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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.

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 subtropical 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

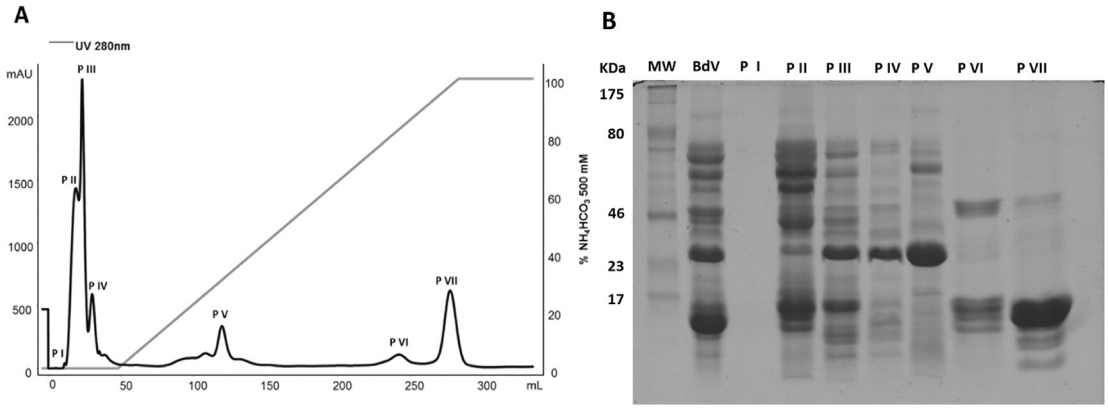

Fig. 1. A) Ion-exchange BdV chromatography. 50 μg of BdV diluted in 1 mL of NH_4CO_3 20 mM pH 8.0 were eluted in ascending gradient of NH_4HCO_3 (20–500 mM), on CM-Sepharose column previously balanced with solution of 20 mM NH_4HCO_3 in 1 mL/min flux. The peaks whose absorbance to 280 nm was relevant was collected and designed PI-P VII. **B) Electrophoretic profile of the BdV and of the peaks obtained by ion-exchange chromatography.** BdV and fractions/peaks (PI to PVII) were submitted to SDS-PAGE 12.5% polyacrylamide gel in reducing conditions and stained with a solution containing 0.08% (m/v) *Coomassie Brilliant Blue*, 8.0% (m/v) aluminium sulphate, 1.6% (m/v) o-phosphoric acid and 20.0% (v/v) methanol for 2 h. The obtained lanes were compared with standard molecular mass.

collected: PI-PVII (Fig. 1A). All peaks and crude venom were submitted to SDS-PAGE on 12.5% gel electrophoresis (Fig. 1B). Two criteria for fractions choice to be processed were considered: buffer B concentration to fraction elution and the molecular mass of PLA_2 that is about 14 kDa. The fractions/peaks PVI and PVII showed these features and were selected to the purification process in reverse phase chromatography on C-18 column. The electrophoretic profile of the BdV and its fractions revealed the possible presence of acidic PLA_2s in fractions P II and P III and proteases in P II, P III, P IV and PVI (Fig. 1B).

Fraction PVII was subjected to a single step of reversed-phase chromatography, from which it has been purified an unpublished basic PLA_2 from BdV, that was termed BdipTX-I (Fig. 2A).

After the chromatographic steps, BdipTX-I was analysed by SDS-PAGE in 15% polyacrylamide gel to determine the approximate molecular mass compared to standard molecular mass (MM), and the purity of the isolated proteins (Fig. 2B). Gel analysis showed that the isolated PLA_2 showed satisfactory degree of purity and the approximate molecular mass of the protein was about 14 kDa.

3.2. Phospholipase activity

Direct phospholipase activity on 4N3OAB as a substrate, confirmed that BdipTX-I is an enzymatically inactive protein, since the two concentrations tested (5 and 46 μg/mL) did not show activity. On the other hand, BdV (42.5 μg/mL) presented enzymatic activity on the tested substrate, which can be seen by the increase of O.D. in 425 nm (Fig. 3).

3.3. BdipTX-I partial sequence

The N-terminal amino acid sequence of BdipTX-I revealed, using the numeral system proposed by Renetseder et al. (1985), the presence of a lysine at position 49, confirming that the isolated protein belongs to the Lys-49 PLA_2 class (Table 1).

The multiple alignment of amino acids of BdipTX-I showed an identity of 90–97% compared to PLA_2s Lys49 isolated from *Bothrops* snake venom. This result showed a conserved primary structure normally found in *Bothrops* species (Fig. 4).

