



Involvement of neutrophils and interleukin-18 in nociception in a mouse model of muscle pain

Molecular Pain
Volume 14: 1–10
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DOI: 10.1177/1744806918757286
journals.sagepub.com/home/mpx


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Abstract

Muscle pain is a common condition that relates to various pathologies. Muscle overuse induces muscle pain, and neutrophils are key players in pain production. Neutrophils also play a central role in chronic pain by secreting interleukin (IL)-18. The aim of this study was to investigate the involvement of neutrophils and IL-18 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. The pressure pain threshold, number of neutrophils, and IL-18 levels were investigated. Furthermore, the effects of the IL-18-binding protein and Brilliant Blue G on pain were investigated. In stimulated muscles, pressure pain thresholds decreased, and neutrophil and IL-18 levels increased compared with that in non-stimulated muscles. The administration of IL-18-binding protein and Brilliant Blue G attenuated hyperalgesia caused by excessive muscle contraction. These results suggest that increased IL-18 secretion from larger numbers of neutrophils elicits mechanical hyperalgesia.

Keywords

Muscle pain, neutrophil, interleukin-18, electrical stimulation, mechanical hyperalgesia

Date Received: 15 August 2017; revised 26 September 2017; accepted: 13 December 2017

Introduction

Musculoskeletal disorders are the most prevalent causes of chronic health problems, disabilities, and health care utilization.¹ Among these health conditions, muscle pain is a common condition² that relates to various pathologies. Muscle pain may manifest as neck and shoulder pain,³ non-specific low back pain,⁴ and myofascial pain syndrome (MPS).^{5–11} Neck and shoulder pain occurs in approximately 30% of the working population^{12,13} and is a risk factor for long-term absenteeism.^{12,13} Lower back pain has a lifetime prevalence of as high as 84% and causes disabilities of 11%–12% in the general population.¹⁴ MPS is very common in the general population, and its incidence may be as high as 54% in women and 45% in men.⁹ Furthermore, MPS is a primary source of pain in between 30% and 85% of patients presenting at a primary care setting or pain clinic.^{6–8} Although numerous

treatment methods, such as pharmacological treatments, stretching, and massage, have been developed to alleviate these diseases, no single successful strategy is available as the underlying pathogenic mechanisms have not been fully determined.^{5,6,10,15,16}

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Muscle overuse, especially with eccentric contraction, induces pain during exercise.^{17,18} Muscle fiber destruction due to muscle overuse induces the release of adenosine triphosphate (ATP).^{2,19} Activation of specific receptors (so-called nociceptors) by ATP and low pH are particularly important in generating muscle pain.^{2,19} Shinoda et al. reported the involvement of ATP in a masseter muscle pain model in rats.²⁰ Furthermore, sustained muscle contraction and chronic muscle ischemia induce a decrease in pH in affected tissues.^{2,19}

Inflammatory responses to exercise-induced muscle damage may also produce muscle pain.¹⁷ Neutrophils and macrophages invade skeletal muscles and are assumed to produce proinflammatory cytokines after exercise-induced muscle damage.^{17,21} Neutrophils, which first invade injured skeletal muscles after exercise, are key players in innate immunity and influence inflammatory and immune reactions through the production of numerous cytokines such as interleukin (IL)-1 β , tumor necrosis factor alpha (TNF- α), and interferon gamma (IFN- γ).²² Dessem et al. reported an increased number of neutrophils in muscles presenting with mechanical hyperalgesia after eccentric contraction.²³

IL-18 is a proinflammatory cytokine that was first described in 1989. Recent findings have revealed that neutrophils are a source of IL-18.^{22,24} The activity of IL-18 is balanced by the naturally occurring IL-18 binding protein (IL-18BP), which shows high affinity for IL-18 in humans.²⁵ IL-18 has been implicated in numerous diseases (e.g. autoimmune diseases, metabolic syndromes, inflammatory bowel disease, sepsis, and acute kidney injury).²⁵ Recombinant IL-18BP has been investigated as a therapeutic cytokine in clinical trials of rheumatoid arthritis and plaque psoriasis.²⁶ Previous investigators reported a relationship between IL-18 and chronic pain.^{27,28} For example, Vasudeva et al. reported that IL-18 functions as a central node associated with neuropathic pain in animal models.²⁷ However, few reports have described the relationship between IL-18 and muscle pain. Eriksson et al. reported that elevated serum IL-18 levels were found in patients with Sjögren's syndrome and myalgia, compared with IL-18 levels in patients with Sjögren's syndrome without myalgia.²⁹

The aim of the present study was to investigate the roles of the increased numbers of neutrophils and IL-18 secretion from these cells in mechanical hyperalgesia induced by repeated, excessive muscle contractions.

