British Journal of Pharmacology (2008) 153, 1043–1053
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Role of complement C5a in mechanical inflammatory hypernociception: potential use of C5a receptor antagonists to control inflammatory pain

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Background and purpose: C5a, a complement activation product, exhibits a broad spectrum of inflammatory activities particularly neutrophil chemoattraction. Herein, the role of C5a in the genesis of inflammatory hypernociception was investigated in rats and mice using the specific C5a receptor antagonist PMX53 (AcF-[OP(D-Cha)WR]).
Experimental approach: Mechanical hypernociception was evaluated with a modification of the Randall–Selitto test in rats and electronic pressure meter paw test in mice. Cytokines were measured by ELISA and neutrophil migration was determined by myeloperoxidase activity.
Key results: Local pretreatment of rats with PMX53 (60–180 μg per paw) inhibited zymosan-, carrageenan-, lipopolysaccharide (LPS)- and antigen-induced hypernociception. These effects were associated with C5a receptor blockade since PMX53 also inhibited the hypernociception induced by zymosan-activated serum and C5a but not by the direct-acting hypernociceptive mediators, prostaglandin E_2 and dopamine. Underlying the C5a hypernociceptive mechanisms, PMX53 did not alter the cytokine release induced by inflammatory stimuli. However, PMX53 inhibited cytokine-induced hypernociception. PMX53 also inhibited the recruitment of neutrophils induced by zymosan but not by carrageenan or LPS, indicating an involvement of neutrophils in the hypernociceptive effect of C5a. Furthermore, the C5a-induced hypernociception was reduced in neutrophil-depleted rats. Extending these findings in rats, blocking C5a receptors also reduced zymosan-induced joint hypernociception in mice.
Conclusions and implications: These results suggest that C5a is an important inflammatory hypernociceptive mediator, acting by a mechanism independent of hypernociceptive cytokine release, but dependent on the presence of neutrophils. Therefore, we suggest that inhibiting the action of C5a has therapeutic potential in the control of inflammatory pain.
British Journal of Pharmacology (2008) **153,** 1043–1053; doi:10.1038/sj.bjp.0707640; published online 17 December 2007

Keywords: inflammatory pain; hyperalgesia; C5a; complement system; neutrophils; cytokines

Abbreviations: BK, bradykinin; CFA, complete Freund's adjuvant; CINC-1, cytokine-induced neutrophil chemoattractant-1; IL-1β, interleukin-1β; i.pl., intraplantar; LPS, lipopolysaccharide; MPO, myeloperoxidase; OVA, ovalbumin; PMX53, C5a receptor antagonist; TNF-α, tumour necrosis factor-α; ZAS, zymosan-activated serum

Introduction

Pain is one the classical symptoms of the inflammatory process. It is now accepted that sensitization of primary nociceptive neurons is the common denominator of inflammatory pain that leads to states known as hyperalgesia/allodynia in humans or hypernociception in animal models (Cunha *et al*., 2007). In experimental studies, this phenomenon has been attributed to the direct action of hypernociceptive inflammatory mediators (mainly prostaglandins and sympathetic amines) on their receptors present in nociceptor membranes, which in the last instance lowers the nociceptor threshold, increasing neuronal membrane excitability (Ferreira *et al*., 1978b; Khasar *et al*., 1999; Coutaux *et al*., 2005). Nevertheless, the release of these

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Received 21 August 2007; revised 15 October 2007; accepted 22 November 2007; published online 17 December 2007

nociceptor direct-acting hypernociceptive mediators is generally preceded by a cytokine cascade (Cunha *et al*., 2005; Verri *et al*., 2006). Following the administration of inflammatory stimuli in the rat paw, the formation of bradykinin (BK) occurs initially, which triggers subsequent release of tumour necrosis factor-α (TNF-α). This key cytokine stimulates two hypernociceptive pathways: interleukin-1β (IL-1β) production, which in turn induces prostanoid production and CXC chemokines (CINC-1/IL-8), which induce the release of sympathetic amines (Ferreira *et al*., 1988; Cunha *et al*., 1991, 1992; Lorenzetti *et al*., 2002). Although these events are critical to the genesis of inflammatory hypernociception, it is important to mention that other inflammatory mediators and cells, such as nerve growth factor (Woolf *et al*., 1994), leukotriene B_4 (Guerrero *et al*., 2008) and neutrophils (Levine *et al*., 1985), also play a crucial role in this inflammatory event.

In the last few decades, the role of the complement system in the genesis of the inflammatory process as well as in inflammatory diseases, including rheumatoid arthritis, has been gaining increasing attention (Vakeva *et al*., 1998; Woodruff *et al*., 2002; Sarma *et al*., 2006). One of the main effector components of the complement system is the anaphylatoxin C5a. It is one of the most potent inflammatory peptide mediators, and its biological effects result in binding to its G-protein-coupled receptor (C5aR1) present in inflammatory cells, such as neutrophils, eosinophils and monocytes (Chenoweth and Hugli, 1978; Gerard *et al*., 1989; Werfel *et al*., 1992). C5a is a potent neutrophil chemoattractant and induces an increase in oxidative burst, phagocytosis and release of granule enzymes by these cells (Hetland *et al*., 1998).

The role of C5a in the genesis of inflammatory pain is not completely understood. In an early study, Levine *et al*. (1985) demonstrated that the intraplantar (intradermal) administration of C5a produces a mechanical hypernociception that is dependent on the presence of polymorphonuclear leukocytes. The interdependence of neutrophil migration and inflammatory hypernociception has also been demonstrated in other studies (de Abreu Castro and Ferreira, 1979; Lavich *et al*., 2006). There is also evidence that the activation of the complement system in spinal cord contributes to mechanical hypernociception during different types of neuropathies (Twining *et al*., 2005). However, to the best of our knowledge, there has been no study investigating the role of C5a in the genesis of hypernociception during innate and adaptive inflammation. In the present study, using a specific C5a receptor antagonist (PMX53; AcF-[OP(D-Cha)WR]), we evaluated the endogenous role of C5a in mechanical inflammatory hypernociception in innate and adaptive immune inflammatory responses and the possible mechanisms by which C5a mediates inflammatory hypernociception. PMX53 belongs to a class of selective small cyclic peptides, which potently and insurmountably inhibit C5a effects on polymorphonuclear leukocytes. For instance, PMX53 inhibits C5a-induced myeloperoxidase (MPO) release from human neutrophils (March *et al*., 2004). This antagonist is also effective in numerous inflammatory disease models in mice and rats in which the physiopathology depends on complement system activation including rheumatoid arthritis (Woodruff *et al*., 2002), inflammatory bowel disease (Woodruff *et al*., 2003), ischaemia–reperfusion injuries (Woodruff *et al*., 2004), and recently in a model of neurodegeneration (Woodruff *et al*., 2006).

