

Conjugated linoleic acid inhibits osteoclast differentiation of RAW264.7 cells by modulating RANKL signaling

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Abstract Bone destruction is a pathological hallmark of several chronic inflammatory diseases, including rheumatoid arthritis, periodontitis, and osteoporosis. Inflammation-induced bone loss of this sort results from increased numbers of bone-resorbing osteoclasts. Numerous studies have indicated that conjugated linoleic acid (CLA) positively influences calcium and bone metabolism. Gene-deletion studies have shown that receptor activator of nuclear factor- κ B ligand (RANKL) is one of the critical mediators of osteoclastogenesis. In this report, we examine the ability of CLA to suppress RANKL signaling and osteoclastogenesis in RAW264.7 cells, a murine monocytic cell line. Treatment of these cells with RANKL activated nuclear factor- κ B (NF- κ B), and preexposure of the cells to CLA significantly suppressed RANKL-induced NF- κ B activation, including phosphorylation of I- κ B α , degradation of I- κ B α , and nuclear translocation of p65. RANKL induced osteoclastogenesis in these monocytic cells, and CLA inhibited RANKL-induced tumor necrosis factor- α production and osteoclast differentiation, including osteoclast-specific genes such as tartrate-resistant acid phosphatase, cathepsin K, calcitonin receptor, and matrix metalloproteinase-9 expression and osteoclast-specific transcription factors such as c-Fos, nuclear factor of activated T-cells expression, and bone resorption pit formation. CLA also inhibited RANKL-induced activation of mitogen-activated protein kinase p38 but had little effect on c-Jun N-terminal kinase activation. Collectively, these data demonstrate for the first time that CLA inhibits osteoclastogenesis by modulating RANKL signaling. Thus, CLA may have important therapeutic implications for the treatment of bone diseases associated with enhanced bone resorption by excessive osteoclastogenesis.—Rahman, M. M., A. Bhattacharya, and G. Fernandes. **Conjugated linoleic acid inhibits osteoclast differentiation of RAW264.7 cells by modulating RANKL signaling.** *J. Lipid Res.* 2006. 47: 1739–1748.

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Bone remodeling depends on a delicate balance between bone formation and bone resorption, wherein bone-forming osteoblasts and bone-resorbing osteoclasts play central roles

(1). Tipping this balance in favor of osteoclasts leads to pathologic bone resorption, as seen in bone diseases such as autoimmune arthritis and postmenopausal osteoporosis (2).

Osteoclasts are bone-resorptive multinucleated cells (MNCs) derived from hematopoietic stem cells (3, 4). Differentiation of osteoclasts is regulated by soluble or membrane-bound molecules expressed by osteoblasts and stromal cells in the bone microenvironment (5). One such factor, receptor activator of nuclear factor- κ B ligand (RANKL), a member of the tumor necrosis factor (TNF) family (6–9), plays an essential role in osteoclastogenesis. The binding of RANKL to its receptor RANK leads to the recruitment of TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK, thereby resulting in the activation of distinct signaling cascades mediated by mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK) and p38 MAPK (p38) (10). It has been shown that JNK1-activated c-Jun signaling in cooperation with nuclear factor of activated T cells (NFAT) is key to RANKL-regulated osteoclast differentiation (11). Stimulation of p38 results in the downstream activation of the microphthalmia/microphthalmia transcription factor, which controls the expression of the genes encoding tartrate-resistant acid phosphatase (TRAP) and cathepsin K, indicating the importance of p38 signaling cascades (10). RANKL also induces c-Fos by an as yet unknown mechanism. Indeed, the essential role of the TRAF6 and c-Fos pathways in osteoclastogenesis as determined by gene targeting studies is well documented (12, 13). In addition, several negative regulators of RANKL signaling have been reported (14–16). A typical example is osteoprotegerin, which functions as a soluble decoy receptor for RANKL, thereby attenuating excessive RANKL signaling. There-

Abbreviations: CLA, conjugated linoleic acid; CTR, calcitonin receptor; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase; LA, linoleic acid; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MNC, multinucleated cell; NFATc1, nuclear factor of activated T-cells; NF- κ B, nuclear factor- κ B; RANKL, receptor activator of nuclear factor- κ B ligand; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase.

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fore, agents that can suppress RANKL signaling can suppress osteoclastogenesis.

Considerable attention over the last several years has focused on the possible beneficial effects of dietary conjugated linoleic acid (CLA), including anticarcinogenic and antitumorogenic effects (17), reduction in the risk of atherosclerosis, hypertension, and diabetes, improvement in feed efficiency, promotion of energy metabolism, and positive effect on immune function (18, 19). CLA is a collective term used to describe a set of 28 distinct positional and geometric isomers of linoleic acid (LA; 18:2) (18–20) and is most commonly found at positions *cis*-9,*trans*-11 (c9t11) and *trans*-10,*cis*-12 (t10c12), both of which possess biological activity (21). CLA isomers occur naturally in ruminant food products (beef, lamb, and dairy) (22). Our recent study showed that CLA significantly reduces body weight and body fat mass in mice, particularly with treadmill exercise (23).

Very recently, it was reported that dietary CLA may positively benefit bone mineral density in postmenopausal women (24). Increased whole body ash in young mice fed a diet with CLA supplementation has been reported (25). CLA increased the bone formation markers osteocalcin and alkaline phosphatase in a murine osteoblastic cell line (26). Anhydrous butterfat, a rich natural source of CLA, also stimulated the rate of bone formation in young growing chicks by modulating prostaglandin E₂, which plays an important role in the local regulation of bone formation and bone resorption (27). To date, studies have been carried out only to define whether CLA affects calcium and bone metabolism using animal models. However, it is not clear whether CLA protects bone loss as a result of the upregulation of osteoblastic factors and bone formation and/or as a result of the downregulation of osteoclastic factors and bone resorption. In light of the prostaglandin E₂-decreasing ability of CLA, it is possible that CLA may have inhibitory effects on osteoclastogenesis. However, the effect of CLA on osteoclastogenesis has not yet been investigated. Hence, this study was designed to examine the effect of CLA on osteoclastogenesis using the mouse myeloid RAW264.7 cell line that differentiates *in vitro* into osteoclasts upon treatment with specific growth factors.

