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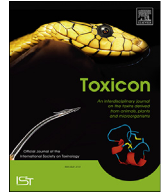
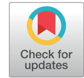


Local and systemic effects of BdipTX-I, a Lys-49 phospholipase A_2 isolated from *Bothrops diporus* snake venom



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ABSTRACT

The present work aimed to isolate a basic phospholipase A_2 (PLA_2) from *Bothrops diporus* snake venom (BdV), evaluate and compare the myotoxic and oedema-inducing activities, as well as the systemic effects caused by both the isolated PLA_2 and BdV on *Swiss* mice. A Lys-49 PLA_2 (BdipTX-I) was obtained through two chromatographic steps: an ion-exchange and a reverse phase. The local (oedema and myotoxicity) and systemic (hepatic and renal functions) effects were then assessed for BdipTX-I and BdV. Results showed that the oedema-inducing activity was significant in all tested doses (5 and 20 μg/paw) for both BdipTX-I and BdV. Myotoxicity was evaluated by the increase of serum CK, CK-MB and LDH, and results showed that BdV effect is more prominent than BdipTX-I effect. The systemic effects were evaluated by determining specific laboratory markers: AST, ALT, GGT, ALP, urea, creatinine, protein and calcium. BdipTX-I and BdV were able to induce renal changes in the experimental model, leading to proteinuria (induced both by BdipTX-I and by BdV) and uremia (induced only by BdV). Thus, it is concluded that the systemic effects of BdV and BdipTX-I occur differently.

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1. Introduction

The World Health Organization (WHO) estimates that at least 2,5 million cases of snakebite envenoming occur annually around the world, which cause approximately 100,000 deaths (Chippaux, 1998; Kasturiratne et al., 2008). This became an important public health problem, particularly in rural areas of tropical and sub-tropical countries, in Africa, Asia, Oceania and Latin America being recently added to the WHO's neglected diseases (WHO - World Health Organization, 2017).

In Brazil, about 27,261 snakebite envenoming was reported in 2014 (Brasil, 2017). Those belonging to the genera *Bothrops* and *Bothrocophias* account for about 90% of the snakebites (Bernarde, 2011). Envenoming by *Bothrops* causes oedema, haemorrhage and necrosis of the muscle tissue. These local effects develop quickly after bite and, consequently, a delay in access to health services often results in tissue damage that can lead to permanent disability. Systemic effects also occur. These include neurotoxicity, respiratory insufficiency, myoglobinemia, hyperkalemia, acute renal failure, cerebral haemorrhage, disseminated intravascular coagulation, cardiovascular shock caused by hypovolemia, vasodilation and direct effects on the myocardium (Del Brutto and Del Brutto, 2012; Gutiérrez et al., 2006).

Bothrops diporus belongs to the Viperidae family, inhabits marshes and preferably deciduous semi-tropical forests and pampas and is widely distributed around Central and South America. It is found in Argentina, Paraguay and Brazil (Silva, 2004). In Brazil, it is widely distributed from the Southwest to the Southern region of habitats (Minoli et al., 2011).

As for the *B. diporus* venom, several activities have already been described including neuromuscular blocking (Abreu et al., 2007) oedema, fibrinogenolytic, haemorrhagic and coagulant activity (Acosta de Pérez et al., 1998; de Oliveira et al., 2011), cytotoxic activity on C2C12 cell line (Bustillo et al., 2009). In addition, two recombinant acidic phospholipases A_2 ($BdsPLA_2$-I and $BdsPLA_2$-II)

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were obtained through cloning and expression (Yunes Quartino et al., 2012). Thus, the present study aimed to isolate and characterize a basic PLA_2 from *B. diporus*.

2. Materials and methods

2.1. Venom fractionation and PLA_2s purification

All the steps of protein isolation were conducted at the Centro de Biomoléculas Aplicadas à Saúde (CEBio, FIOCRUZ-RO/UNIR). *B. diporus* venom (BdV) used during the study was acquired from the Serpentarium Bioactive Proteins Ltda, Batatais-SP and kept dried and refrigerated (2–8 °C) on the Amazon Venom Bank at CEBio (authorization: CGEN/CNPq 010627/2011-IBAMA 27131-1 and 2).

PLA_2 isolation and purification was conducted as described by Soares et al. (1998). About 50 mg of *B. diporus* venom were solubilized in 1.0 mL of ammonium bicarbonate buffer (AMBIC) 20 mM, pH 8.0 and centrifuged at 5,9000 × g for 10 min. The supernatant was submitted to fractionation using an ion exchange chromatography column CM-Sepharose FF® (1 × 40 cm) previously equilibrated with the same buffer used to prepare the venom sample. Elution was carried out with a linear gradient up to a concentration of 0.5 M AMBIC for 10 column volumes under flow of 1 mL/min, in an Akta Purifier 10 (GE) chromatography system. Absorbance of the effluent solution was monitored at 280 nm and the fractions collected manually. The fractions were analysed regarding the molecular mass by 12.5% SDS-PAGE (Laemmli, 1970). A second chromatographic step was used to purify the PLA_2. This step consisted in a reverse phase chromatography using Discovery® C18 column (25 × 4.6 mm, Supelco) previously equilibrated with TFA 0.1% solution (solution A). Elution was carried out with a linear gradient up to a concentration of 70% solution B (acetonitrile + TFA 0.1%) for 5 column volumes, under flow rate of 1 mL/min, in an Akta Purifier 10 (GE) chromatography system. Absorbance of the effluent solution was monitored at 280 nm and relevant fractions collected manually. The obtained samples purity was assessed by 15% SDS-PAGE.

