Effects of low-intensity pulsed ultrasound on osteoclasts: Analysis with goldfish scales as a model of bone

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Analysis with goldfish scales as a model of bone

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Running title: LIPUS moderately activates osteoclasts

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Abstract

The effects of low-intensity pulsed ultrasound (LIPUS) on osteoclastogenesis were examined using fish scales that had both osteoclasts and osteoblasts. The binding of the receptor activator of NF-κB ligand (RANKL) in osteoblasts to the receptor activator of NF-κB (RANK) in osteoclasts induced osteoclastogenesis. Therefore, we focused on RANK/RANKL signaling. After 6 h of incubation following LIPUS treatment, mRNA expression of RANKL increased significantly. Resulting from the increased RANKL mRNA level, the expression of transcription-regulating factors significantly increased after 6 h of incubation, and then the mRNA expression of functional genes was significantly up-regulated after 12 h of incubation. However, the mRNA expression of osteoprotegerin (OPG), which is known as an osteoclastogenesis inhibitory factor, also significantly increased after 6 h of incubation and tended to further increase after 12 h of incubation. At 24 h of incubation, osteoclastic functional genes' mRNA expression decreased to the level of the control. Furthermore, we performed an *in vivo* experiment with goldfish. Two weeks after daily LIPUS exposure, osteoclastic marker enzymes tended to decrease while osteoblastic marker enzymes were activated. The regeneration rate of the LIPUS-treated scales was significantly higher than that of the control scales. Thus, LIPUS moderately activates osteoclasts and induces bone formation.

Low-intensity pulsed ultrasound (LIPUS), a noninvasive remedial measure, promotes the repair of bone fracture and distraction osteogenesis (see a review, 25). Until now, the study of LIPUS has focused on osteoblastic growth and differentiation (3, 8, 10). However, the details of the direct effect of LIPUS on osteoclasts are still not fully understood.

Recently, the mechanism of osteoclastic activation has been clarified (11). Namely, the receptor activator of NF-κB (RANK) and the receptor activator of NF-κB ligand (RANKL) have been identified in mammals (1). Subsequently, it has been found that the fusion of pre-osteoclasts is induced to form multinucleated osteoclasts (active type of osteoclasts) by binding RANKL in osteoblasts to RANK in osteoclasts (11). In addition, osteoprotegerin (OPG), which is produced by osteoblasts, functions as a decoy receptor of RANKL and suppresses osteoclastogenesis (5, 9). Thus, RANK/RANKL and OPG signaling regulate osteoclastic activation (Fig. 1).

Analyzing osteoclastic activation requires an experimental system that contains both osteoclasts and osteoblasts. The teleost scale is a calcified tissue that contains osteoclasts, osteoblasts, and bone matrix (2, 4, 12, 15, 17, 18, 27). Using fish scales, we have developed an original *in vitro* culture system (15, 16) that is quite sensitive to hypergravity loading (17, 19), suggesting

that fish scale is a suitable model for analyzing physical stimuli. In addition, we identified RANK and RANKL sequences from goldfish scales and reported that RANK and RANKL mRNA expressions change in response to parathyroid hormone and prostaglandin E_2 using a scale *in vitro* culture system as parathyroid hormone and prostaglandin E_2 do in mammals (13, 20).

With our original *in vitro* scale culture system, the effect of LIPUS on the osteoclasts of goldfish scales was examined, focusing on the interaction between osteoclasts and osteoblasts on the basis of mRNA expression analyses of RANK/RANKL and OPG signaling. To confirm the *in vitro* data, an *in vivo* experiment with goldfish was performed. We demonstrated that LIPUS moderately activates osteoclasts and promotes the regenerating ratio in the scales of goldfish.

MATERIALS AND METHODS

Animals Goldfish (*Carassius auratus*) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan). Then, we artificially fertilized one pair (a female and a male) of goldfish (20 – 30 g) at the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet for

puffer fish every morning and were maintained in fresh water at 26°C. After growing the fish to 12 – 15 cm in body length, the goldfish were moved to the Noto Marine Laboratory of Kanazawa University and used for the *in vitro* experiments.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

Ultrasound conditions The Sonic Accelerated Fracture-Healing System (SAFHS) 4000J (Teijin Pharma Ltd., Tokyo, Japan) through a transducer (effective area: 3.88 cm^2) at a frequency of 1.5 MHz with a pulsed-wave mode (pulse-burst width: 0.2 s; pulse repetition frequency: 1 kHz; and intensity: 30 mW/cm^2) was used in the present study. This ultrasound generator is the same as Exogen's SAFHS (Exogen Inc., Piscataway, NJ, USA).

