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Original Contribution

Antioxidant α-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-κB DNA binding and prevents in vivo bone resorption induced by receptor activator of nuclear factor-κB ligand and tumor necrosis factor-α

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Abstract

The relationship between oxidative stress and bone mineral density or osteoporosis has recently been reported. As bone loss occurring in osteoporosis and inflammatory diseases is primarily due to increases in osteoclast number, reactive oxygen species (ROS) may be relevant to osteoclast differentiation, which requires receptor activator of nuclear factor-κB ligand (RANKL). Tumor necrosis factor-α (TNF-α) frequently present in inflammatory conditions has a profound synergy with RANKL in osteoclastogenesis. In this study, we investigated the effects of α-lipoic acid (α-LA), a strong antioxidant clinically used for some time, on osteoclast differentiation and bone resorption. At concentrations showing no growth inhibition, α-LA potently suppressed osteoclastogenesis from bone marrow-derived precursor cells driven either by a high-dose RANKL alone or by a low-dose RANKL plus TNF-α (RANKL/TNF-α). α-LA abolished ROS elevation by RANKL or RANKL/TNF-α and inhibited NF-κB activation in osteoclast precursor cells. Specifically, α-LA reduced DNA binding of NF-κB but did not inhibit IKK activation. Furthermore, α-LA greatly suppressed in vivo bone loss induced by RANKL or TNF-α in a calvarial remodeling model. Therefore, our data provide evidence that ROS plays an important role in osteoclast differentiation through NF-κB regulation and the antioxidant α-lipoic acid has a therapeutic potential for bone erosive diseases.

Keywords: Osteoclast differentiation; Cytokines; Antioxidant; α-Lipoic acid; Nuclear factor kappa B; Bone resorption; Free radical

Introduction

Osteoclasts, cells specialized for resorption (dissolution) of calcified matrix, are continuously generated from discrete subpopulations of hematopoietic cells through differentiation processes. The differentiation events are regulated by various systemic and local factors including hormones, growth factors, and immune mediators in endocrine, paracrine, and autocrine manners. However, the tumor necrosis factor (TNF) family cytokine RANKL (receptor activator of nuclear factor-κB ligand) and its receptor RANK are the fundamental components required for osteoclast differentiation [1–4]. Many of the factors regulating osteoclast differentiation exert their effects either by changing the expression of RANKL and osteoprotegerin, a decoy receptor for RANKL that interferes with RANKL binding to RANK, or by positively or negatively influencing RANK-dependent signaling [5–10]. RANKL binding to RANK triggers several intracellular signaling pathways in osteoclast

Abbreviations: RANKL, receptor activator of nuclear factor-κB ligand; α-LA, α-lipoic acid; ROS, reactive oxygen species; TRAP, tartrate-resistant acid phosphatase; IKK, inhibitory κB kinase; NF-κB, nuclear factor κB; IL-1, interleukin-1; NAC, *N*-acetylcysteine; M-CSF, macrophage colony stimulating factor; BMMs, bone marrow macrophages; RT-PCR, reverse transcription-polymerase chain reaction; DPI, diphenyleneiodonium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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precursor cells, ultimately causing the expression of osteoclast-specific genes. These signaling events include the activation of PI3K/Akt, the cascade of mitogen-activated protein kinase activation, and the stimulation of transcription factors nuclear factor κB (NF-κB) and nuclear factor of activated T cells [11]. The TNF receptor-associated factor family adaptor molecules are recruited to RANK and play a pivotal role in these RANKL-induced signaling responses [11]. RANKL also plays an important role in the activation of differentiated mature osteoclasts.

Reactive oxygen species (ROS), initially recognized as important small molecules for defense against invading organisms and as deleterious metabolic intermediates to kill host cells, have later been increasingly documented for the second messenger or modulator roles in signal transduction [12–14]. There have been some studies implicating ROS in bone regulation. Bone resorption stimulated by parathyroid hormone and interleukin-1 (IL-1) was inhibited by removal of superoxide anions while addition of hydrogen peroxide increased bone resorption by isolated osteoclasts [15,16]. Generation of ROS by osteoclasts was shown both in vivo and in vitro, and NADPH oxidase, a ROS generating enzyme, was detected in osteoclasts [15,17,18]. Recently, we also demonstrated that RANKL stimulates hydrogen peroxide production in differentiated osteoclasts [19]. These findings indicate that mature osteoclasts produce ROS probably through activated NADPH oxidase and ROS may in turn contribute to their bone resorption function. The possibility of ROS involvement in osteoclast differentiation has been raised based on the correlation between ROS production and the formation of osteoclasts on bone surfaces in vivo, and in in vitro culture of bone marrow cells [15,20]. However, whether the ROS-mediated contribution was made by osteoblasts/stromal cells or osteoclast precursors and which agonistic factors were directly responsible for stimulating ROS production were not clear in those studies due to the presence of mixed populations of cells. TNF-α is a potential candidate for inducing ROS in osteoclast lineage cells because it exerts strong effects on osteoclast differentiation [7] and has been shown to cause ROS generation in other cell types [13,21].

Use of antioxidants has been suggested to be beneficial in oxidative stress-associated diseases. *N*-Acetylcysteine (NAC) was shown to decrease the risk of colon cancer in patients with previous adenomatous colonic polyps and to increase immune functions in HIV patients [22,23]. It was reported that oxidative stress levels were negatively associated with bone mineral density and antioxidant levels were lower in osteoporosis patients [24,25]. In line with these observations, vitamin C intake showed beneficial effects in increasing bone mineral density in women and, more recently, NAC and vitamin C inhibited ovariectomy-induced bone loss in a rodent osteoporosis model [26,27]. α-Lipoic acid (α-LA) is a powerful antioxidant that has long been used clinically for treating diabetic neuropathy [28,29]. This compound also improves glucose uptake in patients with type 2 diabetes mellitus and has antiobesity effects [30,31]. Recently, interest in the clinical applications of α-LA to many chronic diseases has greatly been elevated, perhaps due to the relative clinical safety and potent antioxidant properties [32]. However, the effect of this antioxidant on bone loss prevention and bone cell activity regulation has not been investigated to date.

In this study, we investigated the effect of α-LA on osteoclast differentiation. We found that α-LA inhibited osteoclastogenesis in bone marrow-derived precursor cell cultures under osteoclastogenic conditions attained with a high-dose RANKL alone or a low-dose RANKL plus TNF-α. The inhibitory effect appeared to be due to suppression of ROS generation and subsequent reduction in the activation of NF-κB induced by those osteoclastogenic stimuli. Furthermore, the potential usefulness of this antioxidant in inhibiting in vivo bone loss was verified by calvarial bone resorption analyses.