Methods

Animals

Adult male Wistar rats (weighing 180–200 g) and male C57BL/6 mice (weighing 20–25 g) were used in this study. The animals were housed (five per cage) in temperature-controlled rooms (22–25 °C), with access to water and food *ad libitum*, until used in the Department of Pharmacology of the School of Medicine of Ribeirão Preto, University of São Paulo. All behavioural testing was performed between 0900 and 1700 hours. Animal care and handling procedures were in accordance with the International Association for Study of Pain guidelines for the use of animals in pain research and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo). Each experiment used up to six rats per group. All efforts were made to minimize the number of animals used and any discomfort.

Mechanical hypernociception test in rat paw

Mechanical hypernociception was tested in rats by using the constant-pressure rat-paw test, as described by Ferreira *et al*. (1978a, b). In this method, a constant pressure of 20 mm Hg (1 mm Hg = 133 Pa) is applied via a syringe piston moved by compressed air to an area of 15 mm^2 on the plantar surface of the hindpaw and discontinued when the rat displays a 'freezing reaction'. The reaction typically comprises a reduction in escape movements (that animals normally make to free themselves), increased vibrissal movements, a variation in the respiratory frequency terminating with a brief apnea concomitant with retraction of the head towards forepaws. The apnea is frequently associated with successive waves of muscular tremor. For each animal, the latency to onset of the freezing reaction is measured before (zero time) and after administration of the hypernociceptive stimuli. In this test, the end point is a behavioural response, the freezing reaction. The constant-pressure rat-paw test over the years has been instrumental in many original observations (Ferreira and Nakamura, 1979a, b; Cunha *et al*., 1992). The drugs were administered by intraplantar (i.pl.) injection using a 100 μl Hamilton microsyringe. The needle was introduced subcutaneously near the third digit, with its tip reaching the middle of the plantar hindpaw. The intensity of hypernociception was measured before the first intraplantar injection by using the values measured at zero hour as control reaction times. The intensity of mechanical hypernociception was quantified as the reduction in the reaction time, calculated by subtracting the value of the subsequent measurements after stimulus injection from the first (zero time) (Ferreira *et al*., 1978a).

Dorsal flexion of the tibiotarsal joint: assessment by a modified electronic pressure-meter test for mice

Experiments were performed as described previously (Guerrero *et al*., 2006). In a quiet room, mice were placed

in acrylic cages ($12 \times 10 \times 17$ cm high) with a wire grid floor 15–30 min before testing for environmental adaptation. Stimulations were performed only when animals were quiet, without exploratory movements or defecation and not resting on their paws. In these experiments, an electronic pressure meter was used, which consists of a hand-held force transducer fitted with a polypropylene tip (IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). For this model, a non-standard large tip (4.15 mm^2) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hindpaw to induce the dorsal flexion of the tibiotarsal joint, followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the animal's hindpaw. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until three successive consistent measurements were obtained (that is, the variation among these measurements was less than 1 g). The flexion-elicited withdrawal threshold is expressed in grams.

Procedures for active immunization in rats

Ovalbumin (OVA) was dissolved in sterile saline to an appropriate concentration (2 mg ml^{-1}) and mixed with an equal volume of complete Freund's adjuvant (CFA) at a concentration of 1 mg ml^{-1} of *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide mono-oleate. CFA was used to augment the efficiency of the immunization procedure (Freund, 1956) by prolonging the lifetime of injected autoantigen and by stimulating its effective delivery to the immune system. Rats weighing approximately 100 g were injected subcutaneously at two different sites on their back to give a total dose of 200 μg of OVA dissolved in an emulsion containing an equal volume of sterile saline plus CFA. Control (sham-immunized) rats were injected with this emulsion without OVA. After 21 days, the rats were challenged with the intraplantar administration of OVA (25 μg in 100 μl of saline) to the right hindpaw (Cunha *et al*., 2003).

Preparation of zymosan-activated serum

Zymosan-activated serum (ZAS), a source of $C5a_{DesArg}$ chemotactic factor, was prepared by incubating fresh rat serum with zymosan (10 mg ml^{-1}) at 37 °C for 30 min. At the end of the incubation, the zymosan was removed by centrifugation (2×10 min at 3000 *g*, room temperature), and the serum was heated to 56 °C for 30 min, aliquoted and kept frozen (−70 °C) until use (Torres and Forman, 1999). The control serum was incubated for 30 min at 37 °C without the addition of zymosan, and the sequence of steps in the procedure was the same as described for ZAS.

Measurement of MPO activity

The accumulation of neutrophils in the rat paw or in the mouse tibiotarsal joint region was measured by means of tissue MPO activity. After the animals had been killed, subcutaneous tissues of the paws or tibiotarsal bone complex were removed and assayed for MPO according to a method described previously (Souza *et al*., 2000). Briefly, tissues were homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 3000 *g* for 15 min, at 4 °C. The pellets were re-suspended in hexadecyltrimethyl ammonium bromide 0.5% buffer (pH 5.4), and the samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were re-centrifuged (3000 *g*, 15 min, at 4 °C) and 2–5 μl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$, 0.5 mM hydrogen peroxide, and the reaction was terminated with 4 M H_2SO_4. The absorbance was read at 450 nm, and the results are expressed as number of neutrophil $\times 10^6$ per mg tissue.

