Involvement of inflammasome activation via elevation of uric acid level in nociception in a mouse model of muscle pain

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Abstract

Muscle pain is a common condition in many diseases and is induced by muscle overuse. Muscle overuse induces an increase in uric acid, which stimulates the nucleotide-binding oligomerization domain-like receptor (NLR). This receptor contains the pyrin domain NLRP-3 inflammasome which when activated, results in the secretion of potent pro-inflammatory cytokines such as interleukin-1β (IL-1β). The aim of this study was to investigate the involvement of inflammasome activation via the elevation of uric acid level in nociception in a mouse model of muscle pain. The right hind leg muscles of BALB/c mice were stimulated electrically to induce excessive muscle contraction. The left hind leg muscles were not stimulated as a control. Mechanical withdrawal thresholds, levels of uric acid, IL-1β, and NLRP3, caspase-1 activity, and the number of macrophages were investigated. Furthermore, the effects of xanthine oxidase inhibitors, such as Brilliant Blue G, caspase-1 inhibitor, and clodronate liposome, on pain were investigated. In the stimulated muscles, mechanical withdrawal thresholds decreased, and the levels of uric acid, NLRP3, and IL-1β, caspase-1 activity, and the number of macrophages increased compared to that in the non-stimulated muscles. Administration of the inhibitors attenuated hyperalgesia caused by excessive muscle contraction. These results suggested that IL-1β secretion and NLRP3 inflammasome activation in macrophages produced mechanical hyperalgesia by elevating uric acid level, and xanthine oxidase inhibitors may potentially reduce over-exercised muscle pain.

Keywords

Muscle pain, inflammasome, uric acid, electrical stimulation, mechanical hyperalgesia

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Introduction

Muscle is a common source of pain that relates to various pathologies, such as neck and shoulder pain,[1] non-specific lower back pain,[2] and myofascial pain syndrome (MPS).[3] Neck and shoulder pain occurs in more than 30% of the working population,[4] and lower back pain has a lifetime prevalence as high as 84% in the general population.[5] MPS is also very common in the general population, with an incidence rate as high as 50%.[4] Although numerous treatment methods, such as pharmacological treatments using non-steroidal

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anti-inflammatory drugs, physiotherapy, and dry needling, have been developed to reduce pain, no single successful strategy is available.[3]

Muscle overuse, especially with eccentric contraction, induces muscle pain due to an increase in adenosine triphosphate (ATP) and a lower pH.[6–8] Muscle fiber destruction through muscle overuse, induces the release of ATP, which activates specific receptors (so-called nociceptors and metaboreceptors).[6,8] Sustained muscle contraction and chronic muscle ischemia result in a decrease in pH in affected tissues.[6,8] Inflammatory responses to exercise-induced muscle damage also cause muscle pain.[7] Neutrophils and macrophages invade skeletal muscle and produce proinflammatory cytokines such as interleukin (IL)-1β, IL-18, and tumor necrosis factor (TNF)-α, after exercise-induced muscle damage.[7] Furthermore, we previously reported that IL-18 is secreted from neutrophils using a muscle pain model.[9]

Muscle overuse induces an increase in the level of uric acid, which is released from damaged cells.[10–15] Kono et al. reported an increase in uric acid in dead cells harvested from various tissues including the brain, heart, lung, liver, kidney, and muscle.[16] Uric acid is the final product of purine metabolism. In this metabolism, xanthine oxidase (XO) converts hypoxanthine to xanthine and subsequently, xanthine to uric acid.[17] The purine metabolism system is extremely active during high-intensity exercise and muscle ischemic conditions.[11] When the uric acid level in serum reaches the solubility limit, monosodium urate (MSU) crystal formation occurs,[18] and the crystal induces inflammasome activation.[19,20]

An inflammasome is a group of protein complexes that was first reported in 2002.[19,21] Inflammasome complexes are composed of several proteins, including the nucleotide-binding oligomerization domain-like receptor (NLR), which contains pyrin domain (NLRP) 1, NLRP3, and NLRP6. It also contains caspase activation and recruitment domain 4 (NLRC4).[19,21] Inflammasomes recognize inflammation-inducing stimuli and form an assembly that results in direct activation of caspase-1, which subsequently induces the secretion of potent pro-inflammatory cytokines such as IL-1β and IL-18, and a form of cell death called pyroptosis.[19] NLRP3 activates the aberrant formation of crystals from endogenous molecules such as MSU.[19,20] Phagocytosis of MSU crystals by macrophages and its recognition by P2 purinergic receptors result in NLRP3 inflammasome activation and release of IL-1β.[12,19,20]

IL-1β is a pro-inflammatory cytokine, which is released from various cells including keratinocytes, synoviocytes, and macrophages.[22,23] Although IL-1β has numerous important functions under normal conditions, the overproduction of IL-1β is implicated in various diseases such as rheumatoid arthritis and osteoarthiritis.[24,25] IL-1β is associated with numerous states of pain.[22] Gout, one of the painful diseases caused by MSU deposition, is related to IL-1β production via NLRP3 inflammasome activation.[22] So et al. reported that Anakinra, a recombinant IL-1 receptor antagonist, rapidly relieved the inflammatory symptoms of gout in gouty arthritis patients.[26] For the onset of neuropathic pain, the interplay between the immune and nervous systems may be important, and IL-1β may contribute to pain.[22] In various animal models of neuropathic pain, IL-1β expression level is increased in the injured nerve, dorsal root ganglia, and spinal cord.[27–29] IL-1β is associated with persistent pain, and injury-induced central sensitization is an important mechanism for its development. IL-1β also acts as a mediator between glia and neurons.[22,30]

Involvement of inflammasome-mediated processes in numerous painful diseases including gout,[20] pseudogout,[31] and osteoarthritis[32] has been reported. However, few reports have described the relationship between inflammasome and muscle pain. Thus, the aim of the present study was to investigate the role of IL-1β secretion due to inflammasome activation in nociception in a mouse model of muscle pain.

Materials and methods

Experimental animals

The protocol for this experiment was approved by the Animal Research Committee of Tohoku University (approval number: 2016MdA-240). Male, 5–7-week-old BALB/c mice (body weight: 20–23 g) were obtained from Japan CLEA (Tokyo, Japan). The mice were housed under a 12:12 h light–dark cycle at $23 \pm 1^\circ$C.

Repeated electrical stimulation of triceps surae muscles

Repeated electrical stimulation was used to induce excessive muscle contractions as previously described.[9] Briefly, two needle electrodes were applied transcutaneously into the triceps surae muscle of the right hind leg after each mouse was anesthetized with an intraperitoneal injection of medetomidine (ZENOAQ, Fukushima, Japan, 0.3 mg/kg), midazolam (SANDZ, Tokyo, Japan, 4.0 mg/kg), and butorphanol (Meiji Seika Pharma Co., Tokyo, Japan, 5.0 mg/kg). Electrical stimulation was performed at 10 Hz with a 10 V amplitude and a 100 μs pulse width for 30 min for 7 days per week. During electrical stimulation, the right hind leg was immobilized. The ankle joint was placed in the dorsal flexion so that the triceps surae muscle was fully extended for isometric contraction. The needle electrodes were

also applied to the contralateral triceps surae muscle without electrical stimulation and immobilization.

Assessment of mechanical nociceptive thresholds

Assessment of the mechanical withdrawal threshold (MWT) was performed using the Randall–Selitto test (MK-201D Pressure Analgesy-Meter, Muromachi Kikai Co., Tokyo, Japan) as previously reported.[9] Briefly, a linear increase in pressure (10 mm Hg/s) was applied to the lateral surface of the triceps surae muscle using a cone-shaped plastic tip attached to a scale with a display. The MWT was defined as the amount of pressure (mm Hg) required to elicit pain-related behaviors such as vocalization, struggling, and leg withdrawal.[9,33,34] Experiments investigating secondary hyperalgesia were performed in the plantar surface of the foot. The cut-off value of the MWT was 250 mm Hg.[34] On day 7 following the initiation of electrical stimulation, an assessment of the MWT data was performed in the morning as circadian rhythm affects pain sensitivity. To avoid bias, assessment of the MWT data was performed by an investigator who was blinded to the experimental conditions.

