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RESEARCH PAPER

The snake venom metalloproteinase BaP1 induces joint hypernociception through TNF-α and PGE_2-dependent mechanisms

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Background and purpose: Matrix metalloproteinases (MMPs) have been implicated in joint tissue destruction in arthritis. However, MMPs have not been assigned a role in joint pain. We investigated the ability of BaP1, a metalloproteinase from *Bothrops asper* snake venom, with structural homology to MMPs, to induce joint hypernociception.
Experimental approach: Animals received intra-articular (i.art.) BaP1. Hypernociception was assessed using the rat-knee joint articular incapacitation test. Cell influx, prostaglandin E_2 (PGE_2), and TNF-α levels were assessed in joint exudates following BaP1 injection.
Key results: BaP1 (5 μg per joint) provoked hypernociception between 1 and 6 h after i.art. injection. Cell influx, mostly neutrophils, was maximal 3 h after BaP1 i.art. injection. BaP1 also led to increase in PGE_2 and TNF-α levels in the joint exudates. Pretreatment with either indomethacin (4 $mg.kg^{-1}$ i.p.) or with an anti-TNF-α antiserum (i.art.) significantly inhibited both BaP1-induced joint hypernociception and cell influx. In isolated rat peritoneal macrophages, BaP1 increased cyclooxygenase (COX)-2 expression, while not altering that of COX-1.
Conclusions and implications: This is the first demonstration that a metalloproteinase promotes joint hypernociception. This effect involves local release of PGE_2 and TNF-α. BaP1-induced increase in PGE_2 is associated to increased COX-2 expression in macrophages. Blocking PGE_2 or TNF-α inhibits BaP1-induced hypernociception. In addition to unravelling a hitherto unknown mechanism whereby TNF blockade provides analgesia in arthritis, the data show, for the first time that MMPs are involved in inflammatory joint hypernociception and induce COX-2 expression.
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Keywords: metalloproteinase BaP1; hyperalgesia; synovial joint; arthritis; TNF-α; prostaglandins

Abbreviations: ADAM, a disintegrin and metalloproteinase; COX, cyclooxygenase; i.art., intra-articular; IL-1, interleukin-1; MMP, matrix metalloproteinase; OA, osteoarthritis; PET, paw elevation time; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; SVMP, snake venom metalloproteinase; TACE, tumour necrosis factor-α-converting enzyme; TNF-α, tumour necrosis factor-α; TIMP, tissue inhibitor of metalloproteinases

Introduction

Inflammatory arthropathies, including rheumatoid arthritis (RA) and osteoarthritis, are among the leading causes of disability, especially in the elderly. Though joint deformities may be prominent in these patients, joint pain is the most frequent complaint and the main reason to seek medical attention.

Articular cartilage homeostasis is maintained, at least in part, by equilibrium between the secretion of matrix metalloproteinases (MMPs) that include aggrecanases, elastases, gelatinases and stromelysin, and that of their counterparts, the tissue inhibitors of metalloproteinases (TIMPs). This equilibrium is tightly regulated and a cytokine imbalance seems to operate in chronic arthritis favouring the secretion of proinflammatory cytokines leading to enhanced MMP release and/or activation, thus contributing to joint destruction (Arend, 2001).

The MMPs encompass a group of zinc-dependent peptidases from the metzincins superfamily, which are involved in the lysis of components of the extracellular space. In addition to their binding sites and cleavage of extracellular molecules, including collagen and aggrecan, MMPs may further amplify their activity through the release of chemokines (Mohammed *et al.*, 2003). Further, MMPs may shed adhesion molecules and stimulate the release of cytokines. Members of another group from the metzincins family, known as a disintegrin and metalloproteinase (ADAM) have been implicated in the control of several physiological

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events including cytokine shedding and cell migration (Blobel, 1997; Peschon *et al*., 1998). For instance, ADAM-17 is also known as the tumour necrosis factor-α-converting enzyme (TACE) from its ability to shed the mature tumour necrosis factor-α (TNF-α) from leucocyte membranes (Gearing *et al*., 1994; Black *et al*., 1997). Hence, ADAM-17 is critically involved in the release of a cytokine closely linked to tissue destruction in RA (Zhang *et al*., 1998; Choy and Panayi, 2001). Interestingly, TIMP-3 is able to inhibit TACE, thereby regulating this enzyme activity (Amour *et al*., 1998).

