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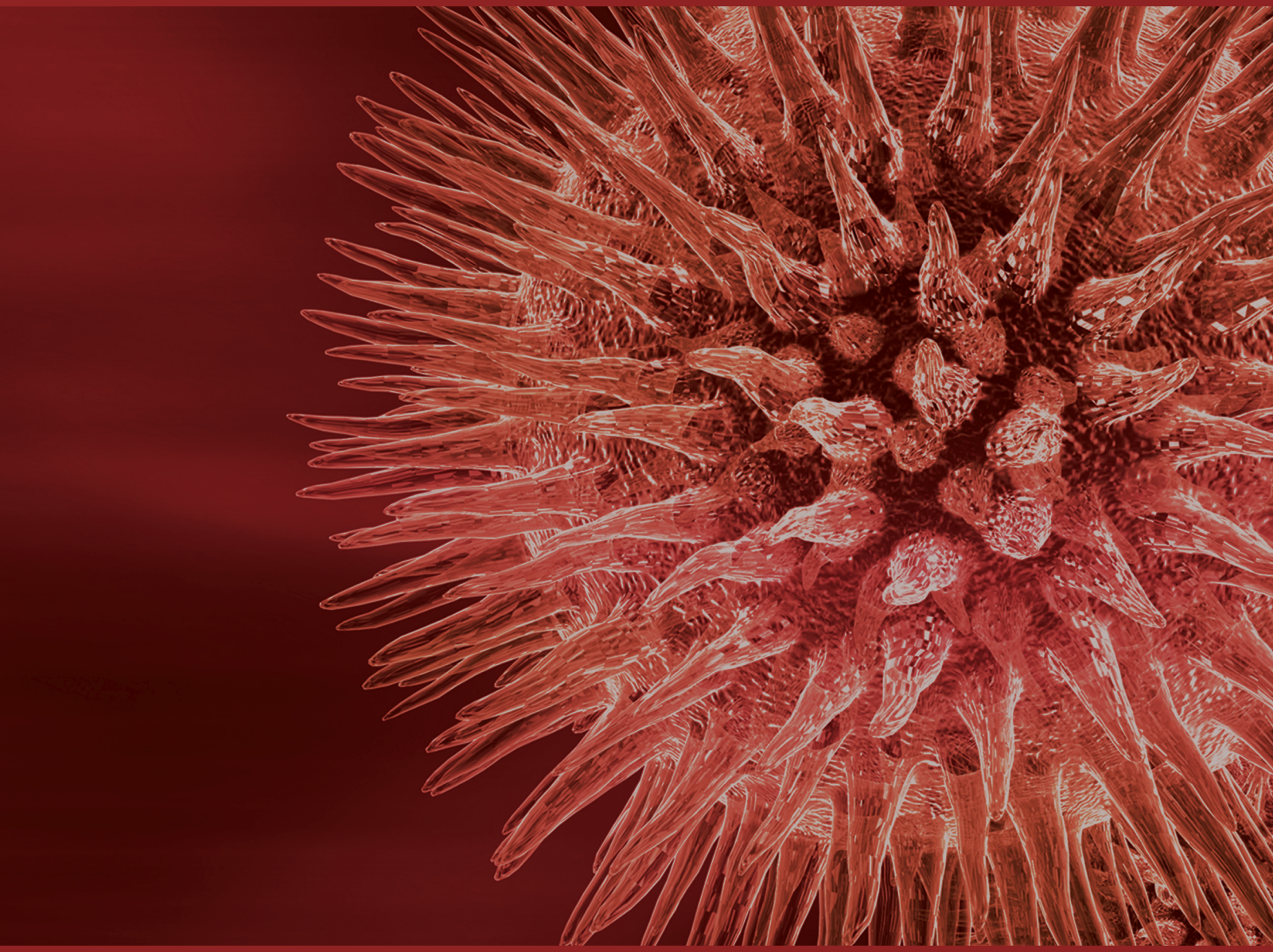
Guest Editors: Luis Alberto Ponce Soto, Laura Leiva, and Elen Cristina Teizem Landucci



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Editorial

Understanding the Molecular Mechanism and Structure-Function Relationship of the Toxicity of PLA_2 and K49 Homologs in Snake Venom

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The snake venom PLA_2s, in association with the catalytic function that defines them, have developed a diverse set of biological functions which go beyond the purely digestive. Striking examples are the PLA_2 that have evolved as toxins, either in conjunction with other proteins or by themselves, and in spite of having a similar structural geometry, they exert an amazing variety of pharmacological effects, which include myotoxic, neurotoxic, hemolytic, edematogenic, hyperalgesic, pro- and anti-inflammatory, hypotensive, anticoagulant, platelet-aggregation inhibitory, and cytotoxic. Furthermore, since the first studies reported by Maraganore et al. [1] to the present, in homologous K49 LA_2 from snake venom, have increased significantly. Despite its inability to bind Ca2 ion co-factor crucial for catalytic activity, it is capable of triggering toxic or pharmacological effects independent of PLA_2 activity, thus opening a new understanding of the involvement of independent regions or domains of the catalytic site, postulated from Condrea et al. [2] and reinforced by Majunatha Kini and Evans [3]. The present work highlights some of the most relevant contributions in the study of venom PLA_2s, including the PLA_2 homólogous K49.

This special issue of contains nine papers describing structural and functional analysis of different PLA_2 and PLA_2 homólogous K49 from snakes. The review by C. V. Carregari and colleagues discusses the importance of the proinflammatory activity that characterized this new venom from *Bothriopsis bilineata* snake venom. The fact that Bbil-TX elicited a stronger inflammatory reaction argues in favor of a role of enzymatic phospholipid hydrolysis in this phenomenon, either through the direct release of arachidonic acid from plasma membranes or through activation of intracellular processes in target cells. I. G. Rodríguez et al. analyze, the PLA_2s isolated from Panama *Bothrops asper* venoms (pMTX-I, II, III, and IV), which are able to induce myotoxic activity, inflammatory reaction, and mainly leukocyte migration to the muscle and induce J774A.1 macrophages activation to start phagocytic activity and superoxide production. J. Nelson et al. make an important contribution to the study of synergistic effects of secretory PLA_2 from the venom of *Agkistrodon piscivorus piscivorus* with cancer chemotherapeutic agents. Healthy cells typically resist hydrolysis catalyzed by snake venom secretory PLA_2. However, during various forms of programmed cell death, they become vulnerable to attack by the enzyme. This observation raises the question of whether the specificity of the enzyme for dying cells could be used as a strategy to eliminate tumor cells that have been intoxicated but not directly killed by chemotherapeutic

agents. This work suggests that exposure of lymphoma cells to these drugs universally causes changes to the cell membrane that render it susceptible to enzymatic attack and that the snake venom enzyme is not only capable of clearing cell corpses but can aid in the demise of tumor cells that have initiated but not yet completed the death process. L. Wei et al. present a valuable contribution to the study of induction in the accumulation of mast cells, promutoxin, a new variant of PLA_2 R49. The action of an R49 PLA_2s, promutoxin from *Protobothrops mucrosquamatus* venom on mast cell accumulation, has not been previously examined. The promutoxin-induced mast cell accumulation was inhibited by cyproheptadine, terfenadine, and ginkgolide B, indicating that histamine and platelet activation factor (PAF) are likely to contribute to the mast cells accumulation. Preinjection of antibodies against adhesion molecules ICAM-1, CD18, CD11a, and L-selectin showed that ICAM-1, CD18, and CD11a are key adhesion molecules of promutoxin-induced mast cell accumulation. Promutoxin, as a novel member of minor subgroup of PLA_2, is an enzymatically inactive enzyme. It induced mast cell accumulation via a PAF and histamine H1 receptor-dependent mechanism and through a CD11a/CD18 and ICAM-1 associated adhesion pathway. F. A. Marangoni et al. present an important contribution to the study structure function of a new PLA_2 isolated from *Bothrops leucurus*. Kinetic and pharmacological studies illustrate a behavior similar to other PLA_2 from snake venom Viperidae; however from the structural point of view, in relation to the few differences in its sequence, the contribution of each region or domain, as well as each amino acid, has been crucial in the understanding of toxic or pharmacological activities.

S. Huancahuire-Vega et al. analyze the use of chemical modifications of a new PLA_2 from *Porthidium hyoprora* snake venom in the study structure-function relationships. The results supported the hypothesis that both the catalytic sites as the hypothetical pharmacological sites are relevant to the pharmacological profile of PhTX-I. K. Giannotti et al. discuss an interesting study on the pathogenesis of the inflammatory process induced by a homologous K49 PLA_2 from the venom of the snake *Bothrops asper*. The MT-II (PLA_2 homologous K49) induces lipid droplet formation in macrophages that depends on distinct signaling pathways and the C-terminal region. MT-II directly activates murine macrophages to form LDs by a mechanism independent of enzymatic activity. This effect is related to the C-terminal loop of the MT-II molecule since a synthetic peptide corresponding to region 115–129 induced LD formation similarly to MT-II. Moreover, MT-II-induced LD formation is related to increased expression and recruitment of PLIN2 from its constitutive pools and regulated by distinct signaling pathways that include PKC, PI3K, ERK1/2, and $iPLA_2$. In addition, MT-II induced synthesis and compartmentalization of PGE2 within LDs. Therefore, LDs may represent an important platform for the synthesis and accumulation of lipid mediators under MT-II stimulus, that takes place in the mechanisms whereby this PLA_2 homologous K49 triggers inflammation. M. A. G. Heleno et al. conduct a major study on the structure-function basis of a new PLA_2 from *Bothrops roedingerii*, indicating that their enzyme profiles as toxic or pharmacological show similar behavior to other PLA_2. BrTX-I caused a neuromuscular blockade in biventer cervicis preparations in a similar way to other *Bothrops* species. BrTX-I induced myonecrosis and oedema-forming activity analyzed through injection of the purified BrTX-I in mice. Since BrTX-I exert a strong proinflammatory effect, the enzymatic phospholipids hydrolysis might be relevant for these phenomena, incrementing levels of IL-1, IL-6, and TNFα.

The best approach to understanding structure-function mechanisms of PLA_2 from snake venoms will present a comprehensive view of toxinologists in different research fields, including biochemistry, biophysics, pharmacology, toxicology, and medicine. We hope that this special issue will encourage researchers to take on this challenge and increasingly elucidate structure-function behavior of PLA_2 and K49 counterparts in snake venom.

Luis Alberto Ponce-Soto
Laura Leiva
Elen Cristina Teizem Landucci

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Review Article

Antitumoral Potential of Tunisian Snake Venoms Secreted Phospholipases A_2

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Phospholipases type A_2 (PLA_2s) are the most abundant proteins found in Viperidae snake venom. They are quite fascinating from both a biological and structural point of view. Despite similarity in their structures and common catalytic properties, they exhibit a wide spectrum of pharmacological activities. Besides being hydrolases, secreted phospholipases A_2 ($sPLA_2$) are an important group of toxins, whose action at the molecular level is still a matter of debate. These proteins can display toxic effects by different mechanisms. In addition to neurotoxicity, myotoxicity, hemolytic activity, antibacterial, anticoagulant, and antiplatelet effects, some venom PLA_2s show antitumor and antiangiogenic activities by mechanisms independent of their enzymatic activity. This paper aims to discuss original finding against anti-tumor and anti-angiogenic activities of $sPLA_2$ isolated from Tunisian vipers: *Cerastes cerastes* and *Macrovipera lebetina*, representing new tools to target specific integrins, mainly, $\alpha 5\beta 1$ and αv integrins.

1. Introduction

Snake venom is a natural biological resource, containing several neurotoxic, cardiotoxic, cytotoxic, and many other different active compounds [1, 2]. Due to this broad range of biological functions, these biomolecules have been the subject of hundreds of scientific articles in different research fields, including biochemistry, biophysics, pharmacology, toxicology, and medicine [2–5]. Viperidae snake venoms contain class II PLA_2s, which share structural features with secreted PLA_2 ($sPLA_2$) of the class II-A present in inflammatory exudates in mammals. A number of venom PLA_2s have been shown to induce a variety of pharmacological effects although comprehensive studies of the actions of venom PLA_2s in the various events of toxicity are scarce [6, 7].

2. Viperidae Snake Venom Phospholipase A_2 Enzymes: Secreted Phospholipases A_2

Secreted PLA_2 constitute a large superfamily of enzymes that are widely distributed in living organisms. The $sPLA_2$ from Viperidae snake venoms fall under group II. They are generally Ca^{2+}-dependant enzymes that catalyze the hydrolysis of the sn-2 fatty acid bond of phospholipids to release free fatty acids and lysophospholipids [7]. These enzymes are small proteins (~13-14 kDa), containing 120–125 aminoacid residues, 7 disulfide bridges, and have a partially conserved

structure that define the PLA_2 fold [8]. Group II snake venom PLA_2 enzymes can also be divided into different subgroups on the basis of the aminoacid residue in the forty-ninth position. Asp49 plays an important role in catalysis and it is conserved in most snake venom PLA_2 enzymes, and hence these are identified as D49 enzymes [9]. However, in some of the group IIA PLA_2 enzymes this aminoacid residue is replaced by lysine, serine, asparagine, or arginine and they are identified as K49 [10], S49 [11], N49 [12], or R49 [13] enzymes, respectively. Substitution of Asp in the forty-ninth position interrupts the binding of cofactor Ca^{2+} to the Ca^{2+}-binding loop, and hence "mutants" show low or no hydrolytic activity [10, 14, 15]. In addition, there are several substitutions in the Ca^{2+}-binding loops of these mutant enzymes.

Secreted phospholipases A_2 constitute major components of snake venoms and have been extensively investigated not only because they are very abundant in these venoms but mainly because they display a variety of relevant toxic actions such as neurotoxicity, myotoxicity, cytotoxicity, cardiotoxicity, edema-inducing, artificial membrane disrupting convulsant, hypotensive and proinflammatory effects [7, 16–19]. Besides, they exert a wide range of biological effects, including anticoagulant, platelet aggregation inhibiting [7, 20, 21], bactericidal [22], anti-HIV [23], antimalarial and antiparasitic [24], antitumor [21, 25, 26], and recently antiangiogenic effect [27–29]. Due to this functional diversity, these structurally similar proteins aroused the interest of many researchers as molecular models for study of structure-function relationships. One of the main experimental strategies used for the study of myotoxic PLA_2s is the traditional chemical modification of specific aminoacid residues and examination of the consequent effects upon the enzymatic, toxic, and pharmacological activities. Furthermore, some venom $sPLA_2$ have no catalytic activity while they exert various toxic and pharmacological effects [17, 21, 26]. The absence of direct correlation between catalytic activity and pharmacological effects has led to the hypothesis that specific actions of $sPLA_2$ are due to the presence of pharmacological sites on the $sPLA_2$ surface overlapping or distinct from the catalytic site. These pharmacological sites would allow the $sPLA_2$ to bind specifically to soluble or membrane-bound proteins that participate to the $sPLA_2$ mechanism of action [30].

Since this hypothesis was proposed, a collection of binding proteins have been identified using several toxic snake venom $sPLA_2$ [31]. Besides β-bungarotoxin [32, 33], early studies with the neurotoxic $sPLA_2$ OS2 from Australian Taipan snake *Oxyuranus scutellatus scutellatus* have led to the identification of two families of binding proteins called N- and M-type receptors [31, 34, 35]. The N-type receptors are present in mammalian brain and other tissues. Neurotoxic $sPLA_2$, such as OS2, bind with N-type receptors with high affinity, while nontoxic $sPLA_2$ including OS1 bind with much lower affinity, suggesting that these receptors are involved in neurotoxicity.

Conversely, the M-type receptors bind with high affinity both toxic and nontoxic $sPLA_2$ including OS1 and OS2 [31]. Importantly, the M-type receptors also bind with several mammalian $sPLA_2$ [31, 36], suggesting that these proteins are the endogenous ligands for these receptors, and possibly for the collection of binding proteins initially identified with venom $sPLA_2$.

3. Tunisian Viperidae Snake Venom Proteins

Snake venom is a natural source for molecules known as modulators of integrin-mediated functions [37]. Pharmacological study of snake venoms reveals structural and functional polymorphisms of proteins they contain. In our laboratory in Pasteur Institute of Tunis, we are interested in studying different pharmacological effects of Tunisian Viperidae venoms, mainly, the horned viper, *Cerastes cerastes*, *Macrovipera lebetina transmediterranea,* and *Cerastes vipera* [38]. Bazaa et al. showed that these venoms contain proteins belonging to a few protein families. However, each venom showed distinct degree of protein composition complexity. The three venoms shared a number of protein classes though the relative occurrence of these toxins was different in each snake species. On the other hand, the venoms of the *Cerastes* species and *Macrovipera lebetina* each contained unique components [38]. The comparative proteomic analysis of Tunisian snake venoms provides a comprehensible catalogue of secreted proteins, which may contribute to a deeper understanding of the biological effects of the venoms and may also serve as a starting point for studying structure-function correlations of individual toxins.

Thereby, disintegrins and C-type lectins (CLPs) are among the most studied proteins proved to be components of medical and biotechnological value [39–42]. Indeed, they are potent and specific antagonists of several integrins, such as $\alpha v\beta 3$ and $\alpha 5\beta 1$ [43, 44] and can thus act in many biological processes including platelet aggregation, angiogenesis, tumor invasion, and bone destruction [39, 45–47]. On the other hand, CLPs were first described as modulators of platelet before their antiadhesive activity was highlighted [48–50]. CLPs are thus able to inhibit integrin-dependent proliferation, migration, invasion, and angiogenesis [26, 44, 51, 52]. Sarray and coworkers have isolated lebectin and lebecetin, two C-type lectins, from *Macrovipera lebetina* snake venom inhibiting $\alpha 5\beta 1$- and αv-containing integrins [43, 44]. Since their initial characterization, snake venom disintegrins have been extensively studied [39, 46], they are potent inhibitors of integrin-ligand interactions. The integrin inhibitory profile of disintegrins primarily depends on the sequence of a tripeptide located at the apex of a mobile loop and constrained in its active conformation by the appropriate pairing of disulfide bonds. So, CC5 and CC8 have been previously characterized as *Cerastes cerastes* dimeric disintegrins targeting $\alpha_{IIb}\beta_3$, $\alpha v\beta 3$, and $\alpha 5\beta 1$ integrins [53]. In addition to dimeric disintegrins, *Macrovipera lebetina* venom includes short disintegrin, namely, lebestatin which targets $\alpha 1\beta 1$ integrin [46].

Recently, phospholipases A_2 (PLA_2s, EC 3.1.1.4) have been demonstrated to modulate integrins which are essential

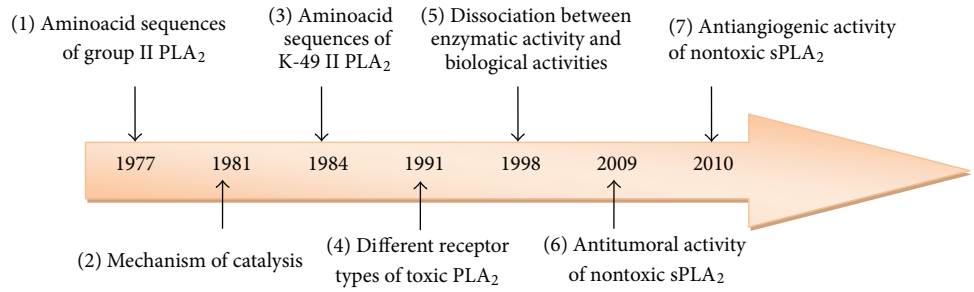


Figure 1: Milestones in PLA_2 enzyme research. (1) Heinrikson et al. [81], (2) Lambeau et al. [56], (3) Maraganore et al. [10], (4) Lambeau et al. [82], (5) Landucci et al. [83], (6) Zouari-Kessenti et al. [21], Bazaa et al. [27], and (7) [27, 28].

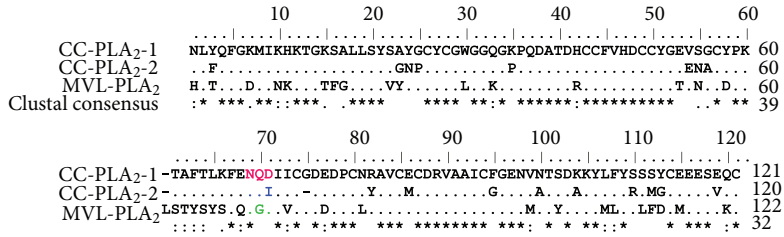


Figure 2: Sequence alignment of Tunisian Viperidae $sPLA_2$: CC-PLA_2-1 (ACO92622) [28], CC-PLA_2-2 (ACO92623) [28], and MVL-PLA_2 (CAR40186) [26]. Gaps (—) have been introduced to optimize alignment.

protagonists of the complex multistep process of angiogenesis, the major target for the development of anticancer therapies [21, 27, 28] (Figure 1).

4. Secreted Phospholipases A_2 from Tunisian Vipers

Three acidic, nontoxic, Asp49 phospholipases A2 have been isolated from Tunisian vipers: CC-PLA_2-1 and CC-PLA_2-2 from *Cerastes cerastes*, and MVL-PLA_2 from *Macrovipera vipera*. They have a molecular weight of 13737.52, 13705.63, and 13626.64 Da, respectively. They contain, respectively, 121, 120, and 122 aminoacids, including 14 cysteines each [21, 26]. The sequences alignment shows similarity as high as 50% (Figure 2). Furthermore, none of the three PLA2s is cytotoxic up to 2 μM.

CC-PLA_2-1 and CC-PLA_2-2 present a high enzymatic activity [21], while MVL-PLA_2 shows a low one. Although they differ greatly in their catalytic properties, these shared many pharmacological activities proving the lack of correlation between enzymatic and pharmacological activities.

5. Pharmacological Activities of $sPLA_2$ from Tunisian Vipers

CCPLA_2-1, CC-PLA_2-2, and MVL-PLA_2 show many pharmacological effects [21, 26]. The most interesting are the antitumor and antiangiogenic activities which involve integrins [27, 28].

5.1. Tunisian Viperidae $sPLA_2$ Effects on Haemostatic System. Snake venom toxins are now regularly used in laboratories for assaying haemostatic parameters and as coagulation reagents [54, 55]. PLA_2 enzymes are known to inhibit blood coagulation. Depending on the dose required to inhibit coagulation, they are classified into strong, weak, and nonanticoagulant enzymes [56, 57]. Strong anticoagulant PLA_2 enzymes inhibit the activation of FX to FXa by both enzymatic and nonenzymatic mechanisms and inhibit the activation of prothrombin to thrombin by nonenzymatic mechanism [58, 59]. In our case, 0.14 μM of both CC-PLA_2s completely inhibited plasma coagulation. Thus, CC-PLA_2s could be considered among the most anticoagulant yet described for PLA_2s snake venom [21]. Lizaro and coworkers showed that myotoxin II, a basic PLA_2 from *Bothrops nummifer*, was unable to inhibit coagulation of the platelet-poor plasma until 3.57 μM [60]. Moreover, it has been shown that BaspPLA(2)-II, an acidic, Asp49 PLA_2 from *Bothrops asper* venom lacks anticoagulant activity [61].

Platelet aggregation plays a role in clot retraction and wound healing. Any alteration in platelet aggregation could lead to debilitation or death. CC-PLA_2-1 and CC-PLA_2-2 showed high antiplatelet aggregation activities induced by arachidonic acid or ADP [21], contrary to b/D-PLA2 which displays high enzymatic and anticoagulant activities but has no platelet aggregation [62]. Moreover, Kashima and coworkers reported that BthA-I-PLA_2, a nontoxic acidic PLA_2 from *Bothrops jararacussu* snake venom, inhibited ADP-induced platelet aggregation with moderate effect [63].

While, OHVA-PLA_2, an acidic PLA_2 from *Ophiophagus hannah*, strongly inhibited platelet aggregation in the presence of ADP or arachidonic acid [64]. It thus appears that PLA2 platelet activity is not directly due to its acidic nature or its anticoagulation activity.

5.2. Tunisian Viperidae $sPLA_2$ Effects on Tumor Cell Behavior. Snake venom $sPLA_2$ present a wide range of pharmacological effects [7], including cytotoxicity on tumor cells [7, 63, 65]. Concerning CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2, concentrations up to 2 μM during 4 days did not induce detectable cytotoxicity on human cell lines IGR39 (melanoma) and HT1080 (fibrosarcoma) [21, 26].

Adhesion and cell migration are two fundamental steps in numerous diseases, like cancer. CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2 inhibit adhesion and migration of human HT1080 and IGR39 cells to fibrinogen and fibronectin. This effect persists even after complete blockage of the catalytic activity suggesting that, contrary to Bth-A-I-PLA_2 whose antitumoral effect appears to be linked to enzymatic site [63], the inhibitory and enzymatic activities are supported by different sites. RVV-7, a cytotoxic basic PLA_2 from *Russsell's viper* venom, inhibits also tumor development [65]. On the contrary, b/D-PLA_2 represents the exception of these enzymes as it stimulates tumor growth [62]. Since Tunisian phospholipases A_2 are not cytotoxic, it seems that their antitumoral activity is exerted by a different mechanism. Using different assays, such as a solid-phase binding assay and a panel of immobilized antibodies, we have proved that CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2 inhibit cell adhesion and migration by interacting directly with αv and $\alpha5\beta1$ integrins [26, 28].

5.3. Tunisian Viperidae $sPLA_2$ Effects on Angiogenesis. Angiogenesis is fundamental to normal healing, reproduction, and embryonic development. However, this process is also important in the pathogenesis of a broad range of disorders such as arthritis and cancer [66]. Angiogenesis is thus required to sustain malignant cells with nutrients and oxygen for tumors to grow beyond a microscopic size. Thus, the microvascular endothelial cell recruited by a tumor is an important target in cancer therapy and has the advantage of being genetically stable. Therefore, treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone.

The role of $\alpha v\beta3$ integrin in the angiogenic process is well documented [67]. In the last decade, several clinical trials evaluating the efficacy of $\alpha v\beta3$ blockers have led to encouraging results in cancer therapy and diagnosis. Similarly, $\alpha5\beta1$ integrin is involved in angiogenesis and more precisely in growing vessels, but its expression disappears in mature vessels [68]. Thereby, when tested *in vitro*, the two CC-PLA_2 and MVL-PLA_2 impaired adhesion and migration of HBMEC (human brain microvascular endothelial cells) and HMEC-1 (human microvascular endothelial cell), respectively, by interfering with integrin function. Moreover, using the CAM assay, an *ex vivo* model, these $sPLA_2$ strongly reduced vasculature development. The treatment reduced the number of new capillaries and branching, without affecting the mature blood vessels, suggesting once again the implication of $\alpha5\beta1$ integrin. Interestingly, CC-PLA_2-1 and CC-PLA_2-2 inhibit spontaneous angiogenesis as well as angiogenesis induced by growth factors such as VEGF or bFGF [28]. The antiangiogenic effect of PLA_2 can be due partly to the blockage of the $\alpha v\beta3$ and $\alpha5\beta1$ integrins functions. However, inhibition of angiogenesis can also result from blockage of VEGF or its receptor. Thus, it has been reported that inactive PLA_2 homologues, such as KDR-bp isolated from *Eastern cottonmouth* venom, are common antagonists of KDR, a VEGF receptor [69].

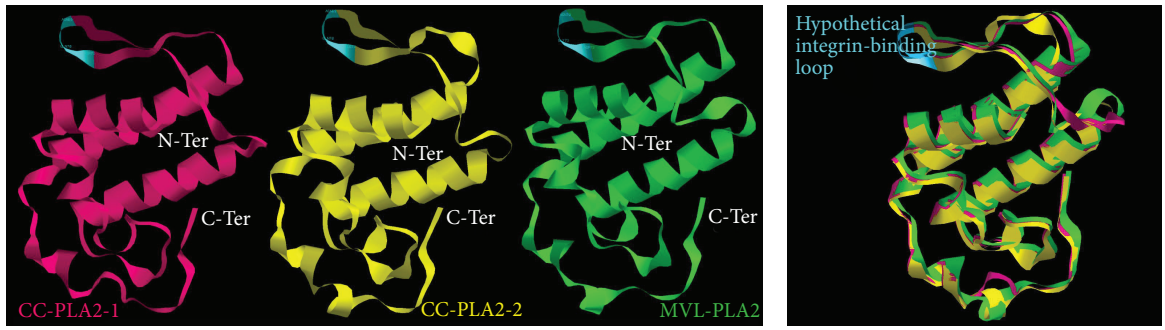
Focal adhesions are specialized sites of attachment of cells where integrins receptors, such as $\alpha v\beta3$, link the extracellular matrix to the actin cytoskeleton, allowing migration [70]. Cell migration is a complex cellular behavior that results from the coordinated changes in the actin cytoskeleton and the controlled formation and dispersal of cell-substrate adhesion sites. While the actin cytoskeleton provides the driving force at the cell front, the microtubule network assumes a regulatory function in coordinating rear retraction. The polarity within migrating cells is further highlighted by the stationary behavior of focal adhesions in the front and their sliding in trailing ends [71].

Treatment of HMEC-1 cells with MVL-PLA_2 induced important changes in cell morphology. Treated cells have a circular shape and actin stress fibers are thinner or absent, with the actin mainly located at the cell periphery. Moreover, MVL-PLA_2 leads to drastic reduction in the size of focal adhesions and their distribution all over the ventral surface of cells, consistent with a decrease in $\alpha v\beta3$ integrin clustering and its absence from lamellipodia [27]. Therefore, it appears that the inhibition of migration is associated with important reorganization of the actin cytoskeleton and focal adhesions. Again, there is a clear dissociation between the anti-angiogenic effect and the catalytic activity.

Furthermore, MVL-PLA_2 strongly increased MT dynamicity in HMEC-1 cells. Because the microtubule cytoskeleton is essential in the orchestration of endothelial cell motility [72, 73], microtubule-targeting agents are known to have antiangiogenic effects through the modulation of cytoskeleton dynamicity [27]. Thus, microtubule-binding drugs are widely used in cancer chemotherapy and also have clinically relevant antiangiogenic and vascular-disrupting properties [74].

6. Importance of the Identification of Pharmacological Sites

The pharmacological sites of PLA_2 enzymes determine the affinity between the PLA_2 and target proteins. The identification of pharmacological sites helps in (1) understanding the structure-function relationships of PLA_2 enzymes, (2) developing strategies to neutralize the toxicity and pharmacological effects by targeting these sites, and (3) developing prototypes of novel research tools and pharmaceutical drugs [7, 8].



(a) (b)

Figure 3: (a) Tertiary model structure of CC-PLA_2-1 (pink), CC-PLA_2-2 (yellow), and MVL-PLA_2 (green). (b) Superimposition of the structural models of CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2. The hypothetical integrin-binding loop (blue) is presented in each PLA_2.

In our studies, we showed that CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2 target the $\alpha 5\beta 1$ and αv integrins, particularly $\alpha v\beta 3$. Moreover, angiogenesis involves expression of the later, which binds to RGD-containing components of the interstitial matrix [75].

To further understand the mechanism of action, we report that endothelial cells are able to adhere on immobilized MVL-PLA_2 and that this adhesion is impaired by RGD peptides [27]. This suggests that interaction between MVL-PLA_2, CC-PLA_2-1, or CC-PLA_2-2 and integrins involves RGD-like sequence which may be responsible for the inhibition of integrin function. This hypothesis is supported by Ramos and coworkers' study, showing that general folding of electrostatic potential is the main intervening of disintegrin-integrin interaction [76].

When MVL-PLA_2 contains a NGD sequence, which could be considered as an RGD-like motif, CC-PLA_2-1 and CC-PLA_2-2 present NQD and NQI, respectively, that may also be responsible for the inhibition of integrin function.

Therefore, bioinformatics study and structural criteria that would allow identifying biologically active RGD-sites on the base of a protein's spatial structure may become a helpful tool for analysis of cellular function of proteins [77]. Furthermore, conformation of the integrin-binding loop in a protein is defined not only by physicochemical properties and conformation of the sequence itself, but also by its structural environment and therefore of the potential biological activity. Besides the RGD-like sequence site should be placed on a loop or a beta-turn to be well exposed. We can cite disintegrin, like applied model, in which we can note a loop accessible stabilized by disulfide bridges [78].

7. Molecular Modeling of CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2

In order to examine the site of the suspected RGD-like sequence, using the SWISS-MODEL Workspace (http://swissmodel.expasy.org/), we have determined the three-dimensional models of CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2.

Firstly, as shown in Figure 3(a), the three models are very similar. Interestingly, we can note the presence of very-well-exposed loop containing the suspected RGD-like motif. This loop is very similar to that of the disintegrins.

In the case of MVL-PLA_2, we find the NGD motif, while for CC-PLA_2-1 and CC-PLA_2-2 there is NQD and NQI, respectively. According to our hypothesis, the residue R in RGD motif is replaced by the N which is hydrophilic and polar residue, it is even more hydrophilic than R residue, this leads to higher affinity towards the $\alpha v\beta 3$ integrin [79]. Besides, the D residue favors recognition of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins [79]. In addition, in CC-PLA_2-1 and CC-PLA_2-2 the RGD-like motif is flanked by two E residues, highly polarized which could enhance the inhibitory effect towards integrins that bind to ligands through RGD sites, including the fibronectin receptor, mainly, the $\alpha 5\beta 1$ integrin [80].

On the other side, based on the study of disintegrins, it is known that integrin-binding ability is apparently more related to the Cys-rich domain. Similarly, CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2 present 14 Cys forming 7 disulfides bridges. We can postulate that disulfide bonds, especially Cys50–Cys86 and Cys57–Cys79, stabilized the hypothetical integrin-binding loop. The superimposition of the structural models of CC-PLA_2-1, CC-PLA_2-2, and MVL-PLA_2 shows that they share similar conformational features (Figure 3(b)).

Nevertheless, further structure-function relationships study must be carried to verify this hypothesis.

8. Conclusion

Secreted phospholipase A_2 enzymes, especially from *Viperidae* Snake venom, exhibit a wide variety of pharmacological effects despite their structure similarity. These enzymes provide a great challenge to protein chemists as subtle and complex puzzles in structure-function relationship. A better understanding will contribute to our knowledge of protein-protein interactions, protein targeting, and protein engineering, and to the development of better-targeted delivery systems. Further research in identifying target proteins will bring details on the mechanisms of the pharmacological effects at the cellular and molecular levels. Studies in these

areas will result in new, exciting, and innovative opportunities in the future, both in finding answers to the toxicity of PLA_2 enzymes and could bring useful tools for developing proteins with novel functions.

Interestingly, we have demonstrated that two isoforms of PLA_2 (CC-PLA_2-1 and -2), from horned Tunisian viper *Cerastes cerastes* and another from *Macrovipera lebetina* MVL-PLA_2 target integrins, a large and very important family of adhesion molecules that promote stable interactions between cells and their environment [26, 28]. Indeed, these $sPLA_2$ exhibit a potent antitumor and antiangiogenic activities. We showed that their effect is likely due to the inhibition of $\alpha 5\beta 1$- and αv-containing integrins [26, 28].

These nontoxic secreted phospholipase A_2 could be new tools to disrupt different steps of tumor and angiogenesis progression through integrins. It is noteworthy that this effect is independent of the enzymatic activity. This finding may serve, on the one hand, as a mean to discuss the molecular regions involved in recognition of tissue targets and, on the other hand, as starting point structure-function relationship studies leading to design a new generation of anticancer drugs.

Abbreviations

$sPLA_2$: Secreted phospholipase A_2
CLP: C-type lectin protein
VEGF: Vascular endothelial growth factor
bFGF: Basic fibroblast growth factor
CAM: Chick chorioallantoic membrane.

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Research Article

Unmasking Snake Venom of *Bothrops leucurus*: Purification and Pharmacological and Structural Characterization of New PLA_2 Bleu TX-III

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Bleu TX-III was isolated from *Bothrops leucurus* snake venom on one-step analytical chromatography reverse phase HPLC, was homogeneous on SDS-PAGE, and was confirmed by Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry in 14243.8 Da. Multiple alignments of Bleu TX-III show high degree of homology with basic PLA_2 myotoxins from other *Bothrops* venoms. Our studies on local and systemic myotoxicity "*in vivo*" reveal that Bleu TX-III is myotoxin with local but not systemic action due to the decrease in the plasmatic CK levels when Bleu TX-III is administrated by intravenous route in mice (dose 1 and 5 μg). And at a dose of 20 μg myotoxin behaves like a local and systemic action. Bleu TX-III induced moderate marked paw edema, evidencing the local increase in vascular permeability. The inflammatory events induced in the mice (I.M.) were investigated. The increase in the levels of IL-1, IL-6, and TNF-α was observed in the plasma. It is concluded that Bleu TX-III induces inflammatory events in this model. The enzymatic phospholipid hydrolysis may be relevant to these phenomena. *Bothrops leucurus* venom is still not extensively explored, and the knowledge of its toxins separately through the study of structure/function will contribute for a better understanding of its action mechanism.

1. Introduction

Snake venom contains a mixture of powerful proteins and peptides that have evolved to be targeted to receptors, ion channels, or enzymes [1], in addition to some carbohydrates, nucleosides, lipids, and metal ions, whose functions are not all known [2, 3]. They interact with a wide variety of mammalian proteins and can disrupt the central and peripheral nervous systems, the blood coagulation cascade, the cardiovascular and neuromuscular systems, and homeostasis in general. These venom proteins act with great precision different toxins recognize different subtypes of certain receptors with only subtle differences and are very biologically active.

Phospholipases A_2 (PLA_2, EC 3.1.1.4) are generally Ca^{2+}-dependent enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of glycerophospholipids. Secreted PLA_2s are small proteins (14–18 kDa) usually containing 5–8 disulfide bonds and possessing a His/Asp dyad required for catalysis. Snake venom PLA_2s are classified into groups I or II, based on their sequence and mode of disulphide pairings. Group I PLA_2s are found in the venoms of Elapidae snakes, whereas group II PLA_2s are present in the venoms of Viperidae snakes [4]. The group II is further divided into two main subgroups: Asp49 and Lys49 (PLA_2 homologues) variants. In the latter, the aspartic acid residue at position 49, critically involved in calcium binding and essential for catalytic activity, is replaced by lysine. Due to this and other critical substitutions, the Lys49 PLA_2s cannot bind calcium efficiently and are considered to be enzymatically inactive [5, 6]. Although catalytic activity has been shown to play a role in the toxic actions of some venom PLA_2s, it is not essential in the case of

Lys49 PLA_2s, which use nonenzymatic mechanisms to alter membrane homeostasis [6].

Several PLA_2s have been identified from *Bothrops leucurus* venom including one acidic [7], one basic phospholipase A_2 and phospholipase A_2 homologous K49 [8]. This diversity of PLA_2, found in the venom of *Bothrops leucurus*, agrees with studies that show marked ontogenetic and individual venom variations [1].

In the present work, a new basic PLA_2 (Bleu TX-III) from the venom of *Bothrops leucurus* has been isolated and characterized, in order to obtain insights into its possible biological roles and its relevance to the pathophysiology of envenomings by this species in the north-eastern Brazil.

2. Material and Methods

2.1. Reverse Phase HPLC. The PLA_2 (Bleu TX-III) from *Bothrops leucurus* snake venom was purified by reverse phase HPLC according to method described by Ponce-Soto et al. [9]. Briefly, 5 mg of whole venom was dissolved in 100 μL of buffer A (0.1% TFA) and 150 μL NH_4HCO_3 50 mM and centrifuged at 4500 g, and the supernatant was then applied on the analytical reverse phase HPLC μ-Bondapack C-18, previously equilibrated with buffer A for 15 min. The elution of the protein was then conducted using a linear gradient of buffer B (66.5% Acetronitrile in buffer A), and the chromatographic run was monitored at 280 nm of absorbance. After elution the fraction was lyophilized and stored at 40°C.

2.2. Electrophoresis. Tricine SDS-PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of the proteins, under reducing and nonreducing conditions [10].

2.3. PLA_2 Activity. PLA_2 activity was measured using the assay described [11] modified for 96-well plates [12]. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, and 100 mM NaCl, pH 8.0), 20 μL of substrate (3 mM), 20 μL of water and 20 μL of PLA_2 (1 mg/mL) in a final volume of 260 μL. After adding PLA_2 (Bleu TX-III) from *Bothrops leucurus* (20 μg), the mixture was incubated for up to 40 min at 37°C, with the reading of absorbance at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase of absorbance after 20 min. All assays were done in triplicate, and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Amino Acid Analysis. Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described [13]. The PLA_2 (Bleu TX-III) from *Bothrops leucurus*, sample (30 μg), was hydrolyzed at 105°C for 24 h, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μL of derivatization solution (ethanol : triethylamine : water : phenylisothiocyanate, 7 : 1 : 1 : 1, v/v) for 1 h at room temperature, after which the PTC-amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture.

2.5. Mass Spectrometry. An aliquot (4.5 μL) of the modified proteins was inject in C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode, and the data acquisition was from *m/z* 100–3.000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data by the mass spectrometer on the *m/z* scale were converted to the mass (molecular weight) scale using Maximum Entropy-based software supplied with Masslynx 4.1 software package. The processing parameters were output mass range 6.000–20.000 Da at a "resolution" of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da, the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.6. Myotoxic Activity. Groups of four Swiss mice (18–20 g) received an intramuscular (i.m.) or an intravenous (i.v.) injection of variable amounts of PLA_2 (Bleu TX-III) from *Bothrops leucurus* in 100 μL of PBS, in the gastrocnemius. A control group received 100 μL of PBS. At different intervals of time (2, 4, 6, 9, and 24 h) blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Sigma 47-UV). Activity was expressed in U/L, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25°C.

2.7. Edema-Forming Activity. The ability of PLA_2 (Bleu TX-III) from *Bothrops leucurus* to induce edema was studied in groups of five Swiss mice (18–20 g). 50 μL of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with venom (10 μg/paw) was injected in the subplantar region of the right footpad. The control group received an equal volume of PBS alone. The swelling of the paw was measured at 0.5, 1, 3, 6, 9, and 24 h after administration. Edema was expressed as the percentage increased in the volume of the treated group to that of the control group at each time interval.

2.8. Cytokines. The levels of cytokines IL-6 and IL-1 in the serum from BALB/c mice were assayed by a two-site sandwich enzyme-like immunosorbent assay (ELISA) as described [14]. In brief, ELISA plates were coated with 100 μL (1 μg/mL) of the monoclonal antibodies anti-IL-6 and anti-IL-1. In 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 hours at room temperature. The

wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 μL of 10% fetal calf serum (FCS) in PBS for 2 hours at room temperature. After washing, duplicate sera samples of 50 μL were added to each well. After 18 hours of incubation at 4°C, the wells were washed and incubated with 100 μL (2 μg/mL) of the biotinylated monoclonal antibodies anti-IL-6 and anti-IL-1 as second antibodies for 45 minutes at room temperature. After a final wash, the reaction was developed by the addition of orthophenyldiamine (OPD) to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines (expressed in picograms per millilitre). The minimum levels of each cytokine detectable in the conditions of the assays were 10 pg/mL for IL-6 and IL-1.

To measure the cytotoxicity of TNF-α present in the serum from BALB/c mice, a standard assay with L-929 cells, a fibroblast continuous cell line was used as described previously [15]. The percentage cytotoxicity was calculated as follows:

$$\left(A_{\text{control}} - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100. \quad (1)$$

Titres were calculated as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayer were lysed. TNF-α activity is expressed as units/mL, estimated from the ratio of a 50% cytotoxic dose of the test to that of the standard mouse recombinant TNF-α.

