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Original Research

Inflammatory effects of patagonfibrase, a metalloproteinase from *Philodryas patagoniensis* (Patagonia Green Racer; Dipsadidae) venom

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Abstract

Patagonfibrase is a P-III class metalloproteinase isolated from the venom of *Philodryas patagoniensis*, a South-American, rear-fanged 'colubrid' snake responsible for accidents with clinical significance. Since local inflammatory reactions are conspicuous signs of snakebites inflicted by this species and taking into consideration that most snake venom metalloproteinases exhibit inflammatory activity, this study deals with the proinflammatory effects evoked by patagonfibrase. Herein, we demonstrate that patagonfibrase causes a time- and dose-dependent hemorrhagic edema when injected into mouse hind paws. The peak of edema occurred at 30 min after injection, and the minimum edematogenic dose was 0.021 μg. By histological analysis, the presence of moderate to marked edema and hemorrhage, and a mild inflammatory infiltrate was observed. When injected subcutaneously into the scrotal bag of mice, patagonfibrase induced cell recruitment with a significant alteration in physiological parameters of leukocyte–endothelium interaction. The presence of 1 mmol/L *o*-phenanthroline, which chelates metal ions, significantly inhibited the proinflammatory effects induced by patagonfibrase. Taken together, these results imply that patagonfibrase is an important contributor to local inflammation elicited by *P. patagoniensis* envenomation, which may pave the way for novel therapeutic strategies to treat this snakebite. Moreover, our findings demonstrate for the first time that a venom metalloproteinase from a rear-fanged snake elicits proinflammatory effects mainly mediated by its catalytic activity.

Keywords: Colubridae, edema, leukocyte–endothelium interaction, rear-fanged snake venom, toxin

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Introduction

Snake venoms are complex mixtures of proteins, peptides and small organic molecules with a variety of potent enzymatic and ligand-based biological activities.[1] Among the enzyme-based toxins, an important class includes the snake venom metalloproteinases (SVMPs), which act synergistically with many other toxins to induce a complex series of local and systemic pathophysiological effects upon envenomation.[2] SVMPs are members of the reprolysin subfamily of the M12 family of metalloproteinases,[3] and they can be divided into three classes (P-I to P-III) that minimally share homologous (P-I) proteinase domains; classes P-II and P-III also contain a disintegrin domain. In the class P-III metalloproteinases, there is an additional cysteine-rich carboxyl terminal domain.[4,5] This third class of SVMPs is particularly interesting, in terms of their diversity of structural features and biological activities.[3]

Some P-III metalloproteinases isolated from venoms of front-fanged viperid snakes are known to induce inflammatory reactions. One of the best studied is jararhagin, which is a SVMP isolated from *Bothrops jararaca* venom. It plays an important role in local tissue damage by promoting potent proinflammatory activity.[6–8] Furthermore, P-III SVMPs are major components of rear-fanged snake venoms and account for several of the main toxic effects observed following envenomation.[9,10] However, to the best of our knowledge, there are no studies on the inflammatory effects of P-III SVMPs found in rear-fanged snake venoms.

Patagonfibrase is a P-III class metalloproteinase isolated from the venom of *Philodryas patagoniensis*,[11,12] a

South-American, rear-fanged snake, which is now considered a member of the family Dipsadidae (formerly Colubridae).[13] Local inflammatory reactions – such as pain, erythema and edema – are conspicuous signs of snakebites inflicted by this species.[14] Taking into consideration that the study of individual snake venom components paves the way for a deeper understanding of the pathophysiology of envenomations, and that most SVMPs, besides inducing hemorrhage and myonecrosis, play a relevant role in the complex and multifactorial inflammatory response characteristic of snakebite envenomation,[15,16] this study aimed to investigate the proinflammatory effects evoked by patagonfibrase.

Material and methods

Animals

Male Swiss mice weighing 18–22 g were supplied by the Animal House, Institute Butantan, São Paulo, Brazil. Animals were maintained for two days at the laboratory before experiments. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996; http://www.nap.edu/readingroom/books/labrats/) and were approved by the Ethical Committee for the Use of Animals of Institute Butantan (certificate 530/08).

