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Bothropoides insularis venom cytotoxicity in renal tubular epithelia cells

Clarissa P. Mello [a], Isabel C.O. Morais [b], Ramon R.P.P.B. Menezes [b], Gustavo J.S. Pereira [c], Alba F.C. Torres [a], Dânya B. Lima [a], Ticiana P. Pereira [a], Marcos H. Toyama [d], Helena S.A. Monteiro [b], Soraya S. Smaili [c], Alice M.C. Martins [a, *]

[a] Department of Clinical and Toxicological Analysis, Federal University of Ceará, Fortaleza 60430370, Ceará, Brazil
[b] Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil
[c] Department of Pharmacology, Federal University of São Paulo (UNIFESP/EPM), Brazil
[d] São Vicente Unit, Paulista Coastal Campus, São Paulo State University (UNESP), São Paulo, Brazil

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ABSTRACT

Bothropoides insularis (jararaca-ilhoa) is a native endemic snake limited to the specific region of Queimada Island, on São Paulo coast. Several local and systemic effects have been described due to envenomation caused by it, such as edema, tissue necrosis, hemorrhage and acute renal failure. Our previous studies have shown that *Bothropoides insularis* venom (*Bins*V) demonstrated important functional and morphologic alterations in rat isolated kidney, especially decrease in tubular electrolyte transport, osmotic clearance and tubular necrosis. In order to elucidate the direct nephrotoxicity mechanism, the aim of the present study was to investigate *Bins*V cytotoxicity effect on renal epithelial cells. The treatment with *Bins*V over MDCK culture decreased cell viability in all concentrations tested with IC_{50} of 9 μg/mL. *Bins*V was able to induce membrane rupture and cell death with phosphatidilserine externalization. Furthermore, *Bins*V induced ROS overproduction and mitochondrial membrane potential collapse, as well as Bax translocation and caspases 3 and 7 expression. Therefore, these events might be responsible by *Bins*V-induced cell death caused by mitochondrial dysfunction and ROS overproduction in the direct cytotoxicity process.

1. Introduction

Snake venoms contain a mix of peptides and proteins, polyamides, histamines and alkaloids used in self-defense and predatory strategies (Oliveira Junior et al., 2013), which effectively perturb vital physiological systems, especially those related to movement, respiration and circulation (Cho and Manjunatha, 2011) causing local and systemic damage, including acute kidney injury, an important component of systemic pathophysiology of envenomation by Viperidae snakes. Acute kidney injury (AKI) induced by snake venom is a frequent complication of *Bothrops* snakebite, showing relevant morbidity and mortality (Sitprija, 2006; Sgrignolli et al., 2011).

The pathogenesis of renal injury in snakebites is complex and many factors are involved in the development of this condition (Sgrignolli et al., 2011). These various factors could be due to a number of specific injuries that occur at systemic and cellular levels. Hypovolemia, hypotension and hypoperfusion, associated to thromboembolic events, are commonly related to its appearance (Albuquerque et al.,

* Corresponding author. Tel.: +55 85 33668263; fax: +55 85 33668292.
E-mail address: martinsalice@gmail.com (A.M.C. Martins).

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2013), leading to tubular cell damage. Additionally, it has been hypothesized that this tubular damage is also caused by direct action of toxin presents in bothropic venoms (De Morais et al., 2013). After the initial kidney injury, the organ develops a molecular response that determines cell fate. Some effects are identified in the cell undergoing renal failure: cells may undergo necrosis, apoptosis, and cell division or behave indifferently to stress, with necrosis and apoptosis currently being the most often studied forms of cell death (Vieira, 2001).

The *Bothrops* genus and the newly designated genera *Bothriopsis*, *Bothrocophias*, *Bothropoides* and *Rhinocerophis*, which belong to the Viperidae family and are popularly known as pit vipers, have a wide distribution and correspond to the most important group of venomous snakes in number of species, population density and number of snakebites occurring in Brazil (Pinho and Pereira, 2001; Queiroz et al., 2008; Oliveira et al., 2010). Specific variations in composition and toxicity of these venoms are usually associated with geographic origin, habitat, climatic variations, diet and age of these snakes (Furtado et al., 2006; Queiroz et al., 2008).

