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A novel fibrinolytic metalloproteinase, barnettlysin-I from *Bothrops barnetti* (barnett´s pitviper) snake venom with anti-platelet properties

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ABSTRACT

Background: Viperid snake venoms contain active components that interfere with hemostasis. We report a new P-I class snake venom metalloproteinase (SVMP), barnettlysin-I (Bar-I), isolated from the venom of *Bothrops barnetti* and evaluated its fibrinolytic and antithrombotic potential.
Methods: Bar-I was purified using a combination of molecular exclusion and cation-exchange chromatographies. We describe some biochemical features of Bar-I associated with its effects on hemostasis and platelet function.
Results: Bar-I is a 23.386 kDa single-chain polypeptide with pI of 6.7. Its sequence (202 residues) shows high homology to other members of the SVMPs. The enzymatic activity on dimethylcasein (DMC) is inhibited by metalloproteinase inhibitors *e.g.* EDTA, and by α2-macroglobulin. Bar-I degrades fibrin and fibrinogen dose- and time-dependently by cleaving their α-chains. Furthermore, it hydrolyses plasma fibronectin but not laminin nor collagen type I. *In vitro* Bar-I dissolves fibrin clots made either from purified fibrinogen or from whole blood. In contrast to many other P-I SVMPs, Bar-I is devoid of hemorrhagic activity. Also, Bar-I dose- and time-dependently inhibits aggregation of washed human platelets induced by vWF plus ristocetin and collagen (IC_{50} = 1.3 and 3.2 μM, respectively), presumably Bar-I cleaves both vWF and GPIb. Thus, it effectively inhibits vWF-induced platelet aggregation. Moreover, this proteinase cleaves the collagen-binding α2-A domain (160 kDa) of α2β1-integrin. This explains why it additionally inhibits collagen-induced platelet activation.
Conclusion: A non-hemorrhagic but fibrinolytic metalloproteinase dissolves fibrin clots *in vitro* and impairs platelet function.
General significance: This study provides new opportunities for drug development of a fibrinolytic agent with antithrombotic effect.

1. Introduction

At the site of vascular injury, circulating platelets adhere to several components of the subendothelial matrix, through the action of a number of adhesive receptors on their surfaces. Thus, platelets play a key role in hemostasis and are also involved in thrombus formation due to their unique ability to form stable adhesion contacts with the damaged vessel wall under conditions of rapid blood flow [1]. This capacity is mediated by the interaction of the platelet membrane glycoprotein (GP)Ib-IX-V complex with the subendothelial von Willebrand Factor (vWF) that can efficiently take place even under high shear flow, in small arteries and arterioles. The vWF-GPIb interaction has unique biomechanical properties that initiate platelet adhesive interactions over a broad range of hemodynamic conditions [1–2]. Furthermore, the integrin alpha2beta1 (α2β1) found in many cells is the receptor for different types of collagens and laminin. Its α2A-domain (α2I-domain) within the α2 subunit, which is homologous to vWF-A-domains, consists of approx. 200 residues and is responsible for mediating binding to collagen [3]. Both, α2β1 integrin and GPIb-IX-V-complex play a key role in platelet activation and aggregation. Therefore, they are naturally targeted by snake venoms. It is well established that viperid venoms have the ability to cause coagulopathy. Hence, some toxins of these venoms are likely to act directly on factors involved in blood

Abbreviations: SVMPs, snake venom metalloproteinases; MMP inhibitor III, matrix metalloproteinase inhibitor III; vWF, von Willebrand Factor; GP, glycoprotein; DMC, dimethylcasein; α2-M, α2-macroglobulin; RT, room temperature; α2β1, alpha2beta1 integrin; r α2β1A-domain, recombinant alpha2beta1 integrin A-domain; ECM, extracellular matrix; Fg, fibrinogen; FN, fibronectin; LM, laminin; BM, basement membrane; Dis, disintegrin.