P. patagoniensis venom

A pool of *P. patagoniensis* venom was obtained from wild specimens captured in northeastern Argentina and maintained at the serpentarium of the local zoo, Corrientes, Argentina. Specimens were extracted by introducing a 100-μL micropipette under each fang, according to a procedure described previously.[17]

Purification of patagonfibrase

Patagonfibrase from *P. patagoniensis* venom was purified in two chromatographic steps: anion-exchange chromatography on a Mono-Q 5/50 GL column (GE Healthcare, Uppsala, Sweden) and affinity chromatography on a HiTrap Blue HP column (GE Healthcare), using a previously described procedure.[11] Protein concentrations were determined by the bicinchoninic acid assay.[18] Standard curves were constructed using bovine serum albumin (Sigma Chemicals, St Louis, MO, USA) diluted in duplicate.

Chemical treatment for inhibition of the enzymatic activity of patagonfibrase

Samples of patagonfibrase were added to 1 mmol/L orthophenanthroline (*o*-Phe) – dissolved in dimethyl sulfoxide – for metalloproteinase inhibition.[12] Samples were incubated for one hour at 37°C. Control samples of patagonfibrase were submitted to incubation without the presence of the inhibitor.

Edematogenic activity

Mice were injected subcutaneously into the subplantar area of the right hind paw with 0.1 μg of patagonfibrase dissolved in 50 μL of sterile 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$. As a control, the left hind paw received the same volume of vehicle. Paw edema was determined by measuring paw thickness with a digital caliper at 0.5, 1, 2, 4, 6 and 24 h after injection, and the results were calculated as the difference in thickness between the right and left paws. Edema was expressed as the percentage increase in paw thickness.[19]

In order to determine the minimum edematogenic dose (MED) of patagonfibrase, doses ranging from 0.006 to 0.05 μg/paw were tested as described above. After 30 min of injection (time corresponding to the peak of edema induced by the enzyme), paw thickness was measured and MED was defined as the minimal dose of patagonfibrase able to induce an increase of 30% in paw thickness.[20]

Hemorrhagic activity

The intensity of local hemorrhage was determined in hind paws used for evaluating the edematogenic activity of venoms.[21] Briefly, groups of mice ($n = 4$) were injected intraplantarly in the right and left hind paws with patagonfibrase and vehicle, respectively, and 24 h later, they were euthanized in a CO_2 chamber. Hind paws were removed at the level of tibiotarsal joint, weighed, fragmented and added to tubes containing 3 mL of Drabkin reagent. Following incubation in the dark at 37°C for 24 h, tubes were centrifuged at 13,000*g* for five minutes, and the supernatants were read at 540 nm. Hemorrhage – i.e. the difference between the hemoglobin content of paws injected with patagonfibrase and those injected with vehicle – was expressed as mg hemoglobin/g of tissue.

Histopathological analysis

Patagonfibrase (0.013, 0.025 or 0.050 μg), dissolved in 50 μL of sterile 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$, was injected in the right paw of mice. As a control, the left hind paw received the same volume of vehicle. At 0.5 h (time corresponding to the peak of edema induced by the enzyme), both paws of mice were removed and fixed in Bouin's solution buffered at pH 7.2 with 0.1 mol/L phosphate buffer for 24 h. The samples were dehydrated in an ethanol ascending series, cleared in xylol and embedded in liquid paraffin. Sections of 5–6 μm were obtained in a Leica RM2155 microtome (Leica Microsystems Inc, Bannockburn, IL, USA) and stained with hematoxylin–eosin.

