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ORIGINAL ARTICLE

Hyperalgesic and edematogenic effects of peptides isolated from the venoms of honeybee (*Apis mellifera*) and neotropical social wasps (*Polybia paulista* and *Protonectarina sylveirae*)

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Abstract Stings by bees and wasps, including Brazilian species, are a severe public health problem. The local reactions observed after the envenoming includes typical inflammatory response and pain. Several studies have been performed to identify the substances, including peptides that are responsible for such phenomena. The aim of the present study is to characterize the possible nociceptive (hyperalgesic) and edematogenic effects of some peptides isolated from the venoms of the honeybee (*Apis mellifera*) and the social wasps *Polybia paulista* and *Protonectarina sylveirae*, in addition to characterize some of the mechanisms involved in these phenomena. For this purpose, different doses of the peptides mellitin (*Apis mellifera*), Polybia-MP-I, N-2-Polybia-MP-I (*Polybia paulista*), Protonectarina-MP-NH2 and Protonectarina-MP-OH (*Protonectarina sylveirae)* were injected into the hind paw of mice. Hyperalgesia and edema were determined after peptide application, by using an electronic von Frey apparatus and a paquimeter. Carrageenin and saline were used as controls. Results showed that melittin, Polybia-MP-I, N-2-Polybia-MP-I, Protonectarina-MP-NH_2 and Protonectarina-MP-OH peptides produced a dose- and time-related hyperalgesic and edematogenic responses. Both phenomena are detected 2 h after melittin, Polybia-MP-I, N-2-Polybia-MP-I injection; their effects lasted until 8 h. In order to evaluate the role of prostanoids and the involvement of lipidic mediators in hyperalgesia induced by the peptides, indomethacin and zileuton were used. Results showed that zileuton blocked peptide-induced hyperalgesia and induced a decrease of the edematogenic response. On the other hand, indomethacin did not interfere with these phenomena. These results indicate that melittin, Polybia-MP-I, N-2-Polybia-MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH peptides could contribute to inflammation and pain induced by insect venoms.

Keywords Hyperalgesia · Inflammation · *Apis mellifera* · *Polybia paulista* · *Protonectarina sylveirae* · Peptides

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Introduction

The Hymenoptera order consists of approximately 100,000 insects, which includes the families Vespidae (hornets, wasps and yellow jackets), Apidae (bees), and Formicidae (fire ants—*Solenopsis* spp.) (Ellis and Day 2005). Many members of this order have poison glands and a stinging apparatus, which are used for prey hunting or defense. Wasp stings are commonly encountered worldwide (Maguire 1998). Stings by bees and wasps, including Brazilian species, are a severe public health problem and a clinical phenomenon well experienced by human beings. People (20–50) die every year in the United States as a result of severe systemic reactions to bees, wasps, and ant stings (Parrish 1963; Hoffman et al. 1983). In Brazil and in South America in general, the official statistic data about this type of incident are very poor; at first glance, the profile of the stinging incidents in Brazil seems to be similar to that observed in North America (Castro and Palma 2009).

It has been reported that human being (0.8–5.0%) suffers a generalized systemic reaction following a Hymenoptera sting (Golden 1989; Charpin et al. 1994). Various manifestations after wasp sting have been described. Stings from these insects may produce a variety of reactions, ranging from mild local irritation to life-threatening anaphylaxis. Unusually, manifestations like vomiting, diarrhea, dyspnea, acute renal failure, hypotension, and collapse may occur. The local reaction observed after envenomation includes typical inflammatory response, characterized by redness, edema or swelling, heat, and pain (Charpin et al. 1994).

Hymenoptera venoms are complex mixtures of biochemically and pharmacologically active components, such as biogenic amines, peptides, and proteins (Nakajima 1986). Mastoparans are the major components of vespid venoms (de Souza et al. 2004; Ho et al. 2001a), presenting from 10 to 14 amino acid residues without cysteine residues in their primary sequence (Nakajima et al. 1986; Mendes et al. 2005). Depending on the environmental conditions these peptides may assume α-helical conformations, with amphipathic feature, which favors electrostatic interactions with the negatively charged phospholipid head groups of the biological membranes. This characteristic may lead to peptide insertion into the membrane bilayer and sometimes to membrane destabilization with its consequent lysis (Rotem et al. 2006). Beside mastoparans, wasp venoms may also contain chemotactic peptides (Mendes et al. 2004), wasp kinins (Nakajima et al. 1986), and antimicrobial peptides (Mendes et al. 2004). Several studies have been performed to identify the substances, especially peptides which are responsible for pain and inflammation phenomena.

It is well known that the stinging honeybee on human skin can induce ongoing pain, hyperalgesia (and allodynia), and inflammation (Chen et al. 1997, 2006a, b). However, the mechanisms underlying the aforementioned events are poorly studied. Melittin is a major constituent of whole bee venom and the most studied one; it produces local pain and inflammatory response upon injection in mice (Hartman et al. 1991). Activation of the capsaicin-sensitive afferent fiber, spinal protein kinase C and A (PKC and PKA), spinal extracellular signaling-regulated kinases (ERK) pathway, and increase in the sympathetic tone were described as the nociceptive mechanism of melittin (Koyama et al. 2002; Li and Chen 2003; Shin and Kim 2004; Yu and Chen 2005; Sumikura et al. 2006). For *Polybia paulista* venom, the single paper found in literature reports that leukotrienes do not participate in the onset of edema formation induced by this venom, but histamine does, particulary H1 histamine receptor-dependent mechanism is involved in this inflammatory process (de Paula et al. 2006). There were no studies about the nociceptive and edematogenic response of peptides obtained from *Protonectarina sylveirae* wasp.

