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NEUTRALIZATION OF THE CYTOLYTIC AND MYOTOXIC ACTIVITIES OF PHOSPHOLIPASES A_2 FROM *BOTHROPS ASPER* SNAKE VENOM BY GLYCOSAMINOGLYCANS OF THE HEPARIN/HEPARAN SULFATE FAMILY

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Abstract—Basic phospholipases A_2 from the venom of *Bothrops asper* exhibit skeletal muscle damaging activity *in vivo*, and cytolytic activity to a variety of cell types in culture. Glycosaminoglycans of the heparin/heparan sulfate family were found to be potent blockers of the cytolytic action *in vitro*, and, as well, to be able to neutralize the muscle damaging activity of purified myotoxins and crude venom *in vivo*. However, the neutralizing effect of heparins was more potent *in vitro* than *in vivo*. The cytolytic activity of myotoxin II (a lysine-49 phospholipase A_2 isoform) and its inhibition by heparin was characterized. The neutralizing effect of heparin did not depend on its anticoagulant activity, since both standard heparin and heparin with low affinity for antithrombin (LA-heparin) had a similar efficiency. Heparan sulfate and low molecular mass heparin (5 kDa) also neutralized myotoxin II. In contrast, different heparin-derived disaccharides were unable to block cytolysis, implying a requirement for a longer carbohydrate chain structure for the interaction with the protein. By affinity chromatography and gel diffusion, it was demonstrated that heparins form a complex with all isoforms of basic venom myotoxins, held at least in part by electrostatic interactions. The phospholipase A_2 activity of myotoxin III, a related aspartate-49 isoform from the same venom, was unaffected by heparins, despite the fact that its myotoxic activity was inhibited, indicating a dissociation of the two actions.

Key words: heparin; phospholipase A_2; cytotoxicity; myotoxin; snake venom

In recent years, heparin has been shown to interact *in vitro* with several types of PLA_2s¶, (EC 3.1.1.4) [1–3] and in some cases, to inhibit their enzymatic activity. PLA_2s are notoriously abundant and widely distributed in snake venoms, in which they have acquired a variety of toxic activities in the course of evolution, such as neurotoxicity, myotoxicity, anticoagulant effect, and edema-forming activity [4, 5].

Non-immunologic inhibitors of myotoxic PLA_2s could be of interest therapeutically, especially considering that antivenom immunoglobulins have a limited efficacy in preventing the muscle damage that follows envenomation [6, 7]. In addition, inhibitors may constitute useful tools for understanding the mechanism of action of myotoxic PLA_2s. The neutralization of myotoxic activity of the venom of *Bothrops jararacussu*, a crotalid species from Brazil, by heparin has been reported [8, 9]. This interesting finding is further investigated in the present work, using myotoxin II, a basic PLA_2 purified from the venom of *B. asper* [10], and with special interest on heparin derivatives with little or no effect on the coagulation system, i.e. heparin with low affinity for antithrombin. Myotoxin II is a natural PLA_2 isoform devoid of enzymatic activity, mainly due to the critical amino acid substitution at position 49 (Asp→Lys) [11], but still displays myotoxic activity [10]. In some experiments, an isoform that has PLA_2 activity, myotoxin III [12], was utilized to investigate the effect of heparins on the enzymatic activity of these proteins. In order to have a well standardized system for the characterization of the neutralizing ability of heparins, an *in vitro* model of cytotoxicity was developed, using two cell types as targets, skeletal muscle myoblasts and capillary endothelial cells.

MATERIALS AND METHODS

Venoms and myotoxic PLA_2s from B. asper. Crude venom of *B. asper* specimens from Costa Rica (Pacific region) kept at the serpentarium of the Instituto Clodomiro Picado (University of Costa Rica) was fractionated by ion-exchange chromatography on CM-Sephadex C-25 as previously described [10] to obtain pure myotoxin II, as evaluated by SDS–PAGE [13] and cathodic native PAGE at pH 4.3 [14]. *B. asper* myotoxin III [12] was kindly provided by Dr J.M. Gutiérrez, University of Costa Rica. Crude venoms of *Agkistrodon piscivorus piscivorus, Bothrops jararacussu, Crotalus durissus terrificus, C. viridis viridis, Naja naja atra,*

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¶ Abbreviations: PLA_2, phospholipase A_2; LA-heparin, heparin with low affinity for antithrombin; CK, creatine kinase; LDH, lactic dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

Trimeresurus flavoviridis, and *Vipera berus*, were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Glycosaminoglycans. Standard heparin (5000 IE/mL), heparin with low affinity for antithrombin (LA-heparin; 7 IU anti-factor Xa/mg, M_r 15,000), and low molecular mass heparin (Fragmin®, 25,000 IE/mL, M_r 5000) were provided by Kabi Pharmacia (Sweden), through the courtesy of Dr L.O. Andersson. Heparan sulfate (from bovine intestinal mucosa, H-7641) and a series of heparin-derived disaccharides were purchased from Sigma. A purified chondroitin sulfate preparation (from bovine cartilage) was kindly provided by M. Maccarana (Uppsala University, Sweden).

Myotoxic activity. Myotoxicity was evaluated in mice (Swiss, 20–24 g) by injecting either crude *B. asper* venom or pure myotoxins, alone or after incubation with varying amounts of heparins (15 min at room temperature), by the i.m. route (gastrocnemius), and then measuring the serum CK (EC 2.7.3.2) activity (kit No. 520, Sigma) after 3 hr [6]. To confirm data from the enzymatic assay, samples of injected tissue were evaluated histologically on hematoxylin-eosin stained sections. In addition, neutralization of *B. asper* venom myotoxicity by heparins was evaluated qualitatively using an intravital microscopic technique to study the effect of local application on the mouse cremaster muscle, as previously described [15].

The ability of LA-heparin to neutralize the myotoxic activity of several snake venoms, in addition to that of *B. asper*, was investigated similarly, using the serum CK levels after 3 hr of injection as an indicator of muscle damage. The ratio of LA-heparin/venom utilized in these screenings was 5 μg/μg.