Intravital microscopy of murine cremaster venules

Intravital microscopy was used to observe leukocyte responses within mouse cremaster venules, at 2, 4 or 24 h after the injection of 100 μL of sterile 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$ (controls) or 0.1 μg of patagonfibrase dissolved in the same buffer, into the subcutaneous tissue of the scrotal bag of mice. The

dose was chosen to minimize hemorrhage over the study period. Animals were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and the cremaster muscle were exteriorized for microscopic examination *in situ* as previously described by Baez.[22] After surgery, animals were maintained on a special board thermostatically controlled at 37°C, which included a transparent platform on which the cremaster was placed, and analyzed in a light microscope (Axioplan II, Carl-Zeiss, Gottingen, Germany; equipped with Achroplan objectives 10.0/0.25 [longitudinal distance/numeric aperture] and 1.60 optovar). Images were captured by a video JVC camera (JVC, Long Beach, CA, USA) and simultaneously transmitted to a TV monitor and to a computer. Images were digitalized in the computer, and recorded on to a DVD for further analysis using the image analyzer software KS 300 (Kontron, Carl Zeiss).

One to three post-capillaries venules (20–40 μm diameter) were selected in an aleatory manner. After the end of the equilibrium period of 10 min, control readings of rolling and adhering leukocytes were taken during 1 min in a 100-μm vascular segment. Firmly adherent leukocytes were considered as those remaining stationary for at least 30 s within a given 100-μm vessel segment. Transmigrated leukocytes were quantified as those in the extravascular tissue within 50 μm of each side of the 100-μm vessel segments studied.

Statistical analysis

Two-way analysis of variance (ANOVA) with repeated measures, followed by Tukey's test, was used to compare results from edematogenic activity over time. One-way ANOVA, followed by Dunnett's test, was used to compare the intensity of mouse paw edema induced by different doses of patagonfibrase and the control. Hemorrhage intensity in hind paws and alterations on leukocyte–endothelium interaction were compared by one-way ANOVA, followed by Tukey's test. Statistical analyses were performed using the software GraphPad InStat, version 3.01 (GraphPad Software Inc, San Diego, CA, USA), and SigmaStat, version 3.5 (Systat Software Inc, San Jose, CA, USA). Differences with $P < 0.05$ were considered statistically significant. Results are presented as mean $\pm$ standard error of mean (SEM) of values obtained with the indicated number of animals.

Results and discussion

Human envenomation by the rear-fanged snake *P. patagoniensis* is mainly characterized by inflammatory reaction on the bitten limb, bearing a striking resemblance to local signs and symptoms of front-fanged *Bothrops* sp. envenomation.[14] The intraplantar injection of *P. patagoniensis* venom in mouse footpad causes a time- and dose-dependent edema,[23,24] which is inhibited by dexamethasone and indometacin and, therefore, mediated for prostaglandins and leukotrienes.[23] Similarly to what happens with *B. jararaca* venom,[16] edematogenic activity of *P. patagoniensis* venom depends on metalloproteinases since it is inhibited by

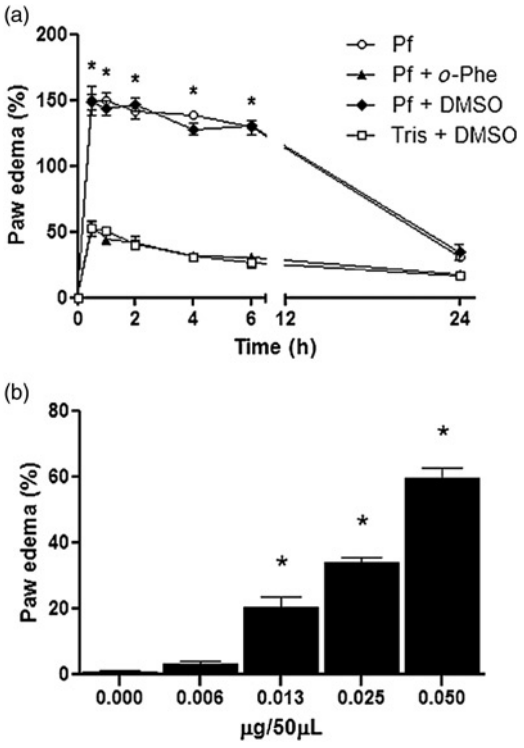

Figure 1 Local edema induced by patagonfibrase (Pf) in mouse paws. (a) Time course of edematogenic activity induced by 0.1 μg Pf. Results are presented as mean $\pm$ SEM of percentage increase in paw thickness between right and left paws ($n = 4$). Asterisks indicate statistically significant differences in comparison with control ($P < 0.001$). (b) Intensity of mouse paw edema induced by different dose of Pf (30 min after injection). Results are presented as mean $\pm$ SEM of percentage increase in paw thickness between right and left paws ($n = 5$). Asterisks indicate statistically significant differences in comparison with control ($P < 0.01$). *o*-Phe, *o*-phenanthroline; DMSO, dimethyl sulfoxide

divalent metal chelators such as ethylenediaminetetraacetic acid.[23,25] Nonetheless, which metalloproteinases of this venom are responsible for the induction of edema is not known.

