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RESEARCH PAPER

Effect of plant neutrophil elastase inhibitor on leucocyte migration, adhesion and cytokine release in inflammatory conditions

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BACKGROUND AND PURPOSE

The serine and cysteine peptidase inhibitor, BbCI, isolated from *Bauhinia bauhinioides* seeds, is similar to the classical plant Kunitz inhibitor, STI, but lacks disulphide bridges and methionine residues. BbCI blocks activity of the serine peptidases, elastase (K_{iapp} 5.3 nM) and cathepsin G (K_{iapp} 160.0 nM), and the cysteine peptidase cathepsin L (K_{iapp} 0.2 nM). These three peptidases play important roles in the inflammatory process.

EXPERIMENTAL APPROACH

We measured the effects of BbCI on paw oedema and on leucocyte accumulation in pleurisy, both induced by carrageenan. Leucocyte–endothelial cell interactions in scrotal microvasculature in Wistar rats were investigated using intravital microscopy. Cytokine levels in pleural exudate and serum were measured by ELISA.

KEY RESULTS

Pretreatment of the animals with BbCI (2.5 mg·kg^{-1}), 30 min before carrageenan-induced inflammation, effectively reduced paw oedema and bradykinin release, neutrophil migration into the pleural cavity. The number of rolling, adhered and migrated leucocytes at the spermatic fascia microcirculation following carrageenan injection into the scrotum were reduced by BbCI pretreatment. Furthermore, levels of the rat chemokine cytokine-induced neutrophil chemo-attractant-1 were significantly reduced in both pleural exudates and serum from animals pretreated with BbCI. Levels of interleukin-1β or tumour necrosis factor-α, however, did not change.

CONCLUSIONS AND IMPLICATIONS

Taken together, our data suggest that the anti-inflammatory properties of BbCI may be useful in investigations of other pathological processes in which human neutrophil elastase, cathepsin G and cathepsin L play important roles.

Abbreviations

BbCI, *Bauhinia bauhinioides* serine and cysteine peptidase inhibitor; CINC-1, cytokine-induced neutrophil chemo-attractant-1; IL-1β, interleukin-1beta; K_{iapp}, apparent inhibitory constant; TNF-α, tumour necrosis factor-alpha

Introduction

A variety of pathological conditions is associated with the release of human neutrophil elastase, a serine peptidase which plays an important role in inflammatory processes. This peptidase is a very damaging enzyme because it degrades insoluble elastin into soluble peptides (Owen and Campbell, 1999; Pham, 2006). Cathepsin G, another serine peptidase, which also has broad substrate specificity, acts in cooperation with human neutrophil elastase. Both peptidases are released during inflammation and can be extremely destructive if their activities are not controlled (Owen and Campbell, 1995). The major endogenous inhibitors of these peptidases are the α_1-peptidase inhibitor (α_1-PI), secretory leucopeptidase inhibitor (SLPI) and α_2-macroglobulin, and when the enzyme/inhibitor balance is in favour of these neutrophil enzymes, their deleterious actions become more obvious (Sallenave, 2000; Moraes *et al.*, 2003).

One sign of acute inflammation is neutrophil [polymorphonuclear (PMN) cells] migration across endothelium and towards the affected tissue. However, the mechanism used by PMN cells to cross the endothelium and enter into the interstitium of inflamed tissue is not entirely clear despite the number of investigations on this subject (Skrzydlewska *et al.*, 2005).

One of the major events in cell migration is leucocyte chemotaxis, which involves two independent, but interrelated processes, such as mobility and directionality that are regulated by extracellular chemo-attractant stimuli (Wu *et al.*, 2005). Many different classes of compounds can act as leucocyte chemo-attractants, including lipids, antimicrobial peptides, chemokines and cytokines (Zabel *et al.*, 2006). In mammalian systems, the complement fragment C5a and the superfamily of small secreted proteins and pro-inflammatory cytokines regulate leucocyte recruitment, infiltration and trafficking, as well as their function (Zlotnik and Yoshie, 2000; Pease and Williams, 2006).

Numerous events are involved in controlling chemo-attractant release. For instance, C5a is generated by serine peptidase activities to which plasmin contributes importantly (Moraes *et al.*, 2003; Kohl *et al.*, 2006), interleukin (IL)1-β is generated by both cathepsin G and elastase activities (Kasama *et al.*, 2005) and IL-8 [the rat orthologue is cytokine-induced neutrophil chemo-attractant-1 (CINC-1)] is generated by IL1-β (Campbell *et al.*, 2003).

Inflammation plays an important role in host defence, and this uncontrolled process results in many human diseases, such as ischaemia–reperfusion injury, allergic reactions and tumorigenesis among others (Coussens and Werb, 2002; Nathan, 2002). The lack of specific therapeutic agents has impaired effective treatment for these inflammatory conditions. Thus, a new approach for a better understanding of the inflammatory processes may provide novel therapeutic targets or treatment strategies.