MATERIALS AND METHODS

Materials

All media components were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA). Fatty acids (CLA c9t11, CLA t10c12, LA) were from Matreya, Inc. (State College, PA). Equal amounts of c9t11 and t10c12 isomers were used for all *in vitro* cultures. Murine sRANKL was from Pepro Tech, Inc. (Rocky Hill, NJ), reagents for mouse TNF- α ELISA were from BD Bioscience, and the RNA extraction reagent TRIZOL was from Invitrogen Life Technologies (Carlsbad, CA). The reverse transcription kit was from Promega Corp. (Madison, WI). The bicinchoninic acid kit for protein determination was from Pierce Chemical Co. (Rockford, IL). TRAP solution was No. 387 from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibodies against phosphorylated p38 (tyrosine 182), phosphorylated JNK (threonine

183 and tyrosine 185), and phosphorylated I- κ B α ; rabbit polyclonal antibodies against nuclear factor of activated T-cells (NFATc1), I- κ B α , and nuclear factor- κ B (NF- κ B) p65; and goat polyclonal antibody against actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Chicken polyclonal antibody against c-Fos was purchased from Abcam, Inc. (Cambridge, MA). [³²P]-ATP was from Perkin-Elmer-NEN (Boston, MA). Oligonucleotides for NF- κ B were from Santa Cruz Biotechnology. T4 polynucleotide kinase and Sephadex G-25 M columns were from Promega. Poly [d(I-C)] was from Roche Diagnostics (Indianapolis, IN). All other chemicals were the highest grade available from Sigma Chemical Co.

Cell cultures

RAW264.7 cells were maintained in DMEM (Sigma) with 10% FCS. All media were supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Sigma). Incubations were performed at 37°C in 5% CO₂ in humidified air. For osteoclast generation and all other experiments, α -MEM medium (Sigma) was used. Fatty acid concentrations used in *in vitro* experiments were optimized by fatty acid incorporation analysis using gas chromatography as described below and by cell viability analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown).

Fatty acid incorporation

RAW264.7 cells were suspended in phenol α -MEM containing 10% FBS and plated at a concentration of 1×10^6 cells/well into a six-well culture dish (Corning) in the presence of different concentrations of LA or CLA for 24 and 48 h. At the end of culture, the plates were washed with PBS. The cells were then collected using a scraper and analyzed for fatty acid incorporation using gas chromatography as described previously (28, 29).

Total TRAP activity

RAW264.7 cells were suspended in phenol α -MEM containing 10% FBS and plated at a concentration of 2×10^4 cells/well into a 48-well culture dish (Corning) in the presence of 50 ng/ml RANKL and incubated for 24 h. Then, different concentrations of LA or CLA were added to the cultures. The medium and factors were replaced after 3 days. After 5 days of culture, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were then lysed with 200 μ l of 0.2% Triton X-100. TRAP activity in cell lysate was determined using TRAP solution. An aliquot of cell lysate was added to 150 μ l of TRAP solution and was incubated at 37°C for 30 min. The reaction was stopped by adding 0.1 N NaOH, and absorbance was measured at 405 nm using a microplate reader.

TRAP-positive cell staining

RAW264.7 cells were suspended in phenol α -MEM containing 10% FBS and plated at a concentration of 2×10^4 cells/well into a 48-well culture dish in the presence of 50 ng/ml RANKL and incubated for 24 h. Then, different concentrations of LA or CLA were added to the cultures. The medium and factors were replaced after 3 days. After 5 days of culture, the cells were fixed and stained for TRAP using the TRAP staining kit according to the manufacturer's instructions. TRAP⁺ cells with more than three nuclei were counted as TRAP⁺ve MNCs.

Pit formation assay

RAW264.7 cells were suspended in phenol α -MEM containing 10% FBS and plated at a concentration of 1×10^4 cells/well on an Osteoclast Activity Assay Substrate plate (OCT USA, Inc.) in

TABLE 1. Fatty acid incorporation in RAW264.7 cells treated with LA (100 μ M) or c9t11 (50 μ M) + t10c12 (50 μ M) CLA for 24 h

Fatty Acids	Untreated	LA	c9t11 + t10c12	P
14:0	2.62 \pm 0.32	1.54 \pm 0.09	2.13 \pm 0.08	
16:0	21.53 \pm 0.18	19.11 \pm 1.34	18.17 \pm 0.40	
16:1 (n-9)	6.46 \pm 0.10a	3.76 \pm 0.24b	2.71 \pm 0.25b	<0.05
17:0	0.41 \pm 0.01	0.47 \pm 0.05	0.51 \pm 0.04	
17:1 (n-9)	0.80 \pm 0.05	0.79 \pm 0.21	0.55 \pm 0.03	
18:0	8.43 \pm 0.32a	14.27 \pm 0.04b	15.84 \pm 0.55b	<0.05
18:1 (n-9)	18.49 \pm 0.45a	10.95 \pm 0.24b	10.47 \pm 0.14b	<0.001
18:1 (n-7)	15.27 \pm 0.00a	10.02 \pm 0.35b	8.22 \pm 0.15c	<0.05
18:2 (n-6) (LA)	2.34 \pm 0.05a	6.90 \pm 0.64b	2.94 \pm 0.08a	<0.001
c9t11 CLA	ND	ND	4.29 \pm 0.20	
c9c11 CLA	ND	ND	3.26 \pm 0.02	
t9t11 CLA	ND	ND	2.27 \pm 0.11	
t10c12 CLA	ND	ND	2.13 \pm 0.02	
20:0	ND	0.28 \pm 0.03	0.31 \pm 0.05	
20:1 (n-9)	1.54 \pm 0.46ab	0.88 \pm 0.15a	1.65 \pm 0.16b	<0.05
20:2 (n-6)	1.76 \pm 0.05a	0.66 \pm 0.05b	0.54 \pm 0.12b	<0.01
20:3 (n-6)	ND	0.68 \pm 0.06	0.48 \pm 0.08	
20:4 (n-6) (arachidonic acid)	6.58 \pm 0.03a	12.44 \pm 1.19b	7.22 \pm 0.23a	<0.001
22:4 (n-6)	0.82 \pm 0.01a	3.04 \pm 0.13b	1.41 \pm 0.01c	<0.01
22:5 (n-6)	ND	2.30 \pm 0.10	ND	
22:5 (n-3)	1.31 \pm 0.01a	1.58 \pm 0.14ab	1.92 \pm 0.01b	<0.01
22:6 (n-3)	3.67 \pm 0.16	2.92 \pm 0.34	3.10 \pm 0.12	