Determination of TNF-α and IL-1β levels

The skin of the plantar area of paws was obtained 3 h after saline, zymosan, carrageenan or lipopolysaccharide (LPS) injection and homogenized in 500 μl of the appropriate buffer containing protease inhibitors, and TNF-α and IL-1β levels were determined as described previously (Safieh-Garabedian *et al*., 1995) by ELISA. Briefly, microtiter plates (Nunc-Maxisorb, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with sheep anti-rat TNF-α or sheep anti-rat IL-1β. After blocking the plates, rat TNF-α or IL-1β standards at various dilutions in medium and 50 μl of samples were added in triplicate and maintained at room temperature for 2 h. Sheep TNF-α or IL-1β-biotinylated polyclonal antibodies were added at a 1:500 dilution, followed by incubation at room temperature for 1 h. Finally, 100 μl of avidin-horseradish peroxidase (1:5000 dilution) was added to each well and, after 30 min, the plates were washed and the colour reagent OPD (40 μg, 50 μl per well) was added. After 15 min, the reaction was terminated with H_2SO_4 (1 M, 50 μl per well) and the absorbance read at 490 nm. The results were obtained by comparing the absorbance with standard curves. In addition, the results were adjusted to 0.5 ml, the volume used to extract the cytokine from the paw skin, and are expressed as picograms of respective cytokine per milliliter. As a control, the levels of these cytokines were determined in animals that received saline (100 μl, i.pl.) or PMX53 (60 μg, 100 μl, i.pl.) 30 min before saline (100 μl, i.pl.) injection.

Experimental protocols

Effect of PMX53 on mechanical hypernociception, neutrophil migration and cytokine release induced by zymosan, LPS, carrageenan or OVA. Rats received saline (100 μl) or PMX53 (60 or 180 μg—zymosan experiments; or 60 μg—LPS, carrageenan or OVA experiments) 30 min before zymosan (30 μg in 100 μl, i.pl.), LPS (0.5 μg in 100 μl, i.pl.), carrageenan (Cg, 100 μg in 100 μl, i.pl.) or OVA (25 μg in 100 μl, i.pl., CFA-immunized animals) stimulus. Mechanical hypernociception was measured 2–24 h and 2–4 days after zymosan injection or 1–24 h after LPS, carrageenan or OVA injection.

We also evaluated the post-treatment effect of PMX53 on zymosan-induced mechanical hypernociception. PMX53 (60 μg in 100 μl, i.pl.) was injected into the right paw on

day 3 after zymosan injection, and hypernociception was measured 1–24 h after the antagonist injection. The groups of animals for MPO and cytokine determination were killed 4 h after zymosan injection or 3 h after LPS, carrageenan or OVA injection, as described above.

In addition, we investigated the effect of PMX53 on zymosan-induced tibiotarsal joint hypernociception. Mice were lightly anaesthetized, and zymosan (30 μg in 5 μl) was administered via a 29 G hypodermic needle inserted into the right tibiotarsal joint region. Control animals received an intra-articular injection of the same volume of sterile saline. PMX53 (0.3, 1 or 3 mg kg^{-1}, s.c.) or PMX53 vehicle (saline) was given systemically (subcutaneously) 30 min before zymosan-induced joint hypernociception, and the responses were measured at 1–7 h. Afterwards, mice were killed and the tibiotarsal bone complex was removed and examined for MPO content as described above.

Effect of PMX53 on mechanical hypernociception and neutrophil migration induced by ZAS or C5a. Rats received intraplantar injection of ZAS (dilution of 1:300 in 100 μl, i.pl.) or C5a (40 ng in 100 μl, i.pl.) 30 min after PMX53 (60 μg in 100 μl, i.pl.) or saline (100 μl, i.pl.) injection, and the mechanical hypernociceptive responses were performed 1–24 h after ZAS or C5a intraplantar injection. A group of animals was killed, and rat paw tissues were collected 3 h after ZAS injection for MPO determination.

Effect of PMX53 on mechanical hypernociception induced by BK, TNF-α, CINC-1, IL-1β, PGE_2 and dopamine in rats. In this set of experiments, PMX53 (60 μg in 100 μl, i.pl.) or saline (100 μl, i.pl.) was injected 30 min before BK (500 ng in 100 μl, i.pl.), TNF-α (2.5 pg in 100 μl, i.pl.), cytokine-induced neutrophil chemoattractant-1 (CINC-1) (100 pg in 100 μl, i.pl.), IL-1β (0.5 pg in 100 μl, i.pl.), prostaglandin E_2 (PGE_2) (100 ng in 100 μl, i.pl.) or dopamine (3 μg in 100 μl, i.pl.) injection, and the hypernociceptive responses were measured 3 h after stimulus challenge.

Additionally, we investigated the effect of PMX53 on TNF-α-induced tibiotarsal joint hypernociception. Mice were pretreated with PMX53 (3 mg kg^{-1}, s.c.) 30 min before TNF-α (100 pg in 5 μl)-induced joint mechanical hypernociception (as described above), and the hypernociceptive responses were measured 1–5 h after TNF-α intra-articular injection.

Effect of vinblastine sulphate on mechanical hypernociception and neutrophil migration induced by zymosan, C5a or carrageenan. Animals (rats) received vinblastine sulphate (0.8 mg kg^{-1}, i.v.) 72 h before intraplantar injection of saline (100 μl), zymosan (30 μg in 100 μl, i.pl.), C5a (40 ng in 100 μl, i.pl.) or carrageenan (100 μg in 100 μl, i.pl.), and the hypernociceptive responses were measured 3 h after intraplantar injection of stimuli. The animals were killed and the rat plantar tissues were collected for MPO assay.

Drugs. The compounds used in this study were PMX53 (Peptech/Promics, Toowong, Queensland, Australia), zymosan, C5a, carrageenan, LPS, CFA, OVA, BK, dopamine, IL-1β, CINC-1, PGE_2 (Sigma-Aldrich, St Louis, MO, USA) and TNF-α (National Institute for Biological Standards and Control, UK). All compounds were dissolved in sterile saline (Halex-Istar, São Paulo, Brazil) except for PGE_2 (DMSO (dimethylsulphoxide), Sigma-Aldrich, 0.2% in saline).

Data analysis. Results are presented as means ± s.e.mean for groups of 3–6 animals. A *t*-test for unpaired groups was used, and one-way ANOVA followed by Bonferroni's test was used as required. The level of significance was set at $P<0.05$.

Results

C5a mediates innate and adaptive inflammation-induced mechanical hypernociception

The importance of C5a in the genesis of inflammatory hypernociception was addressed by treating animals with PMX53, a potent and selective C5a receptor antagonist. The doses of inflammatory stimuli used to induce hypernociception in the rats were as previously determined (Cunha *et al*., 1991, 1992; Ferreira *et al*., 1988, 1993). It was observed that local pretreatment of rats (i.pl.) with PMX53 (60 and 180 μg per paw, 30 min before zymosan injection) inhibited zymosan (30 μg per paw)-induced mechanical hypernociception (Figure 1a). The effect of PMX53 was sustained for 6 h after zymosan injection and decreased after 24 h. The local administration of PMX53 (60 μg per paw) did not alter the nociceptive baseline of the animals (Figure 1a).

