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Relation	

Stimulation of nuclear receptor REV-ERBs alleviates monosodium iodoacetate-induced osteoarthritis pathology of mice and the induction of inflammatory molecules expression in primary cultured chondrocytes

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Abstract

Because inflammation in chondrocytes contributes to the induction of osteoarthritis (OA), regulation of their activity is essential. A previous study showed that stimulation of the reverse erythroblastosis virus (REV-ERB) nuclear receptors in spinal glial cells elicits anti-inflammatory and antinociception effects in animal models of chronic pain. However, the involvement of REV-ERBs in chondrocyte functions and OA pathologies remains to be elucidated. In the current study, we found that pretreatment with the REV-ERB agonist SR9009 significantly blocked the increases in inflammatory molecules [(matrix metalloproteinase (MMP) 3, MMP9, and MMP13] and cytokines (interleukin-1β and tumor necrosis factor) in primary cultured chondrocytes following treatment with lipopolysaccharide. Furthermore, repeated intra-articular treatment with SR9009 significantly prevented monosodium iodoacetate-induced mechanical hypersensitivity and tended to partially reduce knee joint damage in mice. In conclusion, our findings suggest that REV-ERBs have a critical role in alleviating nociceptive hypersensitivity in OA pathologies by negatively regulating inflammation in chondrocytes.

Keywords

REV-ERBs; chronic pain; chondrocyte; osteoarthritis; lipopolysaccharide; monosodium iodoacetate

Abbreviations

DAMPs; damage-associated molecular patterns, GAPDH; glyceraldehydes-3-phosphate dehydrogenase, iNOS; inducible nitric oxide synthase, IL-1β; interleukin-1β, IL-6; interleukin-6, LPS; lipopolysaccharide, MIA; monosodium iodoacetate, MMP; matrix metalloproteinase, OA; osteoarthritis, REV-ERB; Reverse erythroblastosis virus, TLR; Toll-like receptor, TNF; tumor necrosis factor

1. Introduction

Osteoarthritis (OA) is the most prevalent musculoskeletal disorder, affecting approximately 200 per 100,000 individuals worldwide. The main symptom of OA is chronic pain associated with the disease's progression, such as joint destruction. OA pain reduces quality of life, and the loss of social productivity is significant, particularly when OA prevents the elderly from working. Non-steroidal anti-inflammatory drugs, steroids, and opioids are used to treat the chronic pain caused by OA, but their effects are insufficient, and side effects associated with long-term use are frequent. Although joint pathologies are improved by joint replacement, it is highly invasive, and 20%–30% of patients have residual pain [1].

Damage-associated molecular patterns (DAMPs) from degraded cartilage matrix are involved in exacerbation of inflammation by acting on Toll-like receptor (TLR) 4 expressed on chondrocytes and synovial cells, which induce inflammatory responses by increasing the expression of proinflammatory cytokines and matrix-degradative enzymes such as matrix metalloproteinases (MMPs) [2]. These factors further exacerbate the inflammatory response of chondrocytes, ultimately leading to cell death. Therefore, it is very important to regulate the inflammatory response in chondrocytes to control OA pathology.

Reverse erythroblastosis virus (REV-ERB) α and β are orphan nuclear

receptors expressed in various cell types [3]. REV-ERBs exert ligand-dependent inhibitory effects on the transcription of various genes by binding to promoter regions and are involved in a wide range of functions [4]. In particular, REV-ERBs function negatively on the regulation of inflammatory responses, including suppression of proinflammatory cytokine expression [5]. Therefore, REV-ERBs are potentially useful drug targets for inflammatory diseases, including OA. We previously found that REV-ERB agonists exert analgesic effects in chronic pain models, which are also pathologically based on inflammation, by inhibitory regulation of glial cell functions in the spinal dorsal horn [3, 5]. However, the involvement of REV-ERBs and their role in OA pathogenesis, especially in chondrocytes, have been insufficiently investigated. Therefore, in this study, we examined the effect of REV-ERB agonist SR9009 on the lipopolysaccharide (LPS)-induced expression of inflammatory molecules in primary cultured chondrocytes from rat knee joints. Furthermore, the analgesic and protective effects of SR9009 on knee cartilage damage were investigated in an OA model induced by intra-articular injection of monosodium iodoacetate (MIA).

