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Original Article

Low-intensity pulsed ultrasound inhibits RANKL-induced osteoclast formation via modulating ERK-c-Fos-NFATc1 signaling cascades

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Abstract: Low-intensity pulsed ultrasound (LIPUS), which is a noninvasive form of mechanical energy, has been utilized as a clinical therapy for bone fracture healing. However, the mechanism how LIPUS affects osteoclast formation and osteoclast activity, has not been fully detailed. Here we found that LIPUS inhibited RANKL-induced osteoclast differentiation in vitro, characterized by decreased number and area of tartrate-resistant acid phosphatase (TRAP) positive cells. Moreover, the expression levels of osteoclast-specific gene were also suppressed by LIPUS treatment. Interestingly, F-actin staining and resorption pit assay showed that LIPUS did not affect the bone resorptive activity of mature osteoclasts. Mechanistically, LIPUS achieved these inhibitory effects by disrupting the phosphorylation of ERK and subsequent activation of the osteoclastic transcription factors, c-Fos and NFATc1. Collectively, our results demonstrated that LIPUS effectively suppresses osteoclast differentiation and osteoclast-specific gene expression through the inhibition of ERK-c-Fos-NFATC1 cascades.

Keywords: LIPUS, osteoclast, ERK, c-Fos, NFATc1

Introduction

Bone remodeling is a crucial metabolic process regulating bone structure and function. This process is continuously active in skeletal tissue through all stages of life and dyn amically balanced by osteoclastic bone resorption and osteoblastic bone formation [1, 2]. Excess osteoclast activity leads to an imbalance between bone resorption and bone formation during bone remodeling, which underlies the pathogenesis of many skeletal diseases, including osteoporosis, rheumatoid arthritis, periodontal disease and metastatic cancers [3]. Therefore, modulating aberrant osteoclast activity might be the promising therapy for such diseases.

Osteoclasts, which are multinucleated giant cells originating from hematopoietic monocyte/macrophage precursors, are the only cells capable of bone resorption in human body. It is now known that receptor activator for NF-κB-ligand (RANKL), a cytokine released by activated osteoblasts, is indispensable for osteoclast differentiation [4]. Binding of RANKL to its receptor RANK promotes recruitment of adaptor protein TRAF6, which subsequently activates downstream signaling pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) [5]. These signaling cascades lead to the upregulation of c-Fos and nuclear factor of activated T cells c1 (NFATc1), which are the key transcription factors for osteoclast differentiation [6, 7]. Then mature osteoclasts are polarized and undergo structural changes to form a sealing zone. Secretion of the lytic enzymes, tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK), into the sealing zone results in the dissolution and degradation of the underlying bone [8].

Low-intensity pulsed ultrasound (LIPUS), which is a noninvasive form of mechanical energy, has been utilized as a clinical therapy for bone fracture healing [9, 10]. Although numerous

animal studies have shown that LIPUS is able to prevent many skeletal diseases, such as osteoporosis, periprosthetic osteolysis and bone nonunion [11-13], the mechanism how it works during bone remodeling is uncertain. Most studies of LIPUS have focused on osteoblastic growth and differentiation [14-16], however, studies regarding the effect of LIPUS on osteoclast activity are scant. Recently, some researchers reported that LIPUS can suppress osteoclast activity by regulating OPG and RANKL expression in osteoblasts [17, 18]. As the molecular communication between osteoblasts and osteoclasts exists extensively in vivo, it is necessary to in investigate the direct effect of LIPUS on osteoclast formation and activity without the presence of osteoblasts in vitro. In this study, we aimed to investigate the effects of LIPUS on RANKL-induced osteoclast formation and bone resorption activity in vitro, and further figure out the underlying mechanisms.

Materials and methods

Media and reagents

Alpha modification of Eagle's medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). Recombinant mouse macrophage colony-stimulating factor (M-CSF) and RANKL were obtained from R&D Systems (Minneapolis, MN, USA). Specific antibodies against p38 (#9212), phospho-p38 (Thr180/Tyr182) (#4511), extracellular signal-regulated kinase (ERK) (#4695), phospho-ERK (Thr202/Tyr204) (#4370), c-Jun N-terminal kinase (JNK) 1/2 (#9252), phospho-JNK (Thr183/Tyr185) (#4668), inhibitor of nuclear factor kappa-B kinase subunit alpha (IκBα) (#4814), phospho-IκBα (Ser32) (#2859), p65 (#8242), phospho-p65 (Ser536) (#3033), c-Fos (#2250), nuclear factor of activated T cells c1 (NFATc1) (#8032), and β-tubulin (#2146) were obtained from Cell Signaling Technology (Danvers, MA, USA). Tartrate-resistant acid phosphatase (TRAP) staining kit, and other reagents were purchased from Sigma-Aldrich unless otherwise noted.

