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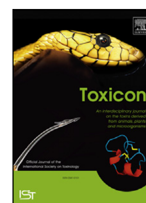

Neutralisation of the pharmacological activities of *Bothrops alternatus* venom by anti-PLA_2 IgGs

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ABSTRACT

Basic phospholipases A_2 (PLA_2) are toxic and induce a wide spectrum of pharmacological effects, although the acidic enzyme types are not lethal or cause low lethality. Therefore, it is challenging to elucidate the mechanism of action of acidic phospholipases. This study used the acidic non-toxic Ba SpII RP4 PLA_2 from *Bothrops alternatus* as an antigen to develop anti-PLA_2 IgG antibodies in rabbits and used *in vivo* assays to examine the changes in crude venom when pre-incubated with these antibodies. Using Ouchterlony and western blot analyses on *B. alternatus* venom, we examined the specificity and sensitivity of phospholipase A_2 recognition by the specific antibodies (anti-PLA_2 IgG). Neutralisation assays using a non-toxic PLA_2 antigen revealed unexpected results. The (indirect) haemolytic activity of whole venom was completely inhibited, and all catalytically active phospholipases A_2 were blocked. Myotoxicity and lethality were reduced when the crude venom was pre-incubated with anti-PLA_2 immunoglobulins. CK levels in the skeletal muscle were significantly reduced at 6 h, and the muscular damage was more significant at this time-point compared to 3 and 12 h. When four times the LD_{50} was used (224 μg), half the animals treated with the venom-anti PLA_2 IgG mixture survived after 48 h. All assays performed with the specific antibodies revealed that Ba SpII RP4 PLA_2 had a synergistic effect on whole-venom toxicity. IgG antibodies against the venom of the Argentinean species *B. alternatus* represent a valuable tool for elucidation of the roles of acidic PLA_2 that appear to have purely digestive roles and for further studies on immunotherapy and snake envenoming in affected areas in Argentina and Brazil.

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1. Introduction

The pit vipers inhabiting Central and South America belong to the genus Bothrops (family Viperidae, sub-family Crotalinae) and are responsible for most cases of deadly snakebites in America. *Bothrops alternatus* is a representative of this group of venomous snakes. Envenomation causes blood coagulation disorders, acute renal and

Abbreviations: PLA_2, phospholipase A_2; i.p., intra-peritoneal; s.c., subcutaneous; i.m., intramuscular; i.d., intra-dermal; MiHD, minimal indirect haemolytic dose; SVMPs, snake venom metallo-proteinases; ABS, antibothropic serum.

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respiratory failures and shock (Cardoso et al., 1993; Fan and Cardoso, 1995; Warrell, 1995; Ribeiro et al., 1998). The venom of Viperidae snakes is a rich source of PLA_2s.

PLA_2s are ubiquitous enzymes that catalyse hydrolysis of the C2 ester bond of 3-sn-phosphoglycerides, producing lysophospholipids and free fatty acids in a calcium-dependent reaction (Scott et al., 1990). These enzymes exert different toxic activities *in vivo*, notably neurotoxicity and myotoxicity (Kini, 2003; Montecucco et al., 2008). Furthermore, both acidic and basic PLA_2s are observed in venom, in variable proportions depending on the species. The basic isoforms appear to have the highest toxicity, especially among the neurotoxic and myotoxic enzymes (Rosenberg, 1986; Krizaj et al., 1993).

All acidic PLA_2s purified from viperid venoms contain a D residue at position 49. These acidic isoforms usually have higher catalytic activity than basic PLA_2s on conventional substrates *in vitro* (Rosenberg, 1986; Rosenberg, 1990; Santos-Filho et al., 2008). However, many acidic PLA_2s are not lethal or weakly lethal in mice (de Araújo et al., 1994; Andriao-Escarso et al., 2002). These enzymes are considered to have solely digestive roles (Fernandez et al., 2010).

We observed previously that isolated PLA_2 and a haemorrhagic metallo-proteinase, acting simultaneously, increases damage on muscle fibres *in vitro*. (Bustillo et al., 2012). Therefore, it is important to examine the effect of pre-incubating the venom with purified anti-PLA_2 IgGs obtained by immunising rabbits with the PLA_2 homologue to test whether all phospholipase A_2 enzymes are inhibited.

