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Proteins from the *Rhinella schneideri* parotoid gland secretion exhibit anti-nociceptive effect against nociception induced by inflammation

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ABSTRACT

As proteins isolated from the *Rhinella schneideri* parotoid gland secretion (RsPP) exhibit anti-inflammatory activity, the goal of this work was to investigate their anti-nociceptive effects using acetic acid-induced writhing, formalin, and hot-plate tests. The intraperitoneal administration of RsPP (2.5 or 5 mg/kg) one hour prior to stimuli significantly reduced the abdominal constrictions induced by acetic acid (73.06 and 72.69% inhibition, respectively) and the inflammatory phase of paw licking time induced by formalin (69.3% inhibition, at 2.5 mg/kg). However, RsPP (1, 2.5 or 5 mg/kg) did not change the latency in response at the hot-plate test. The involvement of inflammatory mediators on the anti-nociceptive effect of RsPP was further demonstrated. RsPP (2.5 mg/kg) significantly inhibited the inflammatory peak of paw edema induced by histamine (44.0%), bradykinin (51.3%), or prostaglandin E2 (53.7%). Our data indicate that RsPP may act on the pain process by inhibiting the effect of inflammatory mediators.

1. Introduction

Parotoid glands are external skin glands found on the back of some amphibians, characterized by the intense production of secretions, which may be discharged in response to a variety of stimuli [1,2]. These secretions are characterized by the presence of biogenic amines, alkaloids, peptides, and proteins [3–6] and are thought to play several roles, including the regulation of physiological functions of the skin or protective mechanisms against predators [7]. Many of the compounds extracted from parotoid gland have exhibited pharmacological properties, such as antibacterial, anticancer, anti-inflammatory, and anti-nociceptive effects [5,8].

In this context, our research group studied the biochemical profile and some biological activities of a water-soluble protein fraction (RsPP) isolated from the parotoid gland secretion of the *Rhinella schneideri* toad [6]. Using mass spectrometry, we demonstrated that RsPP is composed of a complex mixture of proteins, including carbohydrate-binding proteins (galectins), which can exhibit anti-inflammatory properties [9,10]. We showed that RsPP exhibited anti-inflammatory effects by reducing carrageenan-induced edema and myeloperoxidase activity in animal paw tissues, and decreased neutrophil migration and IL-1β release in the peritoneal cavity of animals [6]. Moreover, animals submitted to RsPP sub-chronic toxicity treatment did not present alterations in the blood levels of alanine amino transferase (AST), aspartate amino transferase (ALT), creatinine and urea when compared to the saline control group [6].

Taking into account the anti-inflammatory property of RsPP, the present study aimed to investigate the anti-nociceptive effect of this protein fraction using three different experimental models. Results were discussed based on the participation of different inflammatory mediators.

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2. Material and methods

2.1. Extraction of the parotoid gland secretion and protein isolation (RsPP)

Secretions from the *R. scheneideri* parotoid glands of adult males and females were collected in Petri dishes by means of manual compression of the glands as described by [6]. Both crude materials were pooled and submitted to dialysis against distilled water, for 48 h at 8 °C, using membranes with an 8000 Da cut-off. The lyophilized material was termed proteins from *Rhinella schneideri* parotoid gland (RsPP) and used in all experiments.

2.2. Animals

Male Swiss mice (*Mus musculus*) weighing 20–30 g were obtained from the central animal house of Federal University of Piauí, Brazil. The animals were housed in temperature-controlled rooms (25 ± 2 °C) with free access to food and water and were maintained under a 12-h light–dark cycle.

2.3. Acetic acid-induced writhing

Mice were treated with RsPP (1, 2.5 or 5 mg/kg; i.p.) and the animals received 0.6% acetic acid (10 mL/kg body weight; i.p.) one hour later. After 10 min of acetic acid administration, the number of constrictions was quantified over a period of 20 min by counting the total number of writhes and abdominal muscle contractions and hind paw extensions [11]. The control group received 0.9% sterile saline one hour before acetic acid administration. Subcutaneous (s.c.) administration of Morphine (5 mg/kg) was used as control group for anti-nociception.

2.4. Hot-plate test

Each mouse was placed twice onto a heated plate (55–56 °C), separated by a 30-min interval. The first trial familiarized the animal with the test procedure, and the second trial served as the control for the reaction time (licking the paw or jumping). Animals showing a reaction time greater than 25 s (sec) were excluded. After the second trial (control reaction time) mice were treated with RsPP (1, 2.5 or 5 mg/kg; i.p.) one hour before submitted to a plate heated. Measurements were performed at time zero (0 min) and at 30, 60, 90 and 120 min after RsPP administration. The hot plate cut-off time was 45 s to avoid animal paw lesions [13]. Control groups received 0.9% sterile saline (i.p.) or Morphine (5 mg/kg, s.c.) before the hot plate assay.