Local effect of MSU on hyperalgesia

To confirm the local effects of MSU, recrystallized MSU (Monosodium Urate, No. 133–13432; Wako Pure Chemicals Industries, Osaka, Japan) dissolved in saline was administered to the right triceps surae muscle (MSU group) as previously described.[35] At the same time, saline (only) was administrated to the contralateral triceps surae muscle (Saline group). The solution was injected under the fascia of the lateral head of triceps using a 27-gauge needle. The volume of injected solution was 50 µl in each group. Days 0, 1, 2, and 4 post injection, MWTs were assessed and the triceps surae muscles obtained for measurements of the levels of NLRP3 and IL-1β by enzyme-linked immunosorbent assay (ELISA).

Tissue preparation

On day 7 following the initiation of electrical stimulation, mice were sacrificed by cervical dislocation and the triceps surae muscles isolated. Specimens for ELISA and fluorometric assays were frozen in liquid nitrogen and stored at –80°C. For immunohistochemical staining, the specimens were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and stored overnight at 4°C. After dehydration through a graded series of ethanol solutions, the specimens were embedded in paraffin. The embedded tissues were then cut into 5-µm axial sections.

ELISA experiments

For the measurement of IL-1β levels, tissue samples were disrupted and homogenized using lysis buffer composed of bovine serum albumin (100 µg/ml, A4503; Sigma–Aldrich, St. Louis, MO, USA), Triton X-100 (0.1%, Wako Pure Chemicals Industries), 1 M HEPES (1%, 533–08083; Wako Pure Chemicals Industries), protease inhibitor (1%, P8340; Sigma–Aldrich), and distilled water (DW). After homogenization, samples were centrifuged for 10 min at $9,730 \times g$ and 4°C. The supernatant obtained was stored at -80°C. IL-1β expression levels were analyzed with a Bio-Plex Multiplex Immunoassay System (Bio-Rad, Hercules, CA, USA) and a Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad), according to the manufacturer's instructions. Measurement of NLRP3 level was performed using Mouse NALP3/NLRP3 ELISA Kit (LS-F17336, LifeSpan Biosciences Inc., Seattle, WA, USA). Tissue samples were disrupted and homogenized using phosphate-buffered saline (PBS). Following homogenization, the samples were repeatedly frozen (-20°C) and thawed three times for cell lysing at room temperature. The samples were then centrifuged for 5 min at $5,000 \times g$ and 4°C. The supernatant was utilized for the assay, according to the manufacturer's instructions.

Fluorometric assay experiments

Measurement of the level of uric acid and caspase-1 activity was performed using Uric Acid Colorimetric/Fluorometric Assay Kit (K608-100, BioVision Inc., Milpitas, CA, USA) and Caspase 1 Assay Kit (ab39412, Abcam plc), respectively. Tissue samples were disrupted and homogenized using buffers from each kit. After homogenization, the samples were centrifuged for 5 min at 12,000 rpm and 4°C. The supernatant was used for the assay according to the manufacturer's instructions. Values of caspase-1 activity were normalized to the controls which were not stimulated and administered any drugs.

Immunohistochemistry

Tissue sections were deparaffinized and washed in PBS. They were subsequently incubated with a solution containing Proteinase K (Takara Bio Inc. Shiga, Japan, 25 µl), 0.5 M ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA, USA, 0.5 ml), 1 M Tris-Cl (pH 8.0, 2.5 ml) and 50 ml DW, for 5 minutes at 37°C to induce antigen retrieval. After washing in PBS, endogenous immunoglobulins were blocked by incubation with 10% normal goat serum (Nichirei Biosciences Inc., Tokyo, Japan) for 3 h. The slides were once again washed with PBS and incubated with a polyclonal rabbit anti-mouse NLRP3 antibody (NBP2-12446,

Novus Biologicals, Littleton, CO, USA; dilution, 1:25), polyclonal rabbit anti-mouse caspase-1 antibody (ab1872, Abcam plc, Cambridge, UK, dilution 1: 25), polyclonal rabbit anti-mouse IL-1β antibody (ab9722, Abcam plc, concentration of 10 μg/ml) and a monoclonal rat anti-mouse Cluster of Differentiation (CD) 68 antibody (ab53444, Abcam plc, concentration of 10 μg/ml) in PBS overnight at 4°C. PBS was then used to rinse the slides. Subsequently, the slides were incubated for 1 h in PBS with an Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11034, Life Technologies, Carlsbad, CA, USA; dilution, 1:750) for NLRP3, caspase-1, IL-1β and an Alexa Fluor 555-conjugated goat anti-rat IgG (A-21434, Life Technologies; dilution, 1:750) for CD68 at room temperature. The slides were once again rinsed with PBS. Finally, the slides were incubated with 4,6-diamidino-2-phenylindole (Sigma–Aldrich; dilution, 1:500) for 10 min at 25°C for nuclear staining. Images were captured using a fluorescence microscope (BZ-9000 Biorevo, Keyence, Osaka, Japan). The images were analyzed using Adobe Photoshop (Adobe System Inc., San Jose, CA, USA). At least three images in each slide were captured at 200 × magnification and the number of CD68-positive cells (macrophages) counted. The number was presented as cells/view. To avoid bias, the evaluation was performed by two investigators who were blinded to the experimental conditions. Two animals were used for immunohistochemistry, and two slides/animal were analyzed. After confirming reproducibility, representative images were presented.

Assessment of the effect of drug administration

Several agents were administered intraperitoneally during repeated electrical stimulations of the triceps surae muscles. This was to confirm the suppressing effects of hyperalgesia. Allopurinol (A8003, Sigma–Aldrich; 200 mg/kg/72 h),[36] Febuxostat (F0847, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; 5 mg/kg/24 h),[37] Brilliant Blue G pure (BBG, B0770, Sigma–Aldrich; 45.5 mg/48 h),[38] Caspase-1 inhibitor Z-Trp-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (Z-WEHD-FMK, FMK002, R&D Systems, Inc., Minneapolis, MN, USA; 1 mg/kg/24 h),[39] and Liposomal clodronate (Xygieia Bioscience, Osaka, Japan; 200 μl/body/48 h)[40] were used.

Allopurinol and Febuxostat are XO inhibitors which reduce uric acid formation by inhibiting XO which converts hypoxanthine to xanthine and uric acid.[36,37] BBG is a selective antagonist that attenuates NLRP3 inflammasome activation.[38] Caspase-1 inhibitor Z-WEHD-FMK is a caspase-1 inhibitor used to block caspase-1 activity and subsequently, the production of IL-1β.[39] Clodronate liposome induces macrophage depletion by killing these cells as a result of accumulation and irreversible metabolic damage.[40] Control animals received an equivalent volume of 0.9% saline (saline group).

The MWTs were assessed by performing the Randall–Selitto test on day 7. Measurement of uric acid, NLRP3, IL-1β levels and caspase activity was performed as described above.

Assessment of hyperalgesia in IL-1 knock out mouse

To confirm IL-1β effects on hyperalgesia, homozygous IL-1 knock out (KO) mice were used.[41] Repeated electrical stimulation was performed on IL-1 KO mice as described above, and the MWTs were assessed by performing the Randall–Selitto test on day 7.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 24 (IBM, Armonk, NY, USA). Analysis of the MWT time-course data was performed using 2-way analysis of variance (ANOVA), and repeated measurements compared by the Tukey's post-hoc multiple-comparison test. To compare data from more than three groups from a single day (MWTs, ELISA, and fluorometric assay), 1-way ANOVA with Tukey's post-hoc multiple-comparison test was used for the analysis. The NLRP3 and IL-1β data between the two groups were analyzed using the Wilcoxon signed-rank test. Other data between two groups were analyzed through the use of the paired t test. All data are expressed as the mean ± standard error of the mean. A p value < 0.05 was considered statistically significant.

