Effects of Low-Intensity Pulsed Ultrasound on the Expression of Cyclooxygenase-2 in Mandibular Condylar Chondrocytes

Yasunori Iwabuchi, DDS, PhD
Clinical Fellow

Kotaro Tanimoto, DDS, PhD
Professor

Yuki Tanne, DDS, PhD
Assistant Professor

Toshihiro Inubushi, DDS, PhD
Assistant Professor

Takashi Kamiya, DDS, PhD
Clinical Fellow

Ryo Kunimatsu, DDS, PhD
Assistant Professor

Naoto Hirose, DDS, PhD
Assistant Professor

Tomomi Mitsuyoshi, DDS, PhD
Clinical Fellow

Shaoching Su, DDS
Graduate Student

Department of Orthodontics
Applied Life Sciences
Hiroshima University Institute of Biomedical & Health Sciences
Hiroshima, Japan

Eiji Tanaka, DDS, PhD
Professor
Department of Orthodontics and Dentofacial Orthopedics
Institute of Health Biosciences
The University of Tokushima Graduate School
Tokushima , Japan

Kazuo Tanne, DDS, PhD
Professor Emeritus
Department of Orthodontics
Applied Life Sciences
Hiroshima University Institute of Biomedical & Health Sciences
Hiroshima, Japan

Correspondence to:
Dr Kotaro Tanimoto
Department of Orthodontics
Applied Life Sciences
Hiroshima University Institute of Biomedical & Health Sciences
1-2-3 Kasumi, Minami-ku
Hiroshima 734-8553
Japan
Fax: +81-82-257-5687
Email: tkotaro@hiroshima-u.ac.jp

Aims: To determine the effect of low-intensity pulsed ultrasound (LIPUS) on cyclooxygenase-2 (COX-2) expression and related mechanisms by using cultured articular chondrocytes derived from porcine mandibular condyles after treatment with interleukin-1 beta (IL-1β). **Methods:** Chondrocytes were derived from porcine mandibular condylar cartilage and cultured. The cells were treated with or without 10 ng/mL IL-1β. At the same time, the cells were exposed to LIPUS for 20 minutes. After LIPUS exposure, the conditioned medium was changed to a fresh one without IL-1β, and the cells were incubated for 0 to 24 hours. The effects of LIPUS on IL-1β-treated chondrocytes were examined in terms of the expression of p-integrin β1, COX-2, and phosphorylated extracellular signal–related kinase (p-ERK) 1/2 by real-time polymerase chain reaction (PCR) and Western blot analyses. Differences in the means among multiple groups were examined by one-way analysis of variance (ANOVA) for all groups at each time point, followed by a Scheffé multiple comparison test as a post-hoc test; Student *t* test was also used. **Results:** COX-2 mRNA level was upregulated by the treatment with IL-1β and was suppressed significantly ($P < .01$) by LIPUS exposure. Furthermore, LIPUS enhanced gene expression and phosphorylation of integrin β, and it inhibited the expression of p-ERK 1/2. **Conclusion:** LIPUS exposure inhibited IL-1β-induced COX-2 expression through the integrin β1 receptor followed by the phosphorylation of ERK 1/2. Despite the restricted duration of its effect, LIPUS is suggested to be a potential candidate as a preventive and auxiliary treatment to suppress the degradation of articular chondrocytes in temporomandibular joint osteoarthritis.
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Key words: *cyclooxygenase-2 (COX-2), low-intensity pulsed ultrasound (LIPUS), mandibular condylar cartilage, mechanical stress, osteoarthritis (OA)*

