Critical Role of Protease-activated Receptor 2 Activation by Mast Cell Tryptase in the Development of Postoperative Pain

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ABSTRACT

Background: Studies have indicated that nearly half of all surgical patients still have inadequate pain relief. Thus, it is crucial to understand the mechanisms involved in postoperative pain in order to better treat it. Thus, the aim of this study was to investigate the involvement of mast cell degranulation, tryptase and its substrate, the protease-activated receptor 2, in a model of postoperative pain in mice.

Methods: We evaluated the effect of the compound 48/80 (to cause mast cell mediator depletion), cromoglycate or ketotifen (mast cell stabilizers), gabexate (tryptase inhibitor) or N3-methylbutyryl-N-6-aminohexanoyl-piperazine (protease-activated receptor 2 antagonist) in a postoperative pain model in mice (n = 5–10). Mast cell degranulation and tryptase activity were also evaluated in the operated tissue (n= 5–8).

Results: The pre-treatment with compound 48/80 or ketotifen was able to prevent nociception throughout the postoperative hyperalgesia course (until 5 days after surgery), whereas cromoglycate presented a shorter effect (until 1 day). Gabexate or N3-methylbutyryl-N-6-aminohexanoyl-piperazine also produced a short-lasting effect in preventing postoperative nociception. However, neither gabexate, N3-methylbutyryl-N-6-aminohexanoyl-piperazine nor cromoglycate was capable of reversing nociception when administered after incision. Surgery led to early mast cell degranulation on the incised tissue and increased tryptase activity in tissue perfusates. Cromoglycate fully prevented the tryptase release in the perfusate and the compound 48/80 substantially reduced tryptase activity in the incised tissue.

Conclusion: Thus, the mast cell degranulation with the subsequent release of tryptase and protease-activated receptor 2 activation are potential targets for the development of novel therapies to prevent, but not reverse, postoperative pain.

What We Already Know about This Topic

- When activated, mast cells release tryptase, which activates protease-activated receptor 2
- The role of protease-activated receptor 2 activation in postoperative pain has not been examined

What This Article Tells Us That Is New

- In mice, inhibition of mast cell degranulation and specific antagonism of protease-activated receptor 2 reduced hypersensitivity and guarding behavior, assumed to represent spontaneous nociception
- Targeting mast cells and protease-activated receptor 2 was only effective when given before surgery

DURING the past two decades, the under treatment of acute pain in surgical patients has been widely recognized as an important issue in health care. Patients who have well-controlled pain have an improved health-related quality of life and an overall greater satisfaction with their experience.[1,2] Unfortunately, despite the introduction of new standards, guidelines, and educational efforts, data from around the world suggest that postoperative pain continues to be managed inadequately.[3,4] Of note, the pursuit of postoperative analgesia is frequently complicated by the limited efficacy and undesirable side effects of the currently available analgesic drugs.[5,6] Thus, studying the mechanisms involved in postoperative pain is a useful means of identifying new targets to better treat this pain.

A number of mechanisms are likely involved, including tissue injury related to the incision itself, secondary inflammation, and damage to nerves caused by tissue retraction during the surgery.[7] The site of the incision has been documented to demonstrate signs of inflammation, including local edema, hyperthermia, hyperemia and pain,[8,9] indicating that surgery causes the cellular and vascular release of proinflammatory substances that mediate postoperative pain.[10] Mast cells have been previously shown to degranulate and its

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number are largely reduced following tissue incision.[11] Furthermore, we previously demonstrated that the prevention of mast cell degranulation largely reduced hyperalgesia in a model of postoperative pain.[12] However, the antagonism of histamine or serotonin, two important mast cell mediators, only partially reduce postoperative nociception, indicating that other mast cell components must be involved.

In addition to histamine and serotonin, mast cell degranulation releases tryptase, which has been demonstrated to be an important pronociceptive protease related to some painful diseases such as irritable bowel syndrome.[13–15] Tryptase is known to be a potent activator of protease-activated receptor 2 (PAR-2).[16,17] Tryptase works by cleaving a specific site on the extracellular N-terminal domain of the receptor and releases a new N-terminal domain for the receptor, which acts as a tethered ligand by binding to the second extracellular loop of the receptor to induce intracellular signaling.[18] PAR-2 is a G protein-coupled receptor that is expressed in the peripheral terminals of sensory neurons and seems to play an important role in inflammatory pain.[19–21]

The close association between mast cells and nerves in peripheral tissues, and the fact that large amounts of tryptase are released upon mast cell degranulation, makes tryptase an ideal candidate to activate PAR-2 on peripheral neurons.[10,22] However, the putative role of tryptase or PAR-2 is unknown in postoperative pain. Thus, in this study, we assess the ability of mast cell tryptase to mediate nociceptive responses *via* the PAR-2 receptor in a model of postoperative pain in mice.

Materials and Methods

Animals

The experiments were conducted as described by Oliveira *et al.*[12] using male Swiss mice (25–35 g) and were approved by the Ethics Committee of the Universidade Federal de Santa Maria (process number 45/2010).

Drugs

Gabexate mesylate, N-p-Tosyl-Gly-Pro-Arg p-nitroanilide acetate salt, tryptase from the human lung, compound 48/80, sodium cromoglycate, lidocaine and ketotifen fumarate were obtained from Sigma Chemical Company (St. Louis, MO). N3-methylbutyryl-N-6-aminohexanoyl-piperazine (ENMD-1068) was obtained from Enzo Life Sciences (Farmingdale, NY). Except ketotifen, which was diluted in saline, all other compounds were diluted in a phosphate-buffered saline solution (137 mM NaCl and 10 mM phosphate buffer; pH 7.4). Formaldehyde, paraffin, acetic acid, ethanol, toluidine blue, and xylol were purchased from Merck (Rio de Janeiro, Brazil).

Postoperative Pain Model

Mice were anesthetized with isoflurane *via* a nose cone, and after antiseptic preparation of the right hind paw, a longitudinal incision was made through the skin and fascia of the plantar foot. Surgical procedure was carried out as previously described.[12,23] Sham-operated animals were anesthetized only; no incision was made.

The mechanical threshold of 50% was determined before and after incision with a series of flexible nylon von Frey filaments of increasing stiffness (0.02–10 g) using the Up-and-Down method,[24] as previously described.[12] The mechanical hyperalgesia of all groups was assessed from 0.15 h to 6 days after surgery, when necessary.

For spontaneous nociception measurement, the guarding behavior was observed as described by Xu and Brennan,[25] with a few modifications. The incised and nonincised hind paws were closely observed for a 1-min period and repeated every 30 min from 0.5 h up to 6 h after surgery and twice a day (at 30-min intervals) from 1 up to 6 days after surgery. According to the position of the hind paw at the time of the observation, a score of 0, 1, or 2 was assigned. For each hind paw, a sum score was obtained by adding the two scores every 30 min.