Results

MSU induced mechanical hyperalgesia and inflammasome activation

The MWTs of the MSU group significantly decreased from day 1 following the administration, compared to that before administration. Decrease in MWTs continued at least 4 days after administration. In the saline group, no significant changes were observed during the experimental periods compared to that before administration. Significant differences were observed between the two groups between 1 to 4 days after administration (Figure 1(a)). On days 2 and 4 following administration, NLRP3 and IL-1β induced in the MSU group significantly increased compared to the saline group based on ELISA data (Figure 1(b) and (c)).

Excessive contraction induced mechanical hyperalgesia, elevated uric acid level, and activated inflammasome in muscles

The MWTs of the stimulated muscles significantly decreased compared to that of non-stimulated contralateral muscle (Figure 2(a)). There was no evidence for
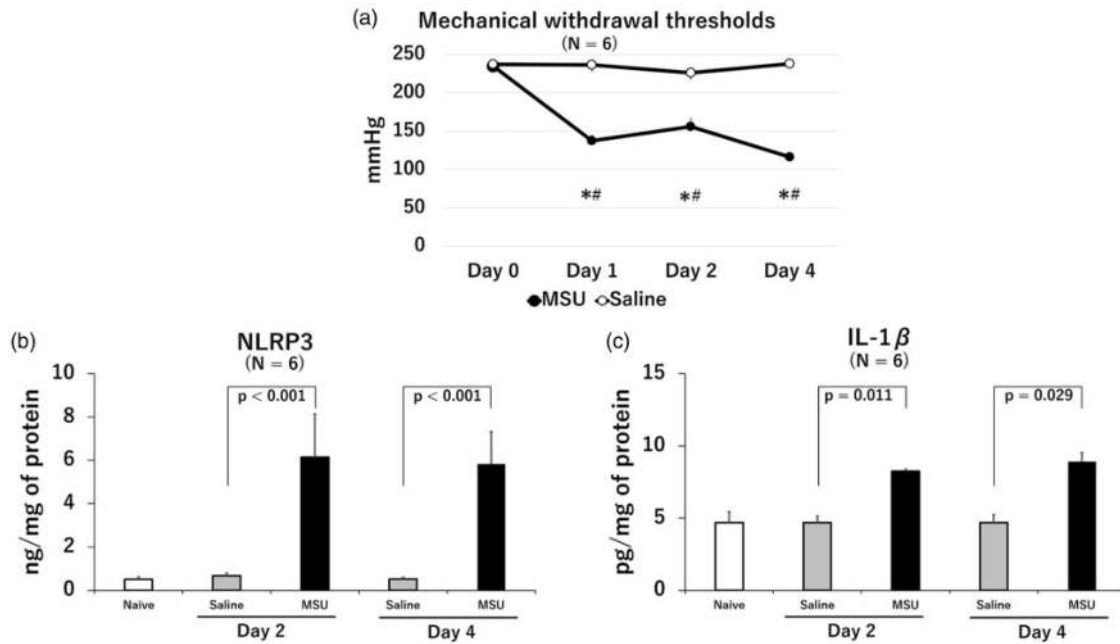
Figure 1. Change in mechanical withdrawal thresholds (MWTs), NLRP3 and IL-1β levels by local administration of MSU. Time course of MWT values in the MSU group and saline group (a). The MWTs of the MSU group significantly decreased compared to those of the saline group, 1 to 4 days after administration. The quantities of NLRP3 (b) and IL-1β (c) in muscle tissues. The protein expression, determined by ELISA, increased significantly in MSU groups compared to saline groups. *p < 0.05: significantly different from the saline group; #p < 0.05: significantly different from the preinjection values; The protein concentration for all samples used in the ELISA was 1.1 mg/ml. MSU: monosodium urate. IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.

secondary hyperalgesia during the experiments. Induction of uric acid, NLRP3, caspase-1 activity, and IL-1β in the stimulated muscles significantly increased compared to that in non-stimulated muscles (Figure 2 (b) to (e)).

Inflammasome activation in macrophages in stimulated muscles

Immunohistochemical staining revealed an increased number of CD68-positive cells (macrophages) in the stimulated muscles compared to those that were not stimulated. Most of these cells were positive for NLRP3, caspase-1, and IL-1β (Figure 3(a) to (r)). The number of cells that were positive for both IL-1β and CD68 in the stimulated muscles significantly increased compared to that of non-stimulated muscles (Figure 3(s)). In the clodronate liposome group, the MWTs significantly increased while NLRP3, caspase-1 activity, and IL-1β levels significantly decreased compared to those in the saline group (Figure 3(t) to (w)). No significant differences were observed between the saline group and naïve group with electrical stimulation.

XO inhibitors (allopurinol and febuxostat) attenuated hyperalgesia in excessively contracted muscles

In both the allopurinol and febuxostat groups, the MWTs significantly increased while uric acid, NLRP3, caspase-1 activity, and IL-1β levels significantly decreased compared to those in the saline group (Figure 4(a) to (e)). No significant differences were observed between the saline group and the naïve group with electrical stimulation. The levels of uric acid and caspase-1 activity of the allopurinol and febuxostat group did not decrease to the same level as the group without stimulation.

BBG attenuated hyperalgesia in excessively contracted muscles

The MWTs significantly increased while NLRP3, caspase-1 activity, and IL-1β levels significantly decreased in the BBG group compared to that in the saline group (Figure 5(a) to (d)). No significant differences were observed between the saline and the naïve groups with electrical stimulation. The caspase-1 activity of the BBG group did not decrease to the same level as the group without stimulation.
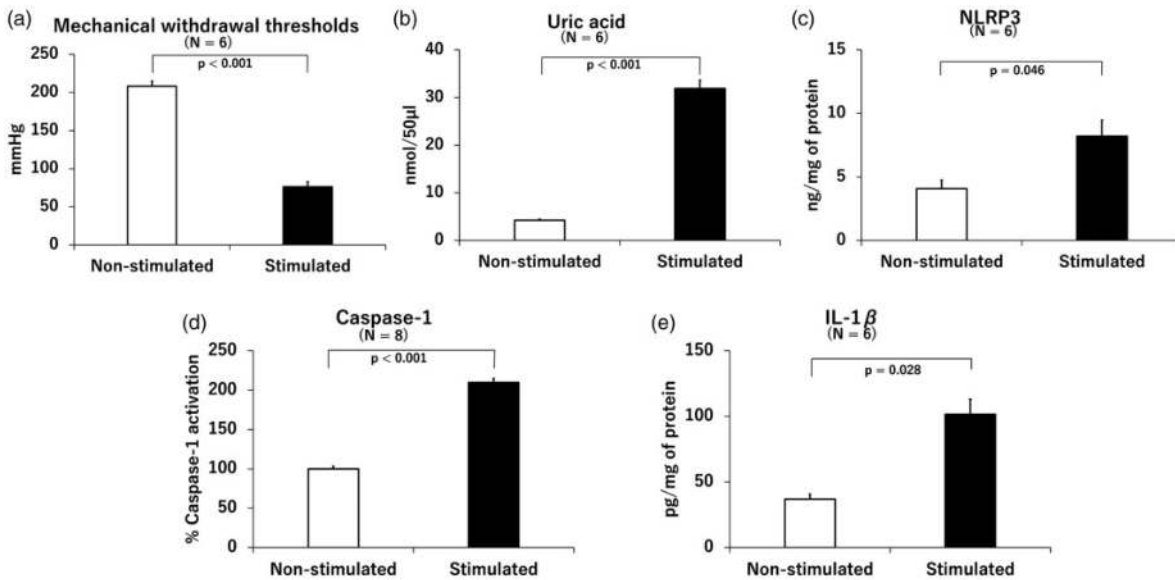
Figure 2. Changes in the MWTs and levels of uric acid, NLRP3, caspase-1, and IL-1β in stimulated and nonstimulated muscles at seven days after initiating electrical stimulation. The MWTs of stimulated muscles significantly decreased compared to those of nonstimulated contralateral muscles (a). The quantities of uric acid (b), NLRP3 (c), IL-1β (e), and fluorescence intensity of caspase-1 activity (d) in muscle tissues. These levels increased significantly in stimulated muscles compared to nonstimulated muscles; the concentrations of NLRP3 and IL-1β were equalized to 1.1 mg/ml. The concentration of uric acid is shown as nmol/50 μl and the fluorescence intensity of caspase-1 activity shown as a ratio to the value of nonstimulated muscles. IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.