LIPUS treatments and RNA isolation We recently developed a culture system using regenerating scales that had active osteoclasts and osteoblasts (19). The present study examined the influence of LIPUS on osteoclasts with

regenerating scales. Goldfish were anesthetized with ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA), and the normal scales that developed on the body were then removed to allow the regeneration of scales. On day 14, goldfish were anesthetized again, and the regenerating scales were removed. Using the removed regenerating scales, the influence of LIPUS on osteoclasts was examined. After LIPUS treatment (20 min), the scales were incubated at 15°C for 3, 6, 12, and 24 h with Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., Aurora, OH, USA) and then frozen at -80°C for mRNA analysis. The LIPUS-untreated scales were incubated in the same conditions as the experimental group and compared with LIPUS-treated scales. Before incubation (0 h), the scales were also frozen at -80°C and compared with incubated scales.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissues (Qiagen GmbH, Hilden, Germany) and treated with DNase I (RNase-Free DNase Kit, Qiagen GmbH) for 15 min at room temperature to remove residual genomic DNA. Complementary DNA synthesis was also performed using a kit (Qiagen GmbH).

Real-time quantitative PCR The PCR amplification was analyzed with a real-time PCR apparatus (Mx3000pTM, Stratagene, La Jolla, CA, USA) using SYBR Premix ExTaq (Takara Bio, Shiga, Japan) (20, 22). Additions of RANK, RANKL, OPG, and functional genes (matrix metalloproteinase-9: MMP-9; cathepsin K) for osteoclasts, TNF receptor-associated factor 6 (TRAF6) and the nuclear factor of activated T-cells and cytoplasmic 1 (NFATc1) were examined as transcription-regulating factors for osteoclasts (Fig. 1). Real-time qPCR assay was performed using the specific primers listed in Table 1. The annealing temperature for RANK, RANKL, TRAF6, NFATc1, MMP-9, cathepsin K, OPG, and elongation factor-1α was 60°C. Each mRNA expression level was normalized to the mRNA expression level of elongation factor-1α.

In vivo *experiment* Goldfish were anesthetized with ethyl 3-aminobenzoate methanesulfonic acid salt (Sigma-Aldrich), and the normal scales that developed on the body were then removed to allow the regeneration of scales. To recover from the wound on the body's surface, the goldfish were kept in the aquarium for 3 days. Thereafter, LIPUS was exposed to the left side of the anesthetized goldfish every evening. Two weeks after daily LIPUS exposure

(3 to 14 days), the scales were taken out from goldfish under anesthesia. The osteoclastic and osteoblastic marker enzyme activities were measured. We examined the influences of LIPUS on the osteoclasts and osteoblasts with tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as respective markers (19). Details of the methods used are the same as those described by Suzuki et al. (2009) (19). We compared the activities of scales (exposure) on the left side of the goldfish with the scales (control) on the right side of the goldfish. Furthermore, to examine the influence of LIPUS on bone formation, regeneration rates (final surface area of scales/ initial surface area of the scales) of the scales on the left side (exposure) of the goldfish were calculated and compared with those of the scales on the right side (control) of the goldfish.

Statistical analysis All results are expressed as means ± SEM. The values of the control scales were compared with those of the experimental scales. Data were assessed using a paired *t*-test, and the significance level chosen was $P < 0.05$.

RESULTS

The mRNA expression of RANK and RANKL

The time courses of mRNA expression for RANK and RANKL after LIPUS treatment are indicated in Figure 2A and 2B, respectively. RANK mRNA levels significantly decreased at 3 h of incubation after LIPUS exposure. Thereafter, RANK mRNA expression recovered to control levels.

In the case of RANKL mRNA expression, LIPUS treatment increased the levels at all incubation times. LIPUS-treated scales and control scales differed significantly after 6 and 12 h of incubation following exposure.

The mRNA expression of transcription-regulating factors

The data after 3, 6, and 12 h of incubation are shown in Figure 3A, 3B, and 3C, respectively. Both TRAF6 and NFATc1, which are transcription-regulating factors related to RANKL signaling, increased significantly after 6 h of incubation following LIPUS exposure. Thereafter, TRAF6 and NFATc1 mRNA levels in the LIPUS-treated scales tended to decrease as compared with those of control scales after 12 h of incubation.