In vitro *cytotoxicity.* The activity of myotoxin II was quantified *in vitro* by a cytotoxicity assay using two cell lines. L6 rat myoblasts (ATCC CRL 1458) were kindly provided by Dr M. Thelestam (Karolinska Institute, Sweden), whereas tEnd cells, a polyoma virus-transformed mouse endothelial cell line of capillary origin [16] were a kind gift from Dr A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Italy). Cells were routinely grown in Iscove's medium (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS; Biological Ind., Haemek, Israel), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 0.05 mg/mL gentamycin. In order to quantify cytotoxicity, cells were seeded at $1–4 \times 10^4$/well in 96-well plates and grown for 2–4 days until almost confluent. At the moment of the assay, culture medium was removed and replaced with 150 μL/well of medium with 1% FCS containing different amounts of myotoxin II. The FCS concentration was lowered to 1% in order to minimize the basal LDH (EC 1.1.1.27) activity of the medium. After 3 hr of incubation at 37° (selected after time-course experiments), 100 μL of supernatant were assayed for LDH released from damaged cells (kit No. 500, Sigma). As reference values for 100 and 0% cytotoxicity, cells were incubated with 0.1% Triton X-100-containing medium, or plain medium, respectively. All samples were assayed in triplicate wells.

For neutralization experiments, myotoxin II (at concentrations that would induce 100% cytotoxicity if uninhibited, i.e. 10 or 20 μg/150 μL/well for tEnd and L6 cells, respectively) was preincubated with the different tested agents for 15 min at room temperature, and then applied to cultures as described above.

In order to determine if the inhibitory activity of heparins on myotoxin-induced cytolysis was due to an effect on the target cells, cultures were preincubated with heparin (170 μg/mL) for 2 hr at 37°, washed, and then exposed to myotoxin II as described above.

A neutralizing monoclonal antibody to *B. asper* myotoxins, MAb-3 [17] was also tested in this cytotoxicity assay, to investigate if a similar neutralizing potency would be obtained in comparison to that observed *in vivo* [18]. MAb-3 was obtained from ascitic fluid in BALB/c mice, and partially purified by ammonium sulfate precipitation. Its final concentration was estimated by radial immunodiffusion [19] using a mouse IgG_1 standard (Sigma).

Edema-forming activity. The ability of LA-heparin to inhibit the edema induced by myotoxin II was tested in the footpads of mice, by injecting 100 μg of the toxin, either alone or after incubation with LA-heparin (5 μg/μg toxin), in 50 μL of PBS by s.c. route. Edema was quantified at different time points by measuring the increase in footpad thickness with a low-pressure spring caliper [20]. Control mice received an injection of 50 μL of PBS.

Phospholipase A_2 assay. An indirect hemolytic assay in suspension, based on the gel diffusion technique described by Gutiérrez *et al.* [21] was utilized. Washed sheep red blood cells were suspended at 1.5% (v/v) in 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 8.1, containing 1% egg yolk as a source of phospholipids and 0.09 mM $CaCl_2$. To 500 μL of this preparation, 5 μL (containing 5 μg) of myotoxin III alone, or after preincubation with heparins (at a ratio of 5 μg heparin/μg toxin), were added and incubated for 30 min at 37°. Then, 3 mL of buffer were added to each tube, and hemolysis was read at 540 nm after centrifugation. To assure the PLA_2 dependency of the lysis and the lack of direct hemolysis, parallel myotoxin samples were run using red blood cells in the absence of egg yolk phospholipids.

Affinity chromatography. Crude *B. asper* venom (25 mg) was applied to a column of heparin-agarose (Sigma) equilibrated with PBS, pH 7.2. After the absorbance at 280 nm of the eluent returned to baseline, elution of the heparin-binding fraction was performed by either a stepwise or a linear gradient to 1 M NaCl. The eluted fraction was analysed by SDS–PAGE and native cathodic PAGE as described above.

Gel diffusion. The interaction between heparins and crude venom or myotoxin II was tested by gel diffusion in 1% agarose-PBS plates [22]. After 24 hr of incubation at room temperature, gels were washed with PBS and the precipitates stained with Coomassie blue R-250.

Cell surface heparan sulfate treatments. The possible role of cell surface heparan sulfate in the

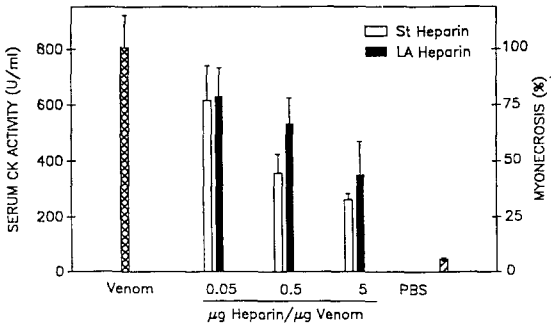

Fig. 1. Heparins neutralize the myotoxic action of whole *B. asper* venom in mice. Venom and different amounts of heparins were mixed, incubated 15 min at room temperature, and then injected i.m. into groups of four mice (50 μg venom/mouse). After 3 hr, CK levels in serum were determined as an indicator of skeletal muscle damage (% myonecrosis). CK values obtained with venom alone were taken as 100%. St heparin: standard heparin (empty bars); LA-heparin: low affinity heparin (filled bars); PBS: phosphate-buffered saline. Bars represent means ± SD of four determinations. All values are significantly ($P < 0.05$) lower than the venom control.

cytotoxic mechanism of myotoxin II was investigated by (a) heparitinase digestion of cell cultures, (b) inhibition of heparan sulfate sulfation with sodium chlorate, and (c) the use of a cell mutant with a defect in heparan sulfate biosynthesis.

For heparitinase treatment, cell cultures were incubated with 1.25 U/well of heparinase III (Sigma), in 100 μL of PBS, for 2 hr at 37° [23]. Control cells were treated similarly but omitting the enzyme. Then, wells were washed twice with culture medium, and the cytotoxicity induced by myotoxin II was determined as described above. For chlorate treatment, cells were plated in microwells with medium containing 10 mM sodium chlorate [24, 25] and grown for 2 days. At the moment of the assay, myotoxin II was added in chlorate-containing medium, and cytotoxicity was determined. Chlorate was omitted in control cultures. Finally, the cytotoxic action of myotoxin II was also quantified on two CHO cell lines, one defective in the synthesis of heparan sulfate (CHO-*pgs*D-677), and the wild type control (CHO-K1) [26], kindly provided by Dr J.D. Esko (University of Alabama-Birmingham, U.S.A.).

RESULTS

When whole *B. asper* venom was preincubated with either standard or LA-heparin, and subsequently injected into mice, its myotoxic action was significantly reduced in a dose-dependent manner (Fig. 1). This result was confirmed by histological evaluation and by the use of intravital microscopy. In the latter system, widespread muscle fiber damage regularly developed 4–6 min after application of venom alone, whereas, when it was mixed with standard or LA-heparin before application, muscle fibers were protected throughout the observation period of 30 min. In contrast to LA-heparin, standard heparin markedly increased the hemorrhage induced by the venom, evident both histologically and intravitally.