Treatments

A selective PAR-2 antagonist (N3-methylbutyryl-N-6-aminohexanoyl-piperazine: ENMD-1068, 1–100 nmol/paw, intraplantar, an inhibitor of tryptase (gabexate mesylate, 0.1–1 nmol/paw, intraplantar) or vehicle (phosphate-buffered saline, 20 μl/paw, intraplantar) was administered 0.5 h before the incision or the sham procedure. Tryptase, a PAR-2 activator (5 ng/paw, intraplantar) was administered 0.5 h before ENMD-1068, gabexate, or vehicle in the sham-operated mice. In other groups of animals, ENMD-1068 (100 nmol/paw, intraplantar), gabexate (1 nmol/paw, intraplantar) or vehicle (phosphate-buffered saline, 20 μl/paw, intraplantar) was also administered 0.5 or 24 h after incision of the mice. Cromoglycate (200 μg/paw), a mast cell membrane stabilizer, was administered daily by intraplantar route 15 min before the incision and 1, 2, 3, 4, 5, and 6 days after incision, always 1 h before a new nociception measurement. Ketotifen (10 mg/kg), another mast cell membrane stabilizer, was administered daily by an oral route for 5 days before the incision and 1, 2, 3, 4, 5, and 6 days after incision. Animals were submitted to surgical procedure 1 h after the last oral injection of ketotifen, always 1 h before a new nociception measurement. Compound 48/80, that promotes depletion of mast cell mediators, was administered daily at increasing doses (1, 3, 10, and 10 μg/paw, intraplantar), and animals were submitted to plantar incision 24 h after the last injection of compound 48/80. The dosages and timing of the drugs were obtained from in-pilot experiments or by previous studies.[12,26–29] To avoid preventable discomfort for the animals, the effects of the drugs were observed just until the end of the antinociception.

To evaluate whether the compounds alone were able to alter the mechanical threshold of animals, sham-operated animals received an intraplantar administration of those compounds. As a positive control, lidocaine was used.[30]

Measurement of Tryptase Activity

To confirm the mast cell depletion produced by repeated treatment with compound 48/80, separate groups of mice were euthanized by cervical dislocation 24 h after the final

injection of compound 48/80. Tryptase activity was measured in the homogenates of the paw skin of animals as described by Hoffmeister *et al.*[27]

Paw skin samples were homogenized and centrifuged, and the resulting supernatants were used to evaluate tryptase activity. The enzymatic activity of tryptase was determined by measuring the hydrolysis of the substrate N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide to its product p-nitroanilide in a spectrophotometer at 405 nm.

Separate groups of animals were submitted to a surgical or sham procedure, and 10, 30, or 60 min after surgery, the animals were euthanized by cervical dislocation. The operated or sham-operated paws were perfused as previously described[31] and tryptase activity was measured in the perfusates.

Histology

We carried out histological analyses in paw tissue samples 10 min after sham or surgical procedures to confirm the mast cell degranulation as described previoulsy.[12] The presence of intact and degranulated mast cells was semi-quantified by mast cell counting in representative slides of the paw tissue of sham-operated and operated animals.[32] Results were represented as the percentage of degranulated mast cells relative to the total number of mast cells.

Statistical Analysis

The results are expressed as means ± SEM, except for the ID_{50} values (*i.e.,* the gabexate or ENMD-1068 dose that reduces nociceptive responses to the order of 50% relative to the control value), which were expressed as geometric means accompanied by their respective 95% confidence limits, and the spontaneous nociception scores, which were reported as medians and interquartile ranges. Spontaneous nociception scores were analyzed with the Mann--Whitney U test. All other data were analyzed using the Student *t* test (two-tailed), a one-way ANOVA followed by a Dunnett's *post hoc* test or a two-way ANOVA followed by a Bonferroni correction when appropriate. To meet parametric assumptions, the data on the 50% mechanical threshold were log transformed before the analysis. All statistical analyses were carried out using GraphPad Software 5.0 (San Diego, CA). *P* values less than 0.05 ($P < 0.05$) were considered significant, and F values presented in the text are demonstrated by treatment *versus* time interactions.

Results

The Prevention of the Mast Cell Degranulation Reduced Postoperative Hyperalgesia

Animals submitted to surgical procedures presented mechanical hyperalgesia from 0.15 h up to 5 days after surgery, when compared with sham-operated animals (fig. 1A). We verified that the depletion of mast cell mediators by repeated treatment with the compound 48/80 (1, 3, 10, and 10 μg/paw, intraplantar) was able to substantially prevent the postoperative hyperalgesia from 0.15 h up to 5 days after surgery [$F(24, 192) = 4.30$, $P < 0.0001$; fig. 1A]. The pre-treatment with the mast cell membrane stabilizers cromoglycate (200 μg/paw, intraplantar) or ketotifen (10 mg × kg^{-1} × day^{-1}, oral route) was also capable of reducing the hyperalgesia of animals [$F(12, 132) = 6.27$, $P < 0.0001$; fig. 1B and $F(12, 120) = 3.21$, $P < 0.001$; fig. 1C, respectively]. However, the antihyperalgesic effect of pretreatment with cromoglycate was shorter (up to 1 day) than with ketotifen, which prevented hyperalgesia from 0.15 h up to 4 days after surgery.

The Inhibition of Tryptase Activity or PAR-2 Antagonism Prevents the Development of Postoperative Nociception

Pre-treatment (30 min before the surgery) with gabexate (1 nmol/paw, intraplantar), a selective tryptase inhibitor, reduced mechanical hyperalgesia in mice from 0.15 to 1 h after surgery [$F(5, 45) = 3.56$, $P < 0.01$; fig. 2A]. Its antihyperalgesic effect occurred at doses of 0.1 and 1 nmol/paw, the calculated inhibitory dose value was 0.30 (0.07–1.22) nmol/paw, and the maximum inhibition was 76 ± 15% 0.5 h after surgery (fig. 2B).

Similarly, pretreatment with the selective PAR-2 antagonist ENMD-1068 (100 nmol/paw, intraplantar) was also able to prevent postoperative hyperalgesia in mice from 0.15 to 2 h after surgery [$F(5, 50) = 5.03$, $P < 0.0001$; fig. 2C]. The antihyperalgesic effect occurred at a dose of 100 nmol/paw,the calculated inhibitory dose value was 59 (27–130) nmol/paw, and the maximum inhibition was 80 ± 13% at 1 h after surgery (fig. 2D).