A diverse array of metalloproteinases has been isolated from the venoms of snakes from the Viperidae family, most of them with haemorrhagic activity. Snake venom metalloproteinases (SVMPs) are members of the metzincin superfamily of zinc metalloproteinases, together with MMPs, astacins and serralysins, all of them exhibiting an identical zinc-binding motif (Bode *et al*., 1993). In turn, SVMPs are part of the reprolysin subfamily of metalloproteinases, which includes the group of mammalian homologous proteins ADAMs (Fox and Long, 1998). Moreover, SVMPs have strong similarities in sequence and domain organization to ADAMs.

A non-haemorrhagic metalloproteinase named BaP1 was isolated from the *Bothrops asper* snake venom, an important venomous snake in Central America (Borkow *et al*., 1993; Gutiérrez *et al*., 1995; Franceschi *et al*., 2000). BaP1, a 22.7 kDa enzyme, belongs to the PI class of the SVMPs, containing only the catalytic domain (Watanabe *et al*., 2003). This SVMP has a potent proteolytic activity and induces inflammation and moderate myonecrosis locally (Rucavado *et al*., 1995, 2002; Fernandes *et al*., 2006). Upon intramuscular injection into mice, BaP1 induces release of cytokines, formation of blisters and infiltration of leucocytes though neither displaying coagulopathy nor toxic reactions after its systemic administration (Gutiérrez *et al*., 1995). BaP1 also induces the expression of a latent form of endogenous MMP-9 (Rucavado *et al*., 2002). Moreover, BaP1 induces cell migration and the local release of interleukin-1 (IL-1) and TNF-α after administration into the peritoneal cavity of mice (Fernandes *et al*., 2006).

Limitation of movement secondary to joint hyperalgesia is a serious burden to patients presenting with inflammatory arthropathies. Despite the prominent role played by MMPs in cartilage destruction, the involvement of these substances in the development of joint pain has not been explored. In the present study, the ability of BaP1 to induce joint hypernociception in rats and related mechanisms were addressed. Herein, we present the first evidence that a SVMP promotes joint hypernociception and cell influx after being injected into the rat knee joint. Further, these SVMP-provoked phenomena involve increases in the joint levels of prostaglandin E_2 (PGE_2), following increased expression of cyclooxygenase (COX)-2, as well as increased release of the proinflammatory cytokine TNF-α.

Methods

Animals

Male Wistar rats (200–250 g) were used. Animals were housed in temperature-controlled rooms and received water and food *ad libitum* until used. All experiments were designed to minimise animal suffering and to use the minimum number associated with valid statistical evaluation. These studies were approved by the Experimental Animals Committee of Butantan Institute (Protocol no. 231/05) in accordance with the procedures laid down by the Universities Federation for Animal Welfare.

Metalloproteinase BaP1

The SVMP BaP1 was isolated from a venom pool obtained from more than 40 adult specimens of *Bothrops asper* snake collected in Costa Rica and kept at the Serpentarium of Clodomiro Picado Institute, Costa Rica. BaP1 was purified by ion-exchange chromatography on CM-Sephadex C-25, gel filtration on Sephacryl S-200 and affinity chromatography on Affi-gel Blue, and the homogeneity of this enzyme was demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions (Gutiérrez *et al*., 1995; Rucavado *et al*., 1998). The enzyme was lyophilised, stored at −20°C and dissolved in 0.15 M NaCl solution just before use.

Induction of arthritis

The animals were anaesthetised with a combination of ketamine (30 mg kg^{-1}) and xylazine (5 mg kg^{-1}) and were subjected to intra-articular (i.art.) injection of a BaP1 solution (5 μg per joint) dissolved in 50 μl of apyrogenic saline. Control animals received bovine serum albumin (BSA, 5 μg per joint) or the same volume of apyrogenic saline.

Collection of synovial exudates and assessment of cell influx

At selected time intervals, the animals were killed by cervical dislocation under halothane anaesthesia, and exsanguinated. The synovial cavity of the knee joints was then washed with 0.4 ml of phosphate-buffered saline (PBS), pH 7.2 containing 5 U ml^{-1} heparin. The synovial exudates were collected by aspiration. Total and differential cell counts were assessed using a Neubauer chamber and stained smears (Hema^3 stain), respectively. The exudates were then centrifuged (500 *g* for 6 min at 4°C) and supernatants were stored at −80°C and later used for the determination of PGE_2 and TNF-α concentrations.

Evaluation of joint hypernociception

The rat knee joint incapacitation test is described in detail elsewhere (Tonussi and Ferreira, 1992; Rocha *et al*., 1999, 2002). Briefly, after BaP1 or BSA injection into their knee joints, animals were put to walk on a steel rotary drum (30 cm wide × 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 r.p.m. Specially designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, the animals were allowed to walk for habituation. The right paw was then connected via a simple circuit to microcomputer data input/output port and the paw elevation time (PET) was measured. PET is the time the animal walks failing to touch the cylinder with the injected hind paw, during a 60 s period, which is

directly proportional to the gait disturbance. In this model, articular incapacitation is used as a measure of joint inflammatory hypernociception. PET was measured before (control time) and 1, 2, 3, 4, 5, and 6 h after i.art. administration of BaP1 or BSA.