Despite the demonstration that melittin induces pain mediated by several mechanisms, the hyperalgesic and edematogenic effects of this peptide, and also of the polycationic peptides Polybia-MP-I, N-2-Polybia MP-I (from the venom of the social wasp *P. paulista*), Protonectarina-MP-NH_2 and Protonectarina-MP-OH (from the venom of the social wasp *P. sylveirae*) have not been determined yet. The present work was undertaken to characterize the hyperalgesic and edematogenic effect of these peptides, and some mechanisms involved in these phenomena.

Materials and methods

Animals

Male Swiss mice, weighing between 25 and 30 g, were used throughout this study. Mice were housed under controlled humidity, at a temperature of 22 ± 1°C subjected to a 12-h light–dark cycle and in a sound-attenuated room. Food and water were available ad libitum and mice were taken to the testing room at least 1 h before the experiment. All behavioral testing was performed between 9:00 am and 4:00 pm. The mice were used only once. All experiments were in accordance with the guidelines for the ethical use of conscious animals in pain research, published by the International Association for the Study of Pain (Zimmermann 1983). The procedures were approved by the Institutional Animal Care Committee at Butantan Institute (CEUAIB, protocol number 567/09). Efforts were made to minimize the number of animals used and their suffering.

Peptide synthesis

The peptides were prepared by stepwise manual solid-phase synthesis using N-9-fluorophenylmethoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (NOVABIOCHEM) (Merrifield 1986; Chan and White 2004). Side-chain protective groups included t-butyl for serine and t-butoxycarbonyl for lysine. Cleavage of the peptide–resin complexes was performed by treatment with trifluoroacetic acid/1.2-ethanedithiol/anisole/phenol/water (82.5:2.5:5:5:5 by volume), using 10 mL/g of complex at room temperature for 2 h. After filtering to remove the resin, anhydrous diethyl ether (SIGMA, USA) at 4°C was added to the soluble material causing precipitation of the crude peptides, which were collected as a pellet by centrifugation at 1,000×*g* for 15 min at room temperature. The crude peptides were solubilized in water and chromatographed under RP-HPLC using a semi-preparative column (SHISEIDO C18, 250 mm × 10 mm, 5 μm), under isocratic elution with 60% (v/v) acetonitrile in water [containing 0.1% (v/v)

trifluoroacetic] at a flow rate of 2 mL/min. The elution was monitored at 214 nm with a UV-DAD detector (SHIMADZU, mod. SPD-M10A, USA), and each fraction eluted was manually collected into 1.5 mL glass vials. The homogeneity was assessed through ESI–MS analysis, while the correctness of the sequence of the synthetic peptides was checked by using automated Edman degradation chemistry as described below.

Peptides purification

Synthetic peptides were purified by using a Capcell Pack C-18 UG120 column (10 mm × 250 mm, 5 μm, Shiseido, Japan) under elution with 38% (v/v) MeCN [containing 0.1% (v/v) trifluoracetic acid (TFA)], at a flow rate of 2.0 mL/min over 60 min; the elutions were monitored at 214 nm, and fractions were manually collected in 5 mL glass vials.

ESI mass spectrometry

Mass spectrometric analysis was performed in a triple quadrupole mass spectrometer (Micromass, mod. Quattro II, UK). The experimental protocol was based on previously described methods (de Souza et al. 2004) and adapted for the present investigation. The mass spectrometer was outfitted with a standard probe electrospray (ESI—Micromass, Altrinchan, UK). The samples were injected into the electrospray transport solvent with a micro-syringe (250 mL) coupled to a micro-infusion pump (KD Scientific, USA) at a flow rate of 4 mL/min. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments to operate at resolution 4,000. The samples were dissolved in 50% (v/v) acetonitrile [containing 0.1% (v/v) formic acid] to be analyzed by positive electrospray ionization (ESI+) using typical conditions: a capillary voltage of 3.5 kV, a cone voltage of 30 V, a desolvation gas temperature of 80°C, and flow of nebulizer gas (nitrogen) about 20 L/h and drying gas (nitrogen) 200 L/h. About 50 pmol of each sample was injected into the electrospray transport solvent. The ESI mass spectra were obtained in the continuous acquisition mode, scanning from m/z 100–2,500 at a scan time of 7 s. The data were acquired and treated using MassLynx software (Micromass, UK).

Amino acid sequencing

The amino acids were sequenced by using a gas phase sequencer PPSQ-21A (Shimadzu, USA) based on automated Edman degradation chemistry.

von Frey electronic pressure-meter paw tests for mice

Mice were placed in acrylic cages (12 × 10 × 17 cm high) with a wire grid floor 15–30 min before testing. During this adaptation period, the paws were poked 2–3 times. Before paw stimulation, the animals were quiet, without exploratory movements or defecation and not resting on their paws. In these experiments, we used a pressure-meter which consisted of a handheld force transducer fitted with a 0.5 mm^2 polypropylene tip (electronic von Frey anesthesiometer, IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). The investigator was trained to apply the polypropylene tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hindpaw. The test consisted of poking a hindpaw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. In the electronic pressure-meter test the intensity of the stimulus was automatically recorded when the paw was withdrawn. The maximal force applied was 18 g. The stimulation of the paw was repeated until the animal presented two similar measurements. If the results were inconsistent (great difference in the baseline response compared to the other animals of the experiment), another animal was used. The results are reported as the Δ (delta) withdrawal threshold (g) which was calculated by subtracting the values obtained after the treatments from the first measurement (before treatment).

Evaluation of edema

Edema was induced by the injection by the intraplantar route (i.pl.) route of carrageenin (300 μg/50 μL) or peptides (10, 30, and 50 μg/50 μL) into one of the hind paws. The volume increase (edema) of paws up to the tibio-tarsal articulation was measured using a digital paquimeter (Mitutoyo, CD-6" CSX-B model, Brazil). Indomethacin and Zileuton were injected into one of the hind paws to evaluate the antiedematogenic effect. The difference between the values obtained for both paws expressed as percent increase in paw volume was used as a measure of edema.