The administration of all compounds tested did not alter the mechanical threshold of sham-operated animals, which demonstrated that the effects of compounds were specific to postoperative hyperalgesia (data not shown). On the other hand, the local anesthesia with lidocaine increased the mechanical threshold of animals (data not shown).

The Inhibition of Tryptase Ativity, the PAR-2 Antagonism, or the Mast Cell Membrane Stabilization Prevents the Development of Spontaneous Nociception

Animals submitted to surgical procedures presented spontaneous nociception from 0.5 h up to 2 days after surgery, when compared with sham-operated animals, with maximal effect from 0.5 to 1 h after surgery (data not shown). In addition to preventing mechanical hyperalgesia, preadministration of either gabexate (1 nmol/paw, intraplantar), ENMD-1068 (100 nmol/paw, intraplantar), cromoglycate (200 μg/paw, intraplantar) or ketotifen (10 mg × kg^{-1} × day^{-1}, oral route) was capable of preventing spontaneous nociception in the operated animals from 0.5 to 1 h after surgery (fig. 3).

The Injection of Tryptase in the Hind Paw Mimics Surgery-inducing Hyperalgesia

Similar to plantar surgery, the intraplantar injection of tryptase (5 ng/paw, intraplantar) was able to produce mechanical hyperalgesia in mice [$F(7, 63) = 6.01$, $P < 0.0001$; fig. 4A].

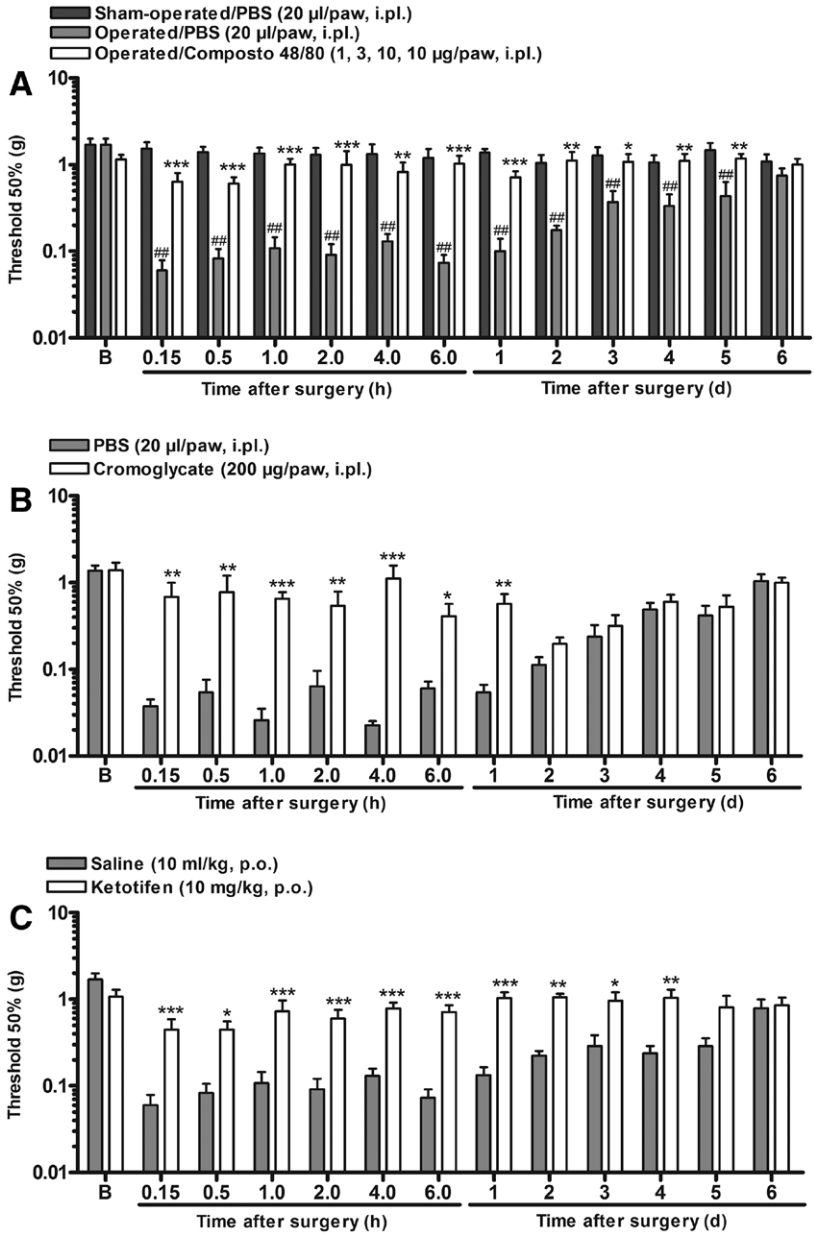

Fig. 1. The effect of mast cell previous depletion or membrane stabilization on postoperative nociception in mice. Time-response curves of the pre-administration of the compound 48/80 (*A*, 1, 3, 10, and 10 µg/paw, intraplantar [i.pl.]), cromoglycate (*B*, 200 µg/paw, i.pl.) or ketotifen (*C*, 10 mg/kg, per os [p.o]) on mechanical hyperalgesia after plantar surgery in mice. *Vertical bars* represent the means + SEM (*A*, *C*, n = 6 to all groups; *B*, n = 7 to phosphate buffered saline [PBS] and n = 6 to cromoglycate groups). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 when compared to the PBS-treated group; ##*P* < 0.001 when compared to baseline (*B*); two-way ANOVA followed by Bonferroni correction test.

This hyperalgesic effect occurred from 10 min to 6 h after its administration. Pretreatment with gabexate (1 nmol/paw, intraplantar F (10, 60) = 5.40, $P < 0.0001$; fig. 4B) or ENMD-1068 (100 nmol/paw, intraplantar F (10, 65) = 5.79, $P < 0.0001$; fig. 4C) largely prevented reduction in the mechanical threshold induced by tryptase from as early as 10 min lasting up to 2 h, with inhibition peaking at 100% and 88 ± 14%, respectively.