2.9. Statistical Analyses. Results were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by Dunnett's test when several experimental groups were compared with the control group. Differences were considered statistically significant if $P < 0.05$.

3. Results

The elution profile of *Bothrops leucurus* venom following RP-HPLC performed on a C18 column showed thirteen fractions (1–12) (Figure 1(a)). The five eluted peaks were screened for PLA_2 activity. Only the fraction labeled in Figure 1(a) presented PLA_2 activity, which was eluted with 59% of buffer B. This peak was further purified by the same chromatography system used in the first fractionation step (RP-HPLC). The result of the repurification showed the presence of only one peak, named Bleu TX-III (Figure 1(a) inserted).

The Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry analysis confirmed the homogeneity of the fraction Bleu TX-III and determined the exact molecular mass of 14243, 4297 Da (Figure 1(b)). This value of molecular mass was used in calculating the molar concentrations of toxin used in the experiments described below.

The amino acid composition determined was D/11, T/9, S/7, E/9, P/4, G/11, A/5, C/14, V/4, M/3, I/5, L/7, Y/11, F/5, K/7, H/3 e R/10, W/Not determined.

Table 1: Sequence obtained by ESI-MS/MS based on the alkylated tryptic peptides derived. The peptide were separated and sequenced by mass spectrometry.

Residue Number	Mass (Da) expected	Amino acid sequence	Mass (Da) calculated
1–11	1377.6510	DLWQFGKMILK	1377.7479
8–15	918.4353	MILKETGK	918.5208
43–53	1504.4404	CCFVHDCCYGK	1504.5356
61–68	1046.4846	TDRYSYSR	1046.4781
69–82	1520.6066	ENGDVVCGGDDPCK	1520.5872
98–106	1110.4915	DNKDTYDIK	1110.5193
107–113	933.4279	YWFYGAK	933.4385

The PLA_2 activity was examined in the *Bothrops leucurus* venom and in Bleu TX-III using the synthetic substrate 4-nitro-3 (octanoyloxy) benzoic. The PLA_2 activity was higher in Bleu TX-III (16,22 ± 0,5268 nmols/mim/mg) and P4 (*b*/D-PLA_2) (15,728 ± 0,3354 nmols/mim/mg), when compared with fraction 2 (P3 *b*/K-PLA_2) (2,856 ± 0,464 nmols/mim/mg), and the whole venom (3,617 ± 0,4144 nmols/min/mg) (Figure 1(c)).

The alkylated protein Bleu TX-III was digested separately with trypsin, and the resulting tryptic peptides were fractionated by RP-HPLC. Each peak numbered in the chromatogram (data not shown) was manually collected and lyophilized, and sequencing of the peptide was done by ESI mass spectrometry. Isoleucine and leucine residues were not discriminated in any of the sequences reported since they were indistinguishable in the low-energy CID spectra. Due to the external calibration applied to all spectra, it was also not possible to resolve the 0.036 Da difference between the glutamine and lysine residues, except for the lysine that was deduced based on the cleavage and missed cleavage of the enzyme.

The deduced sequence and measured masses of alkylated peptides of Bleu TX-III are summarized in Table 1; on the basis of sequence determination, 9 peptides were finding in the protein. The sequence of each peptide was then submitted separately to the NCBI database using the protein search program BLAST-p. Using the position matches of the sequenced peptides with homologous proteins present in the database, it was possible to deduce their original position on the unknown protein Bleu TX-III.

The sequence of those proteins returns high homology with the sequence of a phospholipase A_2 present in the venom of *Crotalus scutulatus scutulatus* (Mojave rattlesnake) (PA2B_CROSS Accession Number P62023; [16]. The partial Bleu TX-III sequence obtained was then resubmitted to BLAST-p, with the search restricted to Crotalinae snakes. Figure 3 shows the result of BLAST alignment between Bleu TX-III with the phospholipase A_2 from human pancreatic (0910150a) [17] and other PLA_2 coming from the venom of snakes of the family Viperidae. P62023 Mtx-b PLA_2 of *Crotalus scutulatus scutulatus* [16]; P0C942_1a LmTX-I de *Lachesis muta muta* [5] and P0C8M1 BmTX-I of *Bothrops moojeni* [18].

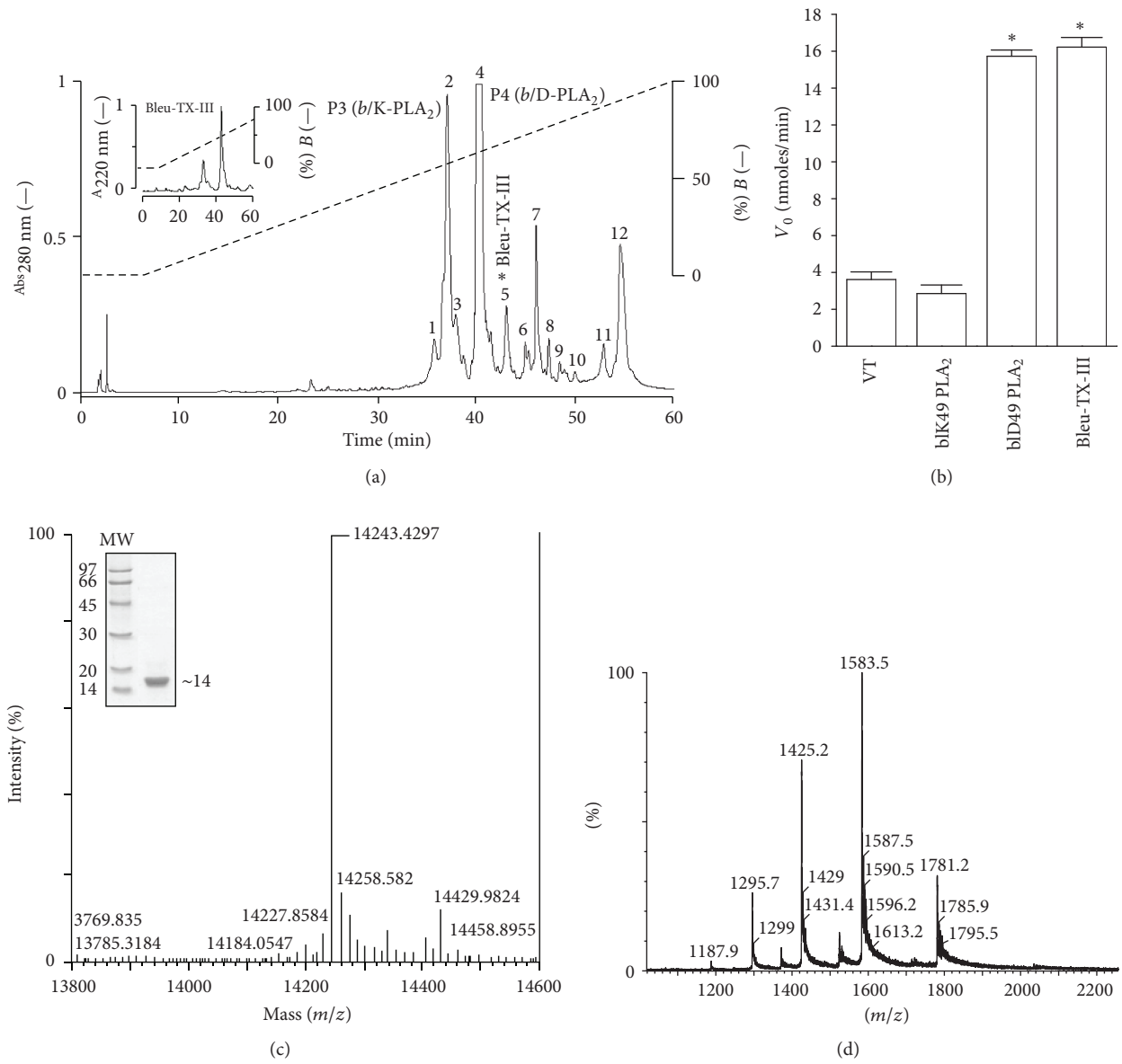

Figure 1: (a) Elution profile of *Bothrops leucurus* venom by RP-HPLC on a μ-Bondapack C18 column. *Fraction 5 (PLA_2 Bleu TX-III) contained PLA_2 and myotoxic activity. Insert: rechromatography on RP-HPLC of Bleu TX-III. (b) PLA2 activity of *Bothrops leucurus* venom, *b*/K49, *b*/D49 [8], and PLA_2 Bleu TX-III. The results of all experiments are the mean ± SEM of three determinations ($P < 0.05$). (c) Mass determination of the native Bleu TX-III by Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry. Insert electrophoretic profile in Tricine SDS-PAGE. (d) Raw electrospray-positive mass spectrum, showing multicharged ions distributions of native Bleu TX-III.

The sequence coverage was high for Bleu TX-III; the protein shared the conserved sequence domains common to this group of proteins, including the 14 cysteines, the calcium-binding site located on Gly30, Gly32, Tyr28, and Asp49, together with the amino acid of active site His48 (SwissProt database http://br.expasy.org/). The tandem mass spectra shown in Figure 2, relative to the peptide eluted in fraction 3 of both digest, having the sequence KCCFVHDCCYG, allow to classify both enzymes as PLA_2. *In vivo*, the Bleu TX-III induced a visible local myotoxic when injected by the i.m. route (Figure 4(a)), but with a regular increase in plasma levels of CK occurred after IV injection in single dose of 20 μg. Time-course analysis showed a maximum increase in plasma CK 3 hours after i.m. injection, returning to normal by 24 h (Figure 4(b)).

Bleu TX-III also induced moderate footpad edema, with a MED of 5 ± 2 μg, evidencing the local increase in vascular permeability. Edema reached its highest point after 3 h, rapidly returning to basal levels thereafter (Figure 4(c)).

To further analyse and compare the mechanisms of the inflammatory events induced by PLA_2 Bleu TX-III, the concentrations of IL-1, IL-6, and TNF-α in the plasma were measured. Bleu TX-III caused a marked increase in levels of both IL-1 and IL-6, 1, 3, and 6 hours, peaking at 6 hours

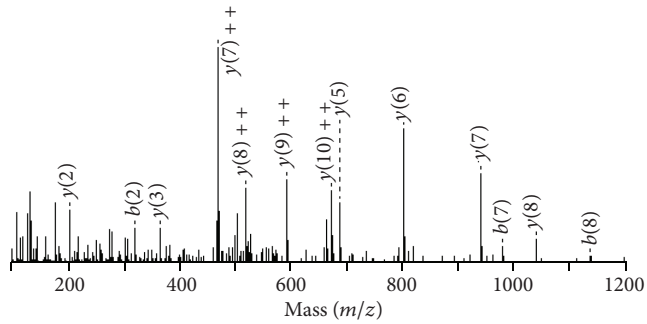

Figure 2: MS/MS spectrum of the doubly-charged tryptic ion of m/z 1504. Ion of the major sequence-specific y-ion serie and of aminor series of the complementing b-ions CCFVHDCCYGK, from which the sequence of Bleu TX-III tag was deduced.

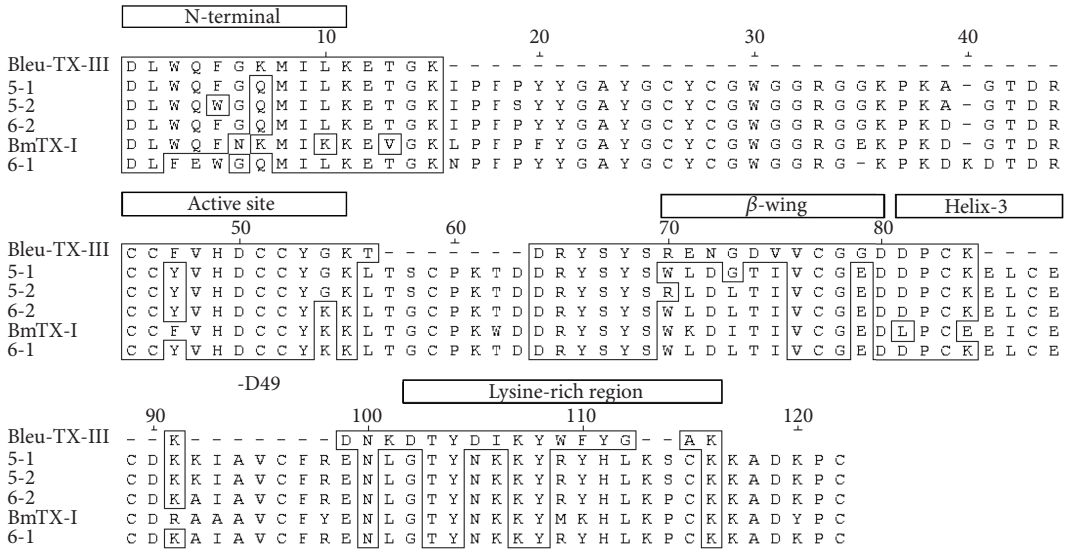

Figure 3: Alignment of the deduced amino acid sequence of the Bleu TX-III PLA_2 with D49-PLA_2. 5_I and 5_II (BmjeTX-I and BmjeTX-II) from *Bothrops marajoensis* [28], 6_1 and 6_2 from *Bothrops jararacussu* [20] and BmTX-I from *Bothrops moojeni* [18].

for IL-1 and 3 hours for IL-6, respectively, (Figures 4(d) and 4(e)). Bleu TX-III caused a significant increase in the TNF-α concentrations only at 1 h (Figure 4(f)).

4. Discussion

PLA_2s are among the most abundant components of snake venoms, showing a wide array of activities in spite of a conserved overall structure [19]. Understanding the structural basis for their diverse toxic activities, including myotoxicity and inflammatory, is still a challenging task. In this work, a new toxin was isolated, and structurally and functionally characterized, from *Bothrops leucurus* venom, showing that it belongs to the family of PLA_2.

The purification procedure for basic PLA_2s developed [9, 20, 21] is shown to be also efficient for the obtainment of the PLA_2 Bleu TX-III myotoxin from *Bothrops leucurus* snake venom. Fractionation protocol of this crude venom using a single pass chromatographic in a column μ-Bondapack C-18 coupled to a system of reverse phase HPLC gave rise to 12 fractions at 280 nm, the two last being the basic myotoxins, named Bleu TX-III PLA_2 (5). This rapid procedure showed high yield, producing 5–10 mg of the proteins with high purity levels (Figure 1(a)) and rechromatography of the major peak by RP-HPLC, what has yielded one main peak (Figure 1(a) inserted). The use of NH_4HCO_3 and acetonitrile (RP-HPLC) as the buffer system is advantageous since these solvents are easily eliminated by lyophilization, thereby eliminating the need for desalting as in the case of ammonium bicarbonate.

The sequences of several tryptic peptides of peaks 2 and 4 (date not shown) were the same as described for P3 (*bl*/K-PLA_2) and P4 (*bl*/D49-PLA_2) [8] (Figure 1(a)). Bleu TX-III was isolated to homogeneity by one chromatographic step. SDS-PAGE under nonreducing conditions showed that it occurs as a monomer, in the range of ~14 kDa after reduction (Figure 1(b) inserted). A subunit molecular mass of 14243.4297 Da was determined by ESI/MS mass spectrometry. The amino acid composition of the toxin revealed a high content of basic and hydrophobic residues, with 14 half-Cys,

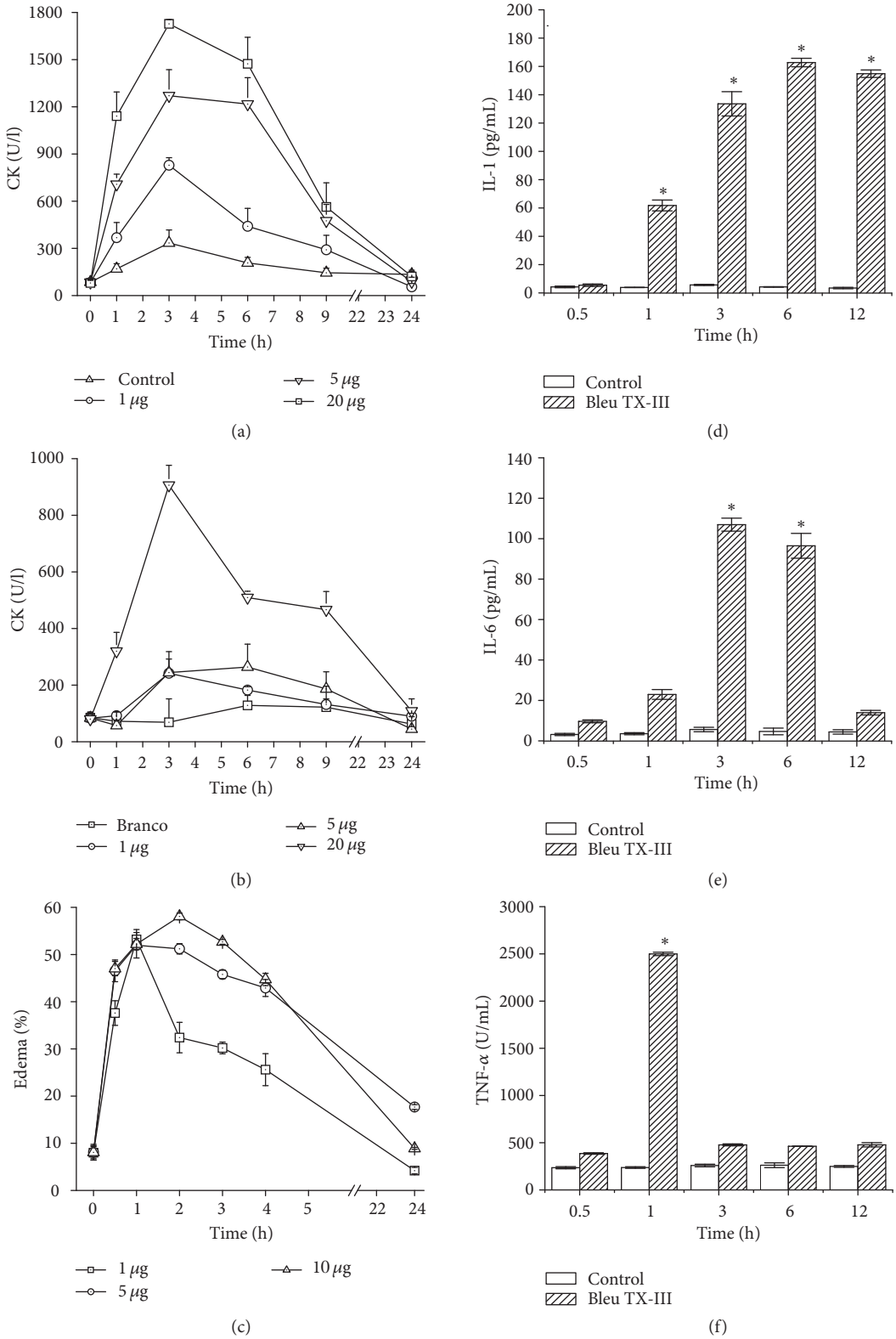

FIGURE 4: (a) and (b) Time-course of the increments in plasma CK activity after intramuscular injection and intravenous of Bleu TX-III PLA_2 (1, 5, and 20 μg). Controls were injected with 100 μL of PBS. At different times, blood was collected, and serum levels were measured. Values are means ± SEM of five mice at each time point. (c) Edema-forming activity of Bleu TX-III PLA_2 (1, 5 and 20 μg). In mice. Induction of edema by Bleu TX-III PLA_2, injected in the footpad of mice. At various time intervals the increase in footpad volume, as compared to controls, was expressed as percent edema. Each point represents the mean ± SEM of five animals. (d), (e), and (f) Cytokines levels in mice. The production of IL-1, IL-6, and TNF-α was assayed in plasma of Bleu TX-III PLA_2. The experiments were performed in triplicate and analyzed statistically by ANOVA or Kruskal-Wallis tests and confirmed by Tukey and Tukey-type tests. Each point represents ±SEM of seven mice $P < 0.05$ between the experimental groups and control group.

in agreement with the reported compositions and primary structures of myotoxic PLA_2s isolated from *Bothrops* venoms [9, 21–23].

This basic PLA_2 showed enzymatic activity on monodisperse substrate, with a strict requirement of Ca^{2+}, and maximum activity at pH 8.0 and 40°C. These characteristics are common to other bothropic and crotalic PLA_2s [21, 24, 25]. The PLA_2 activity is suggested to be higher in Bleu TX-III (16.22 ± 0.5268 nmols/mim) and *bl*/D-49 (4) (15.728 ± 0,3354 nmoles/min) when compared with the whole venom (3.617 ± 0.4144 nmoles/min) and *bl*/K-49 (2.856 ± 0.464 nmoles/min) (Figure 1(c)). The Ca^{2+} ion dependent on the enzymatic activity of Bleu TX-III revealed that the Ca^{2+} ion is an obligatory cofactor for its enzymatic activity. This can be explained by different coordination geometries assumed by the tetrahedral intermediate due to the presence of the Ca^{2+} ion which determine the electrophilic behavior of the catalytic site, as well as stabilizes the otherwise flexible Ca^{2+}-binding loop and appears to optimize the interaction enzyme substrate [26].

Comparison of the N-terminal sequence of Bleu TX-III showed similarity with other myotoxic PLA_2 from *Bothrops* genus (Figure 3).

To Bleu TX-III, no substitution was found in the conserved regions of the catalytic activity, as the channel hydrophobic N-terminal region (1 to 10) is important as a part of the interfacial bonding surface, so as active site (44 to 57) as suggested [26–28]. Considering the 7-peptides sequenced (this work), Bleu TX-III, showed high-level homology with many PLA_2, from different snakes species. The highly conserved sequences XCGXGG and DXCCXXHD responsible for the Ca^{2+}-binding loop and the catalytic site of PLA_2 [29], respectively, are present in the sequence of PLA_2 Bleu TX-III.

The P3 (*bl*/K-PLA_2), P4 (*bl*/D-PLA_2), and fraction 5 (Bleu TX-III) enzymes seemed to be completely separated by reverse phase chromatography, and when some triptic peptides were sequenced by ESI/MS, of three PLA_2, venom showed that *Bothrops leucrurus* have several homologous PLA_2 and PLA_2.

As a result of complications, mainly local edema and necrosis, usually occurred following ophidian accidents with *Bothrops* snakes [30, 31], and studies involving PLA_2s became very important, since they are the main venom components responsible for necrosis and inflammatory response [32].

Histological and ultrastructural studies of the effect of venom PLA_2s on skeletal muscle reveal a common series of pathological alterations which include (1) plasma membrane disruption, (2) formation of "delta-lesions," wedge-shaped areas of degeneration located at the periphery of muscle fibers, (3) hypercontraction of myofilaments, (4) mitochondrial swelling, together with the formation of flocculent densities and rupture of mitochondrial membranes, (5) disruption of intracellular membrane systems, that is, sarcoplasmic reticulum and T tubules, and (6) pycnosis of nuclei [32–35].

Our studies on local and systemic myotoxicity *in vivo* show that PLA_2 Bleu TX-III is not systemic myotoxins in the dose of 5 μg and 20 μg systemic slightly as local action due to decreased plasma levels of CK (Figures 4(a) and 4(b)). This reinforces the hypothesis of differential action of local and systemic myotoxicity proposed [32] and also the specificity and specificity proposed [21, 23, 28].

The snake venom *Bothrops* induced local edema in humans and experimental animals [36]. Besides Bleu TX-III, a number of snake venom PLA_2s, others also induce edema of 30 minutes after injection (Figure 3(c)) [4, 37, 38]. Studies have been conducted trying to understand the mechanisms involved in the inflammatory response induced by the myotoxic PLA_2 from several snake venoms [30]. Studies have been directed trying to understand the mechanisms involved in the inflammatory response induced by myotoxic PLA_2 from several snake venoms [39–41]. However, the relationship between enzymatic activity and edema is contradictory [42]. It is assumed that myotoxic and edematogenic activities can be induced by different structural domains in these PLA_2, or that a partial overlapping of these domains occur [43].

Cytokines, such as IL-1, IL-6, and TNF-α, are also relevant mediators for leukocyte migration and participate in several inflammatory conditions. Our results showed that PLA_2 Bleu TX-III induced a stronger effect on the expression of adhesion molecules by endothelial cells and stimulates the release of both IL-1, IL-6, and TNF-α [44]. Thus, our results suggest that IL-1 may contribute for the leukocyte (Figures 3(d), 3(e), and 3(f)).

In conclusion, Bleu TX-III induces a marked inflammatory reaction in the mouse serum. Since basic myotoxic PLA_2s are abundant in snake venoms, these toxins must play a relevant role in the proinflammatory activity that characterizes this venom. The fact that Bleu TX-III elicited a stronger reaction inflammatory argues in favor of a role of enzymatic phospholipid hydrolysis in this phenomenon, either through the direct release of arachidonic acid from plasma membranes or through the activation of intracellular processes in target cells.

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Research Article

Biochemical, Pharmacological, and Structural Characterization of New Basic PLA_2 Bbil-TX from *Bothriopsis bilineata* Snake Venom

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Bbil-TX, a PLA_2, was purified from *Bothriopsis bilineata* snake venom after only one chromatographic step using RP-HPLC on μ-Bondapak C-18 column. A molecular mass of 14243.8 Da was confirmed by Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry. The partial protein sequence obtained was then submitted to BLASTp, with the search restricted to PLA_2 from snakes and shows high identity values when compared to other PLA_2s. PLA_2 activity was presented in the presence of a synthetic substrate and showed a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0 and 25–37°C. Maximum PLA_2 activity required Ca^{2+} and in the presence of Cd^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} it was reduced in the presence or absence of Ca^{2+}. Crotapotin from *Crotalus durissus cascavella* rattlesnake venom and antihemorrhagic factor DA2-II from *Didelphis albiventris* opossum sera under optimal conditions significantly inhibit the enzymatic activity. Bbil-TX induces myonecrosis in mice. The fraction does not show a significant cytotoxic activity in myotubes and myoblasts (C2C12). The inflammatory events induced in the serum of mice by Bbil-TX isolated from *Bothriopsis bilineata* snake venom were investigated. An increase in vascular permeability and in the levels of TNF-a, IL-6, and IL-1 was was induced. Since Bbil-TX exerts a stronger proinflammatory effect, the phospholipid hydrolysis may be relevant for these phenomena.

1. Introduction

Viperidae snakes are represented in South America by *Crotalus, Bothrops, Bothriopsis* and *Lachesis*. *Bothriopsis bilineata* is the endemic and rare bothropic snake species [1].

The envenomation is characterized by a generalized inflammatory state. The normal reaction to envenomation involves a series of complex immunologic cascades that ensures a prompt protective response to venom in humans [2]. Although activation of the immune system during envenomation is generally protective, septic shock develops in a number of patients as a consequence of excessive or poorly regulated immune response to the injured organism [3]. This imbalanced reaction may harm the host through a maladaptitive release of endogenous mediators that include cytokines and nitric oxide.

PLA_2s are abundant in snake venoms and have been widely employed as pharmacological tools to investigate their role in diverse pathophysiological processes. Viperid and crotalid venoms contain PLA_2s with the ability to cause rapid necrosis of skeletal muscle fibers, thus being referred to as myotoxic PLA_2s [4]. Local inflammation is a prominent

characteristic of snakebite envenomations by viperid and crotalid species [5].

Furthermore, PLA_2 myotoxins are relevant tools for the study of key general inflammatory mechanisms. High levels of secretory PLA_2 ($sPLA_2$) are detected in a number of inflammatory disorders in humans, such as bronchial asthma [6], allergic rhinitis [7], septic shock [8], acute pancreatitis [9], extensive burning [10], and autoimmune diseases [11]. In addition, increased expression and release of $sPLA_2$ have been found in rheumatoid arthritis, inflammatory bowel diseases, and atherosclerosis [12, 13]. Mechanisms involved in the proinflammatory action of $sPLA_2$ are being actively investigated, and most of this knowledge is based on studies using purified venom PLA_2s.

This paper describes the isolation and biochemical and pharmacological characterization of new PLA_2s from *Bothriopsis bilineata* venom, Bbil-TX, and also the study of its various toxic activities, including myotoxicity, cytotoxicity, and inflammation.

2. Materials and Methods

2.1. Venom and Reagents. *Bothriopsis bilineata* venom was donated by Dr. Corina Vera Gonzáles. All chemicals and reagents used in this work were of analytical or sequencing grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Reversed-Phase HPLC (RP-HPLC). Five milligrams of the whole venom from *Bothriopsis bilineata* was dissolved in 200 μL ammonium bicarbonate 0.2 M pH 8.0. The resulting solution was clarified by centrifugation and the supernatant was applied to a μ-Bondapak C18 column (0.78 × 30 cm; Waters 991—PDA system). Fractions were eluted using a linear gradient (0–100%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min over 40 min. The elution profile was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20°C.

2.3. PLA_2 Activity. PLA_2 activity was measured using the assay described by Holzer and Mackessy, [14] modified for 96-well plates. The standard assay mixture contained 200 μL of buffer (10 mM tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μL of substrate (4-nitro-3-octanoyloxy-benzoic acid), 20 μL of water, and 20 μL of Bbil-TX in a final volume of 260 μL. After adding Bbil-TX (20 μg), the mixture was incubated for up to 40 min at 37°C, with the reading of absorbance at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase of absorbance after 20 min. The optimum pH and temperature of the PLA_2 were determined by incubating the enzyme in four buffers of different pH values (4–10) and at different temperatures, respectively. The effect of substrate concentration (0.1, 0.2, 0.3, 0.5, 1, 2, 5, 10, 20, and 30 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min. The inhibition of PLA_2 activity by crotapotins from *Crotalus durissus cascavella* and DAII-2 from *Didephis albiventris* serum was determined by preincubating the protein (Bbil-TX) and each inhibitor for 30 min at 37°C prior to assaying the residual enzyme activity under optimal conditions. All assays were done in triplicate and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Electrophoresis. Tricine SDS-PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of the proteins, under reducing and nonreducing conditions [15].

2.5. Amino Acid Analysis. Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described by [16]. The purified Bbil-TX sample (30 μg) was hydrolyzed at 105°C for 24 h in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μL of derivatization solution (ethanol : triethylamine : water : phenylisothiocyanate, 7 : 1 : 1 : 1, v/v) for 1 h at room temperature, after which the PTC-amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture.

2.6. Determination of the Molecular Mass of the Purified Protein by Mass Spectrometry. An aliquot (4.5 μL) of the protein was inject by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-T of Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode and the data acquisition was from m/z 100–3,000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data produced by the mass spectrometer on the m/z scale were converted to the mass (molecular weight) scale using maximum-entropy-based software (1) supplied with Masslynx 4.1 software package. The processing parameters were: output mass range 6,000–20,000 Da at a "resolution" of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da and the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.7. Analysis of Tryptic Digests. The protein was reduced (DTT 5 mM for 25 min to 56°C) and alkylated (Iodoacetamide 14 mM for 30 min) prior to the addition of trypsin (Promega's sequencing grade modified). After trypsin addition (20 ng/μL in ambic 0.05 M), the sample was incubated for 16 hr at 37°C. To stop the reaction, formic acid 0.4% was added and the sample centrifuged at 2500 rpm for 10 min. The pellet was discarded and the supernatant dried in a speed vac. The resulting peptides were separated by C18 (100 μm ×

100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000 m/z, in order to select the ion of interest; subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode).

Raw data files from LC-MS/MS runs were processed using Masslynx 4.1 software package (Waters) and analyzed using the MASCOT search engine version 2.3 (Matrix Science Ltd.) against the snakes database, using the following parameters: peptide mass tolerance of ±0.1 Da, fragment mass tolerance of ±0.1 Da, and oxidation as variable modifications in methionine and trypsin as enzyme.

2.8. Myotoxic Activity. Groups of four Swiss mice (18–20 g) received an intramuscular (i.m.) or an intravenous (i.v.) injection of variable amounts of the Bbil-TX. Samples (50 μL) containing 0.1, 1, and 5 μg of the PLA_2 Bbil-TX were injected in the right gastrocnemius. A control group received 50 μL of PBS. At different intervals, blood was collected from the tail into heparinized capillary tubes after 2, 4, 6, 9, 12 and 24 hours, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay Ck-Nac, (creatine kinase, Beacon, Diagnostics, Germany). To the reaction mixture 10 μL of the plasma obtained by centrifugation from mice blood was added. The solution is incubated for 2 minutes and reads at 430 nm. The results were expressed as U/L according to the manufacturer.

2.9. Cytotoxicity Assays. Cytotoxic activity was assayed on murine skeletal muscle C2C12 myoblasts and myotubes (ATCC CRL-1772). Variable amounts of Bbil-TX were diluted in assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal-calf serum) and added to cells in 96-well plates, in 150 μL. Controls for 0 and 100% toxicity consisted of assay medium, and 0.1% Triton X-100, respectively. After 3 h at 37°C, a supernatant aliquot was collected for determination of lactic dehydrogenase (LDH; EC 1.1.1.27) activity released from damaged cells, using a kinetic assay (Wiener LDH-P UV). Experiments were carried out in triplicate.

2.10. Edema-Forming Activity. The ability of Bbil-TX to induce edema was studied in groups of five Swiss mice (18–20 g). Fifty microliters of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with Bbil-TX (0.1; 1 and 5 μg/paw) were injected in the subplantar region of the right footpad. The control group received an equal volume of PBS alone. The paw swelling was measured with an Electronic Caliper Series 1101 (INSIZE LTDA, SP, Brazil) at 0.5, 1, 3, 6, 9, and 24 h after administration. Edema was expressed as the percentage increase in the size of the treated group to that of the control group at each time equal to 24 hrs.

2.11. Cytokines. The levels of cytokines IL-6 and IL-1 in the serum from BALB/c mice were assayed by a two-site sandwich enzyme-like immunosorbent assay (ELISA) as described by [17]. In brief, ELISA plates were coated with 100 μL (1 μg/mL) of the monoclonal antibodies anti-IL-6 and anti-IL-1 placed in 0.1 M sodium carbonate buffer (pH 8.2), and incubated for 6 hours at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween 20) and blocked with 100 μL of 10% fetal-calf serum (FCS) in PBS for 2 hours at room temperature. After washing, duplicate sera samples of 50 μL were added to each well. After 18 hours of incubation at 4°C, the wells were washed and incubated with 100 μL (2 μg/mL) of the biotinylated monoclonal antibody anti-IL-6 and anti-IL-1 as a second antibody for 45 minutes at room temperature. After a final wash, the reaction was developed by the addition of orthophenyldiamine (OPD) to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines (expressed in picograms per millilitre). The minimum levels of each cytokine detectable in the conditions of the assays were 10 pg/mL for IL-6 and IL-1.

To measure the cytotoxicity of TNF-α present in the serum from BALB/c mice, a standard assay with L-929 cells, a fibroblast continuous cell line, was used as described previously by [18]. The percentage cytotoxicity was calculated as follows: $(A_{control} - A_{sample}/A_{control}) \times 100$. Titres were calculated as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayer were lysed. TNF-α activity is expressed as units/mL, estimated from the ratio of a 50% cytotoxic dose of the test to that of the standard mouse recombinant TNF-α.

2.12. Statistical Analysis. Results are reported as means ± SEM. The significance of differences between the means was assessed by ANOVA followed by Dunnett's test when various experimental groups were compared with the control group. A value of $P < 0.05$ indicated significance.

3. Results

Fractionation of *Bothriopsis bilineata* venom by RP-HPLC on a μ-Bondapak C18 column resulted in eleven peaks (1–11) (Figure 1). The 11 peaks were screened for myotoxic and PLA_2 activities. Peak 7 caused local myotoxicity at concentrations ranging from 0.1 to 5 μg/mL in mouse gastrocnemius muscle. In addition, peak 7, named Bbil-TX-I (*Bothriopsis bilineata* toxin) showed high PLA_2 activity and was selected for biochemical and pharmacological characterization. The purity of this peak was confirmed by rechromatography on an analytical RP-HPLC μ-Bondapack C18 column, showing the presence of only one peak and by Tricine SDS-PAGE, which revealed the presence of one electrophoretic band with Mr

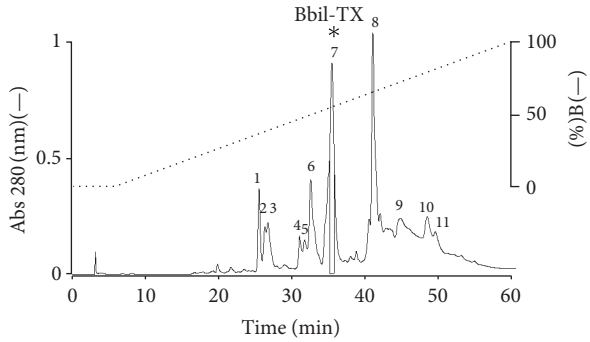

Figure 1: Elution profile of *Bothriopsis bilineata* venom by RP-HPLC on a μ-Bondapack C18 column. Fraction 7 (Bbil-TX) contained PLA_2 activity.

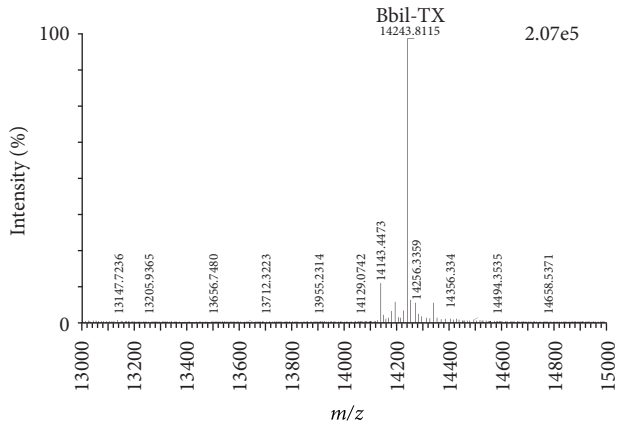

Figure 2: Mass determinations of Bbil-TX by mass spectrometry, using a Q-Tof Ultima API ESI/MS (TOF MS mode).

around 15 kDa, in the absence and presence of DTT (1 M) (data not shown).

Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry analysis confirmed the homogeneity of the peak Bbil-TX and determined the exact molecular mass of 14243.8 Da (Figure 2). This value of molecular mass was used in calculating the molar concentrations of toxin used in the experiments described below.

The alkylated and reduced protein was digested with trypsin and the resulting tryptic peptides (10) were fractionated by RP-UPLC (nanoAcquity UPLC, Waters) (data not shown). Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000 m/z, in order to select the ion of interest; subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). The data files obtained from LC-MS/MS runs were processed using the Masslynx 4.1 software package (Waters) and analyzed using the MASCOT search engine version 2.3 (http://www.matrixscience.com/). Table 1 shows the deduced sequence and measured masses of alkylated peptides obtained for Bbil-TX PLA_2. Isoleucine and leucine residues were not discriminated in any of the sequences reported

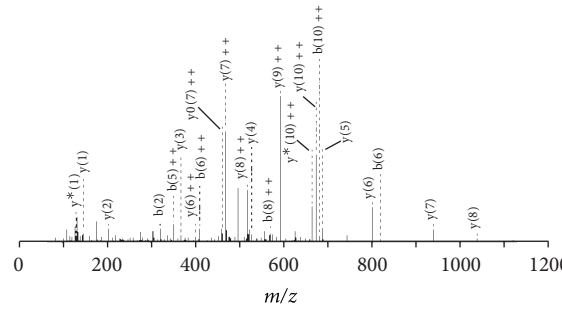

Figure 3: MS/MS spectrum of the doubly charged tryptic ion of m/z 1504.5356. Ion of the major sequence-specific y-ion series and of a minor series of the complementing b-ions CCFVHDCCYGK, from which the sequence of Bbil-TX tag was deduced.

since they were indistinguishable in low energy CID spectra. Because of the external calibration applied to all spectra, it was also not possible to resolve the 0.041 Da difference between glutamine and lysine residues, except for the lysine that was deduced based on the cleavage and missed cleavage of the enzyme.

The ten peptides obtained in Q-Tof Ultima API ESI/MS (TOF MS mode) mass spectrometry of the Bbil-TX PLA_2 were submitted to the NCBI database, using the protein search program BLASTp with the search being restricted to the sequenced proteins from the basic protein with phospholipase A_2 activity family. Based on the positional matches of the de novo sequenced peptides with other homologous proteins, it was possible to deduce the original positions of these peptides in the native protein (Figure 4).

The results of the primary structures show that Bbil-TX PLA_2 is composed of 122 amino acid residues and shares the conserved sequence domains common to PLA_2 group, including the 14 cysteines, the calcium-binding site located on (Y)27, (G)28, (C)31, and (G)32, and the catalytic network commonly formed by (H)48, (D)49, (Y)52, and (D)90. A comparative analysis of the sequence of Bbil-TX PLA_2 with other neurotoxins "*ex vivo*" and myotoxic PLA_2s belonging to Viperidae family, LmTX-I and LmTX-II (*Lachesis muta muta*) [19] and *Crotalus scutulatus scutulatus* (Mojave rattlesnake) (accession number P62023), showed similarity of 81.1–91.0%. (SwissProt database http://br.expasy.org/). The tandem mass spectra shown in Figure 3, relative to the peptide eluted in fraction 3 having the sequence C C F V H D C C Y G K, allows to classify both enzymes as PLA_2.

Amino acid analysis revealed the following composition of Bbil-TX PLA_2: D/11, T/7, S/2, E/12, P/9, G/10, A/7, C/14, V/4, M/2, I/3, L/7, Y/9, F/4, K/12, H/1, and R/6. The PLA_2 activity was examined in the *Bothopsis bilineata* venom and in BbilTX-I using the synthetic substrate 4-nitro-3(octanoyloxy) benzoic acid [14, 19]. The PLA_2 activity was higher in Bbil-TX (24.75 ± 2.68 nmols/min/mg) when compared with the whole venom (8.15 ± 1.24 nmols/min/mg) (Figure 5(a)). Under the conditions used, Bbil-TX showed a discrete sigmoidal behavior (Figure 5(f)), mainly at low substrate concentrations. Maximum enzyme activity occurred at 35–40°C (Figure 5(c)) and the pH optimum was 8.0 (Figure 5(d)). PLA_2s require Ca^{+2} for full activity, with only

Table 1: Sequence obtained by MS/MS based on the alkylated tryptic peptides derived. The peptides were separated and sequenced by mass spectrometry.

Residue number	Mass (Da) expected	Amino acid sequence	Mass (Da) calculated
1–7	898.5293	HLLQFNK	898.5025
16–33	2157.9352	NAIPFYAFYGCYCGWGGR	2157.9189
43–53	1504.5356	CCFVHDCCYGK	1504.5356
61–69	1183.6216	WDIYPYSLK	1183.5913
70–77	884.4277	SGYITCGK	884.4062
78–90	871.8404	GTWCEEQICECDR	1741.6494
91–98	973.5307	VAAECLRR	973.5127
98–104	853.4780	RSLSTYK	853.4657
105–114	1297.5059	YGYMFYPDSR	1297.5437
115–122	965.3514	CRGPSETC	965.3695

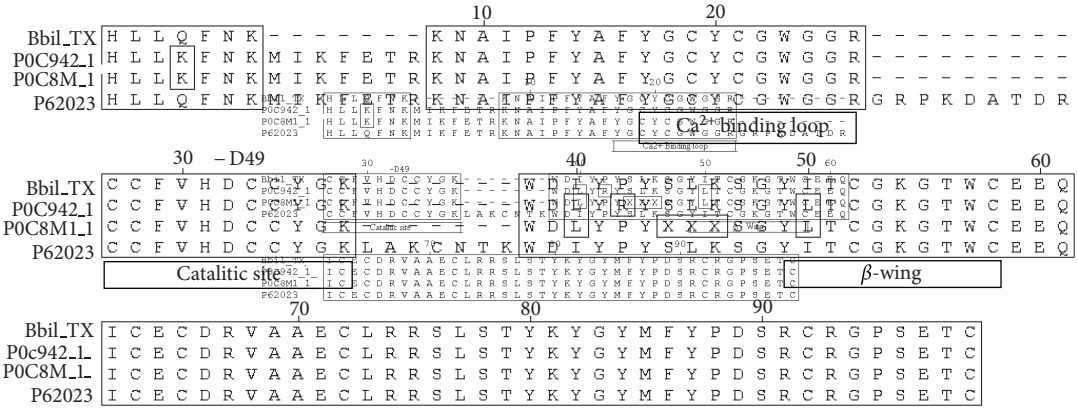

Figure 4: Alignment of the deduced amino acid sequence of the new PLA_2 Bbil-TX with PLA_2 presents in venom of *Lachesis muta muta* (accession number P0C8 M_1 and P0C942_1) [19] and *Crotalus scutulatus scutulatus* (Mojave rattlesnake) (accession number P62023). Nondetermined amino acid residues are indicated by (X); boxed amino acid residues are identical. The highlighted amino acid residues belong to PLA_2 conserved domain Ca^{2+}-binding loop, the catalytic site, and the region β-wing.