3.4. BdV and BdipTX-I biological activity

3.4.1. Oedema

Oedema formation was evaluated several time intervals (0.5, 1, 2, 3, 6 and 24 h) after intraplantar injection of BdV or BdipTX-I in different doses (5 and 20 μg/paw) or endotoxin free saline (control). There was a significant increase in the swelling from 0.5 h at all doses tested (5 and 20 μg/paw) for both BdV and BdipTX-I (Fig. 5), with a peak in 1 h and declining in 24 h, time that it was observed a decline in BdipTX-I to basal line. This effect was not observed at a dose of 20 μg/paw of BdV.

3.4.2. Myotoxic activity

Results showed that compared to control group that received i.m. injection of 150 mM of sterile saline, BdV nd BdipTX-I caused an increase of 284% and 127% of total-CK levels, respectively. The mean of total-CK levels in control group was 859 U/L, in BdV and BdipTX-I groups were 3302.7 U/L and 1954.8 U/L, respectively, statistically different from control animals (Fig. 6A). It was noted also that there was no significant increase in MB-CK levels (Fig. 6B).

However, there was an increase in LDH levels in animals injected with BdV and BdipTX-I (Fig. 6C). The average of the LDH activity of the control group was 194 U/L, whereas BdV presented average of 516 U/L, which corresponds to an increase of 165.9%, and BdipTX-I had an average of 870 U/L, with an increase of 348.3%. The increase of LDH activity caused by BdipTX-I significantly higher relative to the BdV.

3.4.3. Liver function

Fig. 7A showed that AST was the only enzyme that showed change in serum levels. BdV induced an increase of 216.6% (305 U/

$_{1}$SLFELGKMILQETG_KNPAKSYGAYGCNCGVLGRGKPKEATQRCCYVHKCCYKK$_{54}$

$_{55}$LTGCDPKKDRYSYSW$_{69}$

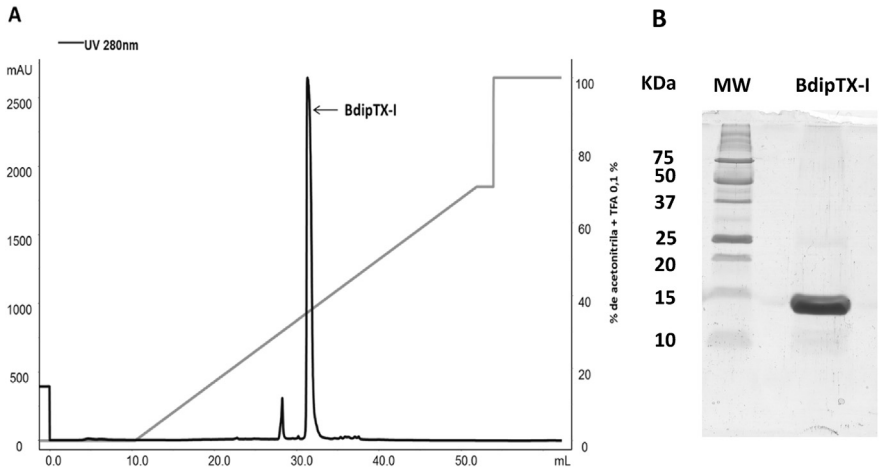

Fig. 2. A) Phase-reverse chromatography BdipTX-I purification. Peak PVII was diluted in 0.1% TFA solution and eluted in 0–70% acetonitrile plus 0.1% TFA gradient on C-18 Discovery® column with 1 mL/min flux. The arrow indicates the basic PLA_2 isolated from *B. diporus* venom, BdipTX-I, which was eluted with 35.4% of acetonitrile plus 0.1% TFA solution. **B) Electrophoresis of isolated PLA_2s from BdV.** BdipTX-I and BdipTX-II from *B. diporus* venom were submitted to SDS-PAGE 15% polyacrylamide gel under reduction conditions and stained with a solution containing 0.08% (m/v) *Coomassie Brilliant Blue*, 8.0% (m/v) aluminium sulphate, 1.6% (m/v) o-phosphoric acid and 20.0% (v/v) methanol for 2 h. The obtained lanes were compared with standard molecular mass.

L) in AST levels and, BdipTX-I induced an increase of 304% (389.3 U/L) compared to control animals (96.3 U/L). The other markers of liver function assessed, ALT, GGT and alkaline phosphatase (Fig. 7B, C and 7D) did not show significant changes. Therefore, since AST is not a marker found only in liver but also cardiac and renal lesions, it can not be affirmed that there was liver damage in the period studied.

3.4.4. *Kidney function*

According to Fig. 8A and B, only the BdV induced excretion of urea in plasma and urine of mice. However, BdipTX-I, as well as BdV, was able to induce a significant increase in urine levels of creatinine and total protein (Fig. 8D and E), indicating possible kidney damage, which can be attributed also to BdV. Serum levels of creatinine and urinary calcium have not changed (Fig. 8C and F). Table 2 showed the average and the increase percentage caused by the change of the renal injury markers by BdV and BdipTX-I.