Osteoarthritis of the temporomandibular joint (TMJ-OA) is characterized by mandibular condylar cartilage degradation due to mechanical overloading.[1] Excessive mechanical overloading of the mandibular condylar cartilage induces the expression of interleukin-1β (IL-1β),[2] an inflammatory cytokine closely related to the progression of TMJ-OA.[3,4] Thus, a large amount of IL-1β has been detected in the synovial fluid of patients with TMJ-OA.[5] A previous study using an experimental model of the early phase of OA in rat knee joints induced by intra-articular injection of monoiodoacetate revealed articular cartilage degradation accompanied by early loss of proteoglycan anabolism, increased matrix metalloproteinase (MMP)-2 activities related to IL-1β expression, and subsequent upregulation of cyclooxygenase-2 (COX-2).[6] In addition, COX-2 and prostaglandin E_2 (PGE_2) are markedly expressed in the mandibular condylar cartilage of patients with TMJ-OA.[7] COX-2 and PGE_2 are induced by IL-1β, and the production of MMPs is enhanced through a PGE_2 receptor (EP4)-related mechanism in fibroblasts, leading to cartilage matrix degradation.[8–10] Thus, COX-2 and PGE_2 play an important role in matrix degradation in the mandibular

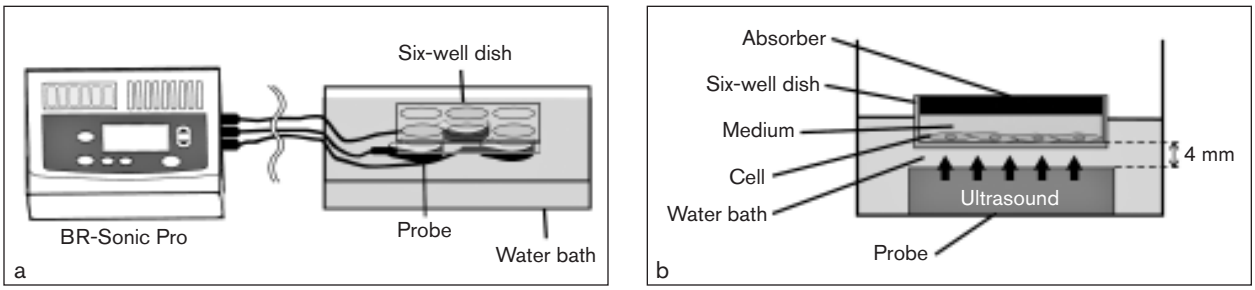

Fig 1 LIPUS exposure assembly. **(a)** Three probes were arranged under a six-well culture plate. **(b)** Cell culture dishes were placed at a distance of approximately 4.0 mm from the surface of transducers, which helped to optimize beam uniformity across the target cell region. The water tank was maintained at 37°C ± 0.5°C, and the US exposure assembly was maintained under a humidified atmosphere of 5% CO_2 at 37°C.

condylar cartilage in TMJ-OA. Mandibular condylar cartilage is an avascular tissue and it has no nutrient supply other than synovial fluid. Therefore, once mandibular condylar cartilage degradation occurs in TMJ-OA, cartilage cannot regenerate itself; this suggests the importance of suppression of cartilage degradation during the early stage of TMJ-OA.

Low-intensity pulsed ultrasound (LIPUS) has been reported to enhance the production of proteoglycan accompanied by increased aggrecan synthesis, but not to affect α_1(I) and α_1(II) procollagen expression levels.[11,12] LIPUS has been reported to affect cartilage matrix metabolism through integrin β1, a possible cell surface receptor for LIPUS in chondrocytes.[13,14] In a previous study, LIPUS was reported to reduce excess production of COX-2 caused by IL-1β in rabbit knee synovial membrane cells.[15] Regarding the mechanism for reduction of COX-2, it has been reported that activation of integrin β inhibited lipopolysaccharide (LPS)-induced extracellular signal–related kinase (ERK) 1/2 phosphorylation in human pulmonary artery endothelial cells.[16] As a result, it is assumed that integrin β1 is activated by LIPUS, while activation of integrin β1 inhibits ERK 1/2 phosphorylation induced by IL-1β in mandibular chondrocytes. In this way, LIPUS is speculated to be able to modulate metabolism of mandibular condylar chondrocytes under inflammation.

The aim of this study was to clarify the effect of LIPUS on COX-2 expression and related mechanisms by using cultured articular chondrocytes derived from porcine mandibular condyles after treatment with IL-1β.