Z-WEHD-FMK attenuated hyperalgesia in excessively contracted muscles

In the Z-WEHD-FMK group, the MWTs significantly increased while caspase-1 activity and IL-1β levels significantly decreased compared to the saline group (Figure 6(a) to (c)). No significant differences were observed between the saline and the naïve groups with electrical stimulation. The caspase-1 activity of the Z-WEHD-FMK group did not decrease to the same level as the group without stimulation.

Excessive contraction did not induce mechanical hyperalgesia in IL-1 KO mice

The MWTs of the stimulated muscles did not decrease compared to those in the non-stimulated contralateral muscle in the IL-1 KO mice (Figure 7(a)). In the IL-1 KO mice, NLRP3 level of the stimulated muscle significantly increased compared to the non-stimulated muscle. Although there was no significant difference, the level of caspase-1 activity in the stimulated muscle tended to increase. For IL-1 KO mice, these levels were lower than those of naïve mice (Figure 7(b) to (c)).

Discussion

The present study revealed the following: 1. The administration of MSU in the triceps surae muscles induced hyperalgesia and IL-1β elevation owing to inflammasome activation, 2. Excessive muscle contraction by electrical stimulation induced mechanical hyperalgesia, elevated uric acid, and activated inflammasome and IL-1β in the muscle, 3. The number of macrophages increased after electrical stimulation and NLRP3 inflammasome activation in these macrophages, 4. Intraperitoneal administration of drugs including XO inhibitors, BBG and Z-WEHD-FMK attenuated hyperalgesia caused by excessive muscle contraction, similar to IL-1 KO mice. The pathway of muscle pain in our model and the effects of inhibitors at each stage are presented in Figure 8.

In our previous study, repeated muscle contraction (7 days) produced mechanical hyperalgesia on days 4–11 following the initiation of contraction; recovery occurred after day 12.[9] In the present study, which was conducted using the same model, MWTs of the stimulated muscles significantly decreased compared to those of non-stimulated contralateral muscles on day 7. Therefore, the stimulated muscles which were harvested on day 7 was appropriate for investigation of muscle pain induced by repeated excessive contraction. Although needle electrodes were also inserted into the contralateral muscle without electrical stimulation, hyperalgesia of the muscle was not observed. This finding demonstrates that the observed muscle hyperalgesia was caused by electrical stimulation, and not by the mechanical irritation of muscle tissue caused by insertion of the needle. In the present study, the muscle on the

contralateral side was used as a control (internal control) to exclude individual differences in mice. If the muscle on the contralateral side is compared to the same muscle in another mouse (external control), some differences should affect the results because the number of circulating leukocytes increases after exercise,[7] which must be dependent on individual specificity.

MSU activates NLRP3 inflammasome.[12,19,20] There are several reports that evaluate inflammasome activation by local administration of MSU.[35,42,43] Ju et al. and Yang et al. reported MSU injection into the soles of mice hindlimb feet, induced elevation of caspase-1 activity and IL-1β.[35,42] Lee et al. reported elevation of NLRP3, caspase-1 activity and IL-1β by MSU injection into the subcutaneous tissue in the back of mice.[43] The data in this study was seen to correspond with these studies.