The Inhibition of Tryptase, the PAR-2 Antagonism, or the Mast Cell Membrane Stabilization Does Not Reverse the Established Postoperative Nociception

In contrast to the results obtained with pretreatment, gabexate (1 nmol/paw, intraplantar), ENMD-1068 (100 nmol/paw, intraplantar) or cromoglycate (200 µg/paw, intraplantar) were not able to reverse the established postoperative hyperalgesia when they were administered 30 min or 24 h

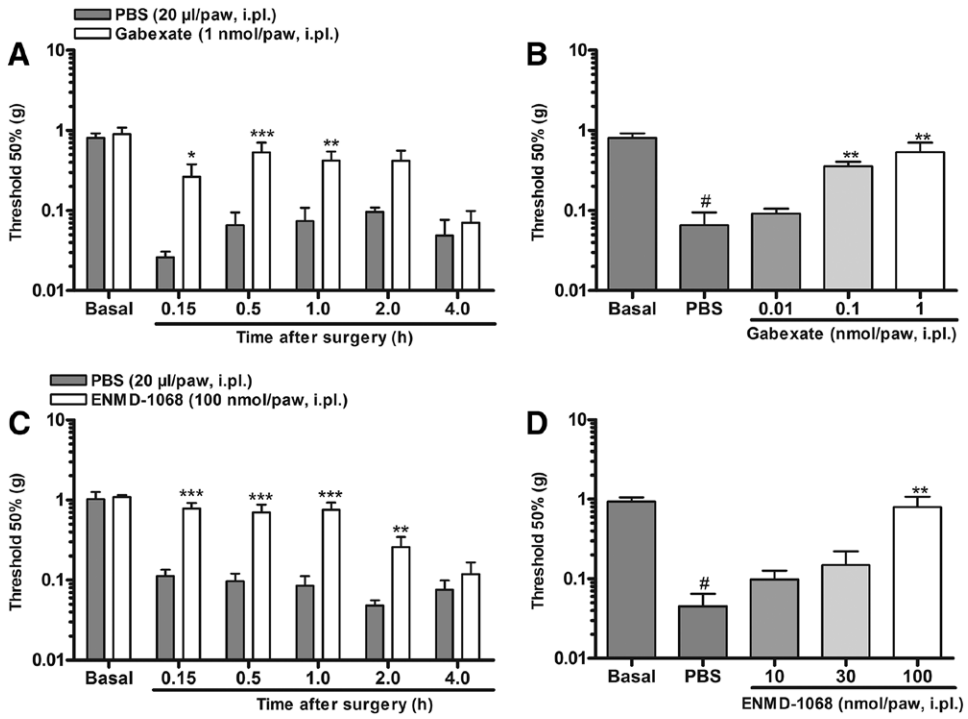

Fig. 2. The effect of pretreatment with gabexate or ENMD-1068 on mechanical hyperalgesia after plantar surgery in mice. Time-response curve of the pre-administration of gabexate (1 nmol/paw, intraplantar [i.p.l.]) (*A*) or ENMD-1068 (100 nmol/paw, i.p.l.) (*C*) and the dose-response curve of the preadministration of gabexate (0.01–1 nmol/paw, i.p.l.) (*B*) or ENMD-1068 (10–100 nmol/paw, i.p.l.) (*D*) on mechanical hyperalgesia at 0.5 and 1 h, respectively, after plantar surgery in mice. The *vertical bars* represent the means + SEM (*A*, n= 6 to phosphate buffered saline [PBS] and n = 5 to gabexate groups; *B*, n = 6 to PBS, basal, and gabexate 0.1 nmol/paw groups and n = 5 to gabexate 0.01 and 1 nmol/paw groups; *C*, n = 6 to PBS and ENMD-1068 groups; *D*, n = 7 to PBS, basal, and ENMD-1068 100 nmol/paw groups and n = 6 to ENMD-1068 10 and 30 nmol/paw groups). #$P < 0.01$ when compared to baseline (*B*); one-way ANOVA followed by Dunnett test. **$P < 0.01$ when compared to the PBS-treated group; one-way ANOVA followed by Dunnett test (*B*, *D*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to the PBS-treated group; two-way ANOVA followed by Bonferroni correction test (*A*, *C*).

after the incision (fig. 5, A–F), [F (6, 78) = 0.62, $P = 0.71$ fig. 5A; F (6, 60) = 0.83, $P = 0.60$ fig. 5B; (F (6, 60) = 1.76, $P = 0.12$ fig. 5C; F (6, 60) = 0.83, $P = 0.54$ fig. 5D; F (6, 60) = 0.32, $P = 0.93$ fig. 5E; F (6, 60) = 0.47, $P = 0.83$ fig. 5F].

Incision Induces An Early Mast Cell Degranulation and Tryptase Release

The plantar surgery increased the activity of tryptase two-fold in the paw tissue perfusate of the operated mice when compared with the sham-operated animals 10 min, but not 30 or 60 min after surgery (fig. 6; F(2,30)= 6.73, $P < 0.01$), indicating an early postoperative tryptase release by mast cells. Accordingly, we also detected mast cell degranulation (percentage of degranulated mast cells was 3.6% and 29.8 ± 3.4% in sham-operated and operated animals, respectively) as early as 10 min after the surgery using histological analyses (fig. 7).

Treatments That Prevent Postoperative Nociception Also Reduced Tryptase Activity In Paw Tissue and Its Release after Incision

We next used two different approaches to confirm the critical role of tryptase in postoperative pain. First, we determined that the depletion of mast cell mediators by repeat treatment with compound 48/80 was able to largely reduce the tryptase activity in the paw skin of mice (inhibition of 81 ± 14%, fig. 8A) as well as reduce the postoperative hyperalgesia (fig. 1A). Second, we also observed that the pre-treatment of the mice with the mast cell membrane stabilizer cromoglycate (200 µg/paw, intraplantar) fully prevented the increase in tryptase activity (100% inhibition, fig. 8B) and the mechanical hyperalgesia after the surgical procedure (fig. 1B).

Discussion

Previously, we showed that surgery causes mast cell degranulation and increases local histamine and serotonin levels.[12] The prevention of mast cell degranulation largely reduces hyperalgesia up to 4 days after surgery. In this study, we extended our previous findings showing that the prevention of mast cell degranulation reduced not only postoperative hyperalgesia, but also spontaneous nociception throughout the postoperative period. The compound 48/80 leads to the degranulation of mast cells, promoting the release of mediators such as histamine, serotonin, and tryptase.[12,33,34] However, the repeat administration of compound 48/80 results in

