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LIPUS suppressed LPS-induced IL-1α through the inhibition of NF-κB nuclear translocation via AT1-PLCβ pathway in MC3T3-E1 cells

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Inflammatory cytokines, interleukin (IL)-1, IL-6, and TNF-α, are involved in inflammatory bone diseases such as rheumatoid osteoarthritis and periodontal disease. Particularly, periodontal disease, which destroys alveolar bone, is stimulated by lipopolysaccharide (LPS). Low-intensity pulsed ultrasound (LIPUS) is used for bone healing in orthopedics and dental treatments. However, the mechanism underlying effects of LIPUS on LPS-induced inflammatory cytokine are not well understood. We therefore aimed to investigate the role of LIPUS on LPS-induced IL-1α production.

Mouse calvaria osteoblast-like cells MC3T3-E1 were incubated in the presence or absence of LPS (*Porphyromonas gingivalis*), and then stimulated with LIPUS for 30 min/day. To investigate the role of LIPUS, we determined the expression of IL-1α stimulated with LIPUS and treated with an angiotensin II receptor type 1 (AT1) antagonist, Losartan. We also investigate to clarify the pathway of LIPUS, we transfected siRNA silencing AT1 (siAT1) in MC3T3-E1. LIPUS inhibited mRNA and protein expression of LPS-induced IL-1α. LIPUS also reduced the nuclear translocation of NF-κB by LPS-induced IL-1α. Losartan and siAT1 blocked all the stimulatory effects of LIPUS on IL-1α production and IL-1α-mediated NF-κB translocation induced by LPS. Furthermore, PLCβ inhibitor U73122 recovered NF-κB translocation. These results suggest that LIPUS inhibits LPS-induced IL-1α via AT1-PLCβ in osteoblasts. We exhibit that these findings are in part of the signaling pathway of LIPUS on the anti-inflammatory effects of IL-1α expression.

KEYWORDS
AT1, IL-1α, low-intensity pulsed ultrasound, NF-κB, PLCβ

1 | INTRODUCTION

Rheumatoid arthritis and periodontal disease are common chronic inflammation bone diseases. World Health Organization (WHO) have been reported that number of affected individual rheumatoid arthritis is more than 23 million people in the world. Periodontal disease is also 47.2% of affected individual in age of 30s and older (Eke et al., 2012).

Abbreviations: α MEM, α-minimum essential medium; AT1, angiotensin receptor type 1; ANOVA, analysis of variance; $[Ca^{2+}]_i$, cytosolic free calcium concentration; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; LIPUS, Low-intensity pulsed ultrasound; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PLCβ, phospholipase C β; RANKL, receptor activator of NF-κB ligand; TLR, toll-like receptor; TNF, tumor necrosis factor.

However, treatment of these diseases still has been elusive. Inflammation bone disease is involved by inflammatory cytokines IL-1, IL-6, and TNF-α (Lopalco et al., 2015). Periodontal disease is characterized by the destruction of alveolar bone by lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P. gingivalis*). LPS is a major constituent of Gram-negative bacterial membrane and activates Toll-like receptor (TLR) 4 and 2 leading to the production of cytokines/chemokines to regulate inflammatory and subsequent adaptive immune responses (Rhee, 2014). LPS, IL-1, and TNF-α response to the differentiation and the activation of osteoclast, which induces bone resorption (Zou & Bar-Shavit, 2002). Thus, LPS and LPS-induced inflammatory cytokines are important factors in the progression of periodontal disease.

Low-intensity pulsed ultrasound (LIPUS) performed 30 mW/cm^2 SATA; intensity, 1.5 MHz, 1 kHz; pulsed an with exposure time of 20 min/day in many previous study (Manaka et al., 2015). LIPUS exerts various effects, including acoustic radiation force, acoustic streaming, and propagation of surface waves; promotion of fluid flow induced circulation; redistribution of nutrients, oxygen, and signaling molecules. In contrast, ultrasound that is very high intensity was damaged biological molecules such as DNA degradation (Elsner & Lindblad, 1989). In addition, LIPUS does not have a cavitation effect on pressure levels or transform acoustic wave energy into heat. LIPUS is a mechanical stimulation that accelerates healing and regeneration of bone fractures; it also promotes osseointegration of dental implant and is therefore used in clinical settings (Gebauer, Mayr, Orthner, & Ryaby, 2005; Harrison, Lin, Pounder, & Mikuni-Takagaki, 2016; Nolte et al., 2001; Roussignol, Currey, Duparc, & Dujardin, 2012; Warden, Bennell, McMeeken, & Wark, 2000). In previous clinical study, the healing index was shortened by 12 days/cm in continues LIPUS stimulation. These results indicated that callus maturation was 27% faster in LIPUS. LIPUS reduced fixator time for 95 days compared to without LIPUS stimulation (Salem & Schmelz, 2014). Previous studies have reported that LIPUS promotes osteoblast differentiation and osteogenesis in vitro (Manaka et al., 2015; Takayama et al., 2007). LIPUS also suppresses LPS-induced inflammatory chemokines in osteoblasts (Nakao et al., 2014). However, it is not known whether continuous stimulation of LIPUS affects in LPS-induced inflammatory cytokine production. We addressed this in the present study and investigated the signaling pathway which is activated by LIPUS in LPS-treated calvarial mouse osteoblast-like cells MC3T3-E1 by evaluating the expression of IL-1α and angiotensin II receptor type 1 (AT1) as well as the nuclear translocation of nuclear factor-κB (NF-κB).