Materials and methods

Experimental animals

Male, five- to seven-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 $23 \pm 1^\circ\text{C}$. All aspects of handling, care, and animal use were approved by the Animal Research Committee of Tohoku University (approval number: 2016Mda-240).

Repeated electrical stimulation of triceps surae muscles

Repeated electrical stimulation was used to induce excessive muscle contraction. 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), two needle electrodes (Single-Stranded Stainless-Steel Wire, A-M System, Sequim, WA, USA) were transcutaneously applied into the triceps surae muscle of the right hind leg. Needle electrodes were placed on the proximal and distal ends of the muscle. After placing the needle electrodes, electrical stimulation of the muscle was performed at 10 Hz with a 10-V amplitude and a 100- μs pulse width for 30 min using a STG4004 multichannel system (MCS GmbH, Reutlingen, Germany), as previously described.²⁰ During electrical stimulation for 30 min, the right hind leg was immobilized with the ankle joint in dorsal flexion so that the triceps surae muscle was fully extended to induce isometric contraction. A fused tetanic contraction was evoked. The electrical stimulation was performed for seven days per week. The needle electrodes were also applied to the contralateral triceps surae muscle without electrical stimulation or immobilization.

Assessment of mechanical nociceptive thresholds

The pressure pain threshold (PPT) was assessed using the Randall–Selitto test (MK-201D Pressure Analgesy-Meter, Muromachi Kikai Co., Tokyo, Japan).³⁰ This test employed a cone-shaped plastic tip (tip diameter: 2.6 mm) attached to a scale with a display. A linearly increasing pressure (10 mm Hg/s) was applied to the lateral surface of the triceps surae muscle. Skin over the triceps surae muscle was carefully shaved bilaterally at the beginning of the experiment in order to find the muscle more easily. Mice were loosely wrapped in a towel to calm them down and were treated gently during the experiment. The PPT was defined as the amount of pressure (mm Hg) required to elicit pain-related behaviors such as vocalization, struggling, and leg withdrawal.^{20,31,32} PPT assessment of both hind legs was performed every day for 13 days after initiation of the electrical stimulation. All experiments investigating mechanical hyperalgesia were performed in the morning as circadian rhythm affects pain sensitivity. Experiments investigating secondary hyperalgesia were

performed in the plantar surface of the foot. The cut-off value of the PPT was 300 mm Hg.³¹ Assessment of the PPT data was performed by a blinded investigator to avoid bias.

Local effect of IL-18 on hyperalgesia

To confirm the local effects of IL-18, recombinant mouse IL-18 protein (B004–5, 50 ng; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) dissolved in saline (rmIL-18 group) or saline alone (saline group) was administered to the triceps surae muscles, as previously described.³³ The volume of injected solution was 25 μ l in each group. At 1, 2, 4, 24, and 48 h post-injection, PPTs were assessed as described above.

Tissue preparation

On day 7 after the initiation of electrical stimulation, mice were sacrificed by cervical dislocation and the triceps surae muscles were obtained. Specimens for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and enzyme-linked immunosorbent assays (ELISAs) 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) overnight at 4°C . After dehydration through a graded series of ethanol solutions, the specimens were embedded in paraffin. The embedded tissues were cut into 5- μ m axial sections. For fluorescence activated cell sorting (FACS) analysis, the specimens were immersed in 1% phosphate-buffered saline (PBS) and analyzed the same day.

qRT-PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was prepared using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland) and primers with the following sequences: IL-18: F5'-TGG TGG GGG TTC TCT GTG GTT-3' and R5'-TTG AGG CGG CTT TCT TTG TCC-3'; EF1 α 1 (internal control primer): F5'-TCG CTT TGC TGT TCG TGA C-3' and R5'-TGG GGT GGC AGG TGT TAG-3'. Relative expression levels of each mRNA were calculated as a function of EF1 α 1 expression, as previously described.³⁴

ELISA experiments

Tissue samples were disrupted and homogenized using lysis buffer composed of bovine serum albumin (BSA; 100 μ g/ml, A4503, Sigma-Aldrich, St. Louis, MO, USA), Triton X-100 (0.1%, Wako Pure Chemicals Industries, Osaka, Japan), 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 $9730 \times g$ and 4°C , and the supernatant was stored at -80°C . IL-18 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.

