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SP2X3 and P2X2/3 receptors play a crucial role in articular hyperalgesia development through inflammatory mechanisms in the knee joint experimental synovitis

Juliana Maia Teixeira[1], **Franciane Bobinski**[2], **Carlos Amílcar Parada**[1], **Kathleen A Sluka**[3], and **Cláudia Herrera Tambeli**[1,*]

[1]Department of Structural and Functional Biology, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil

[2]Department of Physiological Sciences, Center of Biological Sciences, Graduate Program in Neurosciences, Federal University of Santa Catarina (UFSC), Florianopolis, SC, Brazil

[3]Department of Physical Therapy and Rehabilitation Science, Pain Research Program, The University of Iowa, Iowa City, IA, USA

Abstract

Osteoarthritis (OA) is a degenerative and progressive disease, characterized by cartilage breakdown and by synovial membrane inflammation, which results in disability, joint swelling and pain. The purinergic P2X3 and P2X2/3 receptors contribute to development of inflammatory hyperalgesia, participate in arthritis processes in the knee joint and are expressed in chondrocytes and nociceptive afferent fibers innervating the knee joint. In this study, we hypothesized that P2X3 and P2X2/3 receptors activation by endogenous ATP (adenosine 5′-triphosphate) induces articular hyperalgesia in the knee joint of male and female rats through an indirect sensitization of primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines and/or on neutrophil migration. We found that the blockade of articular P2X3 and P2X2/3 receptors significantly attenuated carrageenan-induced hyperalgesia in the knee joint of male and estrus female rats in a similar manner. The carrageenan-induced knee joint inflammation increased the expression of P2X3 receptors in chondrocytes of articular cartilage. Further, the blockade of articular P2X3 and P2X2/3 receptors significantly reduced the increased concentration of TNF-α, IL-6 and CINC-1 and the neutrophil migration induced by carrageenan. These findings indicate that P2X3 and P2X2/3 receptors activation by endogenous ATP is essential to hyperalgesia development in the knee joint through an indirect sensitization of primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines and/or on neutrophil migration.

*Corresponding author: Rua Monteiro Lobato, 255 - Campinas - SP - Brasil - CEP 13083-862 – Brazil., Phone number: +55-19-3521-6195, tambeli@unicamp.br (C.H. Tambeli); ju_maiateixeira@yahoo.com.br (J.M. Teixeira).

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Keywords

articular hyperalgesia; P2X3 and P2X2/3 receptors; knee joint; chondrocytes; pro-inflammatory cytokines; neutrophil migration

Introduction

Osteoarthritis (OA) is the most common form of arthritis occurring more frequently in women than men. It is a progressive and degenerative disease with a higher prevalence in women than in men [1–3]. OA is characterized by inflammation of the synovial membrane (synovitis), cartilage degradation and pain that interferes with function [4,5]. A better understanding of the peripheral processes linking inflammatory pain with OA is necessary for the improvement of the analgesic treatments of this disease [6].

There are seven P2X purinergic receptor subtypes (P2X1–P2X7), which are ligand-gated ionotropic channels that open in response to the binding of extracellular ATP (adenosine 5′-triphosphate) [7] and form as both homomers and heteromers in sensory fibers. P2X3 forms as a homomer or can form as a heteromer with P2X2 [8], and both forms are involved in articular pain and hyperalgesia [9–11]. Specifically, P2X3 and P2X2/3 receptors are localized on peripheral and central terminals of unmyelinated C-fiber and thinly myelinated Aδ sensory afferents [12–14]. Thus, P2X3 and P2X2/3 receptors play a significant role in articular pain and hyperalgesia based on their location on afferent fibers.

Purinergic receptors are also found on non-neuronal cells including those involved in the inflammatory process. In particular, human epidermal keratinocytes express P2X3 receptor mRNA [15], and endothelial cells of thymus [16], urothelial cells [17] and chondrocytes [18] express P2X3 receptors. Further, activation of P2X3 receptors in chondrocytes induced nitric oxide and PGE_2 release [18], suggesting that P2X3 receptors on non-neuronal joint cells may enhance nociception through modulating the inflammatory process. However, it is not known whether the expression of P2X3 receptors on chondrocytes is increased during inflammation or whether inflammatory mechanisms such as pro-inflammatory cytokines and neutrophil migration are involved in the contribution of P2X3 receptors to articular pain

Although the prevalence of articular pain conditions is higher in women than in men [1,3], and the involvement of some receptors in pain and analgesia processes is sex-dependent [19–21], it is not known whether P2X3 and P2X2/3 receptors contributes to articular hyperalgesia in a sex dependent manner.

Therefore, in this study, we used the carrageenan-induced knee joint inflammation model in rats [22–24] to test the hypothesis that: (I) the P2X3 and P2X2/3 receptors contribute to carrageenan-induced articular hyperalgesia in a sex-dependent manner, (II) the carrageenan-induced articular inflammation increases expression of P2X3 receptors in the chondrocytes of knee joint articular cartilage and that (III) the contribution of P2X3 and P2X2/3 receptors activation to carrageenan-induced articular hyperalgesia involves a previous release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine-induced chemoattractant-1

(CINC-1, analog to IL-8 in rats) and/or the migration of neutrophils to the inflamed knee joint.

Materials and Methods

Animals

Male and female Wistar rats (200–250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP, Campinas, SP, Brazil) and from Harlan Laboratories (Madison, WI, USA), were used in this study. The animals (specific pathogen free - SPF) were housed in plastic cages with soft bedding (five/cage) on a 12:12 light-dark cycle (lights on at 06 AM), with food (commercial chow for rodents) and filtered water available *ad libitum,* in a temperature-controlled room (±23°C). Testing sessions took place during light phase (09:00 AM – 5:00 PM) in a quiet room maintained at 23°C [25]. During the tests, the animals had no access to water or food.

Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the State University of Campinas (protocol number: 2049–1) and by the Animal Care and Use Committee at the University of Iowa and were carried out in accordance with the IASP guidelines for the study of the pain in animals [26]. The sample size of this study was determined and calculated in accordance with [27]. The group size (n) for each experimental group is showed in “Results sections” and between parentheses in all the figures. Animals were divided randomly into the groups. The experimenter blinded to the experimental groups made all analyses.

Carrageenan-induced knee joint inflammation (synovitis)

Under brief inhalation of isoflurane anesthesia, the skin around the knee joints was shaved and treated with an antiseptic solution of iodine alcohol. Using a 26-gauge needle connected to a polyethylene catheter and also to a Hamilton syringe (50 μL), rats were subjected to intraarticular (i.a.) injection of λ-carrageenan dissolved in 25 μL sterile 0.9% saline solution into their right knee joints [28,23]. The other drugs were injected intra-articularly in the same manner that carrageenan and the control animals received vehicle or sterile 0.9% saline solution.

Drugs and doses

The following drugs were used: λ-carrageenan (Cg; 300 μg/knee, i.a., [29,23,30] and 5-([(3-Phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl)-1,2,4-benzenetricarboxylic acid (A-317491 - the selective P2X3 and P2X2/3 receptor antagonist [31]: 20, 60, 180, 540 μg/knee, i.a., [32]). The drugs were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in 25 μL sterile 0.9% saline solution.

Estrus phase determination of estrous cycle

Because females rats with lower levels of ovarian hormones, such as estrus females, are the most responsive to some analgesic drugs [19–21] and presented an articular hyperalgesic response of the same magnitude than males rats, they were used in this study.

Estrus phase in female rats was determined by daily microscope examination (9:00 – 10:00 AM) of vaginal smears taken by gentle lavage. Estrus phase was identified by the predominance (80 %) of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles [33,34]. This phase was chosen because it represent phase of low ovarian hormonal level, 17β-estradiol and progesterone [35,36].

Gait disturbance - Rat knee-joint incapacitation test

We used the rat knee-joint incapacitation test (Insight, Ribeirão Preto, SP, Brazil), as described previously [23]. Briefly, 3 hours after drugs injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide × 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed in the cylinder to walk and the right paw was connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that rats walk failing to touch the cylinder surface with the injected hind paw, during a 60 seconds period, which is directly proportional to the gait disturbance. Incapacitation (articular hyperalgesia) was quantified as an increase in the PET. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of A-317491, it was injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint.

Tissue Preparation

Three hours after carrageenan (300 μg/knee) or sterile 0.9% saline solution injection (when carrageenan-induced articular hyperalgesia reaches its maximum), rats were anesthetized with sodium pentobarbital (120 mg/kg i.p.) and transcardially perfused with 4% paraformaldehyde (PFA, in 0.1 M phosphate buffer (PB), pH 7.4). Whole knee joints were rapidly removed and kept in the same fixative for 24 hours at 4°C. The fixed specimens were decalcified for 8 weeks with 10% ethylenediaminetetraacetic acid (EDTA) in 0.01 M phosphate buffered saline (PBS) at 4°C with three fresh solution changes per week [37]. After the complete demineralization, the decalcified specimens were then rinsed thoroughly in PBS, placed in 30% sucrose overnight and embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). All samples were rapidly frozen and stored at −80 °C until being cut on cryostat. Serial sections were then cryosectioned at 20 μm using a cryostat which were obtained at the medial midcondylar region in sagittal plane [38,39].

Immunohistochemistry

Immunohistochemistry labeling was performed using standard immunofluorescence techniques. Nonspecific binding sites were blocked with 10% NGS (normal goat serum) for 30 minutes. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated in primary antibody (1:1000, guinea pig Anti-P2X3 Receptor, AB5896, Merck Millipore, Billerica, MA, USA) diluted in 1% NGS and 0.05% Triton X-100 in 0.1M PBS and applied to the tissues overnight (4°C) in a humid atmosphere. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated in secondary antibody (1:1000, goat anti-guinea pig IgG-Alexa488, Life Technologies, Grand Island, NY, USA) diluted in 1% NGS and 0.05%

Triton X-100 in 0.1M PBS for 1 hour at room temperature. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated with TO-PRO3 (1:4000, 30 minutes; Invitrogen, Carlsbad, CA, USA) for nuclear staining. After a final washing, the sections were cover slipped using using Vectashield (Vector Laboratories, Burlingame, CA, USA). Negative controls were prepared without incubation in primary antibody to confirm that there was no non-specific binding of the secondary antibody.

After staining, sections were examined with the Confocal Bio-Rad MRC 1024 Microscope and images were taken with a 20 × objective lens of three specific regions of the knee joint: the articular cartilage covering the femoral condyle, the articular cartilage covering the tibial plateau and the meniscus. Five randomly selected knee sections were chosen for each sample (six samples per group) and were digitally imaged and stored for later analysis. The density (mean, arbitrary units) of each section and the number of positive cells (chondrocytes) were quantified by manually counting total numbers in a given area using Image J (National Institutes of Health). Specifically, a standard size (average area of 75,190 μm^2 for the layer of cartilage covering the femoral condyle, 62,410 μm^2 for the layer of cartilage covering the tibial plateau and 93,630 μm^2 for meniscus) was applied to each section. Chondrocytes were counted if they were positively stained for P2X3 receptor.

Synovial lavage fluid

Under deep anaesthesia (80 mg/kg ketamine and 20 mg/kg xylazine, i.p.), rats were killed by cervical dislocation, the skin overlying the knee was excised, the patellar ligament was dissected and a 26-gauge needle connected to a Hamilton syringe (100 μL) was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μL of phosphate-buffered saline solution (PBS) containing 4 mM EDTA. The resulting synovial lavages (total volume of 100 μL) were combined and immediately stored at −80 °C for further analysis [28].