Herein, we demonstrate that patagonfibrase, a P-III metalloproteinase found in *P. patagoniensis* venom,[11,12] induces a

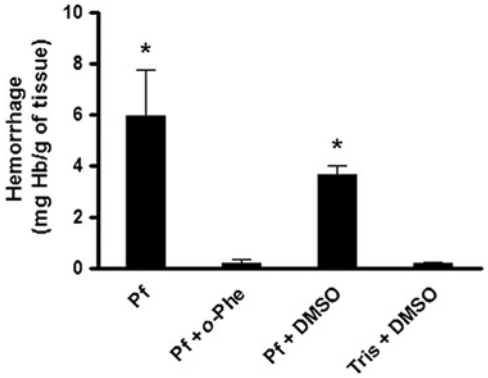

Figure 2 Intensity of local hemorrhage induced by 0.1 μg patagonfibrase (Pf) in hind paws at 24 h. Results are presented as mean $\pm$ SEM of the difference between the hemoglobin content of paws injected with Pf and those injected with 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$ ($n = 4$). Asterisks indicate statistically significant differences in comparison with control ($P < 0.01$). *o*-Phe, *o*-phenanthroline; DMSO, dimethyl sulfoxide

time- (Figure 1a) and dose-dependent (Figure 1b) hemorrhagic (Figure 2) edema when injected into murine hind paws. The peak of edema occurred 30 min after injection of 0.1 μg patagonfibrase, gradually decreasing from one to six hours, and subsiding after 24 h (Figure 1a). Development of edema induced by patagonfibrase over time was similar to that observed by *P. patagoniensis* whole venom[23,24] and some P-III *Bothrops* metalloproteinases.[26] It is important to highlight that a similar behavior was observed when a lower dose of patagonfibrase (0.050 μg) was used (data not shown). In addition, edema with accompanying hemorrhage was observed even at such a low dose as 0.025 μg (Figure 3). The time course of edema induction by patagonfibrase and *P. patagoniensis* whole venom in mice is similar to that observed in human envenomation, where the inflammatory reaction begins within 30 min to a few hours after the accident and takes a few days to decrease – without treatment – if a tourniquet is not used.[14,23,27]

The presence of 1 mmol/L *o*-Phe, which chelates metal ions, significantly inhibited the hemorrhagic edema formation induced by patagonfibrase in the paw (Figures 1a and 2). Therefore, this pathological effect – which had a very rapid onset – is likely to depend on the direct proteolytic activity of the enzyme on extracellular matrix components. This is in accordance with a previous report[28] showing that hemorrhage and edema are directly evoked by jararhagin, a metalloproteinase isolated from *B. jararaca* venom and one of the best studied P-III SVMPs. Moreover, Dale *et al.*[8] have demonstrated that a synthetic peptide derived from the calcium-binding protein S100A9 inhibits jararhagin-induced edema and hemorrhage in rat paws, probably by acting on the catalytic site of this enzyme.

Patagonfibrase exhibited a MED of 0.021 μg, which implies that edema induced by patagonfibrase in murine footpads is much more intense than that induced by *P. patagoniensis* whole venom (MED = 0.26 μg).[25] The amount of patagonfibrase required to induce an increase of 30% in paw thickness is approximately 8.1% of the amount of crude venom required to accomplish the same percentage increase. Taking into account that patagonfibrase represents about 2.8% of total venom proteins,[11] it is likely that the edematogenic activity of crude *P. patagoniensis* venom cannot be solely accounted by the amount of patagonfibrase present in venom. In addition, edematogenic activity of patagonfibrase is nearly 62-fold higher than baltergin (MED = 1.3 μg),[29] a 55-kDa metalloproteinase isolated from the venom of *Bothrops alternatus*, which is the main species responsible for snakebites in northeastern Argentina.

