Analysis of the inflammatory response in the rat paw caused by the venom of *Apis melifera* bee

M. C. Calixto, K. M. Trichês and J. B. Calixto

Department of Pharmacology, Centre of Biological Sciences, Universidade Federal de Santa Catarina, Rua Ferreira Lima, 82, Florianópolis, SC, 88015-420, Brazil, Fax: ++55482 22 4164, e-mail: Calixto@farmaco.ufsc.br or Calixto3@terra.com.br

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Abstract. Objective: This study examines the pro-inflammatory action caused by subcutaneous (s.c.) injection of the bee venom (BV) *Apis melifera* in the rat paw.

Methods: Male Wistar rats were used. The venom of *Apis melifera* was injected s.c. into the rat paw and the oedema formation and the activity of myeloperoxidase (MPO) were measured.

Results: Subcutaneous injection of BV caused dose- and time-dependent paw oedema (ED50 of 1.5 μg/paw) with peak at 30 min. The MPO activity increased about 1.6, 4.2 and 8.9 folds at 0.5, 4 and 6 h after s.c. injection of BV. The mast cell degranulating drug 48/80, pyrilamine or metysergide, inhibited BV-mediated oedema formation (88, 62 and 96%, respectively). Likewise, L-NAME, the NK1 antagonist FK 889, the B2 des-Arg9-[Leu8]-BK or B1 kinin antagonist Hoe 140 also antagonised the paw oedema induced by BV (60, 59, 49% and 49%, respectively). SR48968 and SR14280, respectively NK2 and NK3 antagonists and also indomethacin, inhibited by 31, 29 and 22% respectively BV-induced oedema formation. In contrast, the PAF antagonist WEB 2086 or valeryl salycilate, did not affect the BV-induced paw oedema. The levels of MPO were inhibited by compound 48/80, cyproheptadine, Hoe 140, or by des-Arg9-[Leu8]-BK (85, 61, 59, and 53%, respectively) measured 6 h after.

Conclusion: These results indicate that the BV from *Apis melifera* causes a marked dose-and time-dependent oedema formation in the rat paw, an effect that is accompanied by intense leukocyte migration. The pro-inflammatory response induced by BV is mediated by several mechanisms, namely the release of histamine and/or serotonin from mast cells, activation of H1 histamine receptor, production of nitric oxide, the involvement of kinins through the activation of B1 and B2 receptors, and also tachykinins acting at NK1 receptor or and to a lesser extent at NK2 and NK3 receptors.

Key words: *Apis melifera* – Bee venom – Paw oedema – Leukocyte influx – Myeloperoxidase – Inflammation – Anti-inflammatory drugs

Introduction

The venom of the bee specie of *Apis melifera* (BV) comprises a great mixture of components such as proteins, peptides and small organic molecules. Among the active components present in the BV which account for its toxic effects are melittin, the 26-aminoacid peptide which is the main component of the BV and comprises about 40 to 50% of the crude venom, the antigenic enzymes phospholipase A2 and hyaluronidase, the potent mast-cell degranulating peptide 401, apamine, the antagonist of high conductance calcium-activated potassium channels, the proteases inhibitors etc. In addition, the BV also contains low-molecular-weight organic constituents, namely histamine, dopamine and noradrenaline (see for review [1]).

Recent evidence has shown that the subcutaneous or s.c. injection of BV into rodents causes bilateral and persistent heat and mechanical hyperalgesia. These hyperalgesic effects seem to be mediated by central sensitisation mediated via activation of glutamatergic receptors (both NMDA and non-NMDA) and capsaicin-sensitive primary afferent fibres, and activation of the protein kinase C-dependent mechanisms at the spinal cord level [2–8].

Despite the fact that BV contains several inflammatory components and considering that it can release most pro-inflammatory mediators, so far there has been no scientific study aiming to investigate the mechanisms underlying the BV-induced inflammatory actions. Therefore, in the present study we have investigated, by the use of pharmacological and biochemical procedures, some of the mechanisms by which the BV causes inflammatory response when injected s.c. into the rat paw.