The ability of LA-heparin to neutralize the muscle-damaging activity of venoms from other species was explored. It was found that the venoms of *A. p. piscivorus*, *B. jararacussu*, and *T. flavoviridis*, were also significantly neutralized regarding myotoxic activity, as judged by the reduction in the serum CK levels measured at 3 hr. However, not all venoms tested were susceptible to heparin neutralization of myotoxic activity (Table 1).

To analyse in more detail the neutralizing ability of heparins towards myotoxins of *B. asper* venom, an *in vitro* cytotoxicity assay was developed using purified myotoxin II. This toxin was cytolytic not only to L6 myoblasts, but also to tEnd cells, in the concentration range of 25–150 μg/mL. Unexpectedly, endothelial cells were significantly more susceptible than myoblasts to the cytotoxic action of

Table 1. Effect of LA-heparin on the myotoxic activity of different snake venoms, tested by preincubation and subsequent *in vivo* administration to mice*

Species	Dose (μg)	Serum CK levels at 3 hr (U/mL)		P value†
		Venom alone	Venom + LA-heparin	
Agkistrodon p. piscivorus	50	768 ± 101	354 ± 72	<0.01
Bothrops jararacussu	50	370 ± 60	181 ± 59	<0.01
Crotalus durissus terrificus	5	298 ± 109	382 ± 80	>0.1
Crotalus viridis viridis	50	617 ± 118	484 ± 172	>0.1
Naja naja atra	5	454 ± 326	418 ± 180	>0.1
Trimeresurus flavoviridis	50	1086 ± 220	393 ± 69	<0.01
Vipera berus	50	399 ± 66	408 ± 111	>0.1

* The ratio of LA-heparin/venom in the preincubation mixture was 5 μg/μg. The dose utilized the case of *C. d. terrificus* and *N. a. atra* venoms was 5 μg/mouse (instead of 50 μg/mouse) due to their highly lethal neurotoxic activity.

† Serum CK values are presented as means ± SD, using four animals in each group. Normal CK levels in mice injected with saline were 51 ± 11 U/mL. The means between each pair (venom alone and venom + LA-heparin) were compared by two-tailed Student's *t*-test.

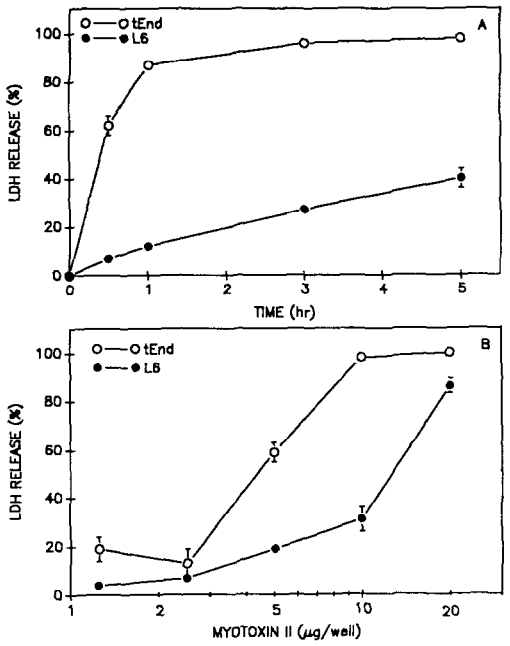

Fig. 2. Myotoxin II is cytotoxic to cultured rat myoblasts (L6) and mouse capillary endothelial cells (tEnd). (A) Time-course of LDH release from damaged tEnd (○) and L6 (●) cells after incubation with myotoxin II (10 μg/well; see Materials and Methods). LDH release is expressed as a percentage, considering the enzyme activity of Triton X-100 treated culture supernatants as 100%. (B) Dose–response curves of cytotoxic activity of myotoxin II on tEnd (○) and L6 (●) cells, measured by release of LDH at 3 hr. Each point represents mean ± SD of triplicate wells.

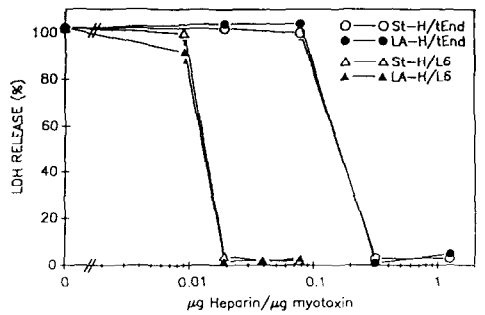

Fig. 3. Standard heparin and LA-heparin block the cytotoxic activity of myotoxin II. Either standard (St-H; empty symbols) or low affinity (LA-H; filled symbols) heparins were mixed with myotoxin II at the indicated proportions, incubated for 15 min at room temperature, and then assayed for cytotoxic activity on tEnd (circles) and L6 (triangles) cells. The toxin challenge for tEnd and L6 cells was 10 and 20 μg/well, respectively. Cytotoxicity was measured by the release of LDH from cells at 3 hr. Each point represents mean ± SD of triplicate wells.

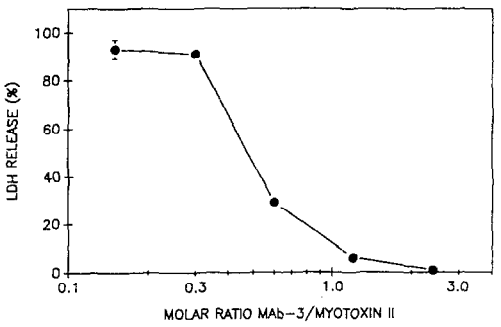

Fig. 4. Neutralization of the cytotoxic activity of myotoxin II on tEnd endothelial cells by monoclonal antibody MAb-3. MAb-3 and myotoxin II mixed at different molar ratios were preincubated for 15 min at room temperature, and then assayed for cytotoxic activity on tEnd cells. Cytotoxicity is estimated by the release of LDH, under the same conditions as in Fig. 3. Each point represents mean ± SD of triplicate wells.

myotoxin II (Fig. 2A and B). Morphologically, both cell types showed, after exposure to the toxin, an abundant cytoplasmic granulation, followed by an apparent dissolution of the membrane, without detachment, similar to the appearance of detergent-treated cultures. An incubation time of 3 hr (Fig. 2A) and a toxin challenge of 20 and 10 μg/well, for L6 and tEnd cells, respectively (Fig. 2B), were selected for all subsequent experiments, to assure 100% cytotoxicity in the absence of a neutralization effect. When preincubated with myotoxin II, standard as well as LA-heparin blocked its cytolytic activity on both cell types. The neutralizing potency of heparins in this assay system was considerably high, since cells were completely protected at approximate ratios of 0.02 and 0.3 μg heparin/μg myotoxin II, for L6 and tEnd cells, respectively, with no observable differences in the inhibitory efficiency of the two heparin types (Fig. 3). Since the average molecular mass of both heparins utilized (15 kDa) is similar to that of myotoxin II, the heparin/toxin ratios expressed as μg/μg roughly correspond to molar ratios. Under identical conditions, the myotoxicity-neutralizing antibody MAb-3 was also able to inhibit the action of myotoxin II in the cell culture system, completely preventing its effect at an approximate molar ratio of 1:1 (Fig. 4).