Cell culture

Monocyte/macrophage precursors were obtained from femur and tibia bone marrow of 6-week-old male C57BL/6 mice as described previously [19], and then differentiated into bone marrow macrophages (BMMs) in complete α-MEM (10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin) supplemented with 30 ng/mL M-CSF for 3 days. Mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in complete DMEM (10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin). All cell cultures were maintained in a humidified environment of 5% CO_2 at 37°C.

Ultrasound application

Cells were stimulated using a LIPUS-generating system (Teijin Pharma Ltd., Tokyo, Japan), which was described previously [20]. The average intensity was 30 milliwatts/cm^2, the pulsed frequency was 1.5 MHz, the repetition rate was 1 kHz, and the pulse lasted 200 μs. The culture plate was placed above these transducers with a thin layer of coupling gel. The pattern and intensity of the LIPUS signal used in this study were essentially the same as that used in clinical practice and in animal model experiments.

In vitro osteoclast differentiation

BMMs were seeded in 96-well plates at a density of 8×10^3 cells/well, in triplicate, in complete α-MEM supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL. Culture medium was replaced every 2 days. During osteoclastogenesis, LIPUS was conducted to treat cells for 20 min per day. After 3 or 5 days of culture, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA) for 15 min, and stained for TRAP activity. TRAP-positive multinucleated cells with more than three nuclei were counted under a light microscope (Olympus BX51, Tokyo, Japan). Similarly, the effect of LIPUS on osteoclast differentiation was also tested on the RAW264.7 cell line.

F-actin ring immunofluorescence and resorption pit assay

To investigate the effect of LIPUS on F-actin ring formation, BMMs were treated with 30 ng/mL M-CSF and 50 ng/mL RANKL for 4 days. An equal number of BMM-derived osteoclasts were seeded into Osteo Assay Surface Multiple

Table 1. Primers used for quantitative real-time PCR

Gene	Forward (F) and reverse (R) primer sequence (5'-3')
GAPDH	F: ACCCAGAAGACTGTGGATGG
	R: CACATTGGGGGTAGGAACAC
CTSK	F: CTTCCAATACGTGCAGCAGA
	R: TCTTCAGGGCTTTCTCGTTC
TRAP	F: CTGGAGTGCACGATGCCAGCGACA
	R: TCCGTGCTCGGCGATGGACCAGA
DC-STAMP	F: AAAACCCTTGGGCTGTTCTT
	R: AATCATGGACGACTCCTTGG
c-Fos	F: CCAGTCAAGAGCATCAGCAA
	R: AAGTAGTGCAGCCCGGAGTA
NFATc1	F: CCGTTGCTTCCAGAAAATAACA
	R: TGTGGGATGTGAACTCGGAA
CTR	F: TGGTTGAGGTTGTGCCCA
	R: CTCGTGGGTTTGCCTCATC

Well Plate (Corning, MA, USA) coated with hydroxyapatite, and allowed to adhere overnight. Cells were then treated with LIPUS for another 2 days. Next, cells were fixed with 4% PFA for 15 min, permeabilized for 5 min with 0.5% Triton X-100, and stained with rhodamine-conjugated phalloidin (1:200; Invitrogen Life Technologies, Carlsbad, CA, USA) diluted in 0.2% bovine serum albumin (BSA)-PBS for 1 h. Fluorescent images were captured with a fluorescence microscope (EU5888, Leica, Wetzlar, Germany) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Then, these plates were washed twice with PBS and incubated with 5% NaClO for 10 min to remove cells adhered to the plates. Resorption pit images were captured using a light microscope (Olympus) and analyzed using ImageJ software.