2. Materials and Methods

2.1. Animals

All experiments using animals were conducted by the “Guidelines for the Care and Use of Laboratory Animals” established by The Japanese Pharmacological Society and Hiroshima University, and the experimental procedure was reviewed and approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University (A18-140-3). Male ddY mice (8 weeks) and pregnant Wistar rats (13 days) were obtained from Japan SLC, Inc. (Shizuoka, Japan). One day old pups of both sexes were obtained from pregnant rats.

2.2. Reagents

MIA was obtained from NACALAI TESQUE (Kyoto, Japan), and dissolved in sterile saline. SR9009 and LPS were prepared as described previously [5].

2.3. Animals model

MIA-induced OA model was developed based on a previous study [6]. Briefly, under anesthesia, MIA (0.75 mg/10 μl) were injected into the left knee joint. Sterile saline was injected as control. SR9009 (300 nmol/5 μl) was injected to the left knee joint 3 times a week starting at 3 days after intra-articular injection of MIA.

2.4. Assessment of pain behavior

Withdrawal thresholds (in grams) of the hind paw to mechanical stimulation were determined as previously described [5]. Withdrawal thresholds were measured before MIA injection (0 day), and 3, 7, 14, 21, 28 days after the MIA injection.

2.5. Safranin-O staining

To evaluate cartilage matrix degradation, the cartilage matrix was stained with Safranin-O as described previously [7]. Sections were examined with a BZ-9000 Biorevo all-in-one fluorescence microscope (Keyence, Elmwood Park, NJ, USA). In the images, the thickness of the cartilage matrix in the femur and tibia was measured from the surface of the articular cartilage to the surface of the subchondral bone in the area stained red using Image J.

2.6. Cell culture

Primary cultured chondrocytes of rat knee cartilage were prepared as described previously [2].

2.7. RT-PCR analysis

Preparation of total RNA, synthesis of cDNA, protocol of RT-PCR assay and the sequences of primers for Rev-erbα and Rev-erbβ were previously described [3]. The sequences of primers for RPL13A were 5'-TTGTGCGGCTGAAGCCTA-3' (forward) and 5'-GCCTCAAGAGCTGCTTCT-3' (reverse). The resulting PCR products had the size (Rev-erbα; 198 bp, Rev-erbβ; 166 bp, RPL13A; 153 bp) expected from the known cDNA sequence.

2.8. Real-time PCR analysis

Analysis of changes in mRNA expression levels in cultured chondrocytes was performed by real-time PCR as described previously [2, 5].

2.9. Statistical analysis

Data are expressed as the mean ± SEM. ROUT test (Q = 10%) was performed to identify and exclude outliers from analyses. Comparisons of mRNA expression were performed using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Tukey-Kramer method. Confirming the significance in the pain assessment were analyzed by two-way repeated-measures ANOVA, followed by the Tukey-Kramer method for multiple comparisons. Differences were considered to be significant when the P value was less than 0.05.

3. Results

3.1. REV-ERB agonist blocks LPS-induced mRNA upregulation of proinflammatory molecules and matrix degradation enzymes in primary cultured chondrocytes

Rat primary cultured knee chondrocytes expressed Rev-erbα and Rev-erbβ mRNAs (Fig. 1a). The PCR-amplified products of these transcripts were absent in control reactions that omitted the RT reaction, which demonstrated that they were derived from their respective mRNAs and not from genomic DNA (Fig. 1a).

We examined the effects of the REV-ERB agonist on inflammatory responses in primary cultured rat chondrocytes using LPS, which activates TLR4 as an inflammatory stimulus. The mRNA expression of proinflammatory molecules [interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF), and inducible nitric oxide synthase (iNOS)] and MMP3, MMP9, and MMP13 was significantly increased at 24 hours after 100 ng/ml LPS treatment (Fig. 1b). Pretreatment with 10 μM SR9009 significantly suppressed the LPS-induced mRNA upregulation of IL-1β, TNF, MMP3, MMP9, and MMP13, but not that of IL-6 or iNOS (Fig. 1b).