Materials and Methods

Cell Isolation and Culture

The experiment was approved by the Board for Animal Experiments of Hiroshima University. The TMJs of female pigs aged 6 to 9 months and weighing 100 to 110 kg were obtained from a Japan Agriculture slaughterhouse (Hiroshima, Japan). Mandibular condylar cartilage was carefully dissected from the mandible, and the condylar cartilage pieces were digested with 0.1% actinase in Dulbecco's modified Eagle medium (DMEM) for 1 hour and 0.02% collagenase in DMEM for 12 hours. Isolated chondrocytes were seeded at a density of 2.0 × 105 cells/well in six-well culture plates (35 mm diameter, Corning). The cultures were maintained in 2 mL DMEM supplemented with 10% fetal bovine serum (Biological Industries) and 60 mg/mL kanamycin under an atmosphere of 5% CO_2 in a humidified incubator at 37°C until 80% confluence.

Stimulation of Cells with IL-1β and LIPUS Exposure

The porcine mandibular condylar chondrocytes were cultured to subconfluence. Then the conditioned medium was changed to a fresh one with or without 10 ng/mL IL-1β (recombinant human IL-1β, R&D Systems). The optimal concentration of IL-1β used in this study for the treatment of mandibular condylar chondrocytes has been described elsewhere.[17]

A LIPUS exposure assembly (BR-Sonic Pro, ITO Company) was employed in a series of experiments (Fig 1). Immediately after the addition of 0 or 10 ng/mL IL-1β, the mandibular condylar chondrocytes were exposed to LIPUS for 20 minutes. The probe of this system consists of a 9.6-cm^2 circular surface transducer area. The sound head of this device has an average beam nonuniformity ratio of 3.1 to 3.5:1 and an effective radiating area of 4.5 cm^2 (46.8%). A pulsed ultrasound signal was transmitted at a frequency of 3 MHz, with a spatial temporal average intensity of 30 mW/cm^2, with pulsing at 20% (2 ms on and 8 ms off). A six-well plate was held in place with the top above water level in a foam-fronted plastic sliding assembly containing an aperture of matching dimensions to the monolayer, and the distance between the transducer and the cells was 4 mm, as described previously.[18,19] The water tank between the transducer and cell flask

was maintained at 37°C ± 0.5°C, and the LIPUS exposure assembly was maintained in a humidified atmosphere of 5% CO_2 at 37°C during all experiments. Foam was placed at the top of the tank to minimize standing waves and unwanted reflection from the flask holder. The tank water was maintained at 37.0°C ± 0.5°C. The machines have an electronic control panel that allows an electronic check whenever it is switched on and an alert signal that is triggered if the coupling gel or liquid has been depleted.

After LIPUS exposure, photomicrographs of the cells were taken with a phase-contrast microscope (BZ8000, Keyence). The conditioned medium was changed to a fresh one without IL-1β, and the cells were incubated for 0 to 24 hours. Control samples were also subjected to the same operations under the same conditions without LIPUS stimulation.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total ribonucleic acid (RNA) was extracted from cultured TMJ chondrocytes according to the instructions from the manufacturer by using a total RNA extraction kit (Pharmacia Biotech) 0 to 24 hours after the LIPUS exposure. A ReverTra Ace first-strand cDNA synthesis kit (Toyobo) was used to synthesize first-strand complementary deoxyribonucleic acid (cDNA) from 1 mg total RNA. Quantitative real-time polymerase chain reaction (PCR) analysis was carried out with a Lightcycler 3.0 System (Roche Diagnostics). The primer sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5-TGT CCC TAC TGC CAA CGT G-3, reverse: 5-TGC TCA GTG TAG CCC AGG ATG-3; integrin β1, forward: 5-CGT TAC TGT CGT GAT GAG AT-3, reverse: 5-GCT CTT CTA CCA CAT ACA GGA-3; COX-2, forward: 5-CTT ACT GGA ACA TGG CAT CAC-3, reverse: 5-CTC TGC TCT GGT CGA TTG A-3. Real-time PCR analysis was assessed in a quantitative manner with a cycle threshold (Ct) value that identifies when the fluorescence of a given sample becomes significantly different from the baseline signal. The integrin 1β and COX-2 signals were evaluated in a qualitative manner relative to the GAPDH signals. Normalized Ct values were expressed relative to the value at 0 hours (control level).

