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Tityus serrulatus venom increases vascular permeability in selected airway tissues in a mast cell-independent way

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ABSTRACT

Tityus serrulatus venom (Tsv)-induced pulmonary edema can occur in severe envenomation and the mechanisms involved are not completely understood. Therefore, we studied the effect of pharmacological modulation of the mast cell activation and the histamine antagonism on airways edema (investigated by Evans blue dye extravasation) and measured 5-hydroxytryptamine (5-HT) concentration in bronchoalveolar lavage fluid (BALF) in rats envenomed by Tsv. Additionally, the *in vitro* effect of Tsv on mast cells was studied using histological method and 5-HT release from mesenteric and peritoneal mast cells. We found that i.v. injection of Tsv increase vascular permeability in trachea, upper and lower bronchi and in lung parenchyma. This was not affected by ketotifen, a mast cell "stabilizer," or by pretreatment with pyrilamine (histamine H1 receptor antagonist). Moreover, 5-HT was not found in BALF of envenomed rats. *In vitro* experiments showed that Tsv did not induce mast cell degranulation nor release of 5-HT by mesenteric or peritoneal mast cells, in sharp contrast to preparations challenged by a mast cell activator, compound 48/80. In conclusion, our results show that Tsv causes strong edema in rat airways which is independent of mast cell activation and show that mast cells are not directly activated by Tsv.

1. Introduction

The human envenoming by scorpion sting is an important public health problem in several countries in tropical and subtropical regions. In Brazil, *Tityus serrulatus* is one of the most dangerous species causing severe envenomation, and when the victims are infants, death may occur (Freire-Maia et al., 1994). Local pain, hyperglycemia, hypertension, cardiovascular manifestations, and acute pulmonary edema are included among clinical signs, and the latter is associated with fatal outcome (Amaral et al., 1993; Bucaretchi et al., 1995). In a clinical report with fatal outcome of *T. serrulatus* scorpionism it was described an abundant plasma protein concentration in the tracheobronchial aspirate (Amaral et al., 1994); thus, it is possible that liquid accumulation into whole airways may have an important impact in respiratory distress developed in severe envenomed subject. In rats, Tsv was shown to induce edema in the whole lung preparation and it was shown that inflammatory mediators such as platelet-activating factor and cyclooxygenase-derived lipid mediators are important in this situation (Freire-Maia and Matos, 1993; De Matos et al., 1997). Although the cellular source of the inflammatory mediators involved in the pulmonary edema induced by *T. serrulatus* venom (Tsv) is not known, mast cells are good candidates. Mast cells are strategically poised in tissues that interface with external environment with ability to react within minutes and over hours to biological and chemical stimuli, producing several inflammatory mediators (histamine, lipid mediators, and cytokines).

We therefore set out to study the mast cell involvement on the increase of vascular permeability in airways selected tissues of rats envenomed by Tsv through the use of an antagonist of histamine H1 receptors because histamine has vasoactive properties promoting the increase in vascular permeability (Majno et al., 1969) and ketotifen, a mast cell "stabilizer" drug (Martin and Romer, 1978). Additionally, 5-HT (a mediator stored in rat mast cell) and 5-HIAA (5-hydroxyindole acetic acid) concentration in bronchoalveolar lavage fluid from envenomed rats also was evaluated. To further analyze the effect of Tsv on mast cells, we have quantified 5-HT release from mesenteric and peritoneal mast cells and evaluated mesenteric mast cell degranulation by histological examination.

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2. Materials and methods

2.1. Venom

Tsv lyophilized crude venom was kept at −20 °C. In the moment of use, it was diluted in sterile isotonic saline. The venom was supplied by Butantan Institute, São Paulo, Brazil.

2.2. Animals

Male Wistar rats (150–180 g) obtained from the Central Bioterium of Butantan Institute were housed at 22 ± 2 °C on a 12 h light/dark cycle with free access to standard chow and water. The animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA).

2.3. Evaluation of vascular permeability in airways of rats envenomed with Tsv

Increase in vascular permeability of airways was assessed using Evans blue dye extravasation method modified by Sirois et al. (1988). The dye (Evans blue, Inlab, Brazil) was diluted in isotonic saline and injected (20 mg/kg) in awake rats by intravenous (i.v.) route into the tail vein together with Tsv (200 μg/kg) or an equal volume of sterile isotonic saline (control group). These dose and route were based in previous studies with Tsv-induced lung edema (Freire-Maia and Matos, 1993; De Matos et al., 1997). Thirty minutes later, the animals were anesthetized by intraperitoneal (i.p.) route with an overdose of chloral hydrate (1000 mg/kg) (Merck, Brazil). The peritoneal cavity was opened, and the animals were killed by exsanguination. Before the isolation of the airways, the lung circulation was perfused with isotonic saline *via* a cannula inserted in the pulmonary artery, removing the intravascular dye. The whole airways were removed, and the trachea, upper and inner bronchi, and lungs were dissected. To avoid a possible and unwanted contamination of the upper airways with dye from lung edema, all tissues were rinsed in isotonic saline and dissected onto a filter paper to remove possible contaminant liquid, especially into trachea and bronchi lumen. The tissues were divided in two parts and weighed: one was placed in formamide (4 mL/g) (Sigma Chemical Co., USA) to extract the dye, and the other part was left to dry at 60 °C (for at least 48 h). The Evans blue dye concentration in formamide was measured spectrophotometrically using a microplate reader at 620 nm. Values of dye extravasation in the tissues were expressed as micrograms of dye per gram dry weight.