3.2. Intra-articular injection of REV-ERB agonist alleviates MIA-induced mechanical hypersensitivity and partially inhibits MIA-induced cartilage

destruction

When administered to the knee joint, MIA induces chondrocyte death by inhibiting glyceraldehyde-3-phosphate dehydrogenase, which leads to inflammation and cartilage destruction in the joint. Administration of MIA to the knee joint of mice also tended to decrease the withdrawal threshold to mechanical stimuli from 3 days after administration, and a significant decrease was observed on day 7, which persisted until 28 days (Fig. 2a). We found that repeated intra-articular administration of 300 nmol SR9009 starting from 3 days after MIA administration significantly suppressed the decrease in withdrawal threshold to mechanical stimuli observed persistently after the 7 days of MIA administration (Fig. 2a). Similar administration of SR9009 alone did not affect the withdrawal threshold to mechanical stimulation (Fig. 2a).

We next examined the effect of SR9009 on cartilage degradation in knee joints after MIA administration. The results showed significantly decreased cartilage matrix in the femur and tibia at 28 days after intra-articular injection of MIA (Fig. 2b). Repeated intra-articular administration of SR9009 tended to prevent MIA-induced cartilage matrix degradation at both sites, but these responses were not significant (Fig. 2b).

4. Discussion

Using primary cultured rat knee chondrocytes, we found that the REV-ERB agonist significantly suppressed the inflammatory responses induced by LPS. Furthermore, intra-articular administration of the REV-ERB agonist significantly improved mechanical hypersensitivity and partially suppressed knee cartilage degradation. These results suggest that the anti-inflammatory effect of REV-ERB is important in controlling OA pathology.

In OA, mechanical stimuli cause cartilage degeneration and wear, which in turn accelerate cartilage degeneration by causing inflammation in the joint. DAMPs from degenerated chondrocytes are involved in the progression and exacerbation of OA pathology by further promoting inflammatory responses in the surrounding tissues [8]. We previously showed that stimulation of primary cultured chondrocytes with LPS induces the expression of inflammatory cytokines, matrix degradation enzymes, and iNOS through the stimulation of TLR4 [2]. These findings suggest that DAMP-TLR4-mediated inflammatory responses in chondrocytes are crucial for the progression of OA pathology. Therefore, the search for mechanisms to inhibit these reactions is of great importance for controlling OA pathogenesis.

Although mRNAs of Rev-erbs were expressed in chondrocytes derived from rat knees, little has been reported on their role and function. REV-ERBs act as nuclear

receptors and inhibitory regulators of the expression of various genes, and the specific REV-ERB agonist suppresses gene expression of inflammatory factors in immune cells [9]. We found that the REV-ERB agonist SR9009 suppressed the expression of various inflammatory factors, which have been reported to be associated with OA pathology, in primary cultured chondrocytes stimulated by LPS to an inflammatory state, suggesting that REV-ERB in chondrocytes also has an anti-inflammatory effect similar to that observed in immune cells. However, the action of the REV-ERB agonist in chondrocytes had no effect on IL-6 or iNOS expression, which differs from its effects in other cells [5, 10]. A previous report has suggested that the genes regulated by REV-ERB may differ among cell types and animal species [4].

The inflammatory factors with upregulated expression by LPS and suppressed expression by REV-ERB stimulation in chondrocytes are all involved in the development of pain associated with OA. Hence, the in vitro anti-inflammatory effect of SR9009 raises the prospect of an analgesic effect through REV-ERB activation in OA models. In this study, repeated intra-articular administration of SR9009 significantly improved hypersensitivity to mechanical stimuli in a MIA-induced OA model, whereas a single dose had no effect (data not shown). Because a continuous inflammatory response is expected to occur in the knee joint during OA, a single stimulation of REV-ERB alone is unlikely to adequately control these responses.

Repeated intra-articular administration of SR9009 exerts analgesic and ameliorative effects on joint damage [11]. Furthermore, pain-related genes upregulated in primary sensory neurons (dorsal root ganglia; DRGs) by OA are suppressed by SR9009 administration. The increased expression of the clock gene Bmal1, which contributes to the expression of various inflammatory factors, in the DRGs of OA model mice is suppressed by SR9009 [11]. Therefore, these responses are related to the modulation of clock genes in DRGs. However, this previous study does not mention the role of REV-ERB in chondrocytes. Furthermore, another report contradicts our findings by indicating that the REV-ERB antagonist prevents the progression of OA pathology by inhibiting cartilage degeneration [12]. This study used a different OA model (destabilization by medial meniscus surgery) and did not assess pain. In our study, the inhibitory effect of SR9009 on cartilage degeneration was weak and not significant, suggesting that the mechanism of action of REV-ERB on chondrocyte defense is complex and independent of its anti-inflammatory action.

