ORIGINAL ARTICLE

P2X3 receptors contribute to muscle pain induced by static contraction by a mechanism dependent on neutrophil migration

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Abstract

P2X3 receptors are involved with several pain conditions. Muscle pain induced by static contraction has an important socioeconomic impact. Here, we evaluated the involvement of P2X3 receptors on mechanical muscle hyperalgesia and neutrophil migration induced by static contraction in rats. Also, we evaluated whether static contraction would be able to increase muscle levels of TNF-α and IL-1β. Male Wistar rats were pretreated with the selective P2X3 receptor antagonist, A-317491, by intramuscular or intrathecal injection and the static contraction-induced mechanical muscle hyperalgesia was evaluated using the Randall–Selitto test. Neutrophil migration was evaluated by measurement of myeloperoxidase (MPO) kinetic–colorimetric assay and the cytokines TNF-α and IL-1β by enzyme-linked immunosorbent assay. Intramuscular or intrathecal pretreatment with A-317491 prevented static contraction-induced mechanical muscle hyperalgesia. In addition, A-317491 reduced static contraction-induced mechanical muscle hyperalgesia when administered 30 and 60 min of the beginning of static contraction, but not after 30 and 60 min of the end of static contraction. Intramuscular A-317491 also prevented static contraction-induced neutrophil migration. In a period of 24 h, static contraction did not increase muscle levels of TNF-α and IL-1β. These findings demonstrated that mechanical muscle hyperalgesia and neutrophil migration induced by static contraction are modulated by P2X3 receptors expressed on the gastrocnemius muscle and spinal cord dorsal horn. Also, we suggest that P2X3 receptors are important to the development but not to maintenance of muscle hyperalgesia. Therefore, P2X3 receptors can be pointed out as a target to musculoskeletal pain conditions induced by daily or work-related activities.

Keywords P2X3 receptors · Muscle · Hyperalgesia · Static contraction · Cytokines · Neutrophils

Introduction

It is estimated that pain affects around 100 millions of Americans, and that annual cost, including treatment of pain and medical leave, is between $560 and $635 billions of dollars [1]. Musculoskeletal pain affects over 40% of the general population and has an important socioeconomic impact [2].

Low back pain, neck pain, and other musculoskeletal pain disorders are some of the main disability conditions in the world population [3]. Specifically, the static contraction is the type of muscle contraction most related to daily and work activities [4–7] and has been associated with development of muscle pain [8–10]. Recently, we developed a new model of muscle pain induced by static contraction and showed that this muscle pain is modulated by inflammatory mediators, such as bradykinin, prostaglandins, sympathetic amines, and neutrophils in rats [11].

It is widely known that ATP is an inflammatory mediator [12] important for several pain conditions [13, 14]. The involvement of ATP on pain is mediated by activation of purinergic P2X receptors (P2X1-P2X7) [15], especially the subtype P2X3, since it is expressed in key structures of the system related to pain signaling, such as superficial laminae of the spinal cord and C- and Aδ-primary afferent neurons [16,

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17]. P2X3 are ionotropic receptors associated to Ca^{2+} and Na^+ channels; therefore, its activation depolarizes or sensitizes nociceptive pathways [18]. P2X3 receptors have an important role on development of several pain conditions, including articular [19–21], cutaneous [22, 23], and muscle pain [24–26]. The intramuscular injection of ATP is able to induce muscle pain in mice [27, 28] and in humans [29]. Moreover, eccentric muscle contraction can increase P2X3 receptor mRNA in the masseter [30] and triceps sural muscle [31] and in afferent nociceptors [32]. The mechanism by which ATP, via activation of P2X3 receptors, contributes to muscle pain was not completely elucidated. We have previously demonstrated that the non-selective P2X3 receptor agonist, α,β-meATP, induces mechanical muscle hyperalgesia by a mechanism dependent on bradykinin, sympathetic amines, prostaglandins, neutrophil migration, TNF-α, and IL-1β [33].

Considering the clinical relevance of static contraction-induced muscle pain, the aim of this study was to evaluate whether P2X3 receptors are involved in the mechanical muscle hyperalgesia and neutrophil migration induced by static contraction. We also evaluated whether static contraction-induced muscle pain is modulated by the pro-inflammatory cytokines TNF-α and IL-1β.