The *Bauhinia* genus belongs to the subfamily Caesalpinoideae and comprises more than 600 species widely distributed in the tropical and subtropical forests (Vaz and Tozzi, 2005). Many proteins have been isolated from their seeds, and particularly in *bauhinioides* species there are two inhibitors named *Bauhinia bauhinioides* kallikrein inhibitor (BbKI) and *B. bauhinioides* cruzipain inhibitor (BbCI) showing distinct structural characteristics and biochemical properties (Oliva *et al.*, 1999a,b; Oliva *et al.*, 2001; de Oliveira *et al.*, 2001; Oliva and Sampaio, 2008). BbCI inhibits the activity of different serine peptidases, such as cathepsin G (K_{iapp} 160.0 nM); human neutrophil elastase (K_{iapp} 5.3 nM); porcine pancreatic elastase (K_{iapp} 2.5 nM); and cysteine peptidases, such as cruzipain (K_{iapp} 1.2 nM), cruzain (K_{iapp} 0.3 nM) and cathepsin L (K_{iapp} 0.2 nM) (de Oliveira *et al.*, 2001). The unexpected and uncommon property of inhibiting two classes of enzymes was confirmed by studies with the recombinant protein (Araujo *et al.*, 2005). Moreover, BbCI lacks cysteine and methionine residues conferring resistance to protein oxidation in contrast to α_1-PI, SLPI and α_2-macroglobulin, which are oxidized at methionine in the P_1 position of the reactive site, impairing the efficacy of peptidase inhibition (Travis and Salvesen, 1983; Jung *et al.*, 1995).

Therefore, in the present series of experiments, we have evaluated the effects of BbCI on elastase, cathepsin G and cathepsin L activities in acute inflammatory models *in vivo*, and have assessed some of the chemical mediators involved.

Methods

Animals

All animal care and experimental procedures were in accordance with the ethical principles in animal research, as adopted by the Brazilian College of Animal Experimentation and approved by the Federal University of São Paulo Ethical Committee for Animals. In this study, adult male Wistar albino rats, weighing 200–250 g, housed at 22 ± 1°C, under 12 h light–dark cycle time were used. The animals had a standard pellet diet and tap water *ad libitum*. The animals were acclimatized to the laboratory for

at least 1 h before testing. They were used only once throughout the experiments.

Inhibitor purification and inhibitory activity

BbCI was obtained from *B. bauhinioides* seeds using standard protein purification techniques previously described (de Oliveira *et al.*, 2001). N-terminal sequence analysis indicated a single protein structure corresponding to SVILDTKGEPVSNAA-DAYYLPVPVSJGEFLALAKVG, confirming the homogeneity of the inhibitor isolated. The activity of BbCI was confirmed on human neutrophil elastase, cathepsin G and on cathepsin L.

Carrageenan-induced paw oedema and coaxial perfusion

The rats were anaesthetized with sodium pentobarbital (40 mg·kg^{-1}, i.p.). Anaesthetic supplementation (20 mg·kg^{-1}) was provided as required to maintain light anaesthesia during the experiment. Carrageenan (100 µg) was injected in the right subplantar region. Paw volumes were determined up to 4 h by plethysmography (H. Basile, Milan, Italy). Each measurement was repeated three times, and the mean value was calculated (Silveira *et al.*, 1995; Oliva *et al.*, 2000). The results were expressed as increases in rat paw volume (%) from 0 to 4 h following carrageenan injection. The inflamed animals were pretreated with BbCI 2.5 mg·kg^{-1} or sterile saline injected intravenously (tail vein), 30 min before oedema was induced by carrageenan injection.

For the determination of the local levels of bradykinin (BK) in inflamed (injected with carrageenan) or non-inflamed groups, rats were submitted to a paw coaxial perfusion as described by Wohlers *et al.* (2005). Briefly, before the carrageenan injection, the paw was cannulated and perfused at 53 µL·min^{-1} with a perfusion solution (NaCl 158 mM, KCl 5.65 mM, $CaCl_2$ 0.54 mM, $MgCl_2$ 2.90 mM, $NaHCO_3$ 178 mM, glucose 1.0 g·L^{-1}) kept in a bath at 36.5°C. Paw perfusate samples were collected during 0–1.5 and 1.5–2.5 h after carrageenan injection, and placed into ice-cold tubes containing 80% ethanol. The samples were centrifuged, and supernatant was evaporated in a flow of nitrogen and stored at –70°C. BK levels were determined by radioimmunoassay (RIA) as described below.

RIA

Kinins were extracted from perfusate by ethanol precipitation (1:4 v/v) and left for 10 min at –70°C. The samples were then freeze-dried and dissolved in 200 µL of buffer (phosphate 0.01 M, NaCl 0.14 M containing 0.1% egg albumin, EDTA 30 mM, orthophenanthroline 3 mM, 0.1% NaN_3), pH 7.0. Aliquots (50 µL) were incubated with 100 µL anti-BK antibody (1:80.000) and 100 µL of [^{125}I-Tyr]-BK for 20 h at 4°C. Then, 400 µL of 0.1% bovine γ-globulin in buffer B (phosphate 0.01 M, 0.14 M NaCl containing 0.1% NaN_3), pH 7.0, and 800 µL of 25% polyethyleneglycol 6000 (w/v) solution were added to the samples, which were incubated for 10 min at 4°C. After incubation, the samples were centrifuged at 2.000× *g* for 20 min at 4°C, and the supernatants were removed. The [^{125}I] in the pellets was counted and the BK released was calculated (Shimamoto *et al.*, 1982; Gozzo *et al.*, 2002).