Enzyme-linked immunosorbent assay (ELISA) procedure

An adaptation of ELISA [40] was used to quantify the cytokines of the rat's knee joint. Briefly, three hours after the drugs injections, the synovial lavage fluid (100 μL) was collected and homogenized in 500 μL of a solution of PBS containing 0.4M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mM phenyl-methylsulfonyl-fluoride, 0.1 mM benzotonic-chloride, 10 mM EDTA, and 2 ng/mL aprotinin (Sigma-Aldrich, St. Louis, MO, USA). The samples were centrifuged (10,000 rpm, 15 minutes, 4° C) and the supernatants were used to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. The cytokines were quantified by the following DuoSet ELISA Kits: TNF-α: Rat TNF-α/TNFSF1A (DY510); IL-1β: Rat IL-1β/IL-1F2 (DY501), IL-6: Rat IL-6 (DY506) and CINC-1: Rat CXCL1/CINC-1 (DY515). All procedures followed the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). All procedures were repeated twice to guarantee the accuracy of the results.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) is one of the enzymes released from neutrophils and associated with tissue injury and is used as a marker of neutrophilic activity in peripheral tissues [41].

Briefly, three hours after the drugs injections, synovial lavage fluid was collected and homogenized in 500 μL of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$,1.015 M NaEDTA) followed by centrifugation (3,000 rpm, 15 min, 4° C). The pellet was resuspended in 500 μL of buffer 1 and subjected to hypotonic lyses by the addition of 500 μL of 0.2 % NaCl followed 30 seconds later by addition of 500 μL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). Samples were then snap-frozen in liquid nitrogen three times and centrifuged (10,000 rpm, 15 minutes, 4° C). The supernatant was used in the assay.

The MPO kinetic-colorimetric assay was conducted as previously described [42]. Fifty microliters of each sample and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. Twenty five microliters of 1.6 mM 3,3′,3,3′-tetramethylbenzidine (TMB) was added in each well and the reaction was initiated by the addition of 100 μL of 0.5 mM H_2O_2. The reaction was stopped 5 minutes later by the addition of 50 μL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340. After that, the results were calculated by comparing the optical density of rat's knee joint synovial lavage fluid supernatant with a standard curve of neutrophil (>95% purity), as previously described [43]. The results were presented as number of neutrophils × 10^4/knee. All procedures were repeated twice to guarantee the results authenticity.

Statistical analysis

Statistical analysis was performed using Prism v5 (GraphPad, La Jolla, CA, USA). To determine if there were significant differences ($p<0.05$) between treatment groups for all measures, one-way ANOVA was performed. If there was a significant between-subjects main effect for treatment group, Tukey's test examined for individual differences between groups. For PET data shown in Figures 1C and 2B a two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

P2X3 and P2X2/3 receptors contribute to carrageenan-induced articular hyperalgesia in males and females

In the current study we show that 300 μg of intraarticular injection of carrageenan increases PET during walking to a similar extent for both male and estrus female rats (Figure 1A and B, second bar, $P>0.05$, one-way ANOVA, Tukey test, n=6 per group). The increased PET begins within 1 hour after carrageenan injection, remains elevated through 6 hours (Figure 2B, $P<0.05$, two-way ANOVA, Bonferroni test, n=6 per group) and returns to baseline 24 hours later (Figure 2B, $P>0.05$, two-way ANOVA, Bonferroni test, n=6 per group).

To determine if P2X3 and P2X2/3 receptors contributed to the enhanced PET, the selective antagonist A-317491 was co-administrated with carrageenan (300 μg/knee) into the knee joint. A-317491 at doses of 60, 180 and 540 μg/knee, but not 20μg/knee (Figure 1A and B, $P>0.05$, one-way ANOVA, Tukey test, n=6 per group), significantly attenuated the carrageenan-induced increase in PET (Figure 1A and B, $P<0.05$, one-way ANOVA, Tukey test, n=6 per group) in males and estrus females knee joint when compared to vehicle controls. The highest dose of A-317491 (540 μg/knee) did not affect the carrageenan-induced elevated PET when applied on the contralateral knee joint (Figure 1A and B, $P>0.05$, one-way ANOVA, Tukey test, n=6 per group) of males and estrus females, confirming its local peripheral action. There were no significant differences in the anti-hyperalgesic effect of the different doses of A-317491 on carrageenan-induced articular hyperalgesia between males and estrus females (Figure 1C, $P>0.05$, two-way ANOVA, Bonferroni test, n=6 per group). Therefore, either males or females could be used to perform the subsequent experiments and we chose to perform them only in male rats due to methodological facilities.

P2X3 and P2X2/3 receptors are crucial to the development of carrageenan-induced articular hyperalgesia

To characterize the period of time at which the activation of P2X3 and P2X2/3 receptors contributes to the development of carrageenan-induced hyperalgesia in the knee joint, A-317491 was co-administrated (0 hour) with carrageenan or administrated ½, 1, 2 or 3 hours after injection of carrageenan. Co-administration of A-317941 (540 μg/knee) with carrageenan (300 μg/knee) (Figure 2A, $P<0.05$, one-way ANOVA, Tukey test, n=6 per group), but not its administration ½, 1, 2 or 3 hours after the carrageenan administration (Figure 2A, $P>0.05$, one-way ANOVA, Tukey test, n=6 per group) significantly prevented the carrageenan-induced PET.

In another set of experiments, A-317491 (540 μg/knee) was co-administrated with carrageenan (300 μg/knee) and the measurements were taken ½, 1, 2, 3, 4, 5, 6 and 24 hours later. The carrageenan-induced articular hyperalgesia reached its maximum 3 hours after injection (Figure 2B). A-317491 prevented the development of the carrageenan-induced PET from 1–6 hours (Figure 2B, $P<0.05$, two-way ANOVA, Bonferroni test, n=6 per group).