Materials and methods

Animals

Male albino Wistar rats (200–250 g) from CEMIB (Multidisciplinary Center for Biological Research) UNICAMP were used and all the procedures followed the guidelines on using laboratory animals from IASP [34] and approved by the Committee on Animal Research of the State University of Campinas (license number 2448-1). The rats were housed in plastic cages with soft bedding (five per cage), in a 12-h light/dark cycle (lights on at 6:00 a.m.). Food and water were available ad libitum and the room was temperature controlled (23 °C). Before the test, rats were habituated to the test room for 1 h.

Muscle hyperalgesia induced by static contraction

The muscle hyperalgesia induced by static contraction was performed as described [11]. Initially, the rats were anesthetized with isoflurane and the static contraction was produced by applying electrical pulses through two electrodes (27 gauge) inserted into the belly of the gastrocnemius muscle. The electrical pulses were generated by a Grass S88X stimulator (Grass Technologies, West Warwick, RI, USA). The parameters used were monophasic current, repeated pulse, frequency of 50 Hz, and pulse duration of 19 ms for 1 h. In the

sham group (control), the electrodes were placed but no stimulation was administered.

Drug

The following drug was used: the selective P2X3 receptor antagonist, 5-([(3-phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] [amino]carbonyl)-1,2,4-benzene-tricarboxylic acid (A-317491). The drug was dissolved in saline (0.9% NaCl) and was obtained from Sigma–Aldrich (St. Louis, MO, USA).

Intrathecal injections

A-317491 or vehicle (NaCl 0.9%) was administered intrathecally [22]. For injections, rats were anesthetized with $^1/_3$ O_2 to $^2/_3$ N_2O and isoflurane at 5% and 1.5% [32], respectively, and a 26-gauge needle was inserted in the subarachnoid space on the midline between the L4 and L5 vertebrae. The drug was injected at 1 μl/s with a total volume of 10 μl. The animals regained consciousness approximately 1 min after discontinuing the anesthesia.

Intramuscular injections

A-317491 or vehicle (NaCl 0.9%) was also administered into the gastrocnemius muscle [33]. For each injection, the needle was connected to a polyethylene catheter as well as to a Hamilton syringe (50 μl). The rats were briefly restrained, for a period no longer than 1 min. There was no evidence of stress and the total volume administered was 50 μl.

Mechanical muscle nociceptive threshold test

It was performed during light phase (9:00 a.m. to 5:00 p.m.) in a quiet room, with controlled temperature (23 °C) [35]. To measure the mechanical muscle hyperalgesia induced by static contraction and the role of the selective antagonist, the Randall–Selitto analgesimeter was used (Insight, Ribeirão Preto, SP, Brazil) [33, 36, 37]. A rounded tip with 2.0 mm of diameter was used to quantify the pain threshold of deep tissues [38]. Initially, the baseline muscle withdrawal threshold was quantified by three tests performed at 5-min intervals before static contraction. The mechanical muscle hyperalgesia was quantified as the change in mechanical nociceptive threshold calculated by subtracting the mean of measurements taken 1 h after the end of contraction [11]. Increases in hyperalgesia were represented by increase in the y-axis. All the experiments were performed by a blinded tester.

Enzyme-linked immunosorbent assay

An adaptation of enzyme-linked immunosorbent assay (ELISA) [39] was used to determine whether the static contraction was able to induce the release of TNF-α and IL-1β into the gastrocnemius muscle of rats. To this end, the gastrocnemius muscles were collected ½, 1, 3, 6, and 24 h after the end of static contraction or sham procedure. The tissues were weighed and homogenized in the same weight/volume proportion in a solution of phosphate-buffered saline (PBS) containing complete protease inhibitor cocktail, at 0.02% (Roche, Indianapolis, USA). The samples were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatants were stored at − 80 °C for posterior use to evaluate the protein levels. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α DuoSet ELISA Kit (R&D Systems, catalog number DY510) and IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501). All procedures were performed following the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA) and were repeated twice to guarantee the authenticity of the results.