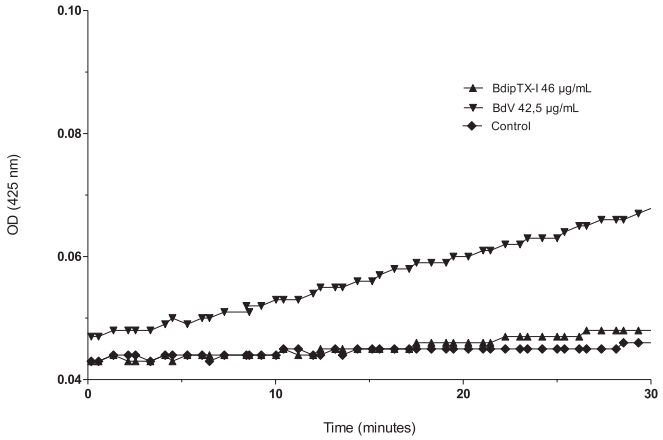

Fig. 3. BdV, BdipTX-I and BdipTX-II phospholipase activity on 4N3OAB substrate. 190 µL of buffer containing the chromogenic substrate N4O3AB and 10 µL of 42.5 µg/mL of BdV, 5 and 42.5 µg/mL of BdipTX-I or water (negative control) was added. The solution was incubated under 37 °C and analysed in spectrophotometer in 425 nm during 30 min with intervals of 30 s. Data was represented in O.D. of kinetic readings.

4. Discussion

PLA_2s are proteins (Supplementary 1) that are present in considerable amounts in snake venoms, particularly in the genus *Bothrops*. They are implicated in the pathophysiology of snakebites because they are responsible for various local and systemic disorders. These include myotoxicity, pro-inflammatory effects such as oedema induction, leukocyte recruitment, activation of leukocytes, the release of inflammatory cytokines (IL-1, IL-6 and TNF-α), the induction of kidney lesions, cardiovascular and neurological disorders (Arni et al., 1995; Calgarotto et al., 2008; Furtado et al., 2014; Gutiérrez et al., 1984; Marangoni et al., 2013; Ponce-Soto et al., 2010; Rueda et al., 2013; Setúbal et al., 2013; Zuliani et al., 2005a, 2005b). In addition, it is known that snake's venom PLA_2s are homologous to the Group II of mammalian PLA_2s (Burke and Dennis, 2009). The study of their mechanisms of action can contribute to the understanding of the role of PLA_2 in various disorders. For these reasons, the present study aimed to isolate a PLA_2 from *Bothrops diporus* venom in order to compare the crude venom local and systemic effects with those evoked by the isolated PLA_2.

In this study, a basic PLA_2 were isolated from *Bothrops diporus* snake venom termed BdipTX-I. Several basic PLA_2s Lys-49 have already been isolated from snake venoms, and most have between

Table 1
Partial amino acid sequence of BdipTX-I.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
S	L	F	E	L	G	K	M	I	-	L	Q	E	T	G	K	N	P	A	K
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
S	Y	G	A	Y	G	C	N	C	G	V	L	G	R	G	K	P	K	E	A
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
T	Q	R	C	C	Y	V	H	K	C	C	Y	K	K	L	T	G	C	D	P
61	62	63	64	65	66	67	68	69											
K	K	D	R	Y	S	Y	S	W											

Species	Short Name		Amino Acid Sequence	Identity	ID
B. diporus	BdipTx-I	1	SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKEATQRCCYVHKCCYKKLTGCDPKKDRYSYSW		
B. neuwiedi	Bneu-I	1	SLVELGKMILQETGKNPVTSYGAYGCNCGVLGRGKPKDAT----------	90.0	Geoghegan et al.(1999)
B. jararacussu	BthTx-I	1	SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCDPKKDRYSYSW	97.1	Q90249.3
B. pirajai	PrTx-II	1	SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGGNPKKDRYSYSW	95.6	P82287.1
B. moojeni	MjTx-II	1	SLFELGKMILQETGKNPAKSYGVYGCNCGVGGRGKPKDATDRCCYVHKCCYKKLTGCDPKKDRYSYSW	94.1	Q9I834.2
B. moojeni	MjTx-I	1	SLVELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTNCDPKKDRYSYDW	92.6	P82114.1
B. leucurus	BlK-PLA2	1	SLFELGKMILQETGKNSVKSYGVYGCNCGVGGRGKPKDATDRCCYVHKCCYKKLTGCDPKKDRYSYSW	91.2	P86975.1
consensus		1	**.************...***.*******.******.**..........................		

Fig. 4. Multiple sequence alignment of the 68 first amino acids of BdipTX-I with others PLA_2 of snake venoms. The residues numbers followed the numeration proposed by Renetseder et al. (1985). "*" indicates a fully conserved residue; ":" indicates conservation between groups of strongly similar properties and "." indicates conservation between groups of weakly similar properties.