The mRNA expression of osteoclast functional genes

The data after 6, 12, and 24 h of incubation are shown in Figure 4A, 4B, and 4C, respectively. The level of MMP-9 mRNA increased significantly after 12 h of incubation following LIPUS exposure. Subsequently, MMP-9 mRNA levels in the LIPUS-treated scales did not change significantly as compared with those in control scales. In the case of cathepsin K mRNA expression, mRNA levels increased significantly after 6 and 12 h of incubation. Thereafter, the mRNA expression of cathepsin K in the LIPUS-treated scales decreased to the control level after 24 h of incubation.

The mRNA expression of OPG

The mRNA expressions of OPG, an osteoclastogenesis inhibitory factor, are indicated in Figure 5. Following LIPUS exposure, OPG mRNA levels increased after 3, 6, and 12 h of incubation. LIPUS-treated scales and control scales were significantly different after 6 h of incubation following exposure. After 12 h of incubation, OPG levels tended to maintain the increased level ($P = 0.071$).

In vivo *experiment*

To confirm the *in vitro* data, we performed an *in vivo* experiment with the

regenerating scales of goldfish. Two weeks after daily LIPUS exposure, an osteoblastic marker enzyme (ALP) had been activated significantly (Fig. 6B), although an osteoclastic marker enzyme (TRAP) tended to decrease (Fig. 6A). Furthermore, bone formation was promoted by osteoblastic activation. The regeneration rate of the treated scales increased significantly as compared with that of the control scales (Fig. 6C).

DISCUSSION

In the present study, we focused on osteoclasts and examined the mRNA expression of RANKL, an osteoclast-activating factor. After LIPUS exposure, we found that the mRNA levels of RANKL were increased at all incubation times. Significant differences between LIPUS-treated scales and control scales were obtained after 6 and 12 h of incubation following exposure. After 6 h of incubation, the mRNA expression of transcription-regulating factors, such as TRAF6 and NFATc1, significantly increased. Then, after 12 h of incubation, the mRNA levels of functional genes (MMP-9 and cathepsin K) in the LIPUS-treated scales were significantly upregulated. These results indicate that LIPUS treatment causes osteoclastic activation *via* RANK/RANKL signaling (see Fig. 1).

OPG is known as an osteoclastogenesis inhibitory factor in osteoblasts (5, 9). We found that OPG mRNA levels increased at all incubation times. As OPG, a decoy RANKL receptor, suppresses osteoclastogenesis that results from being bound to RANKL, this finding suggests that OPG functions to suppress excess bone resorption by LIPUS. Bone is a metabolically active tissue that undergoes continuous remodeling, a process that largely relies on the activity of osteoclasts to remove bone and of osteoblasts to form bone (see a review, 23). In this process, osteoblasts and osteoclasts interact with each other; thus, moderate bone resorption is necessary to promote osteogenesis. Furthermore, regarding RANKL and OPG mRNA expression following LIPUS treatment, a similar phenomenon has been reported in a mouse cementoblast cell line (7). Therefore, the data of mammalian hard tissue support our present results.

Using GeneChip analysis of zebrafish scales treated with LIPUS, we found that cell death-related genes were upregulated (21). Furthermore, the multinuclei of TRAP-stained osteoclasts in the scales were labeled with TUNEL for DNA breaks (21). Our previous report (21) indicates that LIPUS directly functions in osteoclasts and promotes apoptosis in osteoclasts shortly after exposure. In the present study, the mRNA expression of RANK

(osteoclastic marker) decreased after 3 h of incubation following LIPUS treatment. The inhibition of osteoclasts shortly after exposure might be effective for the suppression of excess bone resorption. In an *in vivo* experiment using the tooth of mice, the lengths of root resorption lacunae and the amount of the root resorption area in the LIPUS-treated group were smaller as compared with those in the control group during the 3-week period of daily exposure (7). The numbers of both odontoclasts and osteoclasts were also significantly lower in the LIPUS-treated group (7). The results of *in vivo* experiments with mice (7) suggest the induction of apoptosis in osteoclasts shortly after LIPUS exposure, which should play an important role in inhibiting excess bone resorption. In the present *in vivo* experiment, actually, we demonstrated that after 2 weeks following daily LIPUS exposure, the osteoblastic marker enzyme activated and regeneration rate of the treated-scales was significantly higher than that of the control scales. At 2 weeks after, osteoclastic marker enzyme tended to decrease as the mouse model did.