RNA extraction and quantitative PCR assay

Total RNA from cultured cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using reverse transcriptase (TaKaRa Biotechnology, Otsu, Japan). Quantitative real-time PCR was performed using the SYBR Premix Ex Tag Kit (TaKaRa Biotechnology) and the ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). Each reaction was run for 40 cycles at the following conditions: 95°C for 5 s, 60°C for 20 s, and 72°C for 20 s. GAPDH was used as a housekeeping gene. The mouse primer sequences are shown in **Table 1**.

Western blotting

Western blotting was used to determine the main signaling pathways affected by LIPUS. BMMs or RAW 264.7 cells were seeded in 6-well plates at a density of 8×10^5 cells/well. After pretreatment with or without LIPUS for 20 min, cells were stimulated with 50 ng/mL RANKL for 0, 5, 15, 30 min. To examine the effects of LIPUS on c-Fos and NFATc1, BMMs were plated in 6-well plates at a density of 1×10^5 cells/well and cultured with 30 ng/mL M-CSF and 50 ng/mL RANKL in the presence or absence of LIPUS for 0, 1, 3, or 5 days. Total protein was extracted from cultured cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich). Each protein lysate containing 30 μg protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After non-specific blocking for 1 h, membranes were incubated with primary antibodies at 4°C overnight. After three washes with TBS-Tween, we subsequently incubated membranes with the appropriate secondary antibodies at 4°C for 2 h. The signals were detected by exposure in a Bio-Rad XRS chemiluminescence detection system (Hercules, CA, USA).

Statistical analysis

All data are expressed as mean ± SEM. Each experiment was repeated at least three times separately and the results were analyzed with Prism 6.01 (GraphPad Software, La Jolla, CA, USA). A two-tailed, unpaired Student's t-test was used for the comparisons between two groups. One-way ANOVA with post hoc Newman-Keuls test was used to analyze differences in multiple comparisons. Values of $P < 0.05$ were considered statistically significantly different.

Results

LIPUS inhibits RANKL-induced osteoclast formation in vitro

To investigate the effect of LIPUS on osteoclast formation, BMMs were treated with M-CSF and