Muscle overuse induces an increase in uric acid level in human and mouse.[10,11,13–15] Balsom et al. and Jówko et al. reported an increase in uric acid level in serum after sprint in physical education students.[10,11] Chatzinikolaou et al. and Andersson et al. reported that there is an elevated blood uric acid concentration
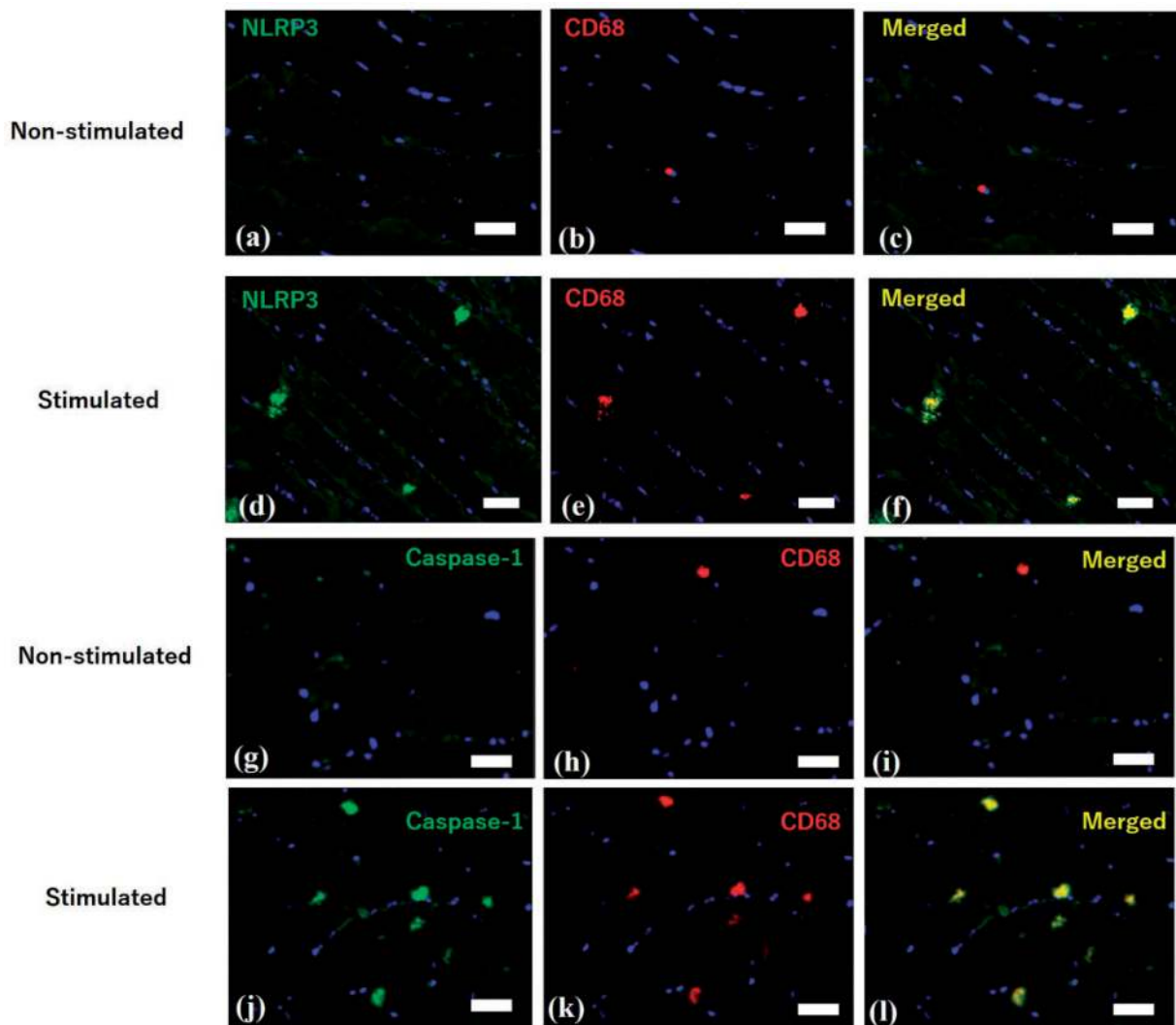
Figure 3. The number of macrophages in muscle, with or without electrical stimulation, and the effect of clodronate liposome (CL) administration. Immunohistochemical staining of NLRP3 (green), caspase-1 (green), IL-1β (green), and CD68 (red) in stimulated and nonstimulated muscles on day 7 is shown ((a) to (r)). The number of CD68-positive cells (macrophages) in stimulated muscles increased and most of these cells are also positive for NLRP3, caspase-1, and IL-1β; scale bar = 50 μm. The number of cells copositive for IL-1β and CD68 increased significantly in stimulated muscles (s). The MWTs increased (t), and levels of NLRP3 (u), caspase-1 activity (v), and IL-1β (w) significantly decreased in the CL group compared to those in the stimulated muscle of the saline group and naïve group. The concentrations of NLRP3 and IL-1β are equalized to 1.1 mg/ml. The fluorescence intensity of caspase-1 activity is shown as a ratio with the value of nonstimulated muscles in naïve group. IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.
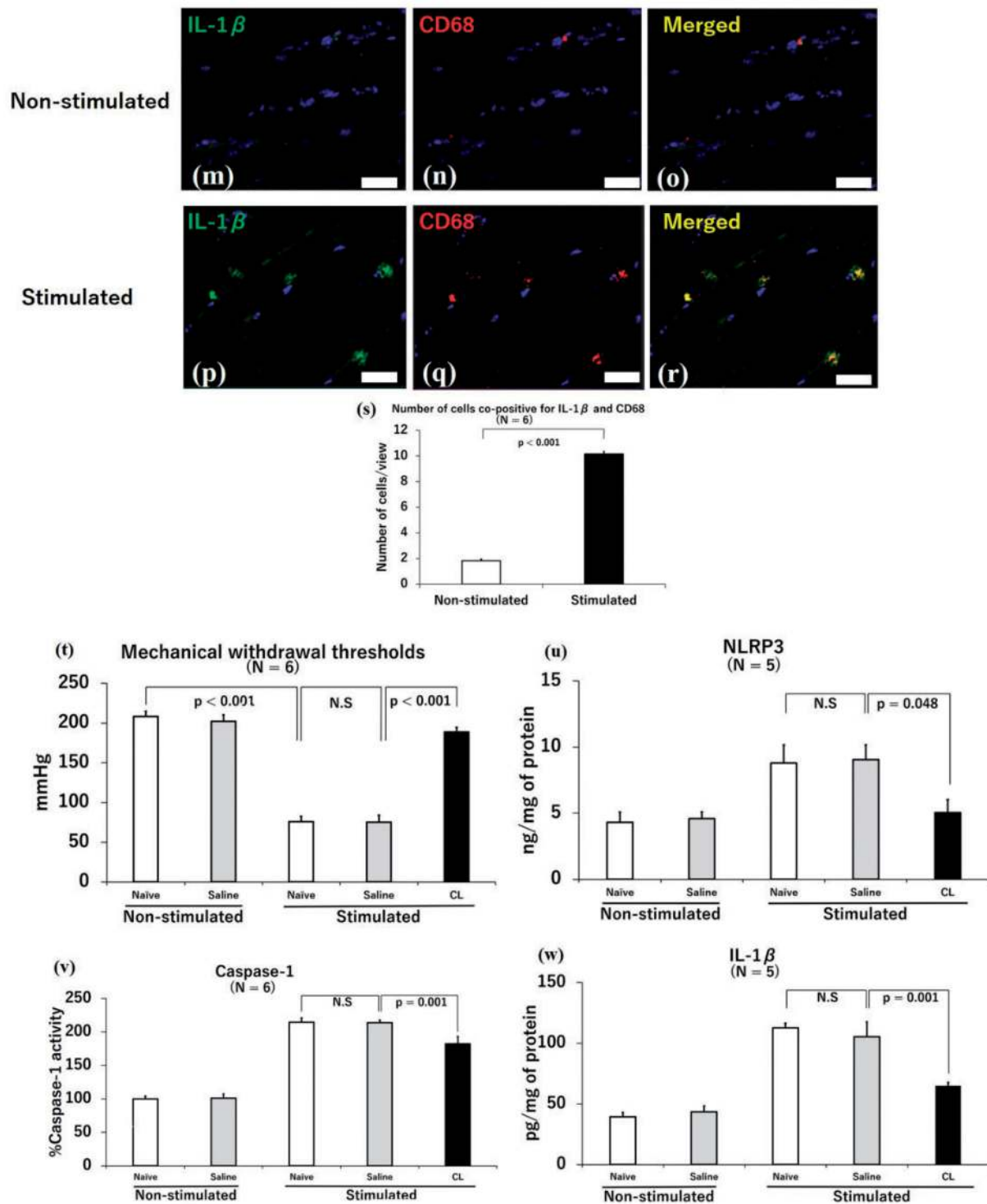
Figure 3. Continued.

and muscle soreness after exercise in team handball players and elite female soccer players, respectively.[13,15] Retamoso et al. reported an increase in uric acid level in the gastrocnemius muscle of mouse after downhill running exercise.[14] These reports supported our findings