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coagulation. Numerous active molecules including serine proteinases (SVSPs), metalloproteinases (SVMPs)/disintegrins, snake C-type lectin-like proteins (snaclecs) and others were identified in snakes and other animal venoms and were investigated in a search for pro- and antithrombotic/coagulant pharmaceuticals depending on the sites of potential intervention [4–5]. Several of these toxins are multifunctional and may act on multiple protein targets of their preys. Moreover, crude snake venoms are natural libraries of biomolecules targeting hemostasis and thrombosis. Several components of a venom synergistically disturb the complete hemostatic system and lead to cardiovascular shock. Particularly venoms from the viperidae (pit vipers and true vipers) family [6–7] cause these severe and detrimental effects. The most abundant proteins in viperid venoms are the SVMPs which damage vascular endothelium and have received special attention due to their associattion with the severe pathological effects [8–10]. SVMPs are zinc endopeptidases phylogenetically most closely related to the mammalian ADAM (**a** **d**isintegrin **a**nd **m**etalloproteinase) family. Together with ADAM and the related ADAMTS (with trombospondin motifs) they constitute the reprolysin subfamily of the metzincin superfamily of metalloproteinases [10–13]. SVMPs are classified into three classes (P-I to P-III), based on their domain structure and are subdivided into several subclasses. P-I SVMPs have an elementary structure as their mature structure consists of the catalytic metalloproteinase (MP) domain only [14–16]. In addition to the MP domain, members of the P-II class have a C-terminal disintegrin domain (Dis). Several disintegrins released from precursors P-II MPs have an RGD motif, which mediates the interaction with integrins and thus may have many potential aplications [7,17]. P-III members contain the MP, disintegrin-like and cysteine rich domains and are the most intriguing proteinases in terms of their complexity and function. Their structure is similar to a group of membrane bound glycoproteins involved in cell-cell and cell-matrix adhesion and signaling [10,12]. P-III SVMPs are further divided into subclasses based on their distinct post-translational modifications including homo-dimerization (P-IIIc) or proteolysis between the MP and disintegrin domains (P-IIIb). The P-IV class SVMPs containing an additional snake C-type lectin-like (snaclec) motif represents another post-translational modification of the P-III structure (subclass P-IIId), however no P-IV mRNA transcripts have been identified [12].

Based on their ability to induce blood vessel damage and consequently hemorrhage, members of P-I class are further divided into a two subclasses: the hemorrhagic subclass P-IA such as acutolysin A from *Agkistrodon acutus* [13], atroxlysin-I from *B. atrox* [14] and others, whereas the members of subclass P-IB have only weak (or no) hemorrhagic effect, *e.g.* leucurolysin-a (leuc-a) from *B. leucurus* [15], mutalysin-II (mut-II) from bushmaster (*Lachesis muta*) [16] and barnettlysin-I (this work). In the present study we have identified a new P-I class SVMP termed barnettlysin-I (Bar-I) from *Barnett's pit viper* venom. The amino acid sequence of Bar-I has been determined and shows high sequence similarity with homologous SVMPs. *In vitro* the enzyme showed hydrolytic activity on some plasma and extracellular matrix (ECM) proteins. Yet, it does not induce hemorrhages using the mice skin model. Acting thrombolytically, Bar-I cleaves fibrin independent of plasminogen activation. In addition, Bar-I inhibited collagen- and plasma vWF-induced platelet aggregation by cleaving not only the vWF and its receptor GPIb but also the collagen receptor $\alpha2\beta1$ integrin. The platelet interactions of both GPIb/IX/V with vWF and $\alpha2\beta1$ integrin mark early events in the activation of platelets, especially under high shear rates of the arterial blood flow. The effective receptor-cleaving antithrombotic effect together with the direct fibrinolytic activity highlights Bar-I as a potential tool for the development of a new anti-hemostatic agent in ischemic diseases.