Drugs

For evaluation of the nociceptive activity of peptides, synthetic Melittin was purchased from Sigma–Aldrich, while the peptides Polybia-MP-I, N-2-Polybia-MP-I, Protonectarina-MP-NH_2 and Protonectarina-MP-OH (manually synthesized on-solid phase) were dissolved in sterile saline (10, 30, and 50 μg in 50 μL) and administered by i.pl. route into one of hind paws of mice. A hypodermic 26-G

needle was inserted into the skin of the second footpad (to avoid back flow) and the tip of the needle was introduced until the central area of the hindpaw, in the same place where filaments or the tip of the pressure-meter were applied. The nociceptive activity was evaluated at different times (0-before treatments, 2, 4, 8, and 24 h) after treatment and compared with control. Carrageenin (Marine Colloids, 300 μg/50 μL) was diluted in sterile saline and was used as positive control. Sterile saline was used as control. In order to evaluate the role of prostanoids in hyperalgesia induced by peptides, the cyclooxygenase inhibitor, indomethacin was used. Indomethacin (Sigma, USA, 100 μg/25 μL) or Tris (control) was administered, by i.pl. route of the mice 30 min before peptide inoculation. Indomethacin (Sigma, USA, 100 μg/25 μL) was diluted in 0.1 M Tris–HCl buffer, pH 8.0. Sterile saline and Tris–HCl buffer alone was used for the control groups (Chacur et al. 2003). To evaluate the involvement of lipidic mediators involved in hyperalgesia induced by peptides, zileuton (Abbott Laboratories, Zyflo®, USA) (100 mg/kg, by oral route) (Horizoe et al. 1998), a lipoxygenase inhibitor, was administered 60 min before injection of the peptides. Zileuton was diluted in hydroalcoholic solution (10% of etilic alcohol). Animals injected with hydroalcoholic solution plus sterile saline were used as control group.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the groups and doses over all times. The factors analyzed were treatments, time and time versus treatment interaction. When a significant time versus treatment interaction was detected, one-way ANOVA followed by the Tukey test was performed for each time in order to distinguish dose effects. One-way ANOVA followed by Tukey test was also used for dose–response curves for a single time point. Results with $P < 0.05$ were considered to be significant.

Results

The peptide melittin (GIGAVLKVLTTGLPALISWIKR KRQQ-NH_2) in its natural form as found in honeybee (*A. mellifera*) venom, was acquired from Sigma–Aldrich Co., USA, while the wasp venom peptides Polybia-MP-I (IDW KKLLDAAKQIL-NH_2), N-2-Polybia-MP-I (INWKKLLD AAKQIL-NH_2), Protonectarina-MP-NH_2 (INWKALLD AAKKVL-NH_2) and Protonectarina-MP-OH (INWKAL LDAAKKVL-OH) were manually synthesized on-solid phase and purified as described in Material and methods. Polybia-MP-I and N-2-Polybia-MP-I are mast-cell-degranulating peptides originally reported in the venom of the social wasp *P. paulista* (de Souza et al. 2004). Protonectarina-MP-NH_2 and Protonectarina-MP-OH are mast-cell-degranulating peptides, previously described in the venom of the neotropical social wasp *P. sylveirae* (Dohtsu et al. 1993); the structural difference among these peptides is in the C-terminal residue, which is in the amidated (–NH_2) form for Protonectarina-MP-NH_2, and in acidic (–OH) form for Protonectarina-MP-OH.

Characterization of the hyperalgesic and edematogenic effect of melittin, Polybia-MP-I and N-2-Polybia MP-I

The effectiveness of peptide toxin doses isolated from animal venoms may differ according to the conformation acquired by each peptide and the pain sensitivity method employed (Picolo et al. 2010; Fernandes et al. 2007; Chen et al. 2006a; Chacur et al. 2004). All peptide doses used in this work were determined by previous studies using a dose–response curve (data not shown) since there are no available data of peptide doses in literature.

The i.pl. injection of melittin, Polybia-MP-I and N-2-Polybia MP-I (10, 30, and 50 μg/50 μL) caused a significant decrease in pain threshold, characterizing the phenomenon of hyperalgesia, detected at 2 h after peptide injection (Fig. 1a, c, e). This phenomenon persists until 8 h. Carrageenin (Cg, i.pl., 300 μg/50 μL) was used as positive control. Control animals were injected with sterile saline (S, control, Fig. 1a, c, e) at same experimental conditions. For N-2-Polybia MP-I, at small doses, the hyperalgesic phenomenon persist only by 2 h. Also, melittin, Polybia-MP-I and N-2-Polybia MP-I (10, 30, and 50 μg/50 μL) induced an increase in paw volume (edema) (Fig. 1b, d, f). The edematogenic effect of melittin, Polybia-MP-I and N-2-Polybia MP-I (30 and 50 μg/50 μL) was observed until 8 h of experimental period. It is important to point out that the peak of the hyperalgesic and edematogenic responses occurred 2 h after peptide injection. After this time both phenomena started to decrease (Fig. 1a–f) and completely disappeared at 24 h. Saline group was significantly different from carrageenan and peptide groups.