A complete neutralization of cytolysis caused by myotoxin II was also achieved with heparan sulfate and the low molecular weight heparin, although not with a variety of heparin-derived disaccharides or with chondroitin sulfate (Table 2). On the other hand, pretreatment of the cells with heparins, followed by washing, had no protective effect on myotoxin II-induced cytolysis (data not shown).

Since myotoxin II induces edema *in vivo*, and was found to be directly cytotoxic to endothelial cells in culture, the ability of heparin to inhibit the edema-forming activity of the toxin was investigated. A significant inhibition of edema in the mouse footpad assay was achieved by preincubation of myotoxin II with LA-heparin (Fig. 5).

Table 2. Heparan sulfate and low molecular mass heparin (Fragmin®), but not heparin disaccharides or chondroitin sulfate, neutralize the cytotoxic activity of myotoxin II on L6 myoblasts and tEnd endothelial cells

Tested agent, ratio	%LDH release, mean ± SD L6 cells	tEnd cells
Myotoxin II control*	96 ± 3	99 ± 2
Heparan sulfate, 0.0075 μg/μg myotoxin	NT	101 ± 1
Heparan sulfate, 0.075 μg/μg myotoxin	95 ± 1	82 ± 2
Heparan sulfate, 0.75 μg/μg myotoxin	4 ± 1	2 ± 1
Chondroitin sulfate, 1 μg/μg myotoxin	NT	96 ± 3
Chondroitin sulfate, 10 μg/μg myotoxin	NT	99 ± 7
Fragmin®, 0.75 μg/μg myotoxin	3 ± 3	5 ± 1
Heparin disaccharides, 0.75 μg/μg myotoxin		
I-A: α-ΔUA-2S-[1→4]-GlcNAc-6S	97 ± 3	NT
II-A: α-ΔUA-[1→4]-GlcNAc-6S	99 ± 2	NT
III-A: α-ΔUA-2S-[1→4]-GlcNAc	101 ± 2	NT
I-H: α-ΔUA-2S-[1→4]-GlcN-6S	102 ± 1	NT
II-H: α-ΔUA-[1→4]-GlcN-6S	102 ± 1	NT
I-S: α-ΔUA-2S-[1→4]-GlcNS-6S	101 ± 1	NT
II-S: α-ΔUA-[1→4]-GlcNS-6S	99 ± 1	NT
III-S: α-ΔUA-2S-[1→4]-GlcNS	100 ± 2	NT
IV-S: α-ΔUA-[1→4]-GlcNS	100 ± 2	NT

NT: not tested.
* The toxin challenge was 10 and 20 μg/well for tEnd and L6 cells, respectively.

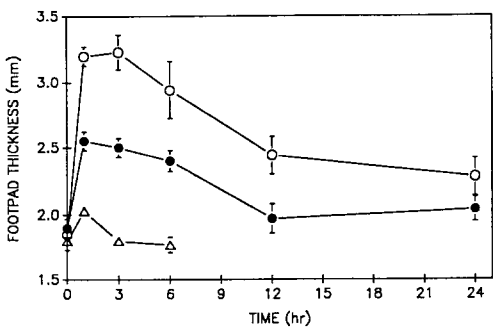

Fig. 5. Neutralization of the edema induced by myotoxin II in the mouse footpad by LA-heparin. Myotoxin II was mixed with either saline (○) or LA-heparin (●) at a ratio of 5 μg heparin/μg myotoxin, and incubated for 15 min at room temperature. Then, 50 μL of each solution (containing 100 μg myotoxin II) were injected s.c. into the footpads of mice. Control mice received 50 μL of saline only (△). Edema was estimated by measuring the footpad thickness at the indicated time points, as described in Materials and Methods. Each point represents the mean ± SD of four animals.

The heparin-binding components in *B. asper* venom were isolated by affinity chromatography, eluting at approximately 0.5 M NaCl concentration. SDS–PAGE of this fraction showed a main band of 14–15 kDa under reducing conditions, which corresponds to the subunit molecular mass of myotoxins, and very small amounts of few other components (Fig. 6A). Native PAGE for basic proteins showed that all described myotoxin isoforms bound to the immobilized heparin column (Fig. 6B). Gel diffusion demonstrated that heparins form a precipitable complex with venom myotoxins (Fig. 6C).

Since affinity chromatography and electrophoresis showed that heparin interacts not only with myotoxin II, but also with other isoforms present in this venom, the effect of heparins on the enzymatic and myotoxic activities of myotoxin III was investigated. Both types of heparins significantly reduced the myotoxic effect of this isoform *in vivo*, without inhibiting its enzymatic activity in an indirect hemolysis assay (Fig. 7). Control erythrocyte suspensions treated with myotoxin III showed no hemolysis in the absence of phospholipids.

Since free heparan sulfate was found to block the cytolytic action of myotoxin II (Table 2), the possible role of cell surface heparan sulfate in the mechanism of cytotoxicity was investigated (Fig. 8). Both heparitinase and chlorate treated L6 and tEnd cells were equally susceptible to myotoxin II, in comparison with their respective controls (Fig. 8A and 8B). In agreement with this, myotoxin II induced a comparable cytolytic effect in heparan sulfate defective mutant cells CHO-*pgs*D-677 and CHO-K1 control cells (Fig. 8C).