Histological analysis of mouse hind paws at 30 min after injection of patagonfibrase showed a typical pattern of the inflammatory process (Figures 3b–d), when compared with the control group (Figure 3a). Edema was observed in the subcutaneous region (Figures 3b–d). The presence of an early but mild inflammatory infiltrate (Figure 3d) is in line with the onset of the leukocyte migratory process. Since results from the intravital microscopy study evidenced increased leukocyte adhesion and migration two hours after patagonfibrase injection (Figure 4), it is plausible to consider

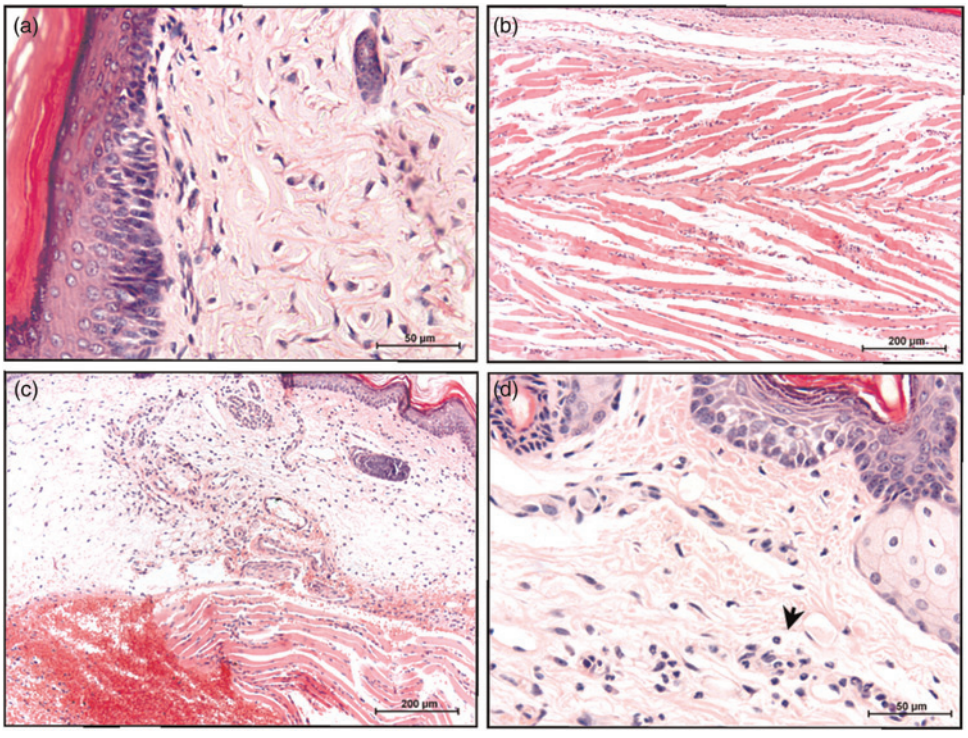

Figure 3 Histological analysis of mouse hind paws 30 min after injection of patagonfibrase (Pf). (a) Normal histological appearance of control mouse hind paws injected with 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$. (b) Edema and hemorrhage induced by 0.025 μg of Pf. (c) Edema, hemorrhage and leukocyte infiltration induced by 0.050 μg of Pf. (d) At the latter dose and with higher magnification, polymorphonuclear leukocytes are visible (arrow). Hematoxylin–eosin stain (A color version of this figure is available in the online journal)

that leukocyte infiltration in mouse hind paws will increment hours later. As previously demonstrated,[11] patagonfibrase is remarkably hemorrhagic, and in virtue of that, hemorrhage was also observed in the connective tissue and in the subjacent skeletal muscle (Figures 3b and c). All these histological alterations were dose-dependent (Figures b and c) and mainly observed in the subcutaneous tissue (the site of injection), which is a well-vascularized region. Similarly, patagonfibrase induced local edema, hemorrhage and inflammatory reaction in mouse gastrocnemius muscle.[11]