1 mM of Ca^{+2} needed for Bbil-TX to present phospholipase activity. The addition of Mg^{2+}, Cd^{2+}, and Mn^{2+} (10 mM) in the presence of low Ca^{2+} concentration (1 mM) decreases the enzyme activity. The substitution of Ca^{2+} by Mg^{2+}, Cd^{2+}, and Mn^{2+} also reduced the activity to levels similar to those in the absence of Ca^{2+} (Figure 5(e)).

The crotapotins are pharmacologically inactive and non-enzymatic acid protein, binds specifically of the PLA_2 inhibited the activity. An isoform of *Crotalus durissus cascavella* F3 and antihaemorragic factor DA2-II from *Didelphis albiventris,* significantly inhibit the Bbil-TX PLA_2 activity (Figure 5(b)).

The local myotoxic effect (i.m.) *in vivo* was observed with PLA_2 Bbil-TX studied. It was observed that the PLA_2 induced a conspicuous effect evidenced by the rapid elevation of plasma CK activity through a time course, reaching its maximum effect 2 h after injection and returning to normal levels after 24 h (Figure 6(a)). Our results showed that the PLA_2Bbil-TX did not show systemic myotoxic effect (i.v.) (Figure 6(b)).

In a concentration of 5, 10, 20, and 40 μg/well (150 μL), the PLA_2 Bbil-TX showed low cytotoxicity in skeletal muscle myoblasts and myotubes (25.49 ± 2.3% and 29.05 ± 3.45%, resp.) in a concentration of 40 mg/well (150 μL) (Figure 6(c)).

Compared to PBS-injected animals, those which received subplantar injections of the Bbil-TX (0.1, 1, and 5 μg/paw) presented marked paw edema (Figure 7(a)). Maximal activity was attained 1 h to the Bbil-TX after injection and receded to normal levels after 24 h. The level of edema induction by 5 μg of PLA_2 1 hour after administration was 61.57%, showing a dose-dependent activity. To further analyze the mechanisms of the inflammatory events induced by Bbil-TX (0.1 μg), TNF-α, IL-6, and IL-1 concentrations were measured in the serum. TNF-α levels were increased 1 h after injection of Bil-TX and no detectable production was observed at the later time intervals studied (Figures 7(b), 7(c), and 7(d)). Bbil-TX caused a significant increase in IL-6 release between 1 and 3 h, respectively, in serum collected after injection of venom compared with the control (Figure 7(c)). However, increased levels of IL-1 were detected between 1, 3, 6, and 12 h, respectively (Figure 7(d)).

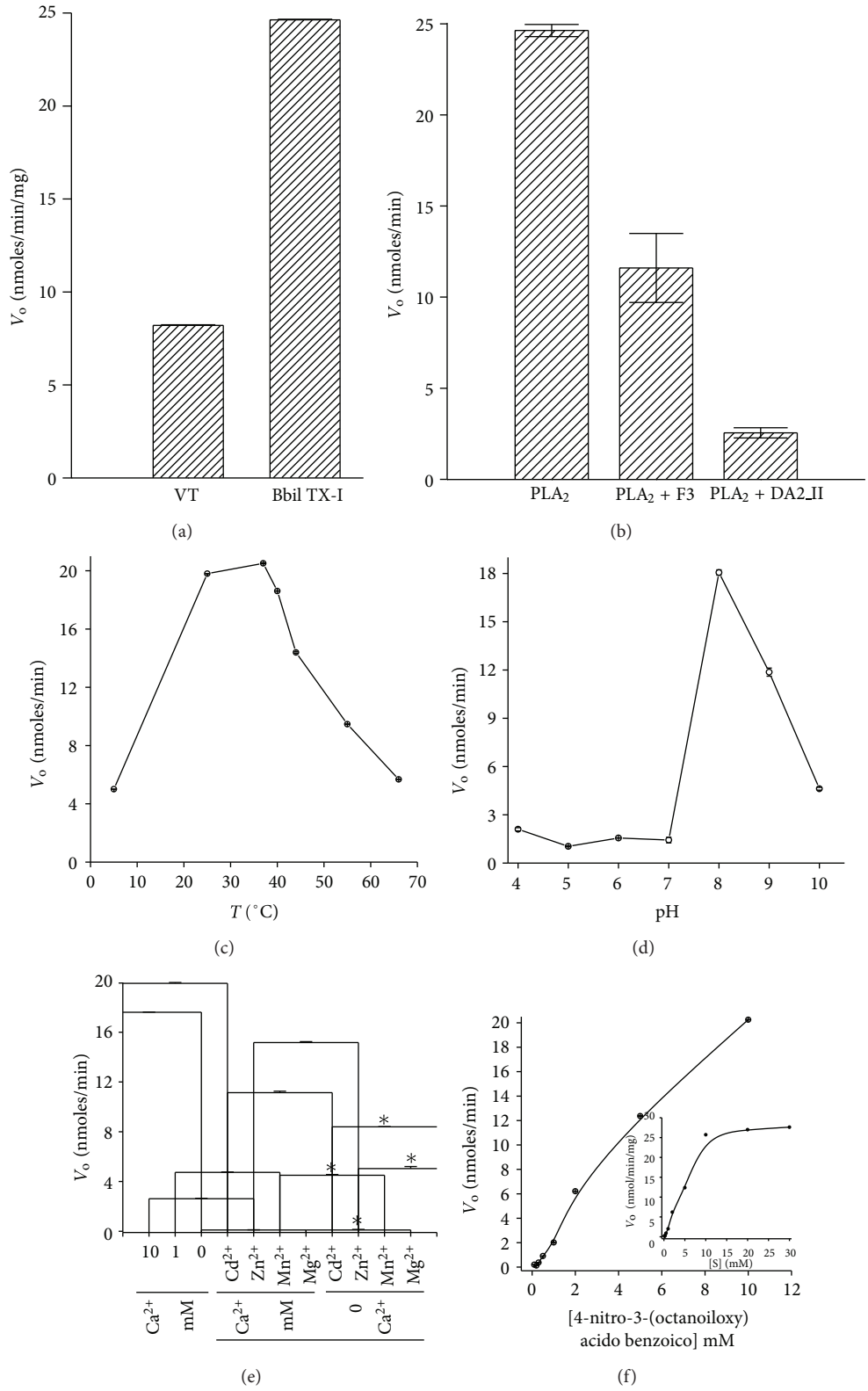

FIGURE 5: (a) PLA$_2$ activity of *Bothriopsis bilineata* venom and peak 7 (Bbil-TX); (b) the inhibitory effect of the antihemorrhagic factor DAII-2 and the crotapotin F3 on PLA$_2$ activity Bbil-TX; (c) effect of temperature on the PLA$_2$ activity of Bbil-TX; (d) effect of pH on Bbil-TX activity; (e) influence of ions (10 mM each) on PLA$_2$ activity in the absence or presence of 1 mM Ca^{2+}; (f) effect of substrate concentration on the kinetics of BbilTX (PLA$_2$) activity. The inset shows the curve shape at low substrate concentrations. The results of all experiments are the mean ± SE, of three determinations ($P < 0.05$).

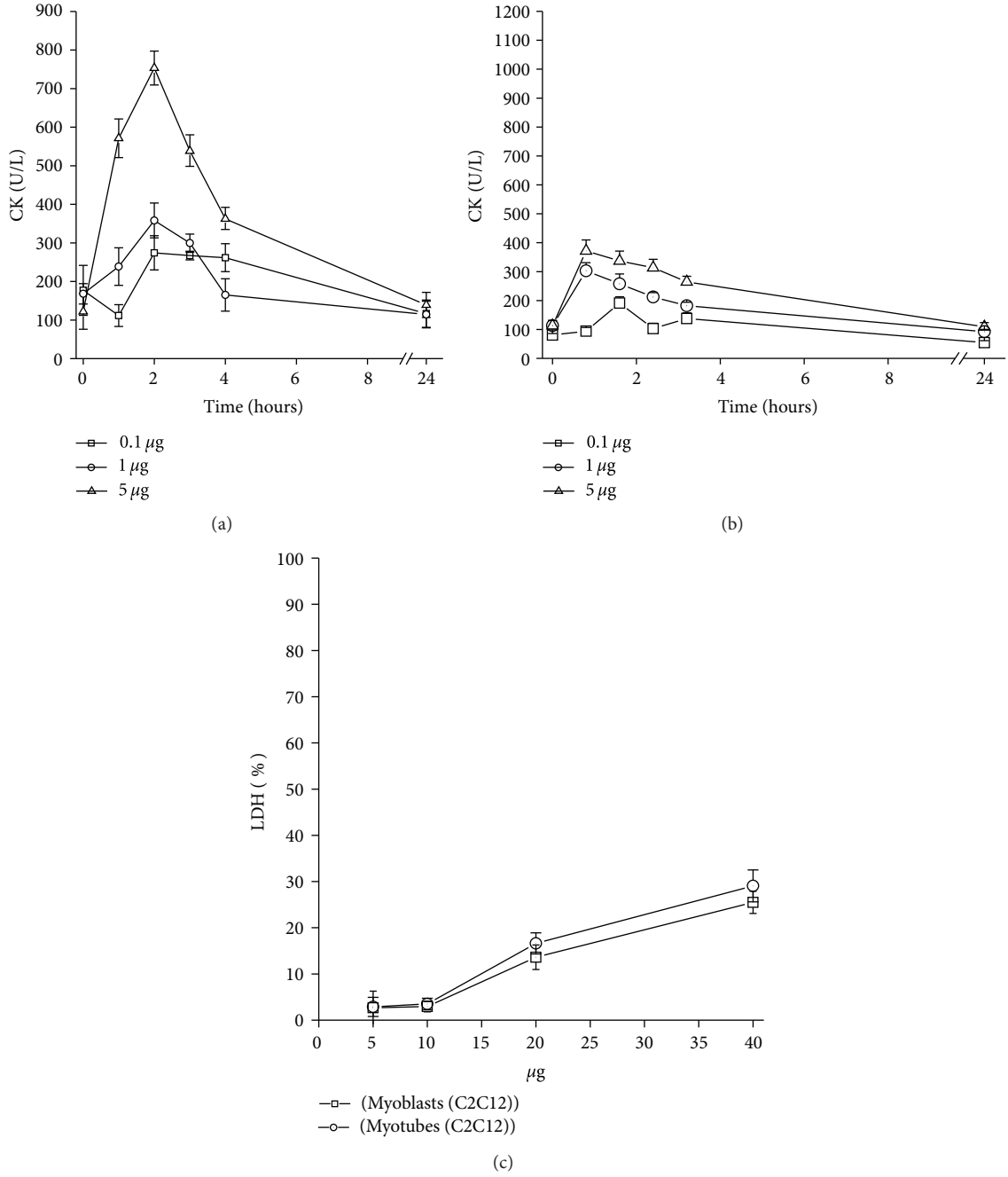

Figure 6: (a, b) Time course of the increments in plasma CK activity after intramuscular (i.m.) or intravenous (i.v.) injection of 0.1, 1, and 5 μg *Bothriopsis bilineata* Bbil-TX; (c) *in vitro* cytotoxic activity of Bbil-TX on murine C2C12 skeletal-muscle myoblasts and myotubes. Cell lysis was estimated by the release of lactic dehydrogenase (LDH) to supernatants, after 3 h of exposure to the toxins. Each point represents mean ± SD of triplicate cell cultures.

4. Discussion

The purification procedure for basic PLA_2s developed by [20–22] showed to be also efficient for the obtainment of neurtoxin "*ex vivo*" and myotoxin from *Bothriopsis bilineata* snake venom. Fractionation of this crude venom by single-step chromatography in a column μ-Bondapack C-18 coupled to a system of reversed-phase HPLC was carried out and as a result of the proposed method, several toxins have been efficiently purified. Fraction 7 was named Bbil-TX (PLA_2). SDS-PAGE showed evidence that Bbil-TX isolated PLA_2s have an Mr of ~14 kDa for the monomers, similar to basic PLA_2s isolated from other venoms (data not shown) [23]. The conserved residues Y28, G30, G32, D49, H48, and Y52 are directly or indirectly linked in the catalyses of the Bbil-TX.

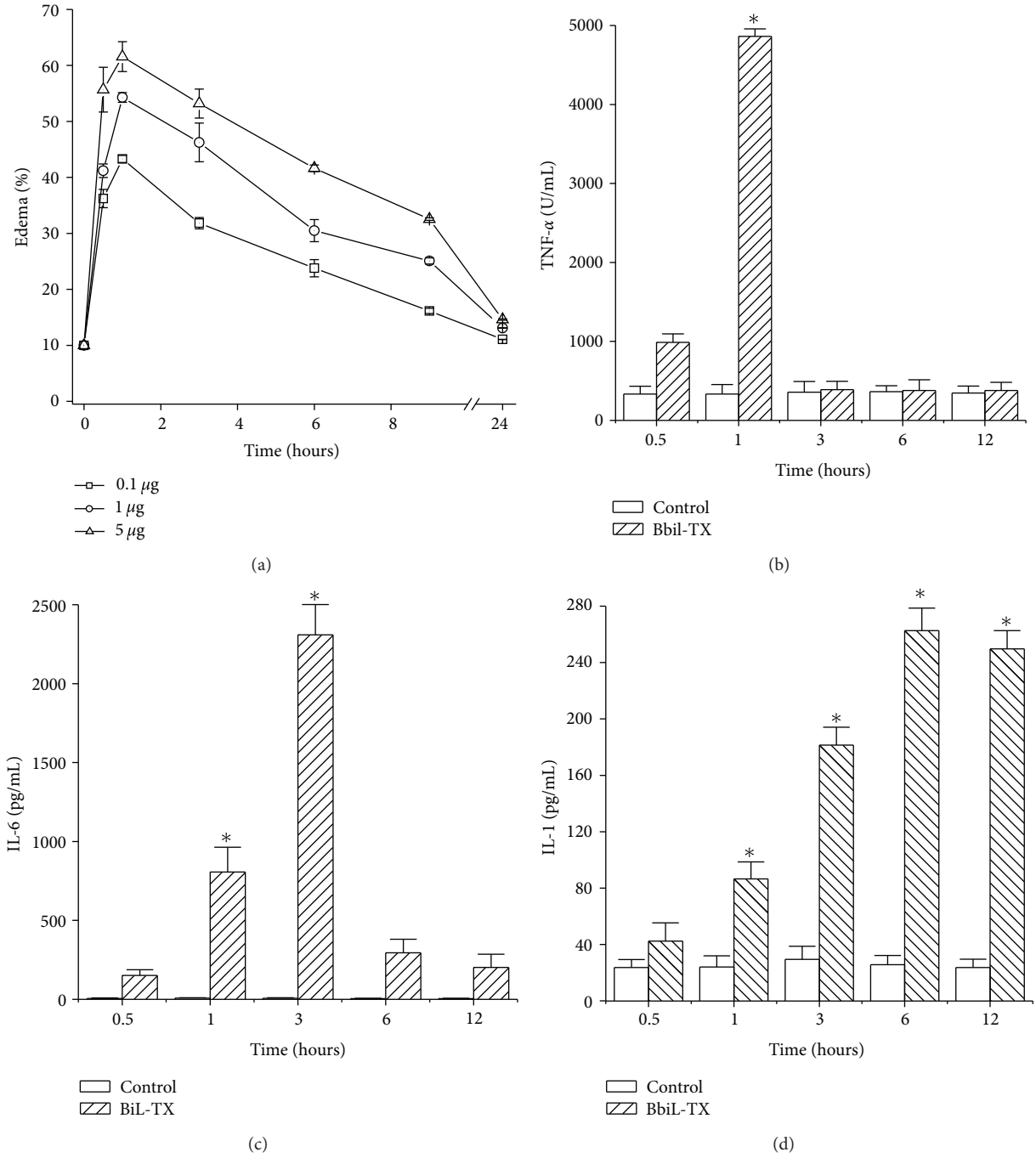

Figure 7: (a) Edema-forming activity of Bbil-TX in mice. Induction of edema by toxins (0.1, 1, and 5 μg/mL), injected s.c. in the footpad of mice. At various time intervals the increase in footpad volume, as compared to controls, was expressed as percent edema. Each point represents the mean ± SD of four animals. Levels of TNF-α (b), IL-6 (c), and IL-1 (d), in the serum after injection of Bbil-TX. Animals were injected i.m. with Bbil-TX (1.0 mg/kg) or sterile saline alone (control) in a final volume of 100 μL. IL-1 and IL-6 were quantified by specific ELISA, and TNF-α was quantified by cytotoxic activity on L929 cells in serum collected at the indicated time intervals after Bbil-TX or saline injection as described in Materials and Methods. Each bar represents mean ± SEM of 7 animals. $^{*}P < 0.05$ when compared with the corresponding control groups.

The molecular masses obtained by mass spectrometry showed to be similar to that of other snake venom PLA_2s [22, 24, 25]. The amino acid composition of the Bbil-TX PLA_2 toxin suggests the presence of 14 half-Cys residues, providing the basis for a common structural feature of PLA_2 in the formation of its seven disulfide bridges [20, 21, 26] and a high content of basic and hydrophobic residues, that provides a explication important in the interaction of the PLA_2 with

negatively charged phospholipids of cells membranes [27]. Such an interaction is important to explain the effect of these enzymes on different cells types, both prokariotes and eukariotes [28, 29].

Comparison of the amino acid sequence of Bbil-TX PLA_2 showed high homology with other neurotoxic and myotoxic PLA_2s from *Lachesis* and *Crotalus* genera (Figure 4). Sequence homology studies had shown that there are extremely conserved positions in the PLA_2s. In positions 1 and 2, there is a predominance of the amino acid sequence (HL), in position 4 (Q), and in positions 5 to 7 (FNK). One of the highly conserved regions in the amino acid sequences of PLA_2 is the Ca^{2+}-binding loop, segment from …YGCYCGXGG… and HD(49)CC. The calcium ion is coordinated by three main chain oxygen atoms from residues (Y)28, (G)30, (G)32, and two carboxylate oxygen atoms of (D)49. Two generally conserved solvent water molecules complete the coordination sphere of the calcium ion forming a pentagonal bipyramidal geometry. It is believed that two disulfide bridges (C)27–(C)119 and (C)29–(C)45 ensure the correct relative orientation of the calcium-binding loop in relation to the amino acids of the catalytic network [30]. The residues (H)48, (Y)52, and (D)99 which are responsible for catalytic activity have an ideal stereochemistry with the presence of the so-called "catalytic network", a system of hydrogen bonds which involves the catalytic triad [30, 31]. Residues forming the Ca^{2+}-binding loop and the catalytic network of Bbil-TX PLA_2 show a high conservation grade, reflecting the nondecreased catalytic activity.

The PLA_2 activity was shown to be higher in Bbil-TX PLA_2 (24.75 ± 2.28 nmoles/min/mg) when compared with the whole venom (8.15 ± 1.24 nmoles/min/mg). PLA_2 enzyme from snake venom shows classic Michaelis–Menten behavior against micellar substrates [32]. With a synthetic substrate, Bbil-TX PLA_2 behaved allosterically, especially at low concentrations, which is in agreement with the results obtained by [23] for the PLA_2 of *Bothrops jararacussu* venom and Damico et al. [19] for the PLA_2 isoform purified from *Lachesis muta muta* venom. Using the same synthetic nonmicellar substrate, it was also possible to observe that the dependence of activity on substrate concentration was markedly sigmoidal for the PrTX-III from *Bothrops pirajai* [33].

PLA_2s from crotalic venoms have showed a similar behavior to the one presented by bothropic PLA_2s with the same substrate used in the kinetic studies to Bbil-TX PLA_2 [14, 34]. Despite the structural and functional differences among bothropic and crotalic PLA_2s, both show allosteric behavior in the presence of the same substrate.

The PLA_2 activity could be verified with different pH levels; the optimum pH of basic PLA_2s is around 7.0 and 8.5 [32, 35]. Bbil-TX PLA_2 can be considered basic since its highest activity is evidenced at pH 8.0. Temperature is another kinetic parameter utilized to characterize the PLA_2 (Asp49). It has been shown that PLA_2 from *Naja naja naja* is very stable in extreme temperatures such as 100°C [35]. The optimum temperature of Bbil-TX PLA_2 was around 37°C, but at 40–45°C, the Bbil-TX PLA_2 activity did not present a huge decrease.

A strict requirement for Ca^{2+} is characteristic of some PLA_2 [5]. Bbil-TX PLA_2 showed typical Ca^{2+}-dependent PLA_2 activity similar to other PLA_2s, and this activity was lower in the presence of other cations. [14, 35–38] observed the same for other PLA_2 from snake venom.

The crotapotin isoform from *Crotalus durrissus cascavella* (F3) venom inhibit significantly the PLA_2 activity of Bbil-TX by approximately 50%. Our results are in agreement with the finding by [14, 21, 39] who reported that highly purified crotapotin can inhibit pancreatic, bee, and other snake venom PLA_2s, and Bonfim et al. [23], who reported that crotapotins from *Crotalus durissus terrificus* (F7), *Crotalus durissus collilineatus* (F3 and F4), and *Crotalus durissus cascavella* (F3 and F4) decreased the catalytic activity of BJ IV (PLA_2 from *Bothrops jararacussu*) by 50%. Together, these results suggest that crotapotin may bind to bothropic PLA_2 in a manner similar to that from crotalic PLA_2.

Bbil-TX PLA_2 increases the plasmatic CK levels after i.m. injection (Figure 6(a)), revealing drastic local myotoxicity. This myotoxicity induced by snake venoms, including *Botrhiopsis bilineata*, may result from the direct action of myotoxins on the plasma membranes of muscle cells, or indirectly, as a consequence of vessel degenerations and ischemia caused by hemorrhagins or metaloproteases. Bbil-X PLA_2 contributes significantly to local myotoxic action *in vivo*. It was already demonstrated that the snake venom PLA_2s are the principal cause of local damage [40]. Myotoxic PLA_2s affect directly the plasma membrane integrity of muscle cells, originating an influx of Ca^{2+} ions to the citosol that starts several degenerative events with irreversible cell injures [41]. The binding sites of myotoxins on the plasma membranes are not clearly established, although two types have been proposed: (a) negatively charged phospholipids [42], present on membranes of several cell types, explaining the high *in vitro* cytotoxic action of these enzymes [28, 43, 44], and (b) protein receptors, which make muscle cells more susceptible to myotoxin action [28].

All these biological effects induced by the toxin occur in the presence of a measurable PLA_2 activity. Although the catalytic activity of PLA_2s contributes to pharmacological effects, it is not a prerequisite [21, 26, 29]. However, further studies are necessary to identify the structural determinants involved in these pharmacological activities.

Some authors, [21, 41, 45, 46], have proposed several models to explain PLA_2 catalytic and pharmacological activities. In these models PLA_2 has two separated places; one is responsible for catalytic activity and the other for biological activity expression. According to them, the pharmacological place would be located on the surface of PLA_2 molecules. According to the model proposed by [47], the anti-coagulant place would be located in a region between the 53 and 76 residues, considering this region charged positively in the PLA_2 with high anticoagulant activity. In PLA_2 with moderate or low anticoagulant activity, there is a predominancy of negative charges.

Further research in identifying target proteins will help determine details of the mechanisms of the pharmacological effects at the cellular and molecular levels [48]. Studies in these areas will result in new, exciting, and innovative opportunities and avenues in the future, both in finding answers to the toxicity of PLA_2 enzymes and in developing proteins with novel functions.

PLA_2s from snake venoms exert a large number of pharmacological activities due to a process of accelerated evolution through which a high mutational rate in the coding regions of their genes has allowed the development of new functions, mainly associated with the exposed regions of the molecules [29]. The integral analysis of the inflammation elicited by Bbil-TX in the mouse serum performed in the present study allowed a parallel evaluation of the increase in microvascular permeability and the production of various inflammatory mediators. Bbil-TX induced an increase in vascular permeability in the paw of mice. This is in agreement with previous observations on the edema-forming activity of similar molecules in the rodent footpad model [49, 50].

The increase of vascular permeability was detected early after Bbil-TX injection and developed rapidly, indicating that the observed plasma extravasation is primarily due to formation of endothelial gaps in vessels of microcirculation. The main edema formation occurred 1 h after the injection of Bbil-TX with constant decrease. Bbil-TX caused paw edema in mice with a time course similar to that reported for other PLA_2s from *Bothrops* venoms in mice and rats, that is, a fairly rapid onset (generally $\leq$ 3 h to peak) followed by a gradual decline over the following 24 h [51–54].

The mediators involved in this effect of Bbil-TX myotoxin were not addressed in this study. However, the immediate plasma extravasation in response to Bbil-TX strongly suggests the involvement of vasoactive mediators derived from mast-cell granules. This strongly suggests that enzymatic phospholipid hydrolysis plays a significant role in this event.

Cytokines, such as IL-1, IL-6, and TNF-α, are also relevant mediators for leukocyte migration and participate in several inflammatory conditions. Our results showed that Bbil-TX induced an increase in TNF-α, IL-6, and IL-1 in the serum [55]. Thus, our results suggest that IL-1 may contribute to the leukocyte influx induced by Bbil-TX. In addition, the similarity observed in the time course of IL-6 and IL-1 increase in the serum may indicate a positive regulatory role for IL-1 on the release of IL-6 induced by Bbil-TX. IL-6, an important mediator of inflammation, causes leukocytosis characterized by a rapid neutrophilia by releasing of PMN leukocytes from the bone marrow [56, 57]. In addition, IL-6 upregulates intercellular-adhesion-molecule-1 (ICAM-1) expression by endothelial cells but decreases the levels of L-selectin on circulating PMN leukocytes contributing to firm adhesion, the next step of cell migration [58].

TNF-α is also likely to be involved in leukocyte infiltration induced by Bbil-TX, since the PLA_2 caused a significant increase of TNF-α levels in the serum. TNF-α is likely to induce the expression of E-selectin, CD11b/CD18, and intercellular adhesion molecule-1 (ICAM-1) and triggers the release of several cytokines such as IL-1 and IL-6 and eicosanoids. Thus, our results suggest that TNF-α may have a role in the expression of CD18 and the release of other cytokines following Bbil-TX injection, thereby being relevant for neutrophil influx and for increase of vascular permeability. It is interesting that TNF-α and IL-6, as well as IL-1, may induce or potentiate the expression and release of group IIA PLA_2s [59, 60].

In conclusion, Bbil-TX induces a marked inflammatory reaction in the mouse serum. Since basic myotoxic PLA_2s are abundant in snake venoms, these toxins must play a relevant role in the proinflammatory activity that characterizes this venom. The fact that Bbil-TX elicited a stronger inflammatory reaction argues in favor of a role of enzymatic phospholipid hydrolysis in this phenomenon, either through the direct release of arachidonic acid from plasma membranes or through activation of intracellular processes in target cells.

Accumulating evidences have strongly shown that venom PLA_2s are among the major mediators of myonecrosis [40], hemolysis, mast cell degranulation, and edema formation [3].

PLA_2s isolated from *Bothrops* venoms are frequently myotoxic [26] and can cause edema in rats and mice [39, 45, 49, 54]. These results suggest that, for some PLA_2s, catalytic activity plays a role in the edematogenic effect.

Ethical Approval

The animals and research protocols used in this study followed the guidelines of the Ethical Committee for use of animals of ECAE-IB-UNICAMP SP, Brazil (protocol number 1931-1) and international laws and policies. All efforts were made to minimize the number of animals used and their suffering.

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Research Article

Biochemical Characterization and Pharmacological Properties of New Basic PLA_2 BrTX-I Isolated from *Bothrops roedingeri* (Roedinger's Lancehead) Mertens, 1942, Snake Venom

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BrTX-I, a PLA_2, was purified from *Bothrops roedingeri* venom after only one chromatographic step using reverse-phase HPLC on μ-Bondapak C-18 column. A molecular mass of 14358.69 Da was determined by MALDI-TOF mass spectrometry. Amino acid analysis showed a high content of hydrophobic and basic amino acids as well as 14 half-cysteine residues. The total amino acid sequence was obtained using SwissProt database and showed high amino acid sequence identity with other PLA_2 from snake venom. The amino acid composition showed that BrTX-I has a high content of Lys, Tyr, Gly, Pro, and 14 half-Cys residues, typical of a basic PLA_2. BrTX-I presented PLA_2 activity and showed a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0, 35–45°C, and required Ca^{2+}. *In vitro*, the whole venom and BrTX-I caused a neuromuscular blockade in biventer cervicis preparations in a similar way to other *Bothrops* species. BrTX-I induced myonecrosis and oedema-forming activity analyzed through injection of the purified BrTX-I in mice. Since BrTX-I exerts a strong proinflammatory effect, the enzymatic phospholipid hydrolysis might be relevant for these phenomena; incrementing levels of IL-1, IL-6, and TNFα were observed at 15 min, 30 min, one, two, and six hours postinjection, respectively.

1. Introduction

PLA_2s (phosphatide 2-acylhydrolase, EC 3.1.14) represent a superfamily of lipolytic enzymes which specifically catalyze the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids resulting in the generation of fatty acid (arachidonate) and lysophospholipids. The PLA_2 superfamily consists of about 15 groups which are further subdivided into several subgroups, all of which display differences in terms of their structural and functional specificities. However, the four main types or classes of PLA_2s are the secreted, the cytosolic, the Ca^{2+}-independent and the lipoprotein-associated PLA_2 [1], PLA_2 structure/function, mechanism, and signaling [2].

Snake venom PLA_2s displays a variety of activities, such as neurotoxicity, myotoxicity, cardiotoxicity, and hemolysis that may be modulated by specific receptors located on target cells [3–6]. Indeed, PLA_2 receptors classified as kinds M and N [7] have been identified in various kinds of cells, including vascular smooth muscle cells, platelets, neutrophils, chondrocytes, fibroblasts, hepatocytes, and mesangial cells, as well as in brain, lung, and skeletal muscle [8, 9]. Snake venom PLA_2 can bind to M receptors, which are the most common kind found in human macrophages and muscle cells, and these may mediate some of the deleterious actions of venom PLA_2s, although that was not conclusively demonstrated [5, 6].

Peru has a rich and diverse herpetofauna that includes venomous snake species of the families Elapidae (16 species

of *Micrurus* and the pelagic sea snake *Pelamis platurus*) and Viperidae (15 species) [10]. Snakebite envenomations represent a public health problem in this country. The vast majority of snakebites in Peru are inflicted by species of the genus *Bothrops* (familyViperidae) [11]. *Bothrops atrox*, *Bothrops brazili*, and *Bothrops bilineatus* are distributed in the tropical rainforests located in the eastern part of the country, whereas *Bothrops barnetti* and *Bothrops roendingeri* are found in the western dry coastal regions [10–12].

This variety of pharmacological roles derives from an accelerated microevolutionary process through which a high rate of amino acid substitutions have occurred in molecular regions located mainly at the surface of these molecules [13–15]. The purpose of this paper is to isolate, biochemically and pharmacologically characterize a basic PLA_2 from *Bothrops roedingeri* venom, BrTX-I.

2. Materials and Methods

2.1. Venom and Reagents. The venom was obtained from the adult specimens of *Bothrops roedingeri* captured in the vicinity of Arequipa-Perú. Swiss mice (18–20 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). All experiments were conduced in accordance with guidelines of the Committee for Ethics in Animal Research, UNICAMP No. 2006-1 (Campinas-Brazil). The reagents used in this work were of analytical or sequencing grade.

2.2. PLA_2 Activity. PLA_2 activity was measured using the assay described in [16, 17], modified for 96-well plates [18]. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μL of substrate (4-nitro-3-octanoyloxy-benzoic acid), 20 μL of water, and 20 μL of PLA_2 in a final volume of 260 μL. After the addition of PLA_2 (20 μg), the mixture was incubated for up to 40 min at 37°C, with the absorbance being read at 10 min intervals. The enzyme activity, expressed as the initial velocity of the reaction (*Vo*), was calculated based on the increase in absorbance after 20 min.

All assays were done three times and the absorbances at 425 nm were measured using a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3. Reversed-Phase HPLC (RP-HPLC). Five milligrams of the venom was dissolved in 200 μL solvent A (TFA 0.1%, pH 3.5). The resulting solution was clarified by centrifugation and the supernatant was applied to a μ-Bondapak C18 column (0.78 × 30 cm; Waters 991-PDA system). Fractions were eluted using a linear gradient (0–100%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min over 40 min. The elution profile was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20°C.

2.4. Electrophoresis SDS-PAGE. The relative molecular mass of the protein was determined by SDS-PAGE [19]. The molecular mass markers were (in kDa): phospholipase B—94, albumin—67, ovalbumin—43, carbonic anhydrase—30, soybean trypsin inhibitor—20, and lysozyme—14.

2.5. Amino Acid Analysis. Amino acid analysis was done on a Pico-Tag amino acid analyzer (Waters Corporation, Massachusetts, USA) as described by [20]. The purified protein (30 μg) was hydrolyzed at 105°C for 24 h in 6 M HCl acid (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μL of derivatization solution (ethanol : triethylamine : water : phenylisothiocyanate, 7 : 1 : 1 : 1, v/v) for 1 h at room temperature after the phenylthiohydantoin (PTC)-amino acids were identified and quantified by HPLC by the comparison of their retention times and peak areas with those of a standard amino acid mixture.

2.6. Reduction and Alkylation. Purified lyophilized protein from RP-HPLC was resuspended in 8 M urea containing 10 mM DTT at pH 8.0 and the disulfide bridges were then reduced by incubation at 37°C for 2 h. Since the number of cysteine residues in the protein was initially unknown, the optimum concentration of iodoacetamide for alkylating the free thiols was derived empirically, based on results obtained from incubations using various concentrations of iodoacetamide and different amounts of protein, with each mixture being analyzed by mass spectrometry [21]. Based on these preliminary experiments, a 30% molar excess of iodoacetamide relative to the total number of thiols was eventually chosen and the mixture was incubated for 1.5 h at 37°C in the dark. The reaction was ceased by injecting the mixture onto a RP-HPLC column followed by lyophilization of the collected peak.

2.7. Enzymatic Hydrolysis. The purified proteins were hydrolyzed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37°C, at an enzyme : substrate ratio of 1 : 100 (w/w). The reaction was ceased by lyophilization.

2.8. Mass Spectrometry. All mass spectra were acquired using a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer Q-TOF Ultima from Micromass (Manchester, UK) equipped with a nano Zspray source operating in a positive ion mode. The ionization conditions of usage included a capillary voltage of 2.3 kV, a cone voltage and RF1 lens of 30 V and 100 V, respectively, and a collision energy of 10 V. The source temperature was 70°C and the cone gas was N_2 at a flow of 80 L/h; nebulizing gas was not used to obtain the sprays. Argon was used for collisional cooling and for fragmentation of ions in the collision cell. External calibration with sodium iodide was made over a mass range from 50 to 3000 *m/z*. All spectra were acquired with the TOF analyzer in "Vmode" (TOF kV = 9.1) and the MCP voltage set at 2150 V.

2.9. Analysis of Native and Alkylated Protein. Lyophilised RP-HPLC fractions of intact native and alkylated protein were dissolved in 10% acetonitrile in 0.1% TFA and was introduced

into the mass spectrometer source with a syringe pump at a flow rate of 500 nL/min. Mass spectra were acquired over the mass range of 1000–2800 *m/z* for the native protein and over the range of 800–2000 *m/z* for the alkylated protein, both at a scan speed of 1 s/scan. The masses were analyzed by the MassLynx-MaxEnt 1 deconvolution algorithm. The data obtained were processed using the Mascot MS/MS Ion Search software http://www.matrixscience.com/.

2.10. De Novo Sequencing of Tryptic Peptides. Alkylated tryptic peptides fractionated by RP-HPLC were lyophilized and re-suspended in 20% acetonitrile in 0.1% TFA prior to injection into the mass spectrometer source at a flow rate of 500 nL/min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 400–2000 *m/z*, in order to select the ion of interest, subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Different collision energies were used, depending on the mass and charge state of the ions. The resulting ion spectra was acquired in the TOF analyser and deconvoluted using the MassLynx-MaxEnt 3 algorithm. Singly charged spectra were processed manually using the PepSeq application included in MassLynx.

2.11. Pharmacological Activity

2.11.1. Young Chicken Biventer Cervicis Preparation. Male chicks (4–8-days-old) were killed with isoflurane and the biventer cervicis muscle was removed [22]. The biventer cervicis muscles were mounted under a tension of 0.5 g, in a 5 mL organ bath (Automatic organ multiple-bath LE01 Letica Scientific Instruments. Barcelona, Spain) at 37°C containing aerated (95% O_2 - 5% CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. Contracture to exogenously applied acetylcholine (ACh; 55 and 110 μM for 60 s) and KCl (20.1 mM for 130 s) was obtained in the absence of field stimulation, prior to the addition of a single dose of BrTX-I (50 μg/mL). A bipolar platinum ring electrode was placed around the tendon, which runs the nerve trunk supplying the muscle. Indirect stimulation was performed with a (MAIN BOX LE 12404 Panlab s.l. Powerlab AD Instruments Barcelona, Spain) stimulator (0.1 Hz, 0.2 ms, 3-4 V). Muscle contractions and contractures were isometrically recorded by force-displacement transducers (Model MLT0201 Force transducer 5 mg–25 g Panlab s.l. AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (OUAD Bridge AD Instruments, Barcelona, Spain).

2.11.2. Myotoxic Activity. Groups of four Swiss mice (18–20 g) received an intramuscular (i.m.) or an intravenous (i.v.) injection of variable amounts of BrTX-I, in 100 μL of PBS, in the gastrocnemius. A control group received 100 μL of PBS. At different intervals of time (2, 4, 6, 9, and 24 h) blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Sigma 47-UV). Activity was expressed in U/L, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25°C.

2.11.3. Edema-Forming Activity. The ability of BrTX-I to induce edema was studied in groups of five Swiss mice (18–20 g) according Ponce-Soto et al. [6, 23, 24]. Twenty microliters of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with BrTX-I (1, 5, 10 and 20 μg/paw) were injected in the subplantar region of the right footpad. The control group received an equal volume of PBS alone. The swelling of the paw was measured at 0.5; 1; 3; 6, and 24 h after administration. Edema was expressed as the percentage increased in the volume of the treated group to that of the control group at each time interval.

2.11.4. Cytokines. The percentage of cytotoxicity was of IL-1, IL-6, and TNF-α in the plasma were collected and measured at 30, 60, 180, and 360 min after i.p. injection of the BrTX-I PLA_2 (1.0 mg/kg) (20 μg/100 μL) or sterile saline. After centrifugation, the supernatants were used for determination of IL-1 and IL-6 levels by a specific EIA. The levels of cytokines IL-1, IL-6, and TNF-α in the serum from BALB/c mice were assayed by a two-site sandwich enzyme-like immunosorbent assay (ELISA). In brief, ELISA plates were coated with 100 μL (1 μg/mL) of the monoclonal antibodies anti-IL-1, in 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 hours at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 μL of 10% fetal calf serum (FCS) in PBS for 2 hours at room temperature. After washing, duplicate sera samples of 50 μL were added to each well. After 18 hours of incubation at 4°C, the wells were washed and incubated with 100 μL (2 μg/mL) of the biotinylated monoclonal antibodies anti-IL-1, anti-IL-6,as second antibodies for 45 minutes at room temperature. After a final wash, the reaction was developed by the addition of orthophenyldiamine (OPD) to each well. Optical densities were measured at 405 nm in a microplate reader were measured using a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines (expressed in picograms per millilitre). The minimum levels of each cytokine detectable in the conditions of the assays were 10 pg/mL for IL-1, IL-6.

2.12. Statistical Analysis. The results are reported as the means ± SEM. The significance of differences among the means was assessed by ANOVA followed by Dunnett's test when various experimental groups were compared to the control group. A value of $P < 0.05$ indicated significance.

3. Results

The elution profile of *Bothrops roendigeri* venom following RP-HPLC performed on a C18 column showed fifteen fractions (1–15) (Figure 1). The fifteen eluted peaks were screened for PLA_2 activity. Only the fraction labeled in figure

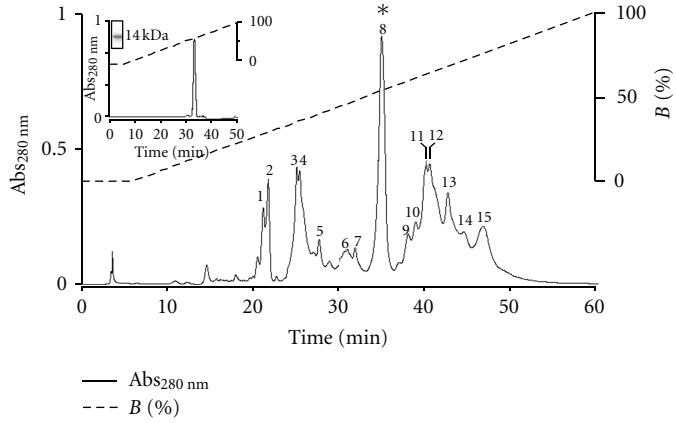


Figure 1: Elution profile of *Bothrops roedingeri* venom by RP-HPLC on an m-Bondapack C18 column. Fraction 4 (BrTX-I) contained PLA_2 activity. Insert Re-chromatography on RP-HPLC chromatography of the BrTX-I and electrophoretic profile of BrTX-I with molecular mass ~14 kDa).

peak 8 presented PLA_2 activity, which was eluted with 58% of buffer B.

To confirm the level of purity, peak 8 was re-purified in a μ-Bondapack C 18 column (0.78 cm × 30 cm; Waters 991-PDA system) in HPLC of the reverse phase, showing a high level of molecular homogeneity (95%), for the presence of a single peak for the peak 8 (BrTX-I, with a very small retention time difference (37.19 ± 0.34 min) (Figure 1 insert). SDS-PAGE show of PLA_2 BrTX-I only band with molecular masses of ~14 kDa (Figure 1 insert) confirmed by MALDI-TOF mass spectrometry in 14,358.69 Da (Figure 2).

The amino acid composition determined was: N, D/10; Q, E/7; S/6; G/6; H/3; R/9; T/6; A/5; P/7; Y/8; V/5; M/1; C/14; I/5; L/7; F/3; K/18; W/Not determined (Figure 5(f)).