FACS analysis

Tissue samples were transferred to sterile Dulbecco's modified Eagle's medium (Wako Pure Chemicals Industries) supplemented with 1% penicillin-streptomycin. Tissues were minced and digested with 0.2% collagenase (Wako Pure Chemicals Industries) and 0.1% DNase I (Sigma-Aldrich) for 1 h at 37°C . PBS was added to the digested muscle tissue samples, which were then filtered through a 70- μ m cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged at $700 \times g$ for 20 min at 4°C . Pellets were resuspended in 1 ml of staining solution composed of 1% BSA (Sigma-Aldrich) in PBS, and then incubated with an Fc receptor-blocking solution (TruStain fcX, 1:50 in staining buffer; BioLegend, San Diego, CA, USA) for 10 min. The samples were then labeled with the following monoclonal antibodies (all at a 1:20 dilution): fluorescein isothiocyanate-conjugated anti-CD45 (clone 30-F11, BioLegend), allophycocyanin (APC)/Cy7-conjugated anti-CD11b (clone M1/70, BioLegend), peridinin chlorophyll a protein/Cy5.5-conjugated anti-Ly6G (clone 1A8, BioLegend), phycoerythrin-conjugated anti-Siglec F (clone E50-2440, BD Biosciences), or APC-conjugated anti-F4/80 (clone BM8, BioLegend). After a 45-min incubation on ice, the cell suspension was washed with staining solution and centrifuged twice for 5 min at $700 \times g$. Neutrophils were defined as single live mononuclear CD45⁺CD11b⁺Ly6G⁺SiglecF⁻F4/80⁻ cells.³⁵⁻³⁷ FACS was performed on a FACS ARIA II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). The sorting gate was set using negative-control cells treated with the Fc receptor-blocking solution.

Immunohistochemistry

Tissue sections were deparaffinized and washed in PBS. Subsequently, they were incubated with Target Retrieval Solution (Agilent Technologies, Santa Clara, CA, USA) for 60 min at 100°C to induce antigen retrieval. After washing in DW and PBS, endogenous immunoglobulins were blocked by incubation with 10% normal goat serum (Nichirei Biosciences Inc., Tokyo, Japan) for 60 min. The slides were washed again in PBS and incubated with a polyclonal rabbit anti-mouse IL-18 antibody

(ab71495, Abcam plc, Cambridge, UK, dilution 1:500) and a monoclonal rat anti-mouse granulocyte receptor-1 (Gr-1) antibody (RB6-8C5, BioLegend, dilution 1:500) in PBS overnight at 4°C, and then rinsed in PBS. Subsequently, the slides were incubated for 60 min in PBS with an Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11034, Life Technologies, Carlsbad, CA, USA, dilution 1:750) for IL-18 and an Alexa Fluor 555-conjugated goat anti-rat IgG (A-21434, Life Technologies, dilution 1:750) for Gr-1 at room temperature, after which they were rinsed in 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 with a fluorescence microscope (BZ-9000 BIOREVO, KEYENCE, Osaka, Japan). The images were analyzed using Adobe Photoshop (Adobe System Inc., San Jose, CA, USA). The localization of Gr-1-positive cells (neutrophils) was evaluated by two blinded investigators to avoid bias. Two animals were used for immunohistochemistry, and two slides/animal were analyzed. After confirming reproducibility, representative images were presented.

Assessment of the systemic effect of BBG on hyperalgesia

Brilliant Blue G (BBG) is a selective P2X7R antagonist that decreases secretion of mature IL-18 by attenuating inflammasome activation in various cell types, including neutrophils.^{38,39} BBG was administered to mice to test the effects of decreased mature IL-18 secretion on hyperalgesia. During repeated electrical stimulation of the triceps surae muscles, BBG dissolved in 0.9% saline (45.5 mg/kg, 0.3 ml/body) was injected intraperitoneally every 48 h (BBG group), as previously described.⁴⁰ Control animals received an equivalent volume of 0.9% saline (saline group). The PPTs were assessed by performing the Randall-Selitto test on day 7. Measurement of IL-18 levels by ELISA was performed as described above.

Assessment of the local effect of IL-18BP on hyperalgesia

To test the local effects of IL-18BP on hyperalgesia, IL-18BP (122-BP-100, R&D Systems, Inc., Minneapolis, MN, USA, 1 µg) dissolved in saline (100 µl) or saline alone (100 µl) was administered to the triceps surae muscles, as previously described.⁴¹ During repeated electrical stimulation, mice were administered either IL-18BP (IL-18BP group) or saline (saline group) in the triceps surae muscles every day of stimulation. PPTs were assessed in the same manner.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 24 (IBM, Armonk, NY, USA). Analysis of PPT time-course data was performed by two-way analysis of variance (ANOVA) with repeated measures with Tukey's post hoc multiple-comparison test. To compare data from four groups from a single day (PPTs and ELISA), one-way ANOVA with Tukey's post hoc, multiple-comparison test was used for the analysis. ELISA data between two groups were analyzed with the Mann-Whitney test. FACS and qRT-PCR data were analyzed using the Wilcoxon signed-rank test. Data were expressed as the mean ± standard error of the mean. A p value of <0.05 was considered to represent statistical significance.