Assessment of TNF-α levels in joint exudates

Synovial washes were collected 30 min, 1, 3 and 6 h after i.art. injection of BaP1 (5 μg per joint) or BSA (5 μg per joint). After centrifugation, the supernatants were used for determination of TNF levels by a standard assay using the fibroblast cell line L929, as described previously (Flick and Gifford, 1984). Briefly, monolayers of L929 cells grown in RPMI-1640 medium were seeded at 3.5×10^4 cells per well in 96-well plates and incubated in humidified air with 5% CO_2 at 37°C for 18 h. Afterwards, 100 μl of each serially diluted supernatant and actinomycin D (2 μg ml^{-1}) were added. Then, 500 ng well^{-1} of anti-TNF-α antibody was added for determination of the assay specificity. After incubation for 24 h at 37 °C, supernatants were removed and viable cells were assessed after fixation and staining with violet crystal (0.2% in 20% methanol). Absorbances at 620 nm were recorded on a microtiter plate reader. Cytotoxicity, expressed as a percentage, was calculated as follows: $(Abs_{control}–Abs_{sample}/Abs_{control}) \times 100$. TNF levels were then expressed as U ml^{-1}, using a standard curve prepared with recombinant TNF.

Determination of PGE_2 in joint exudates

Concentrations of PGE_2 were measured in the synovial exudates at 1, 2, 3 and 6 h after i.art. injection of BaP1 or BSA by enzyme-linked immunosorbent assay (ELISA) after extraction of eicosanoids on Sep Pak C18 columns (Waters Corporation, Milford, MA, USA) eluted with ethanol. In brief, 100 μl aliquots of each extracted sample were incubated with the eicosanoid conjugated with acetylcholinesterase and the specific rabbit antiserum in 96-well microtiter plates, coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbances were recorded at 412 nm in a microplate reader, and concentrations of the eicosanoid were estimated from standard curves.

Expression of COX-1 and -2 in isolated rat peritoneal macrophages

Male Wistar rats were injected intraperitoneally with 4% thioglycolate solution. Peritoneal cells were harvested 96 h later and macrophages, identified under light microscopy (>96% purity), were used for western blotting experiments, as follows. The expression of COX-1 and -2 proteins was measured in rat peritoneal macrophages following 1, 3 and 6 h of incubation with BaP1 (0.5 μM) or RPMI medium (control). For all western blotting experiments, proteins from 10^6 cells incubated in Laemmli buffer were separated on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was incubated for 1 h with blocking buffer (20 mM Tris, 100 mM NaCl, 0.5% Tween 20 and 5% non-fat dried milk) and then probed for 1 h with a mouse antibody against rat COX-1 (1:500), rabbit antibody against rat COX-2 (1:1000), and a rabbit antibody against rat β-actin (1:2000). The membranes were then incubated with peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometry was performed using a GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, São Paulo, Brazil) and analyzed with Quantity One software (Bio-Rad). Actin expression was analysed as an internal control.

Treatments

Groups of rats were pretreated with either the cyclooxygenase inhibitor indomethacin (4 mg kg^{-1} intraperitoneally), dissolved in 0.1 M Tris, pH 8.0 or an antiserum against rat TNF-α (50 μl i.art.), 20 min before i.art. injection of BaP1. Control animals received the vehicle (0.1 M Tris, pH 8.0) or an irrelevant antiserum, respectively.

Statistical analysis

Means and s.e.m. of all data were obtained and compared by analysis of variance. When significant differences were identified, individual comparisons were subsequently made with Tukey's test with significance probability levels set at $P<0.05$.

Chemicals and reagents

Most agents were purchased from either Sigma (St Louis, MO, USA) or Amersham Pharmacia Biotech (Bucks, UK). Other agents were acquired as follows: PGE_2 enzyme immunoassay kit, rabbit anti-rat COX-2 and mouse anti-rat COX-1 antibodies from Cayman Chemical (Ann Arbor, MI, USA); goat anti-rat TNF-α antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-rat β-actin from Sigma; peroxidase-conjugated secondary sheep anti-mouse or donkey anti-rabbit antibodies from Amersham Pharmacia Biotech; Hema3 stain from Biochemical Sciences Inc. (Swedesboro, NJ, USA); Indomethacin was purchased from Merck Sharp and Dohme (Darmstadt, Germany). The mouse antiserum against rat TNF-α was a gift from Dr Fernando Cunha (Faculdade de Medicina, Universidade de São Paulo, Ribeirão Preto, SP, Brazil).