CLA, conjugated linoleic acid; LA, linoleic acid. Values shown are means \pm SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different. ND, not detected.

the presence or absence of 50 ng/ml RANKL and incubated for 24 h. Then, LA (50 μ M) or CLA (50 μ M) was added to the cultures. Half of the medium was replaced with a fresh one every 2 days. After 7 days of culture, the plates were washed in 6% sodium hypochlorite solution to remove the cells. The resorbed areas on the plates were captured with a digital camera attached to the microscope and analyzed by the Metaview Image Analysis System.

RT-PCR analysis of RANKL-stimulated RAW264.7 cells

RAW264.7 cells (1.0×10^4 cells/ml) were cultured in 60 mm tissue culture dishes (Corning) in the presence of RANKL (50 ng/ml). After 24 h of incubation, 100 μ M LA or CLA was added to the culture. Medium was changed every other day. After 4 days of culture, total RNA was extracted using TRIZOL according to the manufacturer's instructions and RT-PCR analysis was performed as described previously (30). The following primers were used: TRAP, 5'-AAATCACTCTTTAAGACCA-3' and 5'-TTATTGAATAGCAGTGACAG-3'; cathepsin K, 5'-GGGCCAGGATGAAAGTTGTA-3' and 5'-CCGAGCCAAGAGAGCATATC-3'; calcitonin receptor (CTR), 5'-CTGCTCCTAGTGAGCCCAAC-3' and 5'-CAGCAATCGACAAGGAGTGA-3'; matrix metalloproteinase (MMP)-9, 5'-TCTGAGGCCTCTACAGAGTCT-3' and 5'-CTCATGGTCCACCTTGTTTAC-3'. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. As an internal control for RNA quantity, the same cDNA was amplified using primers specific for GAPDH mRNA.

TNF- α production in RAW264.7 cell culture

RAW264.7 cells were suspended in phenol α -MEM containing 10% FBS and plated at a concentration of 1×10^5 cells/well into a 24-well culture dish (Corning) for 24 h. Then, they were treated with either CLA (100 μ M) or LA (100 μ M) for another 24 h before adding RANKL (50 ng/ml) to stimulate TNF- α secretion for 16 h. At the end of culture, medium was collected and analyzed for TNF- α using the ELISA kit according to the manufacturer's instructions.

Protein preparation and Western blot analysis

RAW264.7 cells were cultured on 60 mm plates for 48 h with or without 100 μ M LA or CLA. Cells were then treated with sRANKL (50 ng/ml) for 30 min. Cytosolic and nuclear proteins were prepared as described previously (30). In a separate culture, RAW264.7 cells were cultured for 48 h with or without 100 μ M

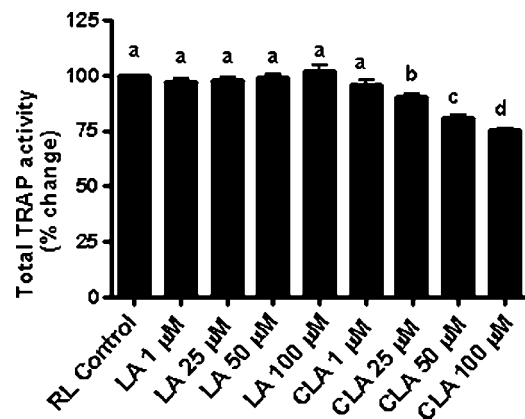


Fig. 1. Conjugated linoleic acid (CLA) dose-dependently inhibited receptor activator of nuclear factor- κ B ligand (RANKL)-stimulated tartrate-resistant acid phosphatase (TRAP) activity in RAW264.7 cells. RAW264.7 cells were cultured with RANKL (RL; 50 ng/ml) for 24 h. Then, different concentrations of CLA or linoleic acid (LA) were added and cultured for another 4 days. The cells were then lysed with 0.2% Triton X-100. TRAP activity in cell lysate was determined using TRAP solution and measured as the optical density at 405 nm. Values shown are means \pm SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: a versus b ($P < 0.05$), a versus c ($P < 0.001$), a versus d ($P < 0.001$), b versus c ($P < 0.001$), b versus d ($P < 0.001$), c versus d ($P < 0.05$).

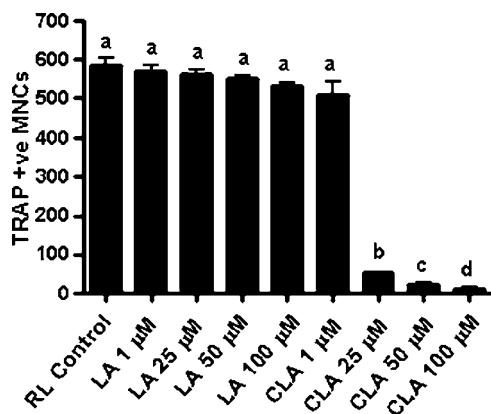


Fig. 2. CLA dose-dependently inhibited RANKL (RL)-stimulated TRAP⁺ multinucleated cells (MNCs) in RAW264.7 cells. RAW264.7 cells were cultured with RANKL (50 ng/ml) for 24 h. Then, different concentrations of CLA or LA were added and cultured for another 4 days. Cells were then fixed and stained for TRAP. The number of TRAP⁺ MNCs was counted in each well. Values shown are means \pm SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: a versus b ($P < 0.001$), a versus c ($P < 0.001$), a versus d ($P < 0.001$), b versus c ($P < 0.05$), b versus d ($P < 0.05$), c versus d ($P < 0.05$).

