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Main pathways of action of Brazilian red propolis on the modulation of neutrophils migration in the inflammatory process

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ABSTRACT

Background: Brazilian propolis is popularly used as treatment for different diseases including the ones with inflammatory origin. Brazilian red propolis chemical profile and its anti-inflammatory properties were recently described however, its mechanism of action has not been investigated yet.
Aim: Elucidate Brazilian red propolis major pathways of action on the modulation of neutrophil migration during the inflammatory process.
Methods: The ethanolic extract of propolis (EEP) activity was investigated for neutrophil migration into the peritoneal cavity, intravital microscopy (rolling and adhesion of leukocytes), quantification of cytokines TNF-α, IL-1β and chemokines CXCL1/KC, CXCL2/MIP-2, neutrophil chemotaxis induced by CXCL2/MIP-2, calcium influx and CXCR2 expression on neutrophils.
Results: EEP at 10 mg/kg prevented neutrophil migration into peritoneal cavity ($p < 0.05$), reduced leukocyte rolling and adhesion on the mesenteric microcirculation ($p < 0.05$) and inhibited the release TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 ($p < 0.05$). EEP at 0.01, 0.1 and 1 µg/ml reduced the CXCL2/MIP-2-induced neutrophils chemotaxis ($p < 0.05$) without affect cell viability ($p > 0.05$).EEP at 1 µg/ml decreased the calcium influx induced by CXCL2/MIP-2 ($p < 0.05$). On the other hand, none of EEP concentrations tested altered CXCR2 expression by neutrophils ($p > 0.05$).
Conclusion: Brazilian red propolis appears as a promising anti-inflammatory natural product which mechanism seems to be by reducing leukocyte rolling and adhesion; TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 release; CXCL2/MIP-2-induced chemotaxis and calcium influx.

Introduction

Natural products from different sources have been widely used in the folk medicine and several biological properties had been scientifically demonstrated (Newman and Cragg, 2012). Furthermore, numerous studies have sought novel anti-inflammatory drugs that act by selectively blockage of neutrophils influx during inflammatory process (Bueno-Silva et al., 2013a; Franchin et al., 2013; Granica et al., 2015; Li et al., 2013). This fact is understandable due to the crucial role of neutrophils in local tissue damage and also because this episode takes place in different inflammatory diseases (Mackay, 2008).

Brazilian propolis has been investigated around the world and its different ethonopharmacological properties such as antimicrobial, anti-inflammatory, anti-nociceptive, hepato-protective and anti-cancer have been extensively investigated (Bueno-Silva et al., 2015; Bueno-Silva et al., 2013a; Bueno-Silva et al., 2013b; da Cunha et al., 2013; Franchin et al., 2012; Machado et al., 2012). Con-

Abbreviations: ANOVA, Analysis of variance; BRP, Brazilian red propolis; BSA, Bovine serum albumin; CEUA, Committee for Ethics in Animal Research; CXCL1/KC, Chemokine ligand 1; CXCL2/MIP-2, Chemokine ligand 2; CXCR2, Chemokine receptor 2; Dexa, Dexamethasone; DMSO, Dimethylsulphoxide; EDTA, Ethylenediamine tetraacetic acid; EEP, Ethanolic extract of propolis; GC–MS, Gas chromatographic-mass spectrophotometric; IL-1β, Interleukin 1 beta; i.p., Intraperitoneal; PBS, Phosphate buffered saline; PI, Propidium iodide; s.c., Subcutaneous; SPF, Specific-pathogen free; TNF-α, Tumor necrosis factor-alpha.

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sequently, it is used by population as food additive and/or dietary complement in products with beneficial properties to human health and in (bio)cosmetics (de Groot, 2013).

Among them, Brazilian red propolis (BRP) (Silva et al., 2008) had previously demonstrated a potent antimicrobial (Bueno-Silva et al., 2013b), antioxidant (Alencar et al., 2007) and anti-inflammatory activity on neutrophil recruitment (Bueno-Silva et al., 2013a; Lima Cavendish et al., 2015) and on lipopolysaccharide-activated macrophage (Bueno-Silva et al., 2015). It is important to note that BRP was recently divided into, at least, two sub-types: one from Sergipe state and another from Alagoas and Paraíba states (Lopez et al., 2014). Furthering, none of the studies above elucidated the mechanism of action on neutrophils migration.

Thus, the objective of this study was to elucidate the main routes of action of Brazilian red propolis on modulation of neutrophils migration during the inflammatory process.

Material and methods

Drugs and reagents

Ethanol, hexane and chloroform were purchased from Merck (São Paulo, SP, Brazil). Carrageenan, dexamethasone, DMSO, Fluo 3- AM, bovine serum albumin (BSA), RPMI-1640 medium, penicillin and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA); Fetal Bovine Serum was from Gibco (Grand Island, NY, USA); ApoScreen^{TM} Annexin V-FITC Kit was acquired from SouthernBiotech (Birmingham, AL, USA); anti-CXCR2, anti-Ly6G and CXCL2/MIP-2 were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of the ethanolic extract of Brazilian red propolis

A sample of propolis was obtained from Maceio, Alagoas State in the Northeast of Brazil (SL 09.40 and WL 35.41). The propolis sample (64 g) was extracted with ethanol 80% (800 ml) in water bath at 70 °C for 30 min and after filtration; the ethanolic extract of propolis (EEP) yielded 47 g (Bueno-Silva et al., 2013a).

