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Inflammatory mediators involved in the paw edema and hyperalgesia induced by Batroxase, a metalloproteinase isolated from *Bothrops atrox* snake venom

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ABSTRACT

Snake venom metalloproteinases have been described as responsible for several inflammatory effects. In this study, we investigated the edema and hyperalgesia induced in rats by Batroxase, a P-I metalloproteinase from *Bothrops atrox* venom, along with possible inflammatory mediators involved in these responses. Batroxase or sterile saline was injected into rat paws and the edema and hyperalgesic effects were evaluated for 6 h by using a plethysmometer and a Von Frey system, respectively. Batroxase induced significant edematogenic and hyperalgesic peak responses in the first hours after administration. The inflammatory mediators involved in these responses were assayed by pretreatment of animals with synthesis inhibitors or receptor antagonists. Peak responses were significantly reduced by administration of the glucocorticoid dexamethasone, the H1 receptor antagonist diphenhydramine and the FLAP inhibitor MK-886. Rat paws injected with compound 48/80, a mast cell degranulating agent, followed by Batroxase injection resulted in significant reduction of the edema and hyperalgesia. However, Batroxase itself induced minor degranulation of RBL-2H3 mast cells *in vitro*. Additionally, the inflammatory responses did not seem to be related to prostaglandins, bradykinin or nitric oxide. Our results indicate a major involvement of histamine and leukotrienes in the edema and hyperalgesia induced by Batroxase, which could be related, at least in part, to mast cell degranulation.

1. Introduction

Snake venoms are complex mixtures composed by proteins and peptides, carbohydrates, lipids and inorganic compounds. Their protein content includes L-amino acid oxidases (LAAOs), phospholipases A_2, metalloproteinases, serine proteinases, lectins and hyaluronidases [46,48]. The envenomation caused by *Bothrops* snake species is characterized by proteolysis, hemorrhage, necrosis, edema and infiltration of leukocytes [31,71]. Systemic effects like coagulopathy, nephrotoxicity, neurotoxicity, cardiotoxicity and hemodynamic changes are also observed [62].

Snake venom metalloproteinases (SVMPs) are zinc-dependent enzymes considered as the main responsible for the hemorrhagic effects of the venoms [4,31]. The hemorrhage induced by SVMPs is due to the lysis of basement membranes of blood vessels [31], causing tissue lesion that can be followed by acute inflammatory reaction, inducing plasma extravasation and leukocyte infiltration [71] and initiating an inflammatory process where hyperalgesia and edema are evident [32].

The inflammatory process is characterized by the cardinal signs of heat, redness, edema and pain or tumor, which are related to various inflammatory mediators such as vasoactive amines, eicosanoids and cytokines [75]. It's well known that several snake venoms and isolated toxins can cause edema, as reported for the venoms of *B. lanceolatus* [34], *B. jararaca* [76] and *B. insularis* [1], and also hyperalgesia, as described for *B. asper* [12] and *B. jararaca* [5] venoms.

Edema is a common sign of the inflammatory response and is dependent on the synergism of mediators that cause local vasodilation resulting in increased blood flow, such as prostaglandins E_2 and I_2, histamine, bradykinin and serotonin, and increased vascular permeability, mostly dependent on platelet activating factor — PAF, histamine, bradykinin, serotonin and cysteinyl-leukotrienes. Among the various mechanisms described for the inflammation induced by venom toxins are: mast cell degranulation with release of histamine and serotonin

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[65], attraction of neutrophils [37], induction of synthesis and release of prostaglandins and leukotrienes from mast cells [24], bradykinin synthesis and induction of nitric oxide release [38].

Hyperalgesia is an increased pain response to a painful stimulus [19]. This response is associated with nociceptive information that was converted by pain sensory receptors found in peripheral tissues in an electrical stimulation deriving from primary afferent fibers, transmitted to neurons of second order on the spinal cord [50]. The activation of nociceptors is achieved by substances that interact with receptors and/or ion channels, leading to changes on the excitability of primary and central sensorial neurons and the induction of nociception in response to a stimulus [78]. Inflammatory mediators such as cytokines and chemokines (TNF-α, IL-1 and IL-8) [20], bradykinin [29,74], complement factors C3a and C5a [40], prostaglandins [28] and leukotrienes [18], sympathomimetic amines [30], PAF, histamine and serotonin [11] are released at the site of inflammation and act on specific receptors and/or ion channels present in the peripheral terminals of nerve fibers, causing sensitization [17,77]. These mediators are released by inflammatory cells, including mast cells [3].

The present study aimed to investigate the capability of Batroxase, a hemorrhagic P-I metalloproteinase from *Bothrops atrox* venom described by Cintra et al. [16], to promote edema and hyperalgesia in rats and evaluate the inflammatory mediators involved in these events. The results obtained in this study could contribute to the elucidation of the inflammatory mechanisms induced by snake venom metalloproteinases as well as the proposition of new therapies to alleviate pain and edema formation after envenomation.