LA or CLA. Cells were then treated with sRANKL (50 ng/ml) for 45 min. Whole cell lysates were then prepared using TNE buffer (10 mM Tris-HCl, pH 7.8, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT, and protease inhibitor cocktail). In a separate culture, RAW264.7 cells were grown on 60 mm plates in the presence of sRANKL. After 24 h of incubation, 100 μ M LA or CLA was added to the culture. Medium was changed every other day. After 4 days of culture, cells were washed twice with PBS and dissolved directly on the plate in TNE buffer. After centrifugation for 10 min, whole cell lysates were collected. Protein concentrations of the nuclear extracts, cytosolic extracts, and whole cell extracts were determined using a bicinchoninic acid protein assay kit. Fifteen micrograms of nuclear extracts and cytosolic extracts and 30 μ g of whole cell extracts were subjected to SDS-PAGE. Proteins were transferred to immunoblot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and subjected to Western blot analysis.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using 5 μ g of nuclear extracts as described previously using a DNA probe (5'-AGTTGAGGGGACTTCCAGGC-3') containing the NF- κ B binding site (*italics*) (29).

Statistics

Results are expressed as means \pm SEM. Data were statistically analyzed among all groups using one-way ANOVA, and $P < 0.05$ for F ratio was considered statistically significant. The Newman-Keuls multiple comparison test was used to test the differences between groups for significance.

RESULTS

CLA incorporation in RAW264.7 cells

To determine whether *in vitro* added fatty acids incorporate into cells, we examined the fatty acid profile in LA/CLA-treated RAW264.7 cells using GC. LA/CLA incorporation was measured in RAW264.7 cells treated with different concentrations for different time points. Fatty acid incorporation data with 100 μ M treatment for 24 h are shown in **Table 1** as an example. LA treatment increased the level of LA and arachidonic acid, whereas CLA-treated RAW264.7 cells showed incorporation of CLA fatty acid isomers (Table 1).

Effects of CLA on RANKL-stimulated, TRAP-positive osteoclast-like cell formation in RAW264.7 cells

As RAW264.7 cells differentiate into osteoclasts in the presence of RANKL, we examined the effect of CLA on the osteoclast differentiation of RAW264.7 cells in the presence of RANKL using TRAP staining. Both mononuclear osteoclast precursor cells and multinucleated osteoclasts are positive for TRAP. At first, we examined whether CLA has any effect on total TRAP activity (**Fig. 1**). CLA dose-dependently inhibited total TRAP activity in RANKL-stimulated RAW264.7 cells (**Fig. 1**). Next, we examined whether CLA has any effect on multinucleated osteoclast-

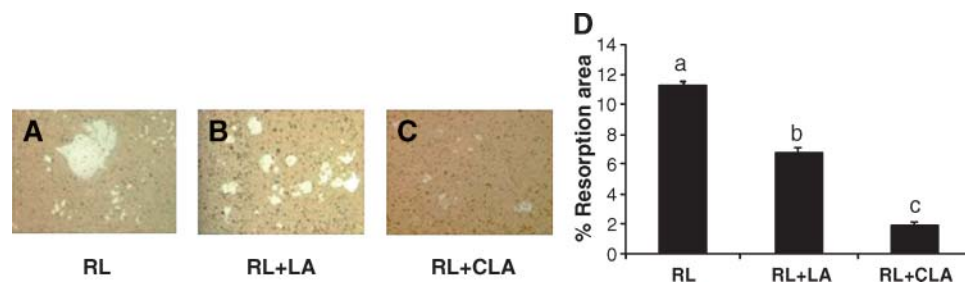


Fig. 3. CLA inhibited RANKL (RL)-stimulated bone resorption of RAW264.7 cells. Demonstration of pit formation on an Osteoclast Activity Assay Substrate plate using RAW264.7 cells stimulated with 50 ng/ml RANKL (A) in the presence of 50 μ M LA (B) or 50 μ M CLA (C). Fatty acids were added after 24 h of incubation of RAW264.7 cells with RANKL. After 7 days of culture, plates were analyzed for resorption pit formation. In D, percentage resorption area per well was measured by image analyzer and plotted. Values shown are means \pm SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: a versus b ($P < 0.001$), a versus c ($P < 0.001$), b versus c ($P < 0.001$).

like cell formation in RANKL-stimulated RAW264.7 cells (Fig. 2). Multinucleated osteoclasts are the active bone-resorbing cells. CLA dramatically inhibited the RANKL-stimulated osteoclast-like cell formation in RAW264.7 cells in a dose-dependent manner (Fig. 2). However, LA did not show any significant difference in total TRAP activity and osteoclast-like cell formation with different doses (Figs. 1, 2).

Effects of CLA on bone resorption in RANKL-stimulated RAW264.7 cells

As we have already seen that CLA dramatically inhibits the formation of osteoclast-like MNCs, which are believed to be responsible for bone resorption, we further examined whether CLA has any effect on the ability of these mature osteoclasts to resorb bone. We used calcium phosphate-coated culture plates to stimulate RAW264.7 cells with RANKL to differentiate mature osteoclasts having

bone-resorbing capacity. RANKL-stimulated RAW264.7 cells showed a number of resorption areas (Fig. 3A). Cultures treated with CLA showed significantly reduced numbers and areas of resorption pits compared with cultures treated with either RANKL alone or together with LA (Fig. 3).

Effects of CLA on osteoclast-specific gene expression

We previously showed that differentiated RAW264.7 cells express high levels of osteoclast-specific genes such as TRAP, cathepsin K, CTR, and MMP-9 (30). To determine whether the inhibitory effect of CLA correlates with the expression of the osteoclast-specific genes, total RNA was prepared and analyzed by semiquantitative RT-PCR. RANKL-stimulated TRAP, cathepsin K, CTR and MMP-9 mRNA levels in RAW264.7 cells were significantly lower in CLA-treated cultures compared with LA-treated or untreated controls (Fig. 4).