Materials and methods

Osteoclast culture

Osteoclast generation was achieved using either primary cultures of mouse bone marrow-derived macrophages or the monocyte/macrophage lineage cell line RAW264.7. For generation of bone marrow-derived osteoclasts, monocytes were isolated from tibiae of ICR mice as previously described [33]. Cells were seeded in 6-well plates (2×10^6/well) and cultured in the presence of 30 ng/ml macrophage colony stimulating factor (M-CSF) for 72 h. Cells at this stage were considered M-CSF-dependent bone marrow macrophages (BMMs) and used as osteoclast precursors [34]. Induction of differentiation to osteoclasts was achieved by culturing the cells either with 100 ng/ml RANKL (Peprotech) and 30 ng/ml M-CSF (R&D Systems) or with 10 ng/ml RANKL, 20 ng/ml TNF-α (R&D Systems), and 30 ng/ml M-CSF. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) activity using an acid-phosphatase kit (Sigma). Typically, TRAP-positive mononuclear cells appeared at Day 3 and TRAP-positive multinuclear cells were observed 5-6 days after RANKL addition. Differentiation of RAW264.7 cells to osteoclasts was conducted by culturing the cells in α-minimal essential medium (α-MEM) containing 10% FBS in the presence of 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α without adding M-CSF. In these cultures, TRAP-positive mononuclear cells appeared in 2-3 days and mature osteoclasts were obtained at Day 4.

Cell toxicity assays

Cytotoxicity assays were conducted by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan), which produces a highly water-soluble formazan dye. Cells were seeded in 96-well plates (1×10^4/well, 200 μl/well) and cultured in the presence of indicated

concentrations of α-LA or the vehicle (0.1% DMSO) for 48 h. In BMM culture, M-CSF (30 ng/ml) was included. At the end of culture period, 20 μl of the CCK-8 reagent was added to each well. After a 1-h incubation at 37°C, the absorbance was determined at 450 nm using a microplate reader with a reference wave length at 655 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analyses

RT-PCR analyses were performed as previously described [35]. The sequences of oligonucleotide primers used were 5′-ACT TCC CCA GAA CTT ACT ACC G -3′ (TRAP forward); 5′- TCA GCA CAT AGC CCA CAG CG-3′ (TRAP reverse); 5′-CAAGGCTGTGGGCAAGGTCA-3′ (GAPDH forward); and 5′-AGGTGGAAGAGTGGGAGTTGCTG-3′ (GAPDH reverse). The amplification cycle comprised a denaturation step for 1 min at 94°C, an annealing step for 1 min at 58°C, and an extension step for 30 s at 72°C. TRAP and GAPDH cDNAs were amplified by 26 and 20 cycles of the thermal reaction, respectively. PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by staining the gel with ethidium bromide.

ROS detection by confocal microscopy

Cells placed on coverslips were serum-starved for 2 h, pretreated with α-LA for 30 min, and loaded with 10 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes) for 1 h at 37°C. Cells were then stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α for 5 min. After fixing the cells, fluorescence images were captured under a confocal microscope (Olympus-FV300). In experiments with diphenyleneiodonium (DPI), the mean fluorescence intensity was measured by the confocal system.

NF-κB reporter assays

RAW264.7 cells were plated at 5×10^5/well in 6-well plates. The next day, cells were transfected with 4 μg of NF-κB-dependent luciferase reporter vector using 10 μl Lipofectamin 2000 (Invitrogen) in DMEM. Four hours after transfection, the medium was replaced by DMEM containing 10% FBS. After incubation for 14 h at 37°C in a CO_2 incubator, cells were collected by scraping, resuspended in α-MEM/10% FBS, and replated in 96-well plates at 2×10^4/well. Cells were stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α in the presence or absence of α-LA for 8 h. Cells were lysed in Reporter Lysis Buffer (Promega) and luciferase activity was measured using a luminometer.

Electrophoretic gel mobility-shift assays

BMM osteoclast precursor cells were serum-deprived for 2 h, pretreated with 0.1 mM α-LA for 30 min, and stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α for 20 min. Nuclear extracts were prepared and NF-κB-binding DNA gel mobility-shift assays were performed as previously described [36].

IKK activity assays

BMM cells were serum-starved for 2 h, pretreated with 0.1 mM α-LA for 30 min, and stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α for 10 min. Cell lysates were prepared and IKK was immunoprecipitated from 700 μg of cell lysates with 2 μg anti-IKK-α antibody (Santa Cruz). In vitro kinase assays were conducted with 2 μg GST-IκB-α and 50 μM ATP at 30°C for 30 min. The extent of IκB phosphorylation was determined by separating the reaction samples by 10% SDS-PAGE and performing Western blotting analyses with anti-phospho-IκB-α antibody (Cell Signaling). The membrane was stripped and reprobed with anti-IKK-α antibody.

Western blotting analyses

BMM cells were serum-starved for 2 h, pretreated with 0.1 mM α-LA for 30 min, and stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α for various times. Cell lysates were prepared as previously described and 20 μg of proteins was separated by SDS-PAGE [36]. Proteins were blotted to PVDF membranes and probed with anti-phospho-IκB-α and anti-IκB antibodies (Cell Signaling) using an enhanced chemiluminescence system. The membranes were stripped and reprobed with anti-actin antibody (Santa Cruz). For NF-κB nuclear translocation experiments, nuclear extracts were prepared as above. Cytosol proteins were obtained from the cell lysate supernatant by centrifugation at 100,000*g* for 1 h. Cytosol and nuclear proteins were resolved and transferred to membranes. The membranes were probed with anti-p65 and anti-p50 antibodies (Abcam). After stripping, the membranes were reprobed with anti-tubulin and anti-histone antibodies (Santa Cruz).

In vivo calvarial bone resorption analyses

All animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University School of Dentistry. Six-week-old ICR mice were used for calvarial bone resorption assays. A collagen sheet (100 mm^2 square) was soaked with PBS, RANKL (2 μg), or RANKL plus α-LA (2 mg) in 30 μl volume and implanted to the center of calvaria. Five mice were used for each group. After 10 days, mice were sacrificed and calvariae were collected. Each calvaria was cut coronally in the middle of the sagittal suture into two pieces. Calvaria pieces were fixed for 2 days in 4% paraformaldehyde and decalcified in 13% EDTA for 4 weeks. After dehydration in ethanol and incubation in chloroform, calvariae were embedded in paraffin. Paraffin blocks were sectioned to 4 μm thickness using a microtome (LEICA RM 2125, Germany). Tissue sections were deparaffinized in xylene, hydrated, and stained with hematoxylin and eosin (H&E). In

more than 10 different H&E-stained sections, bone marrow area and bone tissue (bone marrow plus bone) area were determined using Kappa Image Base Control 2.5 (KAPPA Opto-electronics GmbH, Germany) and the percentage of bone marrow space per bone tissue was calculated.