Carrageenan-induced pleurisy

This experiment included the inflamed and non-inflamed groups. Both groups were submitted to anaesthetic procedure. At 30 min before injection of 100 µg of carrageenan into the pleural space, the inflamed group was pretreated with BbCI (2.5 mg·kg^{-1}) or saline by the tail vein. The non-inflamed rats received the same volume of saline in the pleural space and BbCI (2.5 mg·kg^{-1}) or saline injected via the tail vein.

Carrageenan was injected into the pleural space between the third and fourth ribs on the right side of the mediastinum according to the method of Vinegar *et al.* (1982). Four hours later, the animals were killed and carefully bled from the carotid arteries. The exudate was removed by aspiration from the pleural cavity and collected in 5.0 mL of PBS buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.2), according to the procedure previously described by Harada *et al.* (1996). Any exudates contaminated with blood were discarded.

Total leucocytes in blood were determined using a Neubauer chamber after dilution with Turk's solution (1:20 v/v) before and 4 h after saline or carrageenan injection. Leucocytes in the exudate at the pleural cavity were determined after 4 h of pleurisy. Differential leucocyte counts were carried out on stained slides under oil immersion microscopy. A total of 100 cells were counted and classified as neutrophils, eosinophils, monocytes and lymphocytes based on morphological criteria.

Intravital microscopy of the microcirculation and haemodynamic parameters

The group of inflamed rats were pretreated with BbCI 2.5 mg·kg^{-1} or saline injected via the tail vein, 30 min before the injection of 100 µg of carrageenan into the scrotal chamber, under light ether anaesthesia. Two non-inflamed groups, pretreated with BbCI 2.5 mg·kg^{-1} or saline, were also submitted to anaesthesia. Rolling, adhesion and migration of leucocytes were analysed 2 h after the local injection of carrageenan.

The animals were anaesthetized with sodium pentobarbital (40 mg·kg^{-1}, i.p.), and the internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination *in situ* (Fortes *et al.*, 1991). This procedure was performed through a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum, then opening the cremaster muscle to expose the internal fascia. The procedure does not require extensive surgical manipulation for the observation of the vascular network, and provides a valuable means for trans-illuminating the tissue for quantitative studies of the microcirculation. The animals were kept on a special board thermostatically controlled at 37°C, which included a transparent platform on which the tissue to be trans-illuminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37°C) Ringer–Locke's solution, NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 6 mM, glucose 5 mM, pH 7.2 containing 1% gelatin. The rate of the solution outflow onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of liquid. A 500-line television camera was incorporated with a triocular Zeiss microscope (Carl Zeiss Co, München-Hallbergmoos, Germany) to facilitate the observation of the enlarged image (×3400) on the video screen. Images were recorded on a video recorder using an ×40 long-distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope (Baez, 1973). The image splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image splitter in the phototube, the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image with the other allowed measurement of the vessel diameter. Vessels selected for study were third-order venules, defined according to their branch order location within the microvascular network (Gore and Bohlen, 1977; Rhodin, 1986). These vessels corresponded to post-capillary venules and their diameters ranged from 12 to 16 µm.

Centerline red blood cell velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX, USA) previously calibrated against a rotating glass disk coated with red blood cells. Venular blood flow velocity was calculated using the formula (V_{mean} = centerline velocity/1.6). Venular wall shear rate (γ) was calculated using the Newtonian definition: $\gamma = 8$ (V_{mean}/D_v, where D_v = vessel diameter) (Davis, 1987; Panes *et al.*, 1996).

Leucocytes rolling and adhering to the endothelium (stickers) and migrating into the adjacent perivascular tissue were evaluated using recorded images (Farsky *et al.*, 1995). Two observers independently scored counts. The number of rolling leucocytes was presented as the mean number of cells passing at a designated line perpendicular to the venular axis in 10 min. A leucocyte was considered to be adherent to the venular endothelium if it remained stationary for more than 30 s (Farsky *et al.*, 1995). Adherent cells were counted during a 10 min period in a 100 µm segment of the vessel. A given section of the vascular bed was tested only once for determination of the number of stickers. Three to five determinations were performed on a single animal. These were averaged for each animal. The number of leucocytes accumulating in the connective tissue adjacent to a post-capillary venule was determined in a standard area of 1000 µm^2. This area was defined on the video screen, 10 µm in tissue corresponding to 3.4 cm on the screen (magnification ×3400). Three to five different fields were evaluated on a single animal to avoid variability based upon sampling. Data were then averaged for each animal.

Enzyme immunoassay for cytokines and chemokime

The concentrations of tumour necrosis factor (TNF)-α, IL-1β and CINC-1 were determined in pleural exudates and serum samples by ELISA using commercially available kits, according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA). The sensitivity of the assay was 15 pg·mL^{-1}.

Data analysis

Data were expressed as mean ± SEM and analysed by a computerized package for statistical analysis. A statistically significant difference was determined by the Tukey–Kramer multiple comparison test. The Kruskal–Wallis test followed by Dunn's multiple comparisons test was used when data were not normally distributed. The significance was set at $P < 0.05$.

Materials

ELISA kits for rat TNF-α, IL-1β and CINC-1 were obtained from R&D Systems Inc. All other chemicals used were of reagent grade. λ-Carrageenan type IV and urethane were purchased from Sigma Chemical Company (St Louis, MO, USA). The other chemicals were of analytical grade.