2 | MATERIALS AND METHODS

2.1 | Cell culture

MC3T3-E1 mouse calvarial cell line obtained from Riken Bio Resource Center (Tsukuba, Japan) was used as a model for osteoblasts. Cells were maintained in α-Minimal Essential Medium (MEM; Gibco BRL, Rockville, MD) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% (v/v) penicillin–streptomycin solution (Sigma–Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were treated with 10 μg/ml LPS (*P. gingivalis*) (InvivoGen, San Diego, CA) or left untreated. The medium was replaced every 3 days.

2.2 | Application of low-intensity pulsed ultrasound (LIPUS)

Cells were seeded in 6-well culture plates (Iwaki, Tokyo, Japan) at a density of 2×10^4 cells/cm^2 for 24 hr prior to stimulation with LIPUS (1.5 MHz and a pulsed-wave mode intensity of 30 mW/cm^2), which was applied using a sterilized transducer (OSTEOTRON D^2; Ito Co., Tokyo, Japan) placed on the surface of culture medium so that the distance between the transducer and the cells was approximately 3–4 mm. Untreated control cells were seeded at the same density but were not stimulated with LIPUS.

2.3 | Real-time polymerase chain reaction (real-time PCR)

Cells were seeded on 6-well plates and cultured for up to 14 days. Total RNA was isolated on days 3, 7, and 14 of culture using the RNeasy Mini Kit (QIAGEN, Valencia, CA); RNA concentration was measured by NanoDrop 1000 (ND-1000; Thermo Fisher Scientific, Wilmington, DE). Complementary DNA (cDNA) was synthesized from 0.5 μg of DNase-treated total RNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan), and the resultant cDNA was analyzed by real-time PCR using the SYBR Green kit (Takara Bio). The reactions were performed in a total volume of 25 μl containing 12.5 μl of SYBR premixed Ex Taq, 0.5 μl (20 mM) of each primers (Table 1), 9.5 μl of dH_2O, and 2 μl (0.5 μg) of cDNA. The PCR assays were performed in a Smart Cycler II instrument (Cepheid, Sunnyvale, CA) and analyzed using Smart Cycler software. The cycling conditions included 35 cycles at 95°C for 5 s and

TABLE 1 PCR primers used in the experiments

Target	Primers	GenBank acc.
IL-1α	5′-CACTCTGGCTTTGGGAAGAG-3′	NM_001145920.1
	5′-GCAGTTCCCAAGCATTTCAT-3′	
TLR4	5′-GCCTCGAATCCTGAGCAAACA-3′	NM_021297.2
	5′-CTTCTGCCCGGTAAGGTCCA-3′	
AT1	5′-GGTAGGCGTCCCCCATGGTTT-3′	NM_130458.3
	5′-AGACGGGACAGCCAACCCTAG-3′	
GAPDH	5′-AAATGGTGAAGGTCGGTGTG-3′	NM_008084.2
	5′-TGAAGGGGTCGTTGATGG-3′	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

60°C for 20 s. All real-time PCR experiments were performed in triplicate, and the specificity of the amplified products was verified by melting curve analysis. The calculated values of target gene expression were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control (Shionome et al., 2012).

2.4 | Enzyme-linked immunosorbent assay (ELISA)

After LIPUS stimulation, cells were cultured in serum-free medium for additional 24 hr. The concentrations of IL-1α in the culture medium at 14 days were determined using commercially available ELISA kits (R&D Systems, MN) according to the manufacturers' instructions.

2.5 | Western blotting

Cells were cultured in serum-free medium after LIPUS stimulation and harvested 24 hr later. The total protein concentrations in cell lysates were quantified and 20 μg protein from each sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was then treated with blocking reagent (1% (v/v) BSA) in Tris-buffered saline (TBS) (10 mM Tris, 145 mM NaCl, pH 7.4) for 18 hr at 4°C, washed in Tween 20-containing Tris-buffered saline (TBS) (10 mM Tris, 145 mM NaCl, pH 7.4) (TBS-Tween), and incubated with rabbit polyclonal IgG antibodies or mouse monoclonal IgG antibody specific for AT1, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), ERK1/2 and phosphorylated ERK1/2 (Cell signaling Technology, Danvers, MA) diluted 1:200 or 1,000 in blocking reagent (1% (v/v) BSA) for 1 hr at room temperature. GAPDH was used as an internal standard. Membranes were then washed in TBS-Tween and incubated with biotin-conjugated secondary antibodies diluted 1:5,000 in 1% blocking agent for 15 min at room temperature, followed by a second round of washing and incubation with horseradish peroxidase-conjugated streptavidin in TBS-Tween for 30 min at room temperature. Immunoreactive proteins were visualized using a ChemiDoc XRS (Bio-rad Laboratories, Inc., CA).

