Toxicon 58 (2011) 293–303

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

The effects of Lonomin V, a toxin from the caterpillar (*Lonomia achelous*), on hemostasis parameters as measured by platelet function

Belsy Guerrero [a], Carmen L. Arocha-Piñango [a], Ana M. Salazar [a], Amparo Gil [a], Elda E. Sánchez [b], Alexis Rodríguez-Acosta [c], Sara Lucena [a,*]

[a] *Laboratorio de Fisiopatología, Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 20632, P.O. Box 21827, Caracas 1020A, Republic of Venezuela*
[b] *Department of Chemistry, College of Arts and Sciences, Texas A&M University-Kingsville, MSC 158, Kingsville, TX 78363, USA*
[c] *Sección de Inmunoquímica, Instituto de Medicina Tropical de la Universidad Central de Venezuela, Caracas, Republic of Venezuela*

ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 15 April 2011
Accepted 12 July 2011
Available online 28 July 2011

Keywords:
Lonomia achelous caterpillars
Lonomin V
Platelets
Primary hemostasis

ABSTRACT

Platelets play a central role in hemostasis during vascular injury. Patients affected with the hemorrhagic syndrome caused by contact with *Lonomia achelous* caterpillars (*Lac*) Lepidoptera distributed in various South American countries, show digestive, pulmonary and intraperitoneal bleeding in combination with hematomas and echymosis. In the present study, we have evaluated the effects of Lonomin V (serine protease isolated from *Lac* hemolymph) on some functional properties of platelets, evaluating its importance in primary hemostasis. Platelet adhesion to fibrinogen was reduced by 19, 20, 36, and 37% after pre-treated with 0.2, 2, 20 and 40 nM of Lonomin V, respectively. Pre-incubation of the platelets with 408 nM of Lonomin V, for 4 min at 37 °C, resulted in complete inhibition of the collagen-induced platelet aggregation, in contrast to 56% inhibition of the ADP - induced platelet aggregation. Lonomin V also inhibited anti-$\alpha_{IIb}\beta_3$ integrin binding to platelets by 56, 57, 52 and 54% at concentrations of 0.2, 2, 20 and 40 nM respectively. Additionally, Lonomin V inhibited anti-P-selectin binding to platelets by 28, 37, 33 and 33% at the same concentrations. The platelets tested with Lonomin V did not modify their viability. In summary, Lonomin V inhibited platelet aggregation, probably caused by the degradation of collagen. The anti-platelet activity of Lonomin V has been shown to be unique and a potentially useful tool for investigating cell-matrix and cell–cell interactions and for the development of antithrombotic agents in terms of their anti-adhesive activities.

1. Introduction

Platelets play a central role in hemostasis throughout a vascular injury. The first step involves platelet adhesion to the exposed sub-endothelium, which is mediated by platelet surface glycoproteins (GPIb/V/IX complex, GPVI, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins) engagement, allowing platelets to bind to exposed ligands (subendothelial-bound von Willebrand factor, collagen, fibronectin and laminin) (Kamiguti, 2005). It is a complex process that depends on shear stress, because under low shear conditions, platelets via β1 integrins mostly adhere to collagen, fibronectin and laminin. In contrast, in high shear conditions, platelets adhere to subendothelial-bound von Willebrand factor through the GPIb (Ruggeri and Mendolicchio, 2007). In the latter condition, these interactions initiate platelet activation by increasing calcium influx across the platelet membranes. Afterward, this promotes dense granule ADP secretion, which induces the activation of $\alpha_{IIb}\beta_3$ integrin (via inside-out signaling). Then, platelet–platelet interactions or aggregation are initiated by the binding of fibrinogen or RGD-containing ligands to the activated $\alpha_{IIb}\beta_3$

* Corresponding author. Tel.: +58 212 504 1564x1140; fax: +58 212 504 1086.
E-mail address: selucena@gmail.com (S. Lucena).

0041-0101/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2011.07.003

integrins, which generates signals (via outside-in signaling), ending in irreversible aggregates formation followed by cessation of hemorrhage. Therefore, the key factor for successful platelet function is the decisive activation of the $\alpha_{IIb}\beta_3$ integrin (Yeh et al., 1998; Wei et al., 2009).