Results

Induction of recombinant IL-18 protein production by hyperalgesia

The PPTs of the recombinant IL-18 (rmIL-18) group significantly decreased from 1 to 24 h after administration, compared with that before administration. PPTs recovered by 48-h post-administration. In the saline group, no significant changes were observed during between 1 and 24 h after administration compared with that before administration. Significant differences were observed between the rmIL-18 group and saline group between 1 and 24 h after administration (Figure 1).

Excessive contraction caused by repeated electrical stimulation induced mechanical hyperalgesia and elevated IL-18 production in muscles

The PPTs of the stimulated muscles began to decrease on day 3, and significantly decreased during days 4 to 11

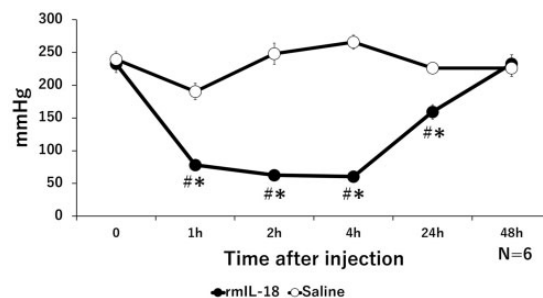


Figure 1. Change in pressure pain thresholds (PPTs) induced by local administration of recombinant IL-18 protein (rmIL-18). Time course of PPT values in the rmIL-18 group and saline group. The PPTs of the rmIL-18 group significantly decreased compared with those of the saline group from 1 to 24 h after administration; * $p < 0.05$, significantly different from the saline group; # $p < 0.05$, significantly different from the pre-injection values.

compared with that of non-stimulated contralateral muscle. Reduced PPTs in the stimulated muscles recovered after the electrical stimulation was completed, and no significant difference was observed in PPTs between stimulated and non-stimulated muscles after day 12. No significant changes were observed in the PPTs of non-stimulated muscles, during the experimental period compared with the values before electrical stimulation (Figure 2(a)). There was no evidence for secondary hyperalgesia during the experiments. IL-18 induction in the stimulated muscles significantly increased compared with that in non-stimulated muscles, as determined by qRT-PCR (Figure 2(b)) or ELISA (Figure 2(c)).

The number of neutrophils increased in hyperalgesic muscles

The number of neutrophils from stimulated muscles (21%) significantly increased compared with that in non-stimulated muscles (14%), as determined by FACS (Figure 3(a), (c), and (d)). However, the number

of macrophages did not increase in the stimulated muscles (Figure 3(b)). Immunohistochemical staining revealed an increased number of Gr-1-positive cells (neutrophils) in stimulated muscle fibers compared with that in non-stimulated Gr-1-positive cells. Most of these cells were also positive for IL-18 (Figure 3(e) to (j)).

BBG and IL-18BP attenuated hyperalgesia in excessively contracted muscles

In the BBG group, the PPTs significantly increased and IL-18 levels significantly decreased compared with those in the saline group (Figure 4(a) and (b)). No significant differences were observed in the PPTs and IL-18 levels between the saline group and the stimulated muscles of non-treated mice or between the BBG group and the non-stimulated muscles of non-treated mice. In the PPTs of the IL-18BP group, no significant changes were found during the experimental period versus before the electrical stimulation. The PPTs of the saline group significantly decreased after day 4 compared those before electrical stimulations.