Carrageenan increased expression of the P2X3 receptor on the chondrocytes in the knee joint

To test if intraarticular carrageenan injection increases P2X3 receptors on chondrocytes in the knee joint, we examined P2X3 receptors expression after induction of inflammation by immunofluorescence. Intraarticular administration of carrageenan (300 μg/knee) significantly increased the number of chondrocytes that express P2X3 receptor in the articular cartilage covering the femoral condyle (Figure 3, $P<0.05$, one-way ANOVA, Tukey test, n=6 per group), the tibial plateau (Figure 4, $P<0.05$, one-way ANOVA, Tukey test, n=6 per group) and on the meniscus (Figure 5, $P<0.05$, one-way ANOVA, Tukey test, n=6 per group) when compared to saline or naive controls. The expression of P2X3 receptors on the chondrocytes after the intraarticular injection of 0.9% NaCl (50 μL/knee) was similar to that

of naive rats in all three regions analyzed (P>0.05, one-way ANOVA, Tukey test, n=6 per group).

P2X3 and P2X2/3 receptors contribute to carrageenan-induced increase in pro-inflammatory cytokines

To verify if P2X3 and P2X2/3 receptors contribute to the release of pro-inflammatory cytokines in the inflamed joint, we examined the cytokines pro-inflammatory concentrations in the inflamed knee joint from animals injected with A-317491 (540 μg/knee) and compared to vehicle controls.

Intraarticular injection of carrageenan (300 μg/knee) significantly increased concentrations of TNF-α (Figure 6A), IL-1β (Figure 6B), IL-6 (Figure 6C) and CINC-1 (Figure 6D) in the knee joint 3 hours after carrageenan injection (P<0.05, one-way ANOVA, Tukey test, n=8 per group). Co-administration of A-317491 (540 μg/knee) with carrageenan significantly prevented increases in TNF-α (Figure 6A), IL-6 (Figure 6C) and CINC-1 (Figure 6D) (P<0.05, one-way ANOVA, Tukey test, n=8 per group), but not IL-1β concentration in the inflamed knee joint (Figure 6B) (P>0.05, one-way ANOVA, Tukey test, n=8 per group). The endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 after the intraarticular injection of 0.9% NaCl was not significantly different from that of naive rats (Figure 6, P>0.05, one-way ANOVA, Tukey test, n=8 per group).

P2X3 and P2X2/3 receptors contribute to carrageenan-induced knee joint neutrophil infiltration

To test if P2X3 and P2X2/3 receptors contribute to the neutrophilic infiltration induced by carrageenan, we examined myeloperoxidase enzyme (MPO) activity after co-administration of A-317491 (540 μg/knee) with carrageenan and compared to vehicle-controls.

Intraarticular injection of carrageenan (300 μg/knee) significantly increased MPO activity 3 hours after injection (Figure 7, P<0.05, one-way ANOVA, Tukey test, n=8 per group). Co-administration of A-317491 (540 μg/knee) with carrageenan showed significantly lower MPO activity when compared to vehicle-controls (Figure 7, P<0.05, one-way ANOVA, Tukey test, n=8 per group). The intraarticular injection of 0.9% NaCl showed similar levels in MPO activity when compared to naive rats (Figure 7, P>0.05, one-way ANOVA, Tukey test, n=8 per group).

Discussion

In this study, we showed for the first time that P2X3 and P2X2/3 receptors contribute to carrageenan-induced articular hyperalgesia in the knee joint of both male and female rats. Uniquely, we showed that carrageenan-induced articular inflammation increases the expression of P2X3 receptors on chondrocytes of the articular cartilage of the knee joint, and that blockade of P2X3 and P2X2/3 receptors prevents carrageenan-induced increases in pro-inflammatory cytokines, and prevents neutrophilic migration to the knee joint. Together these data suggest that P2X3 and P2X2/3 receptors contribute to carrageenan-induced articular inflammatory hyperalgesia indirectly through enhanced release of the pro-

inflammatory cytokines (TNF-α, IL-6 and CINC-1) and stimulation of neutrophilic infiltration to the injured joint.

We showed that the blockade of P2X3 and P2X2/3 receptors in the knee joint significantly reduced carrageenan-induced articular hyperalgesia from 1 to 6 hours. Although spinal P2X3 and P2X2/3 receptors also contribute to hyperalgesia [44], this anti-hyperalgesic effect was arguably the result of blockade of P2X3 and P2X2/3 receptors local to the site of inflammation since A-317941 injection into the contralateral joint did not reproduce the effect.

Likewise in the subcutaneous tissue of rat paw [32], the P2X3 and P2X2/3 receptors antagonist only reduced carrageenan-induced articular hyperalgesia when it was co-administered with carrageenan, but not when it was administered ½, 1, 2 or 3 hours after the carrageenan administration in the knee joint. Therefore, these purinergic receptors in the peripheral tissue seem to be essential to the development of hyperalgesic response, but not for its maintenance. These results may suggest that in this carrageenan-induced synovitis model, ATP may be released only immediately after the carrageenan administration. However, many inflammatory articular conditions (e.g. osteoarthritis) are in a progressive development and ATP may be continuously released, sustaining the articular hyperalgesia in these pathologies. Therefore, P2X3 and P2X2/3 receptors blockers may be effective on ongoing articular inflammatory conditions, although this possibility needs further confirmation by clinical studies in patients with arthropathies.

Our findings are consistent with animal studies showing that during inflammation ATP is released from macrophages, platelets, neutrophils and dying cells [45–51] to the extracellular milieu, where it contributes to the development of inflammatory hyperalgesia in the subcutaneous tissue [32] and in the articular tissue such as the TMJ [11] and the knee joint [9] of rats via P2X3 and P2X2/3 receptors activation. Further, they are also consistent with human studies showing that extracellular ATP is often found in the synovial fluid of patients with arthropathies [52,53].

The chondrocytes, the unique cell type residing in cartilage [54,55], can release PGE_2 [56], a major contributor of inflammatory pain in arthritis conditions, that subsequently acts through a variety of prostanoids receptors expressed in peripheral sensory neurons and spinal cord [57]. A previous *in vitro* study showed that ATP and α,β-meATP (P2X3 agonist, [14] increase PGE_2 production, an effect that was blocked by the selective P2X3 and P2X2/3 receptors antagonist A317491, indicating a role of P2X3 receptors in PGE_2 release by chondrocytes [18]. Therefore, in addition to the P2X3 and P2X2/3 receptors expressed on the peripheral terminals of primary afferent nociceptors that innervate the knee joint [58], the P2X3 and P2X2/3 receptors expressed in chondrocytes may also contribute to carrageenan-induced articular hyperalgesia.