Bar-I is a component of the venom of *B. barnetti* (Barnett's lancehead). This endemic medium size pit viper predominantly inhabits the arid to semi arid tropical scrub of the Pacific coast desert of northern Peru [18–19]. It is an important poisonous snake implicated in human snake bite accidents particularly in rural areas with farmers, herdsmen and children being the major groups at risk [20–22]. Among the Latin American snakes, the genus *Bothrops* (subfamily Crotalinae of Viperidae) comprises 32 or 37 species of neotropical pit vipers commonly referred to as lanceheads, which are widely distributed in tropical and non tropical Latin America [18–19]. They occupy a vast array of habitats, from deserts to tropical lowlands and up to mountains. Moreover, all *Bothrops* snakes are venomous and responsible for more human morbidity in the New World than any other poisonous snake [19–21]. Previously, we have proteomically characterized the toxin composition of *B. barnetti* venom and compared it with the venoms of two other Peruvian pit vipers of medical importance *Bothrops atrox* and *B. pictus* [23]. The main components of *B. barnetti* venom are represented by SVMPs (74.1%) including the P-III and P-I classes, SVSPs (6.7%), phospholipases A_2 (PLA_2s, 6.4%), disintegrins (5.5%), snaclecs (3.3%), and others. A fibrinogen coagulant serine proteinase called barnettobin which acts as a defibrinogenating agent *in vivo* have been described [24]. Deciphering the complex mixture of highly active components of the snake venom and understanding their molecular mechanism increases the chances to utilize the single components in treating hemostatic and thrombotic disorders. Especially, antithrombotic and fibrinolytic molecule, such as Bar-I, which lack hemorrhagic activity, are of medical interest.

2. Materials and methods

2.1. Purification of barnettlysin-I

Bar-I was purified by a two-step purification procedure with size exclusion on Sephacryl S-200 and ion exchange chromatography on CM Sepharose CL-6B. Fractions were analyzed by SDS-PAGE and were tested for enzymatic activity by using dimethylcasein (DMC) and fibrin as substrates. Briefly, one g of crude venom was dissolved in 6 ml of 50 mM ammonium acetate buffer, pH 7.4, containing 0.3 M NaCl and centrifuged at 6000× g (10 min at 4 °C) to remove the insoluble material. The supernatant solution (948 mg protein) was loaded onto a Sephacryl S-200 (2.5 × 100 cm) column and eluted with the above buffer at 6 ml/h at 4 °C. The active fractions containing DMC and fibrin degrading activities were pooled, dialyzed against distilled water containing 1 mM $CaCl_2$ and lyophilized. The active material (94 mg) still containing impurities was applied to a CM Sepharose (1.0 × 20 cm) column, equilibrated with 25 mM Hepes pH 7.4, 1 mM $CaCl_2$ buffer. The bound proteins were eluted with a 0–0.3 M NaCl gradient in the same buffer at a flow rate of 12 ml/h. The purified protein named barnettlysin-I was pooled, dialyzed and lyophilized. The experiments reported here were conducted according to the guidelines established by the Brazilian College for Animal Experimentation and aproved by the local Ethics Committee.

2.2. Protein characterization

For purity analysis, 100 μg of the protein obtained from the CM Sepharose column was applied onto a RP-HPLC C4 (4.6 × 250 mm) column, equilibrated with aqueous 0.1% trifluoroacetic acid (TFA). The protein was eluted with a gradient of acetonitrile (ACN) (0–60%) in 0.1% TFA. The molecular mass was determined by polyacrylamide gel electrophoresis (12%) by the standard method of Laemmli and by matrix-assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry. The amino acid sequence was determined by automatic Edman degradation using a Shimadzu PPSQ-21A protein sequencer according to the manufacturer's instructions.

2.3. Sequence determination

Purified Bar-I (3 mg) was reduced and alkylated with 4-vinyl pyridine as reported [25]. One mg-aliquots of Bar-I was dissolved in 1 ml of 0.1 M Tris–HCl (pH 8.6), 6 M guanidine-HCl. After addition of 30 μl