Alternatively, mice received supracalvarial injections of PBS, TNF-α (2 μg), or TNF-α plus α-LA (2 mg) in 100 μl volume once. Five days after injection, mice calvariae were collected and micro-computerized tomography (μCT) was performed with 1072 Microtomograph (SkyScan, Belgium). A total of 337 tomographic slices were acquired at a 4-μm resolution. 3-D analyses were performed with the V-Works program (Cybermed, Korea).

Statistical analysis

All quantitative experiments were performed in triplicate and repeated three to five times. All data are presented as mean ± SD. Statistical analysis was carried out using the Kruskal-Wallis test for differences among three or more groups and the Mann-Whitney's *U* test for differences between two groups. The SPSS 10.0 package (SPSS Inc., Chicago, IL) was used for statistical analyses.

Results

α-LA inhibits osteoclast formation

In vitro osteoclastogenesis was investigated by culturing bone marrow-derived macrophages and RAW264.7 cells in the presence of the osteoclastogenic cytokine RANKL and scoring the number of TRAP-positive multinuclear cells ($TRAP^+$ MNC) generated. Before examining the effect of α-lipoic acid on osteoclast differentiation, the potential cytotoxicity of α-LA was tested because cell toxicity would decrease cell numbers during culture and consequently would reduce $TRAP^+$ MNC formation irrelative to its effects on differentiation per se. α-LA did not show cytotoxicity to either BMM or RAW264.7 cells at concentrations up to 10 mM (Fig. 1). Therefore, we used α-LA throughout this study at concentrations below 10 mM.

To assess the effect of α-LA on osteoclastogenesis, BMM cells were induced to undergo osteoclastogenesis by culturing in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml). α-LA treatment under this condition decreased the formation of $TRAP^+$ MNC in a dose-dependent manner (Fig. 2A). α-LA inhibited $TRAP^+$ MNC generation by 57.0 ± 2.8 and 96.9 ± 0.8% at 0.01 and 0.1 mM concentrations, respectively (Fig. 2B, left panel). RANKL-dependent osteoclastogenesis has been shown to be greatly enhanced by TNF-α [7]. The simultaneous presence of RANKL and TNF-α occurs during osteoclastogenesis under inflammatory conditions such as rheumatoid arthritis and periodontitis. Under these circumstances a much lower amount of RANKL is required to induce osteoclast differentiation. To test whether α-LA can also block osteoclastogenesis under conditions involving TNF-α, BMMs were cultured with 10-fold lower amounts of RANKL (10 ng/ml) along with M-CSF (30 ng/ml) and TNF-α (20 ng/ml). The

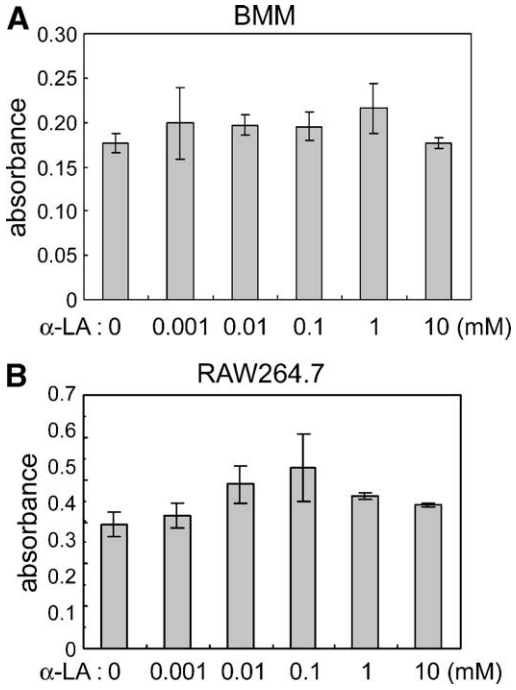

Fig. 1. α-LA has no toxicity to osteoclast precursor cells. BMM cells (A) and RAW264.7 cells (B) were cultured in the presence of the indicated concentrations of α-LA for 48 h. The viable cell activity was determined using CCK reagent as described under Materials and methods.

presence of α-LA greatly reduced the number of $TRAP^+$ MNC generated under this osteoclastogenic condition (Fig. 2B, right panel). The inhibitory effect of α-LA was 48.3 ± 11.0 and 94.3 ± 3.5% at 0.01 and 0.1 mM, respectively.

RAW264.7 cells have been used as a model cell line for osteoclastogenesis studies. These cells generate functional osteoclasts upon treatment with RANKL. α-LA also suppressed the $TRAP^+$ MNC generation from RAW264.7 cells in culture conditions involving RANKL or RANKL plus TNF-α (Fig. 2C).

α-LA affects osteoclast differentiation from the commitment stage

The osteoclast differentiation process proceeds through distinct steps: commitment of precursor cells and multinucleation of the committed cells. The commitment step is accompanied by expression of TRAP and the subsequent fusion of $TRAP^+$ mononuclear cells generates mature osteoclasts. To investigate which stage of osteoclastogenesis is affected by α-LA, BMM cells were cultured in the presence of RANKL while α-LA inclusion was restricted to only some time of the whole 6-day culture period (Fig. 3A). Addition of α-LA (0.1 mM) during the first 2 days reduced $TRAP^+$ MNC formation by 79.3 ± 10.2%. The extents of inhibition when α-LA was included during Days 3-4, and 5-6 were 56.8 ± 15.8 and 52.8 ± 5.2%, respectively. The presence of α-LA throughout the culture period caused 92.6 ± 2.8% inhibition of $TRAP^+$ MNC formation. These results suggest that α-LA suppresses osteoclast differentiation by interfering with the early commitment step as well as further events in later stages of differentiation. To support this notion, we next examined the effect of α-LA on $TRAP^+$