We have previously analyzed scale osteoblastic and osteoclastic responses under 2-, 3-, and 4-gravity (G) loading by both centrifugation and vibration (26). As a result, osteoblastic activity significantly increased under 2- to 4-G loading by both centrifugation and vibration (26), as it did in the

present study. It is known that bone matrix plays an important role in the response to physical stress (6, 14, 24). As teleost scale is a calcified tissue that contains osteoclasts, osteoblasts, and bone matrix (4, 12, 18, 27), we conclude that fish scales are a suitable model for physical stimuli, including LIPUS, to examine the interaction between osteoclasts and osteoblasts.

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Table legend

Table 1 Primer sequences for real-time quantitative PCR

Figure Legends

Figure 1 RANK/RANKL signaling pathway. The binding of RANKL in osteoblasts to RANK in osteoclasts activates several transcription-regulating factors in osteoclasts and then synthesizes cathepsin K or matrix metalloproteinase-9 (MMP-9). On the other hand, osteoprotegerin (OPG) is an osteoclastogenesis inhibitory factor in osteoblasts. OPG, a decoy receptor of RANKL, inhibits osteoclastogenesis by binding to RANKL. LIPUS may promote bone formation by moderate osteoclastic activation regulating the RANK/RANKL and OPG systems. AP-1: Activator protein 1; MAPK: MAP-Kinase.

Figure 2 Effects of LIPUS on the receptor activator of NF-κB (RANK) and the receptor activator of the NF-κB ligand (RANKL) after 0, 3, 6, 12, and 24 h of incubation. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values of the control scales of goldfish. n = 8 samples; one sample from one fish.

Figure 3 Effects of LIPUS on TNF receptor-associated factor 6 (TRAF6), c-Fos, and the nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) after 3 (A), 6 (B), and 12 (C) h of incubation. * indicates a statistically significant difference at $P < 0.05$ from the values of the control scales of goldfish. n = 7 samples; one sample from one fish.

Figure 4 Effects of LIPUS on matrix metalloproteinase-9 (MMP-9) and cathepsin K after 6 (A), 12 (B), and 24 (C) h of incubation. *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, from the values of the control scales of goldfish. n = 7 samples; one sample from one fish.

Figure 5 Effects of LIPUS on osteoprotegerin (OPG) mRNA expression after 0, 3, 6, 12, and 24 h of incubation. * indicates a statistically significant difference at $P < 0.05$ from the values of the control scales of goldfish. n = 7 samples; one sample from one fish.

Figure 6 Effects of LIPUS on osteoclastic (A) and osteoblastic (B) enzyme markers in an *in vivo* experiment 2 weeks after daily LIPUS exposure. The rate of scale regeneration (final surface area/ initial surface area) was calculated and compared between treated scales and control scales (C). * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values of the control scales of goldfish. Each enzyme's activity: n = 5 samples; one sample from one fish. Regeneration rate: n = 10; one sample from one fish.

Table 1. Primer sequences for real-time quantitative PCR

Primer sets for RANK, RANKL, TRAF6, NFATc1, MMP-9, Cathepsin K, OPG, and Elongation factor-1α are presented.

Name	Forward primer	Reverse primer	Accession No.
RANK	GGGAGATGCTGCGAAAAATG	TTTAGGGTTGTGTGGACGAGTG	AB894121
RANKL	CGAGTGTGGCGATTTTGTTG	ATGGGCGTCTTGATTGGAAG	AB894120
TRAF6	TCTGATGGGTCTTCGCTCGGCT	ACTGGACATTTCTGCCCCGTGT	LC149878
NFATc1	CTGTGGCTTTGCTTGTGGATGTC	ACACAAGGCCTTAGTTCTGATGGC	AB685221
MMP-9	GCTTCTGCCCCAGTGAGCTT	GTGGAGCACCAGCGATACCC	AB889498
Cathepsin K	TGGGAGGGCTGGAAACTCAC	CATGAGCCGCATGAACCTTG	AB236969
OPG	CGTGAACACGGTGTGCGAGTGT	CCTCTGCGCAGGCCTCACA	AB970727
Elongation factor-1α	ATTGTTGCTGGTGGTGTTGG	GGCACTGACTTCCTTGGTGA	AB979720

