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Investigating possible biological targets of Bj-CRP, the first cysteine-rich secretory protein (CRISP) isolated from *Bothrops jararaca* snake venom

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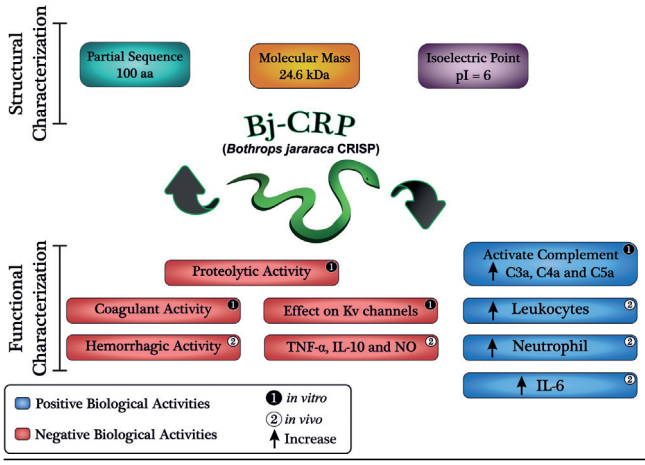
HIGHLIGHTS

- Bj-CRP is the first cysteine-rich secretory protein (CRISP) from *Bothrops jararaca* venom.
- Bj-CRP is an acidic protein of 24.6 kDa with high identity to other snake CRISPs.
- Bj-CRP showed no effects on the hemostatic system, nor acted on potassium channels.
- Bj-CRP induced inflammatory responses *in vivo*, recruiting neutrophils and producing IL-6.
- Bj-CRP affected the complement system, acting on C3 and C4 and generating anaphylatoxins.

GRAPHICAL ABSTRACT



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ABSTRACT

Cysteine-rich secretory proteins (CRISPs) are commonly described as part of the protein content of snake venoms, nevertheless, so far, little is known about their biological targets and functions. Our study describes the isolation and characterization of Bj-CRP, the first CRISP isolated from *Bothrops jararaca* snake venom, also aiming at the identification of possible targets for its actions. Bj-CRP was purified using three chromatographic steps (Sephacryl S-200, Source 15Q and C18) and showed to be an acidic protein of 24.6 kDa with high sequence identity to other snake venom CRISPs. This CRISP was devoid of proteolytic,

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hemorrhagic or coagulant activities, and it did not affect the currents from 13 voltage-gated potassium channel isoforms. Conversely, Bj-CRP induced inflammatory responses characterized by increase of leukocytes, mainly neutrophils, after 1 and 4 h of its injection in the peritoneal cavity of mice, also stimulating the production of IL-6. Bj-CRP also acted on the human complement system, modulating some of the activation pathways and acting directly on important components (C3 and C4), thus inducing the generation of anaphylatoxins (C3a, C4a and C5a). Therefore, our results for Bj-CRP open up prospects for better understanding this class of toxins and its biological actions.

1. Introduction

Snake venoms are constituted by a mixture of biological compounds comprising hydrolytic enzymes, non-enzymatic proteins and inorganic molecules (Aird, 2002). The venom of *Bothrops jararaca* consists of several components, including proteins (about 80% of its dry weight), carbohydrates, lipids, metals, biogenic amines, nucleotides and free amino acids (Escalante et al., 2003). Many toxins have already been isolated and characterized from *B. jararaca* venom, among them phospholipases A_2, L-amino acid oxidases, serine and metalloproteinases. However, although proteomic analyses indicate the presence of cysteine-rich secretory proteins (CRISPs) in this venom (Zelanis et al., 2011, 2012), to date, there are no reports in the literature of CRISPs isolated from it or from any other *Bothrops* venom.

