Low-intensity pulsed ultrasound inhibits lipopolysaccharide-induced IL-6 and RANKL expression in osteoblasts

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Abstract: Periodontal disease is caused by inflammation induced by Porphyromonas gingivalis (P.g.) lipopolysaccharide (LPS) and involves expression of proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor-α, and receptor activator of nuclear factor kappa B ligand (RANKL), which are implicated in bone resorption. Low-intensity pulsed ultrasound (LIPUS) is commonly used in the treatment of bone fracture. However, the mechanisms by which LIPUS inhibits LPS-induced inflammatory cytokines are poorly understood. Therefore, we investigated the effects of LIPUS on LPS-induced expression of the proinflammatory cytokines IL-6 and RANKL. MC3T3-E1 cells were incubated in the presence or absence of P.g. LPS and then stimulated with LIPUS for 30 min/day for a maximum of 14 days. LPS increased mRNA and protein expressions of IL-6 and RANKL on day 14. In addition, mRNA expression of COX-2 LPS was higher after 3 and 7 days of LIPUS treatment. PGE₂ was induced by LPS after 7 and 14 days of culture. LIPUS suppressed all stimulatory effects of LPS. These results suggest that LIPUS inhibits LPS-induced expression of inflammatory cytokines by suppressing PGE₂ production and might thus have potential applications in the treatment of periodontitis.

Keywords: LIPUS; LPS; proinflammatory cytokine; PGE₂.

Introduction

Chronic inflammatory bone diseases such as rheumatoid arthritis (RA) and periodontal disease are common. RA affects more than 23 million people worldwide, and 47% of adults 30 years or older have periodontal disease (1). Periodontal disease is caused by alveolar bone resorption induced by lipopolysaccharide (LPS) from Porphyromonas gingivalis (P.g.). LPS is a constituent of membranes of gram-negative bacteria and stimulates Toll-like receptor (TLR) 4 and 2, which increase inflammatory cytokines/chemokines and regulate subsequent adaptive immune responses (2). Inflammatory bone disease is associated with the inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (3).
LPS, receptor activator of nuclear factor kappa B ligand (RANKL), IL-1, and TNF-α are responses to the differentiation and activation of osteoclasts, which promote bone resorption (4). Furthermore, *P. g.*-associated LPS increases RANKL, IL-1, and IL-6 in osteoblasts (5-7). Thus, LPS and LPS-induced proinflammatory cytokines are important factors in periodontal disease progression.

Low-intensity pulsed ultrasound (LIPUS) is a form of mechanical stimulation that facilitates healing and regeneration of bone fractures and induces osseointegration of dental implants. LIPUS is therefore used in several clinical settings (8-12). In a previous clinical study, continuous LIPUS stimulation shortened the healing index of bone fracture by 12 days/cm; thus, callus maturation was 27% faster during LIPUS. LIPUS reduced fixator time for 95 days as compared with the absence of LIPUS stimulation (13). In previous studies, the usual conditions for LIPUS were 30 mW/cm² SATA; intensity, 1.5 MHz, 1 kHz; pulsed with an exposure time of 20 min for osteoblasts (14). LIPUS exhibits various effects, including acoustic streaming, acoustic radiation force, and propagation of surface waves. Promotion of fluid flow increases circulation and promotes redistribution of nutrients, oxygen, and signaling molecules. In contrast, high-intensity ultrasound damaged DNA and caused degradation (15). In addition, LIPUS does not induce a cavitation effect on pressure levels or transform acoustic wave energy into heat. LIPUS induced osteoblast differentiation and osteogenesis in vitro in previous studies (14,16). LIPUS also inhibited inflammatory chemokines induced by LPS in osteoblasts (17). However, the effects of continuous LIPUS stimulation on LPS-induced inflammatory cytokine expression are poorly understood. In the present study, we investigated the effect of continuous LIPUS on the production of bone resorption-related proinflammatory cytokines in the MC3T3-E1 osteoblastic cell line.

**Materials and Methods**

**Cell culture**
The mouse calvarial cell line MC3T3-E1 was obtained from Riken BioResource Center (Tsukuba, Japan) for osteoblasts. Cells were maintained in α-minimal essential medium (MEM; Wako, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin-streptomycin solution (Wako, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were treated with 10 μg/mL LPS (*P. g.*; InvivoGen, San Diego, CA, USA) or left untreated. The medium was replaced every 3 days.

**LIPUS stimulation**
Cells were plated in 6-well culture plates (Iwaki, Tokyo, Japan) at a density of 2 × 10⁴ cells/cm² for 24 h before LIPUS stimulation (1.5 MHz; pulsed-wave mode intensity, 30 mW/cm²) with a sterilized transducer (Osteotron D²; Ito Co., Tokyo, Japan) placed on the surface of the culture medium. The distance between the transducer and cells was approximately 3-4 mm. Untreated control cells were not stimulated with LIPUS.