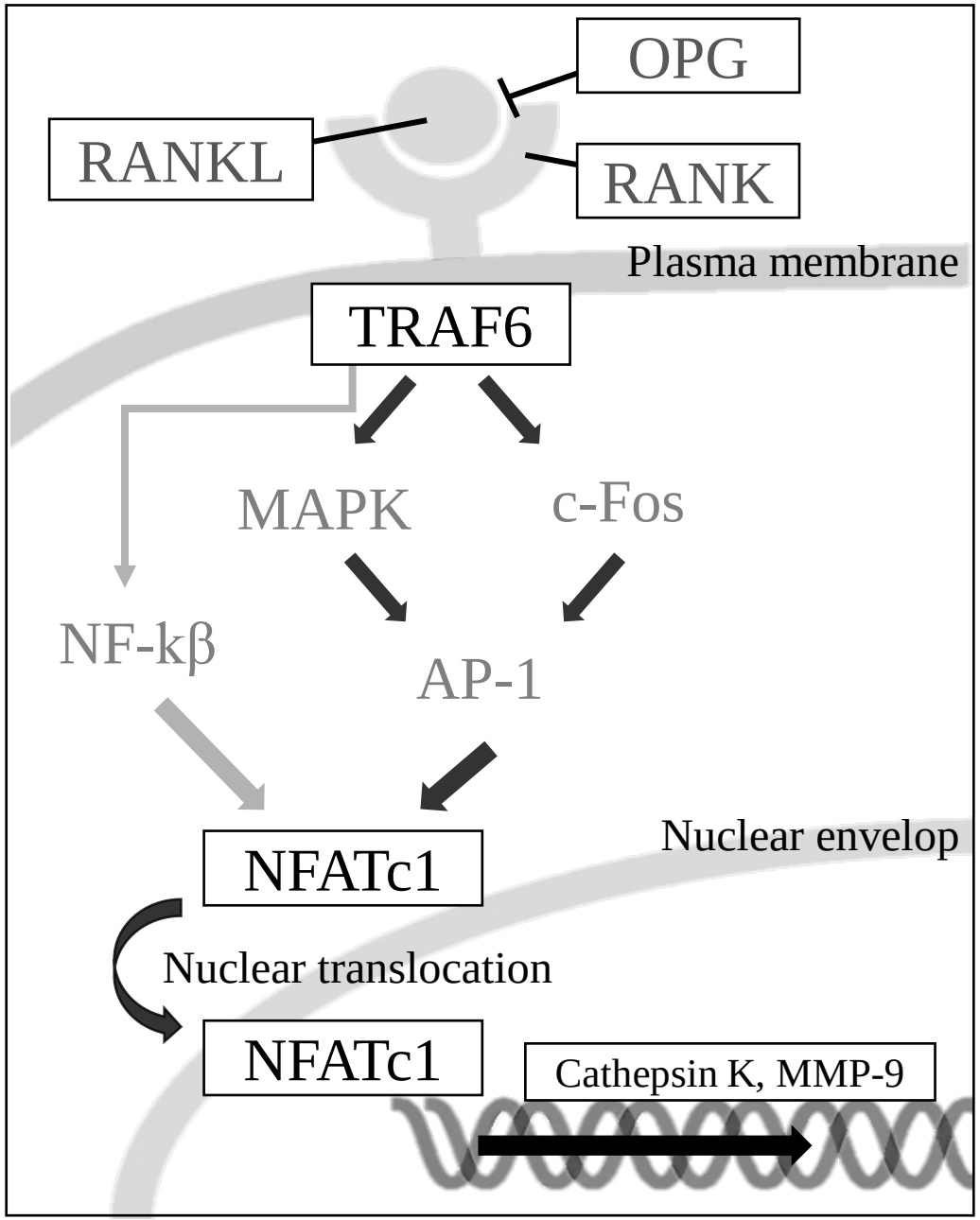

Figure 1 Hanmoto et al.