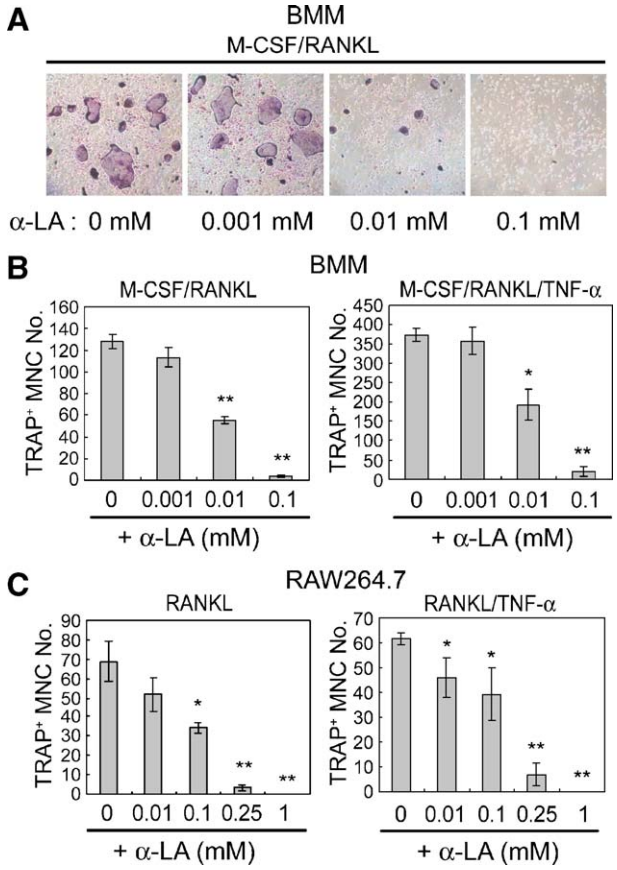

Fig. 2. α-LA inhibits formation of mature osteoclasts driven by RANKL or RANKL/TNF-α. (A) Photographs of TRAP-stained osteoclasts generated from BMMs. (B) α-LA reduced TRAP$^+$ multinuclear osteoclast formation from BMM cells. BMMs cultured for 6 days in medium containing 30 ng/ml M-CSF plus either 100 ng/ml RANKL (left histogram) or 10 ng/ml RANKL and 20 ng/ml TNF-α (right histogram) in the presence of the indicated concentrations of α-LA were stained for TRAP. MNC, multinuclear cell. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated control. (C) α-LA blocked TRAP$^+$ MNC generation from RAW264.7 cells. RAW264.7 cells cultured for 4 days either with 100 ng/ml RANKL (left histogram) or with 10 ng/ml RANKL plus 20 ng/ml TNF-α (right histogram) in the presence of the indicated concentrations of α-LA were TRAP-stained. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated control.

mononuclear cell formation. BMMs were cultured with M-CSF and RANKL in the presence or absence of α-LA for only 3 days and generated TRAP$^+$ mononuclear cells were scored. α-LA reduced the formation of TRAP$^+$ mononuclear cells by 52 ± 4.4% at 0.01 mM and 94.7 ± 1.5% at 0.1 mM (Fig. 3B, left). A similar inhibitory effect of α-LA was also observed in cultures with RANKL plus TNF-α (Fig. 3B, right). Next, whether the decrease in the generation of TRAP$^+$ mononuclear cells by α-LA reflects TRAP mRNA expression levels was addressed. α-LA suppressed the induction of TRAP mRNA by RANKL or RANKL/TNF-α in a dose-dependent manner (Fig. 3C).

α-LA attenuates elevation of ROS levels by osteoclastogenic stimulation

As α-LA is an antioxidant and ROS has been implicated in osteoclast activity [15,16], we investigated whether osteoclastogenic stimuli can elicit ROS production in osteoclast precursor cells and, if they can, whether α-LA has any effect on the level of ROS in those cells receiving such stimulation. Stimulation with RANKL caused an increase in ROS levels in BMM cells within 5 min (Fig. 4A). RANKL plus TNF-α also induced ROS production in BMMs (Fig. 4B). The ROS levels in some but not all cells stimulated with RANKL/TNF-α were higher than in cells treated with RANKL alone (Fig. 4B). More sustained production of ROS was observed in RANKL-treated

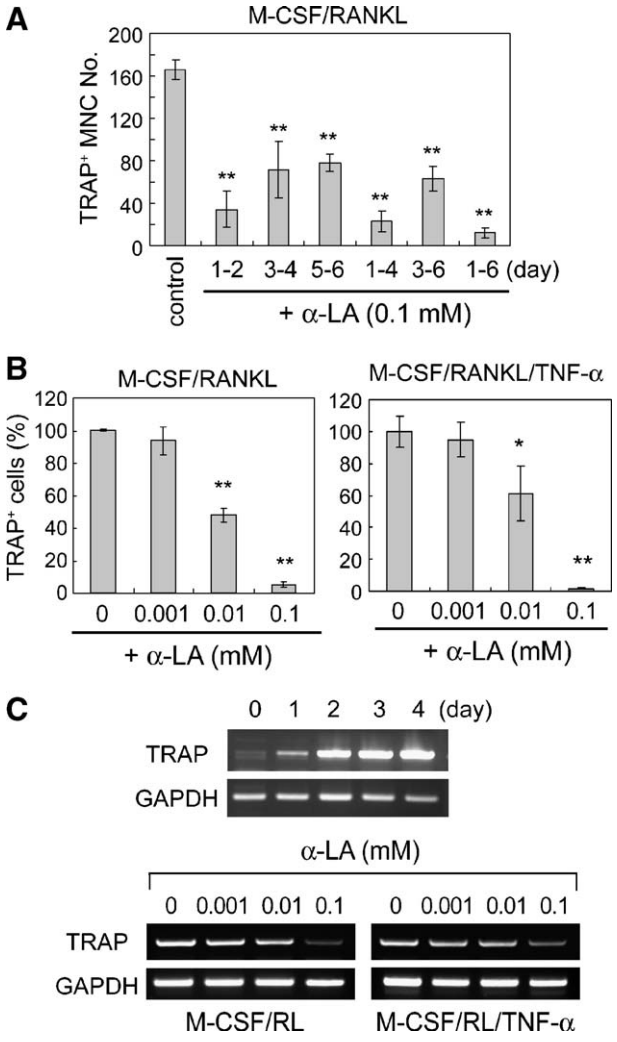

Fig. 3. α-LA blocks osteoclast differentiation from the commitment stage. (A) BMMs were cultured for 6 days in medium containing 30 ng/ml M-CSF plus 100 ng/ml RANKL with inclusion of α-LA during only the indicated days. Cells were stained for TRAP and TRAP$^+$ multinuclear cells were counted. MNC, multinuclear cells. ** $P < 0.01$ compared with vehicle-treated control. (B) α-LA decreased BMM differentiation to TRAP$^+$ mononuclear cells in osteoclastogenic conditions. BMMs cultured for 3 days either with 100 ng/ml RANKL and 30 ng/ml M-CSF (left) or with 10 ng/ml RANKL, 20 ng/ml TNF-α, and 30 ng/ml M-CSF (right) were stained for TRAP. Percentages of TRAP$^+$ mononuclear cells are presented as means ± SD from triplicate samples. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated control. (C) RT-PCR analysis of TRAP mRNA. BMMs were cultured for the indicated days with 100 ng/ml RANKL and 30 ng/ml M-CSF (top panels) or for 2 days with the indicated concentrations of α-LA in the presence of 100 ng/ml RANKL and 30 ng/ml M-CSF (bottom left panels) or 10 ng/ml RANKL, 20 ng/ml TNF-α, and 30 ng/ml M-CSF (bottom right panels). RL, RANKL.