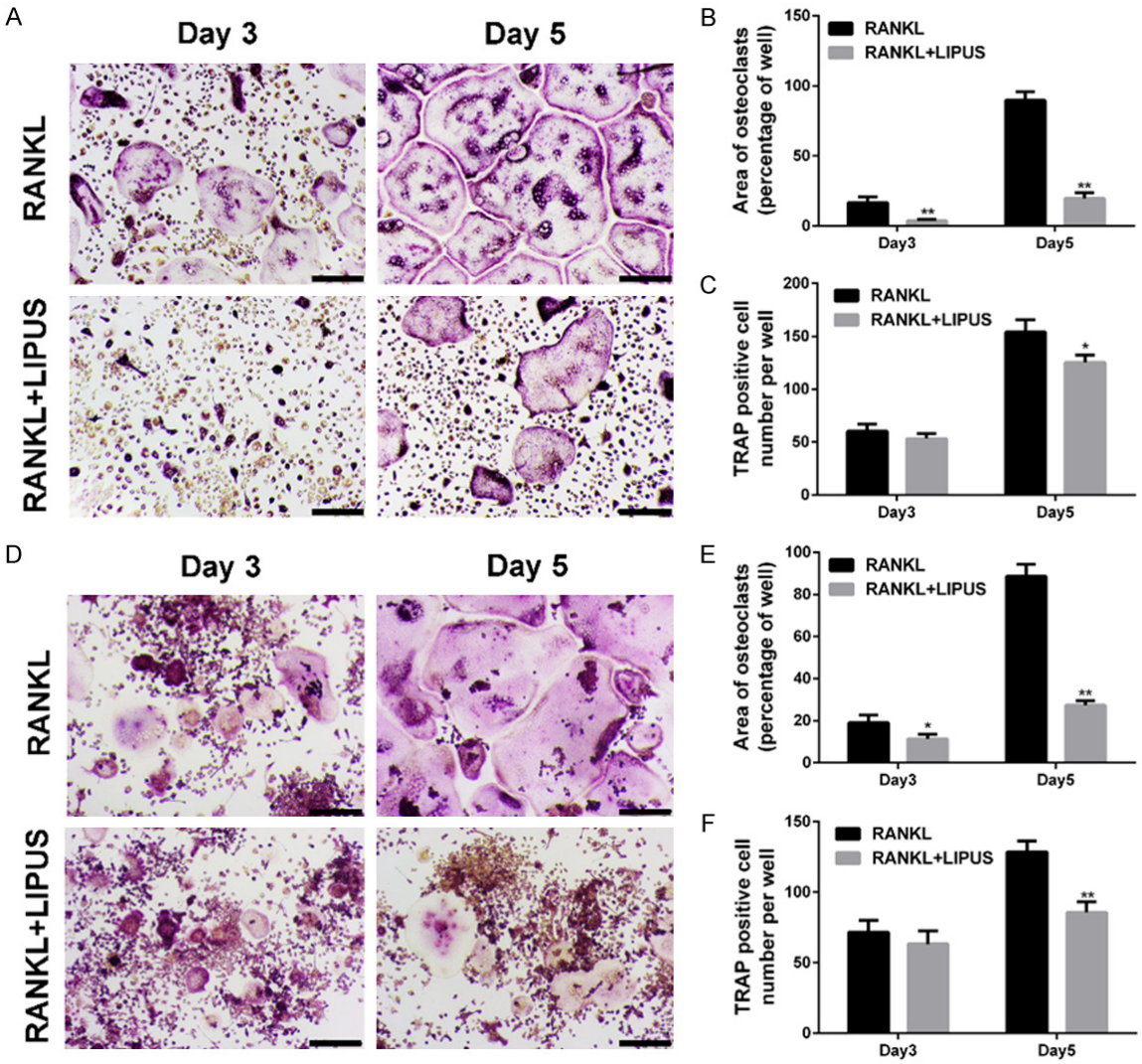

Figure 1. LIPUS suppresses RANKL-induced osteoclast formation in vitro. A. BMMs were stimulated with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence or absence of LIPUS for 3 or 5 days. Cells were then fixed and stained for TRAP activity. B. The area of TRAP positive cells was analyzed. C. The number of TRAP positive cells were analyzed. D. RAW264.7 cells were stimulated with RANKL (50 ng/ml) in the presence or absence of LIPUS for 3 or 5 days. Cells were then fixed and stained for TRAP activity. E. The area of TRAP positive cells was analyzed. F. The number of TRAP positive cells were counted. (Scale bars = 200 μm; *P < 0.05, **P < 0.01).

RANKL, in the presence or absence of LIPUS for 5 days. As shown in **Figure 1A**, TRAP-positive cells appeared after 3 days of RANKL stimulation and differentiated into more numerous and mature osteoclasts in another 2 days. However, osteoclast formation was markedly inhibited by LIPUS treatment. Furthermore, the area and number of osteoclasts were significantly decreased by treatment with LIPUS (**Figure 1B**, **1C**). Meanwhile, the inhibitory effect of LIPUS on osteoclast formation further confirmed in RAW264.7 cell line (**Figure 1D-F**).

LIPUS suppresses RANKL-induced osteoclast-specific gene expression in vitro

With the stimulation of RANKL, osteoclast differentiation is related to the upregulation of several specific genes [21]. Therefore, we used quantitative real-time PCR to examine the inhibitory effect of LIPUS on RANKL-induced mRNA expression of these genes. As expected, RANKL dramatically induced the expression of all evaluated genes, including CTSK, TRAP, calcitonin receptor (CTR), DC-STAMP, c-Fos and