With regard to the specificity of the action by SR9009, it is important to confirm using the REV-ERBs antagonist. On the other hand, in previous study, the REV-ERBs antagonist SR8278 suppressed the effects of REV-ERBs themselves, but the antagonistic effect of SR8278 on the action produced by the REV-ERB agonist was not examined [13]. Therefore, as the relationship between SR9009 and SR8278 may

be different from the previously known agonist/antagonist relationship to G-protein coupled receptors, other methods may need to be investigated in the future.

In various pain models, REV-ERB agonists exhibit analgesic effects on the basis of their anti-inflammatory functions [5, 14]. The present study suggests that the REV-ERB agonist is a novel analgesic agent for OA by negatively regulating inflammatory responses in chondrocytes. Unlike rheumatoid arthritis, which is also a knee inflammatory disease, antibody preparations that target single inflammatory cytokines, such as IL-1β and IL-6, are not successful in treating OA [15]. These findings suggest that OA is a complex and multifaceted condition involving multiple inflammatory factors, and that drugs targeting only a single factor are insufficient. Thus, REV-ERBs inhibit the induction of various inflammatory factors in a multimodal manner and may be a suitable therapeutic target for OA. More detailed analysis of the analgesic effects of REV-ERBs may lead to the development of novel therapeutic strategies for OA.

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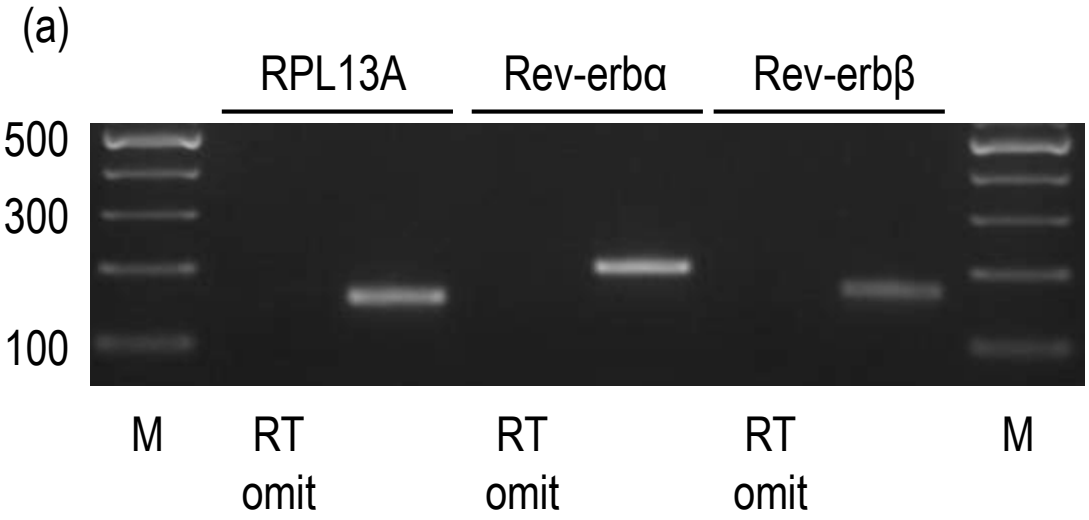
Figure Legends

Fig. 1. (a) Rev-erbs mRNA are expressed in cultured chondrocytes. Molecular size markers are in lane “M” (100 bp ladder). Lanes “RT omitted” represents the PCR products with the reverse transcription reaction omitted. (b) Effects of REV-ERBs agonist on LPS-induced inflammatory factor expression in cultured chondrocytes. Thirty minutes after treatment with SR9009 (10 μM), cells were stimulated with LPS (100 ng/ml) for 24 hours. Data represent means ± SEM of from six to nine independent experiments. **P < 0.01 vs. vehicle treatment. †P < 0.05, ††P < 0.01 vs. LPS treatment alone.