Effects of CLA on RANKL-induced TNF- α production of RAW264.7 cells

TNF- α has the potential to induce osteoclast differentiation (31–33), and TNF- α together with soluble RANKL strongly promotes osteoclast differentiation and activation in vitro (33, 34). Recently, TNF- α has become noteworthy as a target for the treatment of inflammatory bone diseases, and it is expected that understanding of molecules mediating TNF- α signals will provide clues to the development of new therapies for such diseases. Therefore, we examined whether CLA can modulate RANKL-stimulated TNF- α production in RAW264.7 cell cultures. Cultures treated with CLA showed significantly decreased production of TNF- α compared with cultures treated either with RANKL alone or together with LA (Fig. 5).

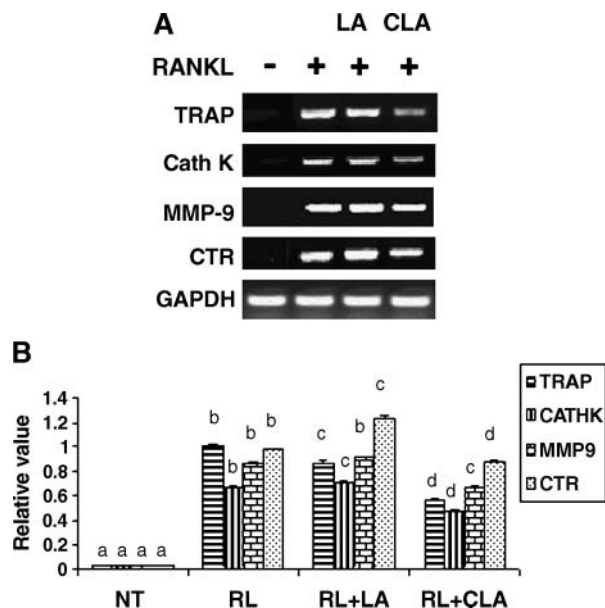


Fig. 4. CLA downregulated osteoclast-specific gene expression in RANKL (RL)-stimulated RAW264.7 cells. A: RAW264.7 cells were cultured with RANKL in the presence or absence of CLA (100 μ M) or LA (100 μ M), and total RNA was prepared, reverse transcribed, amplified by PCR using specific primers designed for genes of TRAP, calcitonin receptor (CTR), cathepsin K (Cath K), matrix metalloproteinase (MMP)-9, and GAPDH, and visualized on agarose gels with ethidium bromide. B: Relative expression of TRAP, CTR, cathepsin K, and MMP-9 is shown. The intensity of the bands was determined by densitometry and normalized by the level of GAPDH. Values shown are means \pm SEM of three independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: TRAP [a versus b ($P < 0.001$), a versus c ($P < 0.001$), a versus d ($P < 0.001$), b versus c ($P < 0.05$), b versus d ($P < 0.001$), c versus d ($P < 0.01$)]; cathepsin K [a versus b ($P < 0.001$), a versus c ($P < 0.001$), a versus d ($P < 0.001$), b versus c ($P < 0.05$), b versus d ($P < 0.01$), c versus d ($P < 0.01$)]; MMP-9 [a versus b ($P < 0.001$), a versus c ($P < 0.001$), b versus c ($P < 0.01$)]; CTR [a versus b ($P < 0.001$), a versus c ($P < 0.001$), a versus d ($P < 0.001$), b versus c ($P < 0.01$), b versus d ($P < 0.01$), c versus d ($P < 0.001$)].

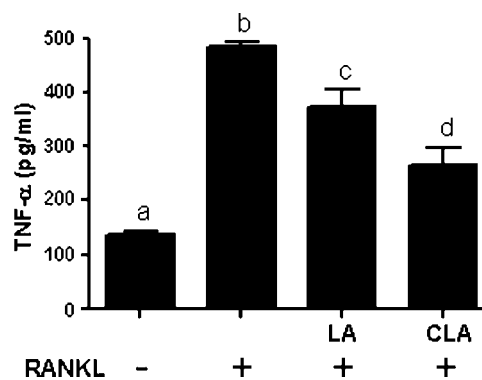


Fig. 5. CLA suppressed RANKL-induced tumor necrosis factor (TNF)- α production of RAW264.7 cells. RAW264.7 cells were cultured for 24 h. Then, they were treated with either CLA (100 μ M) or LA (100 μ M) for another 24 h before adding RANKL (50 ng/ml) to stimulate TNF- α secretion for 16 h. At the end of culture, medium was collected and analyzed for TNF- α using the ELISA kit. Values shown are means \pm SEM of three independent experiments with triplicate cultures. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: a versus b ($P < 0.001$), a versus c ($P < 0.001$), a versus d ($P < 0.01$), b versus c ($P < 0.05$), b versus d ($P < 0.001$), c versus d ($P < 0.05$).

Effects of CLA on RANKL-stimulated activation of NF- κ B and MAPKs

The interaction of RANKL with RANK leads to the activation of the signaling pathways of MAPK (p38 and JNK) and NF- κ B. In an effort to elucidate the intracellular mechanisms underlying the inhibitory effect of CLA, we first focused on the NF- κ B signaling pathway. NF- κ B activation is regulated by three major steps: phosphorylation of I- κ B α , I- κ B α degradation, and nuclear translocation of p50/p65 subunits. I- κ B α phosphorylation, I- κ B α degradation, and p65 NF- κ B nuclear translocation were analyzed using cytosolic extracts and nuclear extracts from RANKL-stimulated RAW264.7 cells. Treatment of RAW264.7 cells with RANKL significantly increased the phosphorylation of I- κ B α , degradation of I- κ B α , and nuclear translocation of p65 NF- κ B (Fig. 6). Pretreatment of RAW264.7 cells with CLA for 48 h significantly reduced I- κ B α phosphorylation, I- κ B α degradation, and p65 NF- κ B nuclear translocation compared with pretreatment with LA or untreated RANKL-stimulated controls (Fig. 6A, B). RANKL-stimulated NF- κ B DNA binding by EMSA was also reduced significantly in CLA-pretreated RAW264.7 cells compared with

LA-treated or untreated controls (Fig. 6C). The JNK and p38 MAPK pathways also play an important role in RANKL-induced osteoclast differentiation. Therefore, we next focused on the effect of CLA on the MAPK family members p38 MAPK and JNK. Western blot analysis using anti-phospho-specific p38 with phosphorylated tyrosine and JNK antibodies with phosphorylated tyrosine and threonine was performed to determine the influence of CLA or LA on the activation of JNK and p38 MAPKs. As shown in Fig. 7, 45 min treatment of RAW cells with RANKL increased the phosphorylation of p38 and JNK. Pretreatment of RAW cells with CLA for 48 h significantly decreased the phosphorylation of p38 and JNK compared with cells pretreated with LA (Fig. 7). However, there was no significant difference between RANKL-stimulated untreated control and CLA-treated cells in the activation of JNK.