Characterization of the hyperalgesic and edematogenic effect of Protonectarina-MP-NH_2 and Protonectarina-MP-OH

The i.pl. injection of protonectarina-MP-NH2 and Protonectarina-MP-OH (10, 30, and 50 μg/50 μL) caused a significant decrease in pain threshold, characterizing the phenomenon of hyperalgesia at 2 h after peptide injection (Fig. 2a, c). These phenomena disappear at 4 h. Carrageenin (Cg, i.pl., 300 μg/50 μL) was used as positive control. Control animals were injected with sterile saline

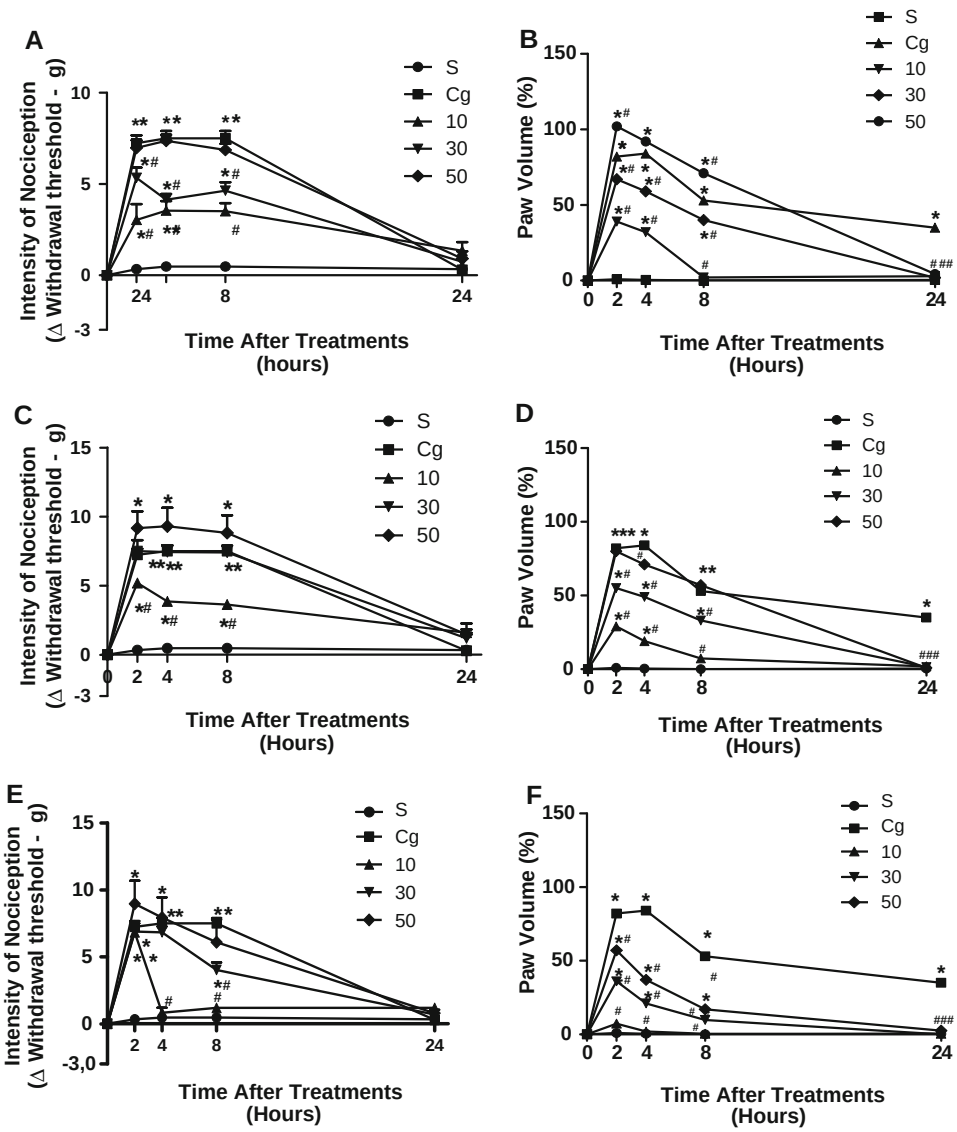

Fig. 1 Evaluation of hyperalgesic and edematogenic effects of melittin and Polybia-MP-I and N-2-Polybia MP-I. Paw threshold was estimated in the electronic von Frey. The force needed to induce paw withdrawal was recorded as the pain threshold represented by delta (Δ) (**a**, **c** and **e**). Edematogenic effect was evaluated using a digital paquimeter (**b**, **d** and **f**). Data were obtained before (time 0), 2, 4, 8, and 24 h after melittin (**a**, **b**), Polybia-MP-I (**c**, **d**) and N-2-Polybia MP-I (**e**, **f**) administration (10, 30, and 50 μg/50 μL, i.pl.) or carrageenin (Cg, 300 μg/50 μL, i.pl.). Control group are animals injected with sterile saline (S). Results are expressed as media ±SEM of five animals per group. $*P < 0.001$ Significantly different from mean values for Saline (S) group; $\#P < 0.001$ Significantly different from mean values for carrageenin (Cg) group

(S, control, Fig. 2a, c) under the same experimental conditions. Also, Protonectarina-MP-NH_2 and Protonectarina-MP-OH (10, 30, and 50 μg/50 μL) induced an increase of paw volume at 2 h (edema) disappearing at 4 h (Fig. 2b, d). The peak of the hyperalgesic and edematogenic responses occurred 2 h after peptide injection. After this time both phenomena started to decrease (Fig. 2a–d).