DISCUSSION

This work demonstrates that glycosaminoglycans of the heparin/heparan sulfate family block the cytolytic action of basic myotoxic PLA_2s from the venom of *B. asper*, both in cell culture and *in vivo*. This inhibition is clearly due to the formation of a complex, which is held, at least in part, by electrostatic interactions between the negatively-charged groups of heparins and the numerous positively-charged amino acid residues of myotoxins, which are highly basic proteins. This is supported

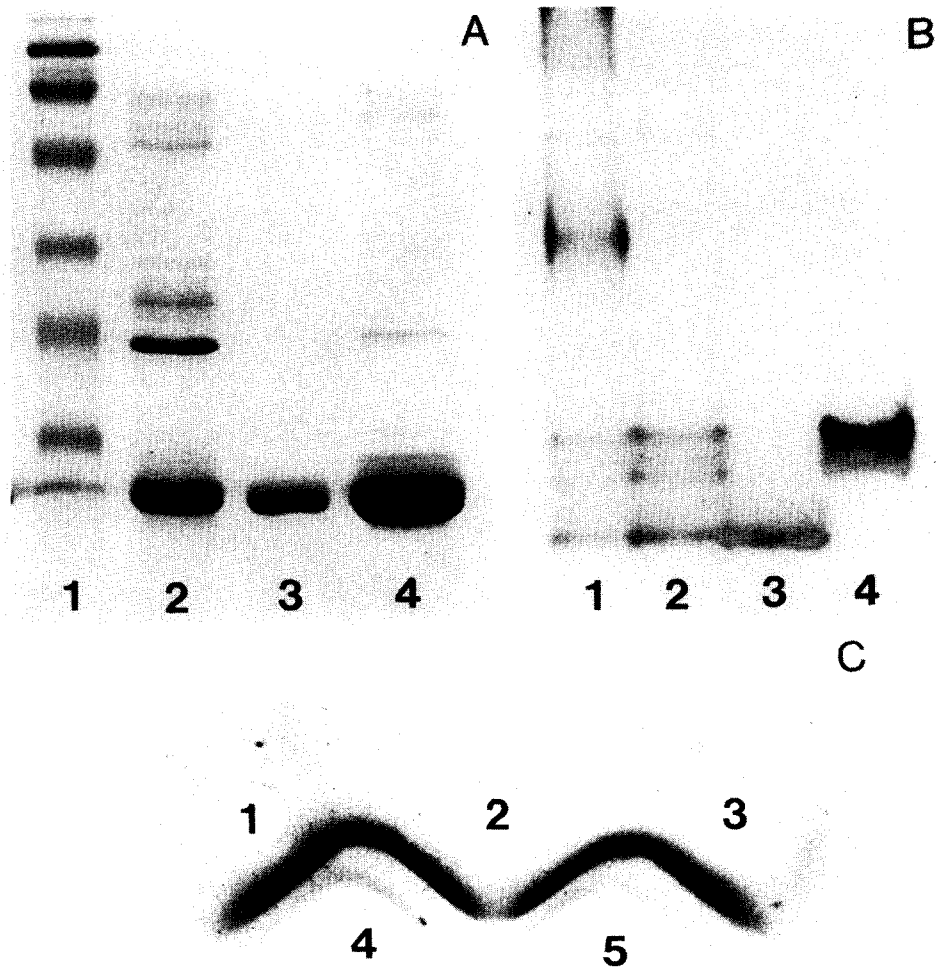

Fig. 6. *B. asper* venom myotoxins bind to heparin. Whole venom was fractionated on a column of heparin-agarose (Sigma) as described in Materials and Methods, and a heparin-binding fraction was obtained by salt elution. (A) Analysis of the heparin-binding fraction by SDS–PAGE, 15% (anode at the bottom, Coomassie blue R-250 stain). Lane 1: molecular mass standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa); lane 2: crude *B. asper* venom, 10 μg; lane 3: myotoxin II, 5 μg; lane 4: heparin-binding fraction, 20 μg. All samples were reduced with 2-mercaptoethanol at 95°. (B) Analysis of the heparin-binding fraction by cathodic PAGE under native conditions (Coomassie R-250 stain, cathode at the bottom). Lane 1: crude *B. asper* venom, 10 μg; lane 2: heparin-binding fraction, 20 μg; lane 3: myotoxin II, 10 μg; lane 4: mixture of myotoxins I and III, 10 μg. (C) Gel diffusion in 1% agarose–PBS. Wells were filled with 20 μL of the following solutions: 1 and 3: crude *B. asper* venom, 20 mg/mL; 2: myotoxin II, 0.5 mg/mL; 4: standard heparin, 0.17 mg/mL; 5: LA-affinity heparin, 0.17 mg/mL. Coomassie blue R-250 stain.

by the direct observation of a precipitate in gel diffusion, and by the dissociation of myotoxins from the heparin affinity column with an increasing ionic strength gradient. Melo *et al.* [9], using gel filtration, also obtained evidence for complex formation between heparin and a myotoxic PLA_2 from *B. jararacussu* venom, that has similar antigenic [27] and physicochemical [28] characteristics to *B. asper* myotoxins. Venoms from many species of *Bothrops*, distributed in Latin America, as well as other crotalids such as *Trimeresurus flavoviridis* from Japan, contain components that cross-react antigenically with *B. asper* myotoxins [27], and therefore are presumably myotoxic PLA_2s with similar properties, as confirmed in several cases [29–31]. Thus, the present results might be relevant also for other myotoxic PLA_2s. Indeed, a significant reduction in the muscle-damaging activity of crude venoms from crotalid species other than *B. asper* was obtained by the use of LA-heparin. Interestingly, the venoms that were inhibited correspond to those containing PLA_2s antigenically-related to those of *B. asper*, *T. flavoviridis* [27] and *B. jararacussu* [27], the latter result confirming the original report by

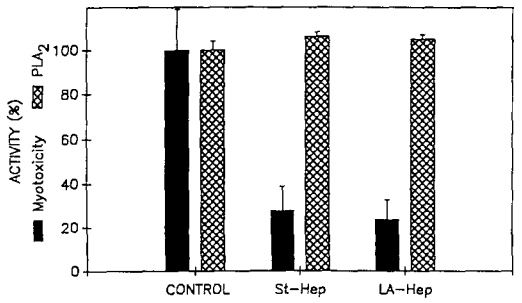

Fig. 7. Heparins block the myotoxic activity of myotoxin III, but not its PLA_2 activity. Either standard (St-Hep) or low affinity (LA-Hep) heparins were mixed with myotoxin III at a ratio of 5 μg heparin/μg myotoxin, and incubated for 15 min at room temperature. Then, mixtures were assayed for *in vivo* myotoxic activity (50 μg myotoxin/mouse) or *in vitro* PLA_2 activity (5 μg myotoxin/tube). Activities are expressed as a percentage, considering the activity of the toxin alone as 100%. Bars represent means ± SD of triplicate determinations.