Samples of the native with mass 14,358.69 Da (Figure 2) and alkylated 15,170.35 Da (Figure 2 inserted) BrTX-I were digested with trypsin and the digests were analyzed by RP-HPLC. Table 1 shows the masses of the tryptic peptides obtained for from the BrTX-I. It is possible to see that these proteins presented five common peptides to the other *Bothrops* snake venoms. The data obtained were processed using the Mascot MS/MS Ion Search software (http://www.matrixscience.com/).

To obtain detailed structural information, the native protein was alkylated and then digested to be analyzed through ESI-MS/MS. The alkylated protein digest was fractionated by RP-HPLC and each chromatographic peak marked in the chromatogram was manually collected and lyophilized. *De novo* sequencing by ESI-MS/MS was carried out for each peptide peak. The sequences were deduced using ESI-MS/MS and 5 peptides were obtained from the alkylated BrTX-I (Table 1).

Ile and Leu residues were not discriminated in any of the sequences, since they were indistinguishable in low-energy collision-induced dissociation spectra. Due to the external calibration applied to all the spectra, it was also not possible to distinguish between Gln and Lys residues based on the 0.035 Da that separates these amino acids, except for Lys, marked in bold in Table 1, which was deduced by

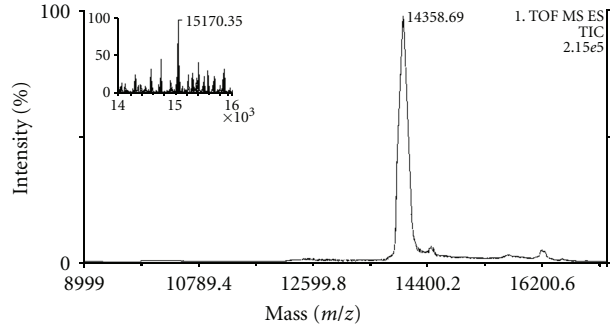


Figure 2: Mass determinations of BrTX-I by mass spectrometry, using a Q-Tof Ultima API ESI/MS (TOF MS mode). Insert mass spectrum, showing multiple alkylation channels of alkylated BrTX-I PLA_2 isolated from *Bothrops roedingeri*.

analysis of the cleavage and missed cleavage sites of the enzyme.

Each *de novo* sequenced peptide of the BrTX-I was submitted separately to the NCBI database, using the protein search program BLAST-p with the search being restricted to the sequenced proteins from the PLA_2 from snake venom family. In order to determine the presence and number of cysteine residues, BrTX-I was reduced and alkylated as described in Section 2.6.

The protein mass registered in peak 1–4 after alkylation was 15170.35 Da; the mass increase of 812 Da indicated the presence of 14 Cys modified residues. The primary structure of the BrTX-I was determined by sequence tryptic digested and deduction of the SwissProt database http://br.expasy.org/. BrTX-I presented a sequence of 54 amino acid residues sequenced, being BrtX-I: DLWQWNKMIK - - - - - - - - - - - - -YGCYCGW GGR- -LTGC P- - - - - - - - - -KDITIVCGE DLPC- - - - - - -KAAAVCFYE NLGTYNKK- - - - - - - - - - - - -

From BrTX-I, five peptides, with molecular masses of 1,360.65 Da (peak 1), 1,404.67 Da (peak 2), 1,791.07 Da

Table 1: Measured molecular masses and deduced amino acid sequences obtained by ESI-MS/MS based on the alkylated tryptic peptides of BrTX-I. The peptides were separated by RP-HPLC and sequenced by mass spectrometry. C = alkylated cysteine, lysine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin. All molecular masses are reported as monoisotopic.

BrTX-I HPLC fraction	Measured mass (Da)	Amino acid Sequence	Theoretical mass (Da)
1	1360.65	DL/IWQ/KWNK/QMI/L**K/Q**	1360.61
2	1404.67	DI/LTI/LVCGEDL/IPC**K/Q**	1404.64
3	1791.07	AAAVCFYENL/IGTYNK/Q**K/Q**	1791.03
4	1120.28	YGCYCGWGGR	1020.25
5	616.79	L/ITGCP**K/Q**	616.75

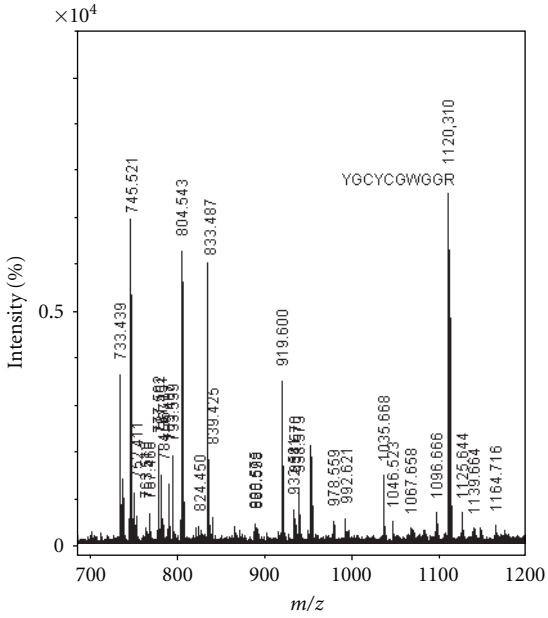


Figure 3: MS/MS spectrum of the peptide tryptic ion of *m/z* 1120.310. Ion of the major sequence-specific peptide of the complementing ions YGCYCGWGGR, from which the sequence of BrTX-I tag was deduced.

(peak 3), 1,120.28 Da (peak 4), and 616.79 Da (Peak 5). After the determination of these molecular masses and with the utilization of iodoacetamide, the cysteines presented in the peptides were alkylated (Table 1).

The peptide eluted in fraction 4 of BrTX-I, having the sequence Y G C Y C G W G G R (tandem MS spectra shown in Figure 3) and the sequence of the BrTX-I protein was deduced and returns high homology with the others PLA_2s from snake from *Bothrops* snake genus present of the venoms snake registered in the date base Blast-p and showed high sequence homology with other PLA_2 in the region associated with the catalytic site (Figure 4).

The PLA_2 activity was examined in the *Bothrops roedingeri* venom and in BrTX-I using the synthetic substrate 4-nitro-3(octanoyloxy) benzoic acid [25]. The PLA_2 activity was higher in BrTX-I c (Figure 5(a)). Under the conditions used, BrTX-I showed a discrete sigmoidal behavior (Figure 5(b) insert), mainly at low substrate concentrations. Maximum enzyme activity occurred at 35–40°C (Figure 5(c)) and the pH optimum was 8.0 (Figure 5(d)). PLA_2s require Ca^{+2} for full activity, being only 1 mM of Ca^{+2} needed for BrTX-I to present phospholipase A_2 activity. The addition of Zn^{2+}, Mg^{2+}, Mn^{2+}, and Cd^{2+} (10 mM) in the presence of low Ca^{2+} concentration (1 mM) decreases the enzyme activity. The substitution of Ca^{2+} by Mg^{2+}, $Cd^{2+}$and Mn^{2+} also reduced the activity to levels similar to those in the absence of Ca^{2+} (Figure 5(e)).

In the neuromuscular activity in chick nerve-muscle preparation, the whole venom concentrations of the 50 μg/mL were tested as well as the concentrations of 5, 20, 50, and 100 μg/mL of BrTX-I. The tested concentration, in both venom and BrTX-I, caused an irreversible dose-dependent blockade of the neuromuscular transmission ($P < 0.05$). The time required for the venom to achieve 50% twitch tension blockade, through an indirect stimulation, was: 22.60 ± 0.61 min (50 μg/mL) (Figure 6). The time required for BrTX-I to achieve 50% twitch tension blockade, also through indirect stimulation only doses of 50 (31.51 ± 0.52 min) and 100 μg/mL (25.29 ± 0.28 min) (Figure 6(a)). The twitch tension records of the control preparation remain stable at 98% to the venom and 97% to the BrTX-I (5 μg) along the 120 min of incubation with Krebs solution.

Regarding the venom, the concentration of 50 μg/mL altered significantly the ACh (110 μM) and KCl (20 mM) induced contractures when compared to the control values. In the concentration of the 50 μg/mL, the complete blockade was not accompanied by significantly inhibition of the response to ACh and KCl (Figure 6(b)). In the control preparations, the contracture to ACh and KCl was kept stable after a 120 min indirect stimulation.

In vivo, BrTX-I induced a conspicuous local myotoxic effect when injected by the i.m. route, only doses 10 and 20 μg (Figure 6(c)), but no increase in plasma CK levels occurred after their i.v. injection even in the same dose of 20 μg. Time-course analysis showed a maximum increase in plasma CK 1 h after i.m. injection, returning to normal by 24 h (Figure 6(d)).

Compared to PBS-injected animals, those which received subplantar injections of the BrTX-I (1, 5, 10 and 20 μg/paw) presented marked paw edema all doses (Figure 7(a)). Maximal activity was attained 2 h to BrTX-I after injection and receded to normal levels after 24 h. The level of edema induction by 20 μg of BrTX-I PLA_2 was similar to the other doses tested.

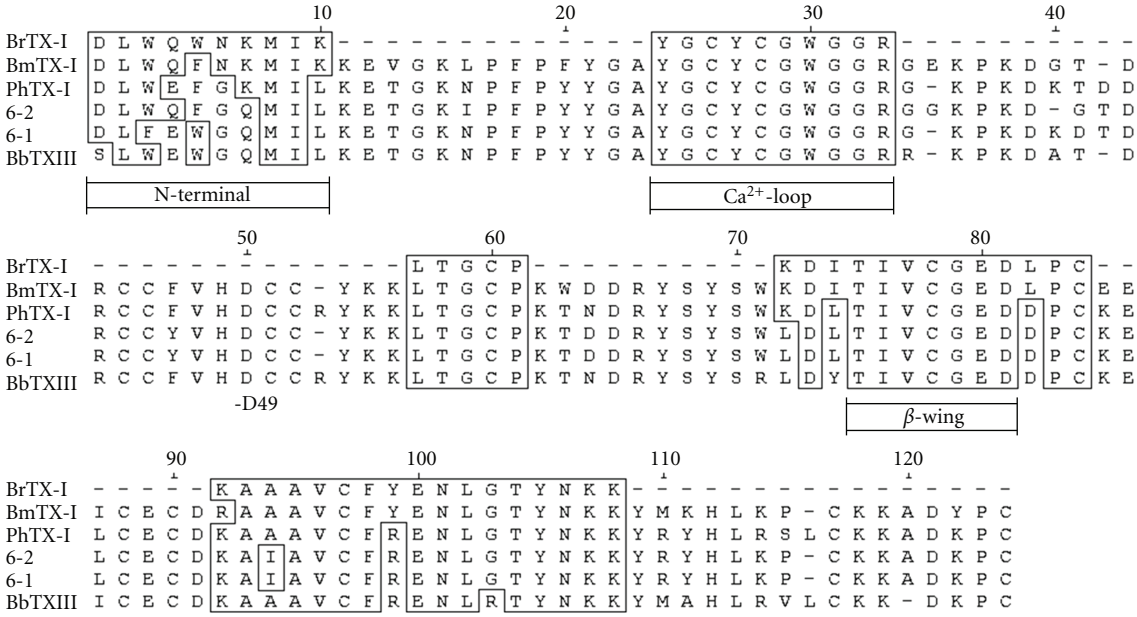


Figure 4: Alignment of the deduced amino acid sequence of the new PLA_2 BrTX-I with PLA_2 present in venom of PLA_2 (BmTX-I) from *Bothrops moojeni* [26], PLA_2 PhTX-I from *Porthidium hyoprora* [27], PLA_2 isoforms (6-1 and 6-2) of the fraction BthTX-II from *Bothrops jararacuçu* [15], and BbTX-III from *Bothrops brazili* [27].

To further analyze and compare the mechanisms of the inflammatory events induced by BrTX-I PLA_2, the concentrations of the IL-1, IL-6, and TNF-α in the serum were measured. BrTX-I caused a marked increase in the TNF-α concentrations only at 1 h (Figure 7(b)). In both the case of IL-1, the maximum peak was recorded at 6 h, on the other hand for IL-6 level the peak was at 3 h (Figures 7(c) and 7(d)).

4. Discussion

The purification procedure for basic PLA_2s developed by Ponce-Soto et al. [6, 15, 23, 24] showed to be also efficient for the obtainment of the Bothropsroedingeritoxin I PLA_2 (BrTX-I) from *Bothrops roedingeri* snake venom. Fractionation protocol of this crude venom using a single pass chromatographic in a column μ-Bondapack C-18 coupled to a system of reverse phase HPLC (0.78 cm–30 cm; Waters 991-PDA system) gave rise to 15 fractions at 280 nm, the eight last being the basic PLA_2 named BrTX-I (Figure 1).

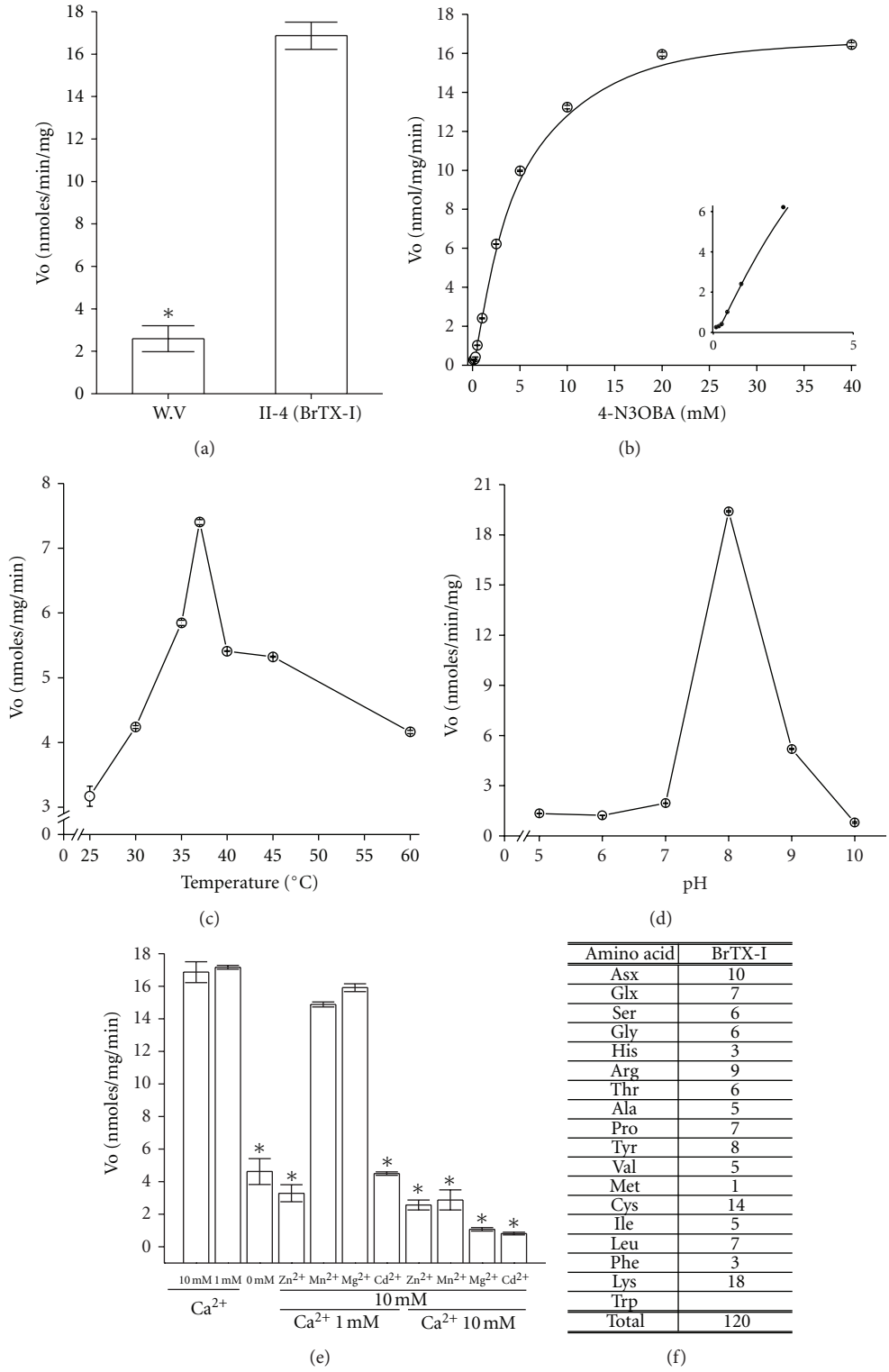
SDS-PAGE showed (Figure 1 insert) the isolated toxin, BrTX-I have Mr of ~14 kDa similarly to basic PLA_2 isolated from other myotoxins from *Bothrops* snake venoms.

The molecular masses obtained by MALDI TOF mass spectrometry showed to be similar to that of other snake venom PLA_2s (14358.69 Da) (Figure 2). Sequence homology studies had showed that there are extremely conserved positions in the PLA_2s. In positions 1 and 2, there is a predominance of the amino acids sequence (DL), in position 4 (Q). One of the highly conserved regions in the amino acid sequences of PLA_2 is the Ca^{2+}-binding loop, segment from…YGCYCGXGG… and HD(49)CC (Figure 3). Residues forming the Ca^{2+}-binding loop and the catalytic network of BrTX-I PLA_2 show a high conservation grade, reflecting the nondecreased catalytic activity.

The primary structure of BrTX-I determined by deduced sequencing (SwissProt database http://br.expasy.org/) method is aligned with the sequences of some other homologous snake venom PLA_2 from snake of the crotalidae family. It was very similar to that of other PLA_2 (Figure 4).

The PLA_2 activity showed to be higher in BrTX-I (16.87 ± 0.643 nmoles/min/mg) when compared with the whole venom (2.59 ± 0.617 nmoles/min/mg) (Figure 5(a)). The PLA_2 from *Crotalus durissus terrificus* venom is a typical PLA_2, since it hydrolyzes synthetic substrates at position 2 and preferentially attacks substrates in their micellar state [28]. They can hydrolyze phospholipids in monomeric, micellar or lipid bilayer phases. PLA_2 enzymes exhibit a large and abrupt increase (up to 10,000 times) in their catalytic activity when monomeric phospholipids aggregate forms micelles at their critical micellar concentration [29]. This is due to the higher efficiency of interfacial catalysis, which depends on the absorption of the enzyme onto the lipid-water interface, strongly promoted by the presence of anionic amphipatic molecules within the membrane [30]. With synthetic substrate, BrTX-I behaved allosterically, especially at low substrate concentrations, which is in agreement with the results obtained by Beghini et al. [31], Bonfim et al. [32, 33], Ponce-Soto et al. [18], Calgarotto et al. [26], Huancahuire-Vega et al. [27] and for other PLA_2 using the same nonmicellar substrate also observed that the dependence of activity on substrate concentration was markedly sigmoidal (Figure 5(b)).

The PLA_2s from snake are highly stable and resistant to heat, acid, and urea, but catalytic activity is inactivated at high



Amino acid	BrTX-I
Asx	10
Glx	7
Ser	6
Gly	6
His	3
Arg	9
Thr	6
Ala	5
Pro	7
Tyr	8
Val	5
Met	1
Cys	14
Ile	5
Leu	7
Phe	3
Lys	18
Trp	
Total	120

(f)

FIGURE 5: (a) PLA_2 activity of *Bothrops roedingeri* venom and peak 4 (BrTX-I); (b) effect of substrate concentration on the kinetics of BrTX-I (PLA_2) activity. (c) effect of temperature on the PLA_2 activity of BrTX-I; (d) effect of pH on BrTX-I activity; (e) influence of ions (10 mM each) on PLA_2 activity in the absence or presence of 1 mM Ca^{2+}. The results of all experiments are the mean ± SE, of three determinations ($P < 0.05$) and (f) amino acid composition of BrTX-I from *Bothrops roendingeri* snake venom.

pH. When micellar substrates are used, maximum catalytic activity occurs at pH 7-8 and 30–55°C [17, 28, 34–36] (Figure 5(c)). BrTX-I showed maximum enzyme activity at 35–45°C and greatest activity at around pH 8.0 (Figure 5(d)).

A strict requirement for Ca^{2+} is characteristic of some PLA_2 [18, 31, 35, 37]. BrTX-I showed typical Ca^{2+}-dependent PLA_2 activity similar to other PLA_2 and this activity was lower in the presence of other divalent cations. Beghini et al. [31] observed the same for PLA_2 from *Crotalus durissus cascavella* venom and Ponce-Soto et al. [18] for PLA_2 from *Crotalus durissus collilineatus* (Figure 5(e)).

The amino acid composition of the BrTX-I PLA_2 toxin revealed a high content of basic and hydrophobic residues, with 14 half-Cys, in agreement with the reported compositions and primary structures of PLA_2 toxins isolated from *Bothrops* venoms (Figure 5(f)), [6, 15, 38, 39]. The pharmacological activities investigated for BrTX-I PLA_2 includes neurotoxicity *ex vivo* in preparation BCP, *in vivo* inducing rapid damaging action to skeletal muscle tissue, paw oedema and increase of IL-1, IL-6 and TNF-α in the mice serum.

Some authors [3, 4, 6, 15, 40–44] have proposed several models to explain PLA_2catalytic and pharmacological activities. In these models PLA_2 has two separated places; one is responsible for catalitic activity and other for biological activity expression. In according to them, the pharmacological place would be located in the surface of PLA_2 molecules.

The BrTX-I caused an irreversible concentration-dependent blockade of the indirectly elicited twitch responses of the chick biventer cervicis muscle preparation (BCP). Only doses 20, 50, and 100 μg/mL caused an irreversible dose-dependent blockade of the neuromuscular transmission (Figure 6(a)). The complete blockade of the muscle contraction all of the doses, was not accompanied by any significant inhibition of the responses to ACh. Inhibition response to KCl was progressive in terms of increasing the dose, suggesting a myotoxic effects due to destabilization of the membrane (Figure 6(b)).

Thus, the neuromuscular blockade produced by BrTX-I may be attributed to presynaptic activity, either by blocking axonal conduction or by affecting transmitter release at the motor nerve-terminal. The fact that the BrtX-I from *Bothrops moojeni* did not significantly affect the response to ACh and KCl, except when high doses were used, suggests that the venom presents a primordial presynaptic nature. Such neuromuscular blockade characteristics have been attributed to presynaptic-acting PLA_2 from snake [45, 46] as those of *Crotalus durissus terrificus* [47], *Micrurus species* [48, 49], and other *Bothrops*, *Bothrops insularis* [50], *Bothrops pauloensis* [47, 51], and *Bothriopsis bilineata smargadina* [52], which did not show any detectable effect on the nicotinic receptor and, in some cases, showed only a mild muscle alteration.

In according to the model proposed by [42], the anticoagulant place would be located in a region between the 53 and 76 residues, considering this region charged positively in the $PLA_2$with high anti-coagulant activity. In PLA_2 with moderate or low anti-coagulant activity, there is a predominancy of negative chargings. This region is placed in a distinct local and separated of foreseen regions by neurotoxicity and myotoxicity.

Local and systemic skeletal muscle degeneration is a common consequence of envenomations due to snakebites and mass bee attacks. PLA_2 is an important myotoxic component in these venoms, inducing a similar pattern of degenerative events in muscle cells. The bothropics PLA_2 myotoxins generally present low systemic toxicity, in contrast to myotoxic PLA_2 that are also strongly neurotoxic [5, 53].

Our studies on local and systemic myotoxicity *in vivo* reveal the BrTX-I is nonsystemic myotoxin with local action due to decrease of the plasmatic CK levels (Figures 6(a) and 6(b)). This fact reinforces the hypothesis of differentiated action of local and systemic myotoxicity proposed by Gutiérrez and Ownby [5] and also the unspecificity and specificity proposed by Kini [3], Ponce-Soto et al. [6] and Gutiérrez et al. [54].

PLA_2s from snake venoms exert a large number of pharmacological activities [35, 54] due to a process of accelerated micro-evolution through which a high mutational rate in the coding regions of their genes has allowed the development of new functions, mainly associated with the exposed regions of the molecules [13]. The integral analysis of the inflammation elicited by BrTX-I from *Bothrops roedingeri* venom in the mouse serum performed in the present study allowed a parallel evaluation of the increase in microvascular permeability, by paw oedema and the production of various inflammatory mediators.

The PLA_2s from snake induced an increase in vascular permeability in peritoneal cavity of mice. This is in agreement with previous observations on the edema forming activity of similar molecules in the rodent footpad model [55, 56]. The increase of vascular permeability was detected after BrTX-I injection and developed rapidly, indicating that the observed plasma extravasation is primarily due to formation of endothelial gaps in vessels of microcirculation (Figure 7(a)). Previous studies have documented polymorphonuclear and mononuclear cellular infiltrate after injection of myotoxic PLA_2s from the venoms of *Bothrops asper* [57], *Bothrops nummifer* [58], and *Bothrops jararacussu* [59] in mouse skeletal muscle, and after intrapleural administration of similar myotoxins from *Bothrops jararacussu* and *Bothrops pirajai* venoms [60]. The mediators involved in this effect of BrTX-I was not addressed in this study. However, the immediate plasma extravasation in response to BrTX-I, strongly suggests the involvement of vasoactive mediators derived from mast cell granules. Previously, the ability of venom PLA_2 to degranulate mast cells has been shown [55].

TNF-α is also likely to be involved in inflammation induced by BrTX-I, since the PLA_2 caused a significant increase of TNF-α levels in the serum. TNF-α is also likely to be involved in leukocyte infiltration induced by BrTX-I, since the PLA_2 caused a significant increase of TNF-α levels in the serum. TNF-α induces the expression of E-selectin, CD11b/CD18 and ICAM-1 and triggers the release of several cytokines such as IL-1 and IL-6 (Figure 7(b)). Thus, our results suggest that TNF-α may have a role in the expression of CD18 and the release of other cytokines

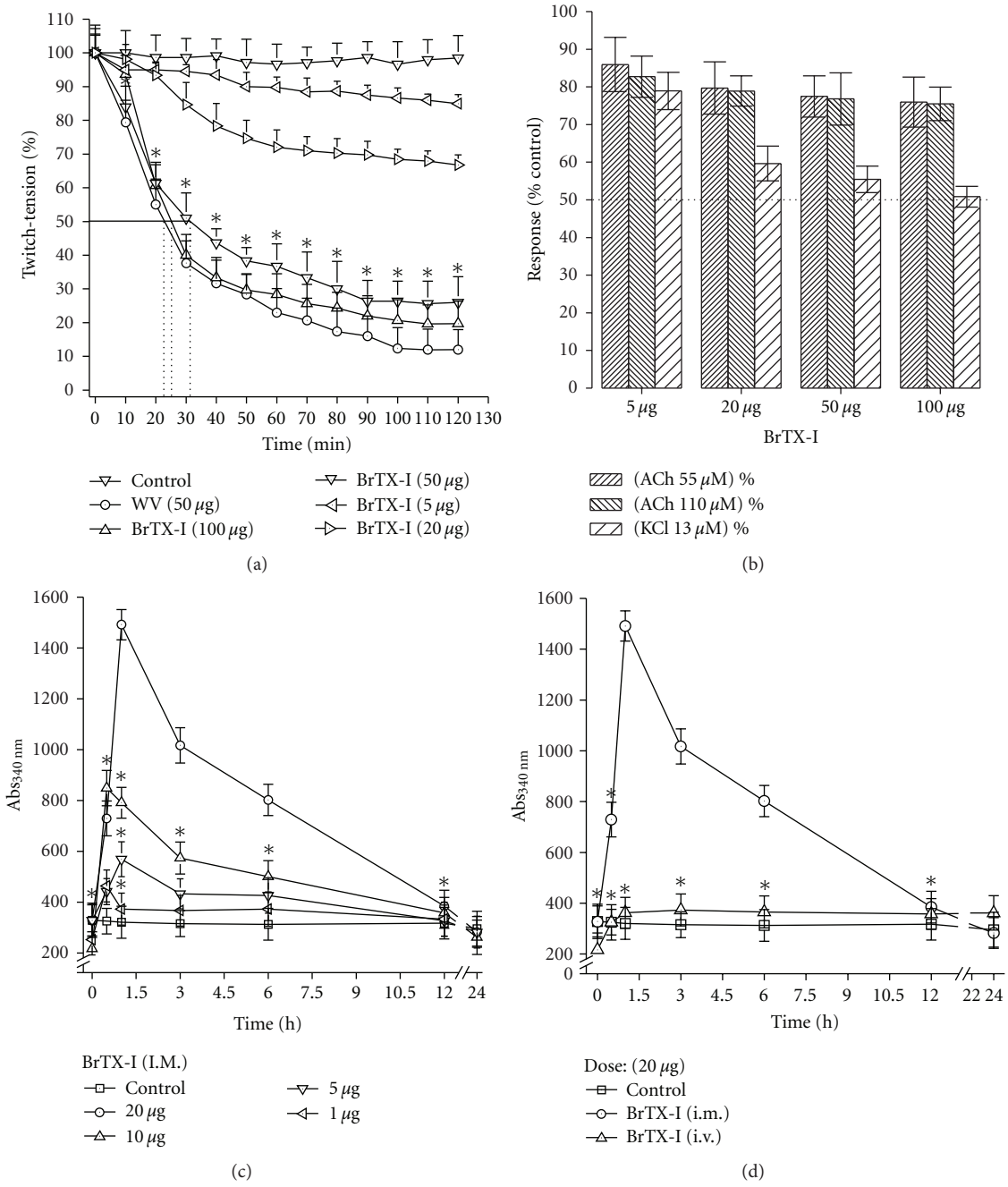


Figure 6: (a) Neuromuscular blockade in chick biventer cervicis muscle preparation (BCP), after addition of *B. roedingeri* whole venom (50 μg/mL), or fraction BII-4 (BrTX-I; 5, 20, 50, and 100 μg/mL). (b) Inhibition of the response to ACh and KCl, after a 120 min incubation with PLA_2 BrTX-I of *Bothrops roedingeri* (5, 20, 50, and 100 μg/mL) in chick biventer cervicis muscle preparation. Each point represents the average from five experiments ± SEM. $P < 0.05$ compared with control. In (c), a group of five Swiss mice (18–20 g) received an intramuscular (i.m.) injection of BrTX-I (1 to 20 μg in 50 μL of PBS), in the gastrocnemius muscle of mice. (d) CK serum levels after control (□) or PLA_2 BrTX-I injection by the i.m. route (○) and i.v route (Δ). At different times, blood was collected, and serum CK levels were measure. Values are means ± SEM of five mice at each point.

following BrTX-I injection, thereby being relevant for neutrophil influx and for increase of vascular permeability on the paw edema.

Cytokines, such as IL-1, IL-6, and TNF-α, are also relevant mediators for leukocyte migration and participate in several inflammatory conditions. Our results showed that BrTX-I induce increase in IL-1 and IL-6 in the serum, exerting a stronger effect (Figures 7(c) and 7(d)). IL-1 induced the expression of adhesion molecules by endothelial cells and stimulates the release of both IL-6 and TNF-α [61]. Thus,

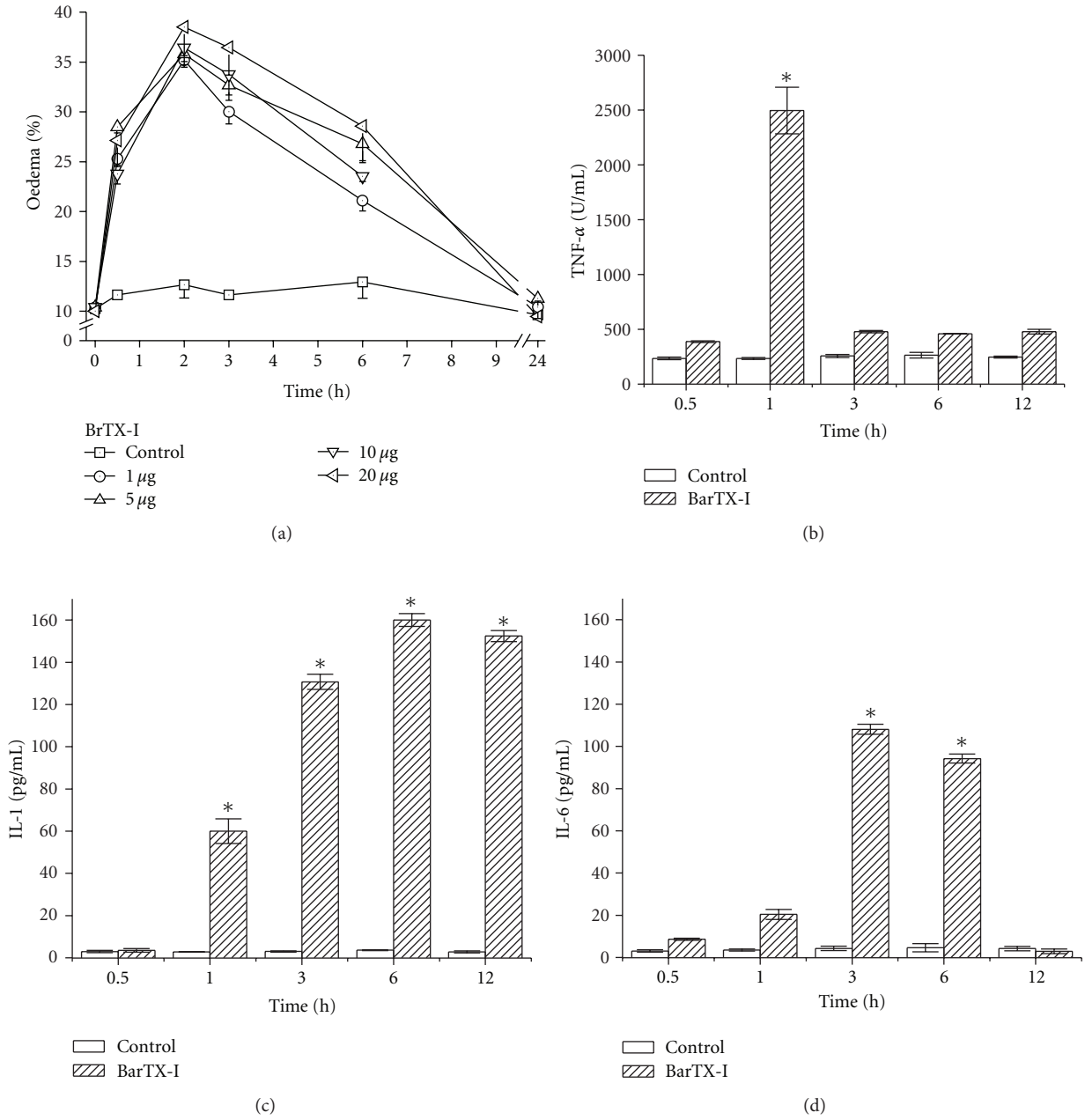


FIGURE 7: In (a), time-course of the mice paw oedema induced by selected doses of BrTX-I (1–20 μg). The oedema, which was expressed as the percentage increased in the volume of the treated group to that of the control group at each time interval, was maximal around 2 h and decreased thereafter. Levels of TNF-α, IL-1 and IL-6 ((b), (c), and (d), resp.) in the serum after injection of BrTX-I. Animals were injected i.m. with BrTX-I (1.0 mg/kg) or sterile saline alone (control) in a final volume of 1 mL. TNF-α, IL-1 and IL-6 ((b), (c), and (d), resp.) were quantified by specific ELISA, in serum collected at the indicated time intervals after BrTX-I or saline injection as described in Section 2. Each bar represents mean GSEM of 5 animals. $^{*}P < 0.05$ when compared with the corresponding control.

our results suggest that IL-1 may contribute for the leukocyte migration.

All these biological effects induced by the BrTX-I occur in the presence of a measurable PLA_2 activity. Although the catalytic activity of PLA_2 contributes to pharmacological effects, it is not a prerequisite [55, 56, 62–64]. However, further studies are necessary to identify the structural determinants involved in these pharmacological activities.

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Research Article

Synergistic Effects of Secretory Phospholipase A_2 from the Venom of *Agkistrodon piscivorus piscivorus* with Cancer Chemotherapeutic Agents

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Healthy cells typically resist hydrolysis catalyzed by snake venom secretory phospholipase A_2. However, during various forms of programmed cell death, they become vulnerable to attack by the enzyme. This observation raises the question of whether the specificity of the enzyme for dying cells could be used as a strategy to eliminate tumor cells that have been intoxicated but not directly killed by chemotherapeutic agents. This idea was tested with S49 lymphoma cells and a broad range of antineoplastic drugs: methotrexate, daunorubicin, actinomycin D, and paclitaxel. In each case, a substantial population of treated cells was still alive yet vulnerable to attack by the enzyme. Induction of cell death by these agents also perturbed the biophysical properties of the membrane as detected by merocyanine 540 and trimethylammonium-diphenylhexatriene. These results suggest that exposure of lymphoma cells to these drugs universally causes changes to the cell membrane that render it susceptible to enzymatic attack. The data also argue that the snake venom enzyme is not only capable of clearing cell corpses but can aid in the demise of tumor cells that have initiated but not yet completed the death process.

1. Introduction

During programmed cell death, the plasma membrane undergoes a series of quantifiable biophysical changes. These include a decrease in membrane order and an increase in interlipid spacing. The degree of change in these membrane properties during apoptosis can be quantified using trimethylammonium diphenylhexatriene (TMA-DPH) and merocyanine 540 (MC540). This "softening" of the membrane has a facilitative effect on the activity of AppD49 snake venom secretory phospholipase A_2 ($sPLA_2$) causing the enzyme to readily hydrolyze membranes of damaged and dying cells while leaving membranes of healthy cells virtually untouched. The connection between cell health and phospholipase activity suggests the intriguing hypothesis that cancer cells treated with antineoplastic agents would become vulnerable to attack by the enzyme resulting in accelerated demise and clearance of these cells.

This study uses four chemotherapeutic agents that initiate apoptosis by different mechanisms (paclitaxel: microtubule inhibitor, methotrexate: inhibitor of thymine synthesis, actinomycin D: transcription inhibitor, and daunorubicin: DNA replication and transcription inhibitor) to answer two critical questions relevant to this hypothesis. First, do cells treated by each type of chemotherapeutic agent become susceptible to hydrolysis by $sPLA_2$? Second, regardless of the induction mechanism, do cellular membranes exhibit the same changes in biophysical membrane properties as the apoptotic program proceeds?

2. Materials and Methods

2.1. Reagents. The monomeric aspartate-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus* was isolated according to the procedure used by Maraganore et al.

[1]. Ionomycin was purchased from Calbiochem (La Jolla, CA, USA). The fluorescent probes TMA-DPH, MC540, and propidium iodide were acquired from Invitrogen (Carlsbad, CA, USA). Methotrexate, daunorubicin, paclitaxel, and actinomycin D were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were purchased from standard sources. Ionomycin, MC540, paclitaxel, and actinomycin D were dissolved in dimethylsulfoxide (DMSO); TMA-DPH in dimethylformamide; methotrexate in slightly basic modified balanced salt solution (134 mM NaCl, 6.2 mM KCl, 1.6 mM $CaCl_2$, 1.2 nM $MgCl_2$, 18.0 mM Hepes, 13.6 mM glucose, pH 7.4, MBSS); daunorubicin in water.

2.2. Cell Culture and Experimental Protocol. Cultures of S49 mouse lymphoma cells were grown in Dulbecco's Modified Eagle Medium at 37°C in humidified air containing 10% CO_2 and prepared for experiments as described [2]. For spectral and kinetic spectrofluorometer experiments (FluoroMax 3, Horiba Jobin-Yvon, Edison, NJ, or PC-1, ISS, Champaign, IL, USA), an aliquot of cells (usually between 0.4×10^6 and 3.0×10^6 cells/mL in MBSS) was transferred to a quartz fluorometer sample cell and allowed 5 min to equilibrate. Spectral bandpass varied between 4 and 16 nm depending on the intensity of the probe and instrument sensitivity. Temperature and sample homogeneity were maintained as described previously [3]. All experiments and incubations were performed at 37°C.

2.3. Membrane Hydrolysis. Membrane hydrolysis due to the action of $sPLA_2$ was assayed by measuring the rate of propidium iodide (37 mM final) uptake by cells as described previously [2, 3]. Use of the propidium iodide assay as an indication of the number of cells hydrolyzed was validated using an intestinal fatty acid binding protein as explained [2]. After beginning data acquisition to obtain a measure of background signal due to scattered light, propidium iodide was added to the cells and allowed sufficient time to equilibrate. Phospholipase A_2 was then added (70 nM final), and data acquisition continued until the intensity of propidium iodide fluorescence was stable. The detergent Triton X-100 was then added to permeabilize any remaining cells at the conclusion of the experiment. Data were analyzed as shown previously [3] and the percentage of cells in the three categories: "already dead;" "alive, but susceptible to $sPLA_2$;" "resistant to $sPLA_2$" was calculated.

2.4. Probes of Membrane Structure. Merocyanine 540 (170 nM final) data were acquired as emission spectra (excitation, 540 nm; emission, 550–700 nm). Spectra were obtained with cell samples before the addition of probe, 5 min after addition, and 10 min after addition of ionomycin (300 nM). The third spectrum after ionomycin treatment was used to establish the maximum response of 100% of the cells as described previously [3, 4]. Spectra were analyzed by subtracting the initial background spectrum from the other two and integrating the intensity from 565 to 615 nm. The integrated intensity of the second spectrum was then expressed as a fraction of that of the third.

Measurements of TMA-DPH steady-state fluorescence anisotropy (excitation = 350 nm; emission = 452 nm, 16 nm bandpass in both cases) were made in the PC-1 fluorometer, with the fluorometer in the "L" configuration and Glan-Thompson polarizers first both in the vertical position (parallel) and repeated with the excitation polarizer in the vertical position and the emission polarizer in the horizontal position (perpendicular). Data were obtained before the addition of the probe to measure background cell fluorescence and after 10 min equilibration with TMA-DPH (240 nM final). TMA-DPH fluorescence was analyzed by calculating the anisotropy (r):

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}, \tag{1}$$

where I_{vv} and I_{vh} are the intensities with parallel and perpendicular polarizer configurations (after subtracting background light), respectively. G is the correction factor for transmission efficiency of the emission monochromator, calculated as follows:

$$G = \frac{I_{hv}}{I_{hh}}. \tag{2}$$

To control the effect of solvent and other factors, control experiments with just the solvent and all other variables intact were run in tandem for all procedures.

3. Results

3.1. Susceptibility to $sPLA_2$. The time needed to induce apoptosis in 100% of S49 cells varied from agent to agent (200 nM paclitaxel, 48 h; 32 nM actinomycin D, 24 h; 10 μM methotrexate, 16 h; 20 μM daunorubicin, 12 h). With each agent, cells became vulnerable to attack by $sPLA_2$ during the process of cell death. Figure 1 shows the rise and fall of the alive and susceptible population for cells that have been treated with methotrexate. Between 5 and 10 h, the proportion of the alive and susceptible population reached a maximum of 13% and then decreased as the cells succumbed completely to the apoptotic process. Results for the other three agents exhibited a similar time-dependent pattern (data not shown). Figure 2 displays the maximum percentage of alive and susceptible cells for each of the four treatments. For paclitaxel and actinomycin D, the maximum occurred at 18 h. For daunorubicin, it was 7 h.

3.2. Physical Changes. Figure 3 illustrates the change in MC540 fluorescence that corresponded in each case to the emergence of the largest alive and susceptible population. For each agent, the fluorescence intensity increased beyond that observed in control samples. However, in no case did it reach 100%, presumably since the entire population of cells was never uniformly synchronized in the program of cell death. The increased MC540 fluorescence intensity is caused by amplified binding of the probe to the cellular membrane, which is reflective of greater interlipid spacing [3, 5, 6].