Fig. 2. Effects of SR9009 on MIA-induced mechanical hypersensitivity and cartilage degradation. (a) Three days after injection of either MIA (0.75 mg) or sterile saline, either SR9009 (300 nmol) or DMSO was injected intraarticularly three times a week, and hind paw withdrawal thresholds were assessed over time. Data represent means ± SEM of ten (vehicle, MIA, MIA+SR) and nine (SR) mice. *P < 0.05, **P < 0.01 vs. saline-injected mice with DMSO at the corresponding time point. †P < 0.05, ††P < 0.01 vs. MIA-injected mice with DMSO at the corresponding time point. (b) Effects of SR9009 on MIA-induced cartilage degradation. After treatment with either MIA or sterile saline, and either SR9009 or DMSO as mentioned above, left knee joint tissues

were stained with Safranin-O. Scale bar = 100 μm. Quantitative analysis of thickness of the cartilage matrix (left; femur, right; tibia) was shown below. Data represent means ± SEM of seven (vehicle, MIA+SR, SR) and eight (MIA) mice. **P < 0.01 vs. saline-injected mice with DMSO.

Figure 1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Rev-erbα
Rev-erbβ
500
300
100
M
RT omit
RT omit
RT omit
M

Figure 1
(b)

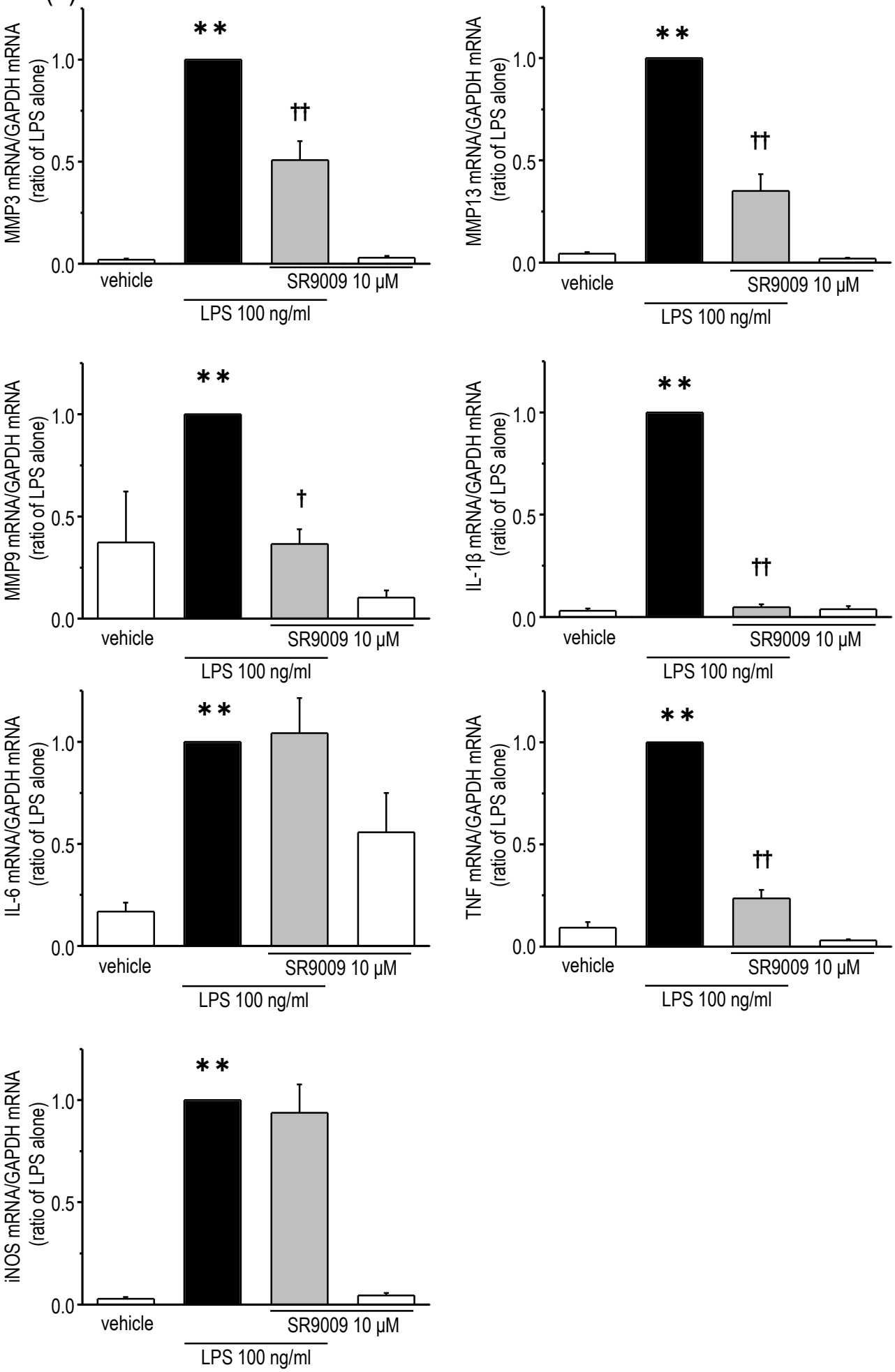
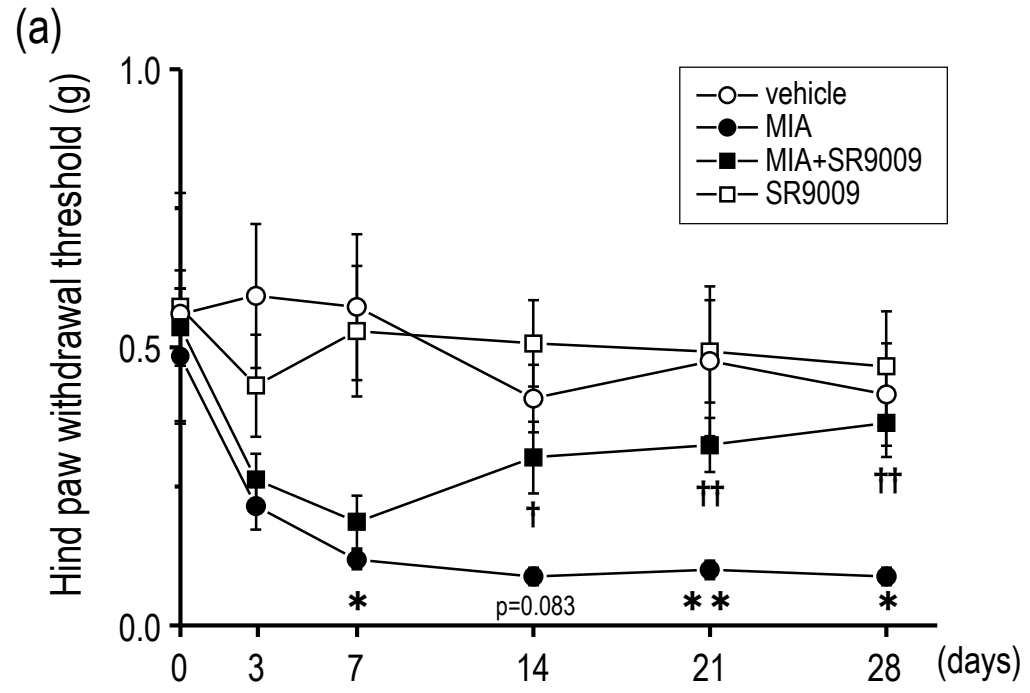

MMP3 mRNA/GAPDH mRNA (ratio of LPS alone)
MMP13 mRNA/GAPDH mRNA (ratio of LPS alone)
MMP9 mRNA/GAPDH mRNA (ratio of LPS alone)
IL-1β mRNA/GAPDH mRNA (ratio of LPS alone)
IL-6 mRNA/GAPDH mRNA (ratio of LPS alone)
TNF mRNA/GAPDH mRNA (ratio of LPS alone)
iNOS mRNA/GAPDH mRNA (ratio of LPS alone)
0.0
0.5
1.0
**
†
††
vehicle
SR9009 10 μM
LPS 100 ng/ml

