Toxicon 109 (2016) 18–25

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Evaluation of the *in vivo* thrombolytic activity of a metalloprotease from *Bothrops atrox* venom using a model of venous thrombosis

Anna L. Jacob-Ferreira [a, *], Danilo L. Menaldo [a], Carolina P. Bernardes [a], Marco A. Sartim [a], Célio D. de Angelis [b], José E. Tanus-Santos [c], Suely V. Sampaio [a, *]

[a] Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil
[b] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil
[c] Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 27 July 2015
Received in revised form
29 October 2015
Accepted 4 November 2015
Available online 7 November 2015

Keywords:
Alpha2-macroglobulin
Batroxase
Metalloproteases
Snake venom
Thrombolytic agents
Venous thrombosis

ABSTRACT

Background: Due to the importance of blood coagulation and platelet aggregation in brain- and cardiovascular diseases, snake venom proteins that interfere in these processes have received significant attention in recent years considering their potential to be used as models for new drugs.
Objectives: This study aimed at the evaluation of the *in vivo* thrombolytic activity of Batroxase, a P–I metalloprotease from *Bothrops atrox* venom.
Methods: *In vivo* thrombolytic activity of Batroxase was tested on a model of venous thrombosis in rats, with partial stenosis of the inferior vena cava, and vessel wall injury with ferric chloride at 10% for 5 min. After formation of the thrombus, increasing amounts of Batroxase were administered intravenously. The prescription medication Alteplase (tissue-type plasminogen activator) was used as positive control for thrombolytic activity, while saline was used as negative control. Bleeding time was assessed with a tail bleeding assay.
Results: Batroxase presented thrombolytic activity *in vivo* in a concentration-dependent manner, with 12 mg/kg of the metalloprotease causing a thrombus reduction of 80%, a thrombolytic activity very similar to the one observed for the positive control Alteplase (85%). The tail bleeding time was not altered by the administration of Batroxase, while it increased 3.5 times with Alteplase. Batroxase presented fibrinolytic and fibrinogenolytic activities *in vitro*, which were inhibited by alpha 2-macroglobulin.
Conclusion: Batroxase presents thrombolytic activity *in vivo*, thus demonstrating a possible therapeutic potential. The inactivation of the metalloprotease by alpha 2-macroglobulin may reduce its activity, but also its potential side effects, as seen for bleeding time.

1. Introduction

The interest in studying the chemical and functional characteristics of toxins isolated from snake venoms is not only due to their relevance in envenomations, but also due to their potential as bioactive molecules that may be used as models for new drugs to the treatment of different pathological processes (Cologna et al., 2009; Rodrigues et al., 2009).

Snake venom metalloproteases (SVMPs) are zinc-dependent enzymes, capable of degrading proteins of vessel membranes, allowing blood extravasation, being one of the main responsible for the hemorrhagic effect characteristic of envenomations by Viperidae snakes (Fox and Serrano, 2005). The toxic effects of these enzymes are also related to the pathogenesis of local myonecrosis and tissue damage (Gutiérrez et al., 1995; Rucavado et al., 1999) and inflammatory reactions (Teixeira et al., 2005; Zychar et al., 2010).

Several SVMPs are able to affect hemostasis by acting on basement membrane components and by degrading or activating

* Corresponding authors. Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, FCFRP-USP, Av. do Café, s/n°, CEP 14040-903, Ribeirão Preto, São Paulo, Brazil.
E-mail addresses: jacob_ferreira@yahoo.com.br (A.L. Jacob-Ferreira), suvilela@usp.br (S.V. Sampaio).

http://dx.doi.org/10.1016/j.toxicon.2015.11.002
0041-0101/

specific factors of the coagulation cascade (Braud et al., 2000). Recent studies have explored the potential of fibrin(ogen)olytic SVMPs as thrombolytic agents. These enzymes act directly on fibrin, degrading clots and preventing the formation of new clots. For this reason, fibrin(ogen)olytic SVMPs have been considered as potential drugs for treatment of patients with vascular diseases, as described for Fibrolase and its recombinant form Alfimeprase (Deitcher and Toombs, 2005; Sajevic et al., 2011).

Batroxase is a metalloprotease isolated from *Bothrops atrox* snake venom, recently purified and biochemically characterized by our research group (Cintra et al., 2012). This enzyme comprises about 1% of the venom of *B. atrox*, has pI 7.5 and molecular mass of approximately 22.9 kDa and 27 kDa (determined by mass spectrometry and SDS-PAGE, respectively), and was classified as a PI-class metalloprotease (formed only by a pro-domain and a proteolytic domain). Batroxase is capable of degrading components of the extracellular matrix, such as type IV collagen and fibronectin, as well as components of the coagulation cascade, as fibrinogen and fibrin. By being able to cleave fibrin independently of the conversion of plasminogen to plasmin, Batroxase would possibly have thrombolytic activity.

Considering the importance of blood coagulation in cardiovascular and cerebrovascular disorders, toxins from snake venoms, especially SVMPs, have received considerable attention in recent years, as being able to interfere with these processes. The study of theses toxins is necessary to start the exploitation of its therapeutic potential for future development and production of new drugs. Thus, the present study aims to assess the potential of Batroxase as a thrombolytic agent *in vivo*, in search of a possible therapeutic application for this molecule.