In addition, the anisotropy of TMA-DPH was used to detect changes in cellular membrane lipid order. As shown in

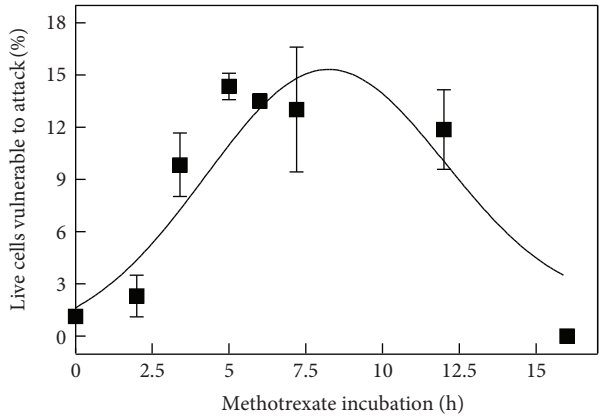

Figure 1: Evolution of "alive and susceptible" cell population during methotrexate-induced cell death. The percentage of the total cell population that was alive and susceptible to $sPLA_2$ was assayed using propidium iodide at 1 h intervals during the apoptotic process (n = 3–5 for each methotrexate time point, n = 36 for control) as explained in Section 2.

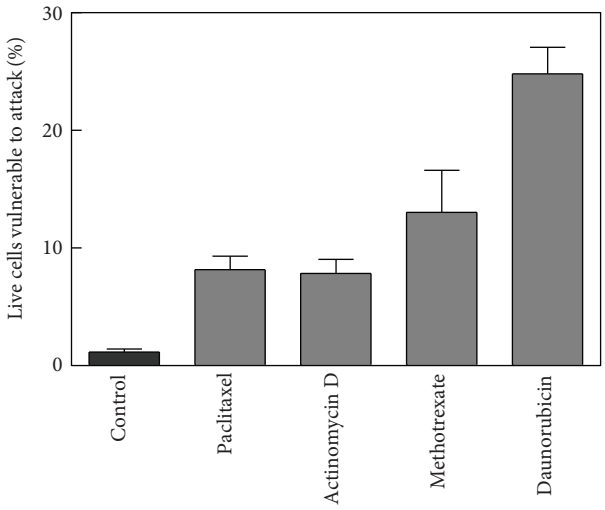

Figure 2: Comparison of maximum percentage of observed "alive and susceptible" population for cells treated with paclitaxel, actinomycin D, methotrexate, and daunorubicin. Data were analyzed by one-way analysis of variance followed by a Dunnett's posttest comparing methotrexate (n = 7), actinomycin D (n = 9), paclitaxel (n = 3), and daunorubicin (n = 4) to control (n = 21). The overall effect of treatment was significant ($P < 0.0001$) with all treatments differing individually from the control ($P < 0.05$).

Figure 4, each drug caused a significant drop in anisotropy. This decrement in anisotropy is generally interpreted as an indication that the membrane had become less ordered or more fluid.

4. Discussion

Prior investigations by our lab using calcium ionophore, glucocorticoid, and thapsigargin have successfully identified

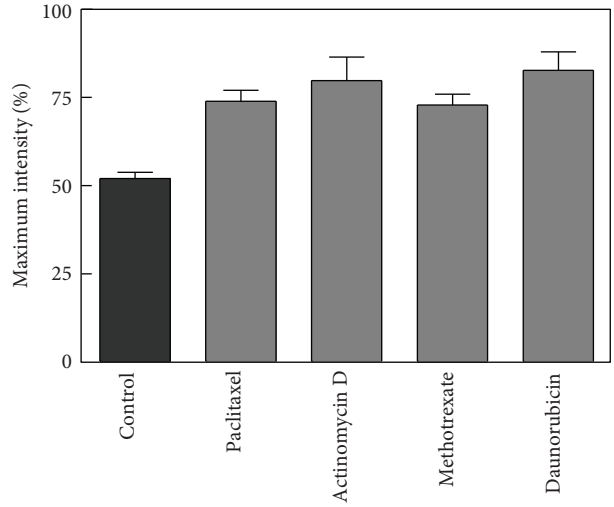

Figure 3: Effect of chemotherapeutic agents on MC540 fluorescence intensity. Cells were incubated with control vehicle or each agent and then harvested at the time when the alive and susceptible population reached a maximum. MC540 was added and assayed as described in Section 2. Data were analyzed by one-way analysis of variance followed by a Dunnett's post test comparing methotrexate (n = 3), actinomycin D (n = 6), paclitaxel (n = 3), and daunorubicin (n = 8) to control (n = 54). The overall effect of treatment was significant ($P < 0.0001$) with all treatments differing individually from the control ($P < 0.05$).

several biophysical changes that occur in the cellular membrane during apoptosis [3, 4, 7–9]. The results of this current study confirm that these changes are a generalizable feature of programmed cell death that occur regardless of the type of apoptotic inducer. Methotrexate, paclitaxel, actinomycin D, and daunorubicin induce cell death by disparate means; however, in each case, the interlipid spacing and fluidity of the membrane increased compared to the control. The idea is that these physical changes improve the likelihood of vertical movements of phospholipids in the membrane sufficient for individual phospholipids to enter the active site of adsorbed enzyme [3, 4, 7, 10–12]. For example, a recent study on thapsigargin-treated cells estimated that the chance of such lipid protrusions from the membrane increases approximately 100 fold when TMA-DPH anisotropy decreases to levels comparable to those displayed in Figure 4 [7]. In each case, the changed biophysical state of the membrane appeared to render it vulnerable to attack by snake venom $sPLA_2$ as evidenced by the emergence of a significant population of cells that were alive and susceptible to hydrolysis.

This ability of snake venom $sPLA_2$ to hydrolyze the membranes of cells dying by administration of antineoplastic agents suggests that it could potentially act as an adjuvant in facilitating the demise of cancer cells. There are two ways by which it could aid in the process. First, the enzyme can directly hydrolyze and permeabilize cells that have initiated apoptosis due to treatment with a chemotherapeutic agent. The fact that many of the cells hydrolyzed were still alive suggested that one could develop a strategy in which the dose of both antineoplastic agent and $sPLA_2$ would be calibrated

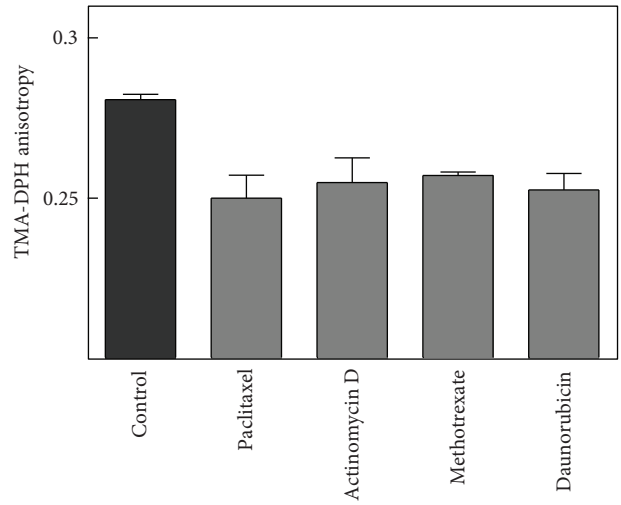

Figure 4: Effect of chemotherapeutic agents on TMA-DPH anisotropy. Cells were incubated with control vehicle or each agent and then harvested at the time when alive and susceptible population reached a maximum. TMA-DPH was added and anisotropy was assayed as described in Section 2. Data were analyzed by one-way analysis of variance followed by a Dunnett's posttest comparing methotrexate ($n = 3$), actinomycin D ($n = 6$), paclitaxel ($n = 3$), and daunorubicin ($n = 3$) to control ($n = 10$). The overall effect of treatment was significant ($P = 0.0006$) with all treatments differing individually from the control ($P < 0.05$).

to maximize the number of cells that remain alive but are vulnerable to enzymatic destruction. In this way, untoward effects of the antineoplastic could perhaps be mitigated. Even if the combined use of enzyme and agent does not change the total number of cells eliminated during the chemotherapy, the data of this study suggest that the clearance of tumor cells could be accelerated, thus allowing the length of the treatment course to be reduced.

The second beneficial effect of combining $sPLA_2$ with traditional chemotherapy is that one of the hydrolysis products released (lysophospholipid) can serve as a "find me" signal for macrophages [13]. This signal recruits macrophages to the vicinity of the cells experiencing hydrolysis. Those cells that additionally express phosphatidylserine on their surface are then eliminated by the macrophage. As shown previously, such expression of phosphatidylserine is a common feature of cells exposed to antineoplastic drugs [14–17]. The caveat to that idea is that the hydrolysis products are also proinflammatory, and failure of the macrophage system to adequately respond could result in unwanted stimulation of the immune system.

This study is not the first to propose use of snake venom as an element of a chemotherapeutic regiment. For example, venom from *Hydrophis spiralis* has been shown to improve Ehrlich ascites carcinoma symptoms in mice treated additionally with 5-fluorouracil [18]. In addition, mammary cancer tumor load was reduced significantly and life span prolonged in mice treated with the venom of *Cerastes cerastes* [19]. Our study differs in using a purified component of the venom, that is, $sPLA_2$ and proposes a mechanism to explain the enhanced susceptibility of cells to the enzyme during chemotherapy. Alternatively, endogenous $sPLA_2$ has been associated with cancer in a variety of other contexts including the etiology of certain tumors [20–23], exacerbation of tumor proliferation [24], as a biomarker for some cancers [24–26], and as a means for delivery of liposome-encased drugs [27–29].

5. Conclusions

We have demonstrated here that lymphoma cells treated with diverse chemotherapeutic agents become vulnerable to enzymatic attack by $sPLA_2$ prior to death. This effect therefore appears to be a general phenomenon of apoptotic cells [3, 4, 30, 31] and could potentially be exploited in designing chemotherapeutic strategies. The results also substantiate the concept that susceptibility to the enzyme relates to physical properties of the cell membrane such as those detected by MC540 and TMA-DPH. Future studies are needed to explore the range of applicability of these observations to various tumor types and to compare them to the behavior of the normal tissues from which the tumors originate. Preliminary results from our work on normal human lymphocytes suggest the possibility that some tumor lines may be substantially more susceptible to the action of snake venom $sPLA_2$ than their normal counterparts [8].

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Research Article

A Lys49 Phospholipase A_2, Isolated from *Bothrops asper* Snake Venom, Induces Lipid Droplet Formation in Macrophages Which Depends on Distinct Signaling Pathways and the C-Terminal Region

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MT-II, a Lys49PLA_2 homologue devoid of catalytic activity from *B. asper* venom, stimulates inflammatory events in macrophages. We investigated the ability of MT-II to induce formation of lipid droplets (LDs), key elements of inflammatory responses, in isolated macrophages and participation of protein kinases and intracellular PLA_2s in this effect. Influence of MT-II on PLIN2 recruitment and expression was assessed, and the effects of some synthetic peptides on LD formation were further evaluated. At noncytotoxic concentrations, MT-II directly activated macrophages to form LDs. This effect was reproduced by a synthetic peptide corresponding to the C-terminal sequence 115–129 of MT-II, evidencing the critical role of C-terminus for MT-II-induced effect. Moreover, MT-II induced expression and recruitment of PLIN2. Pharmacological interventions with specific inhibitors showed that PKC, PI3K, ERK1/2, and iPLA_2, but not $P38^{MAPK}$ or cPLA_2, signaling pathways are involved in LD formation induced by MT-II. This sPLA_2 homologue also induced synthesis of PGE_2 that colocalized to LDs. In conclusion, MT-II is able to induce formation of LDs committed to PGE_2 formation in a process dependent on C-terminal loop engagement and regulated by distinct protein kinases and iPLA_2. LDs may constitute an important inflammatory mechanism triggered by MT-II in macrophages.

1. Introduction

Phospholipases A_2s (PLA2; EC 3.1.1.4) constitute a family of lipolytic enzymes with key roles in several cellular processes by regulating the release of arachidonic acid and lysophospholipids from cell membrane phospholipids. Venoms from snakes of the Viperidae family contain group IIA phospholipases A_2 (PLA_2s), which share structural and functional features with PLA_2s found in inflammatory exudates in mammals [1, 2]. A number of *Bothrops* snake venom PLA_2s have been shown to induce inflammatory events such as edema and leukocyte infiltration and to directly activate inflammatory cell functions [3–6].

Basic PLA_2s are considered the most important venom components responsible for the severe local myotoxicity and inflammation characteristic of the envenomation induced by *Bothrops* genus snakes [7]. These enzymes are further divided into two subgroups, namely, catalytically active variants, presenting a conserved aspartic acid residue at position 49 (Asp49PLA_2s), and catalytically inactive homologues, known as Lys49PLA_2s, which present various substitutions in residues of the Ca^{2+} binding loop, as well as at position

49, where Lys replaces the highly conserved Asp [8, 9]. Such modifications drastically affect the catalytic ability of these proteins rendering these homologues enzymatically inactive [10]. Interestingly, Lys49PLA$_2$ homologues are highly myotoxic, bactericidal, and proinflammatory [9], evidencing that phospholipid hydrolysis is not strictly required for these activities. Studies on synthetic peptides and site-directed mutagenesis identified the C-terminal region of Lys49PLA$_2$s as essential for their biological activities [10, 11]. Thus, Lys49PLA$_2$ homologues constitute interesting models to investigate a series of cellular effects which do not depend on membrane phospholipid hydrolysis.

In the *Bothrops asper* snake venom three myotoxic Lys49-PLA$_2$s have been identified, named MT-II, MT-IV, and M1-3-3, and reported in UNIPROT database. Besides myotoxicity, MT-II, the most studied Lys49PLA$_2$ homologue, has been reported to induce inflammation *in vivo* [5, 12] and to activate some inflammatory functions of macrophages *in vitro*, increasing phagocytosis, respiratory burst, and release inflammatory mediators [4] at noncytotoxic concentrations. However, the knowledge on the effects of this Lys49PLA$_2$ in macrophages functions, is still fragmentary.

Macrophages play key roles in a wide variety of processes associated with tissue maintenance, antigen presentation, inflammation, and tissue repair [13]. Upon inflammatory stimuli, quiescent macrophages become activated and present increased number of lipid rich cytoplasmic organelles named lipid droplets (LDs), also known as lipid bodies. These organelles are functionally involved in biosynthesis, transport, and catabolism of lipids [14, 15] as well as biosynthesis and accumulation of inflammatory mediators, such as eicosanoids and cytokines [16, 17]. Moreover, leukocyte LDs associated with inflammatory responses have been shown to compartmentalize signaling proteins involved in cellular activation and structural proteins, mainly perilipin 2 (PLIN2), also named adipophilin (Adipose differentiation-related protein, ADRP), which has an important role in LD assembly and formation of foam macrophages [18, 19], which are markers of atherosclerotic plaques [19]. Increased numbers of lipid droplets are described in distinct populations of leukocytes during inflammatory and infectious processes [20, 21]. Recently, MT-III, a catalytically active variant Asp49PLA$_2$ from *B. asper* venom, has been shown to activate macrophages to form increased amounts of LDs [22], but no such effect has been described for the action of Lys49PLA$_2$s. Therefore, it is relevant to assess the effects of MT-II on macrophages in terms of LD formation. Such macrophage activation might play a relevant role in the scenario of the local pathological alterations induced by snake venom toxins. Based on these information, in the present study the ability of MT-II to induce LD formation in macrophages was evaluated and the mechanisms involved in this effect were analyzed in terms of recruitment and expression of PLIN2, participation of intracellular PLA$_2$s (cPLA$_2$ and iPLA$_2$) and signaling protein kinases. In light of the absence of catalytic activity in MT-II, the effects of some synthetic peptides related to distinct regions of this Lys49PLA$_2$ molecule on lipid droplet formation were further evaluated in macrophages.

2. Materials and Methods

2.1. Chemicals and Reagents. MTT and L-glutamine were obtained from USB Corporation (Cleveland, OH, USA). H7, LY294002, SB202190, PD98059, and Pyr-2 were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Racemic mixture of BEL and anti-mouse PGE$_2$ was obtained from Cayman Chemical (Ann Arbor, MI, USA). Guinea pig polyclonal antibody anti-mouse PLIN2 and FITC-conjugated donkey anti-guinea pig antibody were obtained from Research Diagnostics Inc. (Flanders, NJ, USA). Secondary antibodies anti-mouse and anti-guinea pig conjugated to horseradish peroxidase and nitrocellulose membrane were obtained from GE Healthcare (Buckinghamshire, UK). Gentamicin was purchased from Schering-Plough, NJ, USA). DMSO and BSA were obtained from Amresco (Solon, OH, USA). Mouse monoclonal antibody anti-β-actin, Nile Red, RPMI-1640, thiocarbohydrazide, OsO_4, and EDAC were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). PFA was purchased from Electron Microscopy Science (USA). Alexa Fluor 488 Goat Anti-mouse IgG was purchased from Life Technologies (Grand Island, NY, USA). DAPI and fluoromount G were purchased from Molecular Probes (Eugene, OR, USA). Donkey serum was obtained from Jackson ImmunoResearch Laboratories (PA, USA). Triton-X was obtained from Union Carbide Corporation (Danbury, USA). GA, thioglycolate, and all salts used were obtained from Merck (Darmstadt, Germany).

2.2. Animals. Male Swiss mice (18–20 g) were obtained from Butantan Institute (São Paulo, Brazil). Animals were housed in a temperature-controlled room (22–24°C) with a 12 h light-dark cycle and fresh water and food *ad libitum* until used. This study was approved by the Butantan Institute Animal Experimentation Ethics Committee (reference number 760/10) in accordance with the procedures laid down by the Universities Federation for Animal Welfare.

2.3. Phospholipase A_2. The Lys49PLA$_2$ homologue (MT-II) was isolated from *Bothrops asper* venom by ion-exchange chromatography on CM-Sephadex C-25 as described [23], followed by RP-HPLC on a C8 semipreparative column (10 × 250 mm; Vydac) eluted at 2.0 mL/min with a 0–70% acetonitrile gradient containing 0.1% trifluoroacetic acid, during 30 min, on an Agilent 1200 instrument monitored at 215 nm. Homogeneity was assessed by analytical reverse-phase HPLC on a C4 column using a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (v/v). The absence of endotoxin contamination in the MT-II preparation was demonstrated by the quantitative *Limulus* amebocyte lysate (LAL) test [24], which revealed undetectable levels of endotoxin (<0.125 EU/mL).

2.4. Synthetic Peptides. The following synthetic peptides were synthesized and used for biological assays: (a) peptide 115–129 (KKYRYYLKPLCKK) corresponding to the original sequence 115–129 of MT-II from *B. asper* snake venom; (b) peptide p115-W3 (KKWRWWLKPLCKK) corresponding to the triple tyrosine-to-tryptophan substitution

of p115-129; (c) peptide pEM-2 (KKWRWWLKALAKK), in which the proline and cysteine residues of p115-W3 were each replaced by an alanine residue; (d) peptide 60–71 (KKDRYSYSWKDK) corresponding to a central region sequence of MT-II; (e) peptide p-Scr (FKFKYKKACKKYK) corresponding to a scrambled peptide version of the sequence 115–129 of ACL myotoxin, a Lys49 PLA_2 homologue from the venom of the snake *Agkistrodon contortrix laticinctus*.

Synthetic peptides were prepared in automated benchtop simultaneous multiple solid-phase synthesizer (PSSM 8 system from Shimadzu Co.) using solid-phase peptides synthesis by the Fmoc procedure [25]. Briefly, sequential couplings of protected amino acids were performed with HOBt, TBTU and NMM on Fmoc-Lys(Boc)-Wang resin (Merck KGaA, Germany). Fmoc group cleavage was performed with 30% piperidine (v/v) in DMF. The resin-bound peptides were cleaved/deprotected with TFA/thioanisole/EDT/phenol/water (82.5 : 5: 2.5 : 5 v/v/v/v) at room temperature for 4 h. After filtration, the filtrate was concentrated under argon stream and precipitated with diethyl ether. All crude peptides were purified by reversed-phase chromatography (Shim-pack Prep-ODS, Shimadzu Co.) semipreparative HPLC, and the purity and identity of the peptide were confirmed by mass spectrometry and by analytical HPLC.

2.5. Harvesting of Macrophages. Peritoneal macrophages were harvested 4 days after i.p. injection of 1 mL of 3% thioglycolate. Animals were killed under CO_2 atmosphere and cells were harvested by washing peritoneal cavities with 3 mL of PBS, pH 7.2, containing 10 IU/mL heparin. Aliquots of the washes were used for total cell counts in a Neubauer chamber after dilution (1 : 20, v/v) in Turk solution (0.2% crystal violet dye in 30% acetic acid). Differential cell counts were performed on smears stained with Hema3. More than 95% of the cell population consisted of macrophages, as determined by conventional morphological criteria. The remaining wash volumes were centrifuged at 500 ×g for 6 min (4°C) and the cell pellets were used for subsequent studies after suitable dilutions.

2.6. Cytotoxicity Assay. Cytotoxicity of MT-II towards elicited macrophages was evaluated using the MTT assay. In brief, 2×10^5 macrophages/well in RPMI-1640 medium supplemented with 40 μg/mL gentamicin sulfate and 2 mM L-glutamine were plated in 96-well plates and incubated with 100 μL of selected concentrations of MT-II (0.4–0.8 μM) diluted in medium or with the same volume of medium alone (control) for 1, 6, 12, and 24 h at 37°C in a humidified atmosphere (5% CO_2). MTT (5 mg/mL) was dissolved in PBS and filtered for sterilization and removal of a small amount of insoluble residue present in some batches of MTT. Stock MTT solution (10% in culture medium) was added to all wells in each assay, and plates were incubated at 37°C for 3 h. One hundred μL of DMSO were added to all wells and mixed thoroughly at room temperature for 30 min. Absorbances at 540 nm were then recorded in a microtiter plate reader. Results were expressed as percentage of viable cells, considering control cells incubated with medium alone as 100% viable.

2.7. Stimulation and Treatment of Macrophages. Macrophages were plated on glass coverslips in 24-well plates at a density of 2×10^5 cells/coverslip and allowed to attach for 30 min at 37°C under a 5% CO_2 atmosphere. Non-adherent cells were removed by washing with PBS. Cell monolayers were cultured for 1 h in RPMI-1640 supplemented with 40 μg/mL gentamicin sulfate and 2 mM L-glutamine at 37°C and 5% CO_2 and were then challenged with selected concentrations of MT-II (0.2–1.2 μM) or synthetic peptides (250 μg/mL) or medium (control). Where appropriate, the following inhibitors were used: 1 μM SB202190, inhibitor of p38MAPK; 1 μM LY294002, inhibitor of PI3 K; 6 μM H7-Dihydro, inhibitor of PKC; 25 μM PD98059, inhibitor of ERK1/2; 1 μM Pyr-2 (Pyrrolidine-2), inhibitor of $cPLA_2$ and 2 μM BEL (bromoenol lactone) an inhibitor of $iPLA_2$. All stock solutions were prepared in DMSO and stored at −20°C. Aliquots were diluted in RPMI-1640 to the required concentration immediately before use. The final DMSO concentration was always lower than 1% and had no effect on lipid body numbers. All pharmacological inhibitors were added between 30 and 60 min before stimulation of macrophages with MT-II or medium (control). Cells treated with the inhibitors were analyzed for viability by the tetrazolium-based (MTT) colorimetric assay. No significant changes in cell viability were registered with any of the above agents or vehicle at the concentrations used (data not shown).

2.8. Lipid Body Staining and Quantification. Analysis of lipid body numbers was performed in osmium-stained cells. In brief, macrophages (2×10^5 cells) adhered to glass coverslips were fixed in 4% PFA in 0.1 M phosphate buffer, pH 7.2, for 15 min, and stained with OsO_4. The coverslips were then rinsed in 0.1 M phosphate buffer, stained in 1% OsO_4 (30 min), rinsed in deionized H_2O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed again in 0.1 M phosphate buffer, restained with 1% OsO_4 (3 min), rinsed with H_2O, and then dried and mounted. The morphology of the fixed cells was observed and round osmiophilic structures were identified as lipid droplets, which were then counted under phase-contrast microscopy using the 100x objective lens in 50 consecutively scanned leukocytes in each coverslip. For assays with fluorescent-labeled lipid droplets, macrophages (2×10^5 cells) adhered to glass coverslips were incubated with Nile Red staining solution freshly prepared in 0.1 M phosphate buffer (10 μg/mL) for 20 min at room temperature and washed with phosphate buffer. After several washes the coverslips were mounted with fluoromount G and examined under a fluorescence microscope equipped with the appropriate filter (Zeiss LSM 510 Meta).

2.9. Electron Microscopy. A standardized protocol for electron microscopy procedure was developed for ultrastructural analysis of lipid droplets. Macrophages (5×10^6 cells) incubated with either MT-II (0.8 μM) or medium alone for 1 h were fixed in a diluted mixture of freshly prepared aldehydes

(4% PFA/1% GA in 0.1 M phosphate buffer) containing 3.5% sucrose for 2 h at room temperature and then washed three times in 0.1 M phosphate buffer. Cells were centrifuged at 129 ×g, and cell pellets were postfixed with OsO_4 (1% in phosphate buffer at room temperature) followed by three washes with saline solution. Uranyl acetate in aqueous solution was then added for 2 h at room temperature before dehydration in a graded series of ethanol (70, 95 and 100% twice for 10 min each). For embedding, aliquots of propylene oxide were added twice for 10 minutes, followed by Spurr resin diluted in propylene oxide (1 : 1) and undiluted Spurr resin for 12 h. Polymerization was carried out for 48 h at 70°C. Samples were then incubated with 4% uranyl acetate and lead citrate for contrast. Thin sections were examined with LEO 906E and Zeiss EM109 transmission electron microscopes.

2.10. Immunodetection of PLIN2 and PGE_2. Detection of PLIN2 in MT-II-stimulated macrophages was performed by PLIN2 immunostaining. In brief, macrophages attached to coverslips and stimulated for 3 h with MT-II (0.8 μM) were fixed in 2% paraformaldehyde (PFA). The cells were permeabilized with 0.2% Triton-X 100 in 0.1 M phosphate buffer and blocked with 0.5% normal donkey serum in 0.1 M phosphate buffer for 90 min. After PBS washes, macrophages were incubated for 1 h with guinea pig polyclonal anti-mouse PLIN2 (1 : 2000) diluted in 0.1 M phosphate buffer with 0.2% Triton-X 100. After three washes with PBS (10 min each), the preparations were incubated for 1 h with secondary FITC-conjugated donkey anti-guinea pig antibody (1 : 500) in the dark for 1 h. After the washes, the slides were mounted with fluoromount G and examined under confocal laser scanning microscope (Zeiss LSM 510 Meta). For analysis of PGE_2 immunostaining, the cell were fixed and permeabilized in 1% N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in $HBSS^{-}/^{-}$. The macrophages were blocked with 0.5% normal donkey serum in 0.1 M phosphate buffer for 60 min. Next, the macrophages were washed with $HBSS^{-}/^{-}$ and incubated for 1 h with anti-PGE_2 (1 : 100). After further washes, cells were incubated with biotinylated rabbit anti-mouse IgG secondary Ab (1 : 250) and Nile red solution (1 : 250) in the dark for 1 h. The cover slips were then washed three times and mounted with fluoromount G containing DAPI (Vector Laboratories, Burlingame, CA) and examined under confocal laser scanning microscope (Zeiss LSM 510 Meta).

2.11. Western Blotting of PLIN2. Aliquots of MT-II-stimulated and -nonstimulated cells (2×10^6 cells) were lysed with 100 μL of sample buffer (0.5 M Tris-HCl, pH 6.8, 20% SDS, 1% glycerol, 1 M β-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 10 min. Samples were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 10% bis-acrylamide gels overlaid with a 5% stacking gel. Proteins were then transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using a Mini Trans-Blot (Bio-Rad Laboratories, Richmond, CA, USA). The membranes were blocked for 1 h with 5% nonfat dry milk in TTBS (20 mM Tris, 100 mM NaCl and 0.5% Tween 20) and incubated with primary antibodies against PLIN2 (1 : 2000 dilution) and β-actin (1 : 3000) for 1 h. They were then washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. Detection was by the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Band densities were quantified with a GS 800 Densitometer (Bio-Rad Laboratories, Richmond, CA) using the image analysis software from Molecular Analyst (Bio-Rad Laboratories, Richmond, CA, USA).

2.12. Statistical Analysis. Data are expressed as the mean ± standard error of mean (SEM) of at least three independent experiments. Multiple comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Values of probability lower than 5% ($P < 0.05$) were considered significant.

3. Results

3.1. Effect of MT-II on Macrophage Viability. Initially, the effect of MT-II on isolated-elicited macrophage viability was assessed by the tetrazolium-based (MTT) colorimetric assay. To this purpose the effect of 24 h incubation with two distinct concentrations of MT-II (0.8 and 1.6 μM) were evaluated. As shown in Figure 1, incubation of macrophages with MT-II at a concentration of 0.8 μM did not affect macrophage viability. At a concentration of 1.6 μM, the $sPLA_2$ homologue partially decreased ($P < 0.05$) macrophage viability.

3.2. MT-II Induces LD Formation in Macrophages. To determine whether stimulation of peritoneal macrophages with MT-II would lead to LDs formation, these cells were incubated with selected concentrations of MT-II (0.2–1.6 μM) for 1 h. As demonstrated in Figure 2(a), incubation of macrophages with MT-II at concentrations from 0.8 to 1.6 μM, but not from 0.2 and 0.4 μM, for 1 h induced a significant increase ($P < 0.05$) in the number of LDs in comparison with control cells incubated with culture medium alone. Maximal LD numbers were observed at 1.6 μM MT-II. To determine the time-course of LD formation induced by MT-II, a submaximal concentration of this $Lys49PLA_2$ was used (0.8 μM), and the number of LDs after 1–24 h of incubation was determined. As shown in Figure 2(b), MT-II caused a significant increase ($P < 0.05$) in the numbers of LDs after 1–24 h incubation compared with control cells. The highest number of LDs was detected after 24 h incubation. As illustrated in Figure 2(c), control macrophages stained with OsO_4 showed very few osmiophilic inclusions in the cytoplasm. In contrast, MT-II-stimulated macrophages exhibited a cytoplasm packed with the osmiophilic organelles, which can be seen as dark punctate structures in Figures 2(d), 2(e), and 2(f).

3.3. Ultrastructural Analysis of LDs Induced by MT-II. To further investigate the stimulatory effect of MT-II leading to LDs formation in macrophages, ultrastructural analysis

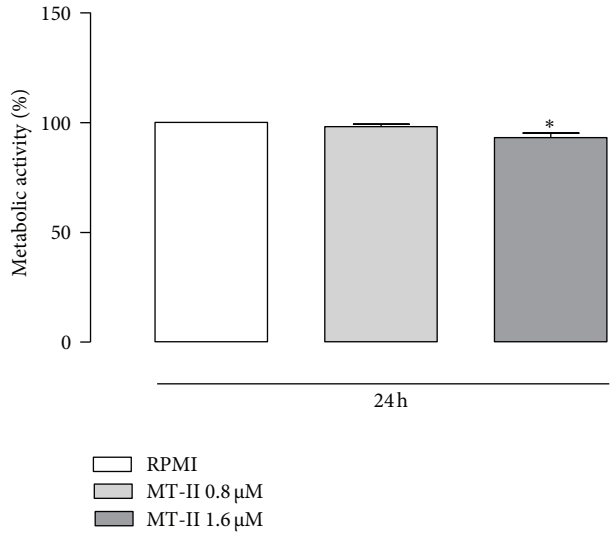

Figure 1: Effect of MT-II on cell viability. The cells were incubated with MT-II (0.8 and 1.6 μM) or RPMI (control) for 24 h, and cytotoxicity was assessed by the tetrazolium-based (MTT) colorimetric assay. Values represent the mean ± SEM from four animals. $^{*}P < 0.05$ compared with control (RPMI).

of LDs was performed using a standardized procedure for TEM. As seen in Figure 3(a) control macrophages showed small, non-membrane-bound, light cytoplasmic LDs. After 1 h incubation, MT-II-stimulated macrophages showed light cytoplasmatic LDs that were present in markedly greater numbers than in the control cells but morphologically similar to LDs in these cells (Figure 3(b)). Also, an enlarged ER was observed in MT-II-stimulated cells in incubation period tested as showed in Figure 3(c).

3.4. Effects of Peptides Corresponding to Selected Regions of MT-II Molecule on LD Formation. The effects of synthetic peptides derived from distinct regions of MT-II protein on LDs formation were investigated in macrophages. Experiments were carried out with macrophages stimulated with peptides corresponding to distinct regions of MT-II molecule for 3 h and then treated as necessary for lipid fixation and stained with OsO_4. Figure 4(a) demonstrates that incubation of macrophages with the C-terminal peptide p115-129 induced a significant increase ($P < 0.05$) in the number of LDs after 3 h of incubation in comparison with nonstimulated control cells. The effect induced by this C-terminal peptide did not differ from that observed in cells stimulated with MT-II native protein for 3 h, which caused a significant increase in the number of LDs in comparison with control cells. On the other hand, neither a scrambled version of the residue sequence used as a control, p-Scr, nor the peptide comprising amino acid residues 60–71 of central region of MT-II modified the basal numbers of LDs after 3 h of incubation as compared with RPMI-treated control cells. As a positive control macrophages were incubated with MT-II native protein for 3 h. In this case, a significant increase in LD number was detected in comparison with RPMI-treated control cells. All peptides tested were used in a concentration (250 μg/mL) previously demonstrated in literature as effective to induce biologic effects (32), but without toxic effect on the viability of macrophages after 3 h of exposure. Figure 4(b) demonstrates that incubation of cells with peptides p115–129, pScr, and p60–71 at a concentration of 250 μg/mL did not affect macrophages viability, whereas incubation of cells with peptides pEM2 (150 μg/mL) and p115-W3 (150 μg/mL) significantly ($P < 0.05$) reduced the viability of macrophages making these peptides unsuitable for the present study. Although MT-II is recognized as a $sPLA_2$ devoid of catalytic activity, we investigated whether a possible residual enzyme activity of MT-II would lead to formation of LDs in macrophages. As shown in Figure 4(c) incubation of macrophages with MT-II (0.8 μM) for 1 h in the presence of a Ca^{2+}-containing medium induced a significant increase ($P < 0.05$) in the number of LDs. This MT-II-induced effect was not modified in the presence of Ca^{2+}-free, EGTA- and Sr_2^{+}-containing medium, with a significant increase of LD numbers observed in comparison with respective control group.

3.5. LD Formation Triggered by MT-II Is Dependent on Distinct Signaling Pathways. To assess the role of kinases in the described actions of MT-II, we determined the effects of the specific inhibitors of p38, PI3 K, PKC, and ERK1/2 (SB202190, LY294002, H7-Dihydro, and PD98059, resp.) on MT-II-induced LDs in macrophages. As seen in Figure 5(a), the PI3 K and PKC inhibitors abolished the LDs formation in MT-II-stimulated macrophages compared with vehicle-treated macrophages stimulated with MT-II. The ERK1/2 inhibitor, in turn, caused 49% reduction in the number of LDs in MT-II-stimulated macrophages when compared with vehicle-treated macrophages stimulated with MT-II (Figure 5(b)). In contrast, the preincubation of macrophages with p38MAPK inhibitor did not change the number of LDs induced by MT-II, in comparison to cells stimulated with MT-II only (Figure 5(b)).

3.6. MT-II Upregulates PLIN2 Protein Expression in MT-II-Stimulated Macrophages. PLIN2 expression can be induced by a variety of inflammatory cells and has been associated with increased numbers of LDs [26, 27]. Therefore, we investigated whether MT-II induces expression of this LD structural protein. Levels of PLIN2 protein expression were analyzed by western blotting in cells incubated and not incubated with MT-II for selected time periods. This analysis revealed increased expression of PLIN2 protein in cells stimulated with MT-II as early as 3 h of incubation, which was sustained up to 12 h. PLIN2 was minimally expressed or absent in control nonstimulated macrophages (Figures 6(a) and 6(b)).

3.7. PLIN2 Colocalizes to LDs in MT-II-Stimulated Macrophages. To better understand the stimulatory effect of MT-II on macrophages that leads to LD formation, cells exposed to MT-II were immunostained with specific antibodies that recognize PLIN2 or neutral lipids from the LD core. As

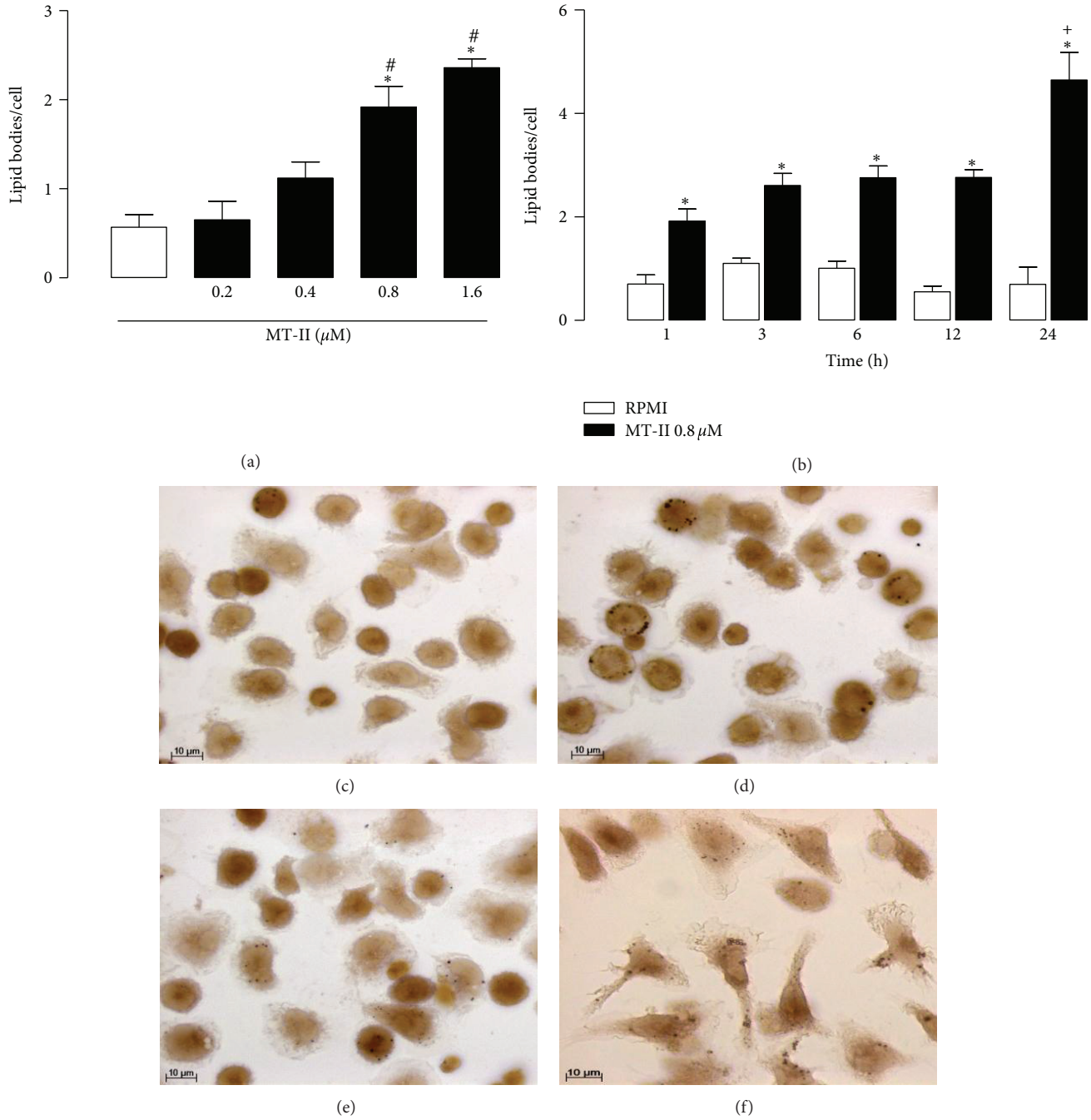

Figure 2: MT-II induces formation of LDs in peritoneal macrophages in culture. (a) Effect of selected concentrations of MT-II on formation of LDs in macrophages which were incubated with various concentrations of MT-II or with RPMI (Control) for 1 h. (b) Time-course of MT-II-induced LD formation. Macrophages were incubated with MT-II (0.8 μM) or RPMI (Control) for 1, 3, 6, 12, or 24 h. LDs were quantified using light microscopy after osmium staining. LDs after osmium staining observed in control (c) or in cells stimulated with MT-II (0.8 μM) for 1 h (d), 12 h (e), or 24 h (f). Each bar represents the mean ± S.E.M. of the number of LDs/cell in 50 counted cells. Values represent means ± S.E.M. for three to five animals. $^{*}P < 0.05$ compared with control cells; $^{\#}P < 0.05$ compared with cells stimulated by 0.2 or 0.4 μM of MT-II; $^{+}P < 0.05$ compared with cells stimulated by MT-II (0.8 μM) for 1, 3, 6 and 12 h.

illustrated in Figure 7 macrophages stimulated with MT-II (0.8 μM) for 3 h exhibited strong fluorescent staining (green) for PLIN2, with a punctate cytoplasmic pattern, which was absent in the nonstimulated control cells. Fluorescent Nile Red-labeled LDs were also visualized 3 h after MT-II-induced stimulation and were virtually absent in nonstimulated control macrophages. After stimulation with MT-II, cytoplasmic-stained PLIN2 matched perfectly with Nile

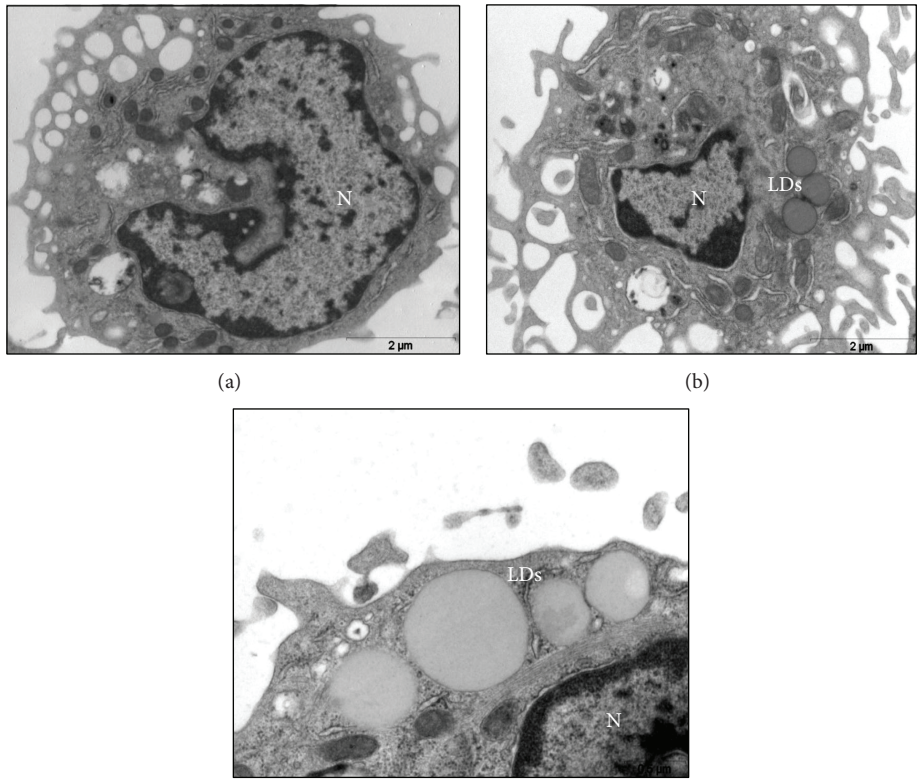

Figure 3: MT-II-induced LDs are morphologically distinct cytoplasmic sites. (a) Control macrophages with typical morphology. (b) and (c) Macrophages incubated with MT-II (0.8 μM) for 1 h showing light and large cytoplasmic lipid droplets (LDs). Also, an enlarged ER can be observed in MT-II-stimulated cells. N: nucleus.