Myeloperoxidase activity measurement

To evaluate the role of P2X3 receptors on static contraction-induced neutrophil migration, the analysis of myeloperoxidase activity was used [40]. The gastrocnemius muscles were collected 1 h after the end of static contraction. The samples were weighed and stored at − 80 °C. The homogenizations were in buffer containing 0.1 M NaCl, 0.02 M NaPO$_4$, and 1.015 M Na EDTA (pH 4.7, 500 μl). After that, the homogenized tissues were centrifuged at 2500g for 15 min. Pellets were resuspended in buffer (500 μl), followed by addition of 0.2% NaCl (500 μl) and, 30 s later, of 1.6% NaCl in glucose (500 μl) to induce hypotonic lyses. After another centrifugation, the pellet was resuspended in buffer containing 0.05 M NaPO$_4$ and 0.5% hexadecyl-trimethylammonium bromide (HTAB) (pH 5.4). Samples were quickly frozen in liquid nitrogen, thawed three times to aid in lysis, and centrifuged for 15 min at 10,000g. The supernatant (50 μl) was added to 96-well microplate with 0.08 M NaPO$_4$, followed by 3,3′5,5′-tetranethylbezidine (25 μl). The reaction was started by the addition of 0.5 mM H$_2$O$_2$ (100 μl) and stopped, 5 min later, by 4 M H$_2$SO$_4$ (50 μl). Optical density was read at 450 nm using an Epoch microplate spectrophotometer (BioTek, USA). Results were calculated by comparing a standard curve of neutrophil (> 95% purity) with the optical density of muscle tissue supernatant and were expressed by the number of neutrophils ×10^8/mg tissue. All the results were repeated twice to guarantee the authenticity of the results.

Statistical analysis

The results were represented by the decrease in mechanical muscle withdrawal threshold in grams (g), reported as mean ± SEM and obtained using GraphPad Prism 5.0 software. All data were analyzed using one-way ANOVA followed by post hoc testing using Tukey's test or the Student's t test. Significance was set at $p < 0.05$.

Results

Mechanical muscle hyperalgesia induced by static contraction is modulated by P2X3 receptors

To verify whether mechanical muscle hyperalgesia induced by static contraction was modulated by peripheral P2X3 receptors, the selective P2X3 receptor antagonist, A-317491, was administered into the gastrocnemius muscle previously to static contraction in the same muscle (right muscle) (Fig. 1a). Pretreatment (5 min) with A-317491 (6.0 and 60 μg/muscle, but not 0.6 μg/muscle) prevented static contraction-induced mechanical muscle hyperalgesia ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 1b). A-317491 (60 μg/muscle) into the contralateral gastrocnemius muscle (left muscle) did not affect the static contraction-induced mechanical muscle hyperalgesia measured in the ipsilateral gastrocnemius muscle (right muscle) ($p > 0.05$, one-way ANOVA, Tukey's test, Fig. 1b).
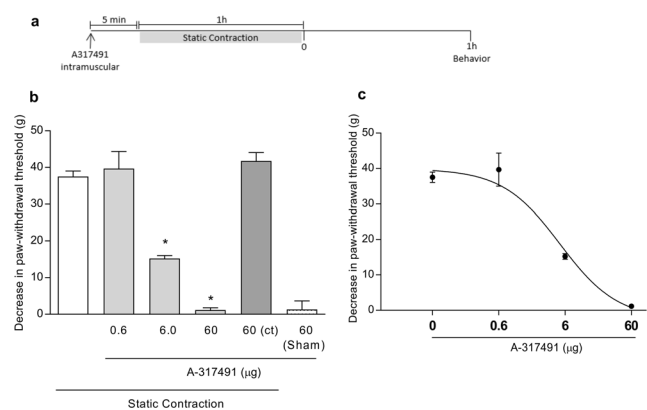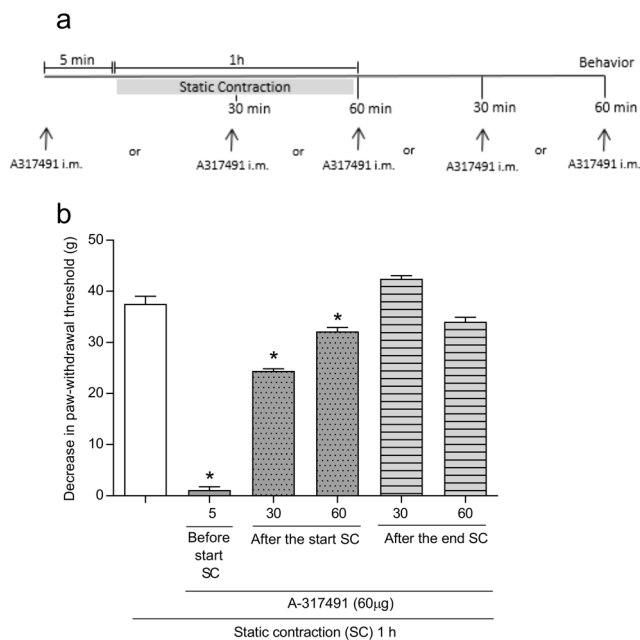