that an increase in uric acid in the stimulated muscles presented hyperalgesia. Although an increase in uric acid level and NLRP3 inflammasome activation was observed in stimulated muscles, MSU crystal formation was not confirmed as the polarizing microscope could
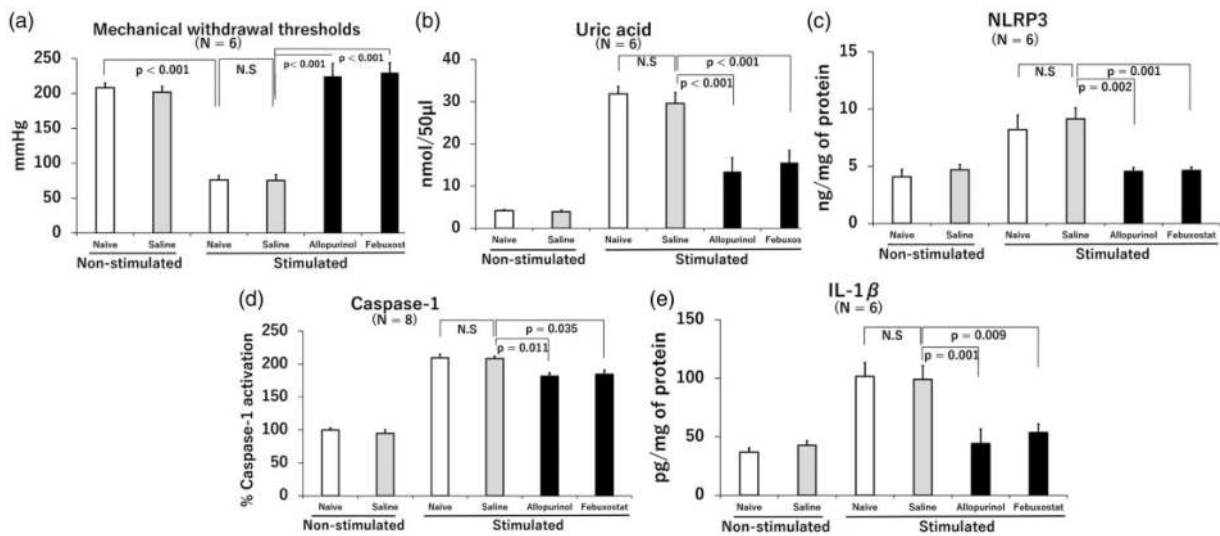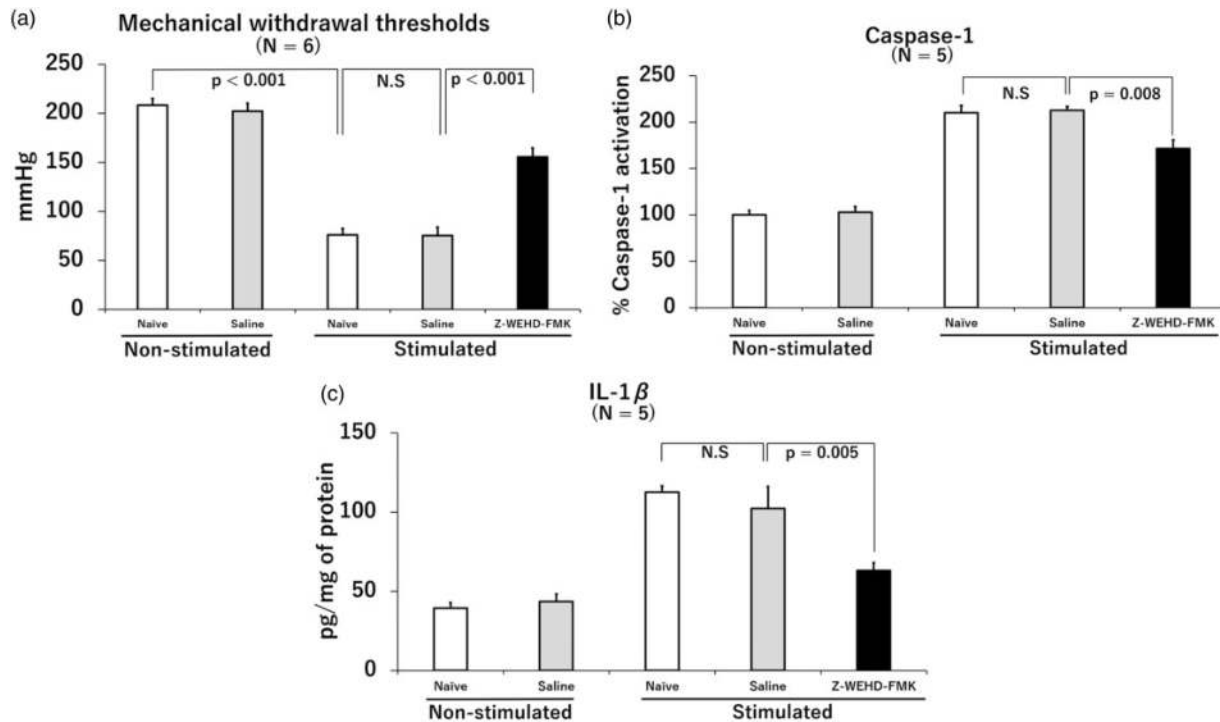
Figure 4. Changes in MWTs and the levels of uric acid, NLRP3, caspase-1 activity, and IL-1β produced after administration of xanthine oxidase (XO) inhibitors. The MWTs increased (a), and levels of uric acid (b), NLRP3 (c), caspase-1 activity (d), and IL-1β (e) significantly decreased in the allopurinol group and febuxostat group when compared to the stimulated muscles of the saline group and the naïve group. The concentrations of NLRP3 and IL-1β are equalized to 1.1 mg/ml and the concentration of uric acid presented as nmol/50 μl. The fluorescence intensity of caspase-1 activity is shown as a ratio with the value of nonstimulated muscles in the naïve group. IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.
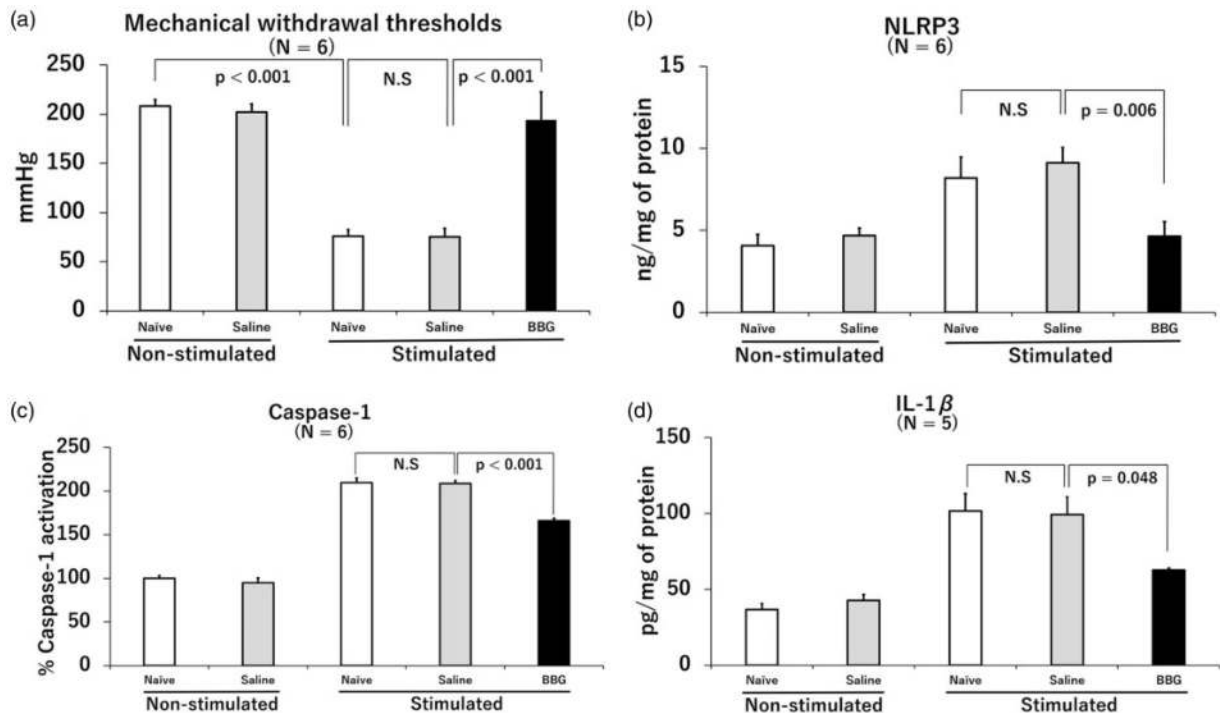
Figure 5. Changes in MWTs and the levels of NLRP3, caspase-1 activity, and IL-1β produced after administration of brilliant blue G (BBG). The MWTs increased (a), and levels of NLRP3 (b), caspase-1 activity (c), and IL-1β (d) significantly decreased in the BBG group compared to the stimulated muscles of the saline group and the naïve group. The concentrations of NLRP3 and IL-1β are equalized to 1.1 mg/ml. The fluorescence intensity of caspase-1 activity is shown as a ratio to the value of nonstimulated muscles in the naïve group. IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.
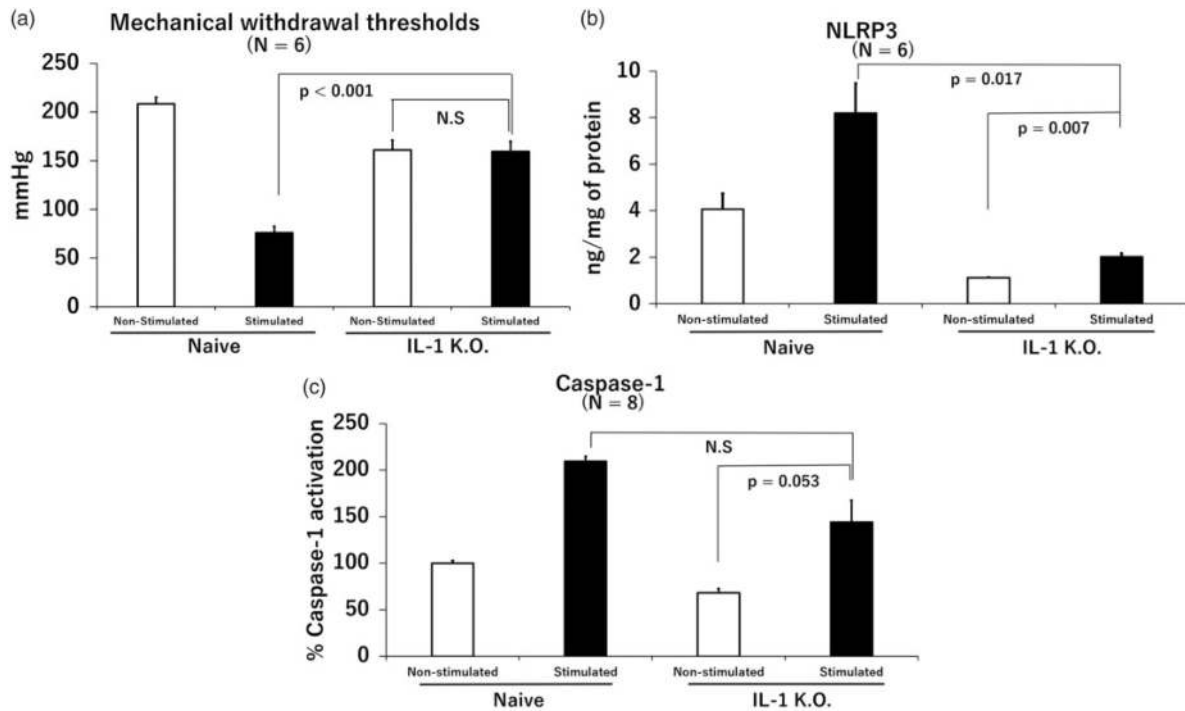
Figure 6. Changes in MWTs and the levels of caspase-1 activity and IL-1β produced after administration of Z-Trp-Glu(OMe)-His-Asp (OMe)-fluoromethylketone (Z-WEHD-FMK). The MWTs increased (a), and levels of caspase-1 activity (b) and IL-1β (c) significantly decreased in the Z-WEHD-FMK group compared to the stimulated muscle of saline group and naïve group. The concentrations of IL-1β were equalized to 1.1 mg/ml. The fluorescence intensity of caspase-1 activity is shown as a ratio with the value of nonstimulated muscles in the naïve group. IL: interleukin.