Red-marked, neutral lipid inclusions. As expected, no significant staining was detected in control macrophages.

3.8. Involvement of Intracellular PLA2s in MT-II-Induced LD Formation in Macrophages. Since a cross-talk between $sPLA_2$ and intracellular PLA_2s for production of prostaglandins has been described, we examined the effects of selective inhibitors of $cPLA_2$(Pyr-2) or $iPLA_2$ (BEL) on MT-II induced formation of LDs. As shown in Figure 8, treatment of macrophages with BEL, but not with Pyr-2 compound caused 51% reduction in the number of LDs in MT-II-stimulated macrophages compared with vehicle-treated cells stimulated with MT-II. These results indicate that $iPLA_2$, but not $cPLA_2$ is involved in MT-II-induced LD biogenesis.

3.9. PGE_2 Colocalizes within LD Induced by MT-II-Stimulated Macrophages. Under inflammatory conditions, lipid mediators are mainly produced within LBs, which compartmentalize both substrate and the enzymatic machinery required for eicosanoid production [28]. Considering that PGE_2 is the major prostaglandin produced in macrophages, we evaluated the subcellular localization of PGE_2 within MT-II-stimulated macrophages. As illustrated in Figure 9 immunofluorescence microscopy revealed that macrophages stimulated with MT-II (0.8 μM) for 3 h exhibited strong fluorescent staining (green) for PGE_2, with a punctate cytoplasmic pattern, which was diffuse in the nonstimulated control cells. Fluorescent Nile Red-labeled LBs were also visualized 3 h after MT-II-induced stimulation and were virtually absent in non-stimulated control macrophages. Overlapping images show that after stimulation with MT-II, cytoplasmic-stained PGE_2 matched perfectly with Nile Red-marked, neutral lipid inclusions. As expected, no significant staining was detected in control macrophages.

4. Discussion

Besides myotoxic activity, MT-II, a Lys49PLA_2 homologue, has been shown to activate some cellular processes in macrophages at noncytotoxic concentrations [4, 5], and these effects may contribute to the overall tissue alterations caused by this toxin. Upon inflammatory conditions, macrophages show increased numbers of cytoplasmic LDs, which have been implicated as key organelles involved in immunity and inflammation.

In this study, we showed that MT-II, a catalytically inactive PLA_2 homologue, was able to directly induce an increase in the numbers of LDs in isolated murine macrophages. This phenomenon was time dependent and had a very fast onset and persisted up to 24 h after stimulation. Within 1 h of MT-II stimulus the presence of weakly osmiophilic LDs, in close association with organelles such as endoplasmic reticulum,

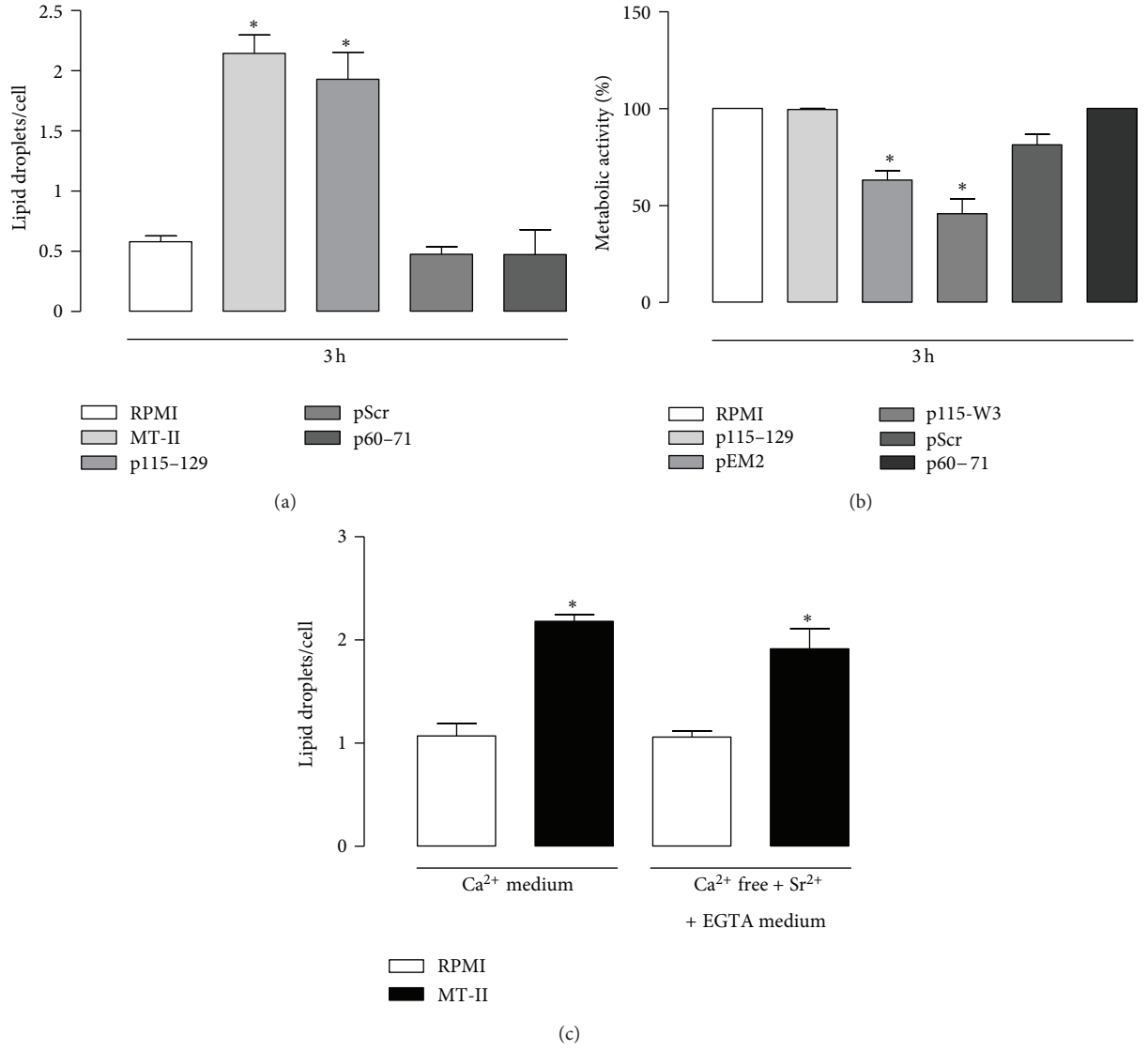

Figure 4: Effects of peptides corresponding to selected regions of MT-II molecule on LD formation. (a) Peritoneal macrophages were incubated with p115-129 (250 μg/mL) or pScr (250 μg/mL) or p60–71 (250 μg/mL) or RPMI (control) for 3 h. LDs were quantified using light microscopy after osmium staining; (b) cells were incubated with p115–129 (250 μg/mL) or pScr (250 μg/mL) or p60–71 (250 μg/mL) or PEM2 (150 μg/mL) or p115–W3 (150 μg/mL) or RPMI (control) for 3 h, after which cytotoxicity was assessed by the tetrazolium-based (MTT) colorimetric assay; (c) effect of MT-II (0.8 μM) on formation of LDs in macrophages in a Ca^{2+} containing medium or Ca^{2+}-free, EGTA (200 μM)-Sr^{2+}-containing medium. LDs were quantified using light microscopy after osmium staining. Each bar represents the mean ± S.E.M. of the number of LDs/cell in 50 counted cells. Values represent means ± S.E.M. for three to five animals. $^{*}P < 0.05$ compared with control cells.

were evidenced by the ultrastructural analysis. According to the current model of LD biogenesis, these organelles arise from endoplasmic reticulum, where the enzymes that synthesize lipids reside [15, 29]. Therefore, endoplasmic reticulum may play a role in LDs biogenesis induced by MT-II.

Because LDs have been associated to regulated inflammatory mediator synthesis with roles in inflammatory and infectious conditions, and macrophages are central elements in the innate immune response it is plausible to consider that biogenesis of LDs induced by MT-II demonstrated herein represents an important mechanism by which this PLA_2 homologue displays an inflammatory response and leads to production and release of inflammatory mediators. Moreover, considering that basic PLA_2s comprise around 30% of *B. asper* venom [30], the fact that MT-II elicited a key inflammatory event in macrophages clearly indicates that this $sPLA_2$ homologue contributes to the local inflammatory response triggered by the whole venom.

In addition, our data demonstrated that the absence of Ca^{2+} and presence of Sr^{2+} in culture medium did not alter LDs formation induced by MT-II, confirming that

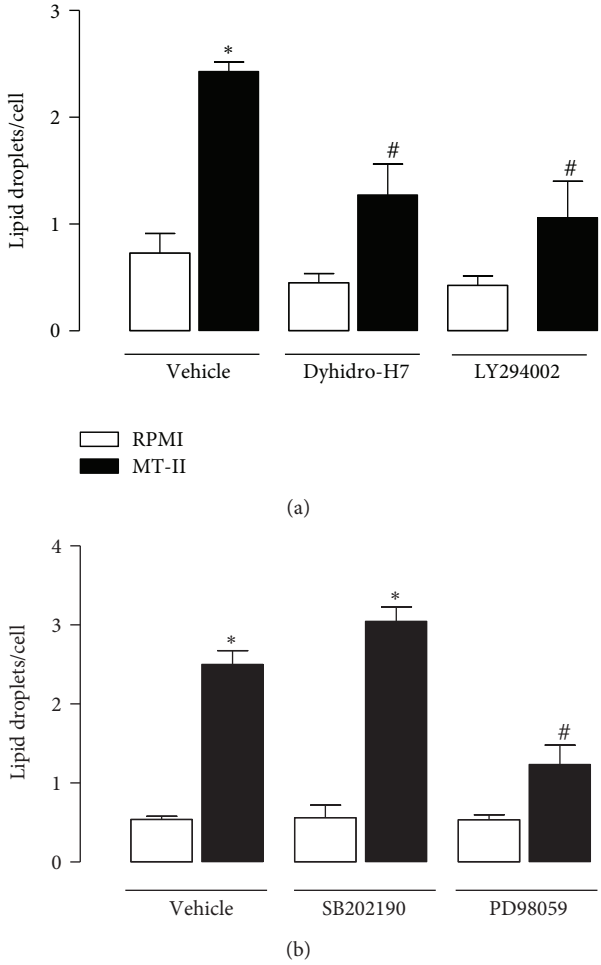

FIGURE 5: Signaling pathways involved in MT-II-induced LD formation. Peritoneal macrophages were incubated with one of the following: (a) PKC inhibitor H7 Dihidro (6 μM) for 1 h or the PI3 K inhibitor LY294002 (1 μM) for 1 h; (b) the p38MAPK or ERK1/2 inhibitors SB202190 (1 μM) or PD98059 (25 μM) for 1 h before stimulation with MT-II (0.8 μM) for 1 h. LDs were counted using light microscopy after osmium staining. Each bar represents the mean ± SEM of the number of LDs/cell in 50 counted cells. Values represent means ± SEM from three to five animals. $^{*}P < 0.05$ compared with control cells; $^{\#}P < 0.05$ compared with MT-II-stimulated cells.

the catalytic activity is not an essential requirement to enhancement of LDs biogenesis by MT-II. A number of experimental evidences suggested that a stretch of residues, located at the C-terminus of the MT-II protein molecule, and involving cationic and hydrophobic amino acids are responsible for myotoxic and cytotoxic effects of this [9, 31, 32] and other Lys-49sPLA$_2$ homologues [31, 33]. Based on these and other studies, a model of Lys49PLA$_2$s-membrane interaction was proposed by Lomonte et al. [9] in which the action of Lys49PLA$_2$s is based on the interaction of the C-terminal positive residues with membrane anionic phospholipids. So far, in the present study we found that the synthetic peptides 115–129 corresponding to MT-II C-terminus peptide induced LD formation in macrophages similarly to the parent protein. This finding indicates for the first time the specific region of MT-II molecule responsible for activation of macrophages, and gives support to the notion that the effects of MT-II in leukocytes are not related to the PLA$_2$ enzymatic activity. The membrane target(s) and the mechanisms by which this C-terminal peptide triggers macrophages activation to form LDs were not addressed in this study, although perturbation of the membrane phospholipid bilayer is likely to be involved.

Perilipin 2 is a protein ubiquitously expressed in a number of cell types, including macrophages, as a major component of intracellular LDs [34, 35]. It has fatty acid-binding properties, contributes to cytoplasmic trafficking of newly synthesized lipids, and plays an important role in assembly of LDs as well as in foam cell formation [35–37]. PLIN2 expression can be induced by a variety of inflammatory stimuli and has been associated with increased numbers of LD [26, 27]. Consistent with its properties, PLIN2 has been considered as a marker of LDs assembly and lipid loading in inflammatory cells, such as macrophages. Accordingly, our results showed that PLIN2 protein expression is upregulated by MT-II, given support to data demonstrating LD formation upon stimulus by this PLA$_2$ homologue. Furthermore, PLIN2 clustering co-localized to LDs was seen indicating that MT-II is also able to recruit this protein from its constitutive pools into LDs, and suggesting a role for PLIN2 as a nucleation site for the assembly of lipids to form new LDs under the stimulus of this Lys49PLA$_2$.

LD biogenesis in leukocytes is a highly regulated process. Studies of the intracellular signaling pathways committed to this process in leukocytes have revealed that distinct pathways can trigger LD biogenesis in a stimulus-dependent manner [38]. To better understand the stimulatory effects of MT-II on LD formation, we herein used pharmacological approaches to identify the critical downstream signaling proteins involved in LD formation induced by this PLA$_2$ homologue and focused on major downstream signaling molecules that have previously been shown to participate in LD biogenesis which follows inflammatory stimuli, such as PKC [39, 40], PI3 K [16, 26] and MAPKs (p38^{MAPK} and ERK1/2) [16, 41]. As a marked LD formation was observed after 3 h of incubation, the effects of pharmacological compounds were evaluated at this time interval. We found that MT-II-induced LD formation is regulated by specific signaling pathways and that PKC, PI3 K, ERK1/2, but not p38^{MAPK} are involved in the formation of LD induced by this PLA$_2$ homologue.

Our finding that macrophage activation by MT-II to form LDs is largely dependent on the PKC agrees with previous reports that PKC activation is implicated in LD formation induced by cys-fatty acid and PAF in leukocytes [39, 42]. Considering that activation of PKC has been associated with increased expression of PLIN2 in macrophages [43], it is possible to suggest that in the present experimental conditions, PKC signaling pathway is important to MT-II-induced up-regulation of PLIN2, and thus to the increased formation of LDs. Moreover, our observation that LDs formation induced by MT-II requires activation of the PI3 K pathway is in line

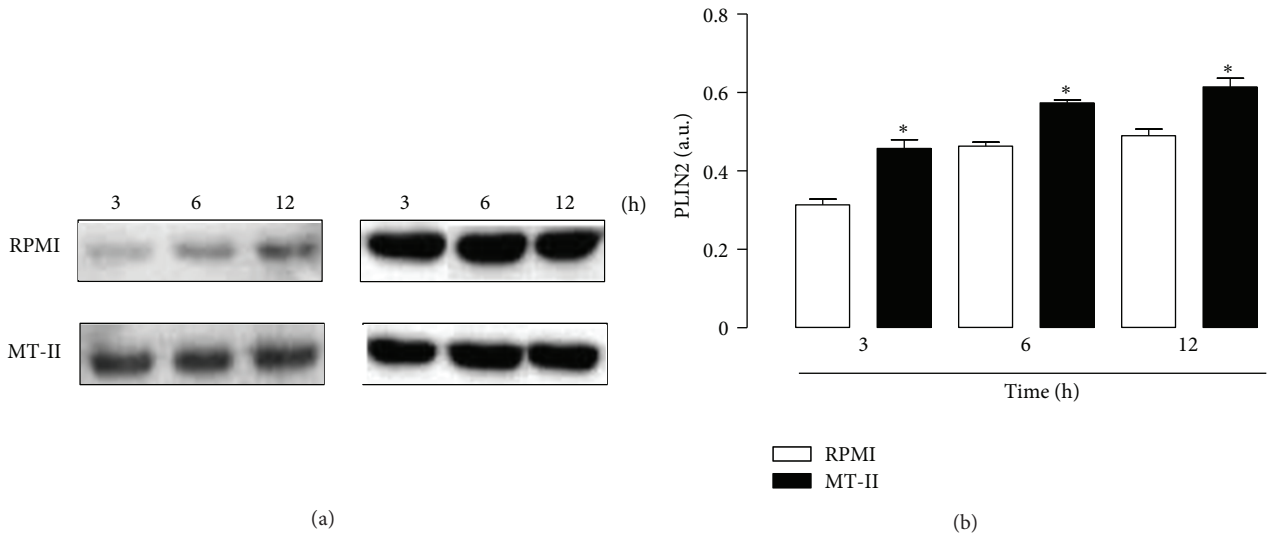

Figure 6: MT-II induces upregulation of PLIN2 expression in macrophages. Peritoneal macrophages were incubated with MT-II (0.8 μM) or RPMI (Control) for 3, 6, and 12 h. (a) Western blotting of PLIN2 and β-actin (loading control) in macrophage extracts. (b) Densitometric analysis of the band intensities of immunoreactive PLIN2. The densities (in arbitrary units) were normalized with those of β-actin. Results are expressed as mean ± S.E.M. from three experiments. $^{*}P < 0.05$ compared with controls.

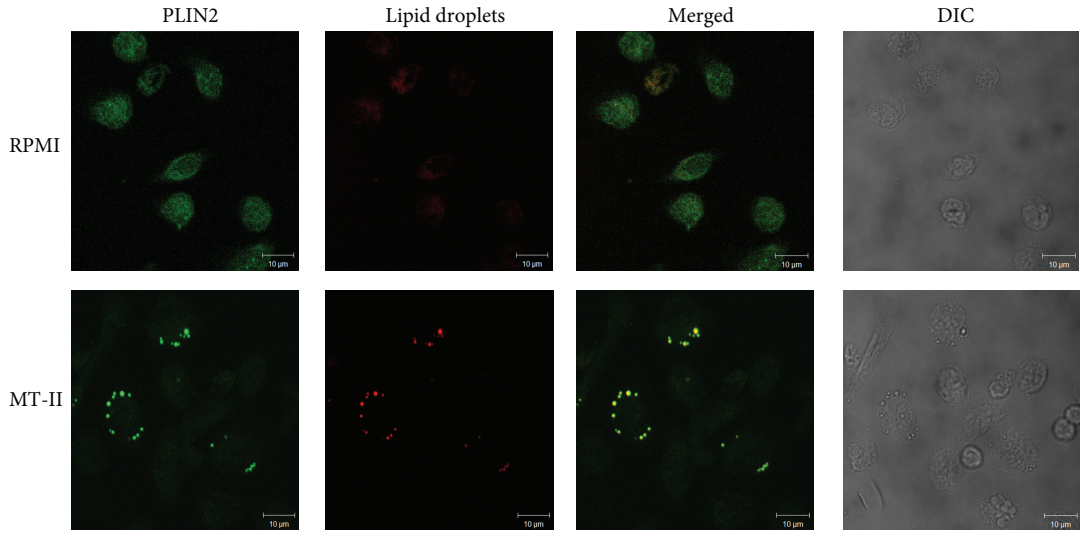

Figure 7: PLIN2 and LD colocalize in macrophages stimulated by MT-II. Macrophages incubated with RPMI (control) or MT-II (0.8 μM) for 3 h were labeled for LDs (fluorescent Nile Red) and for PLIN2 (FITC-conjugated immunocomplex). Merged image shows colocalization of PLIN2 to LDs. Cell nuclei are observed by DIC. The pictures are representative of three independent experiments.

with reports of participation of this signaling protein in processes related to lipid accumulation [26, 44, 45] and in the regulation of PLIN2 which has been largely associated to lipid accumulation into LDs, and to atherosclerosis [26, 45]. Furthermore, our results implicating ERK1/2 signaling in the MT-II effect that leads to LD formation in macrophages are consistent with previous studies demonstrating the involvement of ERK1/2 in LDs biogenesis induced by cytokines and saturated fatty acids in macrophages [16, 46]. Moreover, evidences of the involvement of ERK1/2 in regulation of PLIN2 expression and the growth of LDs [41] give support to our findings of increased protein expression of PLIN2 seen under MT-II stimulus, and is in line with the involvement of ERK1/2 in biogenesis of LDs induced by this PLA_2 homologue. Conversely, the specific inhibitor of $p38^{MAPK}$ failed to inhibit MT-II-induced LD formation, implying that this MAPK element does not contribute to this MT-II-induced effect. Taken together, the above results evidenced that MT-II-induced LD formation is a regulated process associated to activation of selected downstream signaling pathways in

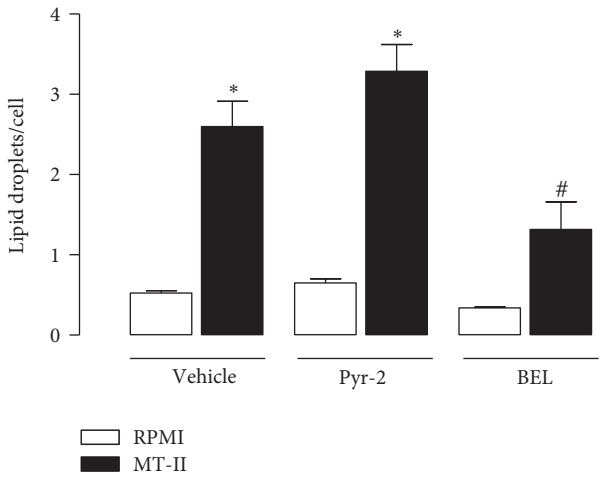

FIGURE 8: Effects of inhibitors of c $cPLA_2$ and $iPLA_2$ on MT-II induced LDs formation. Peritoneal macrophages were incubated with Pyr-2 (1 μM) or BEL (2 μM) compounds for 30 min and then with MT-II (0.8 μM) for 3 h. LDs were quantified using light microscopy after osmium staining. Each bar represents the mean ± sem LDs/cell in 50 counted cells. Values represent means ± SEM from 3–5 animals. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with MT-II-stimulated cells.

macrophages. Of note, despite the lack of enzyme activity, MT-II triggers signaling pathways almost similar to those that signal increased formation of LDs induced by the catalytically active $sPLA_2$ MT-III in macrophages [22], thus providing an additional evidence of functional similarities between these two venom $sPLA_2$variants.

It has been demonstrated that LDs are involved in production of inflammatory mediators [28] and to act as platforms for enhanced PGE_2 synthesis during infection conditions [47, 48]. Moreover, a number of enzymes and signaling proteins were shown to be associated with LDs, including the prostaglandin-forming enzymes named cyclooxygenases [47]. Our findings that MT-II caused an increase of PGE_2 intracellular pools, which colocalized to LDs in macrophages represent the first evidence that a $sPLA_2$ homologue is able to induce synthesis and compartmentalization of a lipid mediator in LDs. These findings suggest that macrophage LD constitutes a relevant site for the synthesis and accumulation of eicosanoids under MT-II stimuli and may represent a rapid and alternative mechanism for PGE_2 production by which macrophages react to activation by this $sPLA_2$ homologue. Moreover, given the importance of PGE_2 in several inflammatory settings, due to its hyperalgesic and edematogenic properties [49, 50], it is reasonable to suggest that LD-derived PGE_2 may have implications for the inflammatory effects of MT-II. This hypothesis is in line with the view that LDs are dynamic organelles integrating lipid metabolism, inflammatory mediator production, membrane trafficking, and intracellular signaling [27, 47, 51].

A number of studies have demonstrated that secreted PLA_2s crosstalk with the intracellular PLA_2s ($cPLA_2$ and $iPLA_2$) to produce arachidonic acid- (AA-) derived inflammatory mediators, such as prostaglandins, in several pathophysiological conditions [52, 53]. $cPLA_2$ is recognized as a key regulator of stimulus-coupled cellular AA release [6, 54]. The $iPLA_2$ in turn has no substrate specificity for the fatty acid residue at *sn-2* position, playing a minor role in eicosanoid synthesis. This intracellular enzyme, however, has a role in membrane phospholipid remodeling through deacylation/reacylation reactions [55]. In this context, $cPLA_2$ and $iPLA_2$ were demonstrated to be involved in LD biogenesis induced by stress in CHO-K1 cells [56] and by the $Asp49PLA_2$ MT-III in macrophages [22]. Taking the above information into account we investigated the participation of both intracellular PLA_2 isoforms in MT-II-induced LD formation. We found that treatment of macrophages with, compound Pyr-2, a specific inhibitor of $cPLA_2$ failed to inhibit MT-II-induced effect, indicating that $cPLA_2$ is not required for LD formation under MT-II stimulus. This finding is in accordance with our observation that $p38^{MAPK}$ is not involved in LD formation induced by MT-II, as upstream $p38^{MAPK}$ is critical for phosphorylation and activation of $cPLA_2$ [57]. In contrast, inhibition of $iPLA_2$ by BEL compound significantly reduced LD formation, thus implying $iPLA_2$ in the mechanisms involved in MT-II-induced LD formation. This finding is supported by recent studies demonstrating participation of $iPLA_2$s in the metabolism of fatty acids and triacylglycerol formation, which are involved in LD formation [56]. We believe that our results are the first demonstration that a $sPLA_2$ devoid of catalytic activity recruits an intracellular PLA_2 ($iPLA_2$) to induce a cellular event, such as LD formation in macrophages. However, the cellular steps involved in such a protein crosstalk were not addressed in the present study and deserve further studies.

5. Conclusions

Taken together, our data show that the venom group IIA $sPLA_2$ homologue MT-II directly activates murine macrophages to form LDs by a mechanism independent on enzymatic activity. This effect is related to the C-terminal loop of the MT-II molecule since a synthetic peptide corresponding to region 115–129-induced LD formation similarly to MT-II. Moreover, MT-II-induced LD formation is related to increased expression and recruitment of PLIN2 from its constitutive pools and regulated by distinct signaling pathways that include PKC, PI3 K, ERK1/2, and $iPLA_2$. In addition, MT-II induced synthesis and compartmentalization of PGE_2 within LDs. Therefore, LDs may represent and important platform for the synthesis and accumulation of lipid mediators under MT-II stimulus that takes place in the mechanisms whereby this $Lys49PLA_2$ triggers inflammation.

Finally, considering that catalytically inactive PLA_2s have been also described in mammalian tissues under normal and pathological conditions [58, 59], this study may shed light on the possible activities of similar proteins on a more general scope, providing insights into the possible roles of human catalytically inactive PLA_2 homologues in

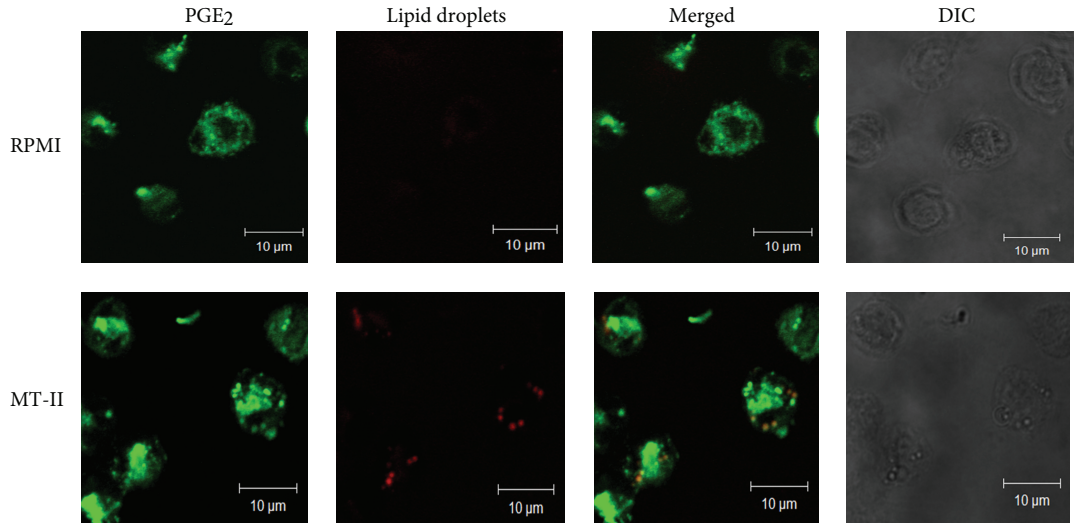

Figure 9: Cytoplasmic lipid droplets compartmentalize PGE_2. Macrophages incubated with RPMI (control) or MT-II (0.8 μM) for 3 h were labeled for LBs (fluorescent Nile Red) and for PGE_2 (Cayman Chemical). Merged image shows colocalization of PGE_2 to LDs. Cell nuclei are observed by DIC. The pictures are representative of three independent experiments.

inflammatory conditions as it has been demonstrated for the snake venom Lys49PLA_2.

Authors' Contribution

K. C. Giannotti and E. Leiguez contributed equally to this work.

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Research Article

Biochemical Characterization, Action on Macrophages, and Superoxide Anion Production of Four Basic Phospholipases A_2 from Panamanian *Bothrops asper* Snake Venom

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Bothrops asper (Squamata: Viperidae) is the most important venomous snake in Central America, being responsible for the majority of snakebite accidents. Four basic PLA_2s (pMTX-I to -IV) were purified from crude venom by a single-step chromatography using a CM-Sepharose ion-exchange column (1.5 × 15 cm). Analysis of the N-terminal sequence demonstrated that pMTX-I and III belong to the catalytically active Asp49 phospholipase A_2 subclass, whereas pMTX-II and IV belong to the enzymatically inactive Lys49 PLA_2s-like subclass. The PLA_2s isolated from Panama *Bothrops asper* venom (pMTX-I, II, III, and IV) are able to induce myotoxic activity, inflammatory reaction mainly leukocyte migration to the muscle, and induce J774A.1 macrophages activation to start phagocytic activity and superoxide production.

1. Introduction

Envenomation resulted from snakebites is the cause of considerable morbidity and mortality in many tropical and subtropical countries, being an important public health problem [1, 2]. According to Kasturiratne et al. [2], the estimative number of deaths per year due to snakebites in 2007 for Central America ranged from 193 to 1,461. Since snakebites affect, in most cases, poor people living in rural parts of tropical countries [3], the World Health Organization (WHO), incorporated snakebite envenoming in its list of neglected diseases (http://www.who.int/neglected_diseases/diseases/en/).

Lance-headed pit vipers belonging the Viperidae family, especially the *Bothrops asper* snake (Figure 1) are responsible for the most severe cases of snakebite envenoming, being the main cause of the largest numbers of bites and fatalities in Central America [1]. Among the American countries, Panama has the highest incidence of snakebite cases, showing an average number of 40 to 65 cases per 100,000 population per year and a estimated total number of 1,300 to 1,800 cases per year [4, 5], which *B. asper* is responsible for 90% of all

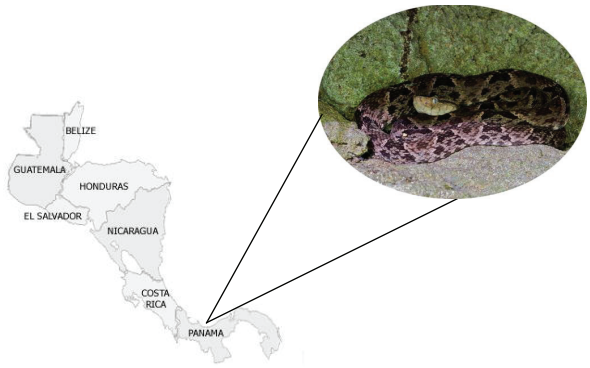


Figure 1: *Bothrops asper* resting in limestone cave, Central Panama.

snakebites cases of major clinical importance [4, 6]. *B. asper* is able to inoculate a relative large amount of venom and is considered extreme aggressive, being able to cause severe accidents [7].

According to Gutiérrez et al. [5], the envenomation produced by *B. asper* induces prominent local tissue damage, characterized by swelling, blistering, prominent oedema, haemorrhage, dermonecrosis, and myonecrosis with clinical manifestations that include bleeding, effects on platelet aggregation, coagulopathy, hypotension, hemodynamic alterations, pulmonary oedema, and acute renal failure. Other less common effects include intravascular haemolysis, acute myocardial damage multiple organ failure, and death. The clinical features of the envenomation are affected by the venom components, which vary according to snake species, geographic region, age, sex, and environment [5, 8, 9].

Snake venoms are characterized as a complex mixture of bioactive molecules, which proteins compose more than 90% of the venom dry weight [10–12]. Many of these proteins are enzymes, in which the most abundant are phospholipases A_2 (PLA_2s; E.C.3.1.1.4) [10]. PLA_2s are members of a protein superfamily that comprise several groups of enzymes with different catalytic mechanisms, as well as different functional and structural features, that cleavage the sn-2 acyl ester bond of glycerolphospholipids producing free fatty acids and lysophospholipids [13, 14]. Snake venom PLA_2s ($svPLA_2$s) have been grouped into four classifications according to minor structural differences as group I and II, both subclassified as type A or B. The group II is found in venoms from Viperidae family, while the group I is found in Elapidae and Hydrophiidae venoms [14]. $svPLA_2$s from Viperidae family are placed into group IIB and are mainly subdivided in two types: Asp49 PLA_2s, which have an Asp residue at position 49, and Lys49 PLA_2s, showing a Lys residue at position 49. Different from Asp49 PLA_2s, Lys49 PLA_2s have low or any catalytic activity upon artificial substrates [13, 15, 16].

This present paper describes the biochemical and toxicological characterization of crude *B. asper* venom from Panama, and the isolation, purification, and biochemical characterization of four basic cytotoxic PLA_2 from this venom and its effects on gastrocnemius muscle and inflammation.

2. Materials and Methods

2.1. Materials. Bothrops asper snake venom was collected from adult specimens, captured in Caldera and Gomez (Provence of Chiriquí, Panama) and in Arraiján (Provence of Panama, Panama). The snakes were maintained in a serpentarium at the Gamboa Rainforest Resort, Panamá, where the crude venom was obtained by inducing the snake to bite a parafilm-wrapped jar. Venoms were centrifuged at 1,000 xg for 15 min, and supernatants were lyophilized and stored at −20°C in Microbiology Department at the Medicine Faculty of Panama University until used. Male albino Swiss mice, weighing 18–22 g, were used for the assays.

The murine macrophage cell lines (J774A.1) were obtained from Rio de Janeiro Cell Bank Collection (Brazil). RPMI-1640, penicillin, streptomycin, and L-glutamine were purchased from Sigma-Aldrich (MO, USA); fetal bovine serum (FBS) was from Cultilab (Brazil). All reagents were low endotoxin or endotoxin-free grades. Animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and was approved by the Committee for Ethics in Animals Utilization of Universidade de São Paulo (CEUA no. 06.1.291.53.3). CM-Sepharose and Phenyl-Sepharose resins were purchased from Amersham Biosciences, Uppsala, Sweden. The Kit CK-UV-Kinetic was purchased from Bioclin, Brazil. The following reagents: ethylenediaminetetraacetic acid (EDTA), molecular weight protein standards, and acrylamide were obtained from Sigma Chemical Co. All other chemicals reagents were of analytical grade from Merck, Aldrich or Pharmacia Biotech.

2.2. Purification and Biochemical Characterization of PLA_2s. B. asper crude venom (300 mg) was dissolved in 1.5 mL of 0.05 M ammonium bicarbonate buffer, pH 8.1 and applied on a CM-Sepharose column (1.5 × 15 cm) according to Soares et al. [17]. All fractions were analyzed by SDS-PAGE, and PLA_2 activity was evaluated *in vitro* by indirect erythrocyte lysis in agar containing human erythrocytes and egg yolk, as previously described [18], being the fraction with PLA_2 activity selected. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) [19]. Isoelectric focusing was performed according to previously described. Buffalyte, pH range 3.0–9.0 (Pierce, IL), was used to generate the pH gradient. To determinate the protein concentration, the microbiuret method was used. The mol. wt of PLA_2s was estimated by mass spectrometry (Quattro II, Micromass). A Procise-491 (Applied Biosystems) automatic sequencer was used for the N-terminal sequencing [17]. The phenylthiohydantoin (PTH) amino acids were identified by comparing their retention times with the 20 PTH-amino acid standard mixture. The peptides obtained were compared with the sequences of other related proteins in the SWISS-PROT/TrEMBL databases using the FASTA and BLAST tools.

2.3. Biological and Pharmacological Characterization

2.3.1. Lethality. Groups of four mice (18–22 g) were injected by IP route with various amounts of crude venom (in a

volume of 0.1 mL) eluted with PBS, (phosphate buffered saline, 0.12 M NaCl, 0.04 M Na_2HPO_4, and pH 7.2). Deaths were recorded at 1, 3, 6, 12, 24, and 48 hours. LD_{50} was calculated using the Spearman-Karber method [20].

2.3.2. Edema-Inducing Activity. Groups of four male Swiss mice (18–22 g) were injected in the subplantar region with various amounts of crude venom (in a volume of 50 μL) prepared with PBS, pH 7.2. Then, the paw increase was measured at different time intervals (30, 60, 120, and 180 min), subtracting the initial paw measure (time 0 h). The paw edema was measured with the aid of a low-pressure pachymeter (Mitutoyo, Japan).

2.3.3. Hemorrhage. Groups of four male Swiss mice (18–22 g) were injected by ID route in the dorsal region with various amounts of crude venom (in a volume of 50 μL) prepared in PBS, pH 7.2. The hemorrhagic activity of the venom was determined as follows. Different doses of venom were injected intradermally, in a volume of 0.1 mL, into groups of four mice (18–22 g); 2 h later, they were sacrificed with CO_2, their skin removed, and the area of the hemorrhagic spot was measured. Diameters were calculated, and the minimum hemorrhagic dose was defined as the dose of venom, which induced a lesion of 10 mm diameter [21].

2.3.4. Coagulant Effect. Platelet-poor plasma was obtained from rabbit citrated blood by centrifuging the plasma twice at 2,500 xg for 15 min at 4°C. Aliquots of 0.5 mL of platelet-poor plasma were incubated with various amounts of crude venom (dissolved in 100 μL of PBS, pH 7.2). Incubation was carried out for 5 min at 37°C. Then, 0.1 mL of 0.25 M $CaCL_2$ was added to each tube, and they were checked for the formation of a clot every 30 seconds for a total period of 2 h. All experiments were carried out in triplicate.

2.3.5. Fibrinolytic Activity. The fibrinolytic activity was measured using 0.6% bovine plasminogen-free fibrin plates [22]. For this purpose, 30 μL of sample was placed on a fibrin plate, and the lysis area was measured after incubation at 37°C for 18 h. PBS was used as negative control. The specific activity was calculated from a standard curve for the lysis area obtained with plasmin on the plasminogen-free fibrin plates. All experiments were carried out in triplicate.

2.3.6. PLA_2 Activity. It was determined by incubating 0.5 mL of crude venom solution (at various amounts) with 50 μL of egg yolk diluted 1 : 5 with 0.1 M Tris, 10 mM $CaCl_2$, and pH 8.5 buffer containing 1% Triton X-100. Incubations were carried out for 10 min at 37°C. The liberated free fatty acids were extracted and titrated according to the method of Dole [23]. Crude venom from *Bothrops asper* (10 μg) and PBS were used as positive and negative controls, respectively. All experiments were carried out in triplicate.

2.3.7. Myotoxic Activity. Groups of four male Swiss mice (18–22 g) were injected in the right gastrocnemius muscle with crude venom (50 μg/50 μL of PBS), MTXs (50 μg/50 μL of PBS), or PBS alone (50 μL). After 3 h, blood was collected from the tail in heparinized capillary tubes and centrifuged for plasma separation. Activity of creatine kinase (CK) was then determined using 4 μL of plasma, which was incubated for 3 min at 37°C with 1.0 mL of the reagent according to the kinetic CK-UV protocol from Bioclin, Brazil. The activity was expressed in U/L, where one unit corresponds to the production of 1 mmol of NADH per minute.

2.3.8. Histological Examination of Myonecrosis. Myotoxic activity was assayed on the basis of the morphologic alterations induced by IM injections of crude venom or MTXs (50 μg) and negative control PBS (50 μL) in the right gastrocnemius muscle of Swiss mice (18–22 g, $n = 4$). After 24 h, the animals were euthanized with CO_2, and a small section of the central region of the muscle was excised and soaked in fixing solution (10% formaldehyde in PBS, v/v). The material was then dehydrated by increasing concentrations of ethanol and processed for inclusion in paraffin. The resulting blocks were sliced in 2.5 μm thick sections, stained with 0.25% (w/v) hematoxylin-eosin and examined under a light microscope [17].

2.3.9. Cytotoxic Assay. Cell viability was measured by Trypan blue exclusion. In brief, monolayers of J774A.1 cells grown in RPMI-1640 medium with 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine were withdrawn and after counting 2×10^5 cells/80 μL were added to plastic vials and incubated with 20 μL of different concentrations of pMTX-I, II, and III (1.5, 3 and 6 μg/mL) diluted in RPMI (control), for 1 h at 37°C in a humidified atmosphere (5% CO_2). Then, 20 μL 0.1% Trypan blue was added to 100 μL of J774A.1 macrophage suspension. Viable cell index was determined in a Neubauer's chamber by counting a total number of 100 cells. Results were expressed as percentage of viable cells.

2.3.10. Colorimetric NBT Assay. The colorimetric NBT assay was conducted in J744A.1 cells. In this assay, the generation of superoxide was estimated by reducing nitroblue tetrazolium (NBT), a yellow liposoluble compound that becomes insoluble and blue in its reduced form [24]. For this test, the cells J774A.1 had their concentration adjusted to 2×10^5/100 μL and were incubated with 100 μL of RPMI containing NBT 0.1% (control) or 100 μL of 2×10^6 zymosan particles suspension diluted in RPMI containing NBT 0.1% (positive control) or 100 μL of different concentrations of pMTX-I, II, and III (1.5, 3 and 6 μg/mL) diluted in RPMI containing NBT 0.1%, and incubated for 1 h at 37°C in humidified atmosphere (5% CO_2). At the end of the incubation period, the vials were centrifugated for 30 seconds at 4,500 xg, and the cells were washed twice with warm PBS. The NBT reduced deposited inside the cells were then dissolved, first by adding 120 μL of 2 M KOH to solubilize cell membranes and then by adding 140 μL of DMSO to dissolve blue formazan with gentle shaking for 10 min at room temperature. The dissolved NBT solution was then transferred to a 96-well plate

and absorbance was read on a microplate reader at 620 nm. Data were expressed as absorbance.

2.3.11. Phagocytic Activity of J774A.1 Cells of Nonopsonized Zymosan. J774A.1 cells were plated on 13 mm diameter glass coverslips (Glass Tecnica, Brazil) in 24-well plates at a density of 2×10^5 cells per coverslip and allowed to attach for 2 h at 37°C under a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with PBS. Cell monolayers were cultured for 1 h with RPMI supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2, and then challenged with RPMI (control) or 6 μg/mL of pMTX-I, II, and III diluted in RPMI. After washing in cold PBS, the monolayers were incubated for 1 h at 37°C and 5% CO_2 with nonopsonized zymosan, prepared as described below, and unbound particles were removed by washing with PBS. Cells were fixed with 2.5% glutaraldehyde for 15 min at room temperature, and the coverslips were mounted in microscope slides. The extent of phagocytosis was quantified by contrast phase microscopic observation. At least 200 macrophages were counted in each determination, and those containing three or more internalized particles were considered positive for phagocytosis [25, 26]. Results were presented as the percentage of cells positive for phagocytosis.

