α-LA suppressed isoprenaline-induced osteoclast formation independently of RANKL expression in vivo.
Fig. 4. Isoprenaline alone increased malondialdehyde (MDA) levels in bone. To determine whether isoprenaline increased oxidative damage in bone or plasma, MDA assay was performed. A: isoprenaline alone increased MDA levels in bone, but not in plasma. In the α-LA treatment group, isoprenaline did not have an effect. B: body weight (g) and heart weight (mg/body weight (g) on the day of death. Data are shown as means ± SE (n = 5) and were analyzed by ANOVA. *P < 0.05 relative to isoprenaline untreated group (either α-LA-treated group or untreated group). Experiments were performed at least two times with similar results.

DISCUSSION

The sympathetic nervous system regulates osteoclastogenesis through the RANKL expression via β2-AR expressed in osteoblasts (11, 40). β2-AR is also expressed in osteoclasts, but it was not well documented whether the sympathetic nervous system regulates bone resorption by osteoclasts directly. Both mature OC derived from bone marrow cells and RAW 264.7 cells expressed β2-AR but not β1-AR and β3-AR (Fig. 1A). We demonstrated previously that β-adrenergic agonists stimulate bone-resorbing activity in osteoclast-like multinucleated cells and osteoclastic activation occurs via adrenergic receptor in osteoclastic cells as a direct response to neuronal activation (3, 38). These observations implied that the sympathetic signaling

α-LA: - - + + +

Isoprenaline: -+ - + - +

B

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<th>Isoprenaline</th>
<th>Vehicle</th>
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<tr>
<td>Body weight (g)</td>
<td>28.8 ± 1.0</td>
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<td>26.9 ± 0.7</td>
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</tr>
<tr>
<td>Heart weight/Body weight (mg/g)</td>
<td>4.2 ± 0.2</td>
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<td>3.8 ± 0.0</td>
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directly regulates bone resorption by osteoclasts. However, the signaling pathway has not been reported yet. In this report we suggest β-adrenergic signaling directly stimulates osteoclastogenesis through β-AR on osteoclast via ROS production.

β-AR agonist induces ROS generation in several cell types (12, 31); therefore, we investigated whether β-AR agonist stimulates intracellular ROS generation on osteoclasts. Isoprenaline induced ROS generation on mature OC derived from bone marrow and RAW 264.7 cells (Fig. 1B). Because ROS is known to stimulate osteoclast differentiation (5, 16, 28), we confirmed that hydrogen peroxide increased osteoclastogenesis from RAW 264.7 cells and BMMs (Fig. 1, C and D). Hydrogen peroxide also increased the expression levels of osteoclast-related genes such as NFATc1, TRAP, and cathepsin K in RANKL-treated RAW 264.7 cells (Fig. 1E). Isoprenaline and hydrogen peroxide increased osteoclastogenesis (Fig. 2, A–F). The antioxidant α-LA and β-blocker propranolol treatment inhibited isoprenaline-increased osteoclastogenesis (Fig. 2, A, B, and E). Isoprenaline also increased expression levels of osteoclast-related mRNAs (NFATc1, TRAP, cathepsin K) in osteoclasts, and α-LA inhibited these increases (Fig. 2, C and F). These in vitro results led to the hypothesis that β-AR signaling directly stimulates osteoclastogenesis without an increase in RANKL expression. Sympathetic nervous signaling suppresses osteoblast proliferation via clock genes (14). Previously, we reported that isoprenaline (10−10 to 10−5 M) increased the expression levels of clock genes in osteoblasts (22). We also reported that isoprenaline (0.1–10 μM) increased osteoclastogenesis (40). In both experiments, the effect of isoprenaline reached a plateau at 1 to 10 μM. Therefore, we selected doses of 1 and 10 μM in the present in vitro experiments.