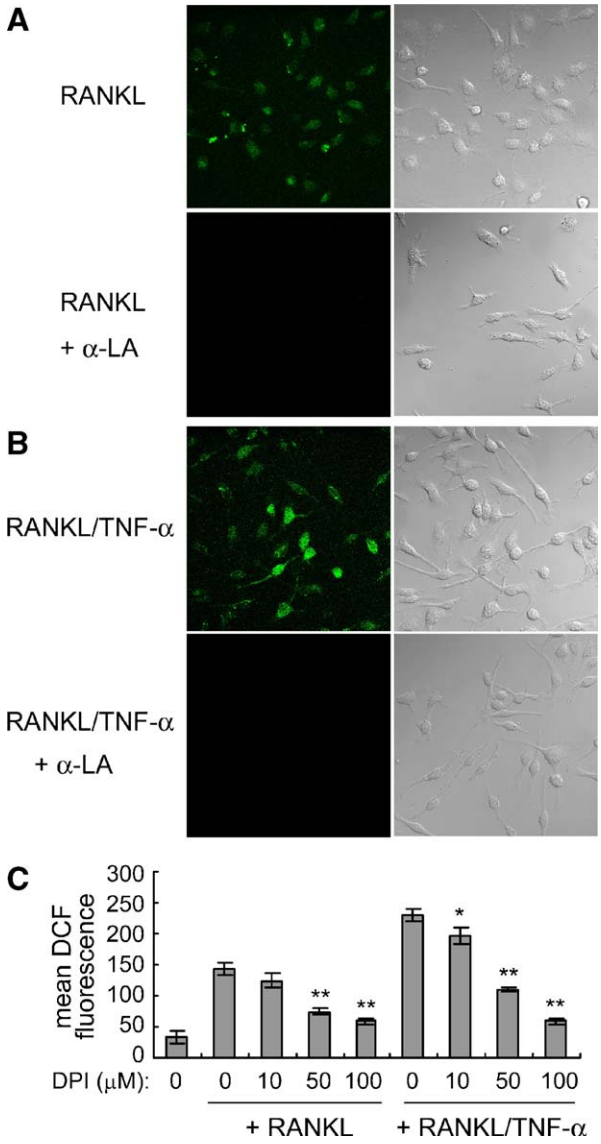

Fig. 4. α-LA suppresses the elevation of ROS levels by osteoclastogenic cytokines. (A and B) BMMs stimulated for 5 min with 100 ng/ml RANKL (A) or 10 ng/ml RANKL plus 20 ng/ml TNF-α (B) in the absence of α-LA (top left panels of A and B) show ROS production. Pretreatment with α-LA (0.1 mM) blocked ROS increases under those stimulatory conditions (bottom left panels of A and B). Differential interference contrast images of the same fields are shown in right panels. (C) BMMs pretreated with vehicle or DPI at the indicated concentrations were stimulated with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α for 5 min. The mean DCF fluorescence intensity was measured by confocal microscopy. * $P < 0.05$, ** $P < 0.01$ compared with the DPI-untreated and factor-stimulated control within the RANKL or RANKL/TNF-α group of samples.

cells than in RANKL/TNF-α-treated cells (data not shown). α-LA treatment almost completely blocked the elevation of intracellular ROS levels by either RANKL or RANKL/TNF-α (Figs. 4A and B). As NADPH oxidase has been implicated in ROS generation in mature osteoclasts [17], we tested the involvement of this enzyme in the RANKL- and RANKL/TNF-α-induced ROS production in BMMs by using the NADPH oxidase inhibitor, diphenyleneiodonium. DPI attenuated the ROS elevation by both RANKL and RANKL/TNF-α in BMMs (Fig. 4C).

α-LA inhibits NF-κB activation by osteoclastogenic stimulation

NF-κB is a transcription factor essential for osteoclast differentiation [37]. In other cell types, NF-κB activation has been documented to require ROS [13,38]. Given these reports and our observation that α-LA blocked ROS production by RANKL or RANKL/TNF-α in osteoclast precursors (Fig. 4), we investigated whether α-LA might have effects on NF-κB activation induced by the osteoclastogenic stimuli. To examine whether NF-κB activation can be stimulated by ROS in osteoclast precursors, we transfected RAW264.7 cells with a reporter plasmid that causes luciferase induction in a manner dependent on NF-κB activity. H_2O_2 treatment increased luciferase activity in these cells (Fig. 5A). RANKL treatment (100 ng/ml, 8 h) of the cells increased the luciferase activity by 10.7 ± 0.06-fold (Fig. 5B). Addition of α-LA led to a dose-

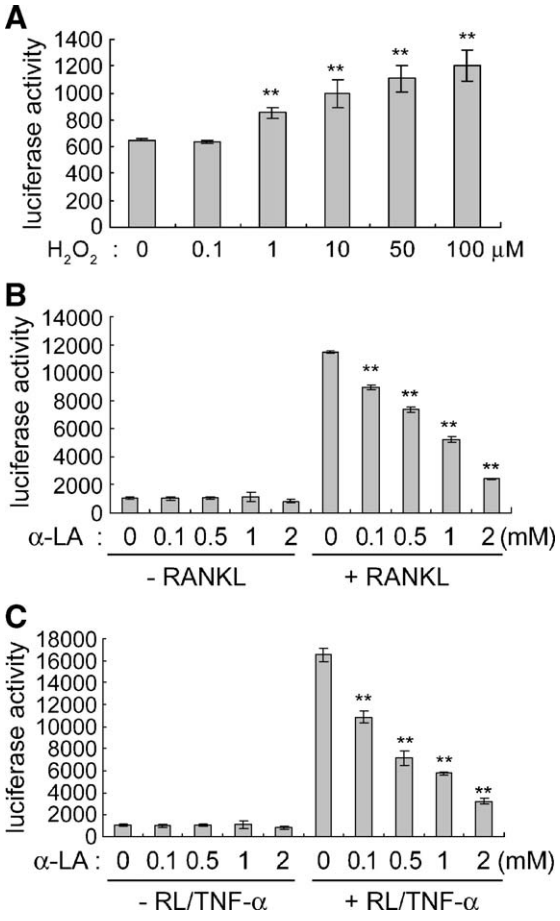

Fig. 5. α-LA inhibits NF-κB activation by osteoclastogenic stimuli. (A) RAW264.7 cells were transfected with a NF-κB reporter luciferase plasmid. Fourteen hours after transfection, cells were treated with indicated concentrations of H_2O_2 for 5 h. Cells were lysed and the luciferase activity and protein concentration were determined. Data represent luciferase activity per μg cell lysate. ** $P < 0.01$ compared with vehicle-treated control. (B) Cells were transfected as in (A) and treated with or without 100 ng/ml RANKL in the presence or absence of α-LA for 8 h. The luciferase activity per μg cell lysate is presented. ** $P < 0.01$ compared with the group treated with RANKL only. (C) Cells were transfected as in (A) and treated with or without with 10 ng/ml RANKL plus 20 ng/ml TNF-α in the presence or absence of α-LA for 8 h. The luciferase activity per μg cell lysate is shown. RL, RANKL. ** $P < 0.01$ compared with the control group treated with RANKL/TNF-α only.

dependent suppression of the RANKL induction of luciferase activity (Fig. 5B). When the cells were stimulated with RANKL (10 ng/ml) plus TNF-α (20 ng/ml) for 8 h, the NF-κB reporter activity increased by 15.3 ± 0.57-fold (Fig. 5C). A dose-dependent inhibitory effect of α-LA was also observed under this osteoclastogenic condition (Fig. 5C).