2. Methods

2.1. Venom

Bothrops atrox snake venom was purchased from Bioactive Proteins Serpentarium (Batatais, São Paulo, Brazil — IBAMA register no. 471301).

2.2. Animals

Experiments were conducted on male Wistar rats weighing 180–200 g. They were housed individually at 24 ± 1 °C under a 12:12 h light–dark cycle (lights on at 06:00 AM) with free access to food and tap water until the night before the experiment when only water was made available. Each animal was used only once. Care and use of the animals were in full compliance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA) and the study was previously approved by the Animal Research Ethics Committee of University of São Paulo, Campus of Ribeirão Preto-SP (Protocol number 02.09.2009).

2.3. Drugs

The drugs DALBK ([des-Arg^9-Leu^8]-BK), HOE-140, L-NAME (N_ω-Nitro-L-arginine methyl ester hydrochloride), dexamethasone, diphenhydramine, methysergide and compound 48/80 were acquired from Sigma-Aldrich. Celecoxib (Celebra®) was purchased from Pharmacia-Pfizer and MK-886 from Merck. The drugs were dissolved in sterile saline (0.9% w/v NaCl in distilled water).

2.4. Purification of Batroxase

The metalloproteinase Batroxase was purified according to Cintra et al. [16] with minor modifications. The isolated protein was lyophilized and stored at −20 °C and dissolved in sterile saline before use.

2.5. Edema

Edema was induced in Wistar rats (5 animals per group, two individual experiments) by intraplantar (i.pl.) injection of Batroxase (10, 20 and 40 μg diluted in 100 μl of sterile saline) in the left hind paw. The right hind paw received the same volume of sterile saline as control. The edema formation was measured using a plethysmometer (Ugo Basile, Italy) and expressed as the difference of volume (ml) between left and right paws [56]. The baseline volume for both paws (time 0) was subtracted from the volumes at 30, 60, 120, 180, 240, 300 and 360 min after sample injections.

To determine if the edema observed was due to Batroxase's activity or to possible contamination of samples with bacterial endotoxins (LPS), a group of rats received 20 μg of Batroxase that was previously incubated at 100 °C for 30 min, while another group received 20 μg of Batroxase that was preincubated with a metalloproteinase inhibitor (20 mM EDTA) for 30 min. Additionally, quantification of LPS was assayed using the *Limulus Amebocyte Lysate* (LAL) QCL-1000® kit (Lonza), according to the manufacturer's instructions. Samples of Batroxase were evaluated after preincubation at 37 °C with 20 mM EDTA for 1 h to ensure that its enzymatic activity would not influence the test. The endotoxin quantification was carried out based on a standard curve with various concentrations of *Escherichia coli* endotoxin (0.1 to 1.0 EU/ml).

2.6. Hyperalgesia

The Von Frey system, an electronic pressure-meter nociception paw test, was used to evaluate hyperalgesia [15,19]. Two investigators assessed the paw withdrawal thresholds and the investigator operating the apparatus was blinded to treatments and to the recorded withdrawal threshold values. Additionally, the same individual assessed all baseline and experimental withdrawal thresholds.

Wistar rats (5 animals per group, two individual experiments) were injected i.pl. in the left hind paw with Batroxase (10, 20 and 40 μg diluted in 100 μl of sterile saline), while the right paw received only sterile saline (100 μl). Each rat was maintained alone for 20 min to accommodate in an acrylic cage that presents an elevated steel mesh platform that allows access to the paws of animals. The Von Frey filaments were applied to the central sub-plantar area of both paws with a gradual increase in pressure, measured in grams. Results are based on a flexion reflex followed by a clear flinch response after paw withdrawal. The results were reported as the variation of withdrawal thresholds (g) based on the difference between right and left paws. The values observed at 60, 120, 180, 240, 300 and 360 min after treatments were subtracted from the baseline values (time 0).

2.7. Treatments

Depletion of preformed mast cell mediators was induced in rat paws by repeated injections of compound 48/80 based on previously described methodologies [66]. Briefly, compound 48/80 was dissolved in sterile saline and injected i.pl. for 4 consecutive days, using 1 μg/paw on the first day, 3 μg/paw on the second day and 10 μg/paw on the third and fourth days. Batroxase (20 μg) was injected 24 h after the fourth injection of 48/80, followed by the evaluation of edema and hyperalgesia.

For the edema measurements, animals were treated 1 h before the injection of Batroxase with the non-steroidal anti-inflammatory COX-2 selective inhibitor, celecoxib (5 mg/kg, per os); 30 min before with the steroidal anti-inflammatory, dexamethasone (0.5 or 2.0 mg/kg, s.c.), the inhibitor of nitric oxide synthesis, L-NAME (5, 10 or 50 mg/kg i.p.), the antagonist of 5-HT$_1$ serotonin receptors, methysergide (5 or 10 mg/kg, s.c.) or the antagonist of H$_1$ histamine receptors, diphenhydramine (5 mg/kg or 10 mg/kg, s.c.); or 15 min before with a lipoxygenase-activating protein (FLAP) inhibitor, MK-886 (1 or 5 mg/kg, s.c.) or the