2.6 | Immunofluorescence localization of NF-κB

Cells were seeded on glass coverslips, fixed with methanol for 15 min at −20°C, and blocked with 1% BSA. Cells were then incubated with rabbit monoclonal antibodies against NF-κB (Cell Signaling Technology) following by Alexa Fluor 488-conjugated goat antirabbit secondary antibody (Thermo Fisher Scientific, Waltham, MA). Signals were detected with an epifluorescence microscope and images were acquired with a Pro600ES digital camera system (Pixera, Osaka, Japan). Osteoblasts were rated positive for nuclear localization if the fluorescence intensity of their nuclei exceeded that of the cytoplasm. The proportion of osteoblasts exhibiting nuclear localization was calculated.

2.7 | Short interfering (si)RNA knockdown

Cells seeded in a 6-well plate were transfected with siRNA against AT1 or a scrambled control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection complex was prepared as follows: 3 μl of 50 μM siRNA were diluted in 150 μl Opti-MEM I without serum and 9 μl of transfection reagent were diluted in 150 μl Opti-MEM I. The two mixtures were combined, and 100 μl were added to each well when the cells had reached 60–80% confluence. After incubation for 6 hr, the medium was replaced and cells were cultured at 37°C in a CO_2 incubator for 24 hr before harvest. AT1 knockdown was confirmed by real-time PCR and Western blotting.

2.8 | Statistical analysis

Data represent the results of three independent experiments with samples were prepared in triplicate. Each value represents the mean ± standard error (SEM). Differences between groups were evaluated with the Student's *t*-test, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, or two-way ANOVA followed by Bonferroni's multiple comparisons test. Differences were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 | LIPUS blocked the upregulation of IL-1α and TLR4 induced by LPS is osteoblasts

We investigated the effect of LIPUS on the LPS-induced expression of IL-1α. We chose to use the 30 min/day stimulation with LIPUS because it had the strongest effect relative to other time courses in osteoblasts (Manaka et al., 2015). LPS increased mRNA levels of IL-1α in osteoblasts by day 14 of culture (by 3.6-fold) compared to untreated control (Fig. 1a). However, the LPS-elicited upregulation of mRNA and protein expression of IL-1α on day 14 was inhibited by LIPUS (Fig. 1a and c). The mRNA expression of TLR4 was also increased by LPS whereas LIPUS blocked the stimulatory effect of LPS (Fig. 1b).

3.2 | Effects of LIPUS on LPS-induced IL-1α expression is exerted via angiotensin receptor type I

The mechanistic basis for the inhibitory effect of LIPUS on LPS-induced IL-1α expression was investigated by evaluating the expression of the mechanosensitive receptor angiotensin receptor type I (AT1) in the presence and absence of AT1 antagonist losartan. LIPUS increased AT1 mRNA expression relative to untreated controls by 1.78, 1.82, and 2.39 fold on days 3, 7, and 14 of culture, respectively (Fig. 2a). LPS stimulation increased IL-1α mRNA expression by 2.9-fold (Fig. 2b) with a similar increase in protein expression (Fig. 2c) on day 14. However, treatment with losartan blocked the stimulatory effects of LIPUS on IL-1α expression at both mRNA and protein levels (Fig. 2b and c).

3.3 | LPS-induced NF-κB nuclear localization is blocked by LIPUS via AT1

To further investigate the signaling pathway by which LIPUS blocks LPS-induced cytokine production, we detected nuclear

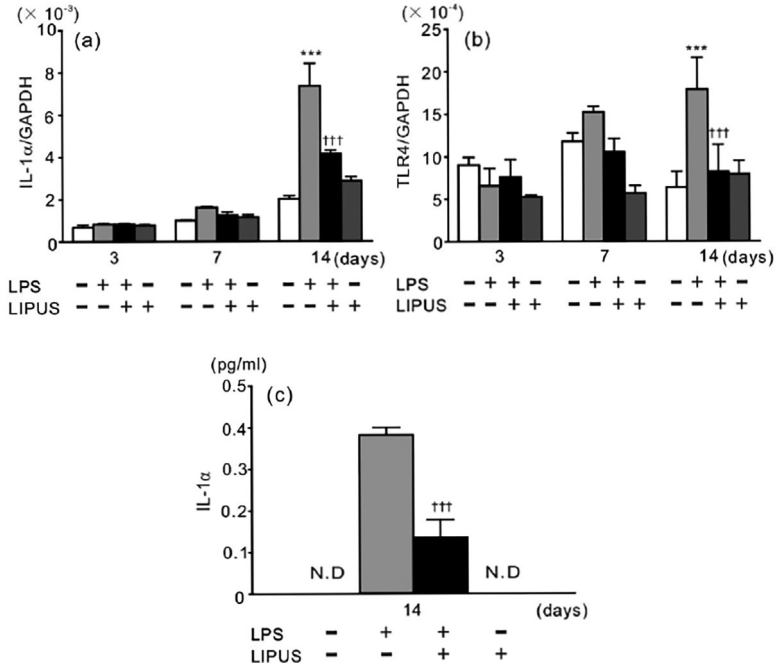

FIGURE 1 Cells were stimulated with LIPUS (30 min/day) and/or LPS (10 μg/ml) or left without stimulation (Untreated) and the mRNA expression of IL-1α (a) and TLR4 (b) was determined on days 3, 7, and 14 of culture using real-time PCR. The protein expression of IL-1α was determined on day 14 of culture using ELISA (c). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; ND, not detected; ***$p < 0.001$ versus control, †††$p < 0.001$ versus LPS