The role of mast cells in the increase of vascular permeability airways induced by Tsv was assessed by treating the animals with a mast cell "stabilizer" agent, ketotifen (5 mg/kg) (Sigma Chemical Co., USA). We investigated the participation of histamine, a vasoactive amine stored in mast cell granules, on Evans blue dye extravasation to airways by treating the animals with pyrilamine (6 mg/kg) (Sigma Chemical Co., USA), a histamine H1 receptor antagonist. The drugs were diluted in sterile NaCl 0.9% and injected i.v. 30 min before envenomation. Additionally, to test the effect of pyrilamine on histamine-induced extravasation of Evans blue in rat airways, a group of animals pretreated with pyrilamine (6 mg/kg, i.v.) received histamine (2 mg/kg, i.v.) (Sigma Chemical Co., USA) 30 min later. Control group received sterile NaCl 0.9% 30 min before histamine injection.

2.4. Assessment of airways mast cell activation in Tsv-envenomed animals

A group of rats received Tsv injection (200 μg/kg i.v.), and after 30 min, they were anesthetized i.p. with an overdose of chloral hydrate (1000 mg/kg) and killed by exsanguination cutting of the abdominal aorta, and the lung circulation was perfused with isotonic saline (20 mL/min) through a cannula inserted into the pulmonary vein. A cannula was inserted into the trachea, and bronchoalveolar lavage was done with 3 mL of isotonic saline; the bronchoalveolar lavage fluid (BALF) was collected, centrifuged (1000 rpm/10 min), and prepared as described below.

2.5. Challenge of peritoneal cells and mesentery

The peritoneal cavity of rats killed by CO_2 inhalation and exsanguinated was washed with 15 mL of cold Tyrode. The peritoneal fluid was collected and centrifuged at 800 rpm for 10 min at 4 °C, and the cell pellet was resuspended in 1 mL of Tyrode. Aliquot of the wash was used to determine total cell count in a Neubauer chamber after dilution (1:20, v/v) in Turk solution (0.2% crystal violet dye in 30% acetic acid); 5×10^5 cells/mL were incubated with Tyrode at 37 °C (negative control), 25 μg/mL of compound 48/80 (a mast cell activator agent, positive control), or 60 μg/mL of Tsv for 30 min at 37 °C atmosphere with 5% of CO_2. The cells were then centrifuged at 800 rpm for 10 min at 4 °C, and the supernatants were collected. A cell viability assay was performed using Trypan blue exclusion; it was done with cell suspension (5×10^5 cells) incubated with Tsv using 0.1% Trypan blue; the cell viability was never less than 95%.

In another set of experiments, the mesentery was removed and divided into three pieces placed each one in a well of a 12-well plate. To each well was added 1 mL of Tyrode solution (137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2 2H_2O$, 0.32 mM $NaH_2PO_4H_2O$, 5.56 mM glucose, and 1.16 mM $NaHCO_3$): in the first one, only Tyrode solution; in the second, Tyrode containing 50 μg of mast cell activating agent, compound 48/80 (Paton, 1951); and the third, Tyrode with 60 μg of Tsv. The plate was incubated at 37 °C atmosphere with 5% of CO_2 and gently shaken at each interval of 5 min. After 30 min, the Tyrode was recovered and centrifuged at 800 rpm for 10 min at 4 °C, and the supernatant was collected. The samples collected from peritoneal cells or mesentery were prepared as described in section sample preparation for 5-HT dosage in high-pressure liquid chromatography.

2.6. Histological assessment of mesenteric mast cell degranulation

The peritoneal cavity was opened as previously described and mesentery from rats were excised and placed in wells containing Tyrode alone or with 48/80 or Tsv and, after 30 min, were stained with a toluidine blue (Sigma Chemical Co., USA) solution composed of 33% water, 50% ethanol, 10% formaldehyde, 5% acetic acid (v/v), and 2% toluidine blue (w/v). After 15 min, the mesentery was blotted dry on filter paper and then divided into approximately 3–4 equal pieces. These were mounted on a glass slide, care being taken not to fold or stretch the tissue. Mast cell degranulation was expressed as the proportion (as %) of mast cells with extruded granules relative to the total mast cells present in 12 microscopic fields (250× magnification), per piece of mesentery. With approximately 35 mast cells per microscopic field, the total number of mast cells counted per mesentery was 900–1000. In rats, degranulated cells can be visualized because the granules are still stained by the dye when they have been discharged from the cell and are extracellular (Mota and Dias da Silva, 1960).

2.7. Evaluation of 5-HT release and 5-HIAA accumulation

The evaluation of 5-HT and 5-HIAA was performed with modification of the method previously described (Chiavegatto et al., 2008; Conceição et al., 2005). Ten microliters of a solution containing 1 M perchloric acid, 0.2% sodium metabisulfite, and 0.2%