Bothropoides insularis (jararaca-ilhoa) is a native endemic snake limited to the specific region of Queimada Island, on São Paulo coast (Valente et al., 2009; Sgrignolli et al., 2011). This venom has similar characteristics to other *Bothrops* and *Bothropoides*, causing local damage and systemic lesions, with a fourteen-fold greater potency (Valente et al., 2009).

Compared with other species of the same genus, *B. insularis* whole venom (*Bins*V) is still poorly studied. Transcriptomic and proteomic analyses have described that it consists mostly of fraction constituents, such as metalloproteinases, bradykinin-potentiating peptides, C-type lectins, serine proteases, phospholipases A_2 (PLA_2), vascular endothelial growth factors, L- amino oxidases (LAAO), cysteine secretory proteins, G_{10} proteins and neurotrophic growth factor (Valente et al., 2009). Recently, it was demonstrated that *Bins*V changes kidney function by promoting alterations in electrolyte transport and osmotic clearance, whereas morphological alterations in renal tubular cells have also been observed (Braga et al., 2006).

However, considering *Bins*V important functional and morphologic alterations in rat isolated kidney, there is scarce knowledge about cellular renal injury mechanism induced by *Bins*V. In this context, this study aimed to characterize the *in vitro* cytotoxic effect of *Bins*V on Madin–Darby Canine Kidney Cells (MDCK).

2. Methods

2.1. Venom, chemicals and drugs

B. insularis venom (*Bins*V) was kindly donated by Dr. Marcos H. Toyama, Universidade Estadual Paulista (UNESP). RPMI medium, Trifluorocarbonylcyanide Phenylhydrazone (FCCP), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Annexin V-FITC kit were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and tetramethylrhodamine ethyl ester (TMRE) was purchased from Molecular Probes (Eugene, OR, USA). For assays, *Bins*V was diluted in sterile Phosphate-Buffered Saline (PBS), pH 7.4. Caspase 3 (#96615) and Caspase 7 (#9492) antibodies were acquired from Cell Signaling (Danvers, Massachusetts, USA), and α-tubulin antibody (#T8203) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

MDCK cells were cultured at 37 °C and 5% of CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10,000 IU/mL) and streptomycin (10 mg/mL). Before each experiment, cells were kept in medium without FBS for 24 h to obtain cells in the G_0 phase of cell cycle. Cells were plated at 10^5 cells/mL and treated with *Bins*V in different concentrations to evaluate biological effects. This cell line maintains morphological and functional characteristics of distal and/or collecting tubule cells, which allows studying the intracellular action mechanisms of bioactive substances (Collares-Buzato et al., 2002).

2.3. Cytotoxicity assays

2.3.1. MTT assay

Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, after 24 h of treatment with *Bins*V, MTT was added (2.5 mg/mL) for 4 h. The solubilization of formazan crystals was performed with Sodium dodecyl sulphate (SDS) 10% in HCl 0.01N. After 17 h, absorbance at 570 nm was performed in a microplate reader (Biochrom® Asys Expert Plus). Cell viability was calculated in comparison with control group. The IC_{50} (venom concentration able to inhibit 50% of cell growth) was determined by non-linear regression.

2.3.2. Lactate dehydrogenase (LDH) assay

In order to evaluate membrane damage induced by the venom, culture supernatant was collected after 24 h of incubation with *Bins*V, to determine Lactate Dehydrogenase (LDH) release, using a commercial kit (TOX 7, Sigma–Aldrich®, St. Louis, USA). In this method, LDH present in samples causes the conversion of lactate into pyruvate, with consequent reduction of NAD^+ to $NADH^+H$. NADH is then used to convert iodotetrazolium chloride (INT) into a colored product (Fotakis and Timbrell, 2006).

2.4. Annexin V-FITC and propidium iodide (PI) staining

After 24 h, *Bins*V-treated cells were stained with fluorescein isothiocyanate (FITC) conjugated to annexinV/propidium iodide (PI) according to the manufacturer's instructions (AnnexinV/FITC Apoptosis Detection Kit, BD Pharmigen, CA, USA). The populations of annexinV^-PI^- viable cells and annexinV^+ apoptotic and annexinV^-PI^+ necrotic cells were analyzed in a FACSCalibur Flow Cytometer (BD, New Jersey, USA) (10^5 events).

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