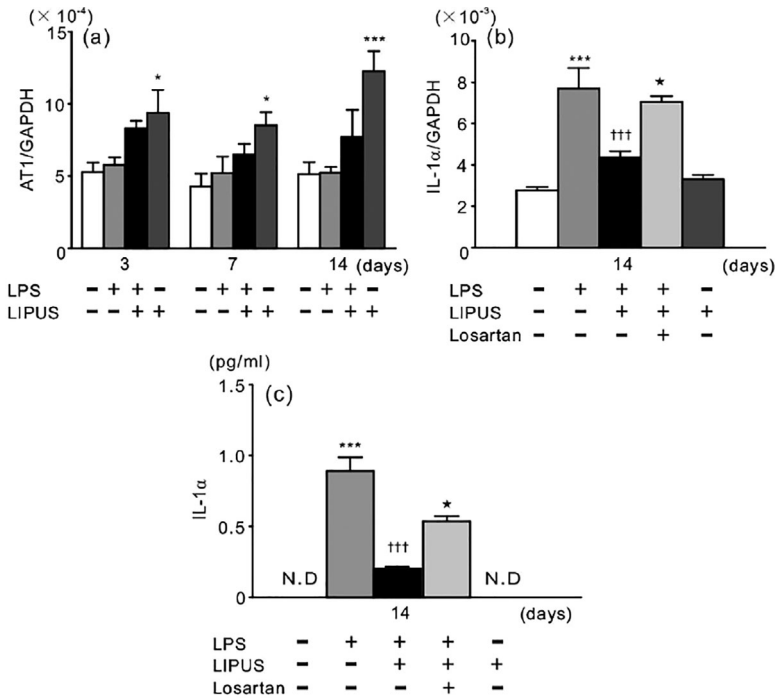

FIGURE 2 Cells stimulated or not with LIPUS for 30 min/day and LPS (10 μg/ml) during 14 days with and/or AT1 antagonist Losartan (5 μM) and the mRNA expression of AT1 (a) was determined by real-time PCR on days 3, 7, and 14 of culture. The mRNA and protein expression of IL-1α was determined by real-time PCR and ELISA on day 14 of culture (b and c). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; ND, not detecte; ***$p < 0.001$ versus control, *$p < 0.05$ versus control, †††$p < 0.001$ versus LPS

localization/activation of NF-κB (Komarova et al., 2003) by immunocytochemistry after stimulation with LPS and/or LIPUS and treatment with losartan. LIPUS blocked the nuclear accumulation of NF-κB induced by LPS at 5 min of culture (Fig. 3a), an effect that was reversed by losartan (Fig. 3a and b).

We next investigated the effect of AT1 on nuclear localization of NF-κB induced by LPS MC3T3-E1 were transfected with siAT1 (siAT1) and control scrambled siRNA (siControl). siAT1 cells significantly reduced mRNA levels of AT1 compared to WT and siControl (control scrambled siRNA) cells (by 0.45 and 0.54-fold, respectively) (Fig. 4a). The protein level of AT1 also expressed the similar effect of mRNA level (by 0.45 and 0.54-fold, respectively) (Fig. 4b and c). The abrogation of the effects of LIPUS in LPS-treated cells was observed upon transfection of AT1-specific siRNA (Fig. 5a and b).

3.4 | Effects of AT1-PLCβ pathway on NF-κB translocation

To clarify the pathway of LIPUS that has inhibitory effects on LPS-induced IL-1α, we used immunofluorescence staining of NF-κB nuclear translocation. We determined the nuclear translocation of NF-κB in the LPS-induced MC3T3-E1 cells stimulated with LIPUS in the presence or absence of PLCβ inhibitor U73122. We determined the nuclear translocation of NF-κB at 5 min of culture. LIPUS reduced the nuclear localization of NF-κB induced by LPS (Fig. 6a). Moreover, U73122 significantly increased the translocation of NF-κB compared to LPS + LIPUS (Fig. 6b and c).

4 | DISCUSSION

Mechanical stimuli such as fluid shear, tension strain, pressure, and LIPUS stimulate a variety of biological responses (Genetos et al., 2005; Kariya et al., 2015; Robling, Castillo, & Turner, 2006;). Particularly LIPUS induces osteogenesis in osteoblasts (Manaka et al., 2015; Takayama et al., 2007), indicating that these cell respond to mechanotransduction. Indeed, osteoblasts express mechanoreceptors in the form of ion channels, integrins, connexins, G protein-coupled receptors, and P2 receptors (Genetos et al., 2005; Ke et al., 2003; Rubin et al., 2006). LIPUS has demonstrated clinical effects on bone fracture healing (Romano, Romano, & Logoluso, 2009), and was found to enhance osteogenesis while inhibiting adipogenesis in cultured mouse bone marrow cells (Kusuyama et al., 2014). LPS induces the secretion of inflammatory factors such as IL-1, IL-6, TNF-α, and prostaglandin (PG) E_2 (Keeting et al., 1991; Page,