α-LA suppresses DNA binding of NF-κB but not IKK activation induced by osteoclastogenic cytokines

Inhibition of NF-κB activity can be the result of interference with various steps in the NF-κB activation pathway. The transactivating potential of transcription factors can be modulated by interacting coactivators or corepressors without affecting DNA-binding potential of the transcription factor. In addition, the translocation of NF-κB from the cytoplasm to the nucleus and the DNA binding of the translocated transcription factor are steps necessary to elicit NF-κB-dependent gene transcription. To gain further insight on the mechanism by which α-LA suppresses the NF-κB activation by RANKL or RANKL/TNF-α in osteoclast precursor cells, we evaluated the DNA-binding activity of NF-κB by electrophoretic gel mobility-shift assays. The nuclear extract from BMM cells stimulated with RANKL (100 ng/ml) caused gel mobility shift of NF-κB-binding DNA oligomers (Fig. 6A, lane 3). Pretreatment of the cells with α-LA attenuated the increase in DNA binding of NF-κB stimulated by RANKL (Fig. 6A, lane 4). Stimulation with RANKL (10 ng/ml) plus TNF-α (20 ng/ml) also induced NF-κB binding to DNA (Fig. 6B, lane 3), which was significantly suppressed by α-LA (Fig. 6B, lane 4). Inclusion of NF-κB p65 and p50 antibodies or excess unlabeled probe blocked the gel mobility shift, indicating that the shifted bands contained NF-κB complexes (Fig. 6, lanes 5 and 6). Therefore, the inhibitory effect of α-LA was exerted at the level of DNA binding or a NF-κB activation event prior to DNA binding.

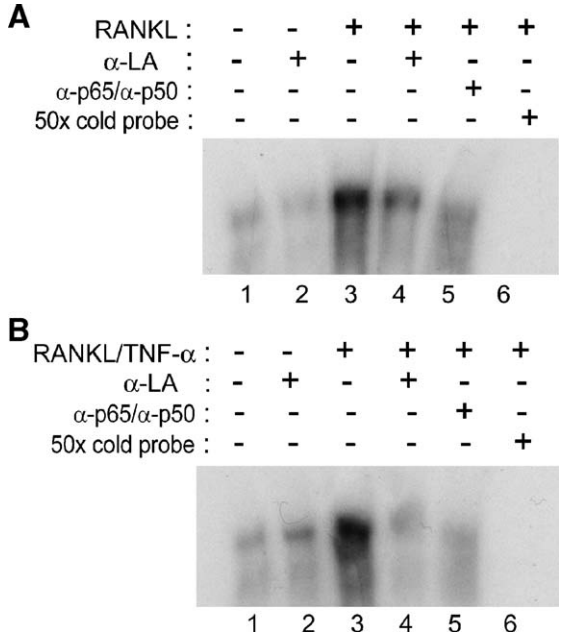

Fig. 6. α-LA mitigates NF-κB activation at the level of DNA binding. BMMs were pretreated with 0.1 mM α-LA or the control vehicle and stimulated with 100 ng/ml RANKL (A) or 10 ng/ml RANKL plus 20 ng/ml TNF-α (B) for 20 min. Nuclear extracts were prepared and the electrophoretic gel mobility-shift assay was conducted with NF-κB-binding DNA probe. To verify the band identity as NF-κB-DNA complex, anti-p65 plus anti-p50 antibodies were included (lane 5) and competition with 50-fold unlabeled probe was performed (lane 6).

We next examined cytosolic events involved in NF-κB activation. IKK is a key enzyme complex that phosphorylates the inhibitory κB (IκB), causing subsequent degradation of IκB and release of NF-κB from the inhibitory subunit. Released NF-κB can translocate to the nucleus and bind DNA. We analyzed the IKK activity changes in osteoclast precursor cells stimulated with RANKL or RANKL/TNF-α in the presence or absence of α-LA. RANKL activated IKK within 10 min while RANKL/TNF-α caused IKK activation within 5 min (data not shown). α-LA did not block the IKK activation by either RANKL or RANKL/TNF-α (Fig. 7A). α-LA also did not inhibit the phosphorylation and degradation of IκB in response to RANKL (Fig. 7B) or RANKL/TNF-α (Fig. 7C). The effect of α-LA on the RANKL-induced nuclear translocation of NF-κB proteins p65 and p50 appeared to be little at 15 min and marginal at 30 min (Fig. 7D). The nuclear translocation of NF-κB induced by RANKL/TNF-α was not affected by α-LA (Fig. 7E). These data indicate that RANKL- and RANKL/TNF-α-induced activation of NF-κB is affected by α-LA mainly at the level of NF-κB binding to its target gene DNA.

α-LA prevents in vivo calvarial bone resorption provoked by RANKL or TNF-α

We next investigated whether the osteoclast inhibitory effect of α-LA is exerted in vivo. Local bone remodeling was induced in mouse calvaria by administering RANKL alone or together with α-LA. Histological sections stained with hematoxylin and eosin showed that RANKL treatment induced bone resorption, resulting in expansion of bone marrow space (Fig. 8A). Simultaneous administration of α-LA significantly reduced the RANKL-induced bone resorption (Fig. 8A). The percentages of bone marrow space to whole bone tissue of calvariae from control, RANKL-treated, and RANKL plus α-LA-treated mice were 3.54 ± 1.41, 18.66 ± 2.45, and 6.05 ± 2.25%, respectively (Fig. 8B). α-LA on calvarial bone resorption was also examined by computerized tomography after TNF-α administration. TNF-α caused a substantial bone resorption and α-LA significantly prevented it (Figs. 8C and D). The calvarial bone volume decreased by 37.61 ± 3.46% in TNF-α-treated mice compared to control mice. In the presence of α-LA, the TNF-α-induced bone volume reduction was only 9.95 ± 0.80% (Fig. 8E).