Involvement of eicosanoids in hyperalgesic and edematogenic effects of melittin, Polybia- MP-I, N-2-Polybia MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH

Since melittin, Polybia-MP-I, N-2-Polybia MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH induced hyperalgesic effect, the aim was to evaluate the involvement of prostanoids (ciclooxigenase pathway) and lipidic mediators (lipoxigenase pathway) in this effect. Mice were injected in one of the hind paws (i.pl.) with melittin (Fig. 3a) (M30, 30 μg/50 μL, i.pl.), Polybia- MP-I (Fig. 3c) (P30, 30 μg/50 μL, i.pl.), N-2-Polybia MP-I (Fig. 3e) (NP30, 30 μg/50 μL, i.pl.), Protonectarina-MP-NH_2 (Fig. 3g) (PNH30, 30 μg/50 μL, i.pl.), and Protonectarina-MP-OH (Fig. 3i) (POH50, 50 μg/50 μL, i.pl.), indomethacin (I, 100 μg/25 μL, i.pl., cycloxigenase pathway inhibitor) and zileuton (Z, 100 mg/kg, by oral route, in 500 μL, lipoxigenase pathway inhibitor) and evaluated in electronic von Frey. Results showed that zileuton blocked peptide-induced hyperalgesia (Fig. 3a, c, e, g, i), when compared with control groups. On the other hand, indomethacin did not interfere with this phenomenon (Fig. 3a, c, e, g, i). Positive groups treated with carrageenan + Zileuton and,

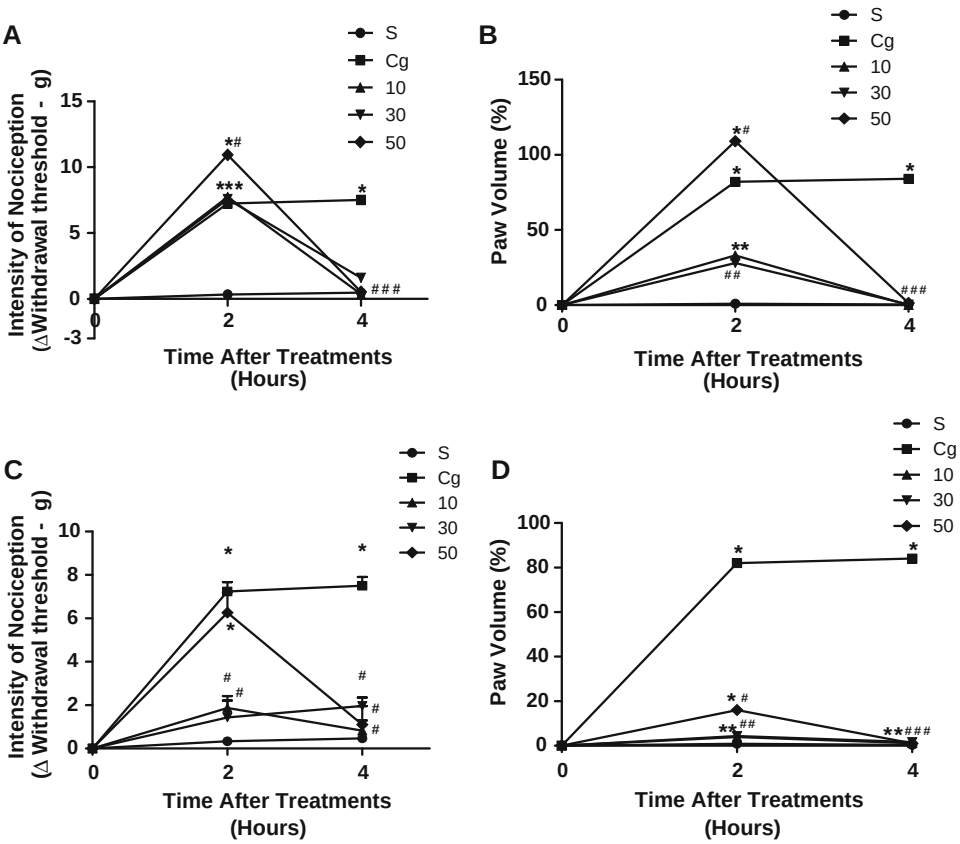

Fig. 2 Evaluation of hyperalgesic and edematogenic effects of Protonectarina-MP-NH2 and Protonectarina-MP-OH. Paw threshold was estimated in the electronic von Frey. The force needed to induce paw withdrawal was recorded as the pain threshold represented by delta (Δ) (**a**, **c**). Edematogenic effect was evaluated using a digital paquimeter (**b**, **d**). Data were obtained before (time 0), 2 and 4 h after protonectarina-MP-NH2 (**a**, **b**) and Protonectarina-MP-OH (**c**, **d**) peptides administration (10, 30, and 50 μg/50 μL, i.pl.) or carrageenin (Cg, 300 μg/50 μL, i.pl.). Control group are animals injected with sterile saline (S). Results are expressed as media ± SEM of five animals per group. $*P < 0.001$ Significantly different from mean values for Saline (S) group; $\#P < 0.001$ Significantly different from mean values for carrageenin (Cg) group

carrageenan + indomethacin showed that zileuton and indomethacin reduced carrageenan-induced hyperalgesia (data not shown). Also, we evaluated the cycloxigenase and lipoxigenase pathway mediators on edematogenic effect of peptides. Zileuton induced a decrease of paw volume when compared with control animals (Fig. 3b, d, f, h, j). It is important to point out that indomethacin did not interfere with paw volume induced by peptides (Fig. 3b, d, f, h, j). Additional control groups treated with saline (vehicle) + Tris (vehicle for indomethacin) or saline + alcoholic solution (vehicle for zileuton) were also performed. Those injections did not interfere either with hyperalgesic effect or with paw volume during all experimental period (data not shown).

Discussion

Hymenoptera venoms have been reported to be responsible for 9.3–28.5% of sensitization cases worldwide (Antonicelli et al. 2002). Their stings cause severe pain, local inflammation, sensitization, local and systemic alterations, and occasionally even death in allergic patients (Antonicelli et al. 2002; Steen et al. 2005).

In this work we evaluated the hyperalgesic (evaluated in von Frey electronic pressure-meter paw) and edematogenic (evaluated by paquimeter) effects of melittin and also of the polycationic peptides Polybia-MP-I, N-2-Polybia MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH. Also, the present work was undertaken to characterize some of the mechanisms involved in these phenomena.