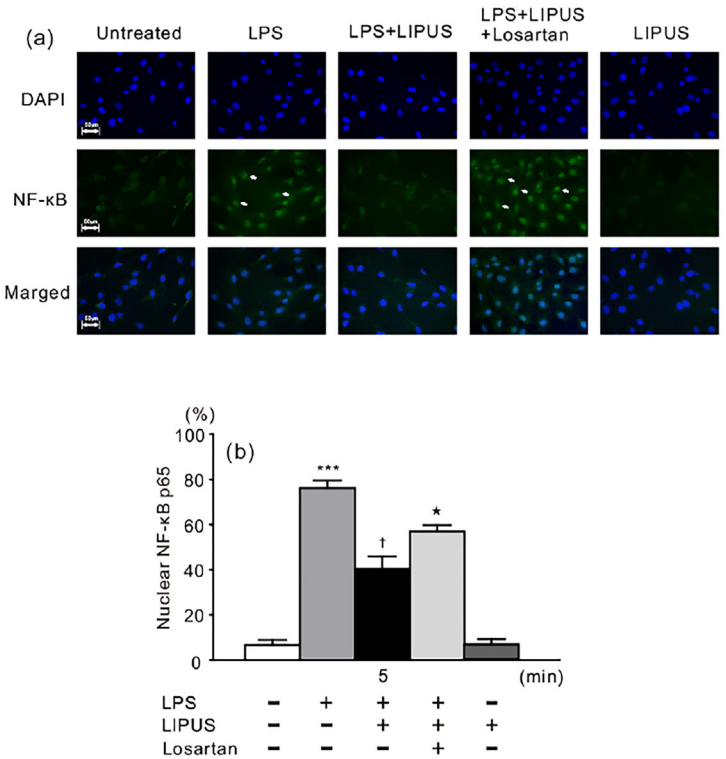

FIGURE 3 Cells plated on coverslips and leave on the 6-well plate, and stimulated or not with LIPUS for 30 min and LPS (10 μg/ml), after 5 min left for the LIPUS stimulation, cells were fixed with methanol. NF-κB localization was assessed by immunofluorescence. Cells plated on coverslips and leave on the 6-well plate, and stimulated or not with LIPUS for 30 min and LPS (10 μg/ml) in the presence or absence of Losartan (5 μM), were fixed with methanol after 5 min left for the LIPUS stimulation, NF-κB localization were assessed by immunofluorescence. The images of LIPUS and LPS + LIPUS were showing predominantly cytoplasmic NF-κB localization. The image of LPS was showing NF-κB localized predominantly in the nuclei (a). The histogram shows the percentage of osteoblasts exhibiting nuclear localization of NF-κB (b). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; $^{***}p < 0.001$ versus control, $^{\dagger}p < 0.05$ versus LPS, $^{\star}p < 0.05$ versus LPS + LIPUS

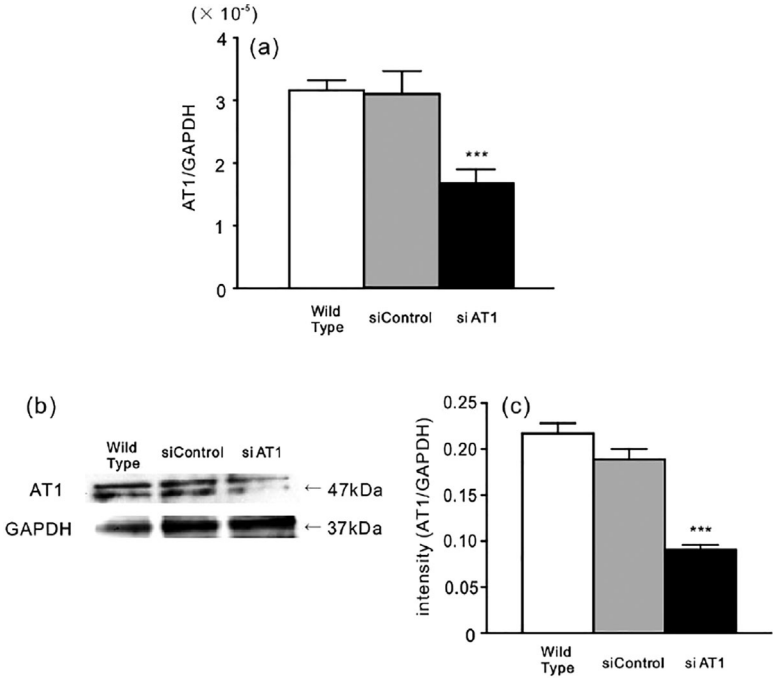

FIGURE 4 Cells were transfected with AT1-specific siRNA (siAT1) or control scrambled siRNA (siControl). Total RNA and protein was purified from confluent cells, and the expression of AT1 mRNA and protein was determined by real-time PCR (a) and Western blotting (b); histograms show the intensity of Western blotting bands under each condition (c). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; ***$p < 0.001$ versus siControl

1991; Shoji et al., 2006); it affects not only gingival epithelium cells but also alveolar bone which includes osteoblasts and osteoclasts (Page, 1991). In this study, we tested the hypothesis that LIPUS can suppress LPS-induced inflammation cytokines release. We found that LPS upregulation of IL-1α (Fig. 1a and b), as well as IL-6 and receptor activator of NF-κB ligand (RANKL) (data not shown), an effect that was abolished by LIPUS.