Effects of CLA on the expression of osteoclast-specific transcription factor

c-Fos is considered one of the most important osteoclast-specific transcription factors (35, 36). Very recently, another transcription factor, NFATc1, was determined as an

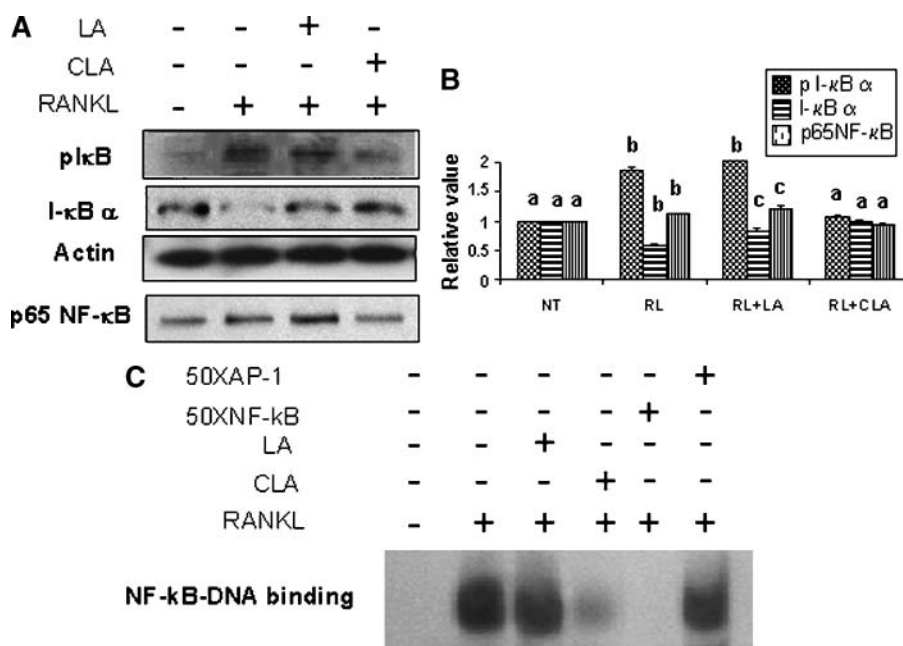


Fig. 6. CLA inhibited RANKL (RL)-induced nuclear factor- κ B (NF- κ B) activation. RAW264.7 cells were treated with or without CLA (100 μ M) or LA (100 μ M) for 48 h, followed by treatment with RANKL (50 ng/ml) for 30 min. Cytosolic and nuclear extracts were prepared and measured using the bicinchoninic acid kit. Equal amounts of proteins [15 μ g of cytosolic extracts and nuclear extracts for Western blot and 5 μ g of nuclear extracts for electrophoretic mobility shift assay (EMSA)] were analyzed. A: Western blot analysis using antibodies to phosphorylated (p) I- κ B α , I- κ B α , and NF- κ B p65. Cytosolic extracts were used for pI- κ B α and I- κ B α analysis, and nuclear extracts were used for NF- κ B p65 analysis. B: Relative expression of pI- κ B α , I- κ B α , and p65 NF- κ B. The intensity of the bands was determined by densitometry, and values of pI- κ B α and I- κ B α were further normalized by the level of actin. Values shown are means \pm SEM of three independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: pI- κ B α [a versus b ($P < 0.001$)]; I- κ B α [a versus b ($P < 0.001$), a versus c ($P < 0.001$), b versus c ($P < 0.01$)]; p65 [a versus b ($P < 0.05$), a versus c ($P < 0.01$), b versus c ($P < 0.05$)]. C: NF- κ B DNA binding activity was determined by EMSA using 5 μ g of nuclear extract. To assess the specificity of binding, a 50 \times excess of cold NF- κ B or AP-1 (irrelevant probe) was added to the reaction. Data are representative of two independent experiments.

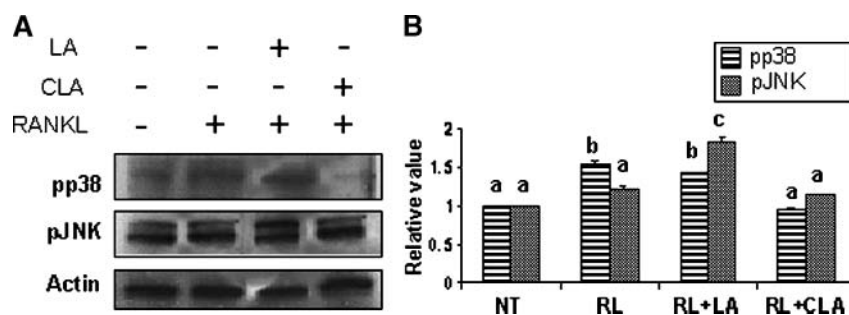


Fig. 7. CLA inhibited RANKL (RL)-induced mitogen-activated protein kinase (MAPK) activation. RAW264.7 cells were cultured for 48 h with or without 100 μ M LA or CLA followed by treatment with RANKL (50 ng/ml) for 45 min. A: Whole cell lysates were prepared, and equal amounts of proteins (30 μ g) were subjected to Western blot using antibodies to phosphorylated p38 MAPK and c-Jun N-terminal kinase (JNK). B: Relative expression of pp38 and pJNK. The intensity of the bands was determined by densitometry and normalized with actin. Values shown are means \pm SEM of three independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: pp38 [a versus b ($P < 0.001$)]; pJNK [a versus c ($P < 0.01$)].

important transcription factor involved in osteoclastogenesis. The NFATc1 gene has been identified as the most strongly induced transcription factor gene after RANKL-stimulated osteoclast differentiation in RAW264.7 cells and is considered to be the master regulator of osteoclastogenesis (16). We examined whether CLA modulates these transcription factors by Western blot analysis using RANKL-stimulated RAW264.7 cells. Pretreatment of RAW264.7 cells with CLA significantly decreased the RANKL-stimulated upregulation of these transcription factors compared with LA-treated or untreated controls (Fig. 8).