2.2. Direct phospholipase activity

For the direct phospholipase activity determination the method described by Holzer and Mackessy (1996) was used and adjusted for microplates. An aliquot of 190 μL of 10 mM Tris, 10 mM $CaCl_2$, 100 mM NaCl buffer, pH 8.0, containing the chromogenic substrate 4-nitro-acid (3-octanoiloxi)-benzoic (4N3OAB) and 10 μL of BdV (42.5 μg/mL) and BdipTX-I (46 μg/mL) or water (negative control) was used. The solution was incubated at 37 °C and the reading was done in Synergy HT spectrophotometer (Biotek) at 440 nm in intervals of 30 s for 30 min. The experiment was conducted with three independent samples.

The protein content of the crude venom or fractions was measured by the Lowry assay method using DC protein Assay (Bio Rad). Results were estimated through a standard curve prepared with bovine serum albumin (BSA, Sigma Aldrich).

2.3. BdipTX-I partial sequence

The N-terminal amino acid sequence was carried out in automatic PPSQ-33A (Shimadzu) which uses the chemical process of sequencing by N-terminal cleavage, derived from the method developed by Edman (1950).

2.4. Biological activities

2.4.1. Animals

Male Swiss mice were used, weighing between 18 and 22 g, provided by FIOCRUZ-RO. The animals were kept in standardized conditions of controlled temperature with light, water and food *ad libitum* until the experiments. The study was approved by Ethics Committee of Animal Use of FIOCRUZ-RO (CEUA, protocol number 2012/08).

2.4.2. Oedema

Groups of four animals were injected in the right posterior paw with 20 μL of 150 mM NaCl sterile, 5 or 20 μg/paw of BdV or BdipTX-I. The contralateral control paw was injected with 20 μL of 150 mM NaCl sterile. The paw volume increase was determined after 0, 0.5, 1, 2, 3, 6 and 24 h injection of BdV or BdipTX-I using an Ugo Basile plethysmometer. Results were expressed as percentage paw volume increase in relation to control paw.

2.4.3. Samples of plasma and urine

The experiments were conducted as described previously by de Souza et al. (2012). Animals (groups of five mice) were inoculated with 50 μg of BdV or BdipTX-I diluted in 20 μL of 150 mM sterile saline or 20 μL of 150 mM sterile saline (negative control) in gastrocnemius right muscle. The inoculated animals were kept individually in metabolic cages during 3 h for the urine collection. The blood collected from orbital plexus with heparinized pipettes was centrifuged at 22 × g for 5 min in order to obtain the plasma. The samples were kept refrigerated (2–6 °C) until the end of each group of mice and used immediately to determine each mediator. The assessment of systemic effect was accomplished using diagnostic kits purchased form Labtest Diagnostica SA (Brazil). The reading was conducted in a Synergy HT spectrophotometer (Biotek).

2.4.3.1. Myotoxic activity. BdV, BdipTX-I (50 μg) or PBS were injected into *Swiss* (18–22 g) mice (groups of five mice) gastrocnemius muscle according to 2.4.3. Myotoxicity activity was evaluated by measuring creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH) liberation and using commercial diagnostic kits purchased form Labtest Diagnostica SA (Brazil).

2.4.3.2. Liver function. BdV, BdipTX-I (50 μg) or PBS were injected into *Swiss* (18–22 g) mice (groups of five mice) gastrocnemius muscle according to 2.4.3. The hepatotoxic activity was evaluated by measuring alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (AP) activity using commercial diagnostic kits purchased form Labtest Diagnostica SA (Brazil).

2.4.3.3. Kidney function. BdV, BdipTX-I (50 μg) or PBS were injected into *Swiss* (18–22 g) mice (groups of five mice) gastrocnemius muscle. The kidney function was evaluated by measuring plasma creatinine and urea biochemical parameters functions and urine total proteins and calcium using commercial diagnostic kits purchased form Labtest Diagnostica SA (Brazil).

2.5. Statistical analysis

Means and S.E.M. of all data were obtained and compared by ANOVA, followed by Tukey test with significance probability levels of p less than 0.05.

3. Results

3.1. Venom fractionation and PLA_2 purification

B. diporus venom was fractionated by ion-exchange chromatography on CM-Sepharose column and seven peaks were

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