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Neurotoxicological effects of a thrombin-like enzyme isolated from *Crotalus durissus terrificus* venom (preliminary study)

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Abstract

A thrombin-like enzyme, purified from the venom of *Crotalus durissus terrificus* by gel filtration and affinity chromatography, showed a single protein band in Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) with a molecular weight of about 33 kDa. Clear cellular morphological changes, deep ganglioside level modifications in some brain areas and behavioral alterations in pup rats injected with this protein were detected. Ganglioside composition, one of the chemical markers of brain maturation, was altered specially in the hypothalamus, hippocampus and prefrontal cortex. The most reliable behavioral effects were a delayed, maturation of the righting reflex, posture and motor response after treatment. These effects were consistent with the histological changes revealed in the cerebellum and prefrontal cortex of treated neonate rats, areas related to motor activities.
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Keywords: Snake venom; *Crotalus durissus terrificus*; Thrombin-like enzymes; Neurotoxicity; Developing rats

1. Introduction

Thrombin-like enzymes (serine-type protease) of the venoms of snakes of the families *Crotalidae* and *Viperidae* snake have been isolated, characterized and widely studied due to their clinical and biochemical importance (Markland and Damus, 1971; Stoker, 1978; Seeger and Ouyang, 1978; Raw et al., 1986; Smolka et al., 1998; Martins et al., 2002).

Gyroxin, a thrombin-like enzyme isolated from the venom of *Crotalus durissus terrificus* (*Cdt*) by Raw et al. (1986) and by Maruñak et al. (2004) produces the "gyroxin syndrome" in mice (barrel rotation) with a variety of symptoms, such as rolling behavior alternated with periods postration (Chon and Chon, 1975), hypoactivity followed by loss of righting reflex, opisthotonus, spastic distortions and

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rotation around the long axis (Alexander et al., 1988), brief periods of hyperexcitation and running, or paleness and chachypnea. Some mice show immobility and stretching of posterior limbs and grooming behavior (Barrabín et al., 1978).

The purpose of this study was to investigate the neurotoxic effects of the thrombin-like enzyme from the venom of *Cdt* from Argentina. We have used a combination of behavioral studies, histology and neurochemistry and concentrated on the period of development marked by extensive neuritogenesis and synaptogenesis (Maccioni et al., 1988; Tettamanti and Riboni, 1993).

2. Materials and methods

2.1. Sample preparation

Cdt venom was pooled from 10 specimens of 8/10-year-old adult snakes held in the serpentarium of the local Zoo, Corrientes, Argentina. The venom was lyophilized and kept frozen at −20 °C.

2.2. Purification of the thrombin-like enzyme

The purification of the thrombin-like enzyme was performed employing a gel filtration method (Alexander et al., 1998) with some modifications. The second step was performed by affinity chromatography in a Benzamidine-Sepharose 6B column, and the thrombin-like enzyme was eluted from the column, with a 0.1 M sodium acetate buffer (pH 5.0) containing 0.15 M NaCl (Camillo et al., 2001). The elution was monitored at 280 nm and enzymatic assays were carried out as described below. From 1.0 g of the whole venom, we obtained 7.0 mg of purified enzyme.

2.3. Analytical procedures

Protein concentrations of venom fractions were determined by measuring the absorbance at 280 nm in a UV–visible CamSpec M 330 spectrophotometer with an optical path of 1 cm, based upon the assumption that the absorption value of 1.0 equals protein concentration of 1 mg/ml.

2.3.1. Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE)

Purified thrombin-like fraction and crude venom samples were diluted with phosphate buffered saline solution, pH 7.2 containing 1% (w/v) SDS, 0.05% (w/v) 2-mercaptoethanol, 5% urea and 0.005% bromophenol blue. Samples were pretreated in reducing conditions at 100 °C for 3 min in order to assay the isolated enzyme for purity, electrophoresis was performed on 12% polyacrilamide slab gel following the method of Laemmli (1970). Protein bands were revealed by Coomassie Brilliant Blue R-250 staining procedure. Molecular weight markers were run in parallel. They were: phosphorylase B (97,400 mol wt), bovine serum albumin (66,200 mol wt), ovoalbumin (45,000 mol wt), carbonic anhydrase (31,000 mol wt), soybean trypsin inhibitor (21,500 mol wt) and lyzosyme (14,400 mol wt).

2.3.2. Thrombin-like activity

Clotting time was determined by mixing 0.2 ml of diluted bovine plasma (3:1 with 20 mM Tris–HCl buffer, pH 8.0) with 0.1 ml of either crude venom or purified venom fractions at 37 °C (Raw et al., 1986) with minimal changes in the quantities and reagents. Clotting times obtained were translated into NIH thrombin-equivalent units by means of a standard thrombin–fibrinogen curve (Lundblad et al., 1976). The active fractions were pooled and characterized using tests for neurotoxic activity in vivo, chemical and histological studies as described below.

2.4. Animals and treatment

Exposure to chemicals during the development period can result in a plethora of effects, ranging from death, gross structural abnormalities or behavioral changes to more subtle effects, so neonatal rats were chosen for the behavioral experiments used in this study. Nulliparous (Wistar rats) 3 months old, were separately placed with fertile males on the proestrus night, and the presence of spermatozoa was checked in a vaginal smear the next morning. That day was denoted as gestation day 0 (GD 0). At this time, pregnant females were individually housed in plastic breeding cages in a temperature-controlled nursery (22–24 °C) and maintained on a 12-h light/dark cycle with access to food and water ad lib. As parturition approached, dams were checked for birth twice daily. Twenty-four hours following parturition (parturition is postnatal day (PND) 0) litters were examined, weighed and culled to eight pups, with equal representation of sex within litter whenever possible and the pups were then randomly divided into two groups of eight pups. The

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