Western Blot Analysis of p-Integrin β1 and Phosphorylated-ERK (p-ERK) 1/2

The chondrocytes were lysed in a ×5 sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris–HCl [pH 6.8], 4% SDS, 10% glycerol). The concentration of the protein was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce Chemical). After the addition of 10% 2-mercaptoethanol and 0.01% bromophenol blue, samples were boiled at 100˚C for 3 minutes. Ten micrograms of protein were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes by using an iBlot gel transfer system (Invitrogen). Membranes were blocked with blocking buffer (LI-COR Biosciences) at room temperature on a shaker for 1 hour. The primary antibodies were incubated with the membranes at room temperature for 2 hours. An anti-p-ERK 1/2 antibody (sc-7383; Santa Cruz Biotechnology) was used at 1:1,000 dilution, and anti-p-integrin β1 (I7533; Sigma Aldrich) was used at 1:500 dilution. After primary antibody incubation, the membranes were washed three times for 5 minutes each with 15 mL of phosphate buffered saline (PBS) and 0.1% Tween-20 before the addition of a secondary antibody conjugated to a fluorescent entity: IRDye 800CW-conjugated goat anti-mouse IgG (dilution 1:15,000 in blocking buffer (LI-COR Biosciences) and IRDye 800 CW-conjugated goat (polyclonal) anti-rabbit IgG (dilution 1:7,000 in blocking buffer (LI-COR Biosciences) for p-ERK 1/2 and p-integrin β1. At the end of the incubation period, membranes were washed twice with 15 mL of PBS and 0.1% Tween-20 and once with 15 mL of PBS. Membranes were dried, visualized, and analyzed on the Odyssey IR imaging system (LI-COR Biosciences).

Statistical Analysis

All experiments were repeated in triplicate. Reproducibility of these results was confirmed by two sets of experiments executed in the same manner. Differences in the mean (± SE) values among multiple groups were examined by one-way analysis of variance (ANOVA) for all groups at each time point, followed by a Scheffé multiple comparison test as a post-hoc test. The mean difference between the control and experimental groups at each time point was examined by Student *t* test. A *P* value of less than .05 was considered as reflecting statistical significance.

Results

Effect of LIPUS Exposure

Morphology of Cultured Mandibular Condylar Chondrocytes in the Presence of IL-1β. There were no obvious changes in the morphology of cultured porcine mandibular condylar chondrocytes by LIPUS exposure for 20 minutes in the presence of IL-1β (Figs 2a and 2b).

Gene Expression of Integrin β1 in Cultured Mandibular Condylar Chondrocytes in the Presence of IL-1β. There were no significant ($P > .05$) changes in integrin β1 mRNA levels 6, 12, and 24 hours after the treatment with IL-1β for 20 minutes in cultured porcine mandibular condylar chondrocytes

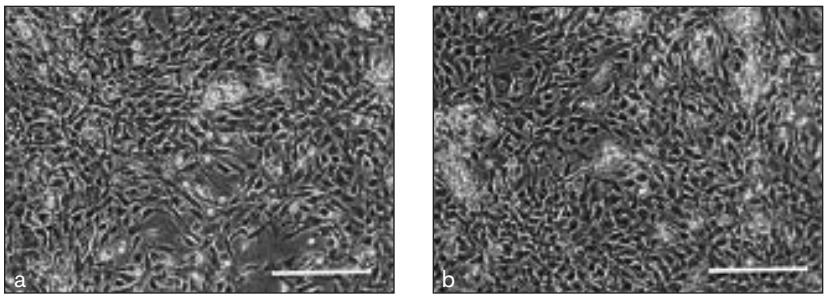

Fig 2 Photomicrographs of cultured porcine mandibular articular chondrocytes with and without LIPUS exposure. Porcine mandibular condylar chondrocytes were incubated **(a)** with or **(b)** without LIPUS exposure for 20 minutes in the presence of IL-1β, and photomicrographs of the cells were taken. There were no obvious changes in the morphology of chondrocytes by LIPUS exposure. Bar: 100 mm.