not be used. According to previous studies, the solubility limit of uric acid is 405 μmol/l in serum.[18] In our results, the average of local uric acid concentration in stimulated muscle was almost 600 μmol/l, which is sufficient to enable crystal formation. Therefore, NLRP3 inflammasome activation in the stimulated muscles must have occurred.

Although there are very few reports of the direct relationship between NLRP3 inflammasome and muscle pain, several studies have reported the relationship between NLRP3 inflammasome and fibromyalgia, and chronic fatigue syndrome, which result in muscle pain and fatigue.[44–46] Bullón et al. and Cordero et al. reported that overactivation of NLRP3 inflammasome occurs in blood mononuclear cells in patients with fibromyalgia.[44,45] Zhang et al. reported the activation of NLRP3 inflammasome in the diencephalons, which is responsible for fatigue sensation. This experiment was conducted using a mouse fatigue model with lipopolysaccharide (LPS) combined with swim stress.[46] These previous reports may support our findings of the increase in NLRP3, caspase-1 activity and IL-1β in the stimulated muscles. Although evaluation of NLRP3 inflammasome activity was performed only in peripheral muscle tissues without any testing of the central nervous system (CNS) in this study, it is quite possible that

NLRP3 inflammasome activation also occurs in the CNS. This is based on the study by Zhang et al.[46]

There are a few reports of NLRP3 inflammasome activation in skeletal muscle cell.[47,48] Ding et al. reported NLRP3 inflammasome activation in interferon gamma (IFN-γ) treated C2C12 cultured myotubes.[47] McBride et al. reported that NLRP3 inflammasome contributes to sarcopenia.[48] However, to the best of our knowledge, there is no previous report of the involvement of NLRP3 inflammasome in muscle pain. NLRP3 is mainly expressed by myeloid cells including monocytes and macrophages.[12] In addition, the activation of NLRP3 inflammasome induced by uric acid occurs in macrophages.[19,20] Martinon et al. and Gicquel et al. reported inflammasome activation in monocyte cell line THP1 and human macrophages which were incubated with MSU crystals.[12,20] These reports support our findings that NLRP3 inflammasome activation occurred in macrophages which invaded the stimulated muscle. There are a few reports that clodronate liposome depletes macrophages and suppresses NLRP3 inflammasome activation and IL-1β release in several disease models such as lung tumor and alcoholic hepatosteatosis.[49,50] As previously mentioned, no report has addressed muscle pain. Gregory et al. reported that clodronate liposome reduced hyperalgesia induced by injection of normal

Figure 7. Changes in MWTs and the levels of NLRP3 and caspase-1 activity in naïve mice and IL-1 knock-out mice. A significant difference was not observed in the MWTs between stimulated and nonstimulated muscles in IL-1 KO mice (a). In the IL-1 KO mice, the levels of NLRP3 (b) and caspase-1 activity (c) in the stimulated muscle tended to increase compared to levels in the nonstimulated muscle. The concentrations of NLRP3 are equalized to 1.1 mg/ml. The fluorescence intensity of caspase-1 activity is shown as a ratio to the value of nonstimulated muscles in the naïve group. KO: knock out; IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain.
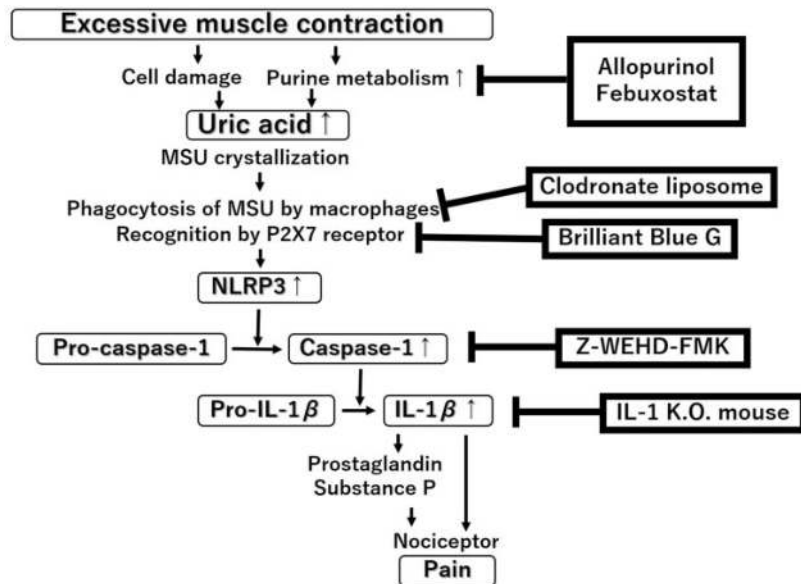
Figure 8. Schema of the pathway of muscle pain in the mouse model, and the effects of inhibitors at each stage. MSU: monosodium urate; KO: knock out; IL: interleukin; NLRP: nucleotide-binding oligomerization domain-like receptor pyrin domain; Z-WEHD-FMK: Z-Trp-Glu (OMe)-His-Asp(OMe)-fluoromethylketone.

saline adjusted to pH 5.0 into muscle when combined with an electrical stimulation. They concluded that muscle fatigue decreases the pH of muscle and activates acid-sensing ion channel 3 (ASIC3) in macrophages. This in turn enhances hyperalgesia of the muscle.[51] Although the detailed mechanisms are different, their report suggests that macrophages are involved in muscle pain and this corresponds with our results.