Figure 2


(a)
Hind paw withdrawal threshold (g)
1.0
0.5
0.0
vehicle
MIA
MIA+SR9009
SR9009
*
p=0.083
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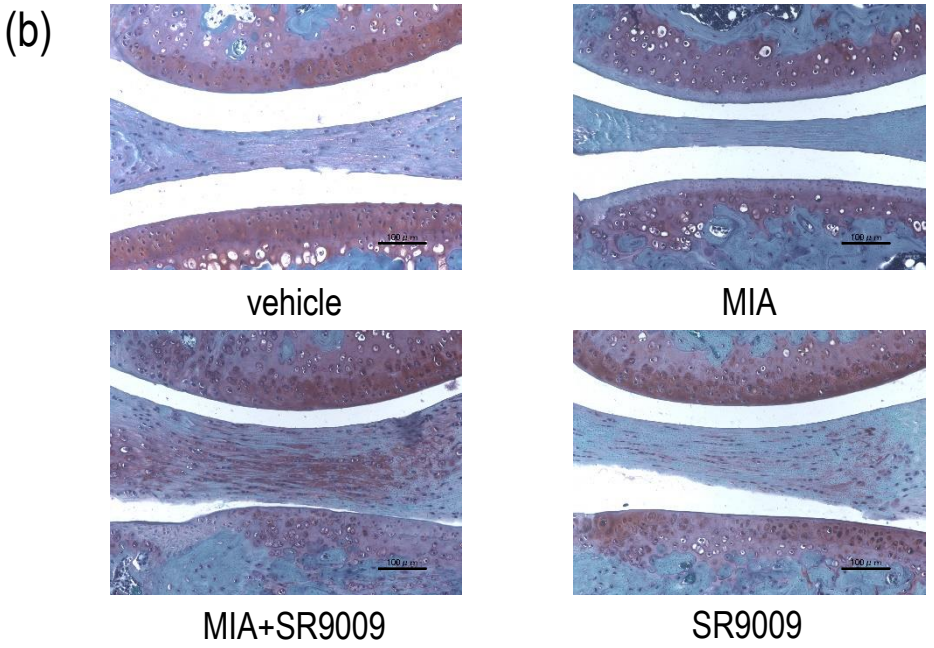
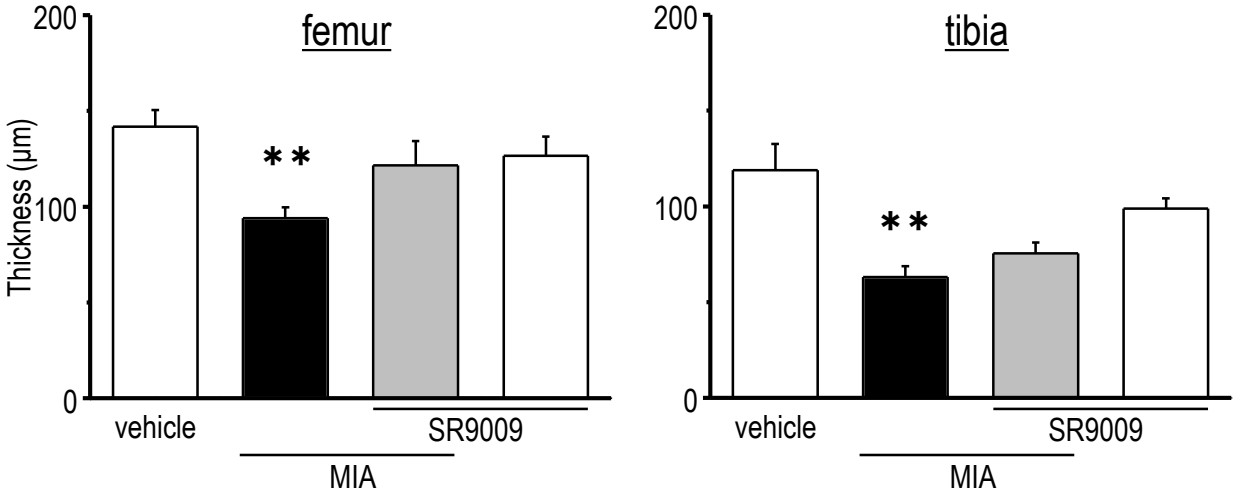
*
†
††
††
0
3
7
14
21
28
(days)


(b)
vehicle
MIA
MIA+SR9009
SR9009


femur
tibia
Thickness (μm)
200
100
0
**
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vehicle
SR9009
MIA
vehicle
SR9009
MIA