Material and methods

Animals

Non-fasted male Wistar rats (180–200 g), kept in a controlled room temperature (22 ± 2°C) under a 12 h:12 h light-dark cycle (lights on 06:00 h) were used. Food and water were freely available. Experiments were carried out in accordance with current guidelines for the care of
laboratory animals and ethical guidelines for experiments in conscious animals [9].

Measurement of rat paw oedema

The animals were slightly anaesthetised with ether and received a 0.1 ml s.c. injection in one hindpaw (right paw) of phosphate buffered saline (PBS, composition mmol/L: NaCl 137, KCl 2.7 and phosphate buffer 10), containing BV (0.1 to 5 µg/paw) alone or in combination with several antagonists. The contralateral paw (left paw) received 0.1 ml of sterile PBS and was used as control. The s.c. injection of PBS alone did not cause any significant increase in the paw volume. Oedema was measured by use of a plethysmometer (Ugo Basile) at several time-points (15, 0.5, 1, 2, 4, 6, and 24 h) after injection of B.V. Oedema is expressed in ml as the difference between the right and left paws.

In separate groups of experiments, in order to investigate the mechanisms by which BV induces paw oedema, animals were treated with one of several inhibitors, namely: rofecoxib (a selective COX-2 inhibitor, i.p., 10 mg/kg), indomethacin (a non-selective COX-1 and COX-2 inhibitor, i.p., 3 mg/kg), valeryl-salicylate (a preferential COX-2 inhibitor, i.p., 10 mg/kg), pyrilamine (a selective H1 histamine receptor antagonist, i.p., 10 mg/kg), cyproheptadine (an antagonist of histamine and serotonin, 20 mg/kg, i.p., 30 min before) or with dexamethasone (an anti-inflammatory steroid drug and phospholipase A2 inhibitor, 0.5 mg/kg, given systemically, s.c., 4 h before). Another group of animals received one of the following drugs in combination with BV: Hoe 140 (a selective B2 receptor antagonist, 10 nmol/paw), des-Arg9-[Leu]7-bradykinin (a selective B2 receptor antagonist, 10 nmol/paw); FK 888 (1 nmol/paw), SR 48968 (3 nmol/paw), SR 142801 (3 nmol/paw), respectively the NK1, NK2 and NK3 selective receptor antagonists; L-NAME (a nitric oxide synthase inhibitor, 0.5 mmol/paw) or WEB 2086 (a PAF receptor antagonist, 15 µg/paw). To deplete the histamine from the mast cells, animals were treated with compound 48/80 (12 µg/paw) 12 h prior experiments. The doses of all antagonists or inhibitors have been chosen on the basis of previous studies [10–14].

Measurement of myeloperoxidase (MPO) activity

The accumulation of neutrophils in the rat paw was indirectly analysed by the measure of the tissue MPO activity. Animals received a 0.1 ml s.c. injection of BV (1.5 µg/paw) in the right paw and were sacrificed at several time points after BV injection. For purpose of comparison, another group of animals received s.c. injection of carrageenan (300 µg/paw) and the activity of MPO was analysed 3 hour later. PBS-treated paws were used as controls. At the time of sacrifice, the subcutaneous tissue of the paws was removed and assayed for MPO according to a previously-described method [15, 16]. Briefly, tissues were homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 9,000 rpm, 15 min, at 4°C. The pellet was re-suspended in hexadecyltrimethyl ammonium bromide 0.5% buffer (pH 5.4) and the samples were frozen and thawed 3 times in liquid nitrogen. Upon thawing, the samples were re-centrifuged (9,000 rpm, 15 min, at 4°C) and 25 µl of the supernatant were used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM NaPO4, and 0.3 mM hydrogen peroxide. The absorbance was measured at 650 nm and the results are expressed in optical density (OD) per mg of dry tissue.