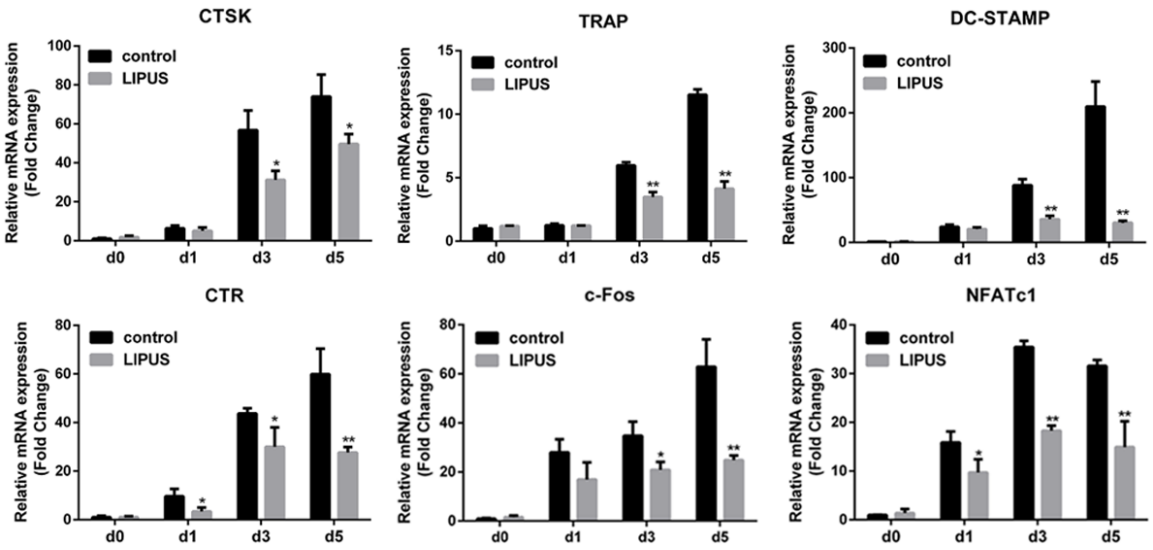

Figure 2. LIPUS inhibits RANKL-induce expression of osteoclast-specific genes. BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) with or without LIPUS treatment for 0, 1, 3, or 5 days. The mRNA expression of osteoclast-specific genes (CTSK, TRAP, DC-STAMP, CTR, c-Fos, NFATc1) was measured using quantitative real-time PCR. Results were normalized to the expression of GAPDH. (*P < 0.05, **P < 0.01).

NFATc1. However, LIPUS significantly inhibited the expression of these osteoclast-related genes in a time-dependent manner (**Figure 2**).

LIPUS does not affect osteoclastic bone resorption activity

Since LIPUS obviously suppressed osteoclast formation, we further examine whether LIPUS could affect osteoclastic bone resorption activity. Mature osteoclasts were plated onto the Osteo Assay Plate in the presence of osteoclastogenic medium and treated with LIPUS for another 2 days. Optical images showed that osteoclasts extensively resorbed the bone surface and no significant difference in resorption activity was observed after LIPUS treatment (**Figure 3A**, **3B**).

A well-polarized F-actin ring is an observable characteristic of mature osteoclasts and an essential prerequisite for osteoclastic bone resorption [22]. Hence, we tested the effect of LIPUS on F-actin ring formation. Under fluorescence microscope, the characteristic F-actin ring structures were observed in the control group and these structures were not impaired by LIPUS treatment (**Figure 3C**). Quantitative analysis also confirmed our observation (**Figure 3D**). Collectively, our findings suggested that LIPUS did not affect osteoclastic bone resorption and F-actin ring formation in vitro.

LIPUS suppresses RANKL-induce activation of ERK signaling pathway in vitro

To elucidate the underlying mechanisms through which LIPUS inhibited osteoclast formation, we investigated the main signaling pathway involved in osteoclast differentiation. Previous studies revealed that the activation of NF-κB and MAPK signaling pathways are crucial for osteoclast formation [6, 23]. As shown in **Figure 4A**, the phosphorylation of ERK was obviously activated in BMMs by RANKL stimulation, while LIPUS pretreatment significantly attenuated the RANKL-induced ERK phosphorylation. Interestingly, LIPUS had no obvious inhibitory effect on the phosphorylation of JNK, p38, p65. Similar results were observed in mouse macrophage cell line, RAW264.7, which further confirmed our findings (**Figure 4B**). Our data suggested that LIPUS impaired RANKL-induced osteoclast differentiation via the suppression of ERK signaling pathway.

LIPUS inhibits RANKL-induced c-Fos and NFATc1 expression in vitro

c-Fos and NFATc1 are considered as the long-term regulators during osteoclastogenesis [23]. Therefore, we investigated the effects of LIPUS on RANKL-induced c-Fos and NFATc1 expression. BMMs were cultured with M-CSF and