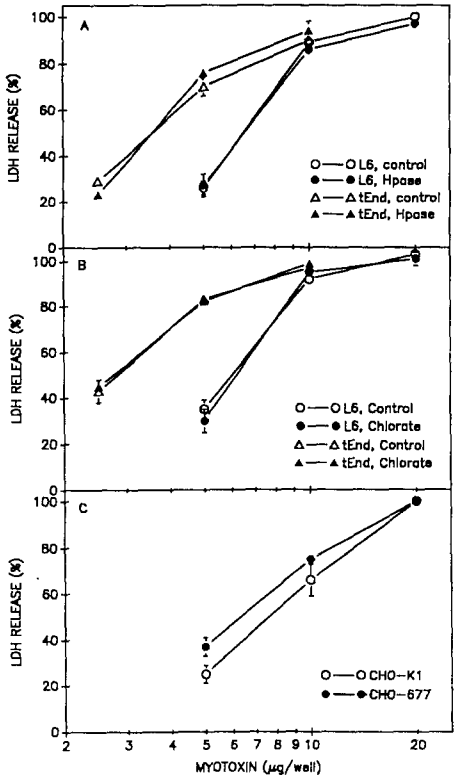

Fig. 8. Cell surface heparan sulfate is not required for the cytolytic activity of myotoxin II. (A) Cell cultures of myoblasts (L6; circles) or endothelial cells (tEnd; triangles) were treated with heparitinase (1.25 U/100 μL PBS/well; Hpase) for 2 hr at 37° (filled symbols) or PBS alone as a control (empty symbols), washed, and then exposed to different amounts of myotoxin II. Cytotoxicity is estimated by the release of LDH, under the same conditions as in Fig. 3. All points represent means ± SD of triplicate wells. No significant differences are found between the two treatments. (B) Cell cultures of myoblasts (L6; circles) or endothelial cells (tEnd; triangles) were grown for 2 days in the presence of 10 mM sodium chlorate-containing medium (filled symbols) or normal medium as a control (empty symbols), and then exposed to myotoxin II. No significant differences are found between the two treatments. (C) Chinese hamster ovary cells were used as targets for myotoxin II action. CHO-*pgs*D-677 (●) is a mutant cell line defective in heparan sulfate synthesis and CHO-K1 (○) is the wild type control. LDH was determined at 3 hr. Only the difference between the values obtained with 5 μg toxin is statistically significant ($P < 0.05$).

Melo *et al.* [8]. Similarly, although the venom of *A. p. piscivorus* has not been tested for the presence of PLA_2s antigenically-related to those of *B. asper*, the closely related species *A. bilineatus* contains such component(s) [32]. Also, a high degree of sequence homology has been found between *B. asper* myotoxin II and a Lys-49 PLA_2 of *A. p. piscivorus* venom [11]. Other venoms tested, despite containing basic PLA_2s with myotoxic activity (*N. n. atra, C. d. terrificus*), or basic myotoxic peptides (*C. v. viridis*) [33], were not susceptible to heparin neutralization. Further studies using purified PLA_2s/myotoxins from these genera are needed, as the experiments were performed with crude venoms. Nevertheless, these findings suggest that the interaction of heparins with some toxic PLA_2s could be based not only on a non-specific electrostatic interaction due to the basic character of the enzymes, but that some specific recognition component might additionally be involved. The lack of myotoxin II neutralization by chondroitin sulfate is also in agreement with this speculation. The structural nature of the interaction between heparins and myotoxic PLA_2s is currently being investigated.

Interestingly from the medical point of view, the ability of heparin to neutralize the cytolytic action of myotoxins did not depend on its anticoagulant activity, since similar results were obtained with conventional heparin and LA-heparin. This is of relevance since *Bothrops* venoms contain potent hemorrhagic toxins and also severely disturb coagulation [34, 35]. Indeed, the potentiating effect of standard heparin on the hemorrhagic action of the venom was evident in the intravital microscopy experiments, emphasizing the potential risks of its use.

As shown by the electrophoretic analyses, all myotoxin isoforms described in *B. asper* venom [36] could be recovered from a heparin affinity column. Of these, myotoxin II, a Lys-49 PLA_2 isoform, was studied in more detail. Since the muscle damaging effect of these PLA_2s is currently considered to be related to their ability to penetrate phospholipid bilayers and alter cell membrane permeability [37–41], an *in vitro* cytotoxicity system was utilized. Myotoxin II was clearly cytolytic to skeletal muscle myoblasts and capillary endothelial cells, the latter cell type being significantly more susceptible. This was unexpected, as it has recently been described that myotoxin III from the same venom is more toxic to cultured L6 myoblasts than to a variety of other cell types, although endothelial cells were not tested [42]. The ratios at which heparins completely

blocked this cytotoxic effect suggest that several toxin molecules may simultaneously be neutralized by one heparin molecule. This could be explained by the polysaccharide nature of heparin, with its linear repeating unit structure [43]. However, the neutralizing efficiency of heparins in the myotoxicity tests *in vivo* was lower than in the cytotoxicity assay *in vitro*. This could be due to several reasons. One possibility to explain this difference would be a partial dissociation of myotoxin/heparin complexes, due to the competition caused *in vivo* by high affinity heparin-binding factors. On the other hand, although the group of basic PLA_2 myotoxins is the main mediator of muscle damage in this venom [18], it is possible that other factors not affected by heparins, for example acidic hemorrhagic toxins causing ischemia, could contribute to some degree to myonecrosis [33]. Nevertheless, even when using purified myotoxins, the neutralizing efficiency of heparin was lower *in vivo* than *in vitro*. Still another alternative, although at present speculative, explanation is that myotoxins would have a significantly higher affinity for mature muscle fibers than for myoblasts (or other cell types) utilized in culture. Although it is clear that several types of cells can be killed *in vitro* by myotoxic PLA_2s of *Bothrops* venoms [41, 42, and present study], the only cell type reported to undergo necrosis *in vivo* is the mature muscle fiber [37]. Immature muscle cell precursors are likely to be less susceptible to myotoxin action, as suggested by the regeneration of affected muscles [44, 45]. The determination of the binding affinity of myotoxins to heparin, and to diverse cell types in culture, particularly to more differentiated muscle cell stages such as myotubes, would shed light on this hypothesis. However, the results obtained with the neutralizing monoclonal antibody MAb-3, do not agree with the concept of a differential affinity/susceptibility to myotoxins of mature muscle, compared to the cultured cell lines: the neutralizing potency of MAb-3 *in vitro* was similar to that reported *in vivo* [18], with a complete inhibition of myotoxin II achieved at an antibody/toxin molar ratio of about 1/1. Further work is needed to clarify the different neutralizing potency of heparins *in vitro* and *in vivo*.

The possibility of an effect of heparins on the target cells, rather than on the toxin, was clearly excluded. The details of the mechanism of myotoxin neutralization by heparins, remain to be determined. Diccianni *et al.* [3] demonstrated that heparin inhibits the hydrolysis of micellar phospholipid substrates by porcine pancreatic PLA_2, by binding to its amino-terminal region, known to act as the interface recognition site. It would be of interest to determine if heparin binds to an analogous region of myotoxic PLA_2s, thereby interfering with penetration and subsequent perturbation of cell membrane homeostasis.