As a single intraplantar injection of zymosan induces mechanical hypernociception that is maintained for 5 days, we also tested the post-treatment effect of PMX53 in this model. Again, the therapeutic treatment with PMX53 (60 μg per paw, given 3 days after zymosan) significantly reduced the zymosan hypernociception, suggesting that C5a is important in maintaining the ongoing hypernociception induced by zymosan (Figure 1a). Furthermore, the post-treatment of the group (3 days after) that was already pretreated with PMX53 (60 μg per paw) also inhibited the zymosan-induced hypernociception (data not shown).

The incubation of rat serum with zymosan triggers the activation of the complement system with the generation of C5a (Issekutz *et al*., 1996; Kodani *et al*., 2000). Thus, to affirm that PMX53 is acting on the C5a receptor, the effect of PMX53 on ZAS- and recombinant C5a-induced hypernociception was tested. Pretreatment of rats with PMX53 inhibited hypernociception induced by either stimulus (Figure 1b). Furthermore, the anti-hypernociceptive effect of PMX53 (60 μg per paw) was also observed on LPS (0.5 μg per paw)- or carrageenan (100 μg per paw)-induced mechanical hypernociception (Figure 1c). These last inflammatory stimuli have been extensively used to investigate the mechanism of innate inflammatory response-induced hypernociception. Besides its role in innate inflammatory response, the complement system also participates in adaptive immune inflammation (Schleimer, 2004). In support of these findings, PMX53 (60 μg per paw) pretreatment also reduced antigen (OVA) challenge-induced hypernociception in previously immunized rats (Figure 1d). Taken together, these results suggest that C5a participates in the

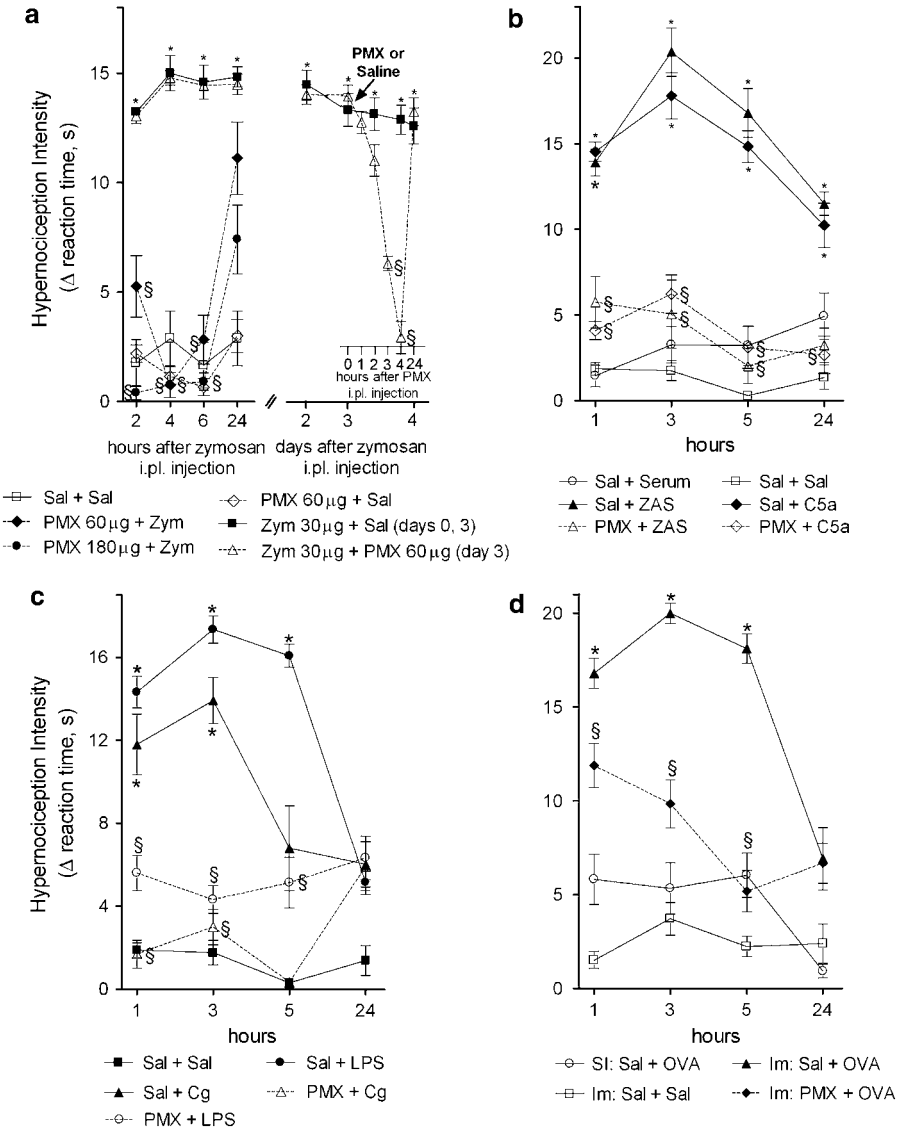

Figure 1 The C5a receptor antagonist (PMX53) inhibits the mechanical hypernociception induced by different stimuli. (**a**) PMX53 (PMX, 60 or 180 μg, i.pl.) was given 30 min before zymosan (Zym, 30 μg, i.pl.) injection, and the hypernociceptive responses were measured at 2–24 h. PMX53 (PMX, 60 μg, i.pl.) or saline was also administered on day 3 after zymosan injection, and hypernociception was evaluated 1–24 h after PMX53 treatment. (**b**) PMX53 (60 μg, i.pl.) was administered 30 min before zymosan activated serum (ZAS, dilution of 1:300, i.pl.) or C5a factor (C5a, 40 ng, i.pl.). (**c**) PMX53 (PMX, 60 μg, i.pl.) was injected 30 min before lipopolysaccharide (LPS, 0.5 μg, i.pl.) or carrageenan (Cg, 100 μg, i.pl.). (**d**) Ovalbumin (OVA, 25 μg, i.pl.) was administered to rats previously immunized as described in Methods. (**b–d**) Hypernociception was evaluated 1–24 h after stimulus injection. The nociceptive baseline of the animals was: 31.0 ± 0.26 s ($n = 30$). *$P < 0.05$ compared with serum control group for ZAS (Sal + Serum 1:300, (**b**) or saline paw injected control group (Sal + Sal, **a–d**); §$P < 0.05$ compared with positive control group (Sal + stimulus); $n = 4$–6 per experiment, representative of two separate experiments.

genesis of hypernociception during the innate and adaptive inflammatory response.