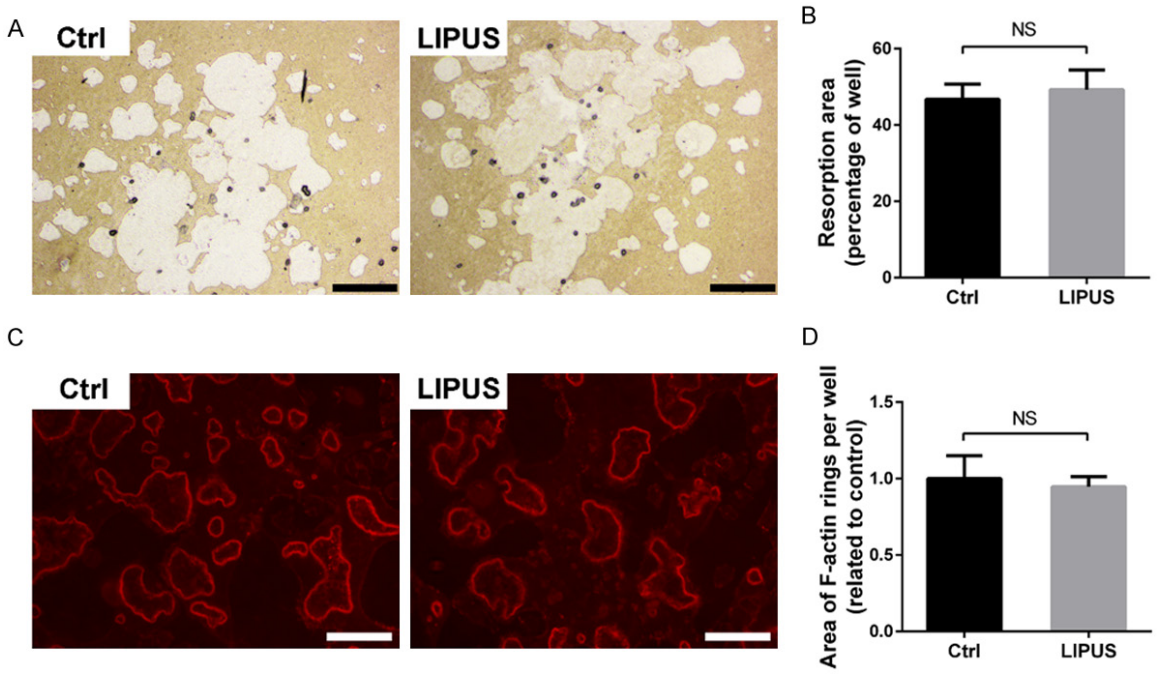

Figure 3. LIPUS does not affect osteoclastic bone resorption activity of BMMs in vitro. An equal number of BMM-derived osteoclasts were seeded into Osteo Assay Surface Multiple Well Plate in the presence or absence of LIPUS for 2 days. A. Representative light microscope images of resorption pits were shown. B. The area of resorption pits was quantified using ImageJ software. C. Representative fluorescence images of F-actin staining with rhodamine-phalloidin. D. The area of F-actin rings was measured using ImageJ software. (Black scale bars = 500 μm; white scale bars = 100 μm; NS, not significant).