To determine that β-AR signaling stimulates osteoclastogenesis independently of RANKL expression, mice were treated with α-LA (0, 0.25, 2.5, 25, and 50 mg·kg body wt−1·day−1 sc) during isoprenaline (5 mg·kg body wt−1·day−1 ip) or vehicle treatment for 2 wk. Isoprenaline treatment alone significantly decreased BV/TV due to decreased Tb.N and increased Tb.Sp and Tb.Spac. Treatment with α-LA did not affect normal bone microarchitecture. α-LA at 0.25 mg·kg body wt−1·day−1 did not prevent isoprenaline-induced Tb.N reduction, and Tb.Sp and Tb.Spac increase. α-LA at 2.5 mg·kg body wt−1·day−1 did not prevent isoprenaline-induced BV/TV reduction. α-LA at 25 and 50 mg·kg body wt−1·day−1 suppressed isoprenaline-induced bone loss. However, 50 mg·kg body wt−1·day−1 α-LA tended to decrease BV/TV and increase Tb.Sp and Tb.Spac. These results indicated that 25 mg·kg body wt−1·day−1 α-LA is the most effective dose to prevent isoprenaline-induced bone loss. These results implied that β-AR agonist reduced bone mass because of increased oxidative damage systemically or locally. To distinguish between these two possibilities, we performed assessment of oxidative damage to lipids of bone and plasma by measuring the levels of MDA. Isoprenaline treatment alone significantly increased MDA levels in bone, but treatment with α-LA suppressed isoprenaline-induced MDA levels in bone (Fig. 4A). On the other hand, isoprenaline treatment did not increase MDA levels in plasma in either α-LA-treated or untreated groups (Fig. 4A), indicating that β-AR agonist caused oxidative damage locally but not systemically. These results suggest that excess ROS generated by β-AR agonists, which cause oxidative damage within skeletal tissues, contribute to bone loss and that exogenous application of α-LA prevents β-AR agonist-induced bone loss. Body weight and the ratio of heart weight/body weight were not altered significantly by drug treatment in our experiment (Fig. 4B). These observations demonstrate that differences in body weight and the cardiovascular system between the groups cannot account for the changes in skeletal microarchitecture. To examine the cellular mechanisms behind the effect of α-LA on β-AR agonist-induced bone loss, histomorphometric analysis was conducted. Isoprenaline treatment alone significantly increased Oc.S/BS and Oc.N/BS. In contrast, treatment with α-LA suppressed isoprenaline-induced Oc.S/BS and Oc.N/BS (Fig. 5, A and B). These data suggest that α-LA improves bone loss by suppressing bone resorption induced by β-AR agonist. Because the sympathetic nervous system regulates osteoclastogenesis through the RANKL mRNA expression via β2-AR on osteoblasts (11, 40) and ROS is known to stimulate RANKL expression in osteoblasts (4), a gene-expression assay was conducted. α-LA did not suppress the isoprenaline-increased RANKL-to-OPG ratio (Fig. 5C). This indicated that α-LA inhibits isoprenaline-increased osteoclast differentiation in a RANKL expression-independent manner. Isoprenaline induces ROS generation in bone, and α-LA inhibits isoprenaline-increased osteoclast differentiation in a RANKL expression-independent manner (Figs. 4A and 5, A–C). These results reveal that β-AR signaling directly stimulates osteoclastogenesis without RANKL expression.

Recently, Kim et al. reported that RANKL stimulation induces a signaling pathway that leads to the generation of ROS and is essential for osteoclastogenesis (21). Consistent with this previous report, we also showed that α-LA inhibits signaling downstream of RANKL. On the other hand, Lee et al. reported that an antioxidant, Torolox (vitamin E analog), suppressed interleukin-1-induced osteoclast formation and bone loss in mouse calvarial bone and prevented osteoclastogenesis by suppressing RANKL expression (29). Our data showed that α-LA inhibits β-AR agonist-increased osteoclast differentiation in a RANKL expression-independent manner. Isoprenaline induces ROS generation in bone, and α-LA inhibits isoprenaline-increased osteoclast differentiation in a RANKL expression-independent manner (Figs. 4A and 5, A–C). These results reveal that β-AR signaling directly stimulates osteoclastogenesis without RANKL expression.

Lean et al. (28) reported that administration of N-acetylcysteine or ascorbate, antioxidants, abolished ovariectomy-induced high-turnover osteoporosis. Rao et al. (36) reported lycopene is a carotenoid commonly found in tomatoes and tomato products that acts as an antioxidant to decrease oxidative stress and the levels of bone turnover markers in postmenopausal women. Furthermore, dietary restriction of lycopene for a period of 1 mo resulted in significantly increased biomarkers of oxidative damage.
stress and bone resorption in postmenopausal women (30). We also demonstrated α-LA treatment suppressed isoproterenol-increased bone resorption. These data indicate the antioxidants mainly suppressed osteoclastic bone resorption. In our study, α-LA treatment suppressed isoprenaline-induced MAR reduction (Fig. 5D). However, it is unclear whether α-LA treatment prevented the isoprenaline-induced reduction in bone formation because MAR tended to decrease by α-LA treatment alone (Fig. 5D). Nojiri et al. (34) reported that intracellular redox imbalance caused by superoxide dismutase 1 (SOD1) deficiency plays a pivotal role in the development and progression of bone fragility. They showed that the surface areas of osteoblasts and osteoclasts were decreased in SOD1-deficient mice. These data indicate the role of ROS on bone formation is different between normal and abnormal conditions. We enhanced osteoclast activity by isoprenaline treatment, but Nojiri et al. (34) did not.

In conclusion, our findings show that α-LA treatment prevents the β-adrenergic signaling-induced bone loss by suppressing osteoclastogenesis, and sympathetic signaling, at least in part, directly stimulates osteoclastogenesis via ROS generation through β2-AR on osteoclasts. We report, for the first time, direct regulation of osteoclasts by the sympathetic receptor, β-AR, which can be attributed to induction of ROS production.

**REFERENCES**


ligand is a cytokine that regulates osteoclast differentiation and activation. 