NF-κB is an important transcription factor response to inflammation and mechanical stimuli which have the five dimers in mammalian: p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (Nf-κB2) (Karin & Ben-Neriah, 2000; Mak & Yeh, 2002). NF-κB p50 and p65 are existed as typical hererodimer (Karin & Ben-Neriah, 2000). NF-κB has with inhibitory proteins IκB in the cytoplasm. The activation of NF-κB kinase leads to IκB kinase (IKK) α and β, which triggers phosphorylate serine residues on IκB for the degradation and the ubiquitination of IκB in the proteasome. (Darnay & Aggarwal, 1999; Karin & Delhase, 2000). The degradation and the ubiquitination of IκB reveals NF-κB translocate to the nucleus to activate gene transcription (Karin & Delhase, 2000; Komarova et al., 2003). We hypothesized that LIPUS responses mechanosensitive molecule such as NF-κB in short time period. In our present study, we continued to stimulate with LIPUS and/or LPS for up to 14 days. LIPUS inhibited the LPS-induced NF-κB nuclear translocation in short time period (5 min after LIPUS stimulation). In addition, LPS increased IL-α production whereas LIPUS inhibited the stimulatory effect at day 14, not but 3 or 7 day. These findings and our results suggest that a repeat of NF-κB nuclear translocation in short time period induced IL-α production on day 14 in LPS-stimulated cells, and LIPUS suppressed LPS-induced IL-1α production via recurrent inhibition of NF-κB nuclear translocation during culture period. Angiotensin II (Ang II) receptor type 1 (AT1) is a receptor of Ang II. AT1-mediated signaling involves G protein dependent and independent pathways. AT1 are with three G protein subfamilies; Gq, Gi, G12 (Hunyady & Catt, 2006). AT1 expression in induced by TNF-α and IL-1β and enhanced by LPS and oxidized low-density lipoprotein, both of which activate NF-κB (Cowling et al., 2002). We found here that LIPUS increased the expression of AT1 (Fig. 2a). A previous study reported that mechanical stress activated AT1 receptor via an independent mechanism of Ang II. Mechanical stress activates extracellular-signal-regulated kinases and increases phosphoinositide production in vitro, but also induces cardiac hypertrophy in vivo without Ang II (Zou et al., 2004). Mechanical stimulation also reduced Ang II in ventricular cells of adult Sprague Dawley rats (De Mello, 2012). We also observed that LIPUS reduced Ang II mRNA expression in present study (data not shown). These results and our results showed that AT1 may be stimulated by a mechanical stress of LIPUS and not in response to Ang II or LPS. Losartan blocked inhibitory effects of LIPUS on IL-1α production and NF-κB activation in our present study (Fig 2 and 3). However, AT1 selective antagonist of losartan is used for treatment of hypertension and cardiac hypertrophy. Losartan also rescued bone formation reduced by Ang II in osteoblasts (Nakai et al., 2015). In contrast, Losartan decreased bone volume/tissue volume (%) whereas rescued blood pressure in hypertension mouse model (Asaba et al., 2009). Losartan such as AT1 selective antagonist may have negative effect in osteoblast or bone. Our results showed that AT1 response to LIPUS which is a mechanical stress, acts anti-inflammatory effect on LPS-induced inflammatory osteoblasts. This might be a one of possible mechanism of AT1 without Ang II in osteoblasts.