There are several previous reports that XO inhibitors inhibit NLRP3 inflammasome activation in various disease models.[52,53] Aibibula et al. reported that febuxostat inhibited NLRP3 inflammasome activation in the mouse model of metabolic syndrome.[52] Similarly, Wan et al. reported that allopurinol inhibited NLRP3 inflammasome activation in the mouse model of non-alcoholic fatty liver disease.[53] Thus, these reports supported our findings that allopurinol and febuxostat inhibited NLRP3 inflammasome activation in the stimulated muscle. In the present study, although the uric acid levels in the allopurinol group and febuxostat group did not decrease to the level seen in non-stimulated muscles, the MWT was recovered to the same level as that of the non-stimulated muscle. There are a few studies suggesting that different mechanisms for uric acid-mediated NLRP3 inflammasome activation is the cause of pain.[36,54] Schmidt et al. reported that administration of allopurinol produced dose-dependent antinociceptive effects in chemical and thermal pain models.[36] They concluded that allopurinol-induced anti-nociception may be related to adenosine accumulation which induces a decrease in the release of painful substances including substance P and glutamate.[36] Ives et al. reported that XO-derived reactive oxygen species, excluding uric acid, is the trigger for NLRP3 inflammasome activation and XO blockade impair it.[54] These mechanisms may explain the dissociation observed in the present study. Allopurinol and febuxostat are used clinically to treat hyperuricemia and gout.[36,37] Since allopurinol and its active metabolite are excreted by the kidneys, a life-threatening toxicity syndrome consisting of an erythematous, desquamative skin rash, fever, hepatitis, eosinophilia, and worsening in renal function can occur if used by patients with renal insufficiency.[55,56] In contrast, febuxostat can be more safely administered to such patients because it does not affect renal excretion.[56] As the safety of these drugs needs to be confirmed before use, it is helpful to use existing medication for chronic pain.

BBG is a selective P2X7 receptor antagonist that attenuates NLRP3 inflammasome activation.[38] The P2X7 receptor is a trimeric ion channel gated by extracellular ATP which present in numerous types of cell including stem, blood, glial, neural, bone, endothelial, muscle, renal and skin cells.[57] BBG attenuates various types of pain including muscle pain and neuropathic pain.[9,58] In our previous study, BBG attenuated hyperalgesia caused by excessive muscle contraction.[9] There are several reports that BBG inhibits NLRP3 inflammasome activation in various disease models.[38,59] Wang et al. reported BBG inhibited NLRP3 inflammasome activation in the bone marrow derived from macrophages. This was observed using an LPS-induced acute lung injury mouse model.[38] Furthermore, Zhong et al.

reported inhibition of NLRP3 inflammasome activation by BBG using graft-versus host-disease model mice produced by allogeneic hematopoietic stem cell transplantation.[59] These reports support our findings that BBG inhibited NLRP3 inflammasome activation in the stimulated muscle. BBG has already been used in clinical settings such as in ophthalmic surgery and confirmed a safe and reliable dye.[60] However, there is no previous report investigating the effects of systemic administration of BBG on the human body.

Z-WEHD-FMK is a caspase-1 inhibitor that inhibits the caspase-1 activity and the subsequent production of IL-1β.[39] There are a few reports that caspase-1 inhibitor attenuated pain.[23,61] Li et al. reported caspase-1 inhibitor attenuated mechanical allodynia in the rat model of complex regional pain syndrome type I.[23] Chen et al. reported caspase-1 inhibitor reduced IL-1β level in a rat model of headache induced by intrathecal injection of inflammatory chemicals.[61] These reports support our findings that Z-WEHD-FMK attenuated pain and reduced IL-1β level in the stimulated muscle. Although several reports have investigated the effect of Z-WEHD-FMK on human cells *in vitro*,[62,63] there is no previous report investigating the effects of Z-WEHD-FMK administration to humans.

In IL-1 KO mice, although NLRP3 and caspase-1 activity levels in the stimulated muscle increased compared to the non-stimulated muscle, the MWTs of these groups were almost equal. This indicates that IL-1β, and not NLRP3 or caspase-1, is involved in the development of muscle pain. In the IL-1 KO mice, NLRP3 and caspase-1 activity levels were lower than in naïve mice. Zasłona et al. reported that pro-IL-1β production induces mature IL-1β production via NLRP3 activation in macrophages (i.e., there is a feed-forward loop among IL-1β production via NLRP3 activation).[64] This may explain the lower levels of NLRP3 and caspase-1 activity in IL-1 KO mice compared to those in naive mice.

From the results of the drug administration tests, caspase-1 activities of treated muscle increased when compared to those of non-stimulated muscle. The activation of inflammasomes occur via various proteins such as NLRP1 and NLRC4, in addition to NLRP3.[19] Recognition of signals by these inflammasome proteins results in the activation of caspase-1.[19] Therefore, caspase-1 activation through other inflammasome proteins than NLRP 3 may occur and might have been the cause of electrode puncture or electrical stimulation.

In the present study, IL-1 KO mice, which are deficient in IL-1α and IL-1β genes, were used instead of the IL-1β KO mice to confirm the effect of IL-1β on hyperalgesia. This is because the activation of NLRP3 inflammasome can induce the secretion of not only IL-1β, but also IL-1α.[65,66] If only the IL-1β gene was deficient, the level of IL-1α can increase as such cytokines display

mutual compensation.[67] The physiological role of IL-1α is not well-defined[65]; however, several studies reported that the progression of inflammatory diseases may not solely be due to IL-1β, but also IL-1α.[66,68] Therefore, IL-1 KO mice were used in this study.

Numerous drugs were used to inhibit inflammasome activation at various stages in the present study. XO inhibitors reduced uric acid formation, P2X7 receptor antagonist attenuated NLRP3 inflammasome activation, and caspase-1 inhibitor attenuated caspase-1 activity. Ultimately, these drugs attenuated the production of IL-1β and pain. Therefore, these drugs may potentially reduce over-exercised muscle pain in humans. In particular, the XO inhibitors can be used to treat human muscle pain as they are already safely used in the human body.

Sjøgaard et al. reported that there are differences between muscle pain such as shoulder and neck pain, which occurs during occupational tasks, and pain that occurs during physical activities at leisure and sports.[69] The former is induced by static sustained and monotonous repetitive muscle contractions, despite a rather low relative muscle load.[69] In contrast, the latter is induced by more dynamic and relatively high muscle forces.[69] As the possibility remains that our model could not completely produce the pathology of chronic muscle pain such as shoulder and neck pain, lower back pain, and MPS, further research is necessary to investigate appropriate electrical stimulation conditions to reflect the pathology of chronic muscle pain. Other limitations are as follows: (1) the evaluation of inflammasome activation and IL-1β levels was performed only in muscle tissues; no additional testing of sensory neurons and the CNS was performed, (2) the evaluation of tissue pH and ATP production was not performed, (3) we did not confirm MSU crystal formation using polarizing microscopes, (4) the cut-off value of uric acid concentration that can induce inflammasome activation was not determined, (5) an analysis of other inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, was not performed, and (6) the evaluation of inflammasome activation was not performed with neutrophils.

Conclusions

IL-1β secretion and NLRP3 inflammasome activation in macrophages due to elevated levels of uric acid produced mechanical hyperalgesia through repeated excessive muscle contractions. Therefore, pharmacological blockade of this process may potentially reduce over-exercised muscle pain in humans.

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Author Contributions

Declaration of Conflicting Interests

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