Fig. 1 Involvement of peripheral P2X3 receptors in the mechanical muscle hyperalgesia induced by static contraction. **a** A-317491 was administered (intramuscular) 5 min before static contraction was performed. Behavior was quantified 1 h after static contraction. **b** Pretreatment with A-317491 (6.0 and 60 μg/muscle) prevented static contraction-induced mechanical muscle hyperalgesia when compared with the sham group, as indicated by the asterisk symbol ($p < 0.05$, Tukey's test, $n = 5$). A-317491 (60 μg/muscle) into the contralateral (ct) gastrocnemius muscle (left muscle) did not affect the static contraction-induced mechanical muscle hyperalgesia measured in the ipsilateral gastrocnemius muscle (right muscle, $p > 0.05$, T test, n = 5). **c** Pretreatment with A-317491 reduced mechanical muscle hyperalgesia in a dose-dependent manner (non-linear regression analysis; ED$_{50}$ = 5.0 μg; $n = 5$)

A-317491 did not change behavioral responses in the sham group ($p > 0.05$, T test, Fig. 1b). The anti-hyperalgesic effect of A-317491 occurred in a dose-dependent manner with an ED_{50} of 5 μg (Fig. 1c).

To verify whether the P2X3 receptors are involved in the development or maintenance of static contraction-induced mechanical muscle hyperalgesia, A-317491 was administered into the gastrocnemius muscle in different periods (Fig. 2a). A-317491 (60 μg/muscle) prevented the mechanical muscle hyperalgesia when administered 30 or 60 min after the beginning of static contraction ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 2b), but not 30 or 60 min after the end of static contraction ($p > 0.05$, one-way ANOVA, Tukey's test, Fig. 2b).

To verify whether static contraction-induced mechanical muscle hyperalgesia was modulated by P2X3 receptors expressed on the spinal cord dorsal horn, A-317491 was administered intrathecally and previously to static contraction (Fig. 3a). Intrathecal administration of A-317491 (60 and 180 μg, but not 20 μg) prevented static contraction-induced mechanical muscle hyperalgesia ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 3b). Intrathecal administration of NaCl 0.9% did not change the behavioral responses of the static contraction group ($p > 0.05$, T test, Fig. 3b). The anti-hyperalgesic

effect of A-317491 occurred in a non-dose-dependent manner with an ED_{50} of 44.12 μg.

Static contraction-induced increase in MPO activity is modulated by P2X3 receptors

To verify whether the static contraction-induced neutrophil migration [11] was modulated by P2X3 receptors, A-317491 was administered previously to static contraction (Fig. 4a). Pretreatment with A-317491 (60 μg/muscle, 5 min) prevented the increase in the myeloperoxidase (MPO) activity when compared with the control group ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 4b).

Static contraction did not increase muscle levels of TNF-α and IL-1β

To verify whether static contraction induces local release of the pro-inflammatory cytokines TNF-α and IL-1β, static contraction was performed in the gastrocnemius muscle of rats and the muscle concentrations of TNF-α and IL-1β were quantified ½, 1, 3, 6, and 24 h after the end of contraction (Fig. 5a). Static contraction did not increase the concentrations of TNF-α (Fig. 5b) and IL-1β (Fig. 5c) when compared with the sham group at any time point measured ($p > 0.05$, T test or one-way ANOVA, Tukey's test).