Results

Effect of BbCI on paw oedema and kinin release

Figure 1 shows the development of paw oedema induced by carrageenan, which was monitored

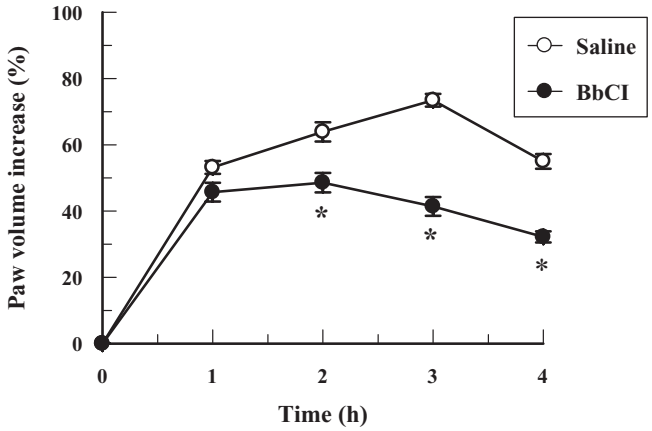

Figure 1

Effect of BbCI on carrageenan-induced paw oedema. Rats were pretreated intravenously with saline (n = 12) or BbCI (2.5 mg·kg^{-1}, i.v. n = 12) 30 min before the local injection of carrageenan (100 µg·0.1 mL^{-1}). Oedema was expressed as increases in rat paw volume (%) from 0 to 4 h following carrageenan injection. Data are expressed as mean ± SEM (*) P < 0.01.

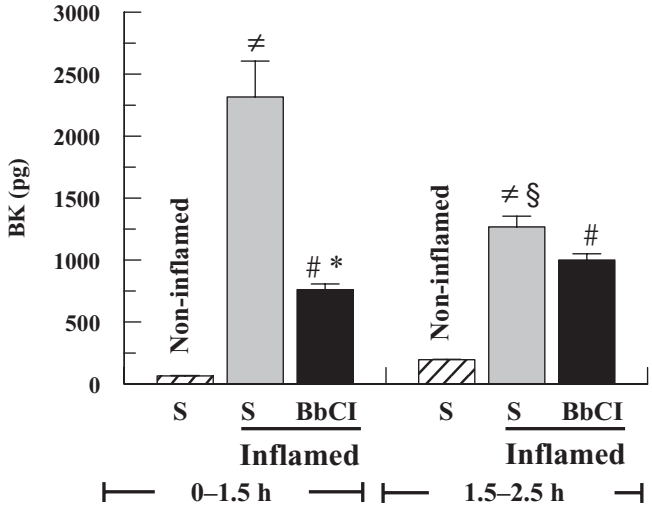

Figure 2

Effect of BbCI on BK release. Rats were pretreated intravenously with saline (S) or with 2.5 mg·kg^{-1} BbCI 30 min before the local injection of saline (non-inflamed) or carrageenan (100 µg·0.1 mL^{-1}). BK levels were determined by RIA in paw perfusate samples collected during 0–1.5 and 1.5–2.5 h after the local injection of saline or carrageenan. Data are expressed as mean ± SEM for six animals in each group. *P < 0.001 versus saline-treated inflamed rats; ≠P < 0.001 and #P < 0.01 versus non-inflamed rats; §P < 0.001 versus corresponding value at 1.5 h.

during 4 h after the subplantar injection of 100 µg carrageenan in rats. Relative to the saline-inflamed group, which showed a time-dependent increase in paw oedema volume, in the inflamed rats pretreated with BbCI (2.5 mg·kg^{-1}), there was a significant reduction in paw oedema at the second (24%), third (44%) and fourth (40%) hours after carrageenan injection.

Compared to non-inflamed rats, the local injection of carrageenan induced significant increases in the levels of BK obtained from the perfusion fluid of the rat paw (Figure 2). Values were higher at 0–1.5 h than at 1.5–2.5 h intervals after carrageenan injection. In contrast, BbCI-treated inflamed rats exhibited a significant reduction in the levels of BK at 0–1.5 h interval. Values attained at 1.5–2.5 h were similar to those observed in saline-treated inflamed group.

Effect of BbCI on leucocyte–endothelial interactions and haemodynamic parameters

Rats pretreated with BbCI or saline were submitted to a local injection of carrageenan (inflamed group) or saline (non-inflamed group), and leucocyte–endothelial interactions at the microcirculation of the spermatic fascia were evaluated 2 h thereafter. Results, illustrated in Figure 3, showed that saline-treated inflamed rats exhibited significant increases in the number of rolling, adherent and migrated leucocytes compared to saline-treated non-inflamed rats. In contrast, BbCI-treated inflamed rats presented a significant reduction on leucocyte–endothelial interactions, including rollers, stickers and migrated cells. Values attained matched those observed in saline- or BbCI-treated non-inflamed animals. Representative photomicrographs are shown in Figure 4. There were no differences in the mean arterial pressure, blood flow velocity and venular wall shear rate between saline- and BbCI-treated inflamed rats (Table 1).