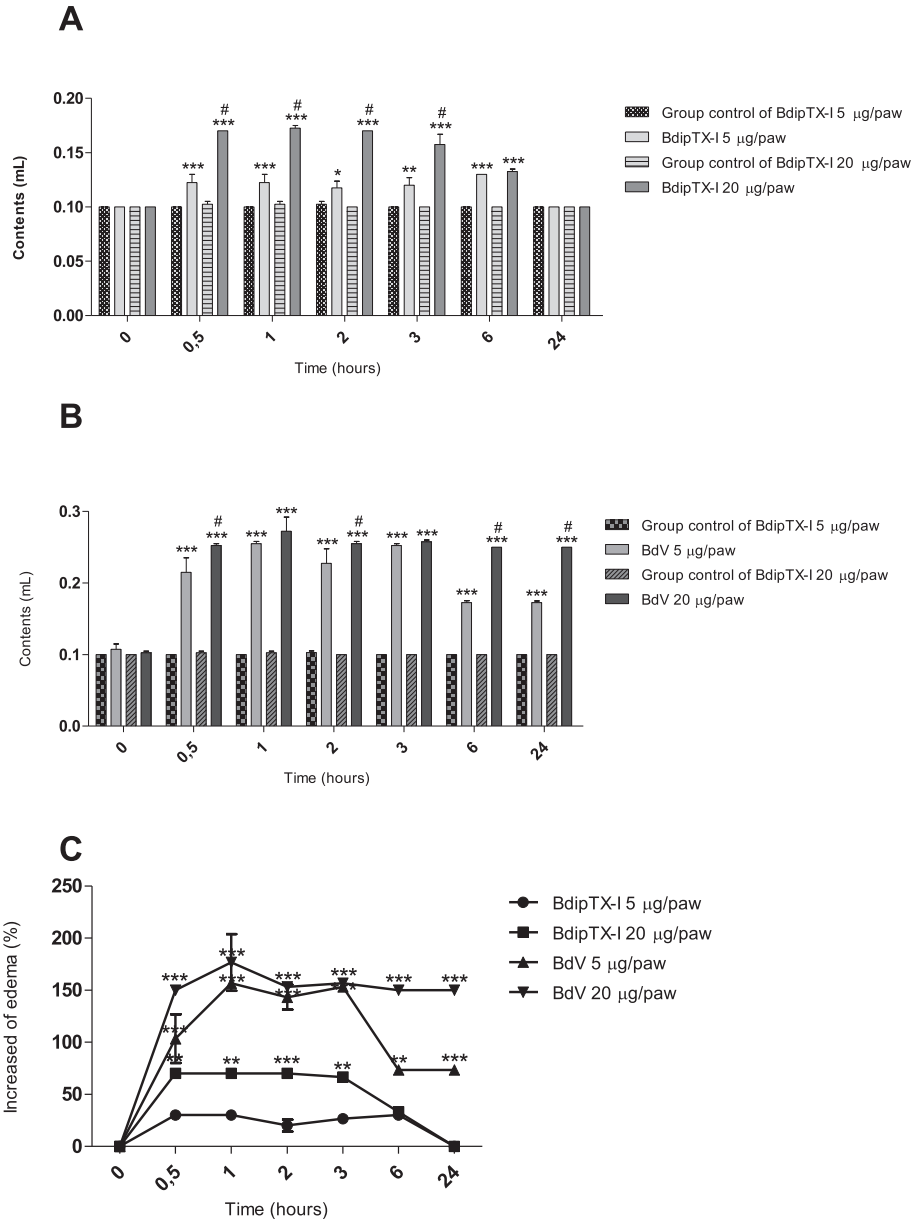

Fig. 5. BdV and BdipTX-I oedema-inducing activity in Swiss mice. Groups of 4 animals were inoculated in the right hind plant with 5 or 20 μg of BdV or BdipTX-I. The left contralateral hind paw, received sterile saline injection (control). The paw increase was monitored with a plethysmometer at 0, 0.5, 1, 2, 3, 6 and 24 h after injections. **A) BdipTX-I oedema-inducing activity. B) BdV oedema-inducing activity. C) Percentage of increased swelling of BdV and BdipTX-I.** Data were expressed as mean ± EPM with $^{*}p < 0.05$ in relation to control paw (A and B) or to BdipTX-I 5ug (C) and $^{\#}p < 0.05$ when compared two tested doses used (ANOVA).