Drugs and reagents

The following drugs were used: Indomethacin, cyproheptadine, compound 48/80, PBS tablets, EDTA, lambda carrageenan grade IV, tetramethylbenzidine (all from Sigma Chemical Company, St. Louis, U.S.A.). WEB 2086 was a gift of Dr. Marco Aurélio Martins, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. NaPO4, NaCl and hydrogen peroxide were all from Merck, Germany. Valeryl salicylate was supplied by the Cayman Chemical Company, Ann Arbor, MI, USA. Rofecoxib was kindly donated by Merk &Co. Inc. (U.S.A) and des-Arg9-[Leu]7-bradykinin (was purchased from Peninsula Belmont, USA). Hoe 140 (des-Arg9-[Hyp3,Thi5,D-Tic7,Oic8]-bradykinin) was kindly donated by Hoecht (Frankfurt Main, Germany) and FK 888 (N-[4-[(1-methyl-1H-indol-3-yl)carbonyl-L-propyl]-N-methyl-N-phenylmethyl-3-(2-naphthyl)-l-alanine-amide was gently donated by the Fuji-sawa Pharmaceutical Co. (Osaka, Japan); SR48968 ((S)-N-[4-[4-(acetylamino-4-phenyl-1-piperidinyl)-2-(3,4-dichloro-phenyl)-butyl]N-methylbenzamide) and SR142801 ((S)-N-[3-benzoyl-3-(4,3-dichlorophenyl)-piperidin-3-yl)[pro-pyl]-4-phenylpiperidin-4-yl]-N-methyl-acetamide) were kindly supplied by SanoFı Recherche (Montpellier, France). The BV of Apis melifera was a gift from Protta, Brazil. All other reagents used were of high grade of purity. The stock solutions for all drugs were prepared in PBS, with the exception of valeryl salicylate which was dissolved in dimethyl sulfoxide, and indomethacin and dexamethasone which were dissolved in 50% absolute ethanol, kept in siliconized plastic tubes, and maintained in a freezer at ~18°C. The final concentration of ethanol or dimethyl sulfoxide did not exceed 5% and did not cause any effect per se.

Statistical analysis

The results are presented as the mean ± s.e. mean of 4 to 5 animals. The percentages of inhibition were obtained in each individual experiment by comparison with the respective control time-point value. Statistical comparison of the data was performed by analysis of variance (ANOVA) followed by Newman-Keuls test or by use of Student’s unpaired t test when necessary. P-values less than 0.05 were considered as indicative of significance. The ED50 value for the BV (i.e. the dose of BV which caused 50% of oedema relative to maximal response) was estimated from individual experiments using the least square method via graphical interpolation.

Results

Paw oedema induced by subcutaneous injection of venom of Apis melifera

The s.c. injection of BV of A. melifera (0.1 to 5 µg/paw) elicited a dose- and time-dependent oedema formation that started at 20 min, peaked at the 30 min and decreased thereafter, being absent 24 h later (Fig. 1). The maximal oedema formation (0.942 ± 0.0 4 ml) was observed at 3 µg/paw at 30 min time-point and the calculated mean ED50 value was 1.5 µg/paw.

The heating of the BV of A. melifera for 5 min at 100°C significantly reduced its ability to induce paw oedema formation. The percentages of inhibition for these effects were 47 ± 1, 43 ± 2, 38 ± 5, 47 ± 4, 63 ± 4% at 0.5, 1, 2, 3 and 4 h time-points, respectively (results not shown).

Effects of several class of drugs on Apis melifera venom-induced paw oedema

The prior treatment of animals with compound 48/80 (12 µg/paw, 12 h beforehand) almost abolished the oedema formation caused by BV (Fig. 2 A). The percentages of inhibitions of the oedema were 85 ± 4, 88 ± 2, 90 ± 2, 88 ± 2 and 85 ± 3%, respectively at 0.5, 1, 2, 3 and 4 h. Pyrilamine (10 mg/kg, i.p. 30 min prior) also significantly inhibited oedema formation induced by BV (Fig. 2 B). The percentages of inhibi-