**Real-time PCR**
Cells were plated on 6-well plates and cultured for up to 14 days. Briefly, total RNA was isolated on days 3, 7, and 14 of culture. We described the methods in detail in our previous report (18). mRNA expression was detected by real-time PCR. The primer sequences of IL-6, RANKL, and COX-2 are shown in Table 1. The cycling conditions were 35 cycles at 95°C for 5 s and 60°C for 20 s. The calculated values for target gene expression were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the internal control.

**Enzyme-linked immunosorbent assay (ELISA)**
Cells were cultured in serum-free medium for an additional 24 h after LIPUS stimulation. Productions of IL-6, RANKL, and PGE₂ in culture media at 14 days were determined with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Statistical analysis**
The data represent the results of three independent experiments; samples were prepared in triplicate. Each value represents a mean ± SEM. Differences between groups were evaluated with one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons test, or with two-way ANOVA followed by the Bonferroni multiple comparisons test. Differences were considered statistically significant at *P* < 0.05.

**Results**
LIPUS inhibited LPS-induced mRNA expressions of IL-6 and RANKL in osteoblasts
We observed the effects of LIPUS on IL-6 and RANKL LPS-induced mRNA levels. LIPUS was administered for 30 min/day, i.e., the duration found to have the strongest effect on osteoblasts (14). On day 14 of culture, LPS-enhanced mRNA expressions of IL-6 and RANKL in osteoblasts were 2.5-fold higher than those in the untreated control (Fig. 1a, b). However, LPS-induced
upregulation of IL-6 and RANKL mRNA levels on day 14 was inhibited by LIPUS (Fig. 1).

**LIPUS inhibited LPS-induced protein expressions of IL-6 and RANKL in osteoblasts**

LPS increased the protein and mRNA expression levels of IL-6 and RANKL (by 1.49- and 3.0-fold, respectively) on day 14; however, LIPUS blocked the stimulatory effect of LPS on IL-6 and RANKL protein expressions (Fig. 2).

**Effects of LIPUS on mRNA expression of COX-2**

As compared with the untreated control, LPS increased mRNA expression of the PGE$_2$ synthesis enzyme COX-2 by 1.42-fold. As compared with the LPS culture, LIPUS inhibited LPS-induced COX-2 mRNA expression on day

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**Table 1** PCR primers used in the experiments

<table>
<thead>
<tr>
<th>Target</th>
<th>5'- Primers</th>
<th>3'- Primers</th>
<th>GenBank Acc.</th>
<th>bp</th>
</tr>
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<tbody>
<tr>
<td>IL-6</td>
<td>5'-CAACGATGATGCACTTGCAGA-3'</td>
<td>5'-CTCCAGGTAGCTATGTTACCCAAGA-3'</td>
<td>NM_031168.1</td>
<td>142</td>
</tr>
<tr>
<td>RANKL</td>
<td>5'-CATGTGCCACTGAGAACCTTGAA-3'</td>
<td>5'-CAGTTGCAAGGCAGGAATGTAAC-3'</td>
<td>NM_011613.3</td>
<td>103</td>
</tr>
<tr>
<td>COX-2</td>
<td>5'-TGAAGGCTCGTGTG-3'</td>
<td>5'-CTACTGAGTACCAGCGGCA-3'</td>
<td>NM_011198.4</td>
<td>148</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AAATGGTTGAAGGCGGTCGTTGATG-3'</td>
<td>5'-CTACTGAGTACCAGCGGCA-3'</td>
<td>NM_008084.2</td>
<td>142</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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7 (by 0.66-fold). LIPUS also reduced COX-2 mRNA expression, as compared with that of the untreated control, on days 3, 7, and 14 (by 0.83-, 0.63-, and 0.70-fold, respectively). In contrast, LPS decreased COX-2 mRNA expression as compared with untreated control on day 14 (by 0.88-fold) (Fig. 3).

Effects of LIPUS on PGE2 production
The mechanism underlying the inhibitory effect of LIPUS on LPS-induced IL-6 and RANKL expression was investigated by evaluating PGE2 expression on days 7 and 14 of culture. LPS-induced PGE2 production was 6.7- and 8.8-fold that of the untreated control on culture days 7 and 14, respectively. LIPUS reduced PGE2 production, as compared with LPS, by 2.1- and 1.76-fold on culture days 7 and 14, respectively. In addition, LIPUS increased PGE2 production, as compared with the untreated control, by 1.6- and 3.0-fold on culture days 7 and 14, respectively (Fig. 4).

Discussion
LPS enhances production of proinflammatory factors such as IL-1, IL-6, TNF-α, RANKL, and prostaglandin PGE2 (19-21). Proinflammatory cytokines affect gingival epithelial cells and alveolar bone, which includes osteoblasts and osteoclasts (21). This study investigated the effects of LIPUS on LPS-induced proinflammatory cytokines in osteoblasts and showed that LIPUS blocked LPS-induced increases in IL-6 and RANKL on day 14 of culture (Figs. 1, 2).