Results

BaP1 promotes joint hypernociception

Figure 1a shows that the i.art. injection of BaP1 (5 μg per joint) provoked hypernociception, illustrated by a marked increase in PET, starting at 1 h and lasting until 6 h after BaP1 injection. Peak values were achieved between 2 and 6 h, as compared to controls. A lower dose of BaP1 (2.5 μg per joint) did not induce hypernociception, whereas doubling the dose (BaP1 10 μg per joint) provoked increases in PET that were

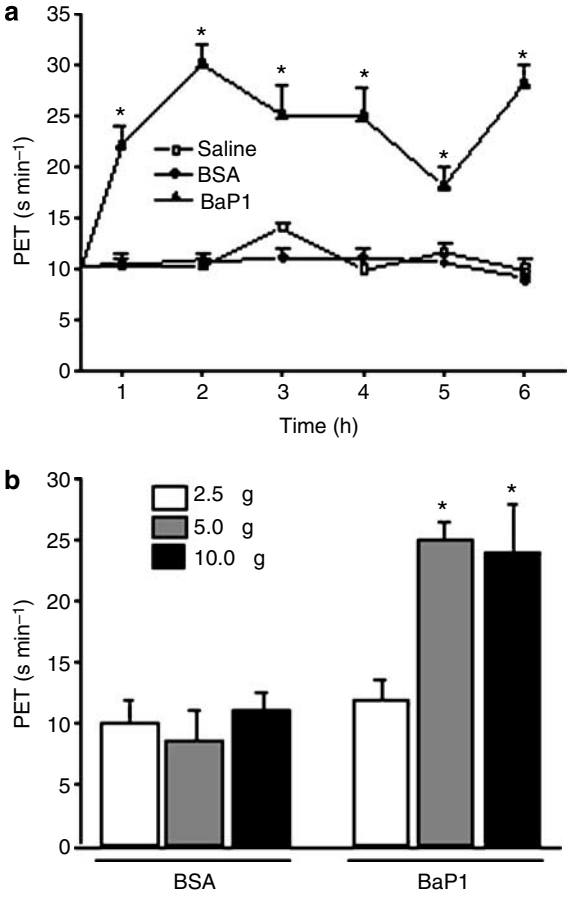

Figure 1 BaP1 promotes joint hypernociception. Groups of rats received i.art. injection of BaP1 or BSA (control). Hypernociception was measured as the increase in paw elevation time (PET) assessed at baseline and 1, 2, 3, 4, 5, and 6 h after the injections. (**a**) The kinetics of joint hypernociception following i.art. injection of 5 μg of BaP1 or BSA, or saline injection. (**b**) A dose–response effect on the maximal PET achieved after BaP1 injection (2.5–10 μg). Results are expressed as the mean $\pm$ s.e.m. of values for each group of six animals. *$P<0.05$, as compared to control. BSA, bovine serum albumin; i.art., intra-articular.

similar to that induced by BaP1 (5 μg per joint) (Figure 1b). Based on these data, we chose the dose of 5 μg BaP1 per joint to use in further experiments.

BaP1 induces cell influx into the joints

Preliminary studies showed that injection of selected doses of BaP1 (2.5, 5 and 10 μg per joint) induced a marked influx of total leucocytes into the synovial cavity 3 h after injection. As observed in joint hypernociception (see above), maximal leucocyte influx was obtained with the dose of 5 μg BaP1 per joint (Figure 2a). This dose was chosen for further experiments. The numbers of total, mononuclear and polymorphonuclear leucocytes in the synovial exudates were determined from 1 up to 6 h after 5 μg per joint BaP1 or BSA i.art. injection. Injection of BaP1 provoked a significant increase in cell counts at all periods studied, being maximal at 3 h (Figure 2b) with a predominance of polymorphonuclear cells (Figures 2c and d).

BaP1 increases PGE_2 and TNF-α levels in joint exudates

As shown in Figure 3a, i.art. injection of 5 μg BaP1 per joint induced a significant increase in PGE_2 levels in the joint exudates from 1 up to 6 h as compared to control (i.art. BSA). Apart from its role in tissue lesion in arthritis, TNF-α is also considered a pivotal cytokine in joint inflammatory hypernociception (Arend and Dayer, 1995; Williams *et al*., 1992; Cunha *et al*., 2005). We measured the levels of this cytokine in the joint exudates. As shown in Figure 3b, when compared to control, significantly increased levels of TNF-α were obtained in the synovial exudates starting at 30 min, with a striking peak at 6 h following BaP1 i.art. injection. TNF-α levels returned to baseline at 12 h after BaP1 injection (data not shown).