Discussion

Abnormal increase in osteoclast differentiation is the major contributing factor in pathologic bone erosion associated with osteoporosis and inflammatory diseases. Based on well-

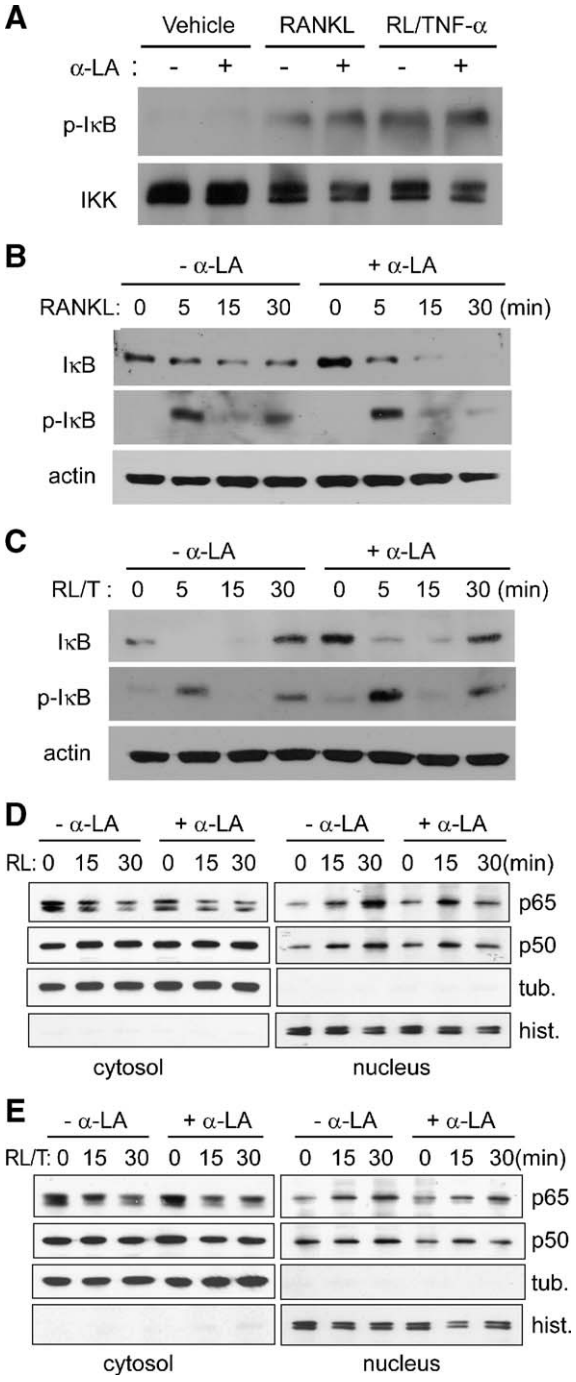

Fig. 7. α-LA does not inhibit IKK activation by osteoclastogenic stimuli. (A) BMMs were pretreated with α-LA (0.1 mM) or the vehicle and stimulated for 10 min with 100 ng/ml RANKL or 10 ng/ml RANKL plus 20 ng/ml TNF-α. IKK was immunoprecipitated from cell lysates and an in vitro kinase assay was conducted with GST-IκB as the substrate. The reaction products were subjected to Western blotting with anti-phospho IκB antibody. (B and C) BMMs were pretreated with α-LA (0.1 mM) or the vehicle and stimulated with 100 ng/ml RANKL (B) or 10 ng/ml RANKL plus 20 ng/ml TNF-α (C) for the indicated times. Cell lysates were prepared and analyzed by Western blotting with anti-phospho IκB, anti-IκB, or anti-actin antibody. (D and E) BMMs were pretreated with α-LA (0.1 mM) or the vehicle, and stimulated with 100 ng/ml RANKL (D) or 10 ng/ml RANKL plus 20 ng/ml TNF-α (E) for the indicated times. Cytosol and nuclear proteins were prepared and analyzed by Western blotting with anti-p65 and anti-p50 antibodies. The membranes were reprobed with anti-tubulin and anti-histone antibodies to confirm fraction identity. RL, RANKL; RL/T, RANKL plus TNF-α. tub., tubulin; hist., histone.

documented evidence for ROS production in inflammatory conditions and recent reports implicating ROS in osteoporosis [25,27], we reasoned that osteoclast differentiation might require ROS as a signaling intermediate and that in vivo bone resorption could be controlled by antioxidants. Recently, we found that a clinical antioxidant drug α-LA has the potential to block RANKL-induced osteoclast differentiation in vitro [39]. In this study, we confirmed the previous result (Fig. 2) and extended the work to analyses of the effect of α-LA on RANKL/TNF-α-induced osteoclastogenesis (Figs. 2 and 3) and to elucidation of the mechanism involved in the antiosteoclastogenic function of α-LA (Figs. 4–7). Furthermore, we observed in vivo efficacy of α-LA by using a mouse calvarial model of bone resorption evoked by RANKL or TNF-α (Fig. 8).

In osteoclast precursor cells, RANKL treatment led to ROS production in a manner dependent on NADPH oxidase (Fig. 4). Implication of ROS in RANKL signaling was documented for the activation of differentiated mature osteoclasts [19]. Similarly, ROS may mediate intracellular signal transduction for the differentiation response of osteoclast precursors to RANKL. In fact, α-LA ablated ROS induction by RANKL- or RANKL/TNF-α and attenuated NF-κB activation in osteoclast precursors (Fig. 5). This demonstrates that the participation of ROS to NF-κB activation described for other cell types and stimulation conditions [14,38] is also evoked in osteoclast precursor cells in response to osteoclastogenic stimuli. Previous reports showing the requirement of NF-κB for osteoclast differentiation [37] support the importance of the NF-κB-activating role of ROS for osteoclastogenesis.

The inhibitory effect of α-LA on NF-κB activation by RANKL or RANKL/TNF-α in osteoclast precursors appears to be mainly at the level of DNA binding of the transcription factor (Fig. 6). Interestingly, IKK suppression could not be detected in RANKL/TNF-α- as well as RANKL-treated cells (Fig. 7A). In this context, Suzuki et al. demonstrated the inhibition of DNA binding of NF-κB by α-LA through a direct redox regulation of the transcription factor [40]. However, in human aortic endothelial cells, α-LA was shown to suppress TNF-induced NF-κB activation by reducing IKK activity [41]. This reflects that more than one mode of action mechanism is operated for α-LA-mediated inhibition of NF-κB depending on cell types. As another example of differential responses, we could not detect in osteoclast precursors the inhibition of phosphorylation and activation of AMPK by α-LA (data not shown), which has been described in other cell types [31]. Therefore, the target employed by α-LA in modulation of intracellular signaling appears to be different depending on cell types and triggering signals.