In an attempt to exclude direct blockage of hypernociception by a mechanism other than C5a receptor inhibition, we tested the effect of PMX53 on the hypernociception induced by the nociceptor direct-acting mediators (prostaglandins and sympathetic amines). Indeed, these hypernociceptive mediators act directly in the membrane of nociceptors triggering their sensitization. PMX53 did not alter PGE_2 (100 ng)- or dopamine (3 μg)-induced hypernociception (Figure 2), supporting our hypothesis that direct antagonism of the C5a receptor is responsible for the effect of PMX53 on inflammatory hypernociception.

C5a does not trigger the hypernociceptive cytokine cascade

It has been demonstrated that inflammatory hypernociception is mediated by a cascade of cytokines initiated by BK in rats (Ferreira *et al.*, 1993). Therefore, it was determined whether the anti-hypernociceptive effect of PMX53 depends on the inhibition of cytokine production. The local pretreatment of rats with PMX53 (60 μg per paw) at a dose that inhibits hypernociception did not reduce the release of TNF-α or IL-1β induced by zymosan (30 μg), LPS (0.5 μg) or carrageenan (100 μg) (Table 1). These results suggest that the inhibition of cytokine production is not the main mechanism involved in the anti-hypernociceptive effect of PMX53.

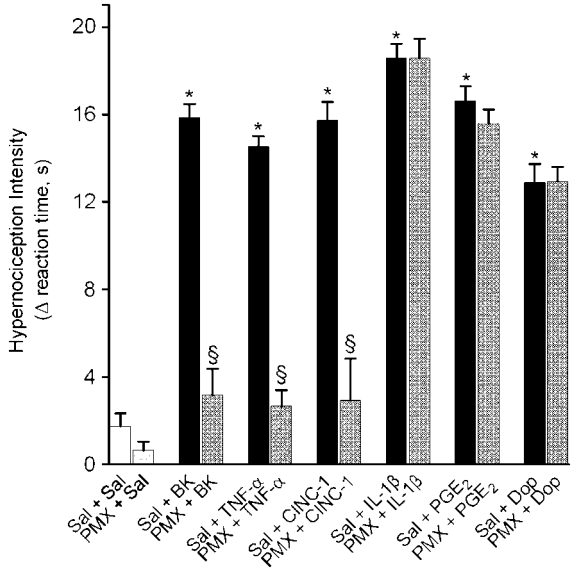

Figure 2 PMX53 treatment inhibits hypernociception induced by BK, TNF-α and CINC-1 but not that induced by IL-1β, PGE_2 or dopamine. Rats were pretreated with PMX53 (PMX, 60 μg, i.p.l.) or saline (Sal, 100 μl, i.p.l.) 30 min before intraplanar injection of saline (Sal, 100 μl), BK (500 ng), TNF-α (2.5 pg), CINC-1 (100 pg), IL-1β (0.5 pg), PGE_2 (100 ng) and dopamine (Dop, 3 μg), and hypernociception was evaluated 3 h after stimulus administration. The data are the means ± s.e.mean; $^*P<0.05$ compared with saline control group (Sal + Sal); $^{\S}P<0.05$ compared with positive control group (Sal + stimulus); $n=4$–5. BK, bradykinin; CINC-1, cytokine-induced neutrophil chemoattractant-1; IL-1β, interleukin-1β; PMX53, C5a receptor antagonist; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor-α.

C5a mediates cytokine-induced hypernociception

Considering that C5a does not mediate the release of cytokines during inflammatory hypernociception, in the next step, we determined whether C5a is involved in the hypernociceptive action of BK and cytokines. To address this question, rats were pretreated with PMX53 (60 μg per paw) 30 min before the intraplantar administration of BK (500 ng), TNF-α (2.5 pg), IL-1β (0.5 pg) or CINC-1 (100 pg), and the intensity of hypernociception was determined 3 h thereafter. The doses of these cytokines and BK used were those previously determined to be hypernociceptive (Cunha *et al*., 1991, 1992; Ferreira *et al*., 1988, 1993). As shown in Figure 2, pretreatment with PMX53 inhibited the hypernociception induced by BK, TNF-α or CINC-1 but not by IL-1β.

Neutrophils participate in C5a-induced inflammatory hypernociception

As demonstrated before, C5a plays a role in neutrophil migration in many inflammatory models. Furthermore, the hypernociceptive effect of C5a depends on neutrophil migration, and neutrophils play a critical role in the genesis of inflammatory hypernociception (Levine *et al*., 1985). Therefore, we investigated whether C5a mediates inflammatory hypernociception by a neutrophil recruitment-dependent mechanism. To test this hypothesis, rats were treated with PMX53 (60 μg per paw) or vehicle (saline) 30 min before

Table 1 Effect of local administration of PMX53 on the levels of cytokines induced by zymosan, carrageenan or lipopolysaccharide

Treatment	*Cytokine (pg per paw)*	
	TNF-α	*IL-1β*
Sal + Sal	25 ± 2	322 ± 28
PMX + Sal	18 ± 1	257 ± 21
Sal + Zym	34 ± 4*	501 ± 27*
PMX + Zym	37 ± 3	480 ± 19
Sal + Cg	42 ± 4*	531 ± 16*
PMX + Cg	42 ± 2	458 ± 35
Sal + LPS	63 ± 4*	608 ± 8*
PMX + LPS	79 ± 5	611 ± 14

Abbreviations: IL-β, interleukin-1β; i.pl., intraplantar; PMX, C5a receptor antagonist; TNF-α, tumour necrosis factor-α.
Rats were pretreated with saline (Sal 100 μl, i.pl.) or PMX53 (60 μg, i.pl.) injection 30 min before zymosan (Zym, 30 μg, i.pl.), carrageenan (Cg, 100 μg, i.pl.) or lipopolysaccharide (LPS, 0.5 μg, i.pl.) injection. The plantar skin was collected 3 h after intraplantar injection of Zym, Cg or LPS, and the cytokine levels (TNF-α and IL-1β) were determined by ELISA. The data are the means ± s.e.mean; $^*P<0.05$ compared with saline control group (Sal + Sal); $n=3$–4.

the intraplantar administration of carrageenan (100 μg per paw), zymosan, ZAS or LPS (Figure 3). At 3 h after inflammatory stimulus injection, the subcutaneous tissues of rat paw were collected for MPO activity assay. This method is used to determine neutrophil content indirectly (Bradley *et al*., 1982). PMX53 treatment reduced neutrophil migration induced by zymosan, ZAS and C5a (Figure 3). Although neutrophils seem to be important in the genesis of inflammatory hypernociception induced by carrageenan and LPS, we found that PMX53 did not reduce the neutrophil migration induced by these stimuli (Figure 3). It is possible that instead of a role in the recruitment of neutrophils, C5a could be activating neutrophils in LPS- and carrageenan-induced inflammation.