Effect of pretreatment with either indomethacin or an antiserum against TNF-α on BaP1-induced joint hypernociception

In an attempt to define the involvement of PGE_2 in BaP1-induced joint hypernociception, a group of animals was pretreated with indomethacin (4 mg kg^{-1} intraperitoneally) or its vehicle 20 min before i.art. injection of BaP1. Indomethacin completely prevented the hypernociception induced by BaP1 (Figure 3c). As observed with indomethacin, injection of the anti-TNF-α antiserum i.art. 20 min before the injection of BaP1 also abolished the hypernociception, as compared to animals that received an irrelevant antiserum (Figure 3c).

Effect of pretreatment with indomethacin or antiserum against TNF-α on BaP1-induced inflammatory cell influx into joints

Increased TNF-α levels have been associated with neutrophil influx in arthritis (O'Dell, 2004). In addition, these cells have been associated with joint hypernociception, so that reducing neutrophil trafficking into joints may lead to pain relief (Bezerra *et al*., 2007). In keeping with these assumptions, Figure 3d shows that pretreatment with indomethacin (4 mg kg^{-1} intraperitoneally) significantly reduced the BaP1-evoked leucocyte influx by 76%, as compared to vehicle-treated rats. Similarly, the i.art. administration of the TNF-α antiserum significantly reduced the influx of leucocytes (78% less cells), as compared to controls (Figure 3d).

Effect of pretreatment with indomethacin or antiserum against TNF-α on BaP1-induced increase in PGE_2 levels in joint exudates

As expected, pretreatment with indomethacin significantly reversed the increase in PGE_2 levels in the joint exudates promoted by BaP1 (less than 0.05 pg ml^{-1}), as compared to values in vehicle-treated controls (Figure 4). However, treatment with the anti-TNF-α antiserum did not affect the increase in PGE_2 levels in the joint exudates induced by BaP1 as compared to animals that received an irrelevant antiserum (Figure 4).

BaP1 induces the expression of COX-2 in isolated rat macrophages

To further explore the mechanism involved in BaP1-induced PGE_2 release, we examined the expression of COX-1 and -2

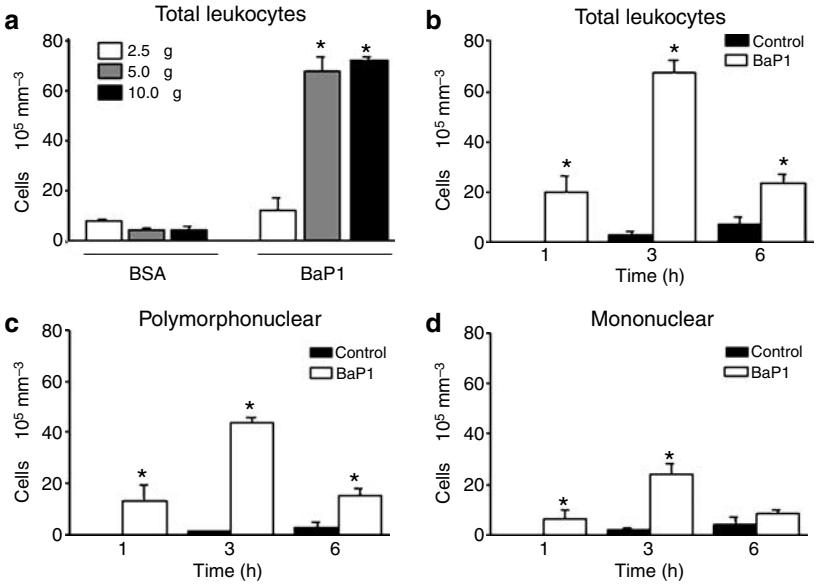

Figure 2 BaP1 promotes cell influx into joints. Groups of rats received i.art. injection of BaP1 or BSA (control). (**a**) The dose–response effect of i.art. injection of BaP1 (2.5–10 μg) on the total cell influx after 6 h, measured in the joint exudates. (**b–d**) The kinetics of total, polymorphonuclear and mononuclear cell influx into the joints measured at 1, 3 and 6 h after the i.art. injection of 5 μg per joint BaP1. Results are expressed as the mean $\pm$ s.e.m. of values for each group of six animals. *$P<0.05$, as compared to control. BSA, bovine serum albumin; i.art., intra-articular.