The interaction of heparin with PLA_2 of different origins has been reported to affect enzymatic activity in some cases [2, 3, 46], but not in others [1, 47, 48]. In the case of myotoxin III, its PLA_2 activity was unaffected by heparins, despite that its myotoxic effect was significantly reduced. Thus, the two toxin actions were clearly dissociated, in agreement with earlier results obtained with neutralizing monoclonal antibodies [18].

LA-heparin was also able to neutralize the edema induced by myotoxin II, in addition to its cytotoxic action. Although the edema-forming effect of PLA_2s has usually been correlated to their ability to hydrolyse phospholipids [49–51], the discovery of Lys-49 PLA_2s devoid of enzymatic activity but still able to elicit edema [10, 52], implies that other mechanisms can also participate in this pharmacological activity. Among these mechanisms might be a direct action on mast cells [52, 53] or on vascular endothelium. In this regard, it is interesting to note that myotoxin II had direct effects on endothelial cells *in vitro*, which were inhibited by heparins. However, since the exact mechanisms by which Lys-49 PLA_2s induce edema *in vivo* are not known, it is difficult to speculate about the mode of heparin neutralization of this effect. Indeed, it would be reasonable to consider the possibility that the edema induced by myotoxin II would be a response to the rapid muscle fiber damage [15], and that neutralization of myotoxicity by heparins would result in an inhibition of this response. However, the observation that some venoms potently induce myonecrosis without a detectable edema response [54] argues against this hypothesis. A similar inhibitory activity of heparin on the edema induced by a PLA_2 from *Trimeresurus mucrosquamatus* has been reported [53].

The ability of free heparan sulfate to block the cytolytic activity of myotoxin II led us to investigate the possible role of cell surface heparan sulfate proteoglycan in the cytotoxic mechanism of these toxins, either as a protecting factor or as a first receptor/susceptibility factor, since their cell binding site(s) have not yet been identified. The results obtained by a combination of approaches, including heparitinase and chlorate cell treatments, as well as the use of CHO-*pgs*D-677 mutant cell line, all indicated that cell surface heparan sulfate is not required by these toxins to exert their cytolytic action.

The present results suggest that the potential of LA-heparin, or even smaller heparin fragments devoid of anticoagulant effects, should be experimentally evaluated as a possible aid in the treatment of snakebites from certain species. Bergonzini *et al.* [55] demonstrated that bioavailability and distribution of heparins in biological compartments depend on their molecular weight. The screening of different heparin-derived disaccharides showed that the neutralizing effect cannot be achieved with such small structures, and therefore requires slightly larger oligosaccharides, as has been shown for the interaction of heparin with other proteins [56]. Thus, the determination of the minimal heparin structure capable to block the cytolytic effect of myotoxic PLA_2s, possibly with a faster tissue distribution than antibodies [57], will point out candidate compounds to be evaluated in future studies.

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REFERENCES

1. Horigome K, Hayakawa M, Inoue K and Nojima S, Purification and characterization of phospholipase A_2 released from rat platelets. *J Biochem* **101**: 625–631, 1987.
2. Diccianni MB, Mistry MJ, Hug K and Harmony JAK, Inhibition of phospholipase A_2 by heparin. *Biochim Biophys Acta* **1046**: 242–248, 1990.
3. Diccianni MB, Lilly M, McLean LR, Balasubramaniam A and Harmony JAK, Heparin prevents the binding of phospholipase A_2 to phospholipid micelles: importance of the amino-terminus. *Biochemistry* **30**: 9090–9097, 1991.
4. Kini RM and Evans HJ, A model to explain the pharmacological effects of snake venom phospholipases A_2. *Toxicon* **27**: 613–635, 1989.
5. Davidson FF and Dennis EA, Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venoms to human secreted forms. *J Mol Evol* **31**: 228–238, 1990.
6. Gutiérrez JM, Chaves F, Bolaños R, Cerdas L, Rojas E, Arroyo O and Portilla E, Neutralización de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon* **19**: 493–500, 1981.
7. Ownby CL, Odell GV, Woods WM and Colberg TR, Ability of antiserum to myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom to neutralize local myotoxicity and lethal effects of myotoxin and homologous crude venom. *Toxicon* **21**: 35–45, 1983.
8. Melo PA and Suarez-Kurtz G, Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. *Toxicon* **26**: 87–95, 1988.
9. Melo PA, Homsi-Brandeburgo MI, Giglio JR and Suarez-Kurtz G, Antagonism of the myotoxic effect of *Bothrops jararacussu* venom and bothropstoxin by polyanions. *Toxicon* **31**: 285–291, 1993.
10. Lomonte B and Gutiérrez JM, A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**: 725–733, 1989.
11. Francis B, Gutiérrez JM, Lomonte B and Kaiser II, Myotoxin II from *Bothrops asper* (terciopelo) venom is a lysine-49 phospholipase A_2. *Arch Biochem Biophys* **284**: 352–359, 1991.
12. Kaiser II, Gutiérrez JM, Plummer D, Aird SD and Odell GV, The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Arch Biochem Biophys* **278**: 319–325, 1990.
13. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
14. Reisfeld RA, Lewis UJ and Williams DE, Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**: 281–283, 1962.
15. Lomonte B, Lundgren J, Johansson B and Bagge U, The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon* **32**: 41–55, 1994.
16. Bussolino F, De Rossi M, Sica A, Colotta F, Wang JM, Bocchietto E, Padura IM, Bosia A, Dejana E and Mantovani A, Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J Immunol* **147**: 2122–2129, 1991.
17. Lomonte B and Kahan L, Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26**: 675–689, 1988.
18. Lomonte B, Gutiérrez JM, Ramírez M and Díaz C, Neutralization of myotoxic phospholipases A_2 from the venom of the snake *Bothrops asper* by monoclonal antibodies. *Toxicon* **30**: 239–245, 1992.
19. Mancini G, Carbonara AO and Heremans JF, Immunochemical quantitation of antigens by radial immunodiffusion. *Immunochemistry* **2**: 235–254, 1965.
20. Lomonte B, Tarkowski A and Hanson LÅ, Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **1**: 93–105, 1993.
21. Gutiérrez JM, Avila C, Rojas E and Cerdas L, An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**: 411–413, 1988.
22. Ouchterlony Ö and Nilsson LÅ, Immunodiffusion and immunoelectrophoresis. In: *Handbook of Experimental Immunology* (Ed. Weir DM), pp. 19.1–19.44. Blackwell Scientific Publications, Oxford, 1978.
23. WuDunn D and Spear PG, Initial interaction of Herpes Simplex virus with cells is binding to heparan sulfate. *J Virol* **63**: 52–58, 1989.
24. Greve H, Cully Z, Blumberg P and Kresse H, Influence of chlorate on proteoglycan biosynthesis by cultured human fibroblasts. *J Biol Chem* **263**: 12886–12892, 1988.
25. Olwin BB and Rapraeger A, Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J Cell Biol* **118**: 631–639, 1992.
26. Lidholt K, Weinke JL, Kiser CS, Lugemwa FN, Bame KJ, Cheifetz S, Massagué J, Lindahl U and Esko JD, A single mutation affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc Natl Acad Sci USA* **89**: 2267–2271, 1992.
27. Lomonte B, Furtado MF, Rovira ME, Carmona E, Rojas G, Aymerich R and Gutiérrez JM, South American snake venom proteins antigenically-related to *Bothrops asper* myotoxins. *Braz J Med Biol Res* **23**: 427–435, 1990.
28. Cintra ACO, Marangoni S, Oliveira B and Giglio JR, Bothropstoxin-I: amino acid sequence and function. *J Protein Chem* **12**: 57–64, 1993.
29. Lomonte B, Gutiérrez JM, Furtado MF, Otero R, Rosso JP, Vargas O, Carmona E and Rovira ME, Isolation of basic myotoxins from *Bothrops moojeni* and *Bothrops atrox* snake venoms. *Toxicon* **28**: 1137–1146, 1990.
30. Kihara H, Uchikawa R, Hattori S and Ohno M, Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipases A_2. *Biochem Int* **28**: 895–903, 1992.
31. Díaz C, Gutiérrez JM and Lomonte B, Isolation and characterization of basic myotoxic phospholipases A_2 from *Bothrops godmani* (Godman's pitviper) snake venom. *Arch Biochem Biophys* **298**: 135–142, 1992.
32. Lomonte B, Moreno E and Gutiérrez JM, Detection of proteins antigenically-related to *Bothrops asper* myotoxin in crotaline snake venoms. *Toxicon* **25**: 947–955, 1987.
33. Ownby CL, Locally acting agents: myotoxins, hemorrhagic toxins and dermonecrotic factors. In: *Hand-*