IL-6 is an important proinflammatory cytokine in RA and is involved in inflammatory bone disease. IL-6 knockout mice exhibit partial inhibition of osteoarthritis development (22-24), which suggests that IL-6 has a direct role in bone (25). IL-6 binds to its soluble receptor, which may promote osteoblast differentiation and activation by increasing expression of alkaline phosphatase or osteocalcin, an osteoblast differentiation marker in osteoblast precursor cells in vitro (26,27). However, the effects of IL-6 on osteoblast proliferation are modest (28). In contrast, IL-6 indirectly induces osteoclastogenesis by stimulating RANKL secretion in osteoblasts (29). Thus, various cell types involved in inflammatory bone disease produce IL-6, including osteoblasts and synovial fibroblasts.

RANKL is a member of the TNF superfamily. It has roles in osteoclast differentiation and survival and is secreted from osteoblasts as a transmembrane ligand. It is also expressed on stromal cells, B-lymphoid lineage cells, and activated T-cells and exists in a soluble form (30-32). RANKL binds to receptor activator of nuclear factor kappa B (RANK), that is as part of the commitment of monocyte/macrophage precursor cells to differentiate...
into the osteoclast lineage and activation of mature osteoclasts (33). RANKL knockout mice exhibit severe osteopetrosis associated with a defect in tooth eruption, which was caused by a complete lack of osteoclasts (34-36). In contrast, RANKL transgenic mice exhibit a decrease in bone mass and an increase in the number of osteoclasts (37). Taken together, these observations indicate that IL-6 and RANKL are important factors in bone resorption leading to inflammation in osteoarthritis and periodontitis.

Mechanical stimulation from pressure, fluid shear, tension strain, and LIPUS result in varied biological responses (38-40). LIPUS induces osteogenesis in osteoblasts (14,16). Previous reports indicate that osteoblasts respond to mechanotransduction. Osteoblasts possess mechanoreceptors in the form of ion channels, integrins, connexins, G protein-coupled receptors, and P2 receptors (38,41,42). An evaluation of the clinical effects of LIPUS on bone fracture healing (43) found that LIPUS induced osteogenesis while inhibiting adipogenesis in mouse bone marrow cells (44). In the present study, LIPUS suppressed expression of IL-6 and RANKL (Figs. 1, 2). These results showed that LIPUS is a response to LPS-induced osteoblasts that are present during inflammation.

PGE_2 is a member of the eicosanoid family of lipid mediators and is produced by nearly all cells. It is synthesized from arachidonic acid, via the actions of COX enzymes, in response to cell-specific stimuli, and signaling molecules. PGE_2 is not stored in cells (45-47). PGE_2 is also an important mediator of inflammatory bone diseases such as osteoarthritis and periodontitis (48). A previous study reported that PGE_2 induced RANKL and IL-6 production. In contrast, the COX-2 inhibitor NS398 reduced production of RANKL and IL-6 in MC3T3-E1 cells (49). These previous findings indicate that PGE_2 stimulates IL-6 and RANKL production via COX-2 in osteoblasts. In the present study, LPS induced COX-2, IL-6, and RANKL, as well as mRNA expression and PGE_2 production. However, the mRNA level of COX-2 was decreased by LPS on day 14 of culture, perhaps because of a biological response, as the maximum effects of LPS on the proinflammatory cytokines IL-6 and RANKL were seen on day 14. MC3T3-E1 cells exhibited progressive inflammation and subsequent anti-inflammatory responses, to recover from inflammation. These results are consistent with those of previous studies. Furthermore, our present study showed that LIPUS suppressed the LPS-induced stimulatory effects on IL-6, RANKL, COX-2, and PGE_2 in osteoblasts (Figs. 1-4). These results suggest that LIPUS exerts anti-inflammatory effects by reducing IL-6 and RANKL production via inhibition of PGE_2 and COX-2 expression in osteoblasts. However, LIPUS also increased PGE_2 production while inhibiting IL-6 and RANKL expression (Fig. 4). PGE_2 affects both bone resorption—which involves inflammation—and bone formation in osteoblasts (48,50,51). Mechanical stimuli also induce osteogenesis via PGE_2 production in osteoblasts (50). Thus, in the present study LIPUS stimulated PGE_2, which might be involved in osteogenesis. Our findings indicate that the molecular mechanism underlying the anti-inflammatory effects of LIPUS is a possible target for future treatments for RA, osteoarthritis, and periodontitis. Furthermore, the pathway mediating the inhibitory effects of LIPUS on IL-6, RANKL, and PGE_2 production should be further investigated.

Acknowledgments

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Conflicts of interest

None declared.

References