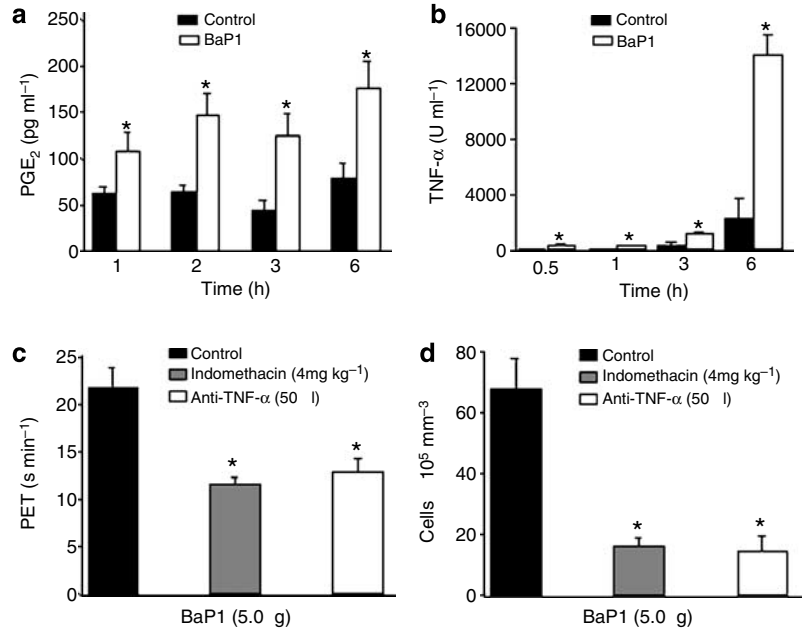

Figure 3 Kinetics of PGE_2 and TNF-α levels following BaP1 injection into rat joints and effect of cyclooxygenase (COX) or TNF-α blockade in BaP1-induced hypernociception and cell influx. Groups of rats received i.art. injection of BaP1 (5 μg per joint) or BSA (control). PGE_2 and TNF-α levels were assessed in joint exudates, using ELISA or the L929 cytotoxicity *in vitro* assay, respectively (see text for details). Joint hypernociception was measured as the increase in PET whereas cell influx was measured in the joint fluids 3 h after i.art. injection of BaP1. Groups were pretreated with either the COX inhibitor indomethacin (4 mg kg^{-1} intraperitoneally) or an anti-TNF-α (50 μl i.art.) antiserum. Indomethacin vehicle or an irrelevant antiserum was used as controls. (**a** and **b**) The kinetics of PGE_2 and TNF-α levels in the joint exudates, respectively. (**c** and **d**) The effect of indomethacin and the TNF-α antiserum on PET and cell influx, respectively. Results are expressed as the mean $\pm$ s.e.m. of values for each group of six animals. *$P<0.05$, as compared to control. BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; i.art., intra-articular; PET, paw elevation time; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor-α.

in isolated rat peritoneal macrophages cultured in the presence of BaP1. Figures 5a–c illustrate a significant increase in the expression of COX-2 induced by BaP1 at all time points tested, being maximal between 1 and 3 h of incubation. On the other hand, COX-1 expression was not influenced by the presence of BaP1.

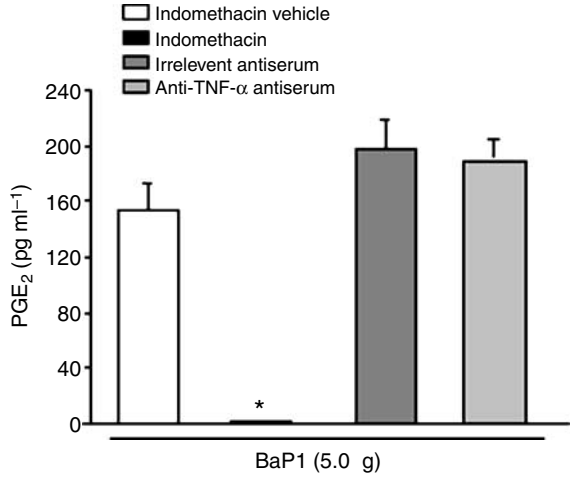

Figure 4 Effect of indomethacin and the anti-TNF-α antiserum on BaP1-induced increase of PGE_2 release. Rats were subjected to i.art. injection of BaP1 (5 μg per joint). Groups were pretreated with indomethacin (4 mg kg^{-1} intraperitoneally), an anti-TNF-α antiserum or the vehicles (control). PGE_2 levels were assessed in joint exudates, 6 h after i.art. injection of BaP1, using ELISA. Results are expressed as the mean $\pm$ s.e.m. of values for each group of six animals. $^*P<0.05$, as compared to controls. ELISA, enzyme-linked immunosorbent assay; i.art., intra-articular; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor-α.