book of Toxinology (Eds. Shier WT and Mebs D), pp. 601–654. Marcel Dekker, New York, 1990.
34. Gutiérrez JM and Lomonte B, Local tissue damage induced by *Bothrops* snake venoms: a review. *Memorias Inst Butantan* **51**: 211–223, 1989.
35. Tan NH and Ponnudurai G, A comparative study of the biological properties of some venoms of snakes of the genus *Bothrops* (American lance-headed viper). *Comp Biochem Physiol* **100B**: 361–365, 1991.
36. Lomonte B and Carmona E, Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. *Comp Biochem Physiol* **102B**: 325–329, 1992.
37. Gutiérrez JM, Ownby CL and Odell GV, Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp Mol Pathol* **40**: 367–379, 1984.
38. Díaz C, Gutiérrez JM, Lomonte B and Gené JA, The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. *Biochim Biophys Acta* **1070**: 455–460, 1991.
39. Rufini S, Cesaroni P, Desideri A, Farias R, Gubensek F, Gutiérrez JM, Luly P, Massoud R, Morero R and Pedersen JZ, Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* **31**: 12424–12430, 1992.
40. Bultrón E, Gutiérrez JM and Thelestam M, Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. *Toxicon* **31**: 217–222, 1993.
41. Brusés JL, Capaso J, Katz E and Pilar G, Specific *in vitro* biological activity of snake venom myotoxins. *J Neurochem* **60**: 1030–1042, 1993.
42. Bultrón E, Thelestam M and Gutiérrez JM, Effects on cultured mammalian cells of myotoxin III, a phospholipase A_2 isolated from *Bothrops asper* (terciopelo) venom. *Biochim Biophys Acta* **1179**: 253–259, 1993.
43. Kjellén L and Lindahl U, Proteoglycans: structures and interactions. *Annu Rev Biochem* **60**: 443–475, 1991.
44. Gutiérrez JM, Ownby CL and Odell GV, Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (fer-de-lance). *Toxicon* **22**: 719–731, 1984.
45. Harris JB and Cullen MJ, Muscle necrosis caused by snake venoms and toxins. *Electron Microsc Rev* **3**: 183–211, 1990.
46. Franson R, Patriarca P and Elsbach P, Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. *J Lipid Res* **15**: 380–388, 1974.
47. Condrea E and de Vries A, Hemolysis and splitting of human erythrocyte phospholipids by snake venoms. *Biochim Biophys Acta* **84**: 60–73, 1964.
48. Ho CL, Tsai IH and Lee CY, The role of enzyme activity and charge properties on the presynaptic neurotoxicity and the contracture-inducing activity of snake venom phospholipases A_2. *Toxicon* **24**: 337–345, 1986.
49. Lloret S and Moreno JJ, Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. *Toxicon* **31**: 949–956, 1993.
50. Vishwanath BS, Fawzy AA and Franson RC, Edema-inducing activity of phospholipase A_2 purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation* **12**: 549–561, 1988.
51. Cirino G, Peers SH, Wallace JL and Flower RJ, A study of phospholipase A_2-induced oedema in rat paw. *Eur J Pharmacol* **166**: 505–510, 1989.
52. Liu CS, Chen JM, Chang CH, Chen SW, Teng CM and Tsai IH, The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim Biophys Acta* **1077**: 362–370, 1991.
53. Wang JP and Teng CM, Comparison of the enzymatic and edema-producing activities of two venom phospholipase A_2 enzymes. *Eur J Pharmacol* **190**: 347–354, 1990.
54. Gutiérrez JM, Lomonte B, Portilla E, Cerdas L and Rojas E, Local effects induced by coral snakes (*Micrurus*) venoms: evidence of myonecrosis after experimental inoculation of venom from five species. *Toxicon* **21**: 777–783, 1983.
55. Bergonzini GL, Bianchini P, Mascellani G, Osima B, Parma B and Volpi N, Different pharmacokinetics, antithrombotic activity and bleeding effects of heparin and two new fragments administered in rat by subcutaneous route. *Arzneim-Forsch/Drug Res* **42**: 1322–1328, 1992.
56. Maccarana M and Lindahl U, Mode of interaction between platelet factor 4 and heparin. *Glycobiology* **3**: 271–277, 1993.
57. Rovira ME, Carmona E and Lomonte B, Immunoenzymatic quantitation of antibodies to *Bothrops asper* myotoxins after polyvalent antivenom administration in mice. *Braz J Med Biol Res* **25**: 23–33, 1992.