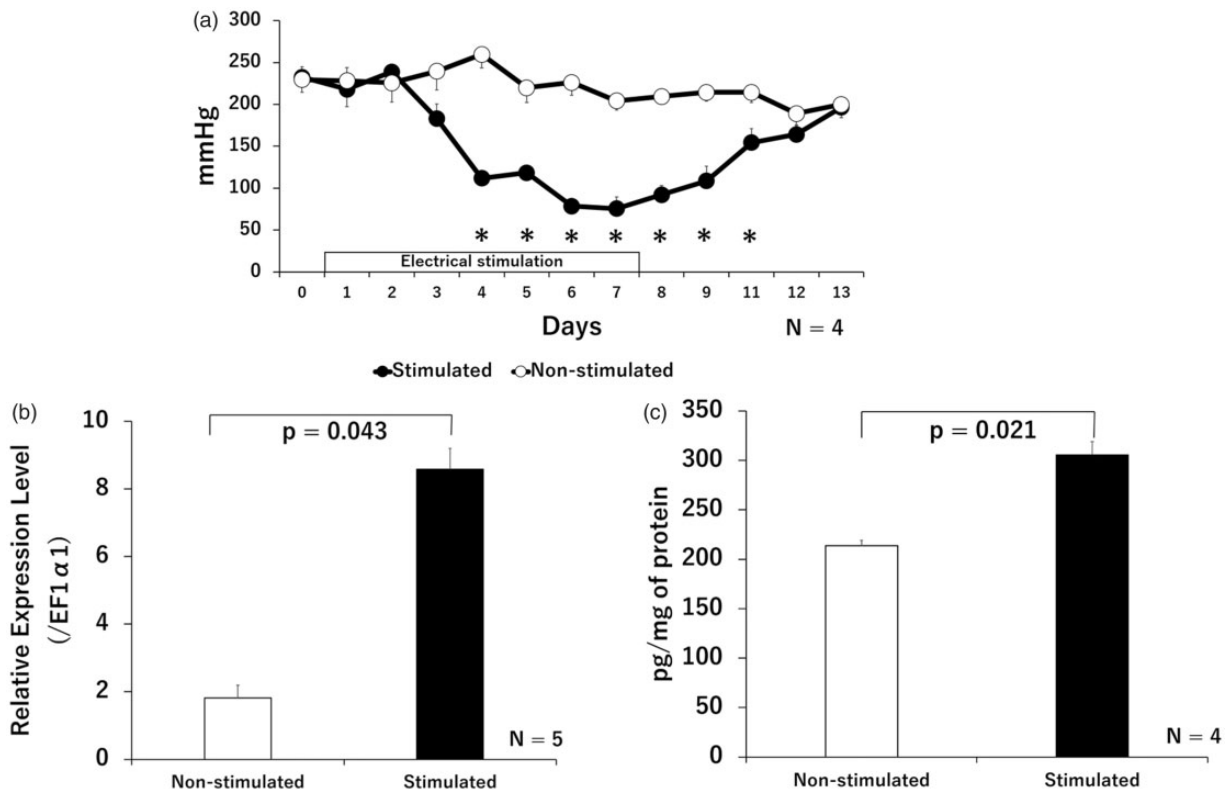


Figure 2. Changes in the PPTs and IL-18 levels in stimulated and non-stimulated muscles. Time course of PPT changes (a). The PPTs of stimulated muscles significantly decreased compared with that of non-stimulated contralateral muscles, during days 4 to 11 after initiating electrical stimulation; * $p < 0.05$, significantly different from non-stimulated muscle. Relative gene expression levels of IL-18 (b) and quantities of IL-18 (c) in muscle tissues, with or without electrical stimulation at day 7; Relative IL-18 mRNA expression (determined by qRT-PCR) and protein expression (determined by ELISA) increased significantly in stimulated muscles. Expression of the IL-18 gene is shown ratiometrically, relative to EF1 α 1 expression. The concentrations of protein of all the samples used for ELISA were equalized to 1.3 mg/ml.