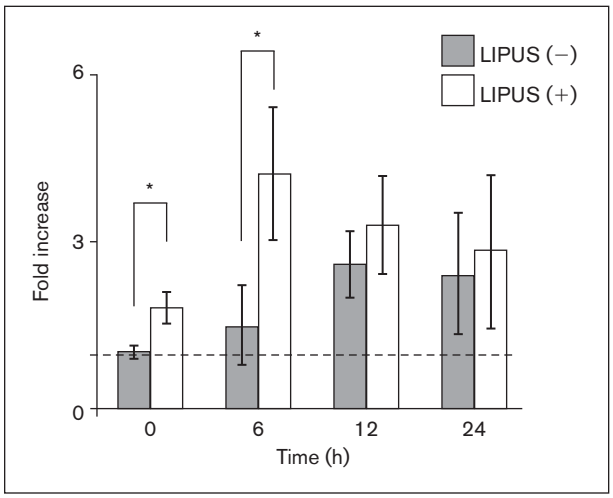

Fig 3 Effect of LIPUS exposure on mRNA expression of integrin β1 in cultured mandibular condylar chondrocytes in the presence of IL-1β. Porcine mandibular condylar chondrocytes were incubated with or without LIPUS exposure for 20 minutes in the presence of 10 ng/mL IL-1β. After 0, 6, 12, and 24 hours of treatment, total RNA was extracted. Integrin β1 gene expression was determined by means of quantitative real-time polymerase chain reaction analysis. The signal intensities of integrin β1 were expressed relative to the value in the cells without LIPUS exposure at 0 hours (control level; dotted line). Data are presented as the mean ± SE. **P* < .05, n = 3.

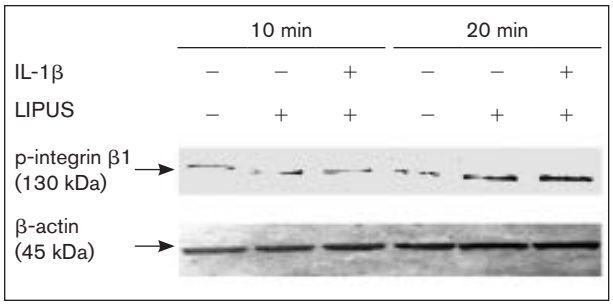

Fig 4 Effect of LIPUS exposure on p-integrin β1 expression in cultured mandibular condylar chondrocytes in the presence or absence of IL-1β. Porcine mandibular condylar chondrocytes were incubated with or without LIPUS exposure for 20 minutes in the presence or absence of 10 ng/mL IL-1β. At 10 and 20 minutes after the treatment, protein was extracted from the cells, and p-integrin β1 was detected by Western blot analysis.

(Fig 3). When the cells were exposed to LIPUS during IL-β treatment, the mRNA level of integrin β1 was upregulated significantly (*P* < .05) after 0 hours and 6 hours as compared to the control group, while at 12 hours and 24 hours after the treatment, no significant differences (*P* > .05) between the two groups were found.

Phosphorylation of Integrin β1 in Cultured Mandibular Condylar Chondrocytes in the Presence or Absence of IL-1β. The expression of p-integrin β1 was substantially enhanced by 20-minute LIPUS exposure in porcine mandibular condylar chondrocytes cultured without IL-1β after 20 minutes (Fig 4). The p-integrin β1 level was also enhanced by 20-minute LIPUS exposure in the presence of IL-1β, and there were no obvious differences in the p-integrin β1 level as compared with the IL-1β untreated group. There were no obvious changes in p-integrin β1 level 10 minutes after LIPUS exposure in all groups.

Gene Expression of COX-2 in Cultured Mandibular Condylar Chondrocytes in the Presence or Absence of IL-1β. The COX-2 mRNA levels in cultured porcine mandibular condylar chondrocytes were upregulated significantly (*P* < .01) at 3, 6, 12, and 24 hours after the treatment with IL-1β as compared to the untreated control groups (Fig 5). Although the COX-2 mRNA levels were not affected by 20-minute LIPUS exposure alone, enhancement of COX-2 mRNA expression by IL-β treatment was inhibited significantly (*P* < .01), but not decreased to the levels in the untreated control groups, by simultaneous LIPUS exposure after 6, 12, and 24 hours.