It is known that metalloproteinases are the main components involved in the inflammatory cell migration induced by *Bothrops* venoms.[16] However, the relative contribution of specific classes of enzymes to the microcirculatory effects induced by *P. patagoniensis* crude venom, which were studied by Lopes,[23] is not known yet. This study demonstrates that at least one metalloproteinase from this venom, patagonfibrase, is involved in such effects. Thus, when injected into the scrotal bag of mice, patagonfibrase induced cell recruitment (Figure 4). It is known that cell accumulation in inflamed tissues results from the generation of chemotactic factors as well as from adhesive interactions between leukocytes and endothelial cells within microcirculation.[30] Thus, two hours after subcutaneous injection of 0.1 μg patagonfibrase, leukocyte rolling decreased significantly, and leukocyte adhesion and migration increased significantly when compared with animals injected with vehicle (Figures 4a–c). Similar results were obtained after four hours of injection (data not shown). However, 24 h after injection, the counts of rolling leukocytes in patagonfibrase-treated animals did not show significant difference in comparison with those of animals injected with vehicle, but the counts of adhered and mostly migrated leukocytes significantly increased (Figures 4d–f). This picture is characteristic of leukocyte–endothelium interactions (LEIs) occurring in an inflamed tissue, and is in accordance with observations obtained by Lopes[23] after subcutaneous injection of *P. patagoniensis* whole venom into the scrotal bag of mice.