Supporting our hypothesis that neutrophils are important for the genesis of inflammatory hypernociception, depletion of rat neutrophils by vinblastine sulphate treatment (72 h before stimulus challenge) reduced mechanical hypernociception induced by zymosan, C5a or carrageenan (Figure 4a). Moreover, the treatment of rats with vinblastine sulphate abolished neutrophil migration induced by these stimuli (Figure 4b).

C5a participates in the genesis of zymosan-induced articular hypernociception in mice

To extend the concept that C5a is important for inflammatory hypernociception, we tested the effect of PMX53 on the articular hypernociception induced by joint administration of zymosan and TNF-α in mice. It was observed that pretreatment of mice with PMX53 systemically (0.3–3 $mg\,kg^{-1}$, s.c.) reduced zymosan- and TNF-α-induced articular hypernociception (Figures 5a and c, respectively). PMX53 (3 $mg\,kg^{-1}$, s.c.) pretreatment also reduced zymosan-induced neutrophil migration to the tibiotarsal joint (Figure 5b).

Discussion

In the present study, we have demonstrated for the first time the involvement of endogenous C5a in the genesis of mechanical hypernociception during the innate and adaptive inflammatory response. Furthermore, PMX53, a selective C5a receptor antagonist, displayed a potent peripheral anti-hypernociceptive effect in different inflammatory models, both in rats and mice. The hypernociceptive activity of C5a was not associated with triggering the hypernociceptive cytokine cascade. However, C5a is involved in the hypernociceptive effect of these cytokines and, moreover, our results demonstrated that neutrophils are involved in the hypernociceptive effect of C5a.

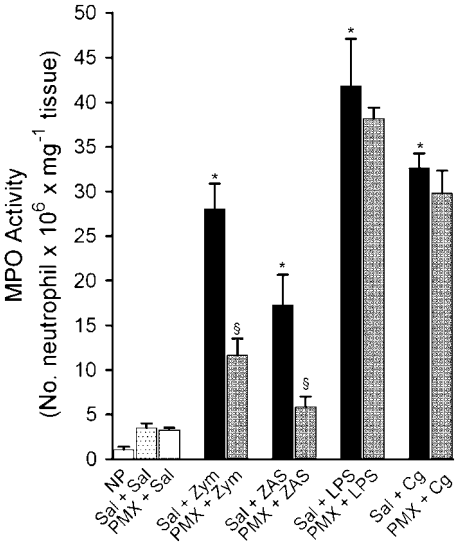

Figure 3 PMX53 inhibits Zymosan- and ZAS-induced neutrophil migration, but not that induced by LPS or Cg. Rats were pretreated with PMX53 (PMX, 60 μg, i.pl.) or saline (Sal, 100 μl, i.pl.) 30 min before intraplanar injection of saline (Sal, 100 μl), zymosan (Zym, 30 μg), zymosan-activated serum (ZAS, dilution of 1:300), lipopolysaccharide (LPS, 0.5 μg) or carrageenan (Cg, 100 μg), and rat plantar skin was collected 4 h after zymosan administration or 3 h after ZAS, LPS or carrageenan administration for assay of myeloperoxidase (MPO) activity (Methods). The naive paw sample (NP) is the control assay. $^{*}P<0.05$ compared with saline control group (Sal + Sal); $^{\S}P<0.05$ compared with positive control group (Sal + stimulus); $n=4$.

The involvement of the complement system in many inflammatory conditions has been demonstrated extensively (Linton and Morgan, 1999; Arumugam *et al*., 2004; Hawlisch *et al*., 2004; Ward, 2004). Initially, the constituents of the complement system were considered mediators mainly of the innate immune response by their ability to enhance phagocytosis and consequently the host defence against pathogens (van Beek *et al*., 2003). Later, the participation of the complement system in the physiopathology of a variety of acute and chronic inflammatory diseases was also demonstrated. For instance, the complement system, especially through C5a, plays a crucial role in the recruitment and activation of neutrophils and other leukocytes in different models of inflammation and diseases such as rheumatoid arthritis (Woodruff *et al*., 2002; Weissmann, 2006). However, the hypernociceptive role of C5a has not been fully elucidated. In one of the first studies that investigated the hypernociceptive activity of C5a, it was demonstrated that C5a injected intraplantarly in rat induces mechanical hypernociception (Levine *et al*., 1985). In the present study, by using the C5a receptor antagonist PMX53, we have shown that C5a acting on its receptor mediates mechanical hypernociception induced by zymosan, carrageenan, LPS or antigen (OVA). We also tested the effect of this drug on the hypernociceptive effect of the nociceptor