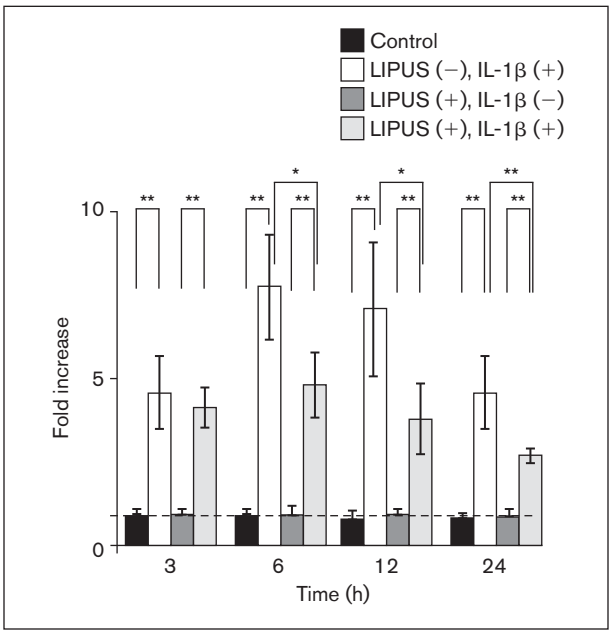

Fig 5 Effect of LIPUS exposure on mRNA expression of COX-2 in cultured mandibular condylar chondrocytes in the presence or absence of IL-1β. Porcine mandibular condylar chondrocytes were incubated with or without LIPUS exposure for 20 minutes in the presence or absence of 10 ng/mL IL-1β. Total RNA was extracted 3, 6, 12, and 24 hours after the treatment. COX-2 gene expression was determined by means of quantitative real-time polymerase chain reaction analysis. The signal intensities of COX-2 were expressed relative to the value at 0 hours (control level; dotted line). Data are presented as the mean ± SE. **P* < .05, ***P* < .01, n = 3.

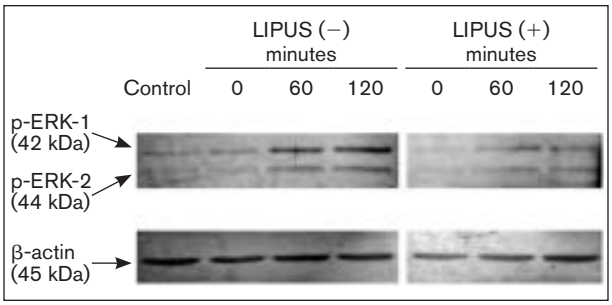

Fig 6 Effect of LIPUS exposure on the expression of p-ERK 1/2 in cultured mandibular condylar chondrocytes in the presence of IL-1β. Porcine mandibular condylar chondrocytes were incubated with or without LIPUS exposure for 20 minutes in the presence of 10 ng/mL IL-1β. The protein was extracted from the cells, and p-ERK 1/2 was detected by Western blot analysis 0, 60, and 120 minutes after the treatment. The band before treatment was regarded as the control.

Expression of p-ERK 1/2 in Cultured Mandibular Condylar Chondrocytes in the Presence of IL-1β. The signal intensities of p-ERK 1/2 were prominently enhanced 60 and 120 minutes after the treatment with IL-1β for 20 minutes in porcine mandibular condylar chondrocytes as compared with the signal before treatment when the cells were not exposed to LIPUS (Fig 6). On the other hand, the signal intensities of p-ERK 1/2 were slightly enhanced 60 and 120 minutes after the treatment with IL-1β and simultaneous LIPUS exposure; however, the enhancement of p-ERK 1/2 was markedly inhibited.