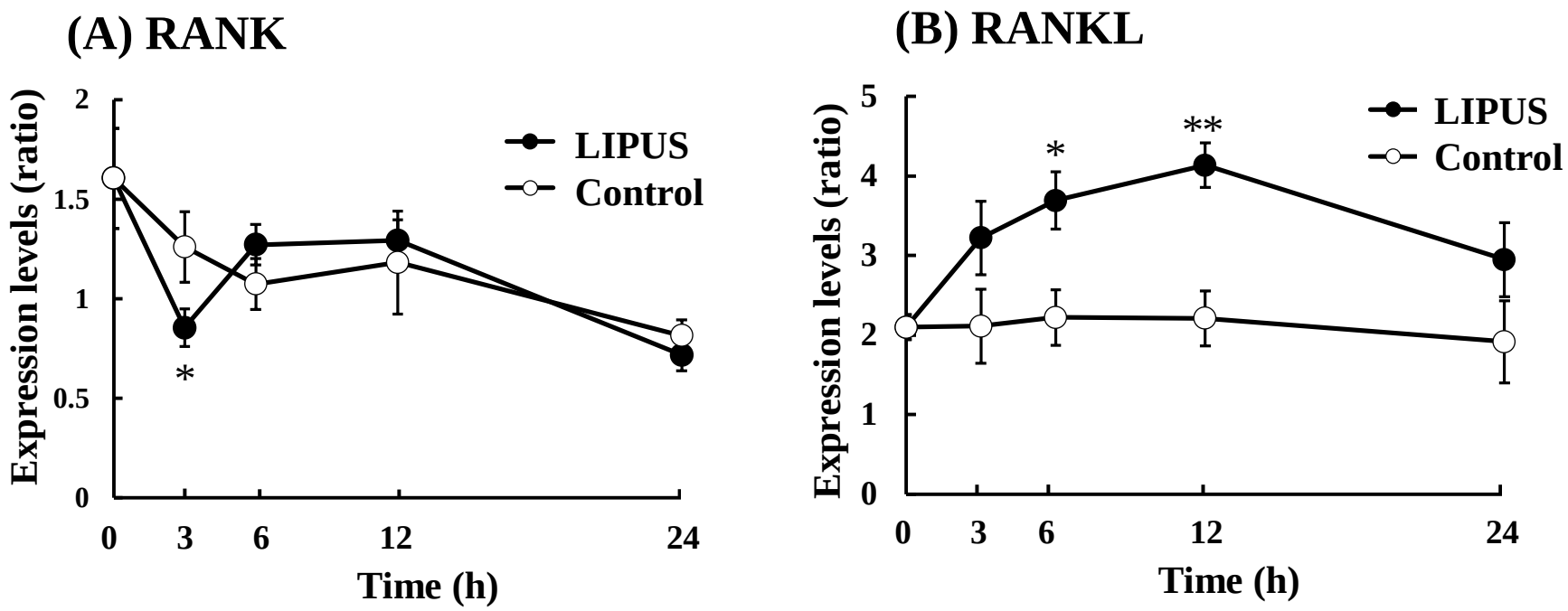

Figure 2 Hanmoto et al.