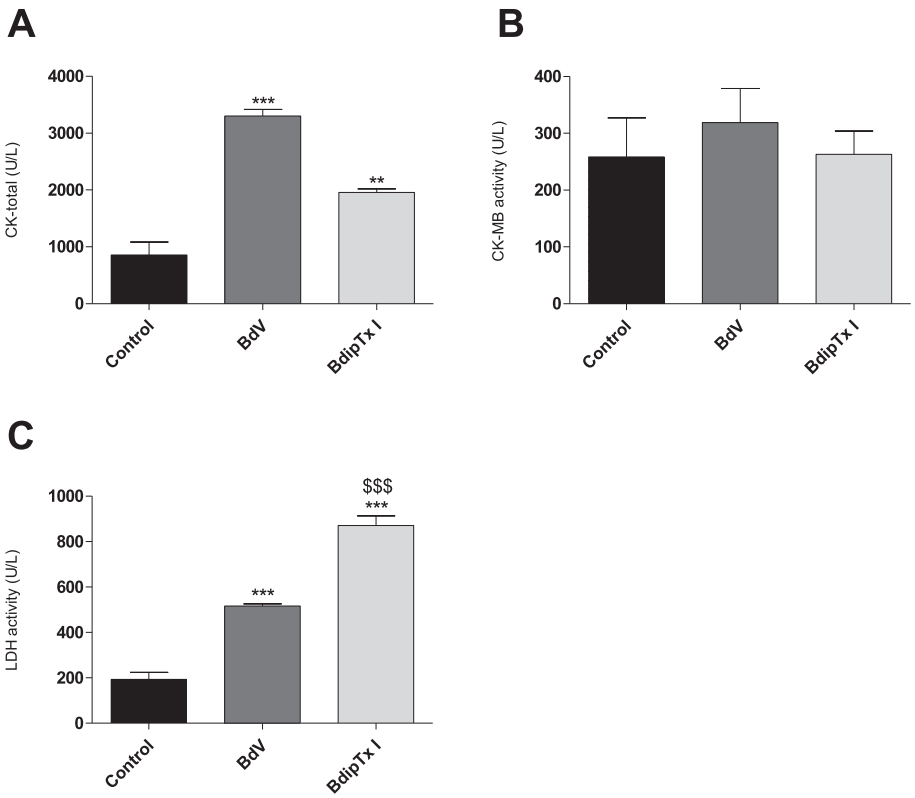

Fig. 6. BdV and BdipTX-I myotoxic effect in *Swiss* mice. 50 μg/20 μL of BdV or BdipTX-I were injected in right gastrocnemius muscle of *Swiss* mice (groups of 5 animals). Control group received 20 μL of sterile 150 mM saline. After 3 h, the blood was collected and the plasma separated for total-CK **(A)**, MB-CK **(B)** and LDH **(C)** determinations using specific diagnostic kinetic kits. Data were expressed as mean ± EPM. $^{*}p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to BdipTX-I in **A** and $^{\$}p < 0.05$ compared to BdV in **C** (ANOVA).

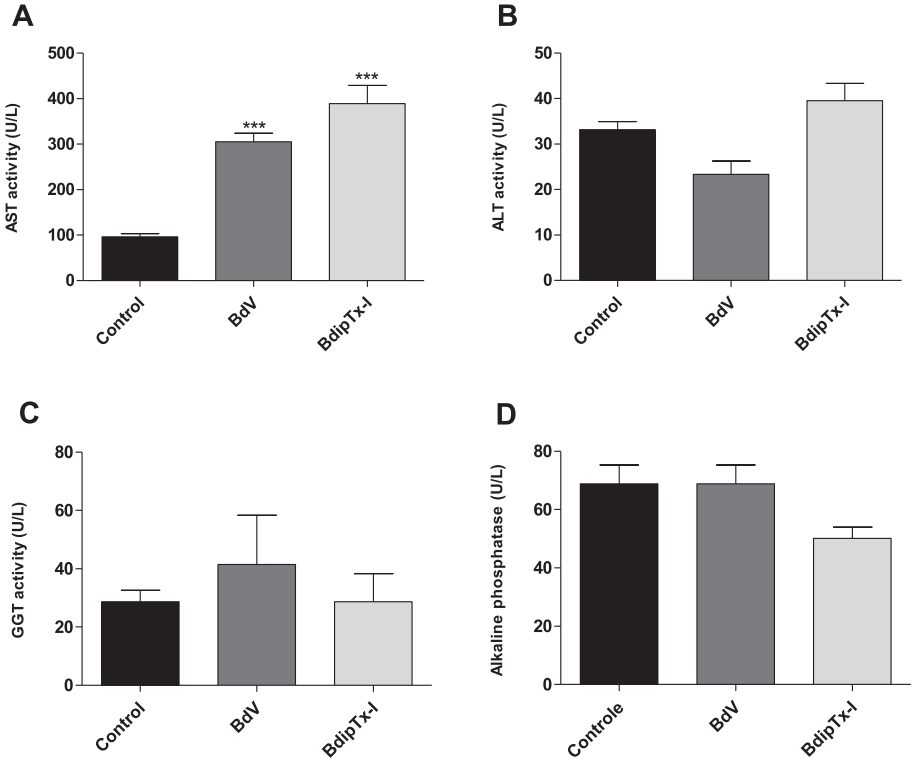

Fig. 7. BdV and BdipTX-I effect on *Swiss* mice liver function. 50 μg/20 μL of BdV or BdipTX-I were injected in right gastrocnemius muscle of *Swiss* mice (groups of 5 animals). Control group received 20 μL of sterile 150 mM saline. After 3 h, the blood was collected and AST **(A)**, ALT **(B)**, GGT **(C)** and alkaline phosphatase **(D)** activities were determined using specific diagnostic kinetic kits. Data were expressed as mean ± EPM. $^{*}p < 0.05$ compared to control (ANOVA).