Our results showed that melittin, Polybia-MP-I, N-2-Polybia MP-I induced hyperalgesic and edematogenic responses 2 h after peptide injection; after this time both phenomena started to decrease and completely disappeared at 24 h.

Bee venom contains a variety of peptides, including melittin, apamin, adolapin, the mast-cell degranulating (MCD) peptide, enzymes (phospholipase A_2), biologically active amines (histamine and epinephrine), and non-peptide components which have a variety of pharmaceutical properties (Son et al. 2007). Melittin contains 26 amino acid residues and is the principal toxin in bee venom (Son et al. 2007); it produces local pain and inflammatory response upon injection in mice (Hartman et al. 1991). The activation of a series of processes such as the capsaicin-sensitive afferent fiber, spinal protein kinase C and A (PKC and PKA), spinal ERK pathway, and increase of the sympathetic tone, were described as part of the nociceptive mechanism of melittin (Koyama et al. 2002; Li and Chen 2003; Shin and Kim 2004; Yu and Chen 2005; Sumikura et al. 2006).

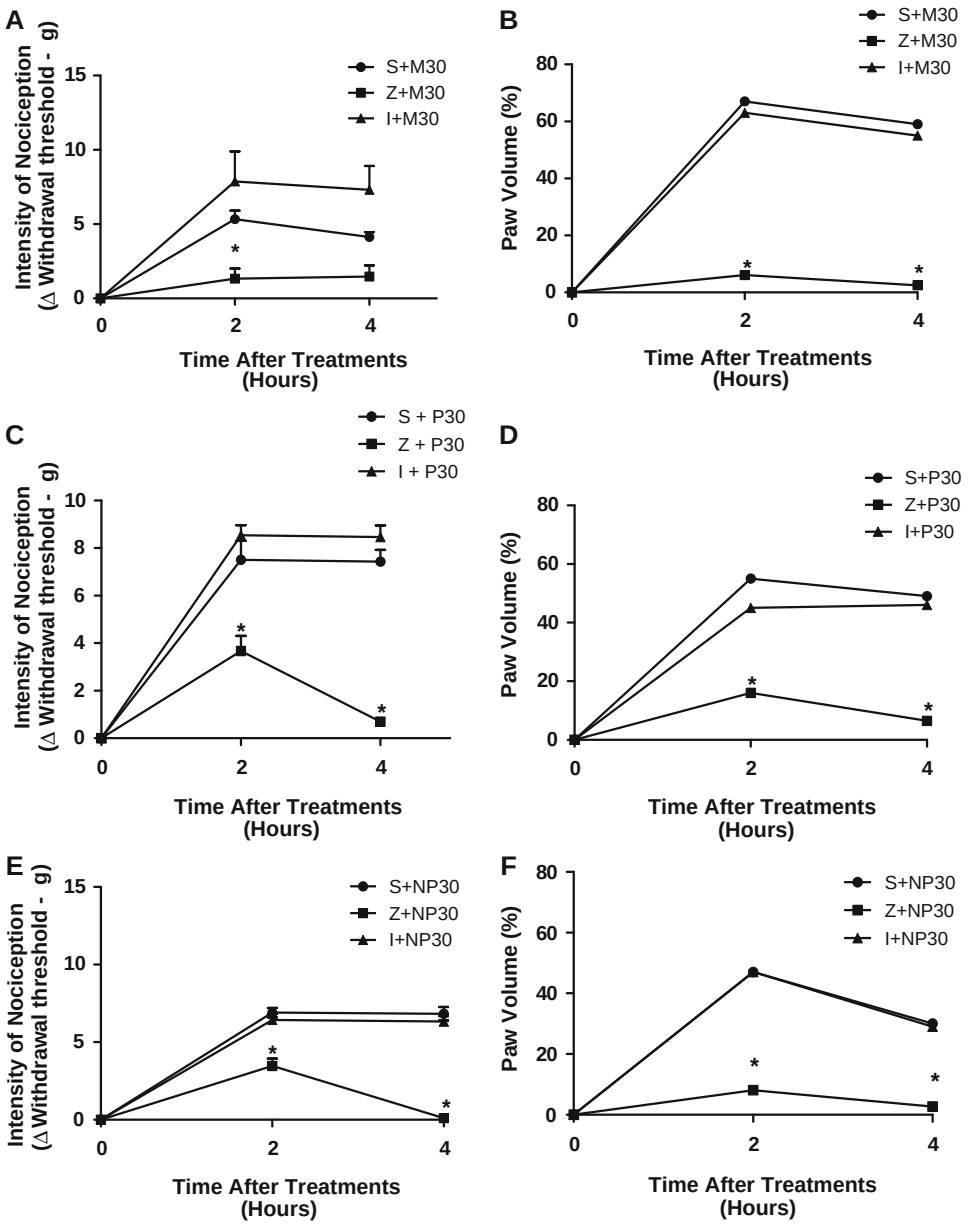

Fig. 3 Involvement of eicosanoids on hyperalgesic and edematogenic effects of melittin, Polybia-MP-I, N-2-Polybia MP-I, Protonectarina-MP-NH2 and Protonectarina-MP-OH. Paw threshold was estimated in the electronic von Frey. The force needed to induce paw withdrawal was recorded as the pain threshold represented by delta (Δ) (**a**, **c**, **e**, **g** and **i**). Edematogenic effect was evaluated using a digital paquimeter (**b**, **d**, **f**, **h** and **j**). Data were obtained before (time 0), 2 and 4 h after melittin (**a** and **b**, M30, 30 μg/50 μL, i.pl.), Polybia- MP-I (**c** and **d**, P30, 30 μg/50 μL, i.pl.), N-2-Polybia MP-I (**e** and **f**, NP30, 30 μg/50 μL, i.pl.), Protonectarina-MP-NH2 (**g** and **h**, PNH30, 30 μg/50 μL, i.pl.) and Protonectarina-MP-OH (**i** and **j**, POH50, 50 μg/50 μL, i.pl.) administration. Indometacin (I, 100 μg/25 μL, i.pl.) and zileuton (Z, 100 mg/kg, by oral route, in 500 μL) were administered 30 min and 1 h before peptide injection, respectively. Control group are animals injected with saline + melittin (S+M30), saline + Polybia-MP-I (S+P30), saline + N-2-Polybia MP-I (S+NP30), saline + Protonectarina-MP-NH2 (S+PNH30), saline + Protonectarina-MP-OH (S+POH50). Results are expressed as media ±SEM of five animals per group. *$P < 0.001$ Significantly different from mean values for control group