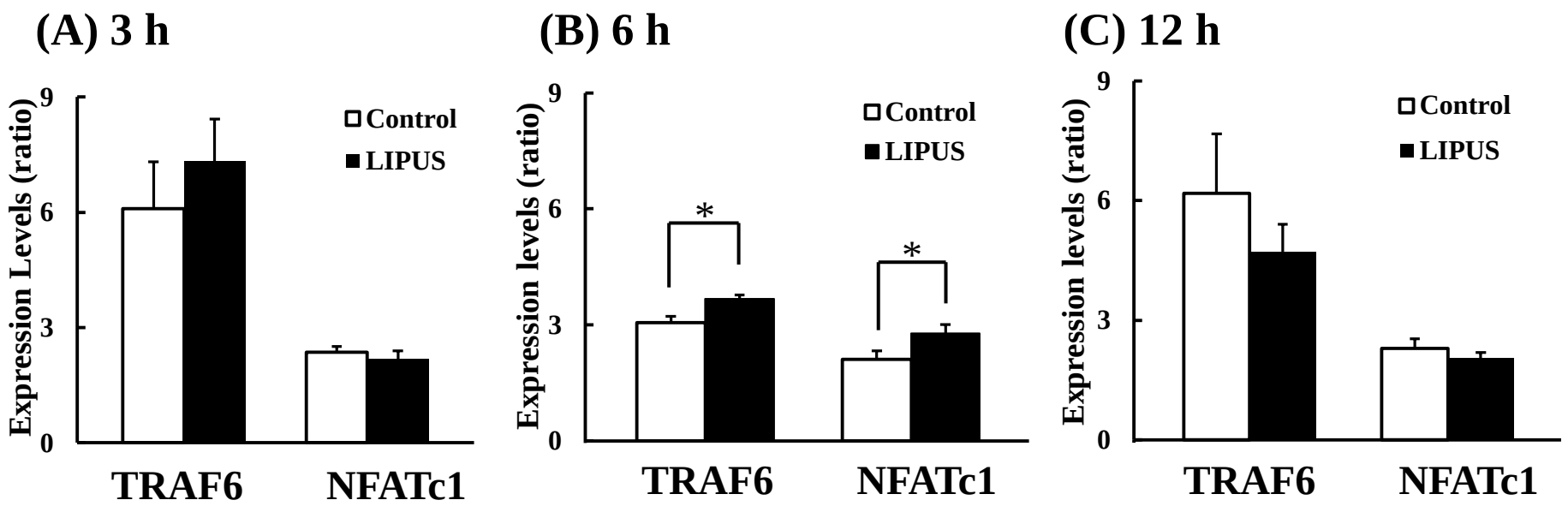

Figure 3 Hanmoto et al.

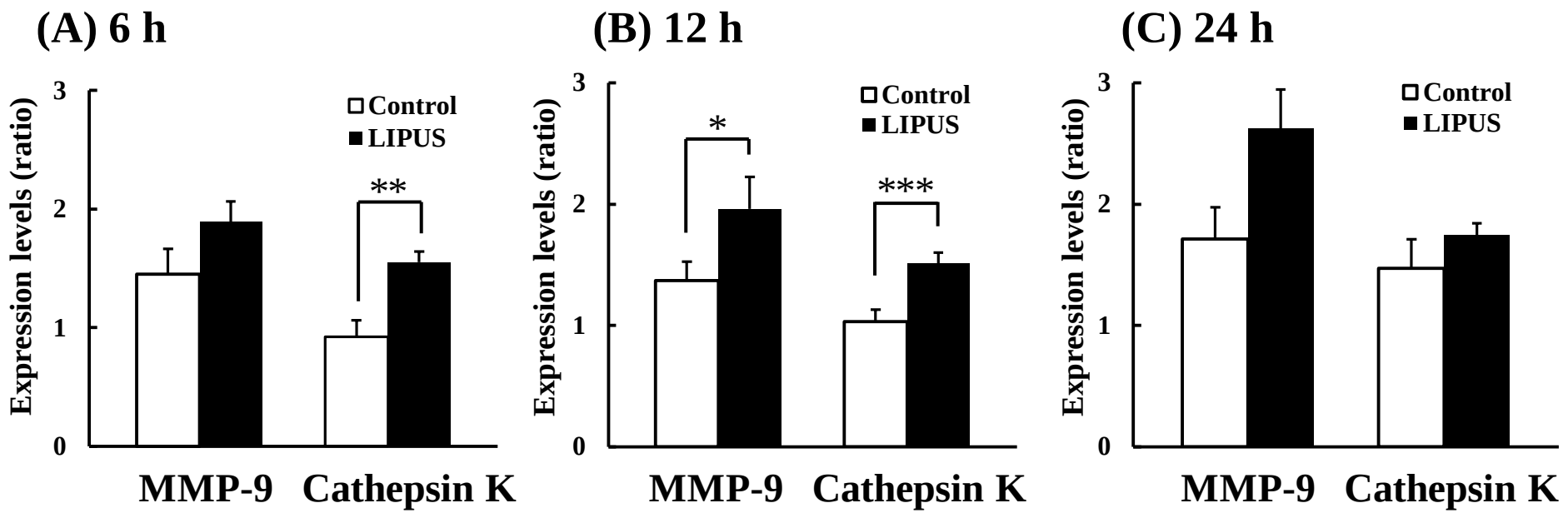

Figure 4 Hanmoto et al.