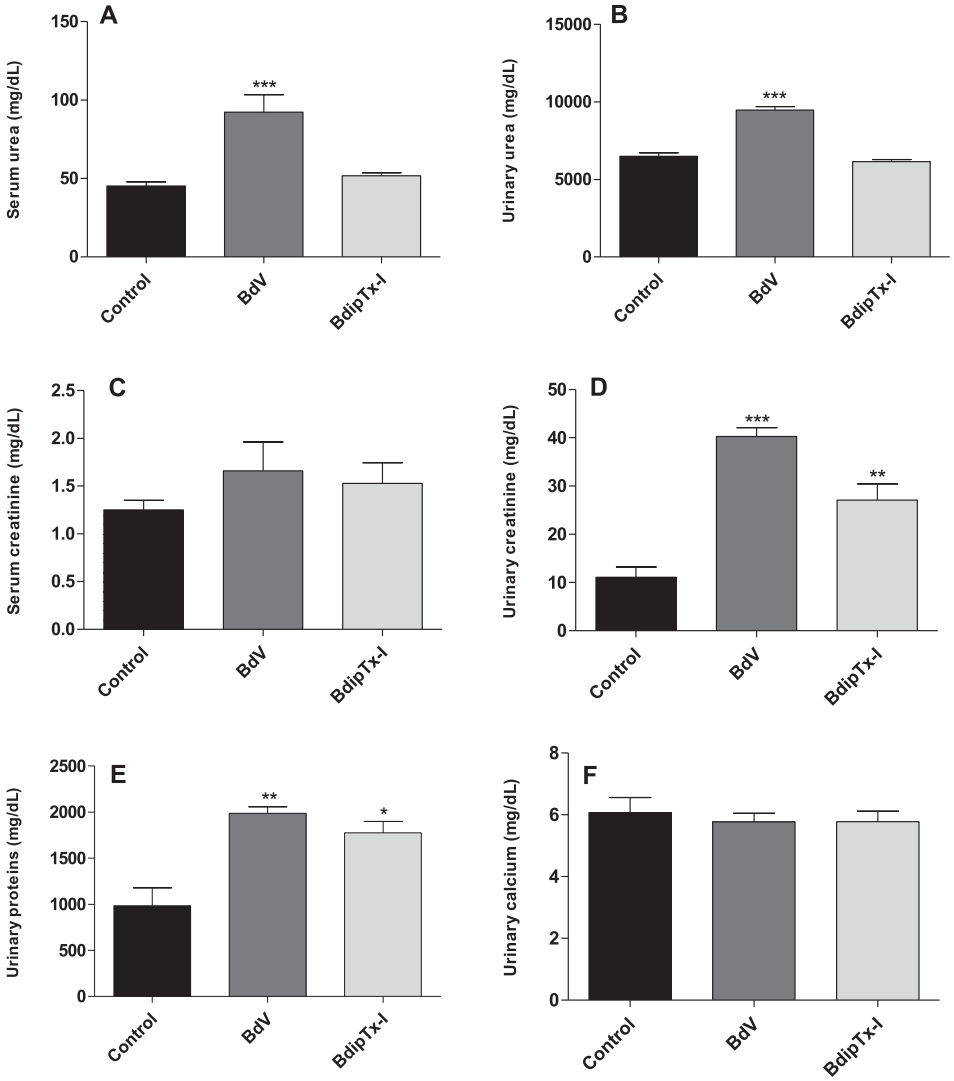

Fig. 8. BdV and BdipTX-I effect on kidney function. 50 μg/20 μL of BdV or BdipTX-I were injected in right gastrocnemius muscle of *Swiss* mice (groups of 5 animals). Control group received 20 μL of sterile 150 mM saline. After 3 h, the blood was collected and serum urea **(A)**, urine urea **(B)**, serum creatinine **(C)**, urine creatinine **(D)**, total protein **(E)** and calcium **(F)** were determined using specific diagnostic kinetic kits. Data were expressed as mean ± EPM. *p < 0.05 compared to control and #p < 0.05 compared to BdipTX-I (ANOVA).

Table 2
Markers of renal damage altered by VBd and BdipTX-I.