In this study, we showed for the first time that the local inflammation induced by carrageenan in the rat's knee joint increases the expression of the P2X3 receptor in the chondrocytes of the articular cartilage. Specifically, we show increases in the cartilage covering the femoral condyle and the tibial plateau, and the meniscus of the rat's knee joint.

However, it is important to point out that the increased P2X3 receptor expression on the chondrocytes was observed 3 hours after the intra-articular administration of carrageenan. Because, at this time, the administration of the P2X3 and P2X2/3 receptors antagonist A-317491 had no effect on carrageenan-induced articular hyperalgesia, the increased P2X3 receptor expression on the chondrocytes may not contribute to carrageenan-induced articular hyperalgesia. Alternatively, it may contribute to carrageenan-induced inflammation associated with cartilage degradation [59], which is consistent with the demonstration that ATP can promote cartilage resorption [60].

Several studies suggest the involvement of pro-inflammatory cytokines in the articular inflammatory processes [61–63]. For example, the concentration of IL-1β, TNF-α, IL-6 and IL-8 is significantly increased in the experimental OA knee joint synovial fluid of rats [64,65] and in the synovial fluid of patients with OA [66]. The importance of TNF-α and IL-6 to the development of arthropathies was demonstrated by the ability of anti-TNF-α and anti-IL-6 antibodies to prevent the progression of bone and cartilage damage, relieving pain symptoms in patients [67–70] and by their ability in the prevention of collagen-induced arthritis in mice [71,72]. In this study, we showed that the pro-inflammatory cytokines TNF-α, IL-6 and CINC-1 mediates, at least in part, the essential role played by articular P2X3 and P2X2/3 receptors in the development of carrageenan-induced hyperalgesia in the knee joint. As showed in the subcutaneous tissue [32], IL-1β is not involved in the contribution of P2X3 and P2X2/3 receptor activation in carrageenan-induced articular hyperalgesia. Thus, we suggest that the activation of P2X3 receptors expressed on chondrocytes may contribute to the release of pro-inflammatory cytokines, which subsequently enhance inflammation and activate nociceptors.

The pro-inflammatory cytokines TNF-α, IL-6 and CINC-1 could contribute to the essential role played by articular P2X3 and P2X2/3 receptors in the development of carrageenan-induced hyperalgesia in the knee joint by stimulating the release of PGE_2 from chondrocytes. Consistent to this idea, these cytokines can act on chondrocytes [73,74], and can release PGE_2 [56]. These pro-inflammatory cytokines could also contribute to the essential role played by articular P2X3 and P2X2/3 receptors in the development of carrageenan-induced hyperalgesia in the knee joint by other mechanisms. For example, TNF-α and IL-6 could direct activate the primary afferent nociceptor [75,76] and CINC-1 could stimulate release of sympathomimetic amines [77,78] from sympathetic nerves that innervate the synovial tissue [79].

In patients with OA, there is an intense neutrophil migration [80] into synovial tissues. Neutrophils are involved in the genesis of inflammatory hyperalgesia [81], and their infiltration into synovial tissues can cause proteolytic enzymes release which contributes significantly to the tissue damage [82]. Neutrophil migration can be induced by carrageenan [28] and by the pro-inflammatory chemokine CINC-1 [83]. In this study, we showed that blockade of P2X3 and P2X2/3 receptors reduces carrageenan-induced neutrophilic migration. This finding highlights an important difference between the pathophysiology of inflammatory hyperalgesia in the articular and subcutaneous tissues, because the neutrophil migration does not mediate the contribution of P2X3 and P2X2/3 receptors to the development of carrageenan-induced hyperalgesia in the subcutaneous tissue [32]. It further

suggests that the inflammatory process is unique to tissue type and may depend on the cell types located in the tissue.

Finally, although sex differences in the action of opioids agonists [84,19,20], β-adrenoceptor antagonist [21] and P2X7 receptor antagonist (unpublished data) have been reported, we showed that there is no sex difference in the anti-hyperalgesic effect induced by the blockade of the P2X3 and P2X2/3 receptors in the rat's knee joint. Similarly, the mechanical analgesia induced by kappa opioid agonist asimadoline is not sex dependent [85]. Therefore, taken together, these findings reinforce our previous suggestion that sex differences in pain and analgesia depend, at least in part, on the particular receptor type under study.

In summary, we have shown in the current study that in the carrageenan-induced knee joint synovitis model, the endogenous ATP through P2X3 and P2X2/3 receptors activation plays a crucial role in the development of carrageenan-induced articular hyperalgesia in the knee joint of males and estrus females in a similar manner. Moreover, during knee joint inflammation the expression of P2X3 receptors is increased in chondrocytes, suggesting that their activation may contribute to the inflammatory process. The essential role played by P2X3 and P2X2/3 receptors in the development of carrageenan-induced articular hyperalgesia is dependent on neutrophil migration and on previous pro-inflammatory cytokines release in the knee joint. Taken together, these findings suggest that selective antagonists for the P2X3 and P2X2/3 receptors could be potential targets for drug development for the symptoms treatment of inflammatory joint diseases.