Discussion

As far as the authors are aware, this is the first attempt to investigate the effects of LIPUS on COX-2 gene expression in mandibular condylar chondrocytes. It is recognized that COX is strongly induced by IL-1β and plays an important role in the pathophysiology of arthritis.[20] The level of COX-2 expression is upregulated in inflamed tissues.[21] Meanwhile, LIPUS has been used extensively as a therapeutic, operative, and diagnostic tool in medicine. It is generally accepted that LIPUS has no deleterious or carcinogenic effects. Therefore, LIPUS is well accepted as a noninvasive and safe therapeutic tool for the treatment of bone fractures.[22] Furthermore, LIPUS promotes matrix metabolism in osteoblasts, fibroblasts, chondrocytes, cementoblasts, synoviocytes, and intervertebral disc cells.[11,19,23,24] It has previously been demonstrated that LIPUS downregulates COX-2 expression in inflamed synovial membranes in vitro and in vivo.[15] Since LIPUS provides mechanical stimulation to the cellular system, it was hypothesized that the mechanical stimulation induced by LIPUS may inhibit COX-2 gene expression induced by IL-1β in the mandibular condylar cartilage as well as the synovial membrane.

LIPUS was found to enhance the phosphorylation of integrin β1 in porcine mandibular condylar chondrocytes within 20 minutes after LIPUS exposure, suggesting the activation of integrin β1 existing on the surface of chondrocytes by LIPUS stimuli.

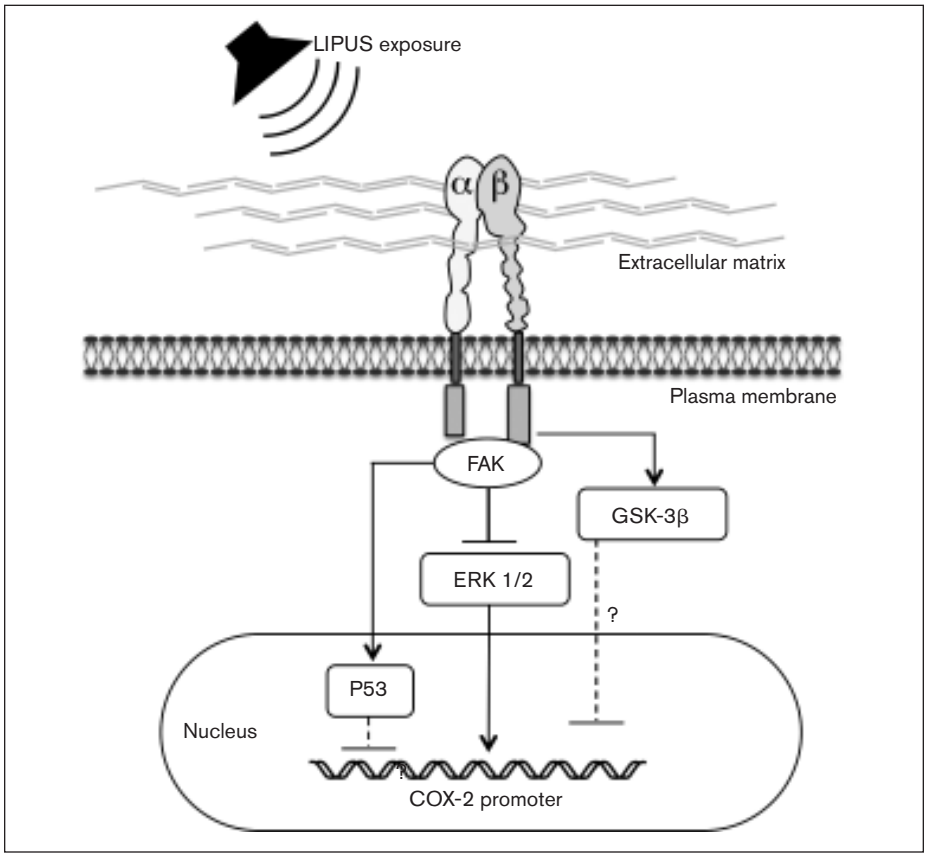

Fig 7 Schematic illustration of possible signaling pathway activated by LIPUS exposure.