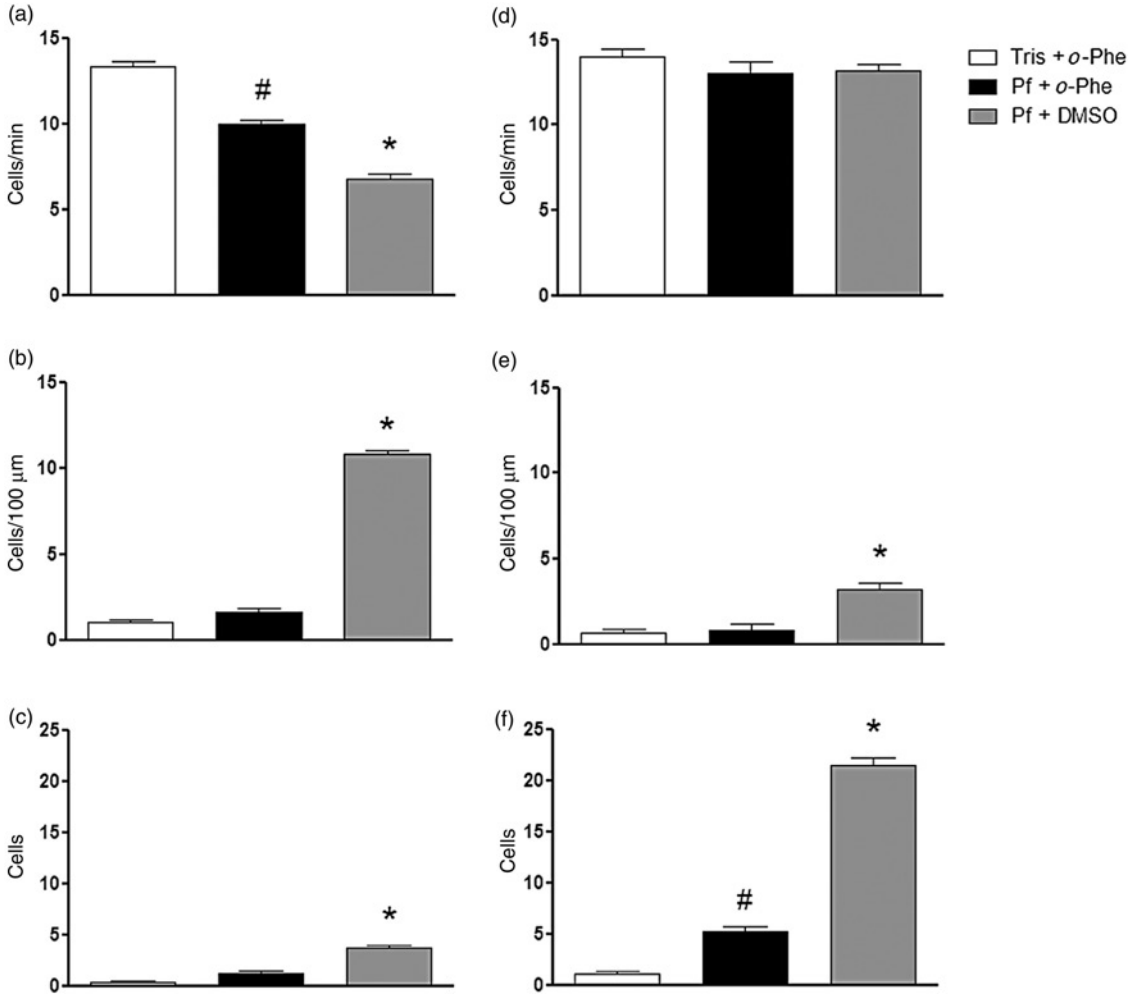

Figure 4 Alterations on leukocyte–endothelium interactions induced by patagonfibrase (Pf) on cremaster muscle of mice. Samples of Pf were treated with 1 mmol/L *o*-phenanthroline (*o*-Phe) for inhibition of its proteolytic activity. Groups of mice were injected with 0.1 μg of dimethyl sulfoxide (DMSO)- or *o*-Phe-treated Pf, or 50 mmol/L Tris-HCl buffer, pH 7.4, containing 1 mmol/L $CaCl_2$ and 1 mmol/L *o*-Phe (100 μL) in the subcutaneous tissue of the scrotal bag. Parameters such as counting of rolling (a and d), and adhered (b and e) or migrated leukocytes (c and f) were evaluated two hours (a, b and c) or 24 h (d, e and f) after the subcutaneous injection of samples. Bars represent mean $\pm$ SEM of events observed in five animals. *Indicates statistically significant differences in comparison with control ($P < 0.05$). #Indicates statistically significant differences in comparison with control and DMSO-treated Pf ($P < 0.05$)

The presence of 1 mmol/L *o*-Phe significantly inhibited the alterations on LEI induced by patagonfibrase (Figure 4). Therefore, the hydrolytic activity of this enzyme on extracellular matrix proteins is likely to be important to leukocyte recruitment occurring at the site of the bite during *P. patagoniensis* envenomation. However, leukocyte rolling at two hours and leukocyte migration at 24 h were not completely inhibited by 1 mmol/L *o*-Phe (Figures 4a and f). These results suggest that these parameters depend not only on the catalytic domain of patagonfibrase, but also on its disintegrin-like and cysteine-rich domains. In agreement with this hypothesis, jararhagin-C – a processed form of jararhagin that exhibits disintegrin-like and cysteine-rich domains, but no catalytic domain – can activate the early events of the acute inflammatory response, such as leukocyte rolling and release of proinflammatory cytokines.[31]

In conclusion, this work demonstrates for the first time the proinflammatory effects evoked by a rear-fanged SVMP, contributing to a better understanding of the structural and mechanistic bases of this class of enzymes that are widely distributed among snake venoms. Furthermore, given the potent activity of patagonfibrase in inflammatory events, our findings imply that this metalloproteinase is an important contributor to local inflammation elicited by *P. patagoniensis* envenomation. Our data are the first to demonstrate that patagonfibrase participates in the genesis of edema and leukocyte recruitment into tissues induced by the whole venom, and that these effects are mainly mediated by the catalytic activity of patagonfibrase. Understanding the role of patagonfibrase within inflammatory pathogenesis will allow us to better understand the action of venom constituents and help to develop more efficacious therapeutic interventions following envenomation.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. MEP, BCZ and FLT conducted the experiments; OA supplied *P. patagoniensis* venom from which patagonfibrase was purified; LRCG and MLS supplied critical reagents, animals and lab equipment; MEP wrote the manuscript; and MLS carefully revised the manuscript.

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