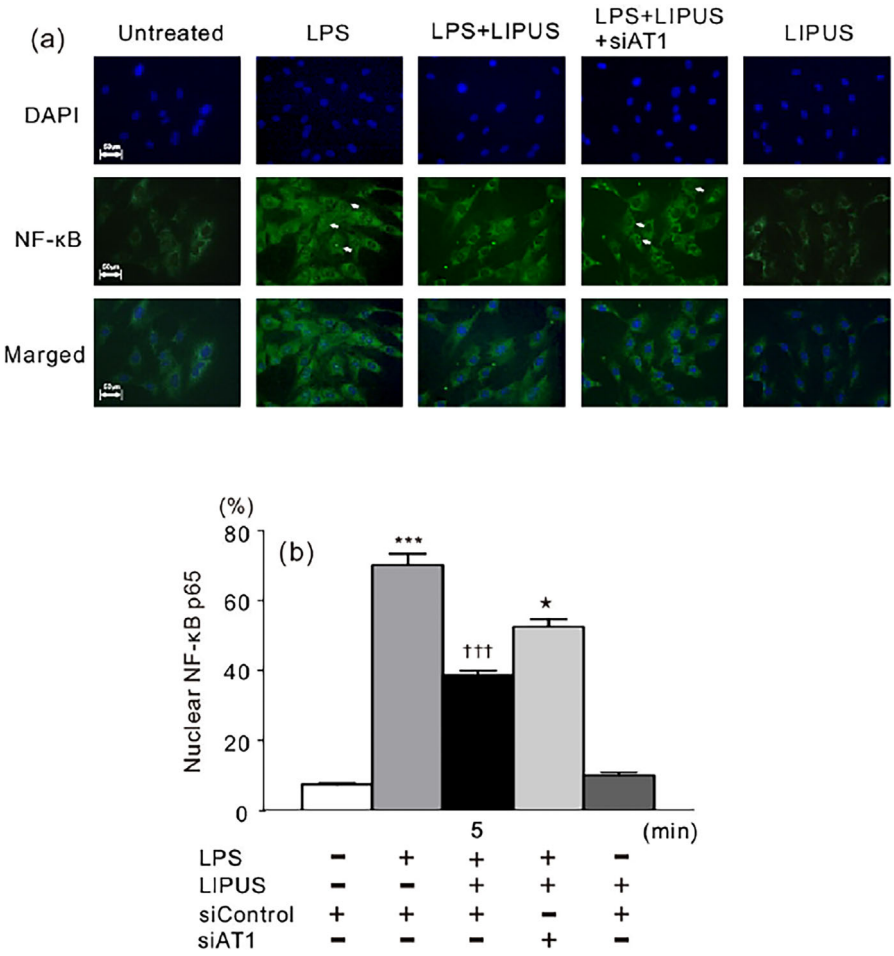

FIGURE 5 Cells were transfected with AT1-specific siRNA (siAT1) or control scrambled siRNA (siControl) stimulated or not with LIPUS for 30 min and LPS (10 μg/ml). After 5 min left for the LIPUS stimulation, cells were fixed with methanol. NF-κB localization was assessed by immunofluorescence (a). The histogram shows the percentage of osteoblasts exhibiting nuclear localization of NF-κB (b). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; ***$p < 0.001$ versus control, †††$p < 0.001$ versus LPS, ★$p < 0.05$ versus LPS + LIPUS

P. gingivalis LPS may activate host cells through either a TLR2- or TLR4-dependent pathway (Darveau et al., 2004). We used 10 μg/ml LPS to stimulate cells, and found that TLR4 mRNA expression was increased by this treatment (Fig. 1b). Especially, 10μg/ml *P. gingivaris* LPS activates TLR4 more than TLR2 (Darveau et al., 2004). The signaling pathway of TLR4 implicates the activation of NF-κB and mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 kinases (Cario et al., 2000; Matsuguchi et al., 2000; Yang et al., 2000; Zhang et al., 1999). Both LIPUS and LPS affect the phosphorylation of ERK1/2 compared to untreated control. However, LIPUS did not affect LPS-induced phosphorylation of ERK1/2 (p-ERK1/2) (Supplemental Fig. 1). Ang II increases p-ERK1/2 via AT1 in osteoblasts. However, shear stress which is one of the mechanical stresses also increased AT1-p-ERK1/2 without Ang II in CHO cells (Barauna et al., 2013; Nakai et al., 2013). In our present study, we showed that LIPUS increased AT1 which acts inhibitory effect on IL-1α production (Fig. 2a). In this regard, p-ERK1/2 might be upregulated by LIPUS stimulation. The expression of TLR4 was not regulated by AT1 (data not shown). LIPUS also did not affect angiotensin II expression (data not shown). In possible mechanism, the activation of ERK1/2 via AT1 by LIPUS might be involved another pathway that is LIPUS-induced osteogenesis (Kusuyama et al., 2014).

We found that LIPUS suppressed the nuclear translocation of NF-κB activation induced by LPS, whereas AT1 inhibition with Losartan or siRNA-mediated knockdown blocked this effect (Figs. 4 and 6). We next examined the implication of AT1 signaling pathway against the inhibition of NF-κB translocation. AT1 is Gq/11-coupled receptors, which responses to mechanical stimuli through the activation of PLCβ-Ca^{2+} pathway (Mederos et al., 2011). We showed that PLCβ inhibitor U73122 was blocked the stimulatory effect of LIPUS on NF-κB translocation in this study. Furthermore, LPS increases intercellular Ca^{2+} in osteoblasts (Tam et al., 1998). Our results exhibited that LIPUS suppresses NF-κB activation via AT1-PLCβ pathway. They were using as samples which were by the one-time LIPUS stimulation for these assays. However, we were using as samples that were continues stimulation of LIPUS in this study. Therefore, we might observe the different effect in our present study.

In summary, LIPUS acts anti-inflammatory effects which inhibited IL-1α-mediated NF-κB activation on LPS-induced inflammatory osteoblasts; this effect was exerted via AT1 (Fig. 7). These findings demonstrated that the molecular mechanism underlying the positive