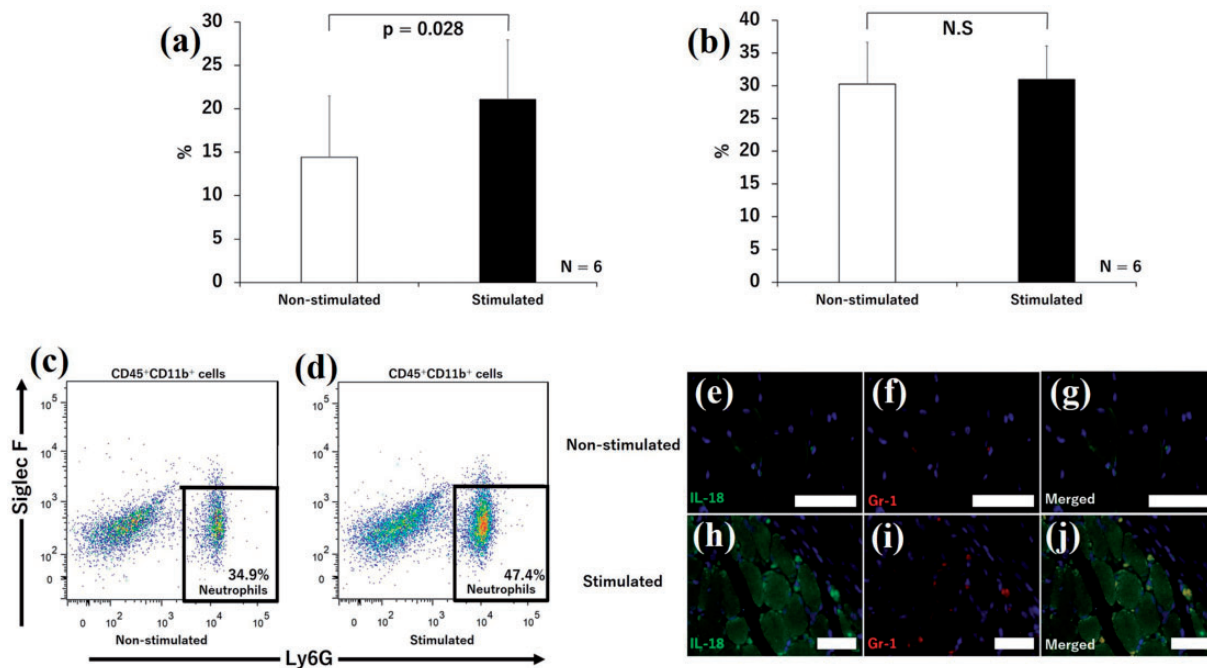


Figure 3. Changes in the number of neutrophils and macrophages in stimulated and non-stimulated muscles. The percentage of the number of neutrophils (a, c, and d) and macrophages (b), and immunohistochemical staining of IL-18 (green) and Gr-1 (red) in stimulated and non-stimulated muscle at day 7 are shown (e to j). The number of neutrophils increased significantly in stimulated muscles. The number of GR-1-positive cells (neutrophils) increased; most of these cells were also positive for IL-18 in stimulated muscles (e to j). The percentage of the number of neutrophils and macrophages are shown as a ratio of the number of CD45-positive cells; scale bar = 50 μm .

Significant differences were observed between the IL-18BP and saline groups from four to seven days after initiating electrical stimulation (Figure 4(c)).

Discussion

Data generated in the present study revealed the following: (1) The administration of rmIL-18 administration into the triceps surae muscles induced hyperalgesia, (2) excessive muscle contraction by electrical stimulation induced mechanical hyperalgesia and IL-18 elevation in the muscle, (3) the number of neutrophils increased after electrical stimulation; these neutrophils secreted IL-18 in painful muscles, and (4) intraperitoneal BBG administration and local IL-18BP administration attenuated hyperalgesia caused by excessive muscle contraction.

Although few reports have shown an association between IL-18 and muscle pain, previous studies reported an association with chronic pain.^{27,28,33} Pilat et al. reported that elevation of IL-18 levels in the spinal cord and intrathecal IL-18BP administration attenuated neuropathic pain in rats.²⁸ Verri et al. reported that intraplantar administration of rmIL-18 induced mechanical hyperalgesia in the planters of mice.³³ These data agree closely with our findings.

It has been reported that muscle contraction in response to electrical stimulation induces mechanical hyperalgesia.^{20,42} This previously reported experimental protocol was followed in experiments using a similar pain model for the rat masseter muscle,²⁰ and the stimuli were set so as to avoid inducing motion or contractions of abdominal muscle. Shinoda et al. reported that repeated muscle contraction (15 days) produced mechanical hyperalgesia 6–15 days following the initiation of contraction in the masseter muscle pain model in rats; these findings were similar to the results of this study. Therefore, the present mouse model is suitable for investigation of triceps surae muscle pain induced by repeated excessive contraction. In order to induce muscle pain, mice were subjected to seven days of contractions in this study. The duration of our experiments was shorter than that in previous studies as hyperalgesia of the muscle was observed at day 4. Although the needle electrodes were also inserted into the contralateral triceps surae muscle without electrical stimulation, hyperalgesia of the muscle was not observed. This finding demonstrates that the observed muscle hyperalgesia was caused by stimulation and not possible injury resulting from repetitive insertion of the needle.