As α-LA inhibits the activity of NF-κB transcription factor, its control of certain gene expression is likely. In support of this, α-LA was reported to inhibit the expression of $\alpha_4\beta_1$ integrin in Jurkat T cells [42] and E-selectin, VCAM-1, ICAM-1, and MCP-1 in endothelial cells [41]. These changes would reduce cell-matrix and cell-cell adhesion. In addition, α-LA inhibited T cell migration and MMP activity [42,43]. Suppressive effects of α-LA on adhesion molecule expression, cell migration, and

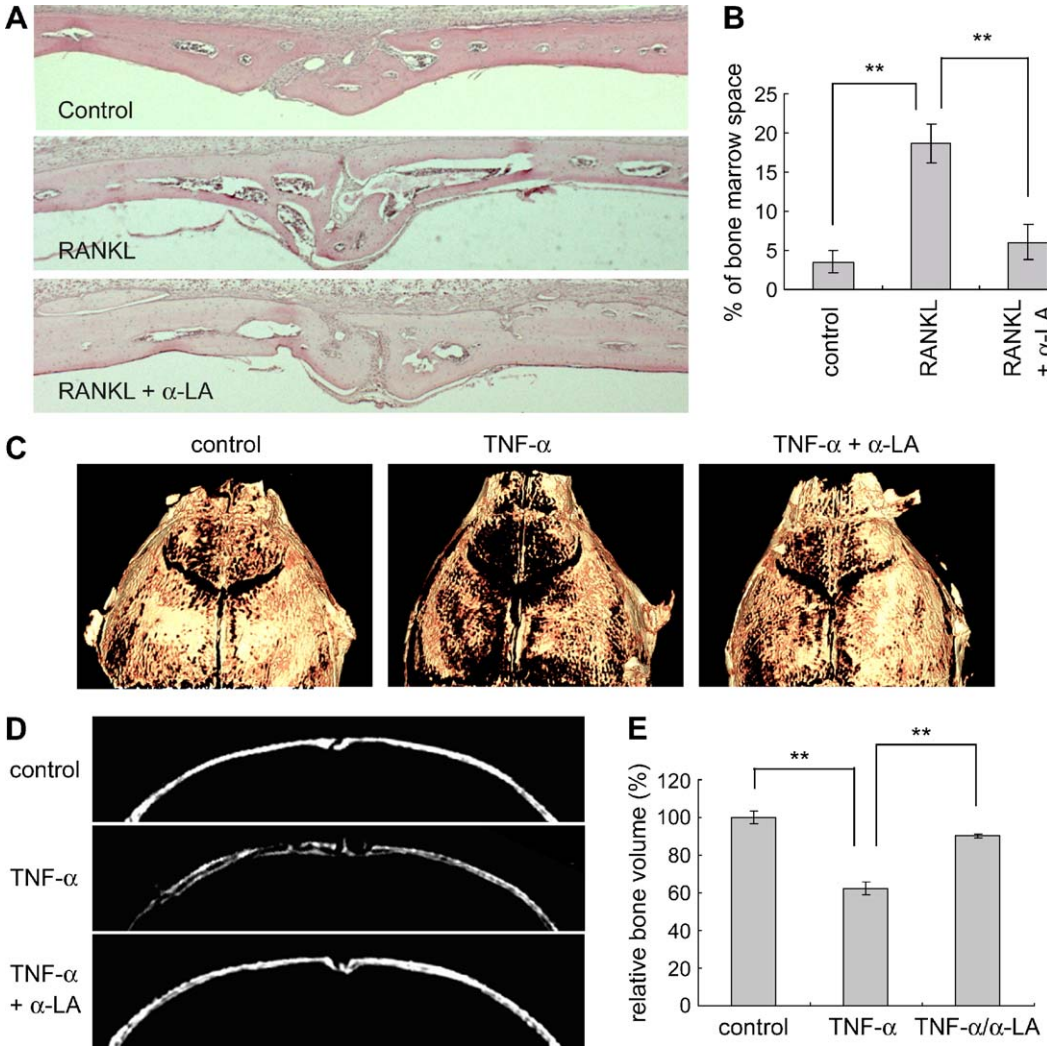

Fig. 8. α-LA inhibits bone resorption in vivo. (A) Mouse calvaria was implanted with a collagen sheet soaked with PBS, RANKL (2 μg), or RANKL plus α-LA (2 mg). Ten days after implantation, calvariae were decalcified and embedded in paraffin. Coronal sections were stained with hematoxylin and eosin. (B) Bone marrow area and bone tissue area were determined from the H&E-stained sections. Percentages of bone marrow space per bone tissue are presented. ** $P < 0.01$ between indicated groups. (C–E) Mice received supracalvarial injection of PBS, TNF-α (2 μg), or TNF-α plus α-LA (2 mg). Five days after injection, calvariae were subjected to μCT analyses. 3D configurations of whole calvaria (C), coronal slices at the midline of sagittal suture (D), and percentages of relative calvarial bone volume (E) are presented. ** $P < 0.01$ between indicated groups.

MMP activity will contribute to its antiosteoclastogenic property because osteoclast differentiation requires migration of precursor and committed cells and homophilic cell-cell interaction for multinucleation. In this context, whether α-LA regulates adhesion molecule expression and MMP activity in osteoclast precursor cells is an intriguing issue.

Using the calvarial bone resorption assay model, we could obtain compelling evidence that α-LA is effective against bone erosion in vivo. Bone resorption induced by RANKL and TNF-α was greatly reduced by α-LA (Fig. 8). Although less efficient, a significant antiresorptive effect of α-LA was also observed when calvariae were treated with IL-1α. In μCT analyses, IL-1α decreased bone volume by 38.3 ± 2.08% in the absence of α-LA and by 23.79 ± 4.98% in the presence of α-LA. Together with the in vitro data showing a suppressive effect on osteoclast differentiation, these in vivo results strongly indicate the therapeutic potential of α-LA for bone erosive diseases.

A study reporting the preventive effect of antioxidants NAC and ascorbate on ovariectomy-induced bone loss in mice [27] supports the potential of therapeutic approaches to reduce oxidative stress in treating osteoporosis. We found that α-LA blocked osteoclast differentiation from BMMs by more than 90% at 0.1 mM concentration (Fig. 2) whereas NAC and reduced glutathione inhibited osteoclastogenesis only by 30% at 20 mM concentration (data not shown). This much more potent effect of α-LA on inhibition of osteoclastogenesis suggests that this antioxidant would be more efficient than the others in preventing bone resorption. Another advantage of α-LA as an antiresorptive drug is that the safety of this compound appears to be warranted as it has been used in humans for more than 30 years in Germany for treatment of diabetes mellitus and polyneuropathy [41]. In addition, our previous work indicated that α-LA has protective effects on TNF-α-induced apoptosis of human bone marrow stromal cells and osteoblasts, which may

be beneficial to bone formation [44]. Therefore, we propose that α-LA is an attractive therapeutic drug candidate for osteoporosis and other bone resorption-associated pathologic conditions.

Acknowledgments

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