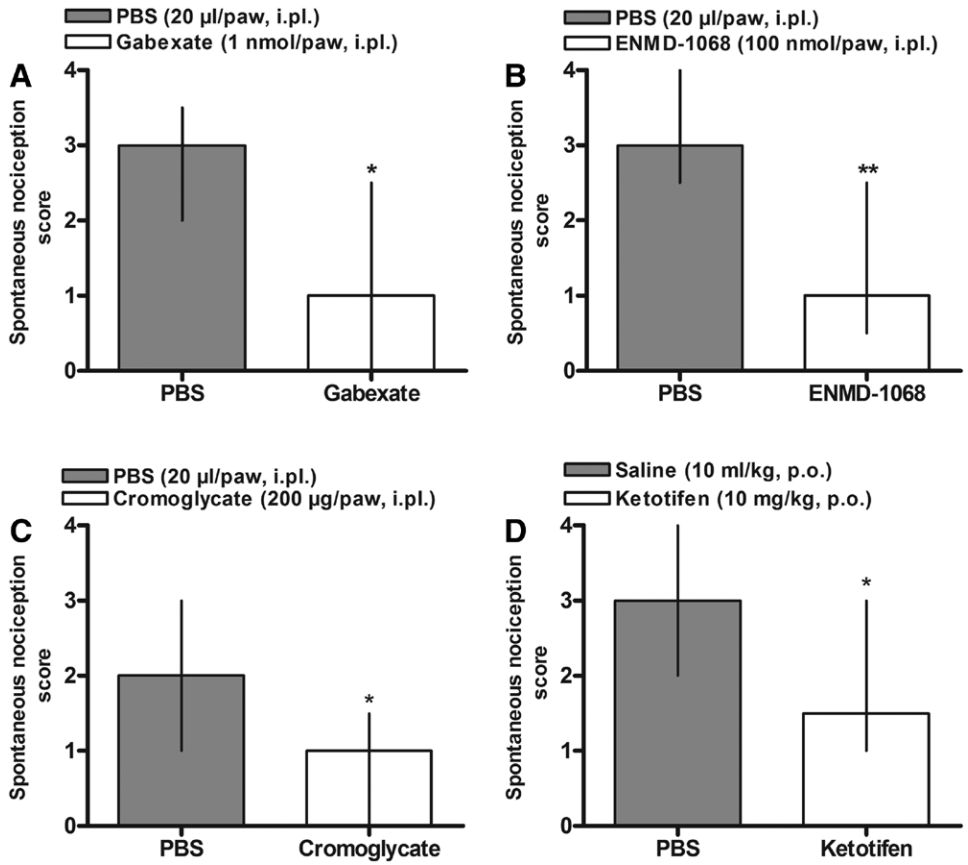

Fig. 3. The effect of pretreatment with gabexate, ENMD-1068, cromoglycate, or ketotifen on spontaneous nociception after plantar surgery in mice. The effect of the preadministration of gabexate (1 nmol/paw, intraplantar [i.pl.]) (*A*), ENMD-1068 (100 nmol/paw, i.pl.) (*B*), cromoglycate (200 µg/paw, i.pl.) (*C*) or ketotifen (10 mg × kg^{-1} × day^{-1}, per os [p.o.]) (*D*) on the sum of spontaneous nociception scores from 0.5 to 1 h after plantar surgery in mice. The *vertical bars* represent the medians and interquartile ranges for (*A*, n=6 to phosphate buffered saline [PBS] and n = 5 to gabexate groups; *B*, n = 10 to both groups; *C*, n = 7 to PBS and n = 5 to cromoglycate groups; *D*, n = 6 to both groups). *P < 0.05, **P < 0.01 when compared to the PBS-treated group; Mann-Whitney U test.

the depletion of these mediators.[12,35] We demonstrated that mast cell depletion in mice was able to prevent nociception of the animals throughout the postoperative period. Moreover, we also verified that cromoglycate and ketotifen were also able to prevent the mechanical and spontaneous nociception of operated animals, in accordance with previous data demonstrating mast cell stabilizers as capable of protecting mast cell membranes, thereby hindering their degranulation.[12,29,36] Cromoglycate presented an antinociceptive effect up to 24 h after surgery. Since cromoglycate presented a shorter antinociceptive effect than compound 48/80, we confirmed the role of mast cells in postoperative pain, testing another mast cell stabilizer, ketotifen. Ketotifen was chosen since it clinically presented an analgesic effect in irritable bowel disease and neurofibroma, as well as improved quality of life and relieved abdominal cramps in patients after extensive abdominal surgery.[37–39] Ketotifen, in the same route and dosage schedule (oral route, treatment before and after surgery) of the clinical studies, was able to prevent postoperative nociception up to 4 days after operation. The longer effect of ketotifen (that was orally pretreated once a day, 5 days before surgery) in relation to cromoglycate (that was locally pretreated only 15 min before surgery) is not unexpected since the repeated pretreatment with this class of drugs seems to cause more long-lasting effects to mast cell stabilization.[29] These new findings reinforce the idea of the critical role of mast cells in postoperative pain and indicate that the pretreatment with mast cell stabilizers, such as ketotifen, could be useful to prevent pain aside from other postoperative problems.

In our previous study,[12] the antagonism of histamine or serotonin receptors only partially decreased postoperative hyperalgesia in mice, suggesting that other mast cell-derived mediators are involved. Histochemical and immunohistochemical techniques have demonstrated that the serine protease tryptase is localized exclusively in mast cells and may be used as an indicator of mast cell activation.[40,41] However, the role of tryptase in painful processes is largely unknown. Clinical studies have demonstrated that tryptases are released in patients with irritable bowel syndrome and they can directly stimulate sensory neurons, generating hypersensitivity symptoms.[15,39]

We also observed that the selective tryptase inhibitor, gabexate, was capable of preventing postoperative