The mRNA level of integrin β1 was also upregulated by LIPUS exposure after 6 hours, leading to an increase in integrin β1 receptor on the surface of the chondrocytes to receive LIPUS stimulation. It is speculated that repeated application of LIPUS exposure may exert an additive effect on chondrocytes by increasing the integrin β1 receptor. Integrin usually receives mechanical stimulation on the cell surface.[25] It has been reported that tensile stress may also increase integrin expression in fibroblasts.[26] The increase in integrin β1 due to tensile stress has been suggested to be related to the increase in cell proliferation.[27] Similarly, it has been reported that LIPUS exposure also affects cell proliferation in fibroblasts, human nucleus pulposus cells, intervertebral disc cells, and chondrocytes.[23,28–30]

Secondly, exogenous IL-1β significantly upregulated the COX-2 mRNA level, while COX-2 mRNA expression induced by IL-1β was reduced by LIPUS exposure. These results were similar to other reports. IL-1β was shown to upregulate COX-2 gene expression in human tendon cells and chondrocytes,[8,15,17] and LIPUS exposure can inhibit production of COX-2 and PGE_2 induced by IL-1β treatment in synovial membrane cells.[15,31] Several suppressive mechanisms for excessive COX-2 gene expression have been reported under specific conditions.[16,32,33] As mentioned above, the upregulation of integrin β4 protein expression inhibited LPS-induced ERK 1/2 phosphorylation in human pulmonary artery endothelial cells.[16] In addition, ERK-1/2 phosphorylation was inhibited through upregulation of integrin α5, αv, and β1 expression by cortisol in synovial fibroblasts derived from knee synovial tissues in patients with OA or rheumatoid arthritis.[34] The activated integrin signaling is mediated by the nonreceptor tyrosine kinase, focal adhesion kinase (FAK), and FAK56 mediates signaling through suppressing ERK activation.[35] A glycogen synthase kinase 3β produced by hypertonic stress negatively regulated COX-2 expression in renal medullary interstitial cells.[33] Activation of p53 resulted in inhibition of COX-2 expression by the v-src expression vector,[32] suggesting that p53 inhibits excessive COX-2 in inflammation. The degree to which the increase in p53 is related to LIPUS stimulation is unclear but warrants further examination.

It was hypothesized that the mechanisms of repression of excessive COX-2 production caused by LIPUS are due to the repression of ERK 1/2, and so the study tested for any changes in ERK phosphorylation caused by LIPUS exposure (Fig 7). First, it was found that p-ERK 1/2 expression was substantially enhanced

by the treatment with IL-1β in mandibular condylar chondrocytes. This result is similar to other reports that IL-1β activates the ERK 1/2 signaling pathway, and that the ERK 1/2 signaling pathway contributes to the induction of COX-2 expression and subsequent PGE_2 production.[17] Second, the IL-1β-induced p-ERK 1/2 was inhibited by LIPUS exposure. This result is in accord with a report that the upregulation of integrin β protein expression inhibited LPS-induced ERK 1/2 phosphorylation in human pulmonary artery endothelial cells[16] and supports the hypothesis. Additionally, this result is similar to a report on the effects of mechanical stress on IL-1β-induced ERK 1/2 phosphorylation in chondrocytes.[36] More recently, it has been shown that LIPUS inhibited MMP-13 expression through downregulation of ERK signaling in a rabbit OA model.[37] Although the detailed mechanism for explaining the inhibition of ERK 1/2 phosphorylation remains unclear, it warrants further examination.

In conclusion, LIPUS exposure inhibited IL-1β-induced COX-2 expression through the integrin β1 receptor followed by the phosphorylation of ERK 1/2. Future studies need to elucidate the effect of LIPUS on the metabolism of TMJ-OA; it will be necessary to determine the effect of LIPUS on the cartilage matrix in TMJ-OA in vivo and elucidate the detailed control mechanism by LIPUS on the matrix metabolism of TMJ-OA. Despite the restricted duration of the LIPUS effect, LIPUS is suggested to be a potential candidate for preventive and auxiliary treatment to suppress the degradation of articular chondrocytes in TMJ-OA.

Acknowledgments

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