Discussion

In the present study, we show that BaP1, a metalloproteinase isolated from the venom of *B. asper*, induced joint hypernociception and cell migration after being injected into the rat joint. These phenomena were associated with increased local (i.art.) levels of PGE_2 that were secondary to increased COX-2 expression. BaP1 also increased local TNF-α levels that were closely associated with the increase in both joint hypernociception and cell influx into the joints.

To our knowledge, this is the first report that a metalloproteinase promotes joint hypernociception. MMPs have long been recognized as proinflammatory substances, being able to promote tissue destruction, through their ability to cleave and degrade matrix molecules such as aggrecan and collagen (Murphy *et al*., 2002).

The kinetics of the joint hyperalgesia promoted by BaP1 showed that it was maximal between 2 and 6 h after i.art. injection of the enzyme. Interestingly, the influx of polymorphonuclear cells (mostly neutrophils) induced by the BaP1 injection paralleled the joint hyperalgesia, being maximal at 3 h and declining thereafter. These results are in agreement with previous studies (Talhouk *et al*., 2000) showing an interplay between upregulation of gelatinases and hyperalgesia induced by endotoxin in the mouse paw. As noted in the Introduction, injection of BaP1 into the mouse peritoneal cavity or the skin promotes a prominent inflammatory cell infiltration (Rucavado *et al*., 1998; Fernandes *et al*., 2006).

Neutrophils are predominant in the synovial exudates of inflammatory arthropathies, including gout, Reiter's disease and RA. In addition, disease severity is closely linked to neutrophil influx (Harris *et al*., 2004), so that therapeutic

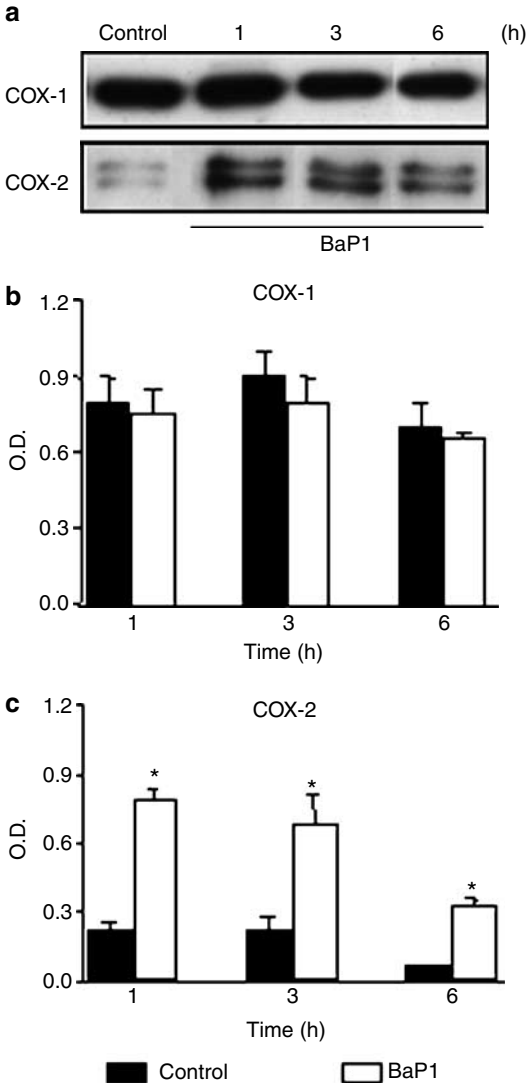

Figure 5 BaP1 induces COX-2 expression in peritoneal macrophages *in vitro*. Macrophages harvested from rat peritoneal cavities elicited with thioglycollate (96 h) were incubated with BaP1 (0.5 μM) or RPMI medium (control). (**a**) Representative illustration of the expression of COX-1 and -2 in homogenates obtained from macrophages incubated for 1, 3 and 6 h with BaP1 (western blot – see text for details). (**b** and **c**) Quantitative analysis of the immunoreactive band intensities for COX-1 and -2 protein, respectively, using densitometry; $n=3$ using different cell samples. $^*P<0.05$, as compared to control. COX, cyclooxygenase.

efficacy is related to a decrease in neutrophil trafficking into joints (O'Dell, 2004). Through the release of inflammatory mediators, such as eicosanoids, cytokines, MMP and reactive oxygen/nitrogen species, neutrophils have been linked to tissue destruction in RA, thus promoting cartilage breakdown and inflammatory bone resorption (Hampton *et al*., 1998). Apart from being an important source of PGs, neutrophils can contribute to hypernociception through the release of other substances, such as leukotriene B_4 (Rocha *et al*., 2004). In addition, we have recently demonstrated that neutrophil-derived peroxynitrite, a reactive nitrogen species, is involved in joint hypernociception in the zymosan arthritis model in rats (Bezerra *et al*., 2007). Also, the

antinociceptive effect of TNF-α blockade, either with antibodies or with the low molecular weight compounds, thalidomide, pentoxyfylline and chlorpromazine, in experimental arthritis was shown to be linked to a reduction in the neutrophil influx into the joints (Bombini *et al*., 2004).