DISCUSSION

CLA is a strong inhibitor of osteoclastogenesis

In the past two decades, CLA had been associated with many potential health benefits (18). Recently, CLA was reported to enhance immunity and bone formation (24). Current evidence suggests that CLA may help to decrease bone loss by reducing prostaglandins in bone tissue (27, 37) or by enhancing calcium absorption (38). Very recently, it was reported that CLA may have a beneficial role in preventing bone loss. Our recent findings also showed higher bone mineral density in young Balb/C male mice fed CLA compared with LA-enriched safflower oil (39). But it was not clear how CLA protected bone loss. The effects of CLA on osteoblastic factors have been primarily explored in animal studies and also in vitro (26, 38). However, no study had been conducted to measure the effect of CLA on osteoclastogenesis. This is the first study to demonstrate the inhibitory effect of CLA on osteoclastogenesis.

In our in vitro study using RAW264.7 cells, we found that CLA dose-dependently inhibited RANKL-induced osteoclast differentiation. Although the inhibition of TRAP-positive MNC formation was most dramatic with CLA treatment, the reduction of total TRAP activity was not as dramatic but remained significant. This might be at-

tributable to the presence of a number of TRAP-positive mononuclear cells in CLA-treated cultures. TRAP, MMP9, cathepsin K, and CTR are well-known markers of osteoclast differentiation (40). Significant but not dramatic re-

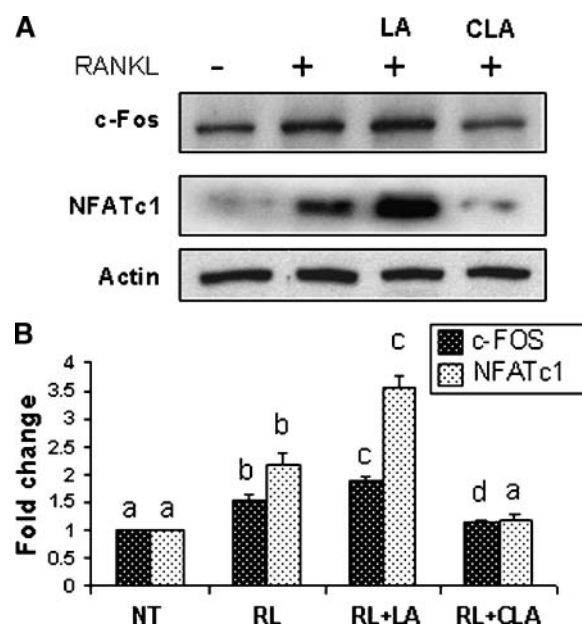


Fig. 8. CLA downregulated the expression of osteoclast-specific transcription factors in RANKL (RL)-stimulated RAW264.7 cells. RAW264.7 cells were cultured with RANKL (50 ng/ml) for 24 h. Then, CLA (100 μ M) or LA (100 μ M) was added and cultured for another 3 days. A: Whole cell lysates were prepared, and equal amount of proteins (30 μ g) were analyzed by Western blotting using antibodies to c-Fos and nuclear factor of activated T-cells (NFATc1). B: Relative expression of c-Fos and NFATc1. The intensity of the bands was determined by densitometry and normalized with actin. Values shown are means \pm SEM of three independent experiments. Statistical differences were evaluated among all groups with one-way ANOVA. Values with different letters are significantly different: cFos [a versus b ($P < 0.001$), a versus c ($P < 0.05$), a versus d ($P < 0.05$), b versus c ($P < 0.05$)]; NFATc1 [a versus b ($P < 0.01$), a versus c ($P < 0.001$), b versus c ($P < 0.01$)].

duction of these gene markers was also noted in CLA-treated RAW264.7 cells stimulated with RANKL, as TRAP-positive mononuclear cells are also positive for these genes. Thus, CLA not only significantly inhibits the differentiation of osteoclast precursors into TRAP-positive mononuclear cells but also prevents its fusion to form multinucleated active osteoclasts. Furthermore, TRAP-positive MNCs but not mononuclear cells are the active bone-resorbing cells. Indeed, a dramatic inhibition of bone resorption by CLA indicates that it strongly inhibits the formation of active TRAP-positive MNCs from its precursor cells to resorb bone. However, we found differences in the effect of CLA on TRAP-positive MNC formation and bone resorption. These variations in the effects of CLA might be attributable to the presence of both functional (with ruffled border) and nonfunctional (without ruffled border) multinucleated osteoclasts in the MNC count, whereas bone resorption represents primarily the effect of only functional multinucleated osteoclasts. These findings suggest that CLA not only inhibits osteoclast differentiation but also inhibits its activation as well.