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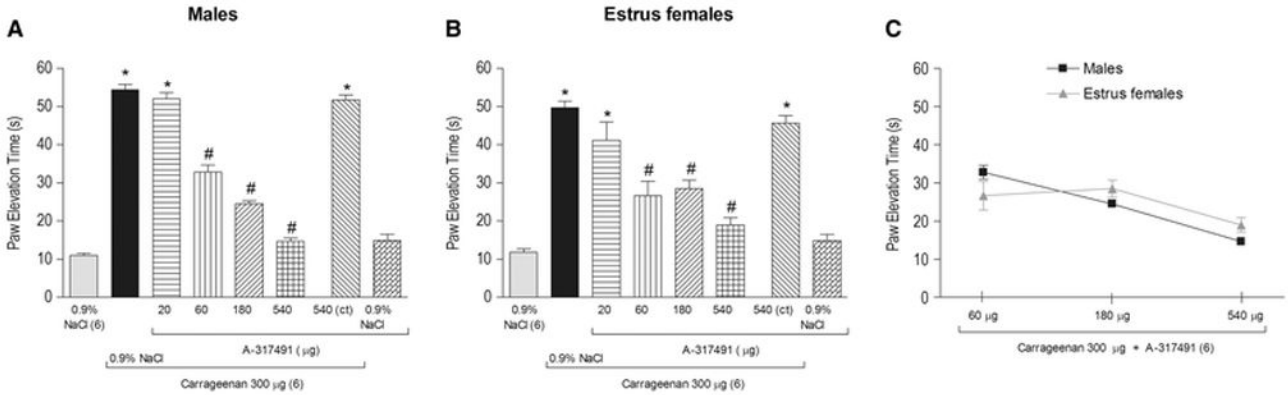

Figure 1. Effect of articular blockade of P2X3 and P2X2/3 receptors on carrageenan-induced hyperalgesia in the knee joint of males and estrus females

Co-administration of the P2X3 and P2X2/3 receptors antagonist A-317491 (60, 180 and 540 µg/knee) with carrageenan (300 µg/knee) significantly reduced carrageenan-induced articular hyperalgesia in males (**A**) and estrus females (**B**), as indicated by the symbol "#" ($P<0.05$, Tukey test) in a similar manner ($P>0.05$, Bonferroni test, **C**). The highest dose of A-317491 (540 µg/knee) injected in the contralateral knee joint (ct) did not affect the carrageenan-induced articular hyperalgesia ($P>0.05$, Tukey test, **A** and **B**). A-317491 injected only with 0.9% NaCl had no effect by itself ($P>0.05$, Tukey test, **A** and **B**). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl (**A** and **B**; $P<0.05$, Tukey test,). In this and in the subsequent figures the articular hyperalgesia was measured 3 hours after the intra-articular (i.a.) drugs administration and the number of rats or samples used are in parentheses.

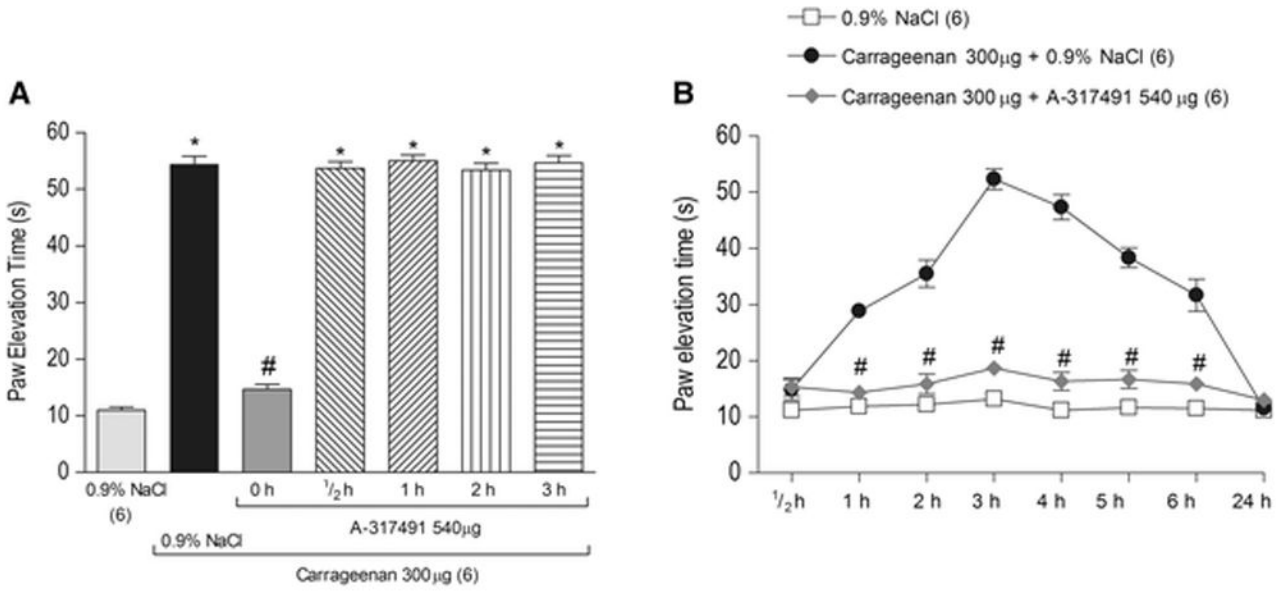

Figure 2. Temporal analysis of the effect of the articular blockade of P2X3 and P2X2/3 receptors on carrageenan-induced articular hyperalgesia

A - Co-administration (0 h) of the P2X3 and P2X2/3 receptors antagonist A-317491 (540 µg/knee) with carrageenan (Cg, 300 µg/knee), but not its administration, 1, 2 or 3 hours after the carrageenan administration ($P>0.05$, Tukey test) significantly reduced carrageenan-induced articular hyperalgesia, as indicated by the symbol "#" ($P<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($P<0.05$, Tukey test). **B -** The temporal analysis of the effect of the co- administration of A-317491 (540 µg/knee) with carrageenan showed that A-317491 blocked the hyperalgesic response 1, 2, 3, 4, 5 and 6 hours after its co-administration, as indicated by the symbol "#" ($P<0.05$, Bonferroni test).