Effect of BbCI on carrageenan-induced pleurisy

The effect of BbCI on leucocyte recruitment was evaluated by measuring total and differential cell counts in pleural exudate samples. As summarized in Table 2, there were no differences in the number of leucocytes [PMN and mononuclear (MN) cells] in the pleural cavity of saline- or BbCI-treated non-inflamed rats. In contrast, treatment of inflamed rats with BbCI reduced the number of leucocytes harvested from the pleural cavity, compared to saline-treated inflamed rats. The number of PMN leucocytes was significantly reduced to 39% of those observed in saline-treated inflamed rats. There were no differences in the number of MN cells between saline- and BbCI-treated inflamed rats. Results, summarized in Table 3, showed that there were equivalent increases in the number of total blood leucocyte counts in saline- and BbCI-treated inflamed rats during the 4 h pleurisy. The number of

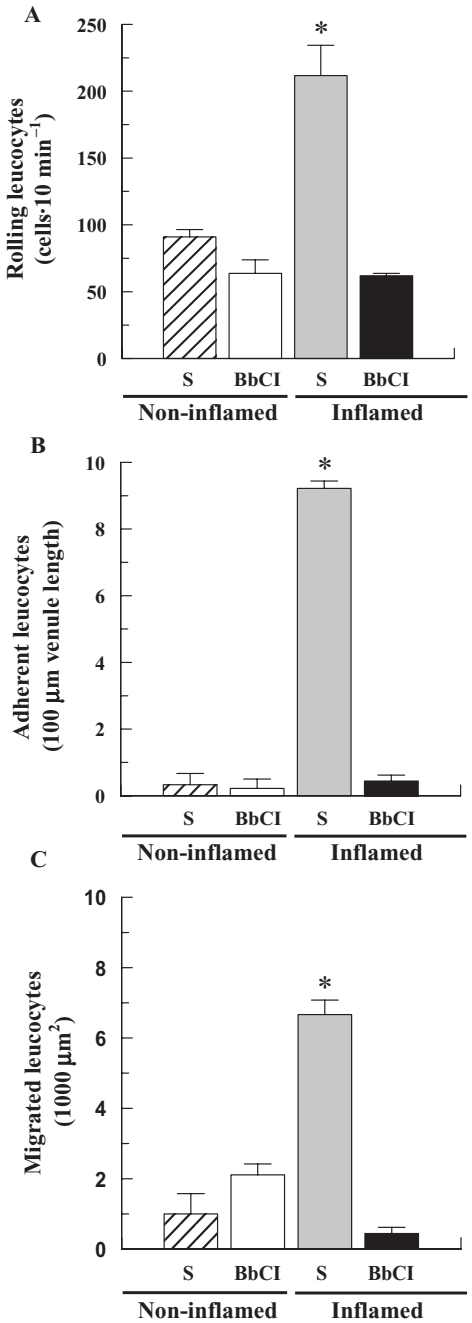

Figure 3

Effect of BbCI on leucocyte–endothelial interactions. Number of rolling leucocytes/10 min (A), adherent leucocytes to the endothelium of a 100 µm segment of post-capillary venules (B) and number of migrated leucocytes to a standard area (1000 µm^2) of the perivascular tissue (C). Rats were pretreated intravenously with saline (S) or BbCI (2.5 mg·kg^{-1}) 30 min before the local injection of carrageenan (100 µg·0.1 mL^{-1}). Leucocyte–endothelial interactions at the spermatic fascia were evaluated 2 h thereafter. Data are expressed as mean ± SEM for six animals in each group *P < 0.001versus other groups.

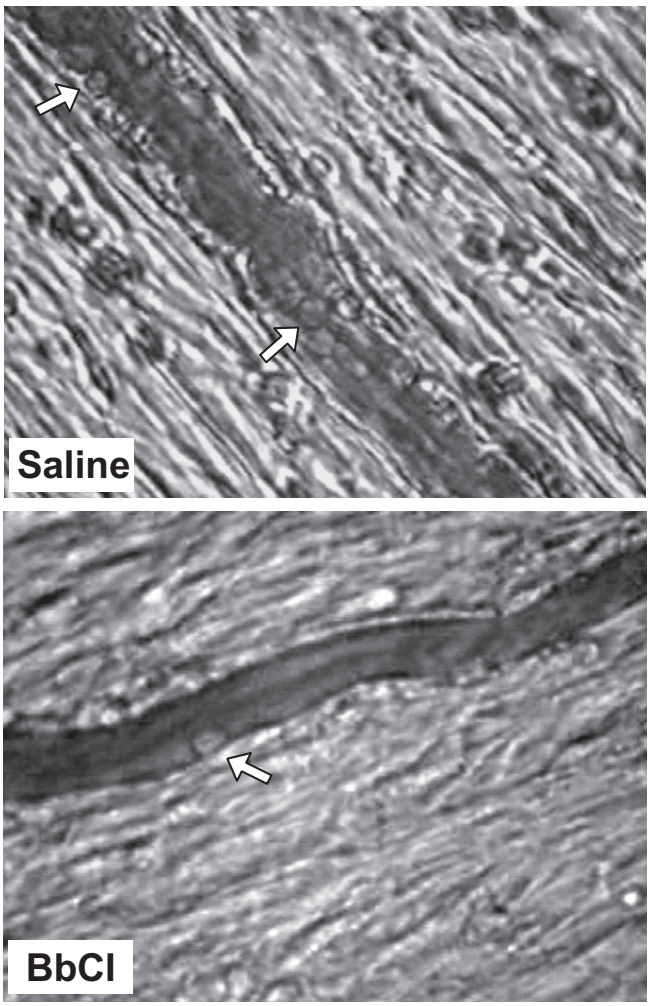

Figure 4

Photomicrographs of a venular segment of the spermatic fascia under intravital microscopy 2 h after the local injection of carrageenan. (100 µg·0.1 mL^{-1}). Rats were pretreated intravenously with saline or BbCI (2.5 mg·kg^{-1}) 30 min before carrageenan injection. Arrows show sites of leucocyte adhesion. Magnification (×3400).