In an attempt to further characterize the pro-nociceptive activity of BaP1, we demonstrated in the joints, an increase of PGE_2, a classical hyperalgesic mediator, starting as early as 1 h after the i.art. injection of BaP1. The fact that indomethacin inhibition of joint hypernociception was correlated with abolition of PGE_2 release into the joints indicates that PGE_2 is a major pro-nociceptive agent in this model. Though indomethacin blocks both COX-1 and -2 isoenzymes, the proinflammatory nature of BaP1 would suggest that the latter enzyme is more relevant. We also demonstrated that BaP1 induces COX-2 expression in isolated peritoneal macrophages. In addition to be the first demonstration of this mechanism, these data support the proposition that the pro-nociceptive effects of BaP1 are associated with increased PGE_2 release, secondary to COX-2 induction. However, it is also likely that the effects of BaP1 are mediated via additional mechanisms.

The cell sources of PGE_2 in the present experimental condition were not completely determined. The reduction of cell influx following the administration of both indomethacin and anti-TNF-α antiserum clearly implicate neutrophils. However, other resident cells are probably involved. Increase in PGE_2 was detected as early as 1 h following BaP1 injection, whereas neutrophil influx occurred later (3 h). Hence, migrated leucocytes are not the sole contributors to the increase in PGE_2. BaP1 induced COX-2 expression in peritoneal macrophages. Since type A synoviocytes closely resembles these intra-peritoneal cells, it is likely that BaP1 would similarly induce COX-2 and subsequent release of PGE_2 from the synoviocytes might well be that BaP1 activity upon synoviocytes leads to the release of PGE_2.

TNF-α is currently considered a final common mediator in the cytokine cascade that operates in some inflammatory diseases, including RA and ankylosing spondylitis (Braun *et al*., 2002). The administration of anti-TNF-α antibodies provides very early and significant pain relief in arthritis patients (Maini *et al*., 1999; Weinblatt *et al*., 2003). These clinical results are supported by experimental data proposing that TNF-α is a final inflammatory pain mediator (Cunha *et al*., 2005).

In the present study, BaP1 injection into the rat joints promoted the release of TNF-α that was maximal 6 h after the injection. Furthermore, administration of anti-TNF-α antiserum prevented not only the cell influx provoked by BaP1, but also the joint hypernociception. However, it did not interfere with the BaP1-induced release of PGE_2 into the joints. Taken together, these effects of BaP1 lead us to speculate that MMPs participate in pain mechanisms in arthritis through TNF-α release. This mechanism appears to be independent of an increase in the i.art. PGE_2 levels. Hence, the analgesia promoted by TNF antagonists could partially be due to a blockade of endogenous MMP activity.

The mechanisms of the increase in TNF-α release induced by BaP1 are not straightforward. As we alluded to above, ADAM-17, a member of the ADAMs group of metalloproteinases, acts as a TNF-α converting enzyme. Moreover, endogenous MMPs, including MMP-1, -2, -3, -7, -9 and -12, are able to process pro-TNF-α to its active form (Parks *et al*., 2004) in addition to their ability to activate pro-TNF-α in macrophages (Haro *et al*., 2000; Churg *et al*., 2003). Therefore, a stimulatory effect of BaP1 on TACE and/or its action in shedding mature TNF-α from its precursor in joint cells, through direct or indirect mechanism via expression of endogenous MMPs, such as MMP-9, could explain this mechanism. This possibility is challenging since it opens up a possibility for an amplifying, proinflammatory loop inside the joint. Based on this premise, we may speculate that MMPs participate in the inflammatory reaction in the very early stages of the insult. Rather than being a player in the later stages, focused on the chronic tissue lesions and destruction that has been usually attributed to them, MMPs could be operative in the initiating inflammatory events, stimulating cell migration and acting in a paracrine way to stimulate these cells.

In conclusion, our data are the first experimental evidence that a metalloproteinase (BaP1) has pro-nociceptive activity in joints. Both PGE_2 and TNF-α contribute to this effect. Targeting MMPs may thus provide both symptomatic and structural benefit in inflammatory arthropathies.

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Conflict of interest

The authors state no conflict of interest.

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