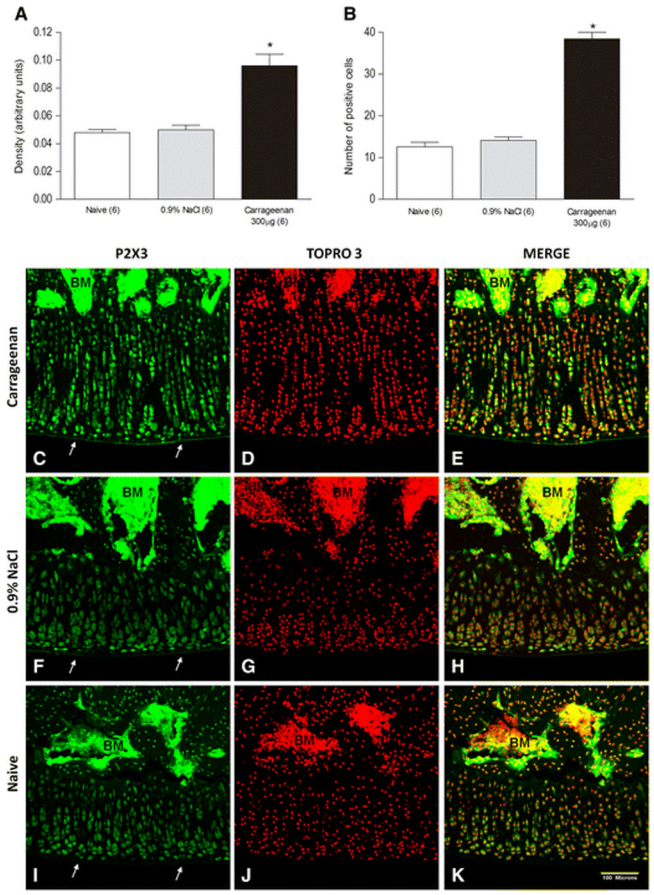

Figure 3. Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the articular cartilage of the femoral condyle of the rats knee joint

Induction of knee joint inflammation with carrageenan (300 µg/knee) significantly increased expression of P2X3 receptor in the chondrocytes of the articular cartilage of the femoral condyle (**A** and **B**). The symbol "*" indicates an expression significantly greater than that of 0.9% NaCl and naïve groups ($P<0.05$, Tukey test). Immunofluorescence confocal micrographs of the articular cartilage (white arrows) of the femoral condyle after carrageenan and 0.9% NaCl injection and of untreated groups. Sections show immunohistochemical labeling for P2X3 receptor (green, columns 1) and labeling for nuclear staining with TOPRO-3 (red, columns 2). Merged pictures in columns 3 show co-localization of P2X3 receptor and nucleus cells (yellow). BM = Bone Marrow. Scale bars = 100 µm.

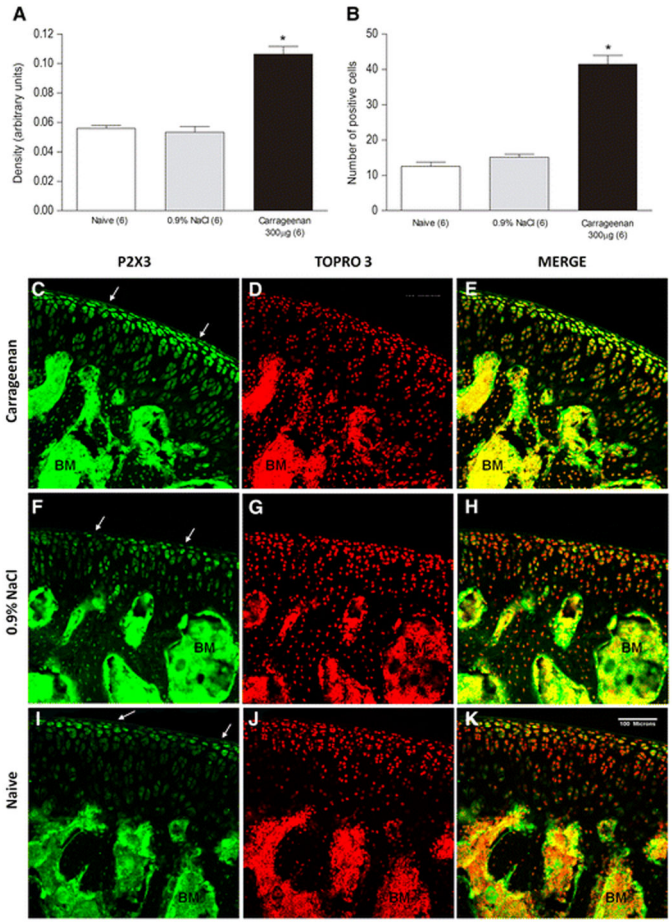

Figure 4. Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the articular cartilage of the tibial plateau of the rat's knee joint

Induction of joint inflammation with carrageenan (300 µg/knee) significantly increased the expression of P2X3 receptor in the chondrocytes of the articular cartilage covering the tibial plateau (**A** and **B**). The symbol "*" indicates an expression significantly greater than that of 0.9% NaCl and naïve groups ($P<0.05$, Tukey test). Immunofluorescence confocal micrographs of the articular cartilage (white arrows) covering the tibial plateau after carrageenan and 0.9% NaCl injection and of untreated groups. Sections show immunohistochemical labeling for P2X3 receptor (green, columns 1) and labeling for nuclear staining with TOPRO-3 (red, columns 2). Merged pictures in columns 3 show co-localization of P2X3 receptor and nucleus cells (yellow). BM = Bone Marrow. Scale bars = 100 µm.