Neutrophils, which immediately migrate to damaged tissues following injury, are involved in inflammatory responses.¹⁷ Infiltration of neutrophils into damaged

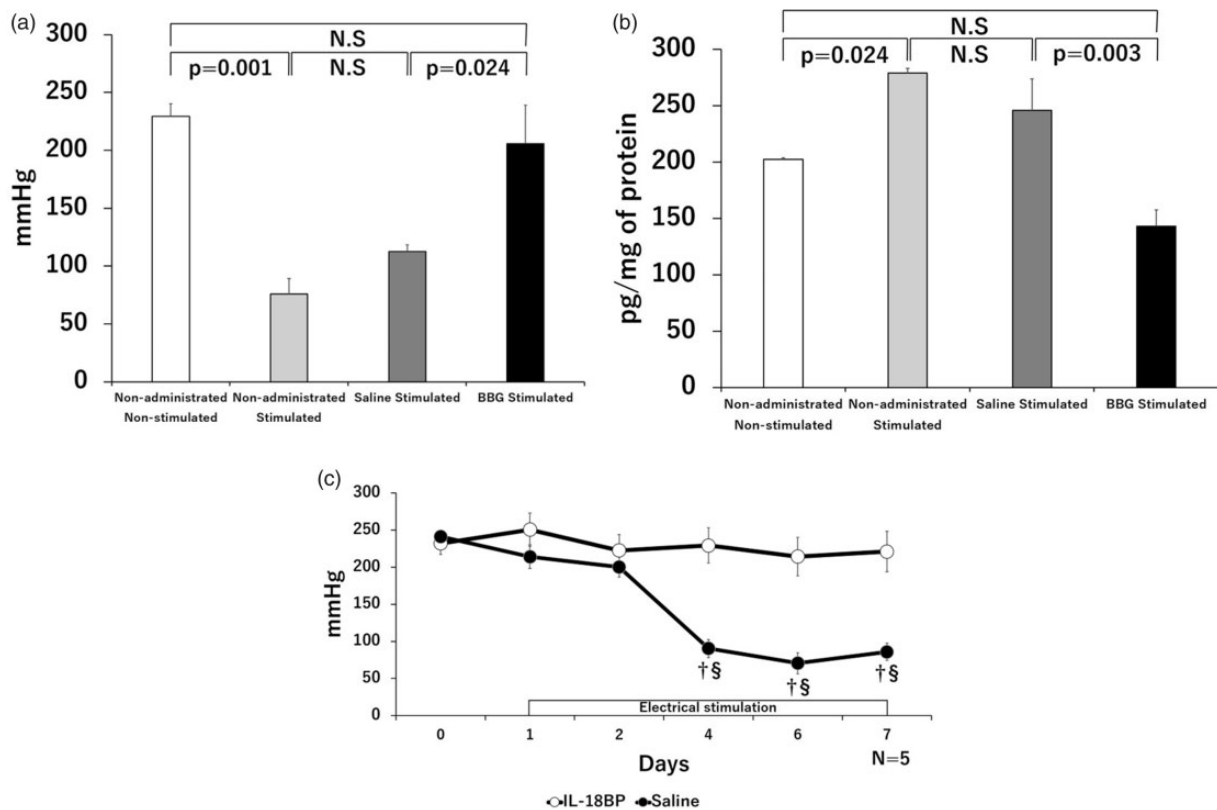


Figure 4. Changes in PPTs and the amount of IL-18 produced after administration of Brilliant Blue G (BBG) and IL-18 binding protein (IL-18BP). The PPTs (a) and quantities of IL-18 (b) in non-stimulated muscles, stimulated muscles of non-treated mice, the saline group, and the BBG group seven days after initiating electrical stimulation are shown. The PPTs increased, and the amount of IL-18 decreased significantly in the BBG group compared with that in the saline group. No significant differences were observed between the saline group and the stimulated muscles of non-treated mice. Time course of the PPT changes in the IL-18BP group and the saline group (c). In the IL-18BP group, PPTs significantly increased compared with those in the saline group; $\dagger p < 0.05$, significantly different from the IL-18BP group; $\S p < 0.05$, significantly different from the pre-stimulation values (day 0). The protein concentrations of protein of all samples used for ELISA were equalized to 1.3 mg/ml.

muscles within several hours of exercise has been demonstrated in various species of mice and rats, as well as in humans.^{23,43,44} After infiltration into damaged muscles, neutrophils phagocytose necrotic tissue⁴⁵ and secrete proinflammatory cytokines.¹⁷ Kanda et al. reported an increased number of circulating neutrophils and their migration to damaged muscle tissues after eccentric exercise, within several hours in humans.⁴³ Dessem et al. and Pizza et al. reported an increase in neutrophil counts in muscle tissues at one to four days after eccentric contraction with electrical stimulation in rat and mouse models, respectively.^{23,44} Furthermore, the relationship between neutrophil invasion and muscle pain has been reported. Schiavuzzo et al. reported that pre-treatment with fucoidan (a leukocyte-adhesion inhibitor) attenuated hyperalgesia induced by local administration of α , β -meATP (a P2X3 agonist) in a rat model.⁴⁶ These reports support the present observation of increased neutrophils in painful muscles. However, some reports indicate that neutrophils are

present for up to 24 h after exercise, and that macrophages subsequently replace the neutrophils.^{17,23} Although the muscle tissues were harvested after repeated stimulation on day 7, we did not observe that the number of macrophages increased in stimulated muscles versus non-stimulated muscles. These results may be explained by the fact that the muscle tissues were harvested approximately 24 h after the last stimulation.