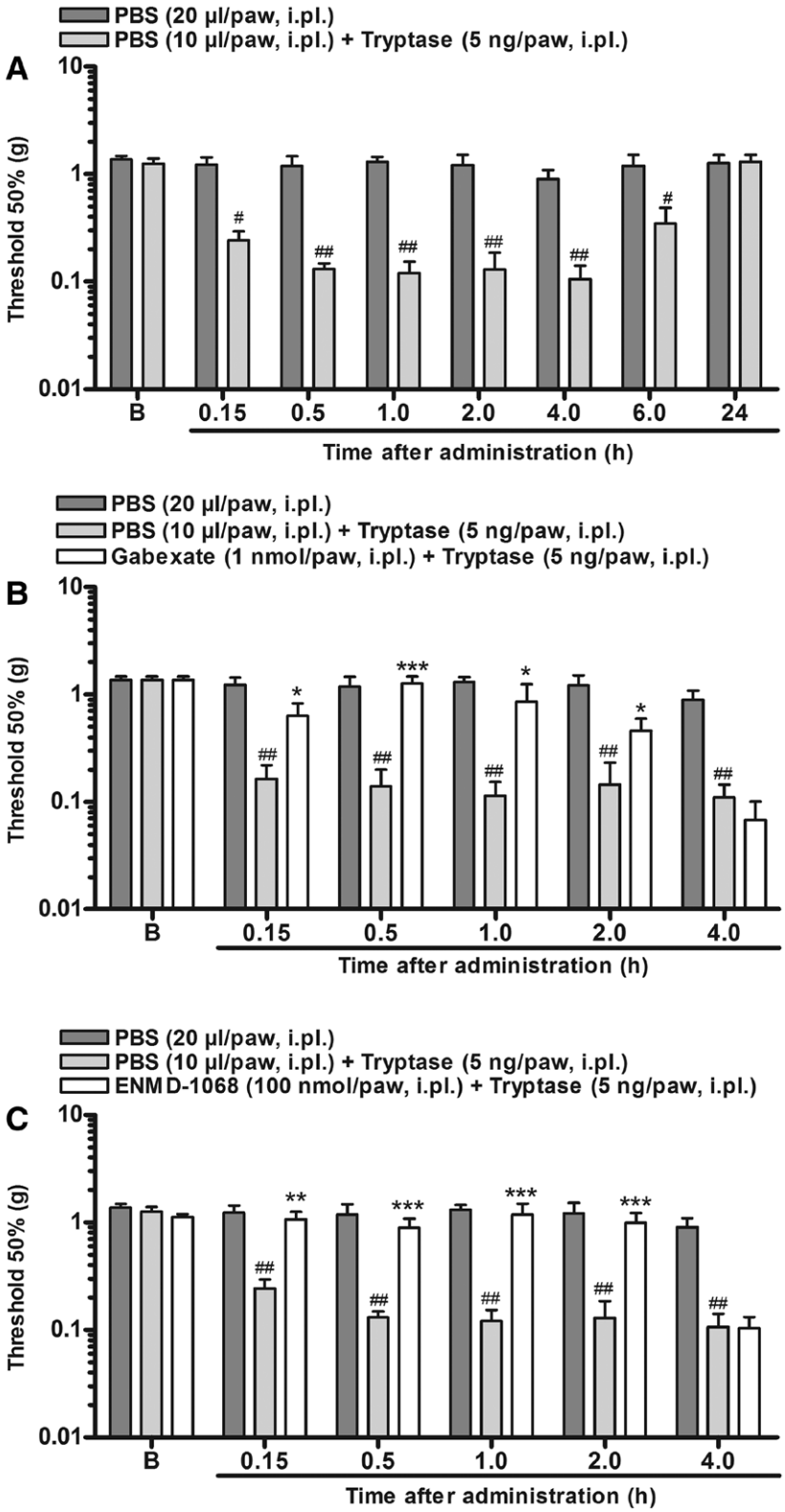

Fig. 4. The effect of pretreatment with gabexate or ENMD-1068 on the reduction in the mechanical threshold induced by intraplantar tryptase in mice. The effect of tryptase (5 ng/paw, intraplantar [i.pl.]) on the mechanical threshold in mice (*A*). The effect of the preadministration of gabexate (1 nmol/paw, i.pl.) (*B*) or ENMD-1068 (100 nmol/paw, i.pl.) (*C*) on the mechanical hyperalgesia induced by intraplantar tryptase in mice. The vertical bars represent the means + SEM (*A*, n = 5 to phosphate buffered saline [PBS] and n = 6 to PBS/tryptase groups; *B*, n = 5 to both groups; *C*, n = 5 to PBS and n = 6 to PBS/tryptase or to ENMD-1068/tryptase groups). ##$P < 0.001$ when compared to baseline (*B*) and *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to the PBS-treated group; two-way ANOVA followed by Bonferroni correction test.

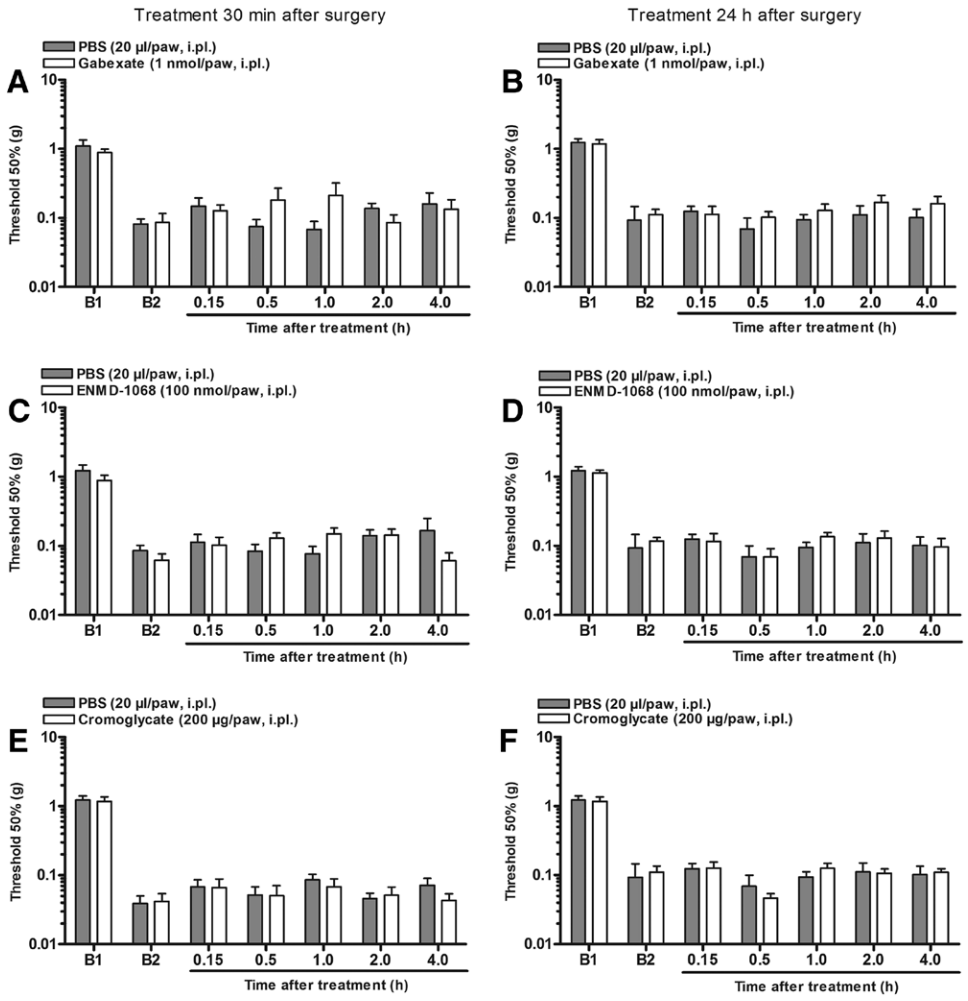

Fig. 5. The effect of posttreatment (30 min after the surgery: *A*, *C*, and *E*; 24 h after the surgery: *B*, *D*, and *F*) with gabexate, ENMD-1068 or cromoglycate on mechanical hyperalgesia after plantar surgery in mice. The effect of post-administration of gabexate (1 nmol/paw, intraplantar [i.pl.]) (*A* or *B*), ENMD-1068 (100 nmol/paw, i.pl.) (*C* or *D*) or cromoglycate (200 µg/paw, i.pl.) (*E* or *F*) on mechanical hyperalgesia after plantar surgery in mice. *B1* indicates the measurement of the baseline mechanical threshold of the animals before plantar surgery, and *B2* indicates the measurement of the baseline mechanical threshold of the animals after plantar surgery and before treatments. The vertical bars represent the means + SEM for (*A*, n = 7 to phosphate buffered saline [PBS] and n = 8 to gabexate groups; *B*, *C*, *D*, *E*, *F*, n = 6 to all groups).