CRISPs are a large family of cysteine-rich secretory proteins related to various biological activities, including reproduction and blockage of ion channels (Gibbs and O'Bryan, 2007; Sunagar et al., 2012). These molecules are single-chain polypeptides of 20–30 kDa found in vertebrates, being particularly present in reptilian venom ducts (Fry et al., 2006; Yamazaki et al., 2003) and mammalian salivary glands, pancreatic tissues and reproductive tracts (Haendler et al., 1993; Roberts et al., 2007). CRISPs have a high degree of sequence similarity and 16 highly conserved cysteine residues that form 8 disulfide bonds, with 10 of these residues forming the cysteine-rich domain (CRD) at the C-terminus (Yamazaki and Morita, 2004). Based on the amino acid sequence homology, the CRISP family can be further classified into four subgroups (CRISP1, CRISP2, CRISP3 and CRISP4), three of which are found in most mammals (CRISP1: acidic epididymal glycoprotein, CRISP2: testis-specific protein 1, and CRISP3: specific granule protein), and a fourth type (CRISP4) that has been described only in mice (Sunagar et al., 2012).

Recent studies have revealed that CRISPs are widespread in snake venoms. Although clear evidences concerning the functions and molecular targets of these toxins are still scarce, some studies have suggested that they may function as ion channel blockers (Nobile et al., 1994, 1996; Brown et al., 1999; Yamazaki and Morita, 2004; Wang et al., 2006). Some venom CRISPs have been shown to interact with ryanodine receptors (RyRs), inhibiting the release of Ca^{2+} ions (Nobile et al., 1994; Morrissette et al., 1995), while other CRISPs were capable of blocking the activity of L-type Ca^{2+} and/or K^+-channels and of cyclic nucleotide-gated (CNG) ion channels, thereby preventing the contraction of smooth muscle cells (Yamazaki et al., 2003, 2002a; Brown et al., 1999; Wang et al., 2006).

Even though ion channels seem to be the main targets of snake venom CRISPs, other biological activities have also been described for these toxins, indicating that they could have many more biological targets to be explored. Patagonin, isolated from *Philodryas patagonienses* venom, demonstrated myotoxic activity when injected into the gastrocnemius muscle, but did not induce edema formation, hemorrhage or inhibition of platelet aggregation. Despite the myotoxicity, patagonin did not induce systemic alterations in mice or histological changes in tissues from the cerebellum, brain, heart, liver and spleen (Peichoto et al., 2009). Crovirin, a CRISP from *Crotalus viridis viridis* venom, showed promising activity against protozoa such as *Trypanosoma* and *Leishmania* (Adade et al., 2014). Natrin from *Naja atra* venom acted in the regulation of the expression of adhesion molecules in endothelial cells, thereby participating in inflammatory processes (Wang et al., 2010).

Considering the lack of information regarding CRISPs and their little known functions in snake venoms, our studies focused on the characterization and evaluation of possible biological targets related to Bj-CRP, a CRISP isolated from *B. jararaca* venom, which is, to the best of our knowledge, the first protein from this class of toxins isolated from the venom of a *Bothrops* species. Thus, besides the biochemical characterization of this molecule, we also investigated its effects on potassium channels, hemostasis, inflammatory processes and the human complement system.

2. Materials and methods

2.1. Materials

Bothrops jararaca venom was donated by the Butantan Institute (São Paulo, Brazil). Bovine serum albumin (BSA), ortho-phenylenediamine (OPD), IgM, mannan, lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Complement components C3, C4, Factor B (FB) were from Quidel Corp. (San Diego, CA, EUA) and C1-Inhibitor (C1-INH) from Genway Biotech Inc. (San Diego, CA, EUA). The chromatographic resins and reagents for the biochemical and enzymatic assays were obtained from GE Healthcare (Chicago, IL, USA), Merck (Kenilworth, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA). Other materials and equipment used were described throughout the methodology and reagents not specified were of analytical grade.

2.2. Animals

Male Swiss or C57BL/6 mice (20–25 g, 6–8 weeks old) were used to evaluate the hemorrhagic activity and inflammatory potential of Bj-CRP, respectively. Sheep blood was obtained from BioBoaVista Bioterium (Valinhos, SP, Brazil). Rabbit blood was collected according to Menaldo et al. (2013). All animals were bred and provided by the animal facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, FCFRP-USP. Animal care procedures were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines and the experimental protocols were approved by the Committee for Ethics on Animal Use (CEUA) from FCFRP-USP (Proc. n° 2014.1.226.53.5).

2.3. Human blood

Serum and plasma used in the experiments were obtained from human blood donated by healthy volunteers who were not using any medications, in accordance with the authorization of the