Although it has been reported that IL-18 is produced by macrophages, dendritic cells, and epithelial cells,^{25,47} recent reports have shown that neutrophils may represent a source of IL-18.^{22,24,48} Robertson et al. reported the constitutive expression of IL-18 in human neutrophils obtained from healthy volunteers and patients with rheumatoid arthritis.²⁴ Spörri et al. reported that neutrophils produced mature IL-18 in a *Legionella pneumophila*-infection mouse model.⁴⁸ These reports support our finding that neutrophils represent a source of IL-18 in painful muscles. Immunohistochemical experiments indicated both neutrophils and muscle fibers were also

IL-18-positive in stimulated muscles, which suggest that IL-18 was secreted from neutrophils in the affected muscle cells.

No previous reports have shown that BBG attenuates pain or suppresses IL-18 production in a model of muscle pain. However, numerous studies have shown that BBG decreases IL-18 secretion by blocking P2X7R in various diseases such as graft-versus-host disease,³⁸ subarachnoid hemorrhage,³⁹ and sleep apnea syndrome.⁴⁹ Furthermore, several reports have shown that BBG attenuates pain in various diseases such as bone cancer,⁵⁰ neuropathic pain,⁵¹ and chronic pancreatitis.⁵² These reports support our results that intraperitoneal BBG administration attenuated hyperalgesia and IL-18 induction by excessive muscle contraction. Our results showed that the saline group tended to have higher PPTs and lower IL-18 levels than the non-treated group. These trends may have occurred due to washing out or dilution of IL-18 in muscles by intraperitoneally injected saline. Although no studies have investigated the treatment of muscle pain by administration of IL-18BP to peripheral tissues, Min reported that subcutaneous IL-18BP administration attenuated acute graft-versus-host disease responses.⁴¹ This report supports the present finding that IL-18BP administration to the triceps surae muscles attenuated hyperalgesia following excessive contraction. Our data from experiments conducted using BBG showed that systemic IL-18 inhibition attenuated muscle hyperalgesia induced by excessive contraction. However, we also found that local administration of IL-18BP to the triceps surae muscles attenuated hyperalgesia. The PPTs of the IL-18BP group (local administration) were almost the same as those of the BBG group (intraperitoneal administration) at day 7 post-initiation of electrical stimulation. These results suggest that increased IL-18 levels affect peripheral tissues, causing muscle hyperalgesia.

Muscle overuse induces ATP release, which generates muscle pain.^{2,19} A few reports have described the relationship between neutrophils, IL-18, and ATP. Perregaux et al. and Eltom et al. reported that ATP enhanced IL-18 production in human blood samples and cigarette smoke-exposed mice, respectively.^{53,54} However, it is not known whether neutrophils produce IL-18 after stimulation by ATP, and therefore whether IL-18 secretion from neutrophils is due to ATP stimulation following muscle contraction. Elton et al. reported that IL-18 enhances the transmigration of neutrophils.⁵⁴ These findings may explain the persistence of muscle pain.

Our study has the following limitations: (1) evaluation of IL-18 levels was performed only in muscle tissues, without additional testing of sensory neurons and the central nervous system; (2) evaluation of ATP production was not performed; (3) assessment of the PPT using the Randall–Selitto test was performed by only one

investigator; (4) evaluation of other inflammatory cytokines such as IL-1 β , TNF- α , and IFN- γ was not performed.

Conclusions

The results of our study suggest that increased neutrophil numbers and IL-18 secretion from neutrophils produce mechanical hyperalgesia induced by repeated excessive muscle contraction. To our knowledge, this report is the first to demonstrate the relationship between IL-18 and muscle pain induced by repeated excessive contraction. These findings reveal a potential therapeutic target for treating various muscle pains, including neck and shoulder pain, lower back pain, and MPS.

Authors' Note

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Author Contributions

SY performed experiments and wrote the article. YH and MT supervised the research design and writing of the article. MS and MK supervised the research design and collection of data. MK, HH, and CC participated in data collection. TY, YS, NI, TS, and YY gave advice regarding the experimental methods used. KS and EI provided advice regarding research design.

Acknowledgment

The authors thank Natsumi Emoto for technical advice and data collection.


Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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