nociception. Notably, a recent study demonstrated that intestinal handling during open gynecological surgery promoted mast cell degranulation with the subsequent local release of tryptase (conventional laparotomy), whereas a minimally invasive technique (laparoscopy) did not result in tryptase release.[41] Knowing that laparoscopy is associated with less postoperative pain compared to conventional laparotomy,[42] our results indicate that mast cells may be involved in postoperative pain, especially where surgical manipulation is more extensive.

Tryptase is a selective and endogenous agonist of PAR-2. Several studies have indicated that PAR-2 is involved in a number of inflammatory diseases, including arthritis, skin inflammation, and inflammatory bowel diseases.[18] However, the role of PAR-2 in painful processes has not yet been fully elucidated. Injections of synthetic PAR-2 agonists have been demonstrated to promote mechanical and thermal hyperalgesia in rats and mice.[26,43] Recently, synthetic agonists of PAR-2 were shown to be unselective, as they also activate Mas-related G protein-coupled receptors.[44] Furthermore, the role of the PAR-2 blockade in the development of nociception is based only on the use of PAR-2-deficient mice, which demonstrate few signs of mast cell degranulation-induced hyperalgesia and formalin-induced hyperalgesia.[26] Unfortunately, these data must be interpreted with caution as PAR-2-deficient mice demonstrate a marked compensatory response to PAR-1 activation.[45] Thus, preclinical trials with PAR-2 antagonists are important tools to reveal the role of PAR-2 in painful processes. Previous studies have shown that the selective PAR-2 antagonist ENMD-1068 has antiinflammatory activity in murine models of arthritis.[46,47] In this study, we observed that pretreatment with ENMD-1068 was able to prevent both mechanical hyperalgesia and spontaneous nociception after surgery, indicating that PAR-2 is a potential therapeutic target for the treatment of postoperative pain.

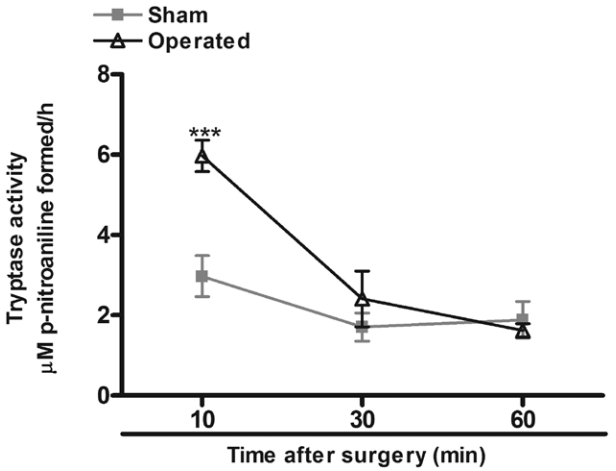

Fig. 6. The effect of plantar surgery on tryptase activity in mice. The effect of surgery on tryptase activity at 10, 30, or 60 min after plantar surgery in mice. Each point represents the mean + SEM (n = 6 to both groups). ***P < 0.001 when compared to the sham group; two-way ANOVA followed by Bonferroni correction test.

Confirming our findings with tryptase inhibition and PAR-2 antagonism in accordance with studies performed in rats,[26] the injection of the selective and endogenous PAR-2 agonist tryptase into the mice's hind paws mimicked postoperative nociception and resulted in hyperalgesia. Moreover, gabexate and ENMD-1068 were able to prevent this tryptase-induced hyperalgesia. This finding is consistent with our results on postoperative pain, where both drugs were capable of reducing postoperative nociception by preventing the activation the PAR-2 receptor. In contrast to the postoperative findings, tryptase-induced hyperalgesia occurs within the first 6 h after its administration, whereas pain due to a surgical procedure lasts about 5 days.[12,23] This finding indicates that tryptase is implicated in early postoperative pain and other pronociceptive mediators are important in maintaining the continuation of pain. In fact, nerve growth factor, interleukins and prostaglandin E_2 have all been demonstrated to be released late after the incision.[48–52]

Confirming the early involvement of mast cells and tryptase in postoperative pain, cromoglycate, ENMD-1068 or gabexate administration 30 min or 24 h after the surgery was unable to alter the intensity of postoperative nociception. Furthermore, we detected early mast cell degranulation and tryptase release 10 min after the surgery, demonstrating an early role of mast cells in postoperative pain. Thus, our results show that tryptase, PAR-2, or mast cell inhibition is not useful in reducing postoperative hyperalgesia once mast cell degranulation, tryptase release, nociceptor activation, and the start of pain have already occurred. Furthermore, possible therapeutic strategies to treat postoperative pain targeting mast cell mediators must be preventative. Of note, recent studies have indicated that preventative analgesia is promising for the treatment of persistent postoperative pain.[53]