Fig. 2 Involvement of peripheral P2X3 receptors in the maintenance mechanical muscle hyperalgesia induced by static contraction. **a** A-317491 was administered (intramuscular) 5 min before and 30 and 60 min after beginning of static contraction and 30 and 60 min after the end of static contraction. Behavior was quantified 1 h after static contraction. **b** Administration of A-317491 5 min before and 30 or 60 min after the beginning of static contraction prevented the mechanical muscle hyperalgesia, as indicated by the asterisk symbol ($p < 0.05$, Tukey's test, $n = 5$). Administration of A-317491 30 or 60 min after the end of static contraction has no effect on mechanical muscle hyperalgesia ($p > 0.05$, ANOVA, $n = 5$)
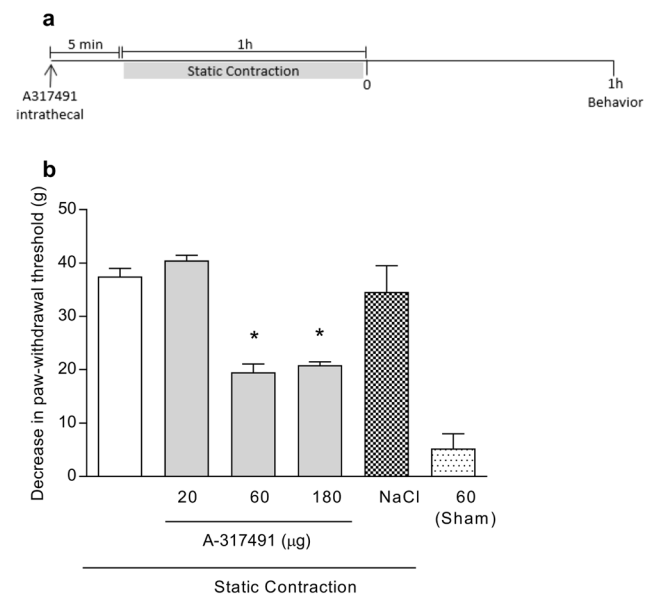

Fig. 3 Involvement of dorsal horn P2X3 receptors in the mechanical muscle hyperalgesia induced by static contraction. **a** A-317491 was administered (intrathecal) 5 min before static contraction was performed. Behavior was quantified 1 h after static contraction. **b** Intrathecal pretreatment with A-317491 (60 and 180 μg) prevented the mechanical muscle hyperalgesia induced by static contraction ($p < 0.05$, Tukey's test, $n = 5$), as indicated by the asterisk symbol, while having no effect in the sham group ($p > 0.05$, T test, $n = 5$)
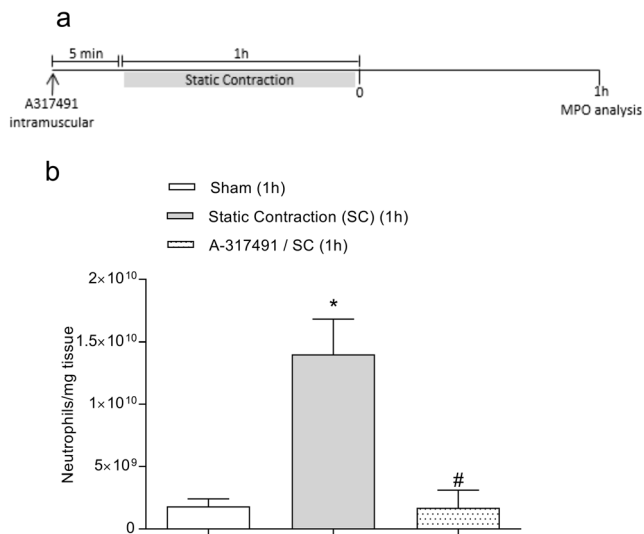
Fig. 4 P2X3 receptors modulate neutrophil migration–induced by static contraction. **a** A-317491 was administered (intramuscular) 5 min before static contraction was performed. The muscle was collected 1 h after static contraction and used to MPO analysis. **b** Static contraction increased MPO activity (expressed by neutrophils/mg of tissue) when compared with the sham group, as indicated by the asterisk symbol ($p < 0.05$, Tukey's test, $n = 5$). Pretreatment with A-317491 (60 μg/muscle) prevented neutrophil migration induced by static contraction when compared with the control group, as indicated by the number symbol ($p < 0.05$, Tukey's test, $n = 5$)

Discussion

In this study, we demonstrated that P2X3 receptors expressed on the gastrocnemius muscle and spinal cord dorsal horn seem to be essential to the mechanical muscle hyperalgesia induced by static contraction in the gastrocnemius muscle of rats. This is because both peripheral (muscle) and central (intrathecal) blockade of P2X3 receptors prevented the static contraction-induced mechanical muscle hyperalgesia. It is widely known that P2X3 receptors are involved in pain signaling of different etiologies and in different tissues, such as neuropathic (partial