Marker	Control	VBd		BdipTX-I	
	Mean (mg/dL)	Mean (mg/dL)	Increase (%)	Mean (mg/dL)	Increase (%)
Serum urea	45,2	92,3	104,0	51,6	14,2
Urinary urea	6475,3	9454,1	46,0	6144,0	−5
Urine Creatinine	11,1	40,3	262,5	27,1	143,7
Urine proteins	983,8	1985,4	101,8	1773,5	80,3

120 and 138 amino acid residues in their primary structure. Their molecular mass varies between 10 and 15 kDa (Supplementary 2). It is important to note that a taxonomic alteration for *Bothrops neuwiedi* snake was published in 2009 by Fenwick et al. These authors proposed by phylogenetic analysis that the *Bothrops diporus* snake, formerly a subspecies from *Bothrops neuwiedi* snake, was elevated to the species level. Thus, due to this reclassification, the data analysis published previously is difficult to be done.

In this context, Geoghegan et al. (1999) isolated and characterized a basic PLA_2 from *Bothrops neuwiedi* venom from Argentina. Nevertheless, the venom used in this work came from *Bothrops neuwiedi diporus* because the geographical distribution analysis of snakes in Argentina showed that in the provinces of Santiago del Estero, Corrientes and Missiones, places where the venoms were obtained, this is the main viper found (Giraudo et al., 2012; Gay et al., 2016).

Among these phospholipases, the multiple alignment of the partial sequence of BdipTX-I showed great identity in their amino acid sequence with other PLA_2s isolated from genus *Bothrops* snakes. These include Bneu-I from *Bothrops neuwiedi*, BthTX-I from *B. jararacussu*, PrTX-II from *B. pirajai,* MjTX-II from *B. moojeni*, MjTX-I from *B. moojeni*, blK-PLA_2 from *B. leucurus* (Fig. 4). Moreover, it was possible to observe that BdipTX-I has highly conserved amino acid

residues at the N-terminal region, which is a constituent of the α-helix region of protein and contains residues involved in the substrate binding site, that is an important element in the myotoxic activity of these proteins (Arni and Ward, 1996; Chaves et al., 1992).

BdipTX-I, as well as BdV, demonstrated oedema-inducing activity. This effect has been observed in other PLA_2 isolated from snake venoms, especially from those that belong to the genus *Bothrops* (Kini and Evans, 1989; Marangoni et al., 2013; Ponce-Soto et al., 2007). The oedematogenic effects caused by both BdV and BdipTX-I were respectively similar to those caused by other venoms and by PLA_2 isolated from *Bothrops* venoms (Huancahuire-Vega et al., 2011; Zychar et al., 2010). The oedema formation was shown to be dose-dependent for BdipTX-I, but not to BdV. A previous study has already shown that BdV has a minimum dose of 2.05 μg/paw for the induction of oedema (Acosta de Pérez et al., 1998). In the same study, the histopathological analysis of animal tissue inoculated with 5 μg of BdV showed necrotizing action and infiltration of polymorphonuclear cells in muscle fibers.

The myotoxic activity of BdipTX-I and BdV was evaluated by the determination of total-CK, CK-MB and LDH in serum after i.m. injection of BdipTX-I or BdV (50 μg/20 μL of sterile saline). When CK levels were compared in relation to BdipTX-I, BdV was significantly more myotoxic, probably due to the presence of the other venom components. In addition to total-CK, the myotoxic effect of BdipTX-I and BdV could also be found by serum LDH determination, in which BdipTX-I demonstrated a significantly greater effect than BdV. When associated with other specific markers, the increased levels of LDH in serum may be due to injuries in the cardiac muscle, liver and kidneys (Cassidy and Reynolds, 1994; Copur et al., 1989).

Likewise, several studies have shown that the snake venoms and PLA_2 induce lesions at the systemic level, affecting the functioning of vital organs such as the heart, liver and kidneys in both human victims and in experimental animal models (Amaral et al., 1986; Barbosa et al., 2002; de Souza et al., 2012; Dias et al., 2012; Gama, 2009; Patil and Bansod, 2012; Peichoto et al., 2006; Shashidharamurthy et al., 2010). For this reason, after finding out that BdipTx-I and BdV induce local effects such as oedema and myotoxicity, the systemic effect from the injury site was investigated. As such, the liver and kidney lesions were evaluated by dosing of specific laboratory markers.

Liver damage was evaluated by AST, ALT, GGT and ALP levels in mice serum. Although these enzymes are not exclusively hepatic, they are released primarily after hepatic injury (Kim et al., 1977; Singer et al., 1995; Shet et al., 1998). It was observed that only AST was significantly altered both by BdipTX-I and BdV. Studies with other snake venoms showed changes in AST and ALT levels, indicating liver injury (de Souza et al., 2012; Shashidharamurthy et al., 2010). The mechanisms involved in this process has not been established yet. In addition to biochemical findings, histological findings demonstrated the presence of necrosis; nuclear and cytoplasmic changes, including cariopicnose of hepatocytes and cytoplasmic vacuolation; congestion of the blood vessels and thrombosis of the portal vein; amyloidosis and atrophy of hepatocytes; and Kupffer cells activation (Jarrar, 2011; Shashidharamurthy et al., 2010; Silva et al., 2012). It was possible to observe that the hepatotoxicity is a side effect of the venom action.