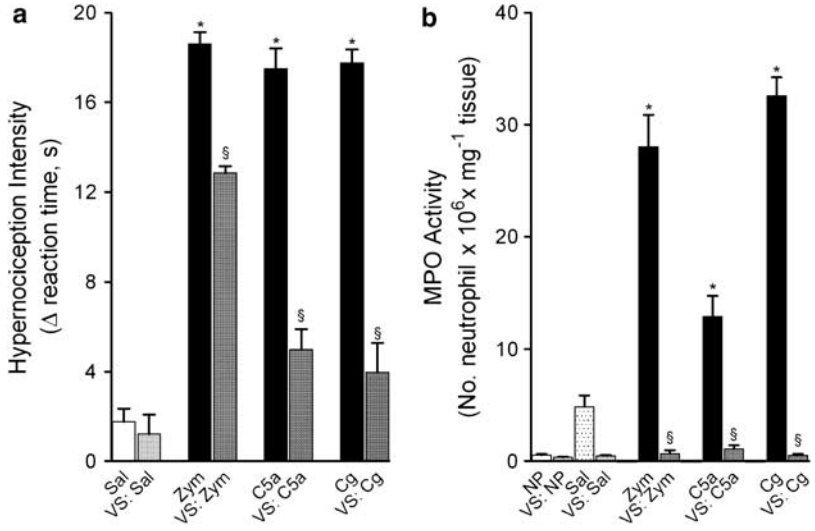

Figure 4 Vinblastine sulphate-induced neutrophil depletion reduces mechanical hypernociception. Rats were pretreated with vinblastine sulphate (VS, 0.8 mg kg^{-1}) or saline (Sal, 200 μl) intravenously 72 h before the injection of saline (100 μl i.pl.), zymosan (Zym, 30 μg i.pl.), C5a factor (C5a, 40 ng, i.pl.) or carrageenan (Cg, 100 μg, i.pl.). (**a**) Mechanical hypernociception was evaluated 3 h after stimulus injection. (**b**) Rat plantar skin was collected 3 h after stimulus administration for MPO activity determination. The naive paw sample (NP) is the control assay for MPO activity. $^{*}P<0.05$ compared with saline control group (Sal + Sal); $^{\S}P<0.05$ compared with positive control group (Sal + stimulus); $n=4$–5. MPO, myeloperoxidase; TNF-α, tumour necrosis factor-α.

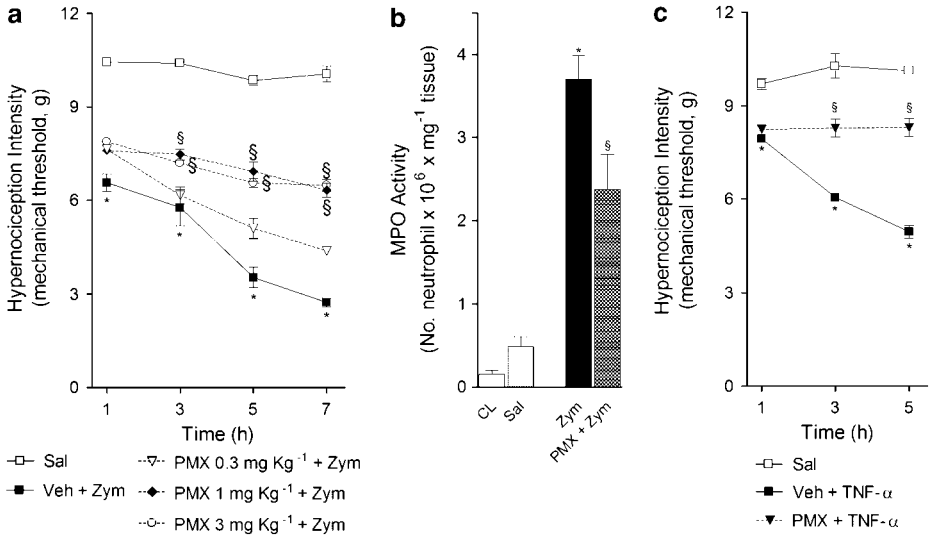

Figure 5 PMX53 inhibits flexion-elicited hypernociception in mouse tibiotarsal joints. (**a**) PMX53 (PMX, 0.3–3 mg kg^{-1}) or vehicle (saline) was injected subcutaneously (systemically) 30 min before intra-articular injection of zymosan (Zym, 30 μg in 5 μl), and the hypernociceptive response was measured 1–7 h after zymosan administration. (**b**) The tibiotarsal joint was collected at 7 h after intra-articular injection of zymosan (Zym, 30 μg in 5 μl) or saline for MPO activity determination. The contralateral joint (CL) is the control assay for MPO activity. (**c**) PMX53 (PMX, 3 mg kg^{-1}, s.c.) or vehicle (saline) was administered 30 min before intra-articular injection of TNF-α (100 pg in 5 μl), and hypernociceptive response measured 1–5 h after TNF-α injection. *$P<0.05$ compared with saline control group (Sal); $^{§}P<0.05$ compared with positive control group (Zym or TNF-α); $n=5$. MPO, myeloperoxidase; TNF-α, tumour necrosis factor-α.

direct-acting hypernociceptive mediators (PGE_2 and dopamine), which act directly on their receptors present on the membrane of primary nociceptive fibres (Cunha *et al*., 1991; Lorenzetti *et al*., 2002). As expected, PMX53 inhibited the mechanical hypernociception induced by ZAS and recombinant C5a, but not that induced by the direct-acting mediators. These results confirm that the antihypernociceptive effect of PMX53 is primarily due to inhibition of C5a receptors. An important finding was that therapeutic (that is post-challenge) treatment with PMX53 also inhibits zymosan-induced sustained hypernociception, suggesting that endogenous C5a release is involved in the maintenance of long-lasting hypernociception induced by zymosan. Thus, it raises the possibility of therapeutic use of C5a receptor antagonists to control chronic inflammatory pain. In support of this notion, oral treatment with PMX53 administered following the onset of experimental arthritis ameliorates disease pathology in rats (Woodruff *et al*., 2002). In this last study, animals that received PMX53 had significant reductions in right knee swelling, gait disturbance, migrated joint cell numbers and right knee histopathology, as well as intra-articular levels of IL-6 and TNF-α and serum levels of TNF-α. Although inflammatory pain is one of the most important symptoms of rheumatoid arthritis, the antinociceptive effect of PMX53 was not addressed in that particular study. In the present study, extending the therapeutic potential of PMX53, we have demonstrated that PMX53 treatment inhibited, in a dose-dependent manner, hypernociception during zymosan- and TNF-α-induced monoarthritis, as well as joint neutrophil migration in mice. In agreement with our findings, it has recently been shown that systemic treatment of rats with PMX53 reduced the hypernociception in an experimental model of pain after surgery, incisional pain (Clark *et al*., 2006).