sciatic ligation and chronic constriction injury) and inflammatory (complete Freund's adjuvant—CFA, carrageenan, formalin-persistent phase) pain in subcutaneous tissue [13, 22, 41], painful diabetic neuropathy [42], cancer-induced bone pain [43], endometriosis pain [44], articular hyperalgesia [19, 45], and visceral pain (colorectal distension, acetic acid–induced abdominal constriction, and trinitrobenzene sulphonic acid colitis) [46, 47]. Similar to subcutaneous tissue [22] and knee joint [19] of rats, in this present study, we also demonstrated that ATP, via activation of P2X3 receptors, is an important mediator on the development but not in maintenance of muscle hyperalgesia, since A-317491 prevented the mechanical muscle hyperalgesia when administered before and 30 or 60 min after the start of static contraction, but not 30 or 60 min after the end of it. Interestingly, the time course effect of A-317491 seems to be dependent on the inflammatory pain etiology, since after 48 h of an inflammation induced by the intraplantar administration of CFA, A-317491 fully blocked thermal hyperalgesia [41]. However, A-317491 before or after a surgery to induce postoperative pain did not decrease the mechanical allodynia observed 2 h and 1 or 2 days after surgery [41]. Taken together, our present results corroborate with previous studies that have demonstrated the involvement of P2X3 receptors on different musculoskeletal pain models, including CFA-induced masseter allodynia [26], eccentric contraction [25, 30, 48], and occlusal interference [24, 49]. Moreover, it was demonstrated that the activation of P2X3 receptors by its agonist (α,β-meATP) mediates musculoskeletal pain [33, 48].

In the present study, we used an animal model of musculoskeletal pain induced by static contraction, which is very similar to musculoskeletal pain frequently related to daily and work activities [11]. Specifically, work-related neck and shoulder pain is associated with static contraction of the trapezius muscle [9, 10, 50–52]. Low back disorders and pain in dental occupations are associated with prolonged static

Fig. 5 Static contraction did not increase muscle levels of TNF-α and IL-1β. **a** A-317491 was administered (intramuscular) 5 min before static contraction was performed. The muscle was collected ½, 1, 3, 6, and 24 h after static contraction and used to ELISA analysis. **b**, **c** Static contraction did not increase muscle levels of TNF-α (**b**) and IL-1β (**c**), at any time point measured, when compared with the sham group ($p > 0.05$, T test or Tukey's test, $n = 5$)
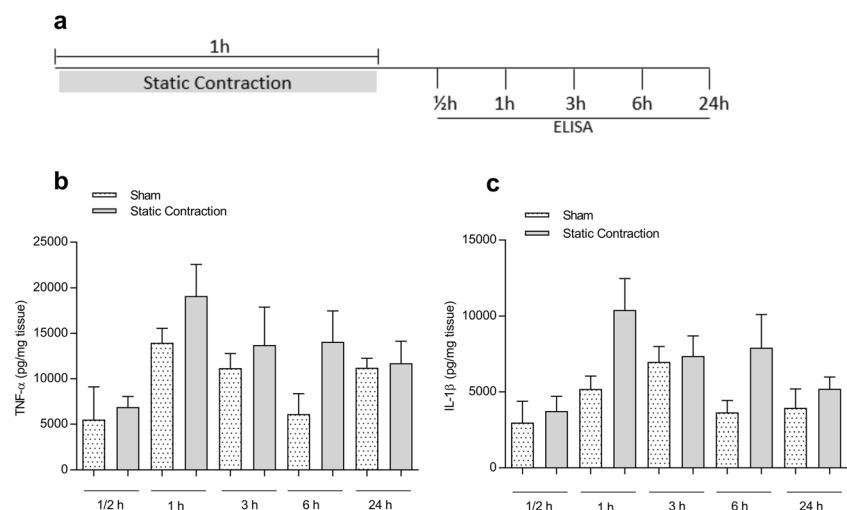
contraction of low back muscle in a sitting posture [53]; back pain during bedrest is primarily caused by low-intensity static contraction of low back muscles [54] and in patients with chronic myalgia or fibromyalgia, the static contraction of a muscle induces a marked increase in pain [55, 56]. We have recently demonstrated that this type of muscle contraction induces musculoskeletal pain by mechanisms that depend on the final inflammatory mediators released, e.g., prostaglandins and sympathetic amines, as well as neutrophil migration [11]. Therefore, our present results lead us to suggest that the static contraction induces ATP release, which in turn activates P2X3 receptors, increasing the susceptibility of muscle nociceptors to the final inflammatory mediators. Some mechanisms by which P2X3 receptors mediate nociceptive responses have already been reported [57, 58]. For example, it has been suggested that the P2X3 receptor is involved in endometriosis pain signal transduction via ERK signal pathway [44]. The peripheral mechanisms underlying the role of P2X3 receptors in inflammatory hyperalgesia are mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous pro-inflammatory cytokines released in different tissues [19, 22] and by a direct sensitization of the primary afferent nociceptors [22]. The vascular endothelial growth factor is involved in neuropathic pain transmission mediated by P2X3 receptors on primary sensory neurons [59]. In addition, the upregulation of P2X3 receptors in different cell types and tissues appears to be a potential peripheral mechanism involved in pain processes [19, 60–64].