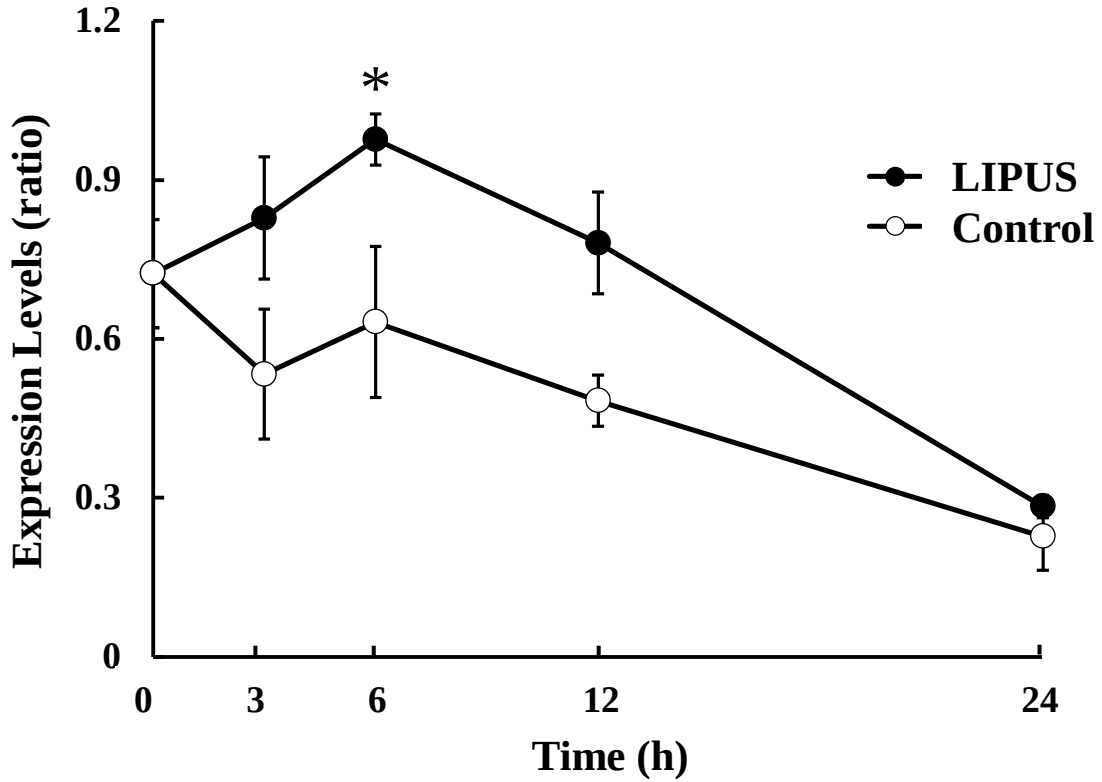

Figure 5 Hanmoto et al.

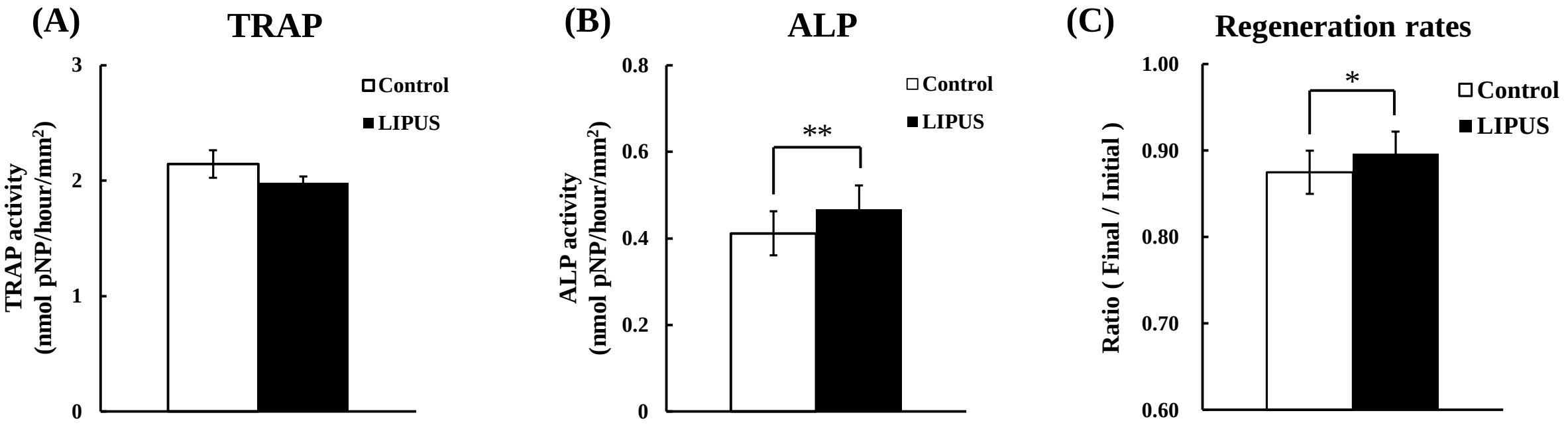

Figure 6 Hanmoto et al.