2.5. Formalin test

Mice were injected with RsPP (2.5 mg/kg; i.p.) and one hour after animals received 20 μL of 2.5% formalin into the right hind paws one hour later. The licking time was determined in two different time ranges after the intraplantar formalin administration: from 0 to 5 min (phase 1, neurogenic) and from 20 to 25 min (phase 2, inflammatory) [12]. Morphine (5 mg/kg, s.c.) was used as a reference drug.

2.6. Paw edema induced by different inflammatory agents

Mice were treated with RsPP (2.5 mg/kg; i.p.) and one hour later the paw edema was induced by injection of 50 μL of 0.9% sterile saline containing, histamine (50 ng/paw), serotonin (10 ng/paw), bradykinin (0.5 ng/paw), or prostaglandin E_2 (0.1 ng/paw) into the right hind paw. Control groups received 50 μL of 0.9% sterile saline before intraplantar injections of phlogistic agents. Paw volume was measured immediately before the irritant injection and at 30, 60, 90, and 120 min after injections of inflammatory mediators. Measurements were performed using a plethysmometer (Panlab, Barcelona, Spain) [14]. The anti-edematogenic effect of RsPP was calculated as inhibition of the edema in relation to the paw volume of the saline-treated animals: % inhibition of edema = [(Vt–Vo) "Control" – (Vt-Vo) "Treated")]/[(Vt-Vo) "Control"] x 100, where Vo and Vt indicate the times immediately before and after injection of inflammatory agents, respectively.

2.7. Statistical analysis

The results are presented as the means ± S.E.M of $n = 5–6$. For all experiments, the statistical analysis was performed through ANOVA followed by Newman Keuls tests. $p < 0.05$ was defined as statistically significant. Data were analyzed using GraphPad Prism 5 software.

2.8. Ethical approval

International ethical guidelines for scientific papers were followed in the preparation of this manuscript. Animals in this study were treated humanely and experiments were performed in accordance with the currently established principles for the care and use of research animals approved by the Ethics Committee of the Federal University of Piauí, Piauí, Brazil (No. 020/2012).

3. Results

The administration of acetic acid in the intraperitoneal cavity of animals induced intense abdominal writhing (43.33 ± 4.76 contortions). Pretreatment of animals with RsPP at doses of 2.5 and 5 mg/kg one hour prior to stimuli significantly reduced the abdominal contortions induced by acetic acid (73.06 and 72.69% inhibition, respectively) (Fig. 1). The dose of 1 mg/kg was not able to inhibit the contortions, evidencing that the inhibitory effect of RsPP on acetic acid-induced writhing was dose-dependent. As expected, morphine showed a potent analgesic effect ($p < 0.05$).

In the hot plate test, the different doses of RsPP (1, 2.5 and 5 mg/kg) did not increase the reaction time at the different intervals tested ($p > 0.05$) (Table 1). At 30, 60, and 90 min, morphine significantly increased the reaction times of mice, by 250.67%, 167.14%, and 123.51%, respectively.

Fig. 2 shows that intraplantar injection of formalin (2.5%/paw) significantly increased the total licking time in the first and second

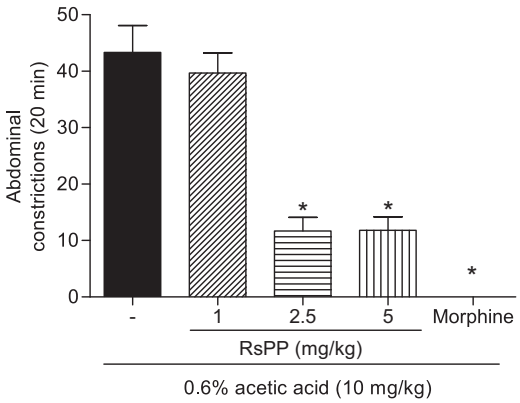

Fig. 1. The anti-nociceptive effect of proteins from *Rhinella schneideri* parotoid gland (RsPP) on acetic acid-induced writhing. Mice were treated with doses of 1, 2.5 or 5 mg/kg (i.p.) of RsPP 1 h prior to stimuli. Morphine (5 mg/kg, s.c.) was used as a positive control. The values are given as the mean ± S.E.M. ($n = 5–6$). *$p < 0.05$ compared to saline group (ANOVA followed by Neuman Keuls test).