The zymosan particles, obtained from yeast cell walls, were suspended in PBS providing a concentration of 3 mg/mL. After that, the zymosan suspension was sonicated for 15 min, and total zymosan particles were determined in a Neubauer's chamber. The ratio of zymosan per macrophage was 1 : 10.

2.4. Statistical Analysis. Results are presented as mean ± S.D. obtained with the indicated number of tests. The statistical significance of differences between groups was evaluated using t student test. A $0.1 < P < 0.05$ value was considered to indicate significance.

3. Results and Discussion

Panamanian *B. asper* snake venom induced hemorrhage, edema, myonecrosis, coagulation, and fibrinolytic activities *in vitro*, and lethality, also presenting PLA_2 activity evidenced by titrimetric and indirect hemolytic assays (Table 1). The toxicological profile was qualitatively similar to that previously described for *B. asper* from Costa Rica [12] and Guatemala [27]. The nature and biological properties of snake venom components are peculiar to each species [8], whereas the presence and concentration of several venom components could vary intraspecifically as a function of geographic distribution, age, sex, feeding, size, season, and the time elapsed between venom extraction [9, 11, 28]. These intraspecific variations have evident clinical and therapeutic implications and can affect the capacity of antivenoms to neutralize venoms from snakes of geographically separated populations [5, 11, 13, 29, 30].

Four basic svPLA_2s were highly purified in a single step purification using an ion-exchange chromatography performed on a CM-Sepharose column. The elution of absorbed proteins with a linear gradient of concentrated buffer resulted in seven fractions (Figure 2(a)), which fractions Ba-4 to Ba-7 were related to PLA_2s. Ba-4 and Ba-5 fraction were related to PLA_2 enzymatically active, whereas Ba-6 and Ba-7 were related to enzymatically inactive PLA_2. The purity degree of the isolated proteins was further demonstrated by SDS-PAGE, mass spectrometry, and N-terminal sequence (Figures 2 and 3) and named as pMTX-I, pMTX-III, pMTX-IV, and pMTX-II, respectively. Different from our study, others research groups have exhaustively purified PLA_2s from *Bothrops* venoms using different combinations of chromatographic methods: gel filtration, ion exchange, RP-HPLC, and affinity with antibodies and heparin [13, 31].

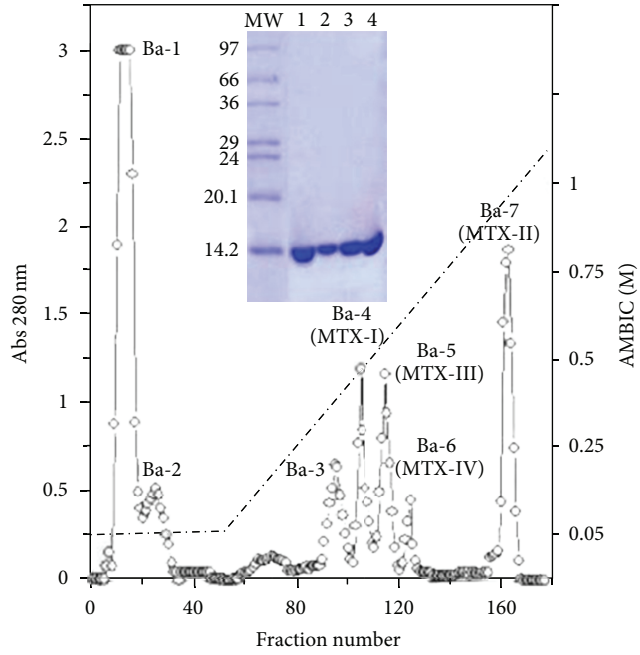
Table 1: Toxic activities induced by *B. asper* snake venom from Panama.

Effect[a]	Activity
Lethal (LD_{50}; μg/mouse)	55
Edema-inducing (DEM; μg)[ab]	1 ± 0.1
Hemorrhagic (MHD, μg)[ab]	1.2 ± 0.2
Coagulant (MCD; μg)[ab]	3.5 ± 0.05
Fibrinolytic (MFD; μg)	0.5
Indirect hemolitic (MHeD; μg)[ab]	7.2 ± 0.01
Phospholipase A_2 (mEq/mg·min)[ab]	27.2 ± 0.45
Myotoxic (MMD, μg)[ab]	10 ± 1

[a] Results are presented as mean ± SD. Except for lethality and fibrinolytic activity.
[b] All experiments were carried out in triplicate.

The purified proteins were characterized as single polypeptide chains, with an isoelectric point ranging from 8.1 to 8.3. The average molecular mass estimated by mass spectrometry was 14,156.5 for pMTX-I, 14,249.5 for pMTX-II, and 14,253.0 for pMTX-III (Figure 3). The N-terminal sequence alignment of pMTX-I and pMTX-III with MTICR myotoxic III PLA_2 (Uniprot accession no.: P20474) from Costa Rican *B. asper* showed, respectively, 96% and 88% of identity, and the sequence alignment of pMTX-II and pMTX-IV showed, respectively, 96% and 94% of identity with *B. asper* MTIICR myotoxic IV PLA_2 (Uniprot accession no.: P24605). Additionally, multiple sequence alignment of pMTX-I, II, III, and IV with Costa Rica *Bothrops* PLA_2s showed highly conserved amino acids, such as cysteine residues involved in disulfide bond formation. Several other conserved residues important to PLA_2 catalytic activity, such as the catalytic site ($D_{42}XCCXXHD_{49}$) and the calcium-binding site ($X_{27}CGXGG_{32}$) [13, 31] were shown. The N-terminal sequences (Figure 2(b)) of the isolated MTXs demonstrated that pMTX-I and III are basic PLA_2s with an aspartate residue at position 49 (Asp49), therefore catalytically active (Figure 4(a)), whereas MTX-II and IV are basic PLA_2s displaying a lysine residue at the same position (Lys49) (Figure 4(a)), therefore, catalytically inactive.

Panamanian *B. asper* crude venom, pMTX-I, II, III, and IV showed a high myotoxic activity (Figure 4(b)). Histopathological analysis revealed a drastic myonecrosis, displaying contracted and clumped fibers in different stages



(a)

MTICR	SLIEFAKMIL-EETKRLPFPYYTTYGCYCGWGGQGQPKDATDRCCFVHDCC	Identity (%)
MTXI	SLIEFAKMIL-EETKRLPFPYYTTTGCYCGWGGQGQPKDARDRCCFVHDCC	96%
MTXIII	SLIEFAKMIL-EETKKLPFPYYTTYGCNCGVGGRGKPKDATDRCCYVHDCC	88%
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MTIICR	SLFELGKMIL-QETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCC	
MTXII	SLFELGKMIL-QETGKNPAKSYGAYGCHCGVLGRGKPKDATDRCCYVHKCC	96%
MTXIV	SLVELGKMIL-QETGKNPVTSYGAYGCNCGVLGRGKPKDATDRCCYVHKCC	94%
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(b)

Figure 2: (a) Purification of myotoxins: ion exchange column of Panama *B. asper* venom (300 mg) on a CM-Sepharose column equilibrated with AMBIC 0.05 M pH 8.0 and eluted with a concentration gradient of AMBIC up to 1 M at a flow rate of 1.5 mL/minute. Inserted: SDS-PAGE 12%. Samples: MW (molecular weight markers); (1) pMTX-I (20 μg); (2) pMTX-II (20 μg); (3) pMTX-III (20 μg); (4) pMTX-IV (20 μg). (b) Comparison of the N-terminal amino acid sequence of phospholipases A_2 isolated from Panama *B. asper* venom: pMTX-I and III belong to the subgroup Asp49, whereas pMTX-II and IV belong to the subgroup Lys49 when compared with myotoxin III (P20474-PA21 BOTAS) Asp49 and myotoxin II (P24605-PA2H2 BOTAS) Lys49 from Costa Rica *B. asper* venom.

of degeneration and leukocyte infiltrate induced by myotoxins. Our results agree with Gutiérrez and Lomonte [32] and suggest that Lys49 myotoxins pMTX-II and pMTX-IV can affect the cell membrane of skeletal muscle fibers by a phospholipid hydrolysis independent mechanism. These results suggest that, moreover the catalytic site, this toxin may possess another molecular region that can bind and disorganize skeletal muscle plasma membrane [31, 32]. Some studies have suggested that myotoxic PLA_2s may induce muscle cell damage by affecting the integrity of plasmatic membranes, thereby leading to hyper contraction and other intracellular effects [13, 31, 32].

In order to evaluate the activation of leukocytes, the toxicity of *B. asper* myotoxins on macrophage J774A.1 cell line were studied. The cells were incubated with different concentrations of pMTX-II, III, and IV during 1 hour. These myotoxins did not affect the macrophage viability, which are in agreement with Zuliani et al. [25], showing their low toxicity on this cell type. Additionally, the effect of the same myotoxins on J774A.1 phagocytosis ability was evaluated via β-glucan receptor, by the uptake of nonopsonized zymosan particles incubated with noncytotoxic concentrations of pMTX-II, III, and IV was investigated. Our data showed that J774A.1 macrophages incubated with RPMI showed an average of phagocytosis of 16.5 ± 0.5%. Incubation of macrophages with pMTX-II, III, and IV, at 6 μg/mL, resulted in phagocytic indexes of 30.6 ± 0.6%, 29.3 ± 5.5%, and 36.5 ± 2.5%, respectively. These results showed that the myotoxins

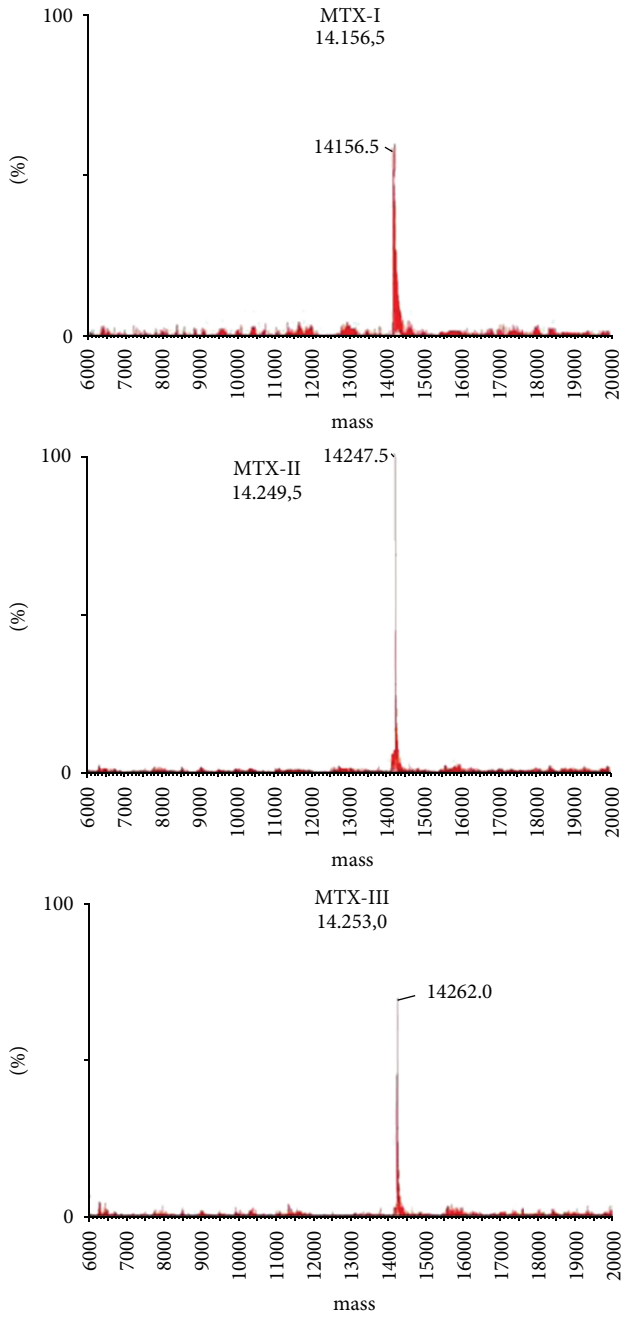

Figure 3: Analysis of mass spectra of PLA_2s isolated: pMTX-I, Mr = 14,156; pMTX-II, Mr = 14,249; pMTX-III, Mr = 14,253.

studied were able to stimulate phagocytosis of non-opsonized zymosan particles by J774A.1 macrophages (Figure 5(a)), which are in agreement with Zuliani et al. [25]. Moreover, these results suggest that phospholipid hydrolysis catalytic activity is not essential for the activity observed and argue with the hypothesis that other molecular regions distinct from the active site may be involved in this effect.

One of the most immediate responses of macrophages during phagocytosis is the production of the potent oxygen free radical, superoxide anion. The enzyme complex primarily responsible for the production of this highly reactive oxygen species is the NADPH oxidase complex [33]. This reaction parallels the release of a variety of inflammatory mediators that play crucial roles in the host defense by microbial killing, but may also cause injury to surrounding tissues [33–35]. In order to investigate the ability of pMTX-II, III, and IV to induce the production of superoxide by a macrophage cell line J774A.1, the cells were incubated with non-cytotoxic concentrations of myotoxins. As shown in Figure 5(b), J774A.1 macrophages incubated with RPMI (negative control) showed a superoxide production average of 0.316 ± 0.05 D.O., and J774A.1 incubated with RPMI plus non-opsonized zymosan (positive control) showed a superoxide production average of 0.455 ± 0.1 D.O. Incubation of macrophages with pMTX-II, III, and IV, at 3 and 6 μg/mL, respectively, induced a significant production of O_2^- in J774A.1 macrophages, showing that myotoxins are able to induce superoxide production by J774A.1 macrophages, indicating the ability of these toxins to activate these cells. Again, these results suggest that the PLA_2 catalytic activity is not important in macrophage activation. Thus, in accordance with our results, increments in hydrogen peroxide (H_2O_2), another reactive oxygen specie generated by a multicomponent enzyme system, NADPH-oxidase, have been described in thioglycollate-elicited macrophages incubated with MTX-II and III from Costa Rica *B. asper* venom [25].

It is important to note that phagocytosis mediated by β-glucan receptors and also by mannose and Fcγ receptors are coupled to the production of both proinflammatory and microbicidal molecules, such ROS [36, 37]. Release of ROS by phagocytic cells has been implicated in microbial killing [38] as well as in the damage to host surrounding tissue [39]. Considering that MT-II and MT-III isolated from *B. asper* venom from Costa Rica display a broad cytolytic activity and affect a variety of cell types in culture [40, 41], our findings suggest the role of O_2^- in the cytotoxicity induced by these myotoxins, a hypothesis that can be addressed with the use of antioxidant agents.

In conclusion, the Panamanian *B. asper* venom has qualitatively a similar toxicological profile to those previously described for *B. asper* from Costa Rica and Guatemala despite the observation of quantitative variations in these activities. The PLA_2s isolated from Panama *Bothrops asper* venom (pMTX-I, pMTX-II, pMTX-III, and pMTX-IV) induced myotoxic activity, inflammatory reaction mainly leukocyte migration to the muscle, activation of macrophages to exert phagocytic activity, and production of superoxide.

Conflict of Interests

The authors declare that there is no conflict of interests.

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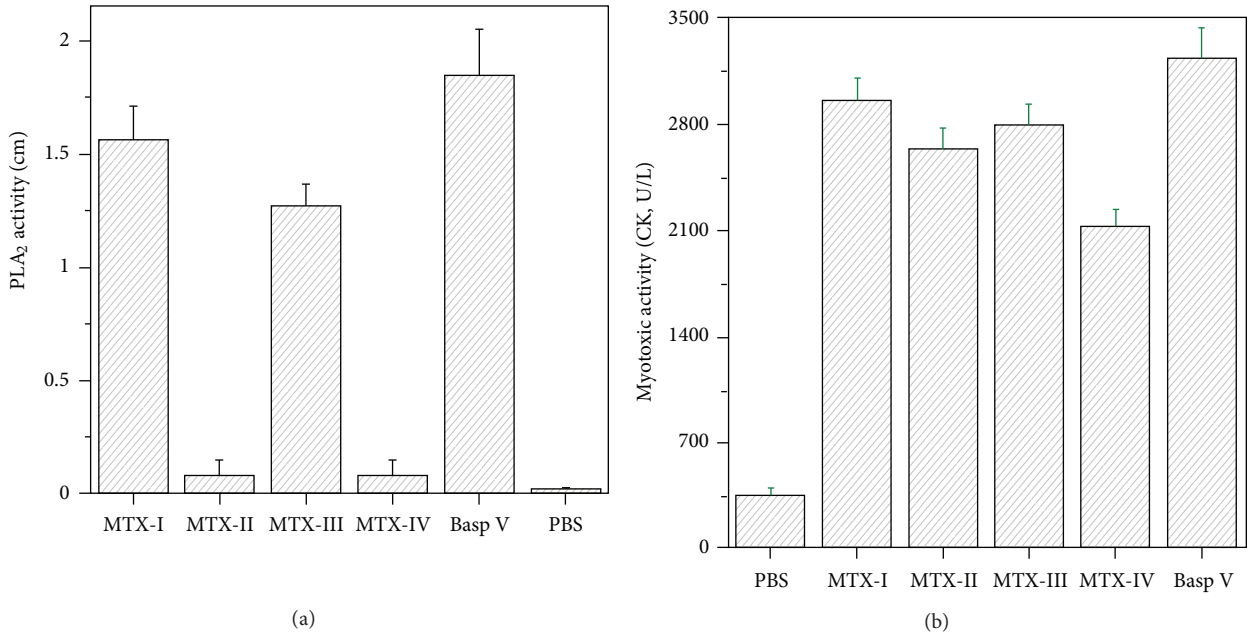

Figure 4: (a) Phospholipase activity (indirect hemolysis) of the isolated enzymes from Panama *B. asper* venom. Samples: pMTX-I (5 μg); pMTX-II (10 μg); pMTX-III (5 μg); pMTX-IV (10 μg). Negative control (PBS) and positive control (VBasp, *B. asper* venom, 10 μg); (b) myotoxic activity of the PLA_2s isolated from Panama *B. asper* venom. *Swiss* mice were injected with 50 μL of the different samples in the right gastrocnemius muscle and, after 3 h, the blood from the tail was collected in capillaries with heparine. Negative (PBS), positive control (BaspV, *B. asper* venom, 25 μg), and isolated myotoxins (50 μg) were used. Values represent the mean ± S.D. from 3 independent experiments.

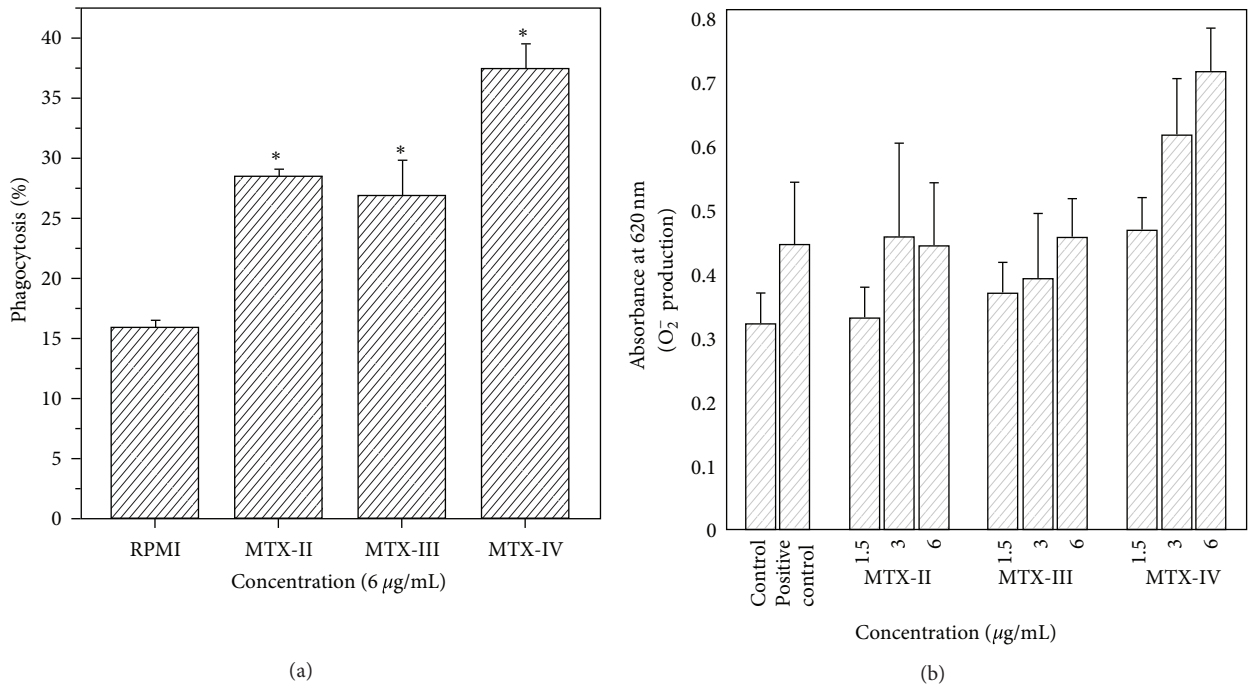

Figure 5: Effect of pMTX-II, pMTX-III, and pMTX-IV on phagocytosis (a) and O_2^- production (b) by J774A.1 macrophages. The phagocytosis of nonopsonized zymosan particles was determined by phase-contrast microscopy. J774A.1 macrophages were incubated with 6 μg/mL of pMTX-II, pMTX-III,pMTX-IV, and RPMI (control) during 60 minutes before addition of non-opsonized zymosan particles. Values represent the mean ± S.D.M. from 3–5 independent experiments. $^*P < 0.05$ compared with control $^\#P < 0.05$ compared with positive control (ANOVA).

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Research Article

Induction of Mast-Cell Accumulation by Promutoxin, an Arg-49 Phospholipase A_2

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Local inflammation is a prominent characteristic of snakebite wound, and snake-venom phospholipase A_2s (PLA_2s) are some of the main component that contribute to accumulation of inflammatory cells. However, the action of an R49 PLA_2s, promutoxin from *Protobothrops mucrosquamatus* venom, on mast-cell accumulation has not been previously examined. Using a mouse peritoneal model, we found that promutoxin can induce approximately-6-fold increase in mast-cell accumulation, and the response lasts at least for 16 h. The promutoxin-induced mast cell accumulation was inhibited by cyproheptadine, terfenadine, and Ginkgolide B, indicating that histamine and platelet-activating factor (PAF) is likely to contribute to the mast-cells accumulation. Preinjection of antibodies against adhesion molecules ICAM-1, CD18, CD11a, and L-selectin showed that ICAM-1, and CD18, CD11a are key adhesion molecules of promutoxin-induced mast-cell accumulation. In conclusion, promutoxin can induce accumulation of mast cells, which may contribute to snake-venom wound.

1. Introduction

snake-venom phospholipase A_2s (PLA_2s) are low-molecular-weight (13,000–14,000 Da), secretory phospholipases containing seven disulfide bonds. Usually, the PLA_2s from Crotalidae or Viperidae venom are divided into two major groups: the Asp-49 PLA_2s (D49 PLA2s) and Lys-49 PLA_2s (K49 PLA_2s), and several minor groups: Ser-49 PLA_2s (S49 PLA_2s) [1–3], Asn-49 PLA_2s (N49 PLA_2s) [4, 5], and Arg-49 PLA_2s (R49 PLA_2s) [6–8]. Besides the digestive function, snake PLA_2s exhibit several other pharmacological properties including antiplatelet [9, 10], anticoagulant [11], hemolytic [9], neurotoxic (presynaptic) [12], and myotoxic [13–15] properties. They have also been involved in various inflammatory processes such as edema, inflammatory cell infiltration, and mast-cell activation [15–20].

Mast cells are primarily located in mucosal and perivascular areas of various tissues, which play an important role in body-defense processes. Mast cells can be activated by snake-venom and release carboxypeptidase A and possibly other proteases, which can degrade venom components [21, 22]. Several snake-venom PLA_2s were reported to be able to activate the rat mast cells and to induce microvascular leakage and inflammatory-cell accumulation at the sites of inflammation [15–20]. Our previous studies showed that TM-N49, an N49 PLA_2 purified from *Protobothrops mucrosquamatus* venom, induces skin edema and mast-cell activation and accumulation [23], and promutoxin, an R49 PLA_2 purified from the same snake, can activate mast cells and induce skin edema [24]. Both TM-N49 and promutoxin are devoid of catalytic activity and are thought to contribute to *Protobothrops mucrosquamatus* venom toxicity [5, 8]. Moreover, both TM-N49 and promutoxin are potent stimuli for induction of neutrophil, lymphocyte, macrophage, and eosinophil accumulation in the mouse peritoneum [25]. These observations suggested that the two novel subgroups of group II PLA_2 may contribute to the inflammatory process caused by snake-venom poisoning. However, the ability of R49 PLA_2 on induction of mast-cell accumulation has not yet been reported. In the present study, we investigated the mechanisms of promutoxin-induced mast-cell accumulation.

2. Materials and Methods

2.1. Reagents. *Protobothrops mucrosquamatus* crude venom was obtained from the stock of the Kunming Institute of Zoology, the Chinese Academy of Sciences. SP-sephadex C-25, heparin sepharose (FF) and superdex 75 were obtained from LKB Pharmacia (Uppsala, Sweden). The following compounds were purchased from Sigma (St. Louis, USA): egg phosphatidyl choline, Triton X-100, trifluoroacetic acid, honey-bee venom phospholipase A_2, platelet-activating factor (PAF), cyproheptadine, and ginkgolide B. Quinacrine was obtained from calbiochem (San Diego, CA, USA). Reagents for sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories Inc. (Hercules, USA). Coomassie Plus assay kit was purchased from Pierce Chemical Co. (Rockford, IL, USA). Fetal-calf serum (FCS) and minimum essential medium (MEM) containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid (HEPES) were purchased from Gibco (Paisley, Renfrewshire, UK). Rat monoclonal antibodies, anti-mouse CD 11a lymphocyte function-associated antigen 1 (LFA-1) α chain, clone M17/4; anti-mouse CD 62L (L-selectin), clone MEL-14; anti-mouse CD18 (integrin β_2 chain), clone M18/2; rat IgG2a isotype standard, clone R35-95; hamster anti-mouse CD54 intercellular adhesion molecule 1 (ICAM-1) monoclonal antibody, clone 3E2; hamster IgG1 isotype standard, clone A19-3 were obtained from BD Biosciences Pharmigen (CA, USA). Hepes and all other chemicals were of analytical grade. BALB/c mice (20–25 g) were bred and reared under strict ethical conditions according to international recommendation.

2.2. Purification of Promutoxin. Promutoxin was isolated from *Protobothrops mucrosquamatus* crude venom following the procedures described previously [8]. Briefly, the lyophilized venom (1.5 g) was dissolved in 20 mL of 0.05 M sodium phosphate buffer (pH 5.8) before being loaded on an SP-Sephadex C-25 column. The absorbed proteins were eluted with a linear gradient (0–0.8 M NaCl). Peak 7 was collected and loaded on Superdex 75 column in 25 mM sodium phosphate buffer (pH 5.8 with 0.15 M NaCl). The main peak was collected, and then loaded on a reverse-phase C_{18} high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA). The main peak fraction, representing the purified promutoxin, was pooled, lyophilized, and stored at –20°C.

Protein concentration was determined by using a Coomassie Plus assay kit with BSA as standard. The PLA_2 activity was routinely assayed by a titration method using egg yolk as substrate [26] and by a colorimetric assay using L-phosphatidylcholine as substrate [27]. Honey-bee PLA_2 was employed as positive control.

2.3. Induction of Mast-Cell Accumulation. Various doses of promutoxin, BSA or normal saline were injected in 0.5 mL volumes into the peritoneum of male BALB/c mice, whose abdominal skin was swabbed with 70% ethanol, a group of 6 mice for each dose. This model was adapted from that described by Thomas and colleagues [28], which complied with the European Community guidelines for use of experimental animals. At 10 min, 2 h, 6 h, or 16 h following injection, animals were sacrificed by inhalation of carbon dioxide, and their peritoneal lavages were collected following a standardized procedure with 5 mL normal saline. After centrifugation at 500 g for 10 min at 4°C, supernatants were collected and stored at −40°C until use, and cells were resuspended in 1 mL of MEM. The total cell numbers were determined by enumerating them with an Improved Neubauer haemocytometer after being stained with 0.1% trypan blue. For the differential cell counting, cytocentrifuge preparations were made, air dried, and stained with modified Wright's stain. Differential cell counts were performed for a minimum of 500 cells. The results were expressed as absolute numbers of each cell type per mouse peritoneum.

For the experiments investigating mast-cell-migration mechanisms, groups of mice were pretreated intravenously (tail vein injection) with monoclonal antibodies against the adhesion molecules L-selectin, CD11a, CD18, and ICAM-1 (all at a dose of 1 mg·kg^{-1}) [29–31], respectively, for 30 min before intraperitoneal injection of 5 μg of promutoxin. Control animals received an equivalent dose of the corresponding normal rat or hamster IgG isotype control. At 6 h following injection, the mice were sacrificed and their peritoneal lavages were processed as described above.

To investigate potential mechanisms involved in promutoxin-induced mast-cell accumulation, several anti-inflammatory compounds including cyproheptadine (2 mg·kg^{-1}) [17], terfenadine (3 mg·kg^{-1}) [32, 33], ginkgolide B (5 mg·kg^{-1}) [34], and quinacrine (10 mg·kg^{-1}) [35] were coinjected into the peritoneum of mice with promutoxin (5 μg per mouse). Control animals received an injection of drug alone. At 6 h following injection, mice were sacrificed and their peritoneal lavages were processed as described above.

2.4. Statistical Analysis. Data are shown as mean ± SE for the number of experiments indicated, and ANOVA followed by Tukey's tests were used for statistical comparison of the data. In all analyses, $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Purification and Characterization of Promutoxin. Approximately 25 mg of promutoxin was obtained from 1.5 g *Protobothrops mucrosquamatus* crude venom following the procedures described above. The purity of the PLA_2 was at least 98% as assessed by SDS-PAGE, HPLC, and mass spectrometry analysis [24].

3.2. Induction of Mast-Cell Accumulation by Promutoxin. As early as 10 min following injection, 5 μg of promutoxin was able to induce significant mast-cell accumulation in the peritoneum of mice. The mast-cell accumulation appeared to last for 16 h (Figure 1). Approximately, up to 6-fold increase

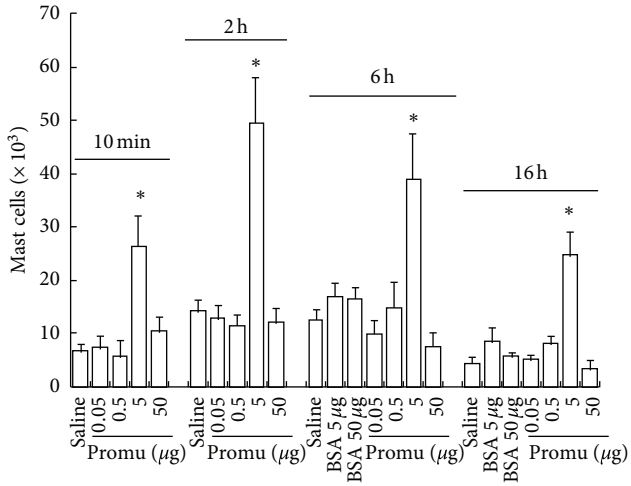

Figure 1: Effect of promutoxin (promu) on mast-cell numbers in mouse peritoneum. Various doses of promu were injected into the peritoneum of mice for 10 min, 2 h, 6 h, or 16 h. Also shown are the responses to BSA and normal saline control. The values shown are mean ± SE for 6 animals in each group. $^{*}P < 0.05$ compared with the response to the corresponding diluent-only control animals.

in mast-cell numbers was achieved at 16 h following injection (Figure 1).

3.3. Effects of Anti-Inflammatory Compounds and Blocking Antibodies on Mast-Cell Accumulation. When coinjected, ginkgolide B, cyproheptadine and terfenadine inhibited 35.9, 71.3, and 92.6% promutoxin-induced mast-cell accumulation in the peritoneum of mice, respectively. However, quinacrine did not significantly alter the extent of promutoxin-induced mast-cell accumulation. At the dose tested, ginkgolide B, cyproheptadine, terfenadine, and quinacrine by themselves failed to induce mast-cell accumulation in the peritoneum of mice (Table 1).

Intravenous injection of monoclonal antibodies against CD18, ICAM-1, and CD11a 30 min prior to intraperitoneal injection of the PLA_2-blocked promutoxin-induced mast-cell accumulation by 87.2, 76.7, and 53.8%, respectively. Monoclonal antibody against L-selectin failed to diminish promutoxin-induced mast-cell accumulation. Normal rat and hamster IgG-isotype controls tested had little effect on promutoxin-induced mast-cell accumulation (Table 2).

4. Discussion

It is found for the first time that promutoxin, a novel member of a minor subgroup (R49 PLA_2) of snake-venom group II PLA_2s, can induce mast-cell accumulation. The observation supports our previous finding that N49 PLA_2, another minor subgroup of snake-venom group II PLA_2s [23] can induce mast-cell accumulation. Obviously, promutoxin does not induce mast-cell accumulation in a concentration-dependent manner [24]. We previously found that promutoxin could activate mast-cells. The reduction of mast-cell

Table 1: The influence of anti-inflammatory compounds on promutoxin- (5 μg) induced mast-cell accumulation in mouse peritoneum.

Compound injected	Number of mast cells ($\times 10^3$)
Saline	14.6 (6.1–23)
Promutoxin	44.6 (21.4–60.8)
Ginkgolide B 5 mg·kg^{-1}	26.2 (9.1–41)
Ginkgolide B 5 mg·kg^{-1} + promutoxin	28.6 (13–50.2)*
Cyproheptadine 2 mg·kg^{-1}	6.2 (1.5–17.2)
Cyproheptadine 2 mg·kg^{-1} + promutoxin	12.8 (4.2–22.2)*
Terfenadine 2 mg·kg^{-1}	11.0 (5.8–20)
Terfenadine 2 mg·kg^{-1} + promutoxin	3.3 (1.6–6.5)*
Quinacrine 10 mg·kg^{-1}	11.5 (5.9–18.1)
Quinacrine 10 mg·kg^{-1} + promutoxin	36.8 (25.3–49.3)

The values shown are medians (range) for six separate experiments. Compounds were injected into the mouse peritoneum for 6 h before peritoneal lavage fluid was collected. $^{*}P < 0.05$ compared with the response to promutoxin alone.

Table 2: The influence of blocking antibodies (Ab) against cell-adhesion molecules on promutoxin- (5.0 μg) induced mast-cell accumulation in mouse peritoneum.

Compound injected	Number of mast cells ($\times 10^3$)
Saline	14.6 (6.1–23)
Promutoxin	44.6 (21.4–60.8)
L-selectin Ab + promutoxin	38.0 (18.6–69.0)
LFA-1 Ab + promutoxin	20.6 (9.1–40)*
CD18 Ab + promutoxin	5.7 (3.1–11.7)*
ICAM-1 Ab + promutoxin	10.4 (2.9–14.5)*
Hamster IgG1 + promutoxin	39.8 (17.0–63.1)
Rat IgG2a + promutoxin	43.3 (19.3–58.0)

The values shown are medians (range) for six separate experiments. Monoclonal antibodies (all at a dose of 1 mg·kg^{-1}) against the adhesion molecules L-selectin, LFA-1, CD18 and ICAM-1 were intravenously injected, respectively, for 30 min before intraperitoneal injection of 5 μg promutoxin for 6 h. $^{*}P < 0.05$ compared with the response to promutoxin alone.

number induced by promutoxin in higher doses may be due to the activation of accumulated mast-cells by promutoxin.

Ginkgolide B, a PAF antagonist, inhibited more than 35.9% promutoxin-induced mast-cell migration, indicating that PAF is likely involved in mast-cell migration by promutoxin. This result appears to agree with a previous work which found that PAF may contribute to mast-cell migration induced by TM-N49 [23]. Inhibition of 71.3 and 92.6% promutoxin-induced mast-cell accumulation by cyproheptadine, a histamine/5-HT antagonist, and terfenadine a selective histamine-H_1-receptor antagonist, implies that histamine is likely to be involved in the above event through its H_1 receptor. Indeed, we have found previously that promutoxin can activate mast cells to release histamine [24] and anticipate herein that released histamine then elicits mast-cell migration. The fact that terfenadine inhibited

promutoxin-provoked mast-cell recruitment to a greater extent than cyproheptadine further implies the selectivity of histamine receptor involved. It was found that snake-venom promutoxin could induce mast-cell accumulation even at 10 min following injection, but the number of lymphocyte, macrophage, eosinophil, and neutrophil migration was not altered at 10 min following injection of promutoxin [25]. It seemed the accumulation of neutrophils, lymphocyte, macrophage, and eosinophil induced by promutoxin could be a secondary event, in which accumulated mast cells are activated by promutoxin and release their chemoattractant factors such as serine proteinases [36, 37], histamine, and PAF to recruit inflammatory cells.

ICAM-1 appears to be a key adhesion molecule of promutoxin-induced mast-cell accumulation as an antibody against ICAM-1 blocked more than 76.7% of the influence of promutoxin on the cell migration. ICAM-1 involved in lymphocyte [38], macrophage [39], eosinophil [40, 41] and mast-cell accumulation [23] has been reported previously, which may support our current observations. An antibody against CD11a (LFA-1) blocked more than 53.8% promutoxin-induced mast-cell accumulation, indicating that CD11a plays an important role in the migration of mast cells, which is consistent with previous reports that TM-N49-induced mast-cell accumulation is mediated by CD18, CD11a, and ICAM-1 [23]. As expected, antibody against CD18 blocked more than 87.2% promutoxin-induced mast-cell accumulation in the present study. Though L-selectin is involved in the neutrophil and eosinophil accumulation provoked by promutoxin, it seemed that L-selectin is not involved in the promutoxin-induced mast-cell accumulation.

Promutoxin, as a novel member of minor subgroup of PLA_2, is an enzymatically inactive enzyme. It induced mast-cell accumulation via a PAF, and histamine H_1 receptor-dependent mechanism, and through a CD11a/CD18-and ICAM-1-associated adhesion pathway. Accumulated mast cells may contribute to snake-venom wound.

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Research Article

Chemical Modifications of PhTX-I Myotoxin from *Porthidium hyoprora* Snake Venom: Effects on Structural, Enzymatic, and Pharmacological Properties

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We recently described the isolation of a basic PLA_2 (PhTX-I) from *Porthidium hyoprora* snake venom. This toxin exhibits high catalytic activity, induces *in vivo* myotoxicity, moderates footpad edema, and causes *in vitro* neuromuscular blockade. Here, we describe the chemical modifications of specific amino acid residues (His, Tyr, Lys, and Trp), performed in PhTX-I, to study their effects on the structural, enzymatic, and pharmacological properties of this myotoxin. After chemical treatment, a single His, 4 Tyr, 7 Lys, and one Trp residues were modified. The secondary structure of the protein remained unchanged as measured by circular dichroism; however other results indicated the critical role played by Lys and Tyr residues in myotoxic, neurotoxic activities and mainly in the cytotoxicity displayed by PhTX-I. His residue and therefore catalytic activity of PhTX-I are relevant for edematogenic, neurotoxic, and myotoxic effects, but not for its cytotoxic activity. This dissociation observed between enzymatic activity and some pharmacological effects suggests that other molecular regions distinct from the catalytic site may also play a role in the toxic activities exerted by this myotoxin. Our observations supported the hypothesis that both the catalytic sites as the hypothetical pharmacological sites are relevant to the pharmacological profile of PhTX-I.

1. Introduction

Phospholipase A_2 (PLA_2; EC 3.1.1.4) enzymes catalyse the hydrolysis of acyl ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides at the *sn*-2 position, with a requirement for a Ca^{2+} [1]. PLA_2 enzymes from snake venom are quite fascinating from both biological and structural point of view. Despite their structure being conserved, they exhibit a wide range of pharmacological activities, including neurotoxicity [2], myotoxicity [3, 4], and cardiotoxicity [5] as well as anticoagulant, hemolytic [6], antiplatelet [7], hypotensive [8], hemorrhagic [9], and edema inducing effects [10]. However, not all the PLA_2 enzymes induce all these pharmacological effects. In general, an individual PLA_2 exhibits one or more specific pharmacological effects [11, 12].

Within the family Viperidae, two distinct types of venom PLA_2 molecules have been described, all of them sharing a high degree of homology both in primary and three-dimensional structure [13]: the "classical" PLA_2, that present an invariant Asp49 residue that plays a key role in catalysis; and the "PLA_2 homologues," devoid of enzymatic activity, that present the substitution of Asp49 by Lys49 or, less frequently, by Ser, Arg, Gln, or Asn [14, 15]. Despite their difference in catalysis, both the Asp49 and the Lys49 proteins are

able to induce various pharmacological effects [16]. Thus the structure-function relationship among this group of proteins is subtle and complicated [1]. In some cases, the pharmacological effects result from their enzymatic activities, probably through the action of the products of hydrolysis, lysophospholipids, and fatty acids, that alter cell membrane shape and permeability [17, 18] but for many of them, the pharmacological effects are independent of their enzymatic activities, such as Lys49 PLA_2 myotoxins, which lack hydrolytic activity and therefore act via another mechanism, which is only partially understood. A site close to the C-terminus, comprising a variable combination of basic and hydrophobic amino acids, has been identified as being responsible for toxicity [19].

In a previous work, we showed that *Porthidium hyoprora* snake venom is a rich source of PLA_2 enzymes. Additionally, we purified a myotoxic PLA_2 (PhTX-I) to homogeneity in reverse-phase HPLC, which constitutes of a single polypeptidic chain, has a molecular mass of 14.249 Da, and whose amino acid sequence exhibits high identity with other myotoxic Asp49 PLA_2 [20]. We also demonstrated that PhTX-I (20 μg/mL) caused edema, *in vivo* creatine kinase release, C2C12 skeletal muscle myoblasts cytotoxicity, and neuromuscular blockade of chick biventer cervicis muscle preparations. However, it is still unknown whether these pharmacological effects were mediated by the phospholipase catalytic activity of PhTX-I or not. One of the strategies employed for the elucidation of the relationship between catalytic activity and pharmacological effects of PLA_2 is based on the chemical modification of specific residues in these enzymes [21]. Using this approach, a dissociation of pharmacological effects and enzymatic activity for various PLA_2 has been observed, suggesting the presence of separate enzymatic and pharmacological active site(s) contained within their amino acid sequences [22, 23]. In the present study, we investigated the effects of chemical modifications of specific amino acid residues (His, Tyr, Lys, and Trp), performed in PhTX-I, on their enzymatic, structural, and pharmacological properties.

2. Material and Methods

2.1. Reagents. 2,4′-Dibromoacetophenone (BPB), 4-nitrobenzenesulfonyl fluoride (NBSF), 2-nitrobenzenesulfonyl chloride (NPSC), 4-nitro-3-(octanoyloxy) benzoic acid, and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA); acetic anhydride (AA) was from Merck.