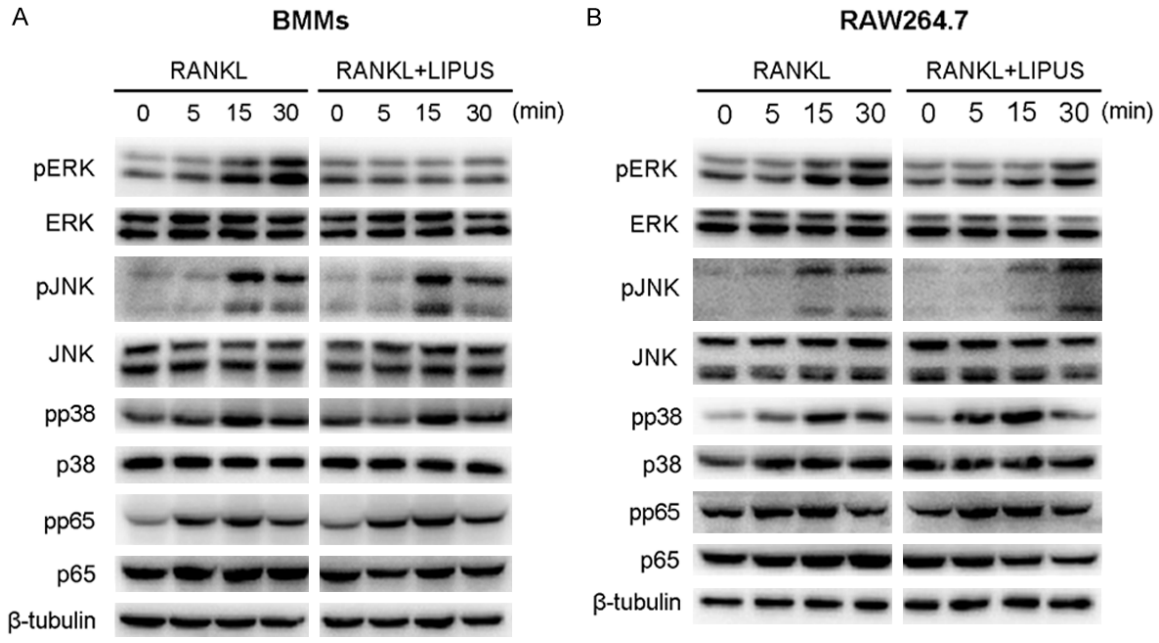

Figure 4. LIPUS inhibits osteoclast formation by specifically impairing RANKL-induced ERK signaling pathway. (A) BMMs or (B) RAW264.7 were pretreated with or without LIPUS for 20 min and then stimulated with RANKL (50 ng/mL) for the indicated periods. Cell lysates were analyzed with the indicated antibodies by Western blotting.

RANKL for 0, 1, 3, and 5 days, in the presence or absence of LIPUS. As expected, the protein levels of c-Fos and NFATc1 increased when the cells were stimulated with RANKL in the

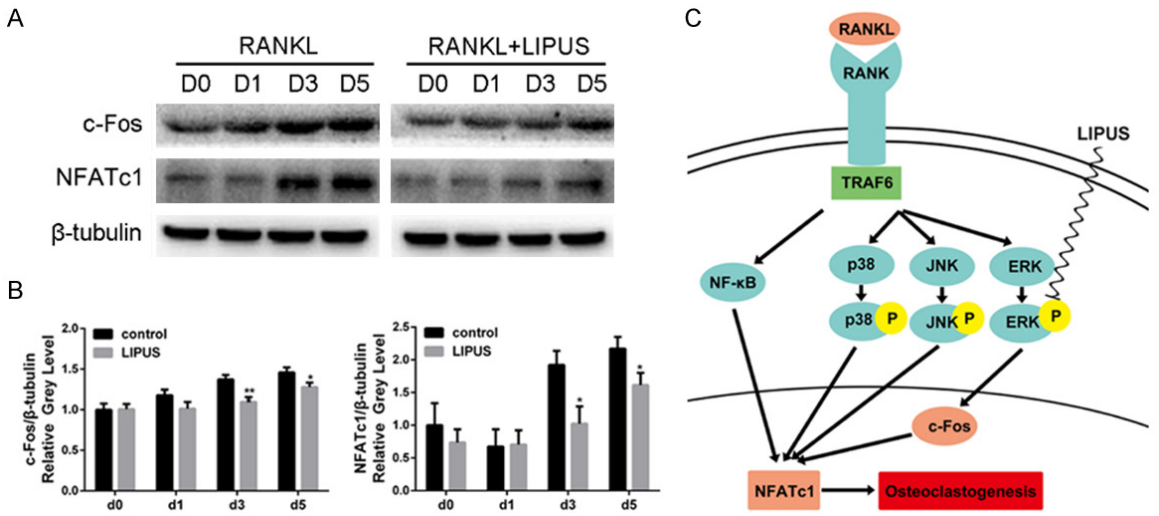

Figure 5. LIPUS suppresses RANKL-induce expression of c-Fos and NFATc1. BMMs were stimulated with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence or absence of LIPUS for 0, 1, 3 or 5 days. A. The protein expression levels of c-Fos and NFATc1 were analyzed using Western blotting. B. The grey levels of c-Fos and NFATc1 were quantified and normalized relative to β-tubulin by Image J. C. Schematic presentation of the inhibitory effects of LIPUS on RANKL signaling. LIPUS inhibited RANKL-induced osteoclast formation through disturbing ERK-c-Fos-NFATc1 signaling cascades. (*P < 0.05, **P < 0.01).

control group. In contrast, RANKL-induced activation and accumulation of NFATc1 and c-Fos were significantly suppressed by the treatment of LIPUS (**Figure 5A**). Quantitative analysis also confirmed our observation (**Figure 5B**). Taken together, these results suggested that LIPUS suppressed osteoclast differentiation and osteoclast-specific gene expression via the inhibition of ERK/c-Fos/NFATc1 cascades (**Figure 5C**).