neutrophils increased twofold in both groups. Monocytes and eosinophils did not change. A slight decrease in the number of lymphocytes was observed in BbCI-treated inflamed rats. In non-inflamed groups, total and differential leucocyte counts in the blood did not change either in saline- or BbCI-treated rats.

Effect of BbCI on IL-1β, TNF-α and CINC-1 levels

IL-1β, TNF-α and CINC-1 concentrations were measured in pleural exudates and serum samples in order to determine whether the release/production of these inflammatory mediators could be modulated by BbCI. Relative to saline- or BbCI-treated non-inflamed rats, equivalent increases in the levels

Table 1

Effect of BbCI on haemodynamic parameters

Treatment	Vessel diameter (μm)	Mean arterial pressure (mmHg)	Blood flow velocity ($mm \cdot s^{-1}$)	Venular wall shear rate (s^{-1})
Saline + carrageenan	19.8 ± 1.7	120 ± 6	2.00 ± 0.05	823 ± 71
BbCI + carrageenan	19.4 ± 1.3	122 ± 4	1.92 ± 0.09	790 ± 58

Rats were pretreated with saline or BbCI (2.5 $mg \cdot kg^{-1}$, i.v.) 30 min before the local injection of carrageenan (100 μg) and evaluated 2 h thereafter. Values are presented as mean ± SEM of six animals in each group.

Table 2

Effect of BbCI on carrageenan-induced 4 h pleurisy

Leucocytes ($\times 10^6$) Group	Total	PMN	MN
Non-inflamed			
Saline (n = 8)	1.65 ± 0.16	0.35 ± 0.1	1.30 ± 0.1
BbCI (n = 8)	1.43 ± 0.15	0.33 ± 0.1	1.10 ± 0.12
Inflamed			
Saline (n = 15)	9.11 ± 0.48	7.33 ± 0.5	1.78 ± 0.15
BbCI (n = 15)	3.55 ± 0.23*	2.85 ± 0.2*	0.70 ± 0.1

Rats were pretreated with saline or BbCI (2.5 $mg \cdot kg^{-1}$, i.v.) 30 min before the intrapleural injection of saline (non-inflamed) or carrageenan (100 μg, inflamed). Pleural exudates were collected 4 h thereafter. PMN and MN (leucocytes) in exudate samples were counted. Values are presented as mean ± SEM. *$P < 0.05$ versus corresponding values in saline-treated inflamed rats.

of IL-1β and TNF-α were observed in pleural exudates obtained from saline- or BbCI-treated inflamed rats (Figure 5A,B). In contrast, levels of these cytokines in the serum did not differ between non-inflamed and inflamed rats in both saline- and BbCI-treated groups (Figure 6A,B).

CINC-1 concentrations significantly increased in both pleural exudate and serum samples from the saline-treated inflamed group, as compared to those from the non-inflamed group (saline or BbCI) as illustrated in Figures 5C and 6C respectively. In contrast, CINC-1 levels in pleural exudate and serum were significantly reduced in the inflamed rats, pretreated with BbCI.

Discussion

Several different proteins isolated from *Bauhinia* seeds have been purified and characterized in terms of their activities on a range of functions, such as haemostasis (Oliva *et al.*, 2003), digestion (Oliva *et al.*, 1999a,b; Sumikawa *et al.*, 2006; Oliva and Sampaio, 2008) and protein–protein interaction (Macedo *et al.*, 2004). Many studies have used crude plant extracts which were neither chemically nor biochemically adequately characterized.

In the present study, we investigated the effect of the sequenced and crystallized proteinase inhibitor, BbCI, on the course of the inflammatory response. BbCI is a potent inhibitor of human neutrophil elastase (K_{iapp} 5.3 nM), cathepsin G (K_{iapp} 160.0 nM) and cathepsin L (K_{iapp} 0.2 nM; de Oliveira *et al.*, 2001). These enzymes degrade components of endothelial cell junctions, allowing PMN migration (Owen and Campbell, 1999; Korkmaz *et al.*, 2008).