The activation of P2X3 receptors produces pro-nociceptive effects in both primary afferent nociceptors and spinal cord. Although the spinal cord activation of P2X3 receptors have significant contributions to both inflammatory and neuropathic hyperalgesia [65], the peripheral activation of P2X3 receptors contributes to the development of inflammatory hyperalgesia but not of neuropathic hyperalgesia [65]. A dose comparison in the present study shows that the intramuscular blockade of P2X3 receptors appears to be more effective than the intrathecal blockade to reduce mechanical muscle hyperalgesia induced by static contraction. This difference may be related with the possible muscle but not central increase of ATP, which would be sufficient to bind to P2X3 receptors leading to membrane depolarization and cell excitation in the nociceptive primary afferent neurons. On the other hand, the activation of P2X3 receptors in supraspinal regions plays an inhibitory role in pain transmission [66].

It is important to clarify that although central and peripheral activation of P2X3 receptors are involved in nociceptive signaling of different etiologies and in different tissues [22, 45, 65, 67], the mechanisms by which these receptors contribute to pain process are not necessarily the same. For example, the present study demonstrated, for the first time, the involvement of P2X3 receptors in neutrophil migration induced by static contraction. It is well known that neutrophils have a role on different pain conditions [16, 17, 65, 67], including muscle pain [11, 30, 33, 68, 69]. Neutrophils invade skeletal muscles and are assumed to produce pro-inflammatory cytokines after exercise-induced muscle damage [70]. We have demonstrated that the non-selective P2X3 receptor agonist, α,β-meATP, induces muscle pain by a mechanism dependent on neutrophil migration [33]. Together, these results reinforce that endogenous ATP activates P2X3 receptors of muscle tissue and trigger the neutrophil migration to contribute to inflammatory muscle pain induced by different stimuli. Interestingly, the role of P2X3 receptors on neutrophil migration associated to inflammatory pain seems to be tissue dependent, since P2X3 receptors are involved with neutrophil migration induced by carrageenan on knee joint [45], but not with subcutaneous tissue [22] of rats. The mechanism by which P2X3 receptors modulate static contraction-induced neutrophil migration is unknown. However, considering that neutrophils modulate inflammatory pain by release of prostaglandins [71] and that static contraction-induced muscle pain is modulated by prostaglandins [11], we suggest that static contraction-induced release of ATP, which via activation of P2X3 receptors, contributed to release of prostaglandins triggered by neutrophils on muscle tissue.

Finally, we demonstrated that there was no increase in muscle levels of TNF-α and IL-1β in a period of 24 h in the end of static contraction. These data are supported by evidences that administration of carrageenan into the gastrocnemius muscle of rats does not increase muscle levels of TNF-α and the muscle levels of IL-1β only increase at 24 h after carrageenan [72]. It is important to point out that, similar to carrageenan, the static contraction induces muscle pain modulated by inflammatory mechanisms [11]. We cannot exclude a possible increase of these cytokines in periods after 24 h. In addition, static contraction is the type of contraction most related to fatigue in humans [73–75]. Therefore, it is possible to hypothesize that static contraction increased muscle blood flow [76, 77] by fatigue mechanisms [78–80] and promoted the clearance of muscle cytokines during contraction [81].

Conclusion

The findings of the present study demonstrated that P2X3 receptors are involved in the mechanical muscle hyperalgesia and neutrophil migration induced by static contraction. In addition, P2X3 receptors are essential to development, but not maintenance of this kind of muscle pain. Considering the static contraction-induced muscle pain has a functional characteristic and, therefore, is clinical and socioeconomic relevant, we point out the P2X3 receptors as important targets to control muscle pain induced by daily or work-related activities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Male albino Wistar rats (200–250 g) from CEMIB (Multidisciplinary Center for Biological Research) UNICAMP were used and all the procedures followed the guidelines on using laboratory animals from IASP [34] and approved by the Committee on Animal Research of the State University of Campinas (license number 2448-1).

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