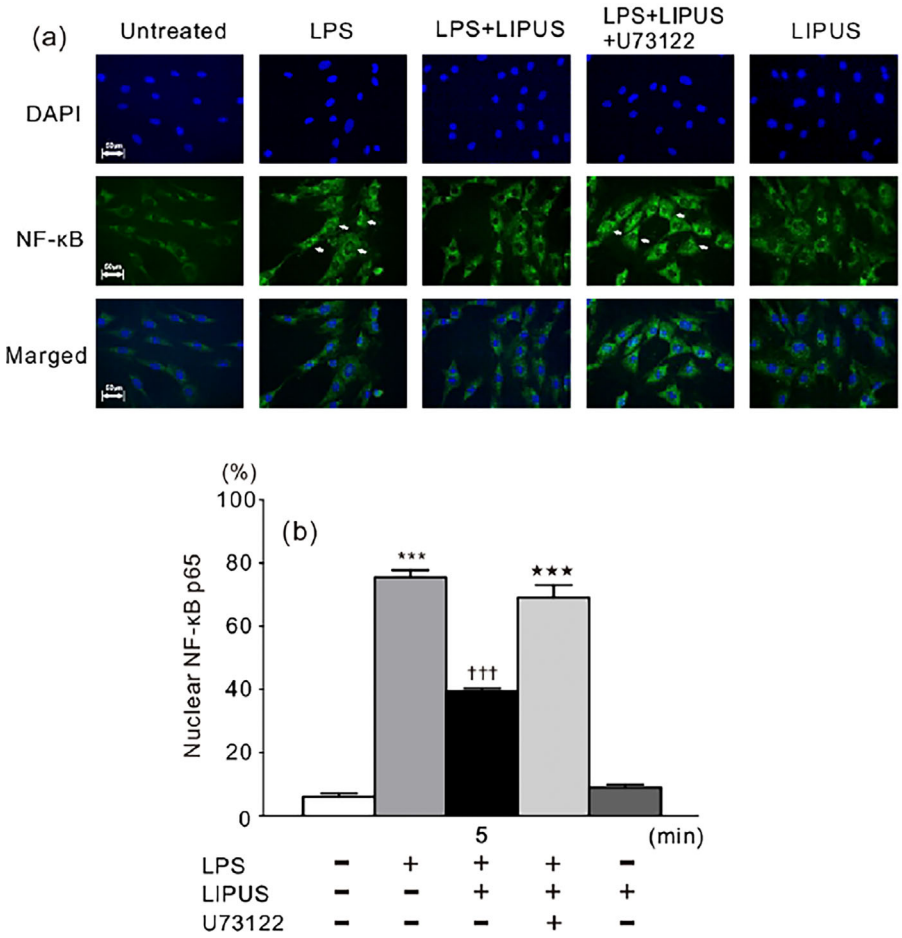

FIGURE 6 Cells plated on coverslips and leave on the 6-well plate, and stimulated or not with LIPUS for 30 min and LPS (10 μg/ml), were fixed with methanol. NF-κB localization was assessed by immunofluorescence. Cells plated on coverslips and leave on the 6-well plate, and stimulated or not with LIPUS for 30 min and LPS (10 μg/ml) in the presence or absence of U73122 (10 μM), were fixed with methanol after 5 min left for the LIPUS stimulation, NF-κB localization were assessed by immunofluorescence. The image of LIPUS and LPS + LIPUS were showing predominantly cytoplasmic NF-κB localization. The image of LPS was showing NF-κB localized predominantly in the nuclei (a). The histogram shows the percentage of osteoblasts exhibiting nuclear localization of NF-κB (b). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate; ***$p < 0.001$ versus untreated, †††$p < 0.001$ versus LPS, ★★★$p < 0.001$ versus LPS + LIPUS

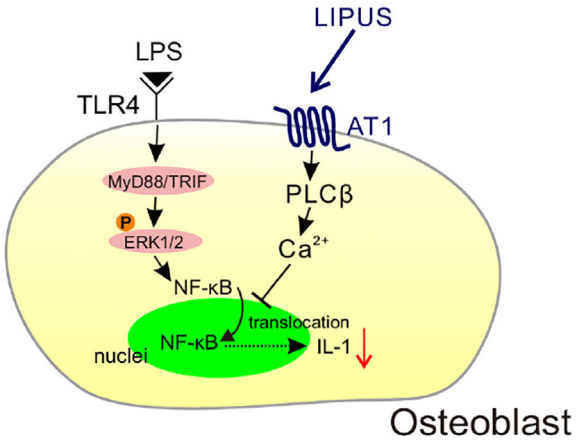

FIGURE 7 Proposed mechanism of LIPUS-AT1 signaling in osteoblasts. LPS induces the translocation of NF-κB, increases IL-1α production in osteoblasts. LIPUS acts AT1 which activated PLCβ, and inhibited the activation of NF-κB induced by LPS. Inhibition of NF-κB reduced IL-1α production

effects of LIPUS, it may suggest as a one of the treatment tools for inflammatory bone disease; rheumatoid osteoarthritis and periodontitis in the future. However, Losartan and siAT1 did not recover the translocation of NF-κB completely in this study. Thus, this mechanism of LIPUS on LPS-induced IL-1α production which we showed might be a part of that. In the future study, we should examine how LIPUS affects another LPS-induced inflammatory factors such as IL-6, RANKL, and PGE_2 production.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

MN, NT, and SS conceived and designed the experiments. MN, SM, and MN performed Cell culture experiments and prepared transfection siRNA. MN and JS performed Real-time PCR experiments. SK, TT and TK helped in Western blotting. MN, NT, NS, and MM analyzed the data. GT, NT, and MN helped in providing regent/materials/analysis tools. MN, NT, and SS wrote the paper.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All study participants provided informed consent, and the study design was approved by the appropriate ethics review boards.

CONSENT FOR PUBLICATION

All the authors have approved the manuscript and agree with submission to your esteemed journal.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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