Endogenously, α_1-antipeptidase, α_2-macroglobulin and SLPI effectively regulate both extracellular neutrophil elastase and cathepsin G activities (Weiss, 1989; Skrzydlewska *et al.*, 2005). The proteolytic–antiproteolytic balance of plasma peptidases and their inhibitors is critically affected by reactive oxygen species (ROS) generated by activated neutrophils, as these inhibitors contain methionine in their structure and are thus susceptible to inactivation by oxidizing compounds, resulting in enhanced proteolysis and destruction of extracellular matrix (Weiss, 1989; Vogt, 1995; Skrzydlewska *et al.*, 2005). The structure of BbCI is unlike these inhibitors, as it lacks methionine and cysteine residues, and this lack suggests a potential resistance to oxidizing conditions and thus a therapeutic value for this inhibitor, which could control the activity of

Table 3

Effect of BbCI on blood leucocyte counts

Cells·mm^{-3} Group	Total	Lymphocytes	Neutrophils	Monocytes	Eosinophils
Non-inflamed					
Saline (n = 8)					
0 h	12.485 ± 432	9.505 ± 360	2.495 ± 133	326 ± 31	159 ± 26
4 h	13.387 ± 325	10.257 ± 317	2.572 ± 139	370 ± 53	188 ± 25
BbCI (n = 8)					
0 h	12.745 ± 494	9.458 ± 472	2.694 ± 189	362 ± 34	231 ± 19
4 h	13.005 ± 547	9.544 ± 415	2.872 ± 120	390 ± 42	199 ± 29
Inflamed					
Saline (n = 15)					
0 h	12.222 ± 347	9.333 ± 294	2.288 ± 98	449 ± 32	152 ± 18
4 h	15.402 ± 394*	9.687 ± 325	4.989 ± 148*	538 ± 32	188 ± 18
BbCI (n = 15)					
0 h	12.458 ± 281	8.901 ± 235	2.962 ± 129	369 ± 32	226 ± 28
4 h	15.026 ± 307*	7.449 ± 224*	6.846 ± 230*	508 ± 46	223 ± 21

Rats were pretreated with saline or BbCI (2.5 mg·kg^{-1}, i.v.) 30 min before the intrapleural injection of saline (non-inflamed) or carrageenan (100 μg, inflamed). Blood samples were collected from the cut tip of the tail of the animals before and 4 h thereafter. *P < 0.05 versus corresponding values before pleurisy.

these peptidases in the presence of ROS (de Oliveira *et al.*, 2001; Araujo *et al.*, 2005).

In this work, carrageenan was used to induce inflammation. It is known that the oedematogenic effect of carrageenan is triphasic (i.e. the first phase involves the release of histamine and 5-HT; the second phase is related to kinin release, especially BK; and the third phase is attributed to local production of prostaglandin E_2) (Di Rosa, 1972; Morris, 2003).

The pro-inflammatory properties of kinins include vasodilation, increased microvascular permeability, release of nitric oxide and a modulating effect on the release of histamine and prostaglandin E_2 (Moreau *et al.*, 2005). The anti-oedematogenic effect of BbCI was seen in the second and third phases of carrageenan-induced paw oedema, which suggests a major influence of BbCI on kinin production. Indeed, a significant reduction in the levels of BK was observed in BbCI-treated inflamed rats, probably due to the inhibitory action of BbCI on kinogenase enzyme activity such as cathepsin L, which can also release BK (Desmazes *et al.*, 2001; Puzer *et al.*, 2004).

Neuhof *et al.* (2003) have shown that the pulmonary oedema in isolated rabbit lungs caused by neutrophil elastase is significantly decreased by 100 μmol·L^{-1} BbCI. In the present study, the strong inhibitory effect of BbCI on leucocyte rolling (70%), adhesion (95%) and migration (90%) in inflamed tissues was demonstrated. This inhibitory effect could be due to haemodynamic changes resulting from BbCI pretreatment. However, there were no differences in blood flow velocity and venular shear rate between saline-treated and BbCI-treated groups, under the inflammatory conditions we have studied here.

Similar results were observed in carrageenan-induced pleurisy. A significant decrease (60%) of neutrophil migration to the pleural cavity was demonstrated in BbCI-treated rats. This inhibitory effect apparently was not related to a defect on neutrophil mobilization from bone marrow to the circulation because equivalent increases in the number of blood neutrophils were observed in both saline- and BbCI-treated rats during the 4 h of pleurisy.

Although the levels of TNF and IL-1β in pleural exudate were not modified by BbCI, a significant decrease in CINC-1 levels occurred and may explain the effect of BbCI on neutrophil migration. This chemokine can be activated by elastase and cathepsin G (Mezyk-Kopec *et al.*, 2005; Soehnlein *et al.*, 2009). Elastase enhances the expression of adhesion molecules such as the intercellular adhesion molecule-1 and chemokine receptors (Zimmermann *et al.*, 2005; Ishihara *et al.*, 2006). Thus, BbCI by inhibiting elastase and cathepsin G might reduce CINC-1 release and neutrophil migration.