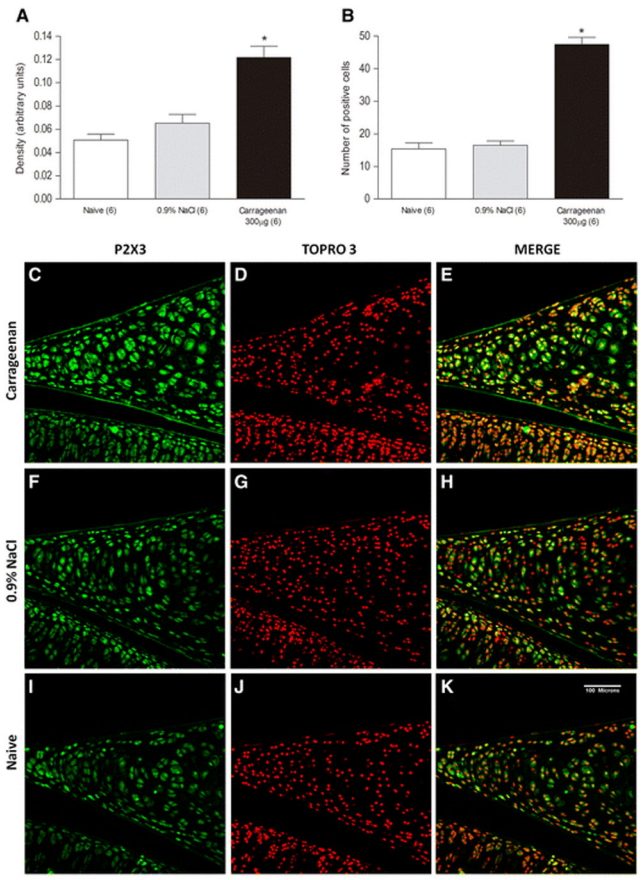

Figure 5. Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the cartilage that forms the meniscus of the rat's knee joint

Induction of joint inflammation with carrageenan (300 µg/knee) significantly increased expression of P2X3 receptor in the chondrocytes of the cartilage that forms the meniscus (**A** and **B**). The symbol "*" indicates an expression significantly greater than that of 0.9% NaCl and naïve groups ($P<0.05$, Tukey test). Immunofluorescence confocal micrographs of the meniscus after carrageenan and 0.9% NaCl injection and of untreated groups. Sections show immunohistochemical labeling for P2X3 receptor (green, columns 1) and labeling for nuclear staining with TOPRO-3 (red, columns 2). Merged pictures in columns 3 show co-localization of P2X3 receptor and nucleus cells (yellow). Scale bars = 100 µm.

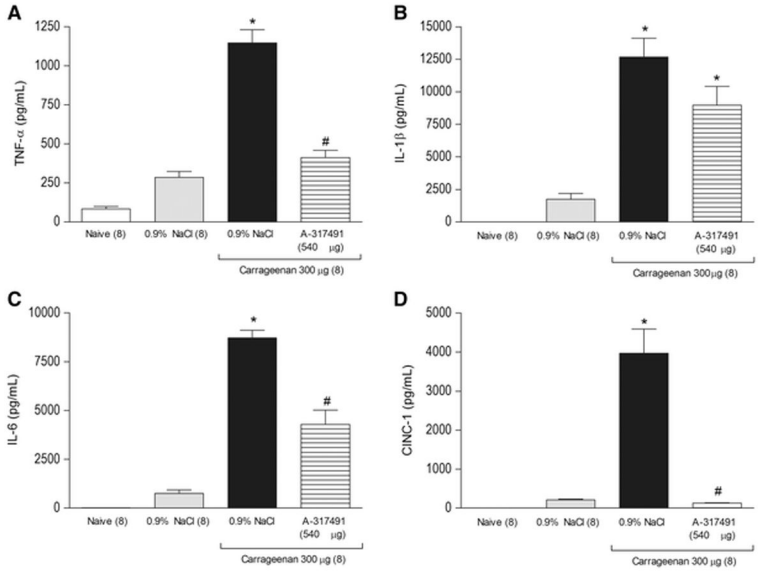

Figure 6. Effect of the articular blockade of P2X3 and P2X2/3 receptors on carrageenan-induced pro-inflammatory cytokine release

Induction of joint inflammation with carrageenan (Cg, 300 µg/knee) significantly increased local concentrations of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**) 3 hours after carrageenan injection. The co-administration of the P2X3 and P2X2/3 receptors antagonist A-317491 (540 µg/knee) with carrageenan significantly reduced the local concentration of TNF-α (**A**), IL-6 (**C**) and CINC-1 (**D**), as indicated by the symbol "#" ($P<0.05$, Tukey test), but not the local concentration of IL-1β (**B**) ($P>0.05$, Tukey test). The intra-articular injection of 0.9% NaCl alone did not significantly affect the local endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive rats ($P>0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that of 0.9% NaCl and naive groups ($P<0.05$, Tukey test).

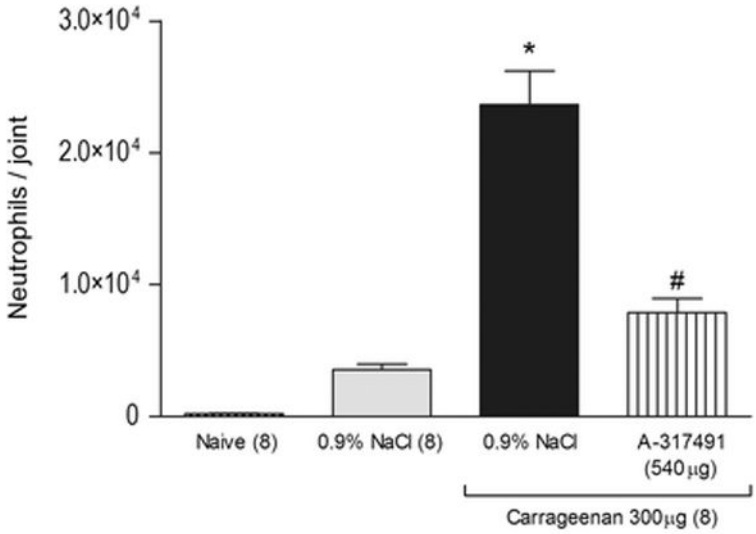

Figure 7. Effect of the articular blockade of P2X3 and P2X2/3 receptors on carrageenan-induced neutrophil migration

Induction of joint inflammation with carrageenan (Cg, 300 µg/knee) significantly increased the neutrophil migration into the rat's knee joint 3 hours after carrageenan injection. The co-administration of the P2X3 and P2X2/3 receptors antagonist A-317491 (540 µg/knee) with carrageenan significantly reduced the carrageenan-induced increase of neutrophil migration, as indicated by the symbol "#" ($P<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that of 0.9% NaCl and naive groups ($P<0.05$, Tukey test).