This study examined whether acidic PLA_2 in *B. alternatus* venom had pharmacological effects in co-operation with other enzymes in the venom. Antibodies obtained against a non-toxic acidic Ba SpII RP4 PLA_2 were used to study venom activity in *in vitro* and *in vivo* assays under partial blocking conditions.

2. Materials and methods

2.1. Reagents and venom

B. alternatus venom was obtained from several snakes kept in the serpentarium at Corrientes, in north-eastern Argentina. The venom was desiccated and stored at 20° C. When required, the venom was diluted with ammonium bicarbonate (1 M, pH 8.0). The small amount of insoluble material was centrifuged at 3000 rpm for 10 min, and the clear supernatant was used for assays. The purified phospholipase A_2, named Ba SpII RP4, was isolated previously (Garcia Denegri et al., 2010). The Sepharose affinity column (HiTrap Protein G HP 1 ml) and ÄKTAprime plus were purchased from GE Healthcare, and sodium phosphate 20 mM (pH 7.00), Glycine-HCl 0.1 M (pH 2.5), Tris–HCl 1 M (pH 9.00) and Tween 20 were purchased from Sigma Chemical Co.

2.2. Animals

The adult CF-1 male mice (20 g ± 5 g) and New Zealand white rabbits (3 kg) were obtained from the Animal House, University of Veterinary Sciences from University of North-eastern Argentina. Food (chow mice diet) was withdrawn 12–14 h before the experiment, but the animals had free access to water. The animals were maintained in a temperature-controlled room (23 ± 2° C), and the relative humidity was between 35% and 65%. The animal room was lit from 6 a.m. to 6 p.m. Rabbits were housed in single cages, and food and water were freely available. This study was approved by the Ethics and Biosafety Committee of the University of Veterinary Sciences at the University of North-eastern Argentina.

2.3. Production of specific antiserum

Anti-PLA_2 serum was obtained by successive immunisations of rabbits (3–4 kg weight) with an initial dose of 1 mg of the purified PLA_2, either intramuscularly or subcutaneously. The first injections were provided i.m. and included Freund's complete adjuvant in a 1:1 ratio. The subsequent boosters were provided s.c. at weekly intervals and contained 3-mg doses in incomplete adjuvant.

Rabbits were bled 10 days after the last PLA_2 antigen injection. Their sera were separated and stored in aliquots at 20° C. The antibody levels in the serum were monitored by gel immuno-diffusion (Ouchterlony, 1949) and ELISA (Chavez-Olortegui et al., 1997) analyses. The serum was then used for purification of specific antibodies by affinity chromatography.

2.4. Isolation of IgG by protein-G affinity chromatography

IgG antibodies were purified from the serum of rabbits immunised with Ba SpII RP4 PLA_2, using a Sepharose-protein G column (HiTrap Protein G HP 1 ml, GE Healthcare) in an ÄKTAprime plus system (GE Healthcare). The column was equilibrated with 20 mM sodium phosphate (pH 7.0), and 0.5 ml of serum (diluted 1:5 in PBS) was applied. Non-adsorbed proteins were removed by washing the column with the same buffer, and the IgGs were eluted with 0.1 M glycine-HCl (pH 2.5). The elution profile was monitored at 280 nm, and 1 ml fractions were collected and immediately neutralised with 70 μl of 2 M Tris–HCl (pH 9.0) prior to pooling and desalting by extensive dialysis against PBS (pH 7.4) for 24 h. After dialysis, the anti-PLA_2 IgG solution was concentrated by ultra-filtration using Amicon® membranes, and the rabbit IgG protein content was determined by the Biuret reaction. The Ouchterlony precipitation test was used to monitor the presence of IgG in the solution.

To verify the absence of other anti-toxins, specifically antibodies against metallo-proteinases, the ability of the anti-toxin to neutralise the major physiopathological properties of *B. alternatus* venom metallo-proteinases was examined, e.g., proteolytic activity was determined using the azocasein assay, and the haemorrhagic activity was monitored as described by Gonçalves and Mariano (Gonçalves and Mariano, 2000) with modifications (Peichoto et al., 2007). Then, 7.2 μg of venom and the corresponding dose of 25 mg/ml of IgG anti-PLA_2 with the MiHD selected were injected i.d. into each mouse. The animals were sacrificed two hours after injection, and the skin was removed. Haemorrhagic halos were immediately excised, fragmented, and added to tubes containing 4 ml of

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