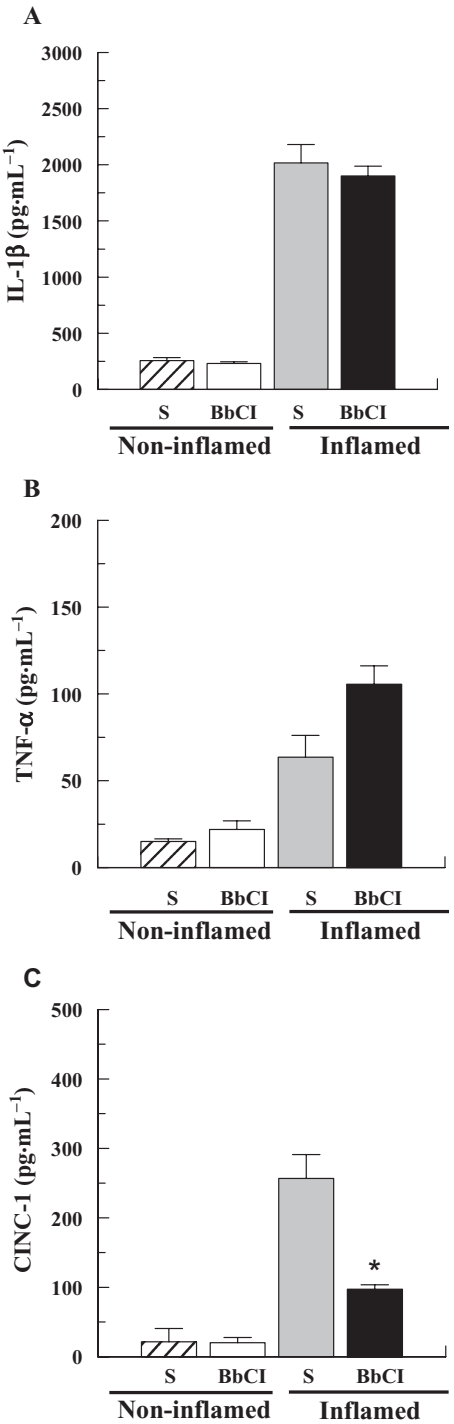

Figure 5

Effect of BbCI on cytokine in the pleural exudate. Rats were pretreated intravenously with saline (S) or BbCI (2.5 mg·kg^{-1}) 30 min before the intrapleural injection of saline (non-inflamed) or carrageenan (100 μg·0.1 mL^{-1}, inflamed). Concentrations of IL-1β (A), TNF-α (B) and CINC-1 (C) were determined by ELISA in pleural exudate 4 h after the induction of pleurisy. Data are expressed as mean ± SEM for six animals in each group. There was a significant decrease in CINC-1 levels in the exudate from BbCI-treated inflamed rats.*$P < 0.05$ versus corresponding values in saline-treated inflamed rats.

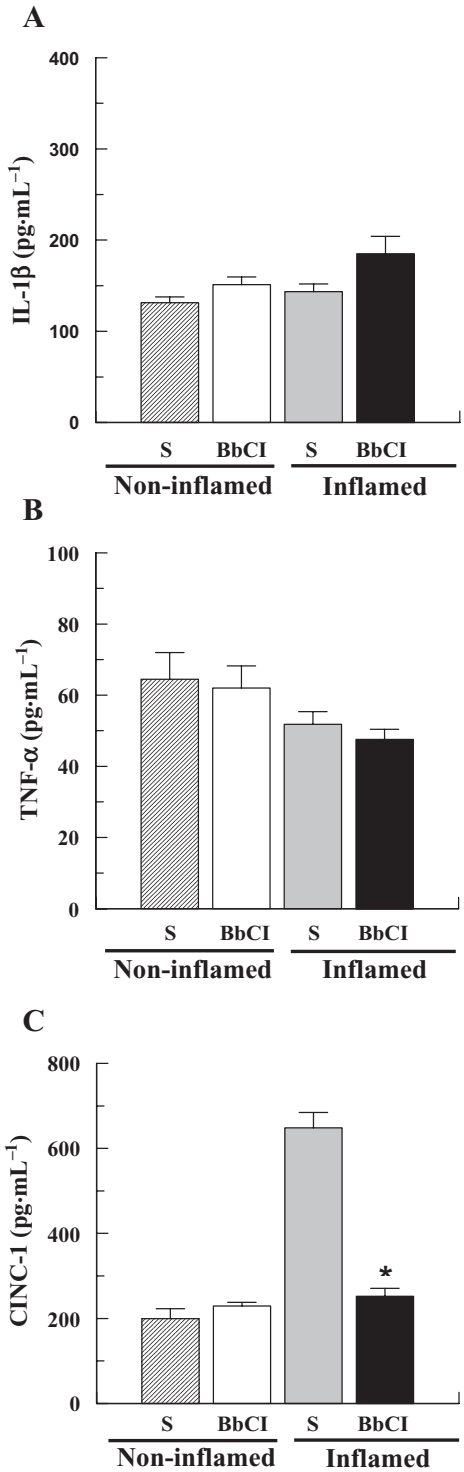

Figure 6

Effect of BbCI on serum cytokine levels. Rats were pretreated intravenously with saline (S) or BbCI (2.5 mg·kg^{-1}) 30 min before the intrapleural injection of saline (non-inflamed) or carrageenan (100 μg·0.1 mL^{-1}, inflamed). Concentrations of IL-1β (A), TNF-α (B) and CINC-1 (C) were determined by ELISA in serum 4 h after the induction of pleurisy. Data are expressed as mean ± SEM for six animals in each group. There was a significant decrease in CINC-1 levels in the serum from BbCI-treated inflamed rats.*$P < 0.05$ versus corresponding values in saline-treated inflamed rats.

Our studies led to the conclusion that BbCI has potential anti-inflammatory actions by inhibiting the activity of proteolytic enzymes in the inflammatory site, and the release of BK and CINC-1, and thus decreasing neutrophil migration during the course of the inflammatory response. We should point out that BbCI had no effect in non-inflamed animals. Thus, this inhibitor may be relevant for investigation of other pathological processes in which neutrophil elastase, cathepsin G and cathepsin L are involved.

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Conflict of interest

The authors declare no conflict of interest.

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