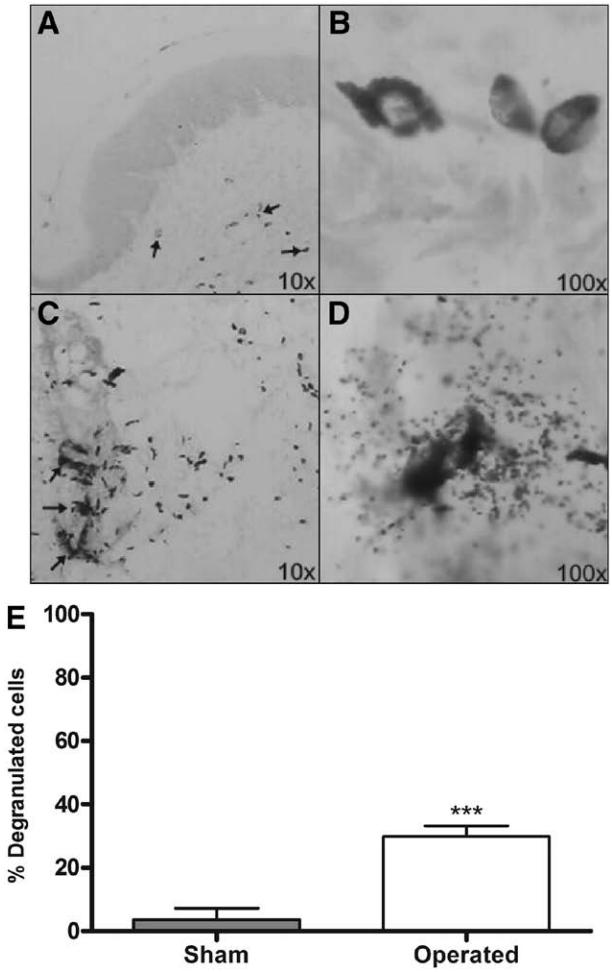

Fig. 7. Representative light microphotographs showing the presence of mast cells after the sham or surgical procedure in the paw tissue of mice. A section was obtained from the paw tissue 10 min after sham (*A*, *B*) or surgical (*C*, *D*) procedure. Percentage of degranulated mast cells relative to intact mast cell (*E*). The *vertical bars* represent the means + SEM (n = 5 to sham-operated group and n =6 to operated groups). ***P < 0.001 when compared to the sham group; Student *t* test.

To provide additional evidence for the critical role of tryptase in postoperative pain, we employed different approaches for confirmation. We demonstrated that the cromoglycate fully prevented the tryptase release in the perfusate, and the compound 48/80 substantially reduced the tryptase activity in the incised tissue. Furthermore, they were able to prevent postoperative hyperalgesia in the animals. These findings are in accordance with previous results demonstrating cromoglycate as capable of protecting mast cell membranes, and of preventing the nociception induced by surgical injury.[12] Thus, the early degranulation of mast cells and the release of their pro-nociceptive mediators appear to play a critical role in the development of postoperative nociception in mice.

In addition to hyperalgesia, patients undergoing surgery also have ongoing, unprovoked pain that is measured as pain at rest and is a common patient complaint.[54,55]

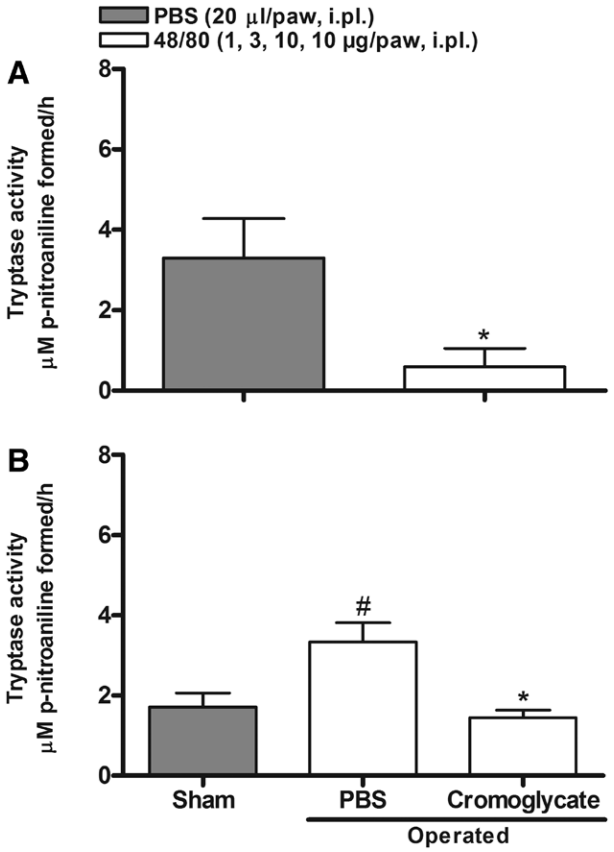

Fig. 8. The effect of pretreatment with compound 48/80 or cromoglycate on tryptase activity in the paw tissue or paw perfusates of mice. The effect of pretreatment for 4 days (1+ 3+ 10+ 10 µg/paw, intraplantar [i.pl.]) with compound 48/80 on tryptase activity in the paw skin of mice (*A*) or the effect of pre-treatment with cromoglycate (200 µg/paw, i.pl.) on tryptase activity in the paw perfusates of mice after plantar surgery (*B*). The vertical bars represent the means + SEM for (*A*; n = 5 in both group) and (*B*; n = 8 to phosphate buffered saline [PBS]-operated, n = 6 to sham-operated and n = 5 to cromoglycate-operated groups). #$P < 0.01$ when compared to the sham group; *$P < 0.05$ when compared to the PBS group; Student *t* test (*A*) or one-way ANOVA followed by Dunnett test (*B*).

Unprovoked pain-related behavior (namely, guarding) after a rodent plantar incision has been described and suggested to correlate to the pain at rest in patients.[25,56,57] Here, we have demonstrated that tryptase inhibition, PAR-2 antagonism, or mast cell stabilizing not only reduced hyperalgesia but also prevented the guarding behavior induced by surgery. This finding reinforces the important role of mast cell tryptase and PAR-2 in several painful symptoms that occur in the postoperative period.

Many conditions associated with mast cell degranulation, such as allergies, and some drug administration do not result in pain. However, many painful conditions are associated with the degranulation of mast cells, such as irritable bowel syndrome,[13,15] laparoscopy,[39,41] chronic pancreatitis,[58] and migraines,[59] among others. These differences may be observed because in painful conditions, other cellular and vascular events that facilitate nociceptor activation and mast cell activation must occur. Besides mast cell mediators, the tissue damage causes the release of intracellular contents including protons, adenosine triphosphate, and mediators, such as nerve growth factor, important in inducing nociceptor and mast cell activation as well postoperative pain.[48–50,60–63] Another important point is the fact that mast cell mediators are released in proximity to nerves in painful conditions, which is relevant to pain/discomfort development.[13] Thus, mast cell degranulation alone seems not to be sufficient to promote pain, but it may contribute to pain after tissue damage.

The local administration of certain drugs has been suggested to be able to exert nonspecific antinociceptive effects by acting as local anesthetics.[64] We have detected that the drugs used in this study did not alter the mechanical threshold of the sham-operated animals at the doses that result in the prevention of postoperative nociception. However, lidocaine, used as a positive control, increased the mechanical threshold of the sham-operated animals. Moreover, our results clearly demonstrate that the effect of mast cell stabilization, tryptase inhibition, or PAR-2 antagonism is specific to postoperative hyperalgesia and not to the detection of normal mechanical stimuli.

Taken together, our findings suggest that mast cell degranulation with the subsequent release of tryptase and PAR-2 activation are potential targets for the development of novel therapies to prevent, but not reverse, postoperative pain.

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