2. Methods

2.1. Snake venom and other materials

B. atrox venom was acquired from Centre of Extraction of Animal Toxins (Morungaba-SP, Brazil). The equipment and other materials were described in the course of the methodology, and unspecified reagents used were of analytical grade.

2.2. Animals

Male Wistar rats (250–270 g) were obtained from the Central Animal Facility of USP (Ribeirão Preto-SP), and kept under controlled conditions of temperature (24 °C) and brightness (12 h light/dark cycles), with free access to food and water. In the previous day from the venous thrombosis experiments, rats had their food removed, for a 16 h period (overnight), to allow the emptying of their intestine and favor the handling of the viscera during the surgical procedure. All experiments involving animals were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines and experimental protocols were approved by the Ethics Committee on Animal Use of Ribeirão Preto campus, University of São Paulo, protocol number 12.1.1809.53.2.

2.3. Isolation of Batroxase from B. atrox venom

Chromatographic fractionation of *B. atrox* venom to obtain the metalloprotease of interest, Batroxase, as well as its biochemical characterization, is described on our previous study (Menaldo et al., 2015). Briefly, the process is initiated by a size exclusion step on Sephacryl S-200, followed by anion exchange chromatography on DEAE Sepharose, both chromatographic resins were obtained from GE Healthcare. The fraction containing the metalloprotease was then ultrafiltered in concentrator tube with polyethersulfone membrane with cut-off of 3000MWCO, Vivaspin® 20 (Sartorius). The isolated protein was quantified using Bradford reagent (Sigma–Aldrich), according to the manufacturer instructions, separated in aliquots of 1 mg/tube, lyophilized and stored at −20 °C until its use in the experiments.

2.4. Inhibition of Batroxase's enzyme activity

2.4.1. Interaction between Batroxase and α2-macroglobulin

The interaction between Batroxase and α2-macroglobulin was tested according to the methodology described by Souza et al. (2001), with modifications.

Batroxase (23 kDa) was incubated with human α2-macroglobulin (725 kDa, Sigma) in stoichiometric proportion of 1:1 (0.15 μg Batroxase: 4.735 μg α2-macroglobulin) for 10 min at 37 °C. The reaction was stopped by addition of reducing sample buffer, and the samples were then boiled for 5 min in a water bath. Samples were loaded on a 7% SDS-PAGE gel for the analysis of the interaction of Batroxase with α2-macroglobulin. Samples of isolated Batroxase and α2-macroglobulin were used as controls. These experiments were performed in 3 separate times.

2.4.2. Inhibition of Batroxase's fibrinogenolytic activity by α2-macroglobulin

The fibrinogenolytic activity of Batroxase was determined according to the methodology described by Edgar and Prentice (1973), with modifications.

Bovine fibrinogen solution in 2 mM Tris–HCl, pH 7.4 (Sigma) (25 μg) was incubated for 60 min at 37 °C with Batroxase (0.333 μg), preincubated or not with α2-macroglobulin in stoichiometric ratio of 1:1 (0.333 μg Batroxase: 10.5 μg α2-macroglobulin) for 10 min at 37 °C. The reaction was stopped with the addition of denaturing sample buffer followed by boiling for 5 min in a water bath. Samples were loaded on a 10% SDS-PAGE gels for analysis of the inhibition of fibrinogenolytic activity of Batroxase by α2-macroglobulin. Isolated samples of fibrinogen (25 μg), Batroxase (5 μg) and α2-macroglobulin (5 μg), besides the fibrinogen solution incubated with α2-macroglobulin without Batroxase (25 μg fibrinogen: 10.5 μg α2-macroglobulin) were used as control. These experiments were performed in 3 separate times.

2.4.3. Inhibition of Batroxase's fibrinolytic activity by rat serum

The inhibition of the fibrinolytic activity of Batroxase by rat serum globulins was evaluated by fibrinolytic activity on Petri dishes. The fibrinolytic activity assay was performed according to a previously described method, with modifications (Leitão et al., 2000).

Petri dishes were prepared with agarose (0.95%) containing 0.3% fibrinogen solution and 1 mg/ml thrombin in 50 mM barbital buffer pH 7.8. Batroxase (2.5 μg) was preincubated with increasing amounts of rat serum (4–16 μL) for 30 min at 37 °C, and then applied to the wells drilled in Petri dishes (5 mm diameter). These plates were incubated for 24 h at 37 °C to enable the formation of the halo of fibrinolysis. These experiments were performed in 3 separate times.

To obtain the rat serum utilized in this experiment, blood of an anesthetized male Wistar rat (250 g) was collected by cardiac puncture. The blood remained for 1 h at ambient temperature for retraction of the clot, and then was centrifuged at 3000 × g for 10 min to obtain serum.

2.5. Thrombolytic activity of Batroxase

2.5.1. Analysis of the thrombolytic activity of Batroxase, in vitro

The thrombolytic activity of Batroxase was tested in preformed