Vespinae venoms contain protein components that are biochemically and pharmacologically active, such as phospholipases, hyaluronidases, acid phosphatases, proteases, nucleases, and peptides (Nakajima 1986; Nicolas et al. 1997; de Oliveira and Palma 1998; Konno et al. 2001). The neotropical social wasp *P. paulista* is a very aggressive endemic species in southeastern Brazil, where it frequently causes stinging accidents (de Souza et al. 2004; de Oliveira and Palma 1998).

Literature shows that mastoparans, the major components of vespid venoms (Murata et al. 2009; Ho et al. 2001b; de Souza et al. 2004), chemotactic peptides

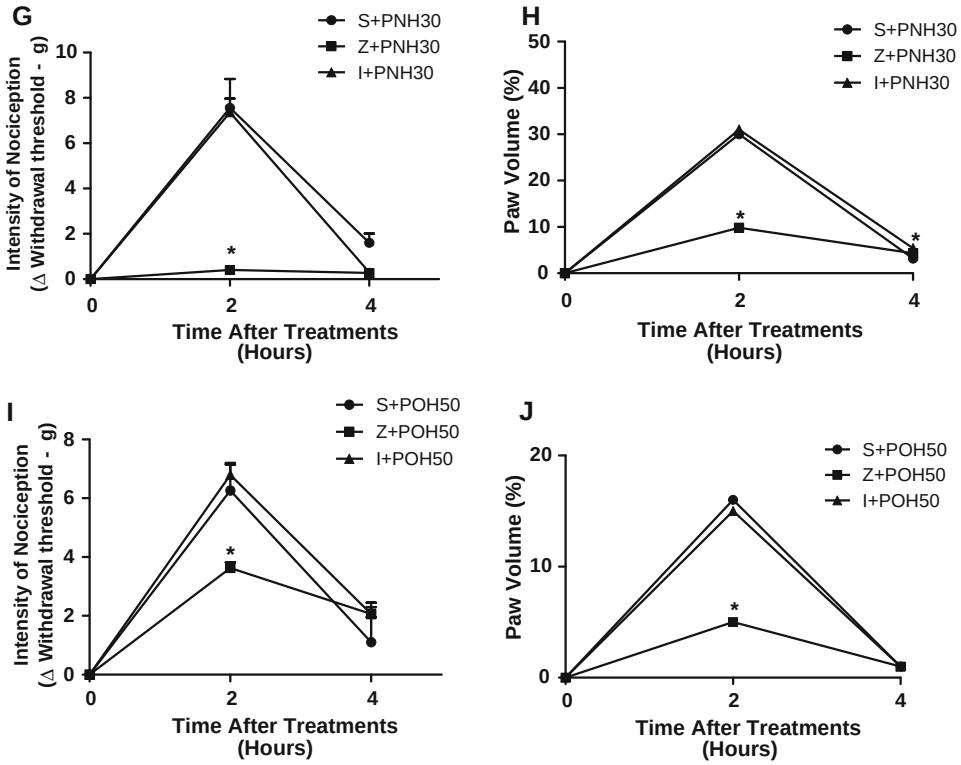

Fig. 3 continued

(Mendes et al. 2004), wasp kinins (Nakajima 1986), and antimicrobial peptides (Mendes et al. 2004), have been identified among the peptides from social wasp venoms. They constitute the most abundant group of peptides in the venoms of social wasps (Hirai et al. 1979).

Studies of the regulatory mechanism of mastoparans have shown that these peptides are involved in the modulation of the activities of proteins such as phospholipase A_2 and phospholipase C (Higashijima et al. 1990; Song et al. 1993). Some mastoparans bind to G-protein coupled receptors (GPCRs) (Jones and Howl 2006), which are involved in the activation of different types of basophils, chemotaxis of leukocytes, and neurotoxicity (de Oliveira et al. 2006).

There is no literature characterizing the hyperalgesic and edematogenic effect of *P. paulista* venom. The single paper found in literature reports that leukotrienes do not participate in the onset of edema formation induced by this venom, but histamine does, particulary H_1 histamine receptor-dependent mechanism is involved in this inflammatory process (de Paula et al. 2006).

Also, there is no literature evaluating the hyperalgesic effect of melittin and Polybia-MP-I, N-2-Polybia MP-I peptides. Electronic von Frey is a commercially available instrument similar to that successfully used for to evaluate the inflammatory hypernociception in rats (Vivancos et al. 2004). The electronic method has several advantages over classical experimental approaches to evaluate hyperalgesic/analgesic effects, such as reduction of the number of attempts required to evaluate the nociceptive threshold; elimination of the problems of standardization; stimulation of areas of equal size, and the end-point is automatically recorded (Jensen et al. 1986).

Another step of this work was to evaluate the nociceptive and edematogenic response of peptides obtained from the venom of *P. sylveirae* wasp. Our results showed that Protonectarina-MP-NH_2 and Protonectarina-MP-OH peptides induced a significant decrease in pain threshold, characterizing the phenomenon of hyperalgesia, 2 h after peptide injection. These phenomena disappeared at 4 h.