NF- κ B/MAPKs may be involved in the CLA-mediated inhibition of osteoclastogenesis

The differentiation and activation of osteoclasts depend on the signal through RANK stimulated with RANKL. Genetic studies using gene-targeting technology have clearly demonstrated that NF- κ B and MAPKs, including JNK and

p38, which are elicited by RANKL, are essential molecules for osteoclast differentiation (11, 16, 41). NF- κ B knockout mice and transgenic mice expressing dominant-negative c-Jun or dominant-negative JNK1 exhibited osteopetrosis (11, 42). In addition, it has been reported that the JNK inhibitor SP600125 and the p38 MAPK inhibitor SB203580 strongly inhibit osteoclastogenesis (11, 16). Chung et al. (43) reported that CLA inhibits LPS-induced inflammatory events in RAW264.7 cells by negatively regulating inflammatory mediators and NF- κ B activation. In contrast, NF- κ B activation by the t10c12 CLA isomer was noted in human adipocytes (43). These variations might be attributable to isomer and/or tissue specificity. Interestingly, in our study, CLA inhibited RANKL-induced NF- κ B activation in RAW264.7 cells. NF- κ B is considered to be involved not only in the process of differentiation but also in the maturation of osteoclasts (44). Thus, the inhibitory action of CLA on osteoclastogenesis seems to involve the inhibitory effect of this agent on NF- κ B activation. In this study, we also found that the phosphorylation of p38 MAPK was decreased after treatment with CLA, suggesting that the inhibition of the p38 MAPK pathway may also be involved in the inhibitory action of this agent. We also tried to determine the effect of CLA on the activation of the JNK pathway, but we could not find strong inhibition of RANKL-stimulated JNK activation by CLA. However, JNK activation was significantly lower in CLA-treated cells compared with LA-treated controls. These findings clearly demonstrate that

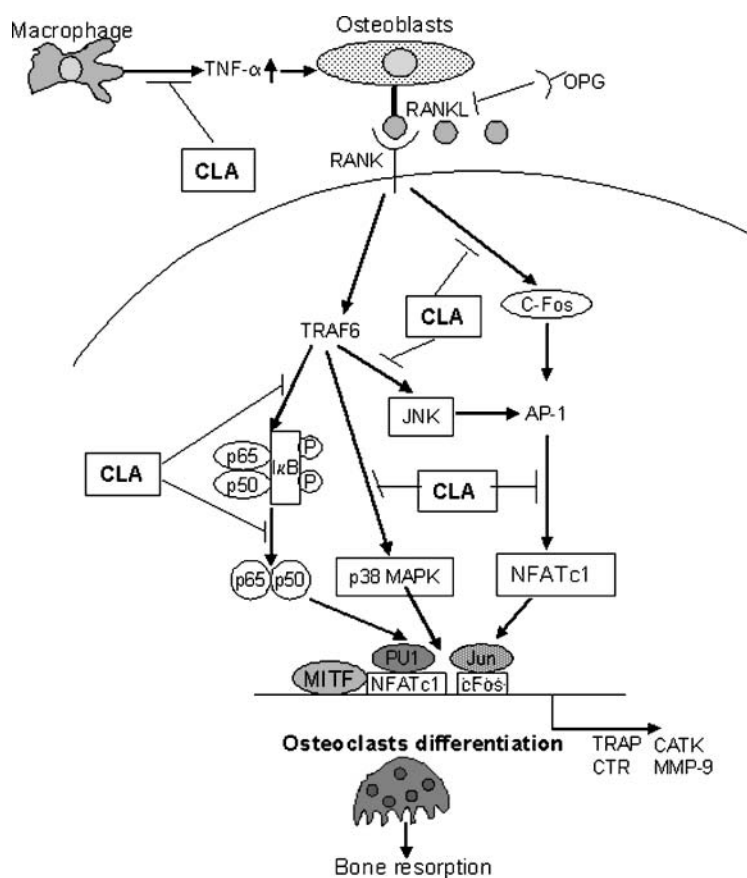



Fig. 9. Possible mechanisms of CLA action on osteoclast differentiation. Several possible inhibitory actions of CLA on osteoclast differentiation are shown. By inhibiting TNF- α production, CLA may suppress the production of RANKL. CLA inhibited the downstream molecules of RANKL signaling such as NF- κ B, JNK, and p38. CLA also suppressed the production of important downstream regulators of osteoclast formation, including c-Fos and NFATc1. Thus, CLA inhibits osteoclast differentiation by modulating various cytokines, signaling molecules, and transcription factors. CATK, cathepsin K; MITF, microphthalmia associated transcription factor; OPG, osteoprotegerin.

CLA-mediated inhibition of osteoclastogenesis induced by RANKL reflects the blocking of specific components of these intracellular signals.

c-Fos/NFATc1 may be involved in the CLA-mediated inhibition of osteoclastogenesis

The binding of RANKL to RANK also results in a cascade of intracellular events that, in turn, increases the transcription of the c-Jun and c-Fos genes, and osteopetrosis is a feature of c-Fos-deficient mice (35, 36, 45). NFATc1 is also a key transcription factor for osteoclast formation. Stimulation of NFATc1 in bone marrow macrophage or RAW264.7 cells by RANKL demonstrates the necessity of NFATc1 in osteoclastogenesis in vitro (11). Most notably, the introduction of NFATc1 promotes the differentiation of bone marrow macrophage cells into TRAP⁺ osteoclast-like cells even in the absence of sRANKL (11, 16). The osteoclastogenic activity of NFATc1 was enhanced by overexpression of c-Jun and c-Fos but was inhibited by overexpression of either dominant-negative c-Jun or c-Fos, indicating that the partnership between c-Jun/c-Fos and the NFAT family is crucial for osteoclast differentiation (11). Interestingly, CLA inhibited the induction of both c-Fos and NFATc1 by RANKL in RAW264.7 cells, thereby attenuating the process of osteoclastogenesis. **Figure 9** summarizes the effect of CLA on osteoclastogenesis. CLA seems to inhibit osteoclastogenesis by modulating various cytokines, signaling molecules, and transcription factors.

In summary, in this study, we present the novel action of CLA on osteoclastogenesis. Osteoclasts have a crucial role in physiologic bone remodeling and also in the pathologic bone loss that is closely associated with inflammatory bone diseases such as rheumatoid arthritis, periodontal disease, and postmenopausal osteoporosis (30). Our findings open up an additional avenue to search out a possible alternative to drugs to prevent the bone loss associated with excessive and progressive osteoclastic bone resorption. We believe that our findings provide clear evidence that CLA may become a novel dietary therapeutic agent after successful clinical trials not only against obesity but also against osteoporotic bone diseases. 

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