To assess whether BdipTX-I and BdV cause kidney toxicity, levels of urea, creatinine and protein excretion were evaluated both in serum and urine whereas levels of calcium were assessed only in urine. A significant increase in urea levels both in serum and in urine were caused by BdV, but not by BdipTX-I. Urinary creatinine levels were increased both by BdipTX-I and BdV, accompanied by a high proteinuria. These data indicate that a nephrotoxic effect can be provoked both by BdipTX-I and BdV. However, the fact that BdipTX-I does not present the same effect of BdV regarding the elevation of urea levels in mice's serum indicates the action of other venom components in the induction of a possible kidney injury.

The mechanisms through which PLA_2 Lys-49 lead to nephrotoxicity have not been properly clarified yet. The literature reveals that the PLA_2s may have direct renal cell cytotoxic effect, which would be associated to the C-terminal region rich in Lys residues of these myotoxins and/or to the release of inflammatory mediators. Barbosa et al. (2002) conducted a study with two PLA_2 of *B. moojeni* (Bmtx-I and Bmtx-II), in which only the PLA_2 Lys-49 showed nephrotoxic activity. In another study Barbosa et al. (2005) showed that BthTX-I and BthTX-II of *B. jararacussu* are nephrotoxic. In addition, the administration of indomethacin, suggests the presence of lipid mediators in nephrotoxicity induced by BthTX-I, a PLA_2 Lys-49.

As for the renal injury caused by bothropic venom generally relates to hemodynamic changes that end up inducing hypotension, hypoperfusion and ischemia (Rezende et al., 1989; Rodrigues Sgrignolli et al., 2011). There may be disseminated intravascular coagulation, nephrotoxicity directly attributed to proteolytic action of venom and/or renal vessel spasm due to induction of vasoactive substances by venom (Amaral et al., 1986; de Morais et al., 2013). Barbosa et al. (2002), using a model of renal perfusion, showed that the *B. moojeni* venom induced renal lesion evidenced by decreases in perfusion pressure on renal vascular resistance and in the transport of sodium and potassium, indicating a tubular injury. In another similar work performed by Mello et al. (2014), it was observed that *B. insularis* venom and the isolated PLA_2 induced nephrotoxicity in perfused isolated kidneys model and in renal tubular cell culture, with indicative of cell death by apoptosis.

In comparison, the acute renal failure is one of the most damaging effects of the venom caused by snakes of the genus *Bothrops* (Amaral et al., 1986; Patil and Bansod, 2012). Studies in human victims reveal presence of cortical necrosis and acute tubular necrosis as the main causes of acute renal failure (Amaral et al., 1986). Experiments with animals showed vacuolar degeneration of glomerular congestion, tubular cells, haemorrhagic areas in the medulla, degeneration of proximal tubular cells and cortical necrosis (Chaves et al., 1992, 1989).

Taken together, the data obtained provide additional information on the activity of the *B. diporus* venom and showed the isolation of basic phospholipase A_2 from this venom. Biological activity assessments showed that it is possible to conclude that the PLA_2 Lys49 BdipTX-I and the BdV induce the formation of oedema, myotoxicity and possible changes in renal function. The myotoxic and oedema-inducing action of BdV were more pronounced when compared to those of BdipTX-I. In addition, the measurement of kidney markers points a possible kidney damage that can be related to injury due to tubular proteinuria, which is one of the first signs of this change. There was a differentiation in the action of the venom against the BdipTX-I since BdV-induced uremia, but not BdipTx-I. These data indicate the action of other toxins present in the venom, such as proteases and LAAO. However, the information collected does not lead to the conclusion that there were no cardiac and hepatic changes caused by BdipTX-I or BdV in the period evaluated. Additional studies are needed to delineate a systemic effect more detailed profile.

Authorship

J.P.Z. and L.F.T. designed the study; L.F.T., L.H.C., O.B.C., J.S.F., G.A.O., A.M.K. and N.M.N. performed the experiments; A.M.K., G.A.O. and A.M.S. performed and supervised the biochemical procedures; J.P.Z., L.F.T. and L.H.C. collected and analysed the data; J.P.Z. and A.M.S. provided reagents; J.P.Z. and L.F.T. wrote the manuscript. All of the authors discussed the results and implications and commented on the manuscript at all stages.

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Conflict of interest

There is no conflict of interest statement.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2017.11.007.

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