Also, it is important to point out that hyperalgesic effect induced by Protonectarina-MP-NH_2 is more severe when compared with those observed for the same peptide in its acidic form (Protonectarina-MP-OH). Studies with many peptide toxins from different biological origins toxins have shown that there is an increase in the biological activity following amidation or, alternatively, a biological activity decreases when the amide is removed (Ali et al. 2001; Katayama et al. 2002). Also, the enhanced potency of amidated inflammatory peptides is often attributed to their increased positive charges (Pouny et al. 1992; Cociancich et al. 1993; Steiner et al. 1988). It has been postulated that

positive charges of the peptides promote interactions with the negative groups of the membranes, facilitating the interaction of these peptides with the cell membranes (Tossi et al. 2000), contributing to its permeabilization.

In mammalian cells, eicosanoid biosynthesis is usually initiated by the activation of phospholipase A_2 and the release of arachidonic acid (AA) from membrane phospholipids. The AA is subsequently transformed by cyclooxygenase (COX) and lipoxygenase (LO) pathways to prostaglandins, thromboxane, and leukotrienes collectively termed eicosanoids. Eicosanoid production is considerably increased during inflammation. Both COX and LO pathways are of particular clinical relevance. The COX pathway is the major target for non-steroidal anti-inflammatory drugs (NSAIDs), the most popular medications used to treat pain, fever, and inflammation. Although their anti-inflammatory effects are well known, their long-term use is associated with gastrointestinal complications such as ulceration (Khanapure et al. 2007).

The 5-Lipoxygenase pathway results in the formation of leukotrienes, including leukotriene B4 (LTB4), 5-oxo-6E,8Z,11Z,14Z-eicosatetranoic acid and the cysteinyl leukotrienes (LTC4, LTD4 and LTE4) and activates all four leukotriene receptors, BLT1, BLT2, cysLT1, and cysLT2. Leukotrienes are produced by multiple inflammatory cells, particularly mast cells, basophils, eosinophils, neutrophils, and macrophages (Berger et al. 2007). Zileuton, a benzothiophene *N*-hydroxyurea, is the only commercially available inhibitor of the 5-Lipoxygenase pathway (Berger et al. 2007). Some cyclooxygenase products can arise as a result of the effect of 5-LO products on other cells and/or tissues or as a result of the physiologic effects of leukotrienes on airway smooth muscle and mucus glands. In these instances, one could hypothesize that 5-LO inhibition may result in a decrease in the production of cyclooxygenase products (Kane et al. 1995). Additionally, in cellular, in tissue and in vivo models, cyclooxygenase inhibition has been shown to result in an increase or shunting of 5-LO products while other models have shown no effect and in vitro and in vivo data are conflicting (Kane et al. 1995).

Since melittin, Polybia-MP-I, N-2-Polybia MP-I, Protonectarina-MP-NH_2 and Protonectarina-MP-OH induced hyperalgesic effect, the aim was to evaluate the involvement of prostanoids (cyclooxygenase pathway) and lipidic mediators (lipoxigenase pathway) in this effect. Indomethacin, a cyclooxygenase inhibitor, failed to reduce hyperalgesia induced by peptides. This suggests that prostaglandin generated by both type 1 and 2 cyclo-oxygenase activities does not play a significant role in the phenomenon evoked by peptides in the present study. It is important to point out that indomethacin (100 μg/25 μL, i.pl) and zileuton (100 mg/kg, by oral route, in 500 μL), blocked the hypernociceptive stage induced by carrageenin (300 μg/paw) (data not shown). On the other hand, zileuton, an inhibitor of lipoxygenase pathways, blocked the hyperalgesia-inducing activity of the peptides. Together, these data indicate that leukotrienes may be important mediators of wasp Hymenopteran peptides-induced hyperalgesia. Leukotriene B4 sensitizes nociceptors, causes hyperalgesia and may contribute to the component of hyperalgesia resistant to nonsteroidal anti-inflammatory agents (Rackham and Ford-Hutchinson 1983; Levine et al. 1984, 1993; Martin 1990; Martin et al. 1988; Madison et al. 1992).

There are few reports about cyclooxygenase and lipoxygenase pathway involved in Hymenoptera venom pain-mediated. Mustafa et al. (2008) investigated leukotriene production using a honeybee venom secretory phospholipase A2. A cyclooxygenase-1 (COX-1) and 15-lipoxygenase inhibitor named, 9–12 octadecadiynioc acid (OCA), abrogated leukotriene production. These results indicate that honeybee venom secretory phospholipase A2 induces leukotriene production, but not histamine release from human basophils.

De Paula et al. (2006) evaluated leukocyte recruitment and edema formation in the peritoneal cavities of rats injected with *Polybia paulista* venom. Administration of MK 886, a leukotriene synthesis inhibitor, completely abolished granulocyte recruitment to the peritoneal cavity. Also, indomethacin had no effect on this venom-induced edema formation suggesting that prostaglandin did not play an important role in this phenomenon. Edema formation, however, was significantly inhibited by treatment with pyrilamine, indicating that the histamine H1 receptor plays a critical role in *Polybia paulista*-induced formation of acute edema.

In conclusion, we have shown that melittin, Polybia-MP-I, N-2-Polybia-MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH peptides produced a dose- and time-related hyperalgesic and edematogenic responses. Also we evaluated the role of prostanoids and the involvement of lipidic mediators in hyperalgesia induced by the peptides. Lipoxygenase pathway, but not cycloxygenase, is involved in peptide-induced hyperalgesia and edematogenic effect. These results indicate that melittin, Polybia-MP-I, N-2-Polybia-MP-I, Protonectarina-MP-NH_2, and Protonectarina-MP-OH peptides could contribute to inflammation and pain induced by *A. mellifera, P. paulista, and P. sylveirae* venoms.

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