Discussion

Although LIPUS has been approved to be an effective form of physiotherapy in clinical application, the mechanism how it works during bone remodeling is uncertain. As both osteoblasts and osteoclasts have been reported to be responsible for bone remodeling, it is important to know the effects of LIPUS on them. A variety of researches have shown that LIPUS could enhance bone formation by promoting osteogenesis in vitro and in vivo [14, 24]. However, the effects of LIPUS on osteoclast formation and activity is still controversial [17, 25-27]. In this study, we have demonstrated that LIPUS suppressed RANKL-induced osteoclast formation and osteoclastic related gene expression via inhibiting the ERK-c-Fos-NFATc1 signaling cascades in vitro, further indicating that osteoclasts may be responsible for the effectiveness of LIPUS.

Previous studies have demonstrated that LIPUS is potential to be a noninvasive mechanical treatment for local bone diseases by modulating various cells in vivo to improve bone repairing and bone remodeling [13]. LIPUS, which is regarded as an acoustic form of mechanical energy, exerts its positive effects by transmitting the energy through and into living cells and tissues as a pressure wave with compression [28]. Several sensing receptors have been identified to response to mechanical stress and subsequently activate the following intracellular signaling cascades for the induction of cell proliferation, apoptosis, differentiation and functions [29-31]. Meanwhile, LIPUS has been verified to exert different effects on different cells. A positive effect of LIPUS has been observed on the osteogenic differentiation of osteoblasts and bone marrow stem cells (BMSCs) [14, 32]. Some studies also have exhibited that LIPUS exerts an inhibitory effect on adipogenesis and a facilitated effect on angiogenesis [14, 33]. As previous studies shown, LIPUS prevents osteolysis by suppressing osteoclast formation and activity in vivo [12, 17]. In this study, we observed that LIPUS significantly inhibited RANKL-induced osteoclast formation using primary BMMs, which is consistent with previous in vivo studies. Some recent studies showed that different intensities of LIPUS exerts opposite effects on osteoclasts. The application of 100 mW/cm^2 and

125 mW/cm^2 decreased osteoclast activities [34, 35], but 30 mW/cm^2 LIPUS for 20 minute stimulation showed a promotive effect on osteoclastic resorptive activity of RAW264.7 cells [26]. However, in our study, no difference on osteoclastic resorptive activity was observed with or without the application of 30 mW/cm^2 LIPUS. This might be due to the difference between primary macrophages and cell line.

The phosphorylation of ERK is a crucial signaling event which controls the differentiation of multiple cell types, including RANKL-induced osteoclastogenesis [36, 37]. LIPUS has been reported to inhibit adipogenic differentiation via the suppression of ERK activation and promote osteogenesis via the enhancement of ERK activation, which indicated that LIPUS could regulate ERK signaling in different cell types [14]. In this study, LIPUS treatment showed a significant inhibitory effect on RANKL-induced ERK phosphorylation. In osteoclastogenesis, the binding of RANKL to RANK results in the phosphorylation of the MAPK signaling pathway, including ERK, JNK and p38 [38]. Subsequently, phosphorylated ERK promotes the activation of c-Fos, helping the formation of AP-1, an essential translation factor for osteoclast formation [39]. Additionally, c-Fos also showed a critical effect on the induction and translocation of NFATc1. NFATc1 is confirmed to be a master transcription factor for the formation and activities of osteoclasts [38]. Our results demonstrated that LIPUS markedly inhibited the mRNA and protein levels of NFATc1 and c-Fos, leading to the suppression of osteoclast-related genes expression, including TRAP, CTSK, CTR and DC-STAMP. Hence, it can be deduced that LIPUS suppressed osteoclast formation by the inhibition of the ERK-c-Fos-NFATc1 signaling pathway (**Figure 5C**), but it did not show any effects on mature osteoclast function.

In summary, our study has presented that LIPUS exerts an inhibitory effect on osteoclast formation by the inhibition of the ERK-c-Fos-NFATc1 signaling pathway, but no effect on mature osteoclast function. Our results reveal the exact effect and relative mechanism of LIPUS on osteoclasts, further helping the application of LIPUS clinically.

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Disclosure of conflict of interest

None.

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