HPLC quantification of BRP compounds

The extract was filtered (0.22 µm diameter Millipore, Billerica, MA, USA) and then 20 µl of sample was injected in the RP-HPLC system equipped with a Shimadzu ODS-A column (RP-18, column size 4.6 × 250 mm; particle size 5 µm) and a photodiode array detector (SPD-M10AVp, Shimadzu Co.). The column was eluted using a linear gradient of water/acetic acid (99.5/0.5 v/v) (solvent A) and methanol (100%) (solvent B), starting with 30% B and increasing to 40% B (15 min), 50% B (30 min), 60% B (45 min), 75% B (65 min), 75% B (85 min), 90% B (95 min), and decreasing to 30% B (105 min), at a solvent flow rate of 0.8 ml/min. Chromatograms were recorded at 260 nm (Alencar et al., 2007). The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: formononetin, daidzein, biochanin A, catechin, epicatechin, rutin, propyl gallate, ferulic acid and *p*-coumaric acid.

Animals

Animals used in every *in vivo* method described below were male, SPF (specific-pathogen free), Balb/c, mice weighing 18–22 g, were purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil). The mice were maintained in a room with controlled temperature (22 ± 2 °C), 12 h light/12 h dark and humidity of 40–60% with access to water and food *ad libitum*. Groups of *in vivo* experiments were composed by five animals. Dexametasone 2 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control while vehicle DMSO 0.1% as negative control. All experiments were conducted in accordance with the approval of the Institutional Committee for Ethics in Animal Research (CEUA/UNICAMP protocol number: 1484-1) and animals were scarified by cervical dislocation.

Experimental procedure to evaluate neutrophil migration

Neutrophil migration to peritoneal cavity experiment was carried out by EEP (1, 3 or 10 mg/kg) or dexamethasone 2 mg/kg administration by subcutaneous (s.c.) injection, 15 min before the inoculation of inflammatory stimuli by intraperitoneal (i.p.) injection of carrageenan at 500 µg/cavity in naive mice. The vehicle DMSO 0.1% was used as negative control. Mice were killed 4 h after the challenge and the peritoneal cavity cells were harvested by washing the cavity with 3 ml of phosphate buffered saline (PBS) containing EDTA 1 mM. In order to count the total number of cells, a Neubauer chamber was used. Smears were prepared using a Cytospin 4 (Thermo Fisher Scientific, Waltham, MA, USA), stained with Solution Panotic Kit (Laborclin, Pinhais, PR, Brazil) and cells were counted (100 cells total) using an optical microscope (100x). Results were expressed as the number of neutrophils per cavity (Dal Secco et al., 2006).

Intravital microscopy

Based on results of neutrophil migration experiment, we chose EEP 10 mg/kg to further analyze its anti-inflammatory properties. Initially, mice were pretreated with EEP 10 mg/kg and negative control as described before (s.c.) and after 30 min, all groups received the i.p. injection of carrageenan 500 µg/cavity. Leukocytes rolling and adhesion were rated by intravital microscopy after 2 or 4 h of the inflammatory stimulus, as previously described (Baez, 1969; Fortes et al., 1991).

Cytokines

Mice were treated with EEP (10 mg/kg, s.c.) and vehicle as the negative control, 30 min prior the injection of carrageenan 500 µg/cavity. Levels of TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 were determined by ELISA using the protocols supplied by the manufacturers (R&D Systems). The results were expressed as pg/ml (Franchin et al., 2013).

Isolation of neutrophils from mice bone marrow

Isolation of neutrophils from mice bone marrow was performed by Percoll gradient. Total bone marrow cells were taken from femur and tibia of mice. Then cells were washed 2 times, suspended in 2 ml Hanks (Sigma-Aldrich, St. Louis, MO, USA) and transferred to a Falcon tube containing 72% and 65% Percoll gradient. After centrifugation (1200 g, 35 min at 18 °C), the band formed between the gradient was collected and the cells were quantified using a Neubauer chamber. The purity was assessed using smear prepared with the aid of a Cytospin 4 (approximately 95% purity neutrophils) (Boxio et al., 2004).

Cell viability assay by flow cytometry

The flow cytometry analysis was performed on neutrophils isolated from mice bone marrow as described previously. Neutrophils were placed in sterile Eppendorf tubes at concentration of 1×10^6 cells/well pretreated with EEP (0.1; 1; 10 and 100 µg/ml) and incubated at 37 °C, 5% CO2, 1.5 h. Subsequently, the cells were washed,