In previous studies, we demonstrated that inflammatory hypernociception is mediated by a cascade of cytokines triggered by BK (BK triggers TNF-α→IL-6→IL-1β→PGE_2; and TNF-α→CINC-1→sympathetic amines) (Cunha *et al*., 1991, 1992; Ferreira *et al*., 1993; Verri *et al*., 2006). Taking into account that C5a induces the release of several pro-inflammatory cytokines such as TNF-α and chemokines (O'Barr and Cooper, 2000; Laudes *et al*., 2002) and that PMX53 inhibits cytokine production in an experimental model of nociception after incisional surgery (Clark *et al*., 2006), as well as in experimental rheumatoid arthritis (Woodruff *et al*., 2002), we investigated whether the effect of PMX53 depends on the inhibition of cytokine production, using our models of inflammatory hypernociception. At a dose that significantly inhibits hypernociception, PMX53 did not affect TNF-α and IL-1β production induced by LPS, carrageenan and zymosan. Thus, it seems that the inhibition of cytokine production is not responsible for the marked antihypernociceptive effect of PMX53. It is important to note that there is also evidence in the literature that C5a does not directly induce the production of pro-inflammatory cytokines (Haynes *et al*., 2000). In fact, C5a alone failed to induce the release of IL-1β, TNF-α and IL-6 from human monocytes (Haynes *et al*., 2000). The differences between models could explain these contradictory data.

The inability of C5a to trigger the hypernociceptive cytokine cascade led us to test whether it could mediate the hypernociceptive action of these cytokines. It was observed that PMX53 reduces BK-(which acts through release of cytokines, see above), TNF-α- and CINC-1-induced mechanical hypernociception. However, it was not able to inhibit IL-1β-induced hypernociception. This suggests that the role of C5a in inflammatory hypernociception fits in a downstream position of the described cytokine cascade. The

inability of PMX53 to inhibit IL-1β hypernociception is expected, as this cytokine induces hypernociception via the direct production of prostanoids by COX (Ferreira *et al*., 1988, 1997).

Our group and others showed that neutrophil migration to inflammatory sites and/or their activation play a crucial role on the genesis of inflammatory hypernociception (de Abreu Castro and Ferreira, 1979; Levine *et al*., 1985; Lavich *et al*., 2006; Cunha *et al*., unpublished observation). Recently we demonstrated that neutrophils, instead of being responsible for initiating the cytokine cascade, are critical for cytokine-induced hypernociception (Cunha *et al*., unpublished observation). Thus, we investigated whether C5a contributes to inflammatory hypernociception through a neutrophil-dependent mechanism. In agreement with a previous study (Levine *et al*., 1985), it was observed that the hypernociceptive action of C5a is associated with the presence of neutrophils, as its hypernociceptive effect was reduced (93% compared with control group) by neutrophil depletion in vinblastine-treated rats. Vinblastine treatment also reduced the hypernociception induced by carrageenan and zymosan, confirming the participation of neutrophils in the onset of hypernociception. Taking into account this information and that in inflammatory disease models, such as rheumatoid arthritis and ischaemia/reperfusion, the effectiveness of PMX53 is closely related to the inhibition of neutrophil migration or activation (Woodruff *et al*., 2003). Therefore, we examined whether the antinociceptive effect of PMX53 is a consequence of the inhibition of neutrophil accumulation. It was seen that PMX53 reduced neutrophil migration induced by zymosan, ZAS or C5a, but not that induced by carrageenan or LPS. Firstly, these results confirm that the main mediator present in ZAS and generated *in vivo* after zymosan administration that mediates neutrophil migration is C5a, whereas other chemotactic mediators could participate in carrageenan- and LPS-induced neutrophil recruitment. For instance, there is evidence that TNF-α, leukotriene B_4 and chemokines mediate neutrophil migration induced by these inflammatory stimuli (da Rocha *et al*., 2004; Vale *et al*., 2004). Secondly, the results also suggest that the anti-nociceptive effect of PMX53 is not mainly due to the inhibition of the migration process. Thus, although the recruitment of neutrophils is essential for the genesis of inflammatory hypernociception, in the absence of their activation by C5a, hypernociception is not triggered even though they are present in the inflammatory focus. It is possible that C5a generated in the inflammatory focus activates the recruited neutrophils to produce permissive hypernociceptive products yet to be determined (Hetland *et al*., 1998; Castellheim *et al*., 2005). For instance, there is evidence that the activation of neutrophils leads to the production of prostaglandins and of 15-HETE, which also shows a hypernociceptive effect (Levine *et al*., 1985; Akama *et al*., 1990; Cunha *et al*., unpublished observations). Furthermore, the activation of C5a receptors on neutrophils promotes oxidative burst with free radical production and the release of enzyme granules, which could also be involved in inflammatory hypernociception (Bazargani, 2005). Although the neutrophil seems to be the main inflammatory cell involved in the hypernociceptive action of C5a, as mentioned before, its receptors are also expressed in other immune cells such as macrophages and mast cells (Kiener *et al*., 1998; Fayyazi *et al*., 1999), which have also been observed to be involved in a variety of chronic pain models (Marchand *et al*., 2005). Therefore, further studies are needed to investigate whether these cells also contribute directly or indirectly to the pro-nociceptive activity of C5a.

Besides the peripheral role of C5a described above, we could not discard the possibility that C5a could also participate in the genesis of inflammatory hypernociception by an effect on the central nervous system. In this context, in a recent study, Griffin *et al*. (2007) showed that during different models of neuropathic pain, there is an increase in the production of complement system components by micraglial cells at the spinal cord. Further supporting the role of the complement system for the genesis of neuropathic pain, more specifically of C5a, it was demonstrated that disrupted C5 signalling using C5-deficient mice, as well as by intrathecal treatment with a C5aR1 antagonist, ameliorates nerve injury-induced allodynia (Griffin *et al*., 2007). Furthermore, intrathecal administration of C5a induces cold allodynia in a dose-dependent manner (Griffin *et al*., 2007).

In summary, the present study demonstrates the importance of endogenous C5a in the genesis of mechanical hypernociception in several inflammatory models and in two different animal species. The role of C5a in inflammatory hypernociception does not involve the triggering of hypernociceptive cytokine production, but seems to be dependent on the presence of neutrophils at the inflammatory site. In conclusion, these results point to the blockade of the C5a receptor as a promising pharmacological approach to control inflammatory pain of different origins. Certainly, PMX53 merits further studies on its possible therapeutic use for the control of pain.

Acknowledgements

We thank Ieda Regina dos Santos Schivo, Sérgio Roberto Rosa and Giuliana Bertozi Francisco for excellent technical support. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Technológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We are very grateful to Promics/Peptech Ltd for supplying us with PMX53 for these studies.

Conflict of interest

SMT was the Scientific Director, CSO (2001–2006), and a founder of Promics Ltd. TMW was a Scientific Consultant to Promics/Peptech 2003–2007.

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