2.2. Purification of PhTX-I. The PhTX-I PLA_2 from *Porthidium hyoprora* venom was purified by reverse phase HPLC [20]. Briefly, 5 mg of whole venom was dissolved in 200 μL of buffer A (0.1% TFA) and centrifuged at 4500 g; the supernatant was then applied to a μ-Bondapak C18 column (0.78 × 30 cm; Waters 991-PDA system), previously equilibrated with buffer A for 15 min. The elution of the protein was then conducted using a linear gradient (0%–100%, v/v) of buffer B (66.5% Acetonitrile in buffer A) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm of absorbance, and after elution the fraction was lyophilized and stored at −40°C.

2.3. Chemical Modifications. Modification of His residues with 2,4′-Dibromoacetophenone (BPB) was carried out as previously described [21]. Briefly, 3 mg of PhTX-I were dissolved in 1 mL of 0.1 M Tris-HCl containing 0.7 mM EDTA (pH 8.0) and 150 μL of BPB (1.5 mg/mL, in ethanol), and the mixture incubated for 24 h at 25°C. Modification of Lys residues with acetic anhydride (AA) was performed at a protein : reagent molar ratio of 1 : 50 [21]. PhTX-I (3 mg) was dissolved in 1.5 mL of 0.2 M Tris-HCl buffer at pH 8.0, and 10 μL of AA was added and the mixture was incubated for 1 h at 25°C. Tyr residues were modified by treatment with 4-nitrobenzenesulphonyl fluoride (NBSF) as previously described [24]. Briefly, 1 μmol of PhTX-I (10 μmol of Tyr) was dissolved in 14 mL of 0.1 M Tris-HCl (pH 8.0) and incubated with 10 μmol, of NBSF for 20 h at 25°C. Modification of Trp residues was performed according to Takasaki et al. [25]. Briefly, 9 mg of PhTX-I were dissolved in 4 mL 50% acetic acid containing 1 mg of 2-nitrobenzenesulfonyl chloride (NPSC) and incubated for 1 h at 25°C. In all cases, excess reagent was removed by ultrafiltration through a Millipore's Amicon Ultra-15 membrane and washed with distilled water, followed by lyophilization.

2.4. Amino Acid Analysis. Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described by Heinrikson and Meredith [26]. Native PhTX-I PLA_2 and their modified derived samples (30 μg) were hydrolyzed at 105°C for 24 h, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolysates were reacted with 20 μL of derivatization solution (ethanol : triethylamine : water : phenylisothiocyanate, 7 : 1 : 1 : 1, v/v) for 1 h at room temperature, after which the PTC amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture.

2.5. Electrophoresis. Native PhTX-I PLA_2 and their modified derivatives were examined by Tricine SDS-PAGE in a discontinuous gel and buffer system, under reducing and nonreducing conditions [27]. The molecular mass markers used were (in kDa): phosphorylase B—97.0, albumin—66.0, ovalbumin—45.0, carbonic anhydrase—30.0, soybean trypsin inhibitor—20.1, and α-lactalbumin—14.4.

2.6. Mass Spectrometry. An aliquot (4.5 μL) of the modified proteins was injected in C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. The gradient was 0%–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode, and the data acquisition was from m/z 100–3.000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data by the mass spectrometer on

the *m/z* scale were converted to the mass (molecular weight) scale using maximum entropy-based software supplied with Masslynx 4.1 software package. The processing parameters were output mass range 6.000–20.000 Da at a "resolution" of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da; the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.7. Circular Dichroism. Circular dichroism (CD) spectra of native PhTX-I PLA_2 and their modified derivatives were recorded with a JASCO model J-720-ORD 306 spectropolarimeter equipped with a thermoelectric sample temperature controller (Peltier system) following standard procedures previously described [28]. After centrifugation at 4000 g for 5 min, samples (1–4 μM protein in 10 mM sodium phosphate, pH 8) were transferred to a 10-mm path-length quartz cuvette. Circular dichroism spectra in the wavelength range 260 to 200 nm were collected, using a bandwidth of 1 nm and a response time of 1 s. Data collection was performed at 25°C with 50 nm/min scanning speed. At least ten scans were accumulated for each sample, and all spectra were corrected by subtraction of buffer blanks. The estimation of secondary structure elements was performed using the CDNN Deconvolution software (version 2.1), and Origin 7.5 (OriginLab) was used for graphics and analysis.

2.8. PLA_2 Activity. PLA_2 activity was measured using the assay described by Cho and Kezdy [29] and Holzer and Mackessy [30] modified for 96-well plates. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, and 100 mM NaCl, pH 8.0), 20 μL of substrate 4-nitro-3-(octanoyloxy) benzoic acid (3 mM), 20 μL of water, and 20 μL of PhTX-I PLA_2 or their modified derivatives (1 mg/mL) in a final volume of 260 μL. After adding proteins (20 μg) the mixture was incubated for up to 40 min at 37°C, measuring absorbance at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase of absorbance after 20 min. All assays were done in triplicate, and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

2.9. Inhibition. The inhibitory effects of EDTA or low molecular weight heparin from porcine intestinal (Mr 6.000 Da) on pharmacological and enzymatic activities of PhTX-I were assessed by incubating the enzyme with 1 mM solution of this chelating agent or a heparin : toxin molar ratio of 2 : 1 for 30 min at 37°C. The inhibition of PLA_2 activity of PhTX-I by crotapotins F2 and F3 from *Crotalus durissus collilinetaus* also was evaluated by incubating the two proteins (1 : 1, w/w) for 30 min at 37°C and then assaying the residual enzyme activity.

2.10. Chick Biventer Cervicis Muscle Preparation (BCP). Animals were anesthetized with halothane and sacrificed by exsanguination. The biventer cervicis muscles were removed and mounted under a tension of 0.5 g, in a 5 mL organ bath (automatic organ multiple-bath LE01 Letica Scientific Instruments. Barcelona, Spain) at 37°C containing aerated (95% O_2-5% CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.70, KCl 4.70, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.00, and glucose 11.65. Contracture to exogenously applied acetylcholine (ACh; 110 μM for 60 s) and KCl (20 mM for 130 s) was obtained in the absence of field stimulation, before and after the addition of a single dose of PhTX-I PLA_2 (1.4 μM) or their modified derivatives (1.4 μM). A bipolar platinum ring electrode was placed around the tendon, which runs the nerve trunk supplying the muscle. Indirect stimulation was performed with a (MAIN BOX LE 12404 Panlab s.l. Powerlab AD Instruments Barcelona, Spain) stimulator (0.1 Hz, 0.2 ms, 3-4 V). Muscle contractions and contractures were isometrically recorded by force-displacement transducers (Model MLT0201 Force transducer 5 mg–25 g Panlab s.l. AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (OUAD Bridge AD Instruments, Barcelona, Spain).

2.11. Myotoxic Activity. Groups of five Swiss mice (18–20 g) received an intramuscular injection (i.m.) of 20 μg of PhTX-I PLA_2 or their modified derivatives dissolved in 100 μL of PBS, in the gastrocnemius. A control group received 100 μL of PBS (0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2). Three hours after injection, blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined by a kinetic assay (Sigma 47-UV). Activity was expressed in U/L, one unit defined as the phosphorylation of 1 μmol of creatine/min at 25°C.

2.12. Edema-Forming Activity. The ability of PhTX-I PLA_2 and their modified derivatives to induce edema was studied in groups of five Swiss mice (18–20 g). Fifty μL of phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) with toxins (1 μg/paw) were injected in the subplantar region of the right footpad. The left footpad received 50 μL of PBS, as a control. The paw volume was evaluated plethysmographically (Model 7140 Plethysmometer, Ugo Basile, USA), immediately before the injection (basal) and after 1 h. Edema-forming activity was expressed as the percentage of the increase in volume of the right foot pad in comparison to the left foot pad (control). The equation for calculation of the percentage of edema in toxins injected paw was

$$\%\text{edema} = \left[\left(\frac{T_x \times 100}{T_0}\right) - 100\right], \quad (1)$$

where T_x is the edema (volume) measured at each time interval and T_0 is the volume of the paw (intact, zero time before toxins injection). The percentage of edema calculated was subtracted from the matched values at each time point in the saline injected hind paw (control).

Table 1: Amino acid composition of native PhTX-I and their chemically modified derivatives.

Amino acid	PhTX-I native[a]	PhTX-I modified[b]	PhTX-I modified[c]	PhTX-I modified[d]	PhTX-I modified[e]
Asp	11	10.92 (11)	10.9 (11)	10.72 (11)	10.56 (11)
Glu	6	5.98 (6)	6.12 (6)	5.92 (6)	6.37 (6)
Ser	3	3.37 (3)	3.15 (3)	3.21 (3)	3.03 (3)
Gly	11	11.43 (11)	11.25 (11)	11.39 (11)	11.13 (11)
His	2	1.74 (2)	1.94 (2)	1.6 (2)	0.65 (**1**)
Arg	7	7.62 (7)	6.56 (7)	7.15 (7)	7.42 (7)
Thr	6	6.34 (6)	6.09 (6)	6.35 (6)	6. 34 (6)
Ala	5	4.81 (5)	5.29 (5)	5.34 (5)	5.44 (5)
Pro	6	5.82 (6)	6.01 (6)	6.05 (6)	5.74 (6)
Tyr	10	10.24 (10)	9.79 (10)	5.94 (**6**)	10.21 (10)
Val	3	3.33 (3)	3.34 (3)	3.49 (3)	3.49 (3)
Met	1	1.47 (1)	1.17 (1)	1.18 (1)	1.45 (1)
Cys	14	13.96 (14)	13.98 (14)	13.74 (14)	13.69 (14)
Ile	2	2.47 (2)	2.35 (2)	2.27 (2)	2.22 (2)
Leu	8	8.29 (8)	8.44 (8)	8.12 (8)	8.01 (8)
Phe	4	3.58 (4)	4.49 (4)	4.74 (4)	4.21 (4)
Lys	17	10.02 (**10**)	16.93 (17)	17.35 (17)	17.2 (17)
Trp	3	ND	ND	ND	ND

[a]Amino acid sequence. Amino acid analysis of chemical modifications with [b]AA (Lys), [c]NPSC (Trp), [d]NBSF (Tyr), and [e]BPB (His). ND: nondetermined.

2.13. Cytotoxic Activity

2.13.1. Maintenance of NG97 and NCIH-3T3 Cell Culture. Cytotoxic activity was assayed on NG97 and NCIH-3T3 cells, which were grown in plastic flasks (25 cm^2) with RPMI 1640 medium (Cultilab, Campinas, SP, Brazil), supplemented with 2% L-glutamine, 120 μg/mL garamycin, and 13% inactivated fetal bovine serum (complete medium). The cultures were incubated at 37°C in an atmosphere containing 5% of CO_2. Medium was changed every 48 h, and when the culture reached confluence, the subculture was performed by treatment with trypsin and versene (Adolfo Lutz, São Paulo, SP, Brazil). Variable amounts of native PhTX-I and chemically modified derivatives were diluted in assay medium and added to cells in 96-well plates. Experiments were carried out in triplicate.

2.13.2. Cellular Viability by Neutral Red Uptake Assay. This assay was done according to the method described by Borenfreund and Borrero [31]. After a 4 h incubation with serum-free medium containing 50 μg of neutral red/mL the cells were washed quickly with PBS and then 0.1 mL of a solution of 1% (v/v) acetic acid : 50% (v/v) ethanol was added to each well to extract the dye. After shaking for 10 minutes on a microtitre plate shaker, the absorbance was read at 540 nm. The cell death was determined in comparison of the absorbance obtained from nontreated cells.

2.14. Statistical Analyses. Results were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by Dunnett's test, when several experimental groups were compared with the control group. Differences were considered statistically significant if $P < 0.05$.

3. Results

Table 1 shows the result of analysis of amino acid composition of the modified proteins of PhTX-I PLA_2 compared to the amino acid sequence of native PhTX-I. After chemical treatment a single His, 4 Tyr, and 7 Lys residues were modified by BPB, NBSF, and AA, respectively. With the methodology employed it was not possible to determine the changes in Trp residues. In all these cases the exact position of the groups modified is unknown. The homogeneity of the modified proteins was evaluated by SDS-PAGE, as shown in Figure 1. Both native PhTX-I and modified forms migrated similarly by electrophoresis, indicating the presence of a single band electrophoretic for each protein, demonstrating the high homogeneity of the samples as well as no detectable change in the apparent molecular weight of the modified forms of the PhTX-I.

Molecular mass values of the modified proteins were determined by ESI mass spectrometry. Figures 2(a), 2(b), 2(c), and 2(d) show the mass spectrum of modified forms of PhTX-I, by BPB, AA, NPSC, and NBSF, respectively; each peak represents the protein carrying a different number of charges (protons). Figures inserted in each spectrum show the deconvolution of the spectra obtained of the modified proteins. The modified protein in His, Lys, Trp, and Tyr residues had molecular mass of 14440.7, 14537.9, 14470.1, and 15068.9 Da, respectively.

To determine whether the chemical modifications in PhTX-I caused changes in protein secondary structure, far-UV circular dichroism (CD) was used. Figure 3 shows

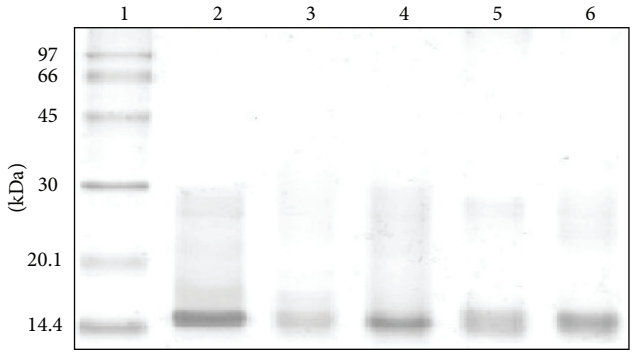

Figure 1: Electrophoretic profile in Tricine SDS-PAGE of native PhTX-I and their chemically modified derivatives. (1) Molecular mass markers, (2) native PhTX-I, (3) AA-treated PhTX-I, (4) NPSC-treated PhTX-I, (5) NBSF-treated PhTX-I, and (6) BPB-treated PhTX-I.

CD spectra of native PhTX-I and their modified forms which demonstrate two negative bands of similar magnitude ($-11{,}000$ to $-10{,}500\ \text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) at 208 and 222 nm and a positive one at ~190 nm (data not shown), indicating a consistent content of α-helical structures. The exception was NBSF modified PhTX-I that lad lower signal at 222 nm than the unmodified protein. Based on the CDNN program analysis of the native PhTX-I spectrum, the contents of α-helices, β-sheets, and β-turns were 32%, 18%, and 17%, respectively. The contents of secondary structure of PhTX-I chemically modified by BPB, NPSC, or AA were very similar to native PhTX-I, with no significant changes in the CD spectrum between them, suggesting that secondary structure of protein remains practically unchanged. However, NBSF treatment resulted in a significant change ($-10{,}500$ to $-9{,}000\ \text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) in molar ellipticity of the negative band at 222 nm, which by CDNN software analysis indicates an altered content of α-helices, β-sheets, and β-turns (30%, 19% and 18%, resp.).

The catalytic activity of native PhTX-I and their modifications were studied using the chromogenic substrate 4-nitro-3-(octanoyloxy) benzoic acid. The catalytic activity of native PhTX-I was almost completely abolished by BPB, but only partially reduced after modification of Tyr or Lys residues; NPSC did not cause a significant decrease in this activity (Figure 4). The incubation of native PhTX-I with crotapotins F2 and F3 from *C. d. collilinetaus* and EDTA diminished the activity; heparin did not significantly inhibit the catalytic activity (Figure 4).

Figure 5 shows the graphical representation of the blockade of the contractile response in the neuromuscular transmission (BCP) of native PhTX-I and modified derivatives. The native PhTX-I at a concentration of 1.4 μM blocked the indirectly evoked contractions reaching 50% of the block in about 20 min. This activity was markedly diminished for all the PhTX-I chemically modified derivatives, except for modification of Trp where no significant change in force of contraction was produced by the modification.

Figure 6 shows the effect of the different chemical modifications on the myotoxic activities of PhTX-I, by time-course measurement of plasma levels CK after intramuscular injection of proteins. It can be seen that even when BPB and AA were the most effective reagents altering this activity, most of the treatments had some effect. Three hours after treatment started, the myotoxic effect was reduced by 85%, 80%, and 72% by treatment with AA, BPB, and NBSF, respectively. EDTA and heparin inhibited around 74% and 50% of this activity.

Regarding the edema-inducing effect, after alkylation with BPB, PhTX-I lost around 70% of its activity one hour after treatment, while modification of Lys and Tyr residues caused only a partial decrease of this activity (55% and 40%, resp.); however, NPSC did not inhibit this activity, as can be seen in Figure 7.

Native PhTX-I was cytotoxic to NIH-3T3 fibroblast cell line and to a lesser extent for the NG97 cell line derived from a human astrocytoma grade III (Figure 8). The cytotoxic activity of PhTX-I was independent of enzymatic activity, since BPB-treated PhTX-I was able to produce cytotoxicity in both cell types. After treatment with NPSC the cytotoxic activity was partially reduced. On the other hand, acetylation and sulfonylation of Lys and Tyr residues, respectively, reduced strongly the cytotoxic activity in both cell types.

4. Discussion

We recently described the isolation of a basic PLA_2 (PhTX-I) from *P. hyoprora* using reverse phase HPLC [20]. This toxin exhibits high catalytic activity, shares various structural similarities with other "bothropics" PLA_2, and has the conserved and essential Asp residue in position 49. PhTX-I induces *in vivo* myotoxicity, moderates footpad edema (at concentrations up to 10 μg/mL and 0.5 μg/mL, resp.), and causes *in vitro* neuromuscular blockade in chick biventer cervicis muscle preparations (at concentrations of 1.4 μM). Here, we have described the chemical modifications of specific amino acid residues (His, Tyr, Lys, and Trp), performed in PhTX-I and how theses modifications affected the structural, enzymatic, and pharmacological properties of this myotoxin.

A first important observation was that after chemical treatment a single His, 4 Tyr, 7 Lys, and one Trp residues were modified by BPB, NBSF, AA, and NPSC, respectively (Table 1). These results were confirmed by mass spectrometry (Figure 2). The mass of native PhTX-I, 14249.22 Da [20], after treatment with NBSF (15068.96 Da) increased 819.74 Da (Figure 2(d)), demonstrating modification of fourth Tyr residues (up to 187.16 Da in each residue corresponding to reagent NBS that would be incorporated). Similarly, only three Tyr residues (7, 70, and 77) with the highest exposed surface areas in the notexin were modified by NBSF, suggesting that the seven remaining residues are "buried" within the molecule [32]. Acetylation of Lys residues of basic PLA_2 myotoxins three (PrTX-I,-III and BnSP-7) caused a complete loss of basicity, being demonstrated by electrophoretic analysis [22, 33]; however AA-treated PhTX-I (14537.9 Da) (Figure 2(b)) shows that only seven Lys residues were modified, due to which there is an increase of 288.60 Da, equivalent to seven times the mass of acetyl radical (42 Da) incorporated into

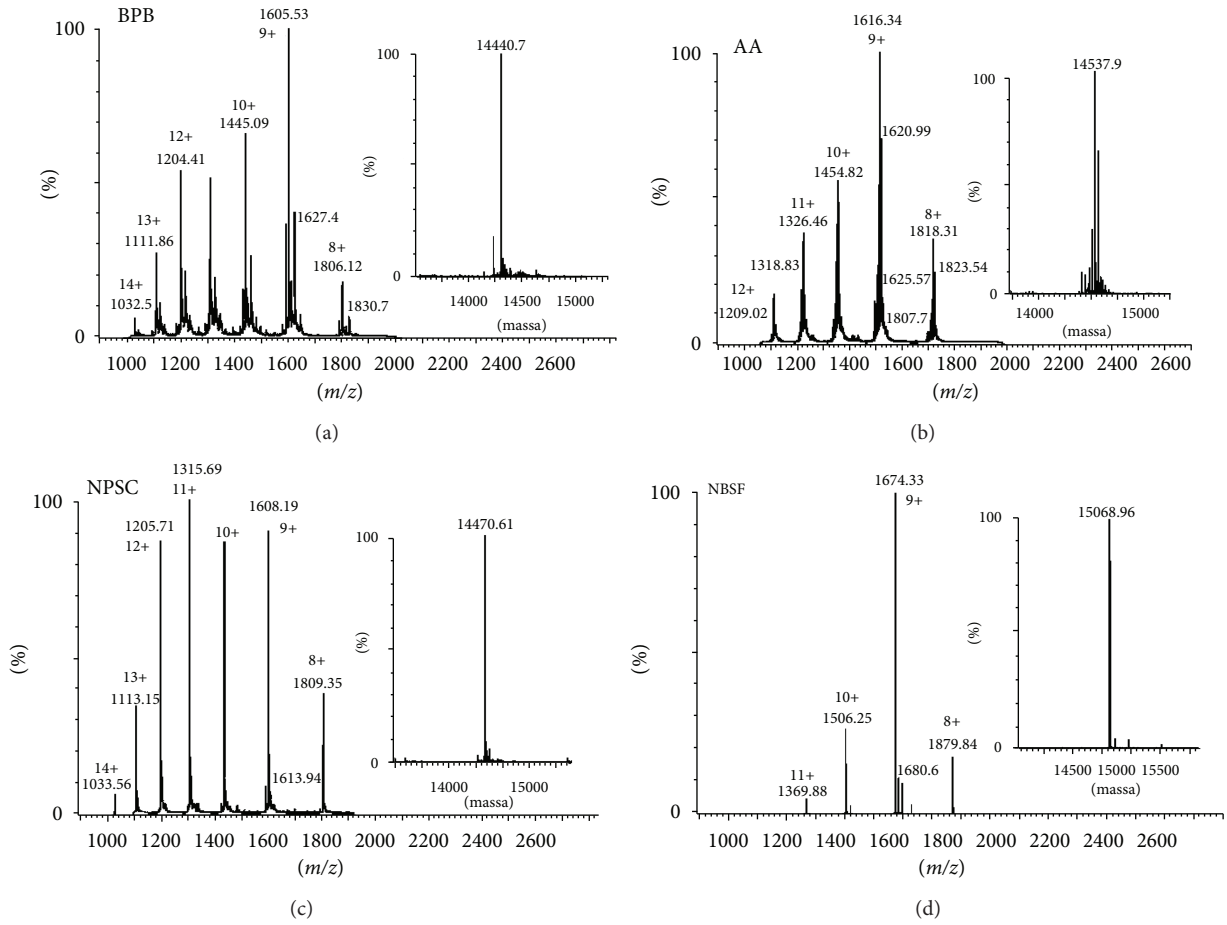

Figure 2: Mass determination by ESI mass spectrometry of the chemically modified derivatives of PhTX-I. Raw and deconvoluted mass spectra of BPB-treated PhTX-I (a), AA-treated PhTX-I (b), NPSC-treated PhTX-I (c), and NBSF-treated PhTX-I (d).

the amino group of K residues, showing similar behavior to the native protein in electrophoresis gel (Figure 1).

Similarly, an increase of 221.39 Da in NPSC-treated PhTX-I (Figure 2(c)) indicated a modification of a single Trp residue. By analogy with other results using PrTX-I and -III from *B. pirajai* [22], we would expect that, with the three Trp residues present in the structure of PhTX-I, this reagent should modify the residue with a larger area of exposure; it would be more easily attacked by the NPSC. Alkylation of His by BPB has been widely used to assess the role of enzymatic activity in the pharmacological actions of PLA_2 [22, 23, 33–36]. PhTX alkylated with BPB had a molecular mass of 14440.7 Da (Figure 2(a)), which confirmed the modification of only one residue of His. His48 is a highly conserved amino acid residue in PLA_2, which has a vital role in catalysis [37]. Since the enzymatic activity of the PhTX-I was almost completely abolished after this modification, His48 was likely the residue modified, because this amino acid is part of the catalytic triad of this protein family.

Examination of native PhTX-I by CD spectroscopy indicated that the predominant secondary structure of this PLA_2 consisted of alpha-helices (Figure 3), in agreement with the results obtained for others as PLA_2 from Taiwan cobra [38], PLA2A from *C. d. ruruima* [39], and BnIV from *B. newidii* [40]. The secondary structure of PhTX-I chemically modified derivatives did not alter significantly after modifications as evidenced by the CD spectra, which exhibited almost the same profile as that of native toxin. Only NBSF-treated toxin revealed detectable changes in secondary structure composition when compared to native toxin (Figure 3). This difference could be due to partial helix unfolding but the small change suggested that such unfolding was minimal. Kini [1] described that the alkylation by BPB does not affect the three-dimensional structure of PLA_2 or its ability to bind phospholipids, but may alter the ability to interact with specific proteins or ligands. Thus, it is suggested that the chemical modifications performed in this study mainly affected the specific residues involved in such modifications and did not result in drastic conformational changes in the molecule.

The PhTX-I PLA_2 is a Ca^{2+}-dependent enzyme, with maximum activity at pH 8 and 40°C, reaching V_{max} and K_m of 11.76 nmoles/min and 1.96 mM, respectively[20]. Heparin slightly decreased enzymatic activity of PhTX-I, (25%) (Figure 4); similarly, this polyanionic compound resulted acting as negative allosteric modulator of PLA_2 *C. d. cascavella*

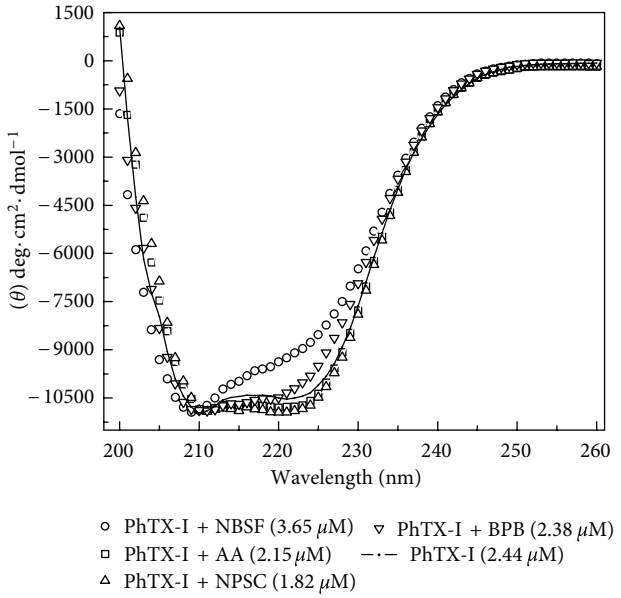

FIGURE 3: Far-UV circular dichroism spectra of native PhTX-I and their chemically modified derivatives. Native PhTX-I (continuous lines) and their chemically modified derivatives (symbols): NBSF-treated PhTX-I (circles), BPB-treated PhTX-I (inverted triangles), NPSC-treated PhTX-I (open triangles), and AA-treated PhTX-I (squares).

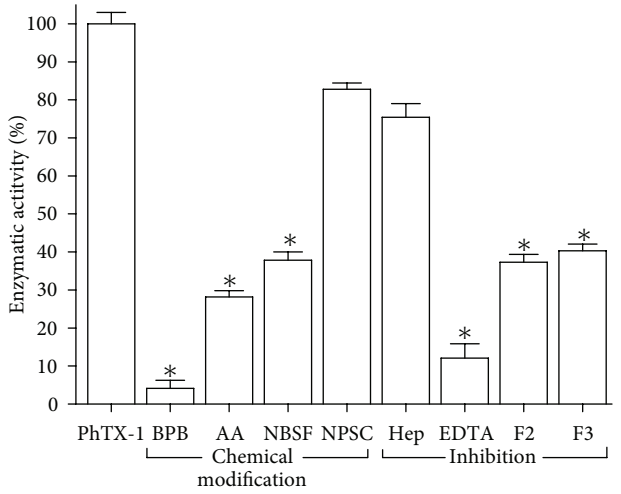

FIGURE 4: PLA_2 activity of native PhTX-I PLA_2 and their chemically modified derivatives with BPB, AA, NBSF, NPSC and inhibitory effect of heparin, EDTA, and crotapotins (F2 and F3) upon 4-nitro-3-(octanoyloxy) benzoic acid substrate $^*(P < 0.05)$.

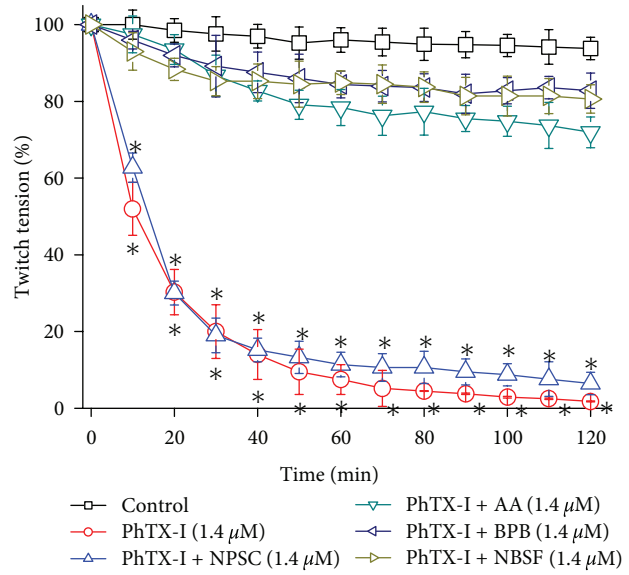

FIGURE 5: Neuromuscular blockade of chick biventer cervicis preparations incubated at 37°C with native PhTX-I and their chemically modified derivatives with BPB, AA, NBSF, and NPSC, all in concentration 1.4 μM. The points are the mean ± SEM of six experiments. $^*(P < 0.05)$ compared to the twitch-tension before toxin addition.

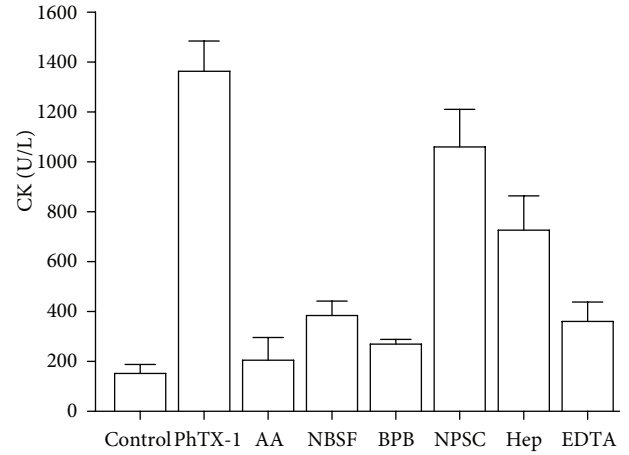

FIGURE 6: Increments in plasma CK activity after intramuscular injection of native PhTX-I their chemically modified derivatives with BPB, AA, NBSF, and NPSC (all 20 μg/100 μL) and incubation of PhTX-I (20 μg) with heparin and EDTA. Controls were injected with 100 μL of PBS. Three hours after injection, blood was collected, and serum levels were measured. Values are mean ± SEM of five mice at each time point.

[41]. On the other hand, EDTA greatly decreased catalytic activity of PhTX-I (88%), as β-bungarotoxin and notexin, which were inhibited by EDTA even in the presence of an excess of Ca^{2+} [42]. The F2 and F3 crotapotins from *C. d. collilineatus* significantly inhibited the enzymatic activity of PhTX-I at approximately 55% (Figure 4), in agreement with BjIV PLA_2 of *B. jararacussu*, which was inhibited by 50% in its catalytic activity by F7, F3, and F4 crotapotins from *C. d. terrificus*, *C. d. collilineatus,* and *C. d. cascavella* [43]. These results suggest that crotapotins can bind to bothropics PLA_2 a manner similar to that of crotalics PLA_2, and raise the possibility that bothropic venoms may contain crotapotin-like proteins which inhibit the catalytic activity of PLA_2.

Acetylation of Lys residues significantly reduced the enzymatic activity; however, a residual activity was detected,

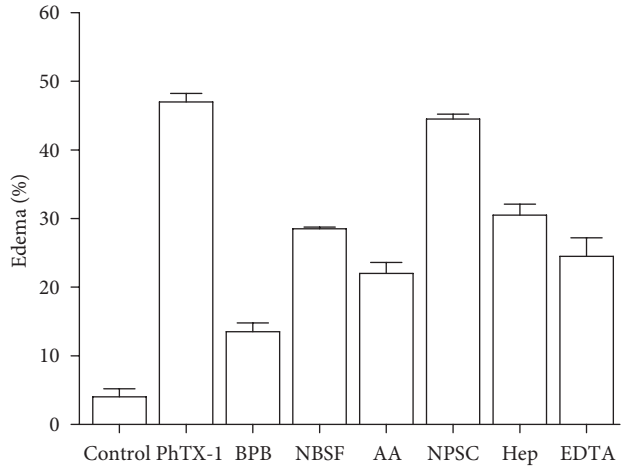

Figure 7: Edema-forming activity of native PhTX-I and their chemically modified derivatives with BPB, AA, NBSF, and NPSC (all 1 μg) and incubation of PhTX-I (1 μg/50 μL) with heparin and EDTA. The toxins were injected s.c. in the footpad of mice. Edema was evaluated after 1 h injection and expressed as percent edema. Each point represents the mean ± SEM of five animals.

corresponding to 26% (Figure 4), similarly to MT-III (*B. asper*) and MT-I (*B. godmani*) [21]. The mode of specific acetylation is not clear, but there is some evidence of reduction of the calcium-binding capacity, thereby reducing the enzymatic activity of PLA_2 [44]. In contrast, both myotoxic and cytotoxic effects were totally abolished, whereas a residual edematogenic effect remained (Figures 6, 7, and 8). These observations agree with previous studies in which the Lys of PrTX-I, PrTX-III, and BnSP-7 PLA_2 were modified by acetylation [22, 33] and drastically decreased myotoxic, edema-inducing, and bactericidal activities. Acetylation of Lys residues in PhTX-I also decreased the blockade of the contractile response in the neuromuscular transmission, more than enzymatic activity (Figure 5). This greater effect on some pharmacological properties than on the enzymatic activity of Lys-modified PhTX-I demonstrates the dissociation between enzymatic and pharmacological activities and evidence of the existence of molecular regions, distinct from the catalytic site, which may be responsible for at least some of the pharmacological properties of these toxins, in agreement with previous studies [23, 34, 35].

Soares et al. [22] suggested that the bactericidal effect of PrTX-III and myotoxin III from *B. pirajai* and *B. asper*, respectively, might be related to overall basicity of the protein from the N-terminal helix and residues the 115–129 in the C-terminal region that is rich in aromatic and basic residues. This C-terminal region was identified as a structural determinant of this effect [45]. Short synthetic peptides representing the C-terminal region of PLA_2 myotoxins showed cytolytic and muscle damaging activities similar to their parent proteins, although they display a lower potency [46–48]. Significant decrease of myotoxic activity after incubation with heparin confirms the involvement of C-terminal region of PhTX-I which is a basic myotoxin with high content of Lys residues [20].

His48 is a highly conserved residue in PLA_2, which has an important role in catalysis [37]. Alkylation of His by BPB has been widely used to assess the role of enzymatic activity in the pharmacological actions of PLA_2 [21, 35, 36, 49]. Here, alkylation of His at the active site of PhTX-I markedly abolished enzymatic activity (<4% residual activity) (Figure 4). Others have reported residual enzymatic activity following alkylation of His48, as βBuTX and notexin from *N. nigricollis* and *N. n. atra* PLA_2, with values close to that found for BPB-PhTX-I (5%–8% of residual activity) [34]. Myotoxic, neurotoxic, and edema-forming activities of PhTX-I, were drastically reduced by this modification (Figures 5, 6, and 7); EDTA by treatment also affected myotoxicity and edematogenic activity (Figures 6 and 7), strengthening the hypothesis that phospholipid enzymatic hydrolysis is involved in these effects. Similarly, neurotoxic and myotoxic activities were inhibited almost completely after alkylation of His48 of PLA_2 Basp-III (*B. asper*), PrTX-III (*B. pirajai*), BthTX-II (*B. jararacussu*), Cdc-9, and Cdc-10 (*C. d. cumanensis*) [21, 22, 35, 49], showing that these pharmacological effects are dependent on the catalytic activity. Since alkylation of the active site His48 completely abolished the catalytic activity and strongly attenuated these three pharmacological effects, Kini [1] suggested the hypothesis that PLA_2 activity potentiates these pharmacological effects induced by *Bothrops* and *Crotalus* myotoxins.

In contrast, cytotoxic activity upon NG97 and NCIH-3T3 cells not was affected by His modification (Figure 8), suggesting that enzymatic activity is not required for this effects and that there are other molecular regions involved in cellular membrane perturbation. Our results agree with MTX-I and II PLA_2 from *B. brazili*, which displayed cytotoxic activity against Jurkat lines independently of catalytic activity [50]. Some authors propose that cytotoxic activity on tumor cell lines is associated with apoptosis induction, considering the fact that PLA_2 enzymes have been proposed to play a role in mediating apoptosis in various models, including cell lines [51]. The PLA_2 activity is proposed to accelerate turnover of phospholipids, which may influence membrane changes that occur during apoptosis [52]. We suggested important role of Lys residues in cytotoxic effect, because PhTX-I treatment with AA abolished this activity.

Studies have been directed trying to understand the mechanisms involved in the inflammatory response induced by myotoxic PLA_2 from several snake venoms [53–55]. However, the relationship between enzymatic activity and edema is contradictory [56]. It is assumed that myotoxic and edematogenic activities can be induced by different structural domains in these PLA_2, or that a partial overlapping of these domains occurs [55, 57].

Residual enzymatic activity after sulfonylation of Tyr residues was 38% (Figure 4). Tyr52 and Tyr73 are part of the catalytic site of PLA_2; giving structural support to stabilize the catalytic system [37], changes in this system would affect the enzymatic activity. Tyr-modified PhTX-I decreased myotoxic and neurotoxic activities more than the enzymatic activity (Figures 4, 5, and 6), once again indicating the dissociation between enzymatic and pharmacological activities.

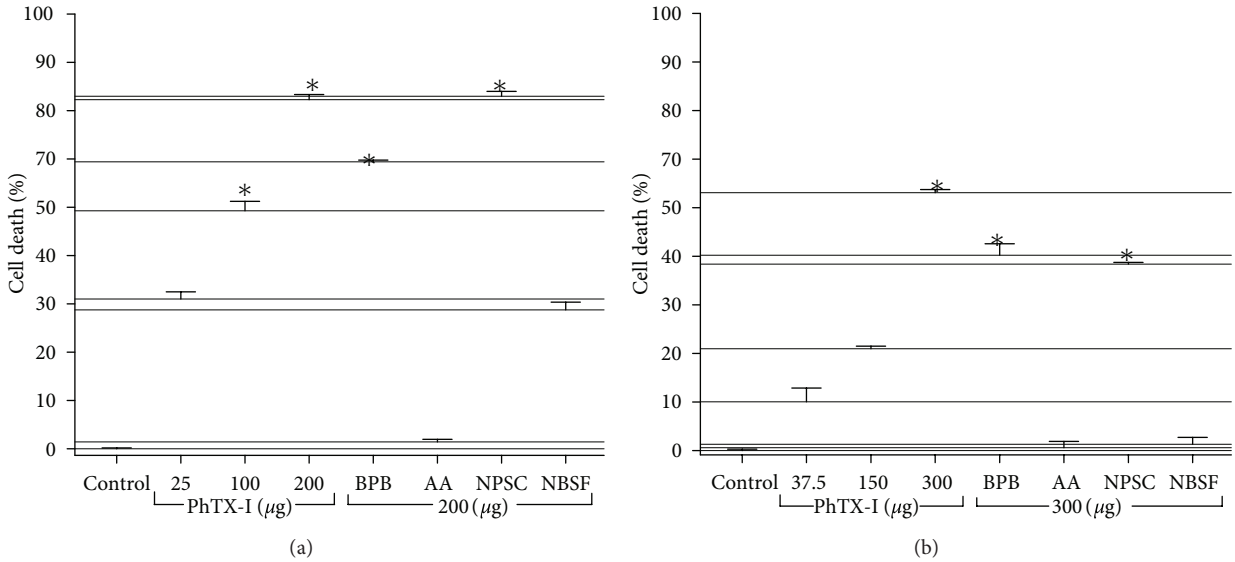

Figure 8: *In vitro* cytotoxic activity of native PhTX-I PLA_2 and their chemically modified derivatives with BPB, AA, NBSF, and NPSC on NCIH-3T3 (a) and NG97 (b) cell culture. Cell lysis was estimated by neutral red uptake assay. The cell death was determined in comparison of absorbance obtained from nontreated cells. Experiments were carried out in triplicate. $^*(P < 0.05)$.

Cytotoxic activity of PhTX-I also was reduced drastically after modification by NBSF (Figure 8), indicating that Tyr residues would also be involved in this process. Zhao et al. [58] observed that myotoxic PLA_2 have a set of Tyr residues located at the C-terminal region of the molecule. These Tyr may contribute to the hydrophobic-cationic combination proposed to play a role in myotoxicity and cytotoxicity [19, 46, 59]. PhTX-I show a Tyr residue in C-terminal region; alterations of this acid amino in this region of the molecule are causing reduction of toxicity of PhTX-I. However, the influence of conformational changes induced by NBSF in these effects cannot be discarded (Figure 3).

NPSC-treated PhTX-I did not significantly decrease enzymatic activity (Figure 4), suggesting that the modified Trp residue is not related to the catalytic system. In the same way, this modification showed no changes as compared to native PhTX-I, in edematogenic and cytotoxic activities (Figures 7 and 8); the myotoxic effect was minimally affected (Figure 6). In this sense Trp residues of PhTX-I have little or no direct action on the muscle. The Trp modifications of PhTX-I also maintained the action upon blockade of the contractile response in chick biventer cervicis muscle preparation (Figure 5). In contrast, modified Trp affected only the neurotoxic effect caused by MjTX PLA2-II [60], indicating the relevant role of this residue in this activity and suggesting that chemical modification could be interfering with the stability of the interaction between the monomers of this dimeric toxin, since Trp77 helps to maintain the homodimeric interaction. This suggests that the shift from dimeric to monomeric form of myotoxin may reduce the ability to affect the plasma membrane [60]. Because PhTX-I is a monomeric toxin, modified Trp would not affect the pharmacological activity of this toxin.

5. Conclusions

Our results indicate the critical role played by Lys and Tyr residues in myotoxic, neurotoxic activities and mainly in the cytotoxicity displayed by PhTX-I. His residue and, therefore, catalytic activity of PhTX-I are relevant for edematogenic, neurotoxic, and myotoxic effects, but not for its cytotoxic activity. Our observations supported the existence of pharmacological sites, distinct from the catalytic site, that contribute to the development of toxicity of these toxins and the hypothesis that the catalytic activity would potentiate the myotoxic and neurotoxic effects induced by snake venom PLA_2. Finally, although a partial dissociation is shown, both the catalytic sites as the hypothetical pharmacological sites are relevant to the pharmacological profile of PhTX-I.

Abbreviations

PLA_2: Phospholipase A_2
PhTX-I: *Porthidium hyoprora* myotoxin-I
EDTA: Ethylenediaminetetraacetic acid
BPB: 2,4′-Dibromoacetophenone
NBSF: 4-Nitrobenzenesulfonyl fluoride
AA: Acetic anhydride
NPSC: 2-Nitrobenzenesulfonyl chloride
CD: Circular dichroism
PBS: Phosphate-buffer saline
CK: Creatine kinase.

Ethical Approval

The animals and research protocols used in this study followed the guidelines of the Ethical Committee for use of animals of ECAE-IB-UNICAMP SP, Brazil (protocol number

1860-1) and international law and policies. All efforts were made to minimize the number of animals used and their suffering.

Acknowledgments

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