Platelet adhesion and aggregation are crucial for primary hemostasis in order to prevent posttraumatic blood loss by providing a platelet plug that leads to the closure of smaller defects of the vessel wall (Nieswandt and Offermanns, 2004; Varga-Szabo et al., 2008). Activated platelets via the integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) provide a guide for the assembly of coagulation factors and the cross-linked fibrin meshwork formation (secondary hemostasis) (Mc Michael, 2005; Palomo et al., 2008). Platelets are vital in the hemostasis and fibrin formation processes. Platelets are also involved in thrombogenesis and fibrinolysis even though endothelial cells are primarily involved in these processes (Bradley et al., 2007).

Patients affected by the hemorrhagic syndrome caused by contact with *Lonomia achelous* caterpillars, show digestive, pulmonary and intraperitoneal bleeding in combination with hematomas and ecchymosis (Lucena et al., 2006). Blood coagulation tests show prolongation of PT (Prothrombin Time), aPTT (activated Partial Thromboplastin Time) and ThT (Thrombin Time). There is a decrease of fibrinogen (Fg), factor V (FV), factor XIII (FXIII), plasminogen (Pg) and α2-antiplasmin. Factor VIII and von Willebrand factor are increased while the platelet count is unaffected. Fibrin Degradation Products (FDPs) are increased and D-dimers are present in most of the cases. Recently, it was demonstrated that Lonomin V, a serine protease of 24 kDa isolated from hemolymph of this caterpillar, degraded fibronectin, a protein implicated in platelet adhesion, with concomitant impairment of its biological properties (Lucena et al., 2006, 2008). Until now, besides coagulation and fibrinolysis disturbances, the probable effects of caterpillar *L. achelous* venom on platelets have not been yet studied. In the current study, we have evaluated the effects of Lonomin V on several functional properties of platelets evaluating its importance in primary hemostasis.

2. Materials and methods

2.1. Reagents

Benzamidine-Sepharose and Phenyl Sepharose were from Pharmacia Biotechnology (Uppsala, Sweden). DEAE-Trisacryl was from Sepracor S.A. (Villeneuve la Garenne, France). Protein-Pak 300 column was from Waters (Milford, MI, USA). ADP, collagen, thrombin and ristocetin as platelet aggregation inducer were purchased from Chrono-log (Havertown, USA). Anti-P-Selectin-FITC, anti-$\alpha_{IIb}\beta_3$-PE, and anti-$\alpha_5\beta_1$, secondary antibody conjugated with FITC and non-specific immunoglobulin G (IgG) were purchased from Becton Dickinson (FACStar, Braintree, MA, U.S.A.). Bicinchoninic acid, *p*-nitrophenyl phosphate, prostaglandin E_1, apyrase, 3-(4,5-dimethylthiazol-2-methyl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), benzamidine/HCl and other chemicals and solvents were from Sigma Chemical Co (St. Louis, MO, USA). Human fibrinogen was acquired from American Diagnostica Inc. (Stamford, CT, USA). Chromogenic substrate (S-2444) was purchased from Chromogenix AB (Milano, Italy). Molecular mass standards for gel filtration were from Bio-Rad (Bio-Rad Laboratories Ltd. California, USA). P-selectin and platelet membrane glycoprotein IIb-IIIa were adquired from Calbiochem (California, USA).

2.2. Isolation of Lonomin V

Lonomin V was isolated from *L. achelous* hemolymph by a modification of the Guerrero et al., method (Guerrero et al., 2001). Briefly, the hemolymph was dialyzed (2 kDa molecular weight cut-off) against 0.021 M Tris-HCl, pH 8.6 (buffer A) and centrifuged for 5 min at 10,000 *g* to remove insoluble material. The supernatant was applied on a DEAE-Trisacryl column (27 × 1.5 cm), previously equilibrated with buffer A. Elution was carried out at a 1 mL/min flow rate first with buffer A and later with a 0.15 M NaCl and 0.5 M, in the same buffer. The first fraction with elevated urokinase-like activity (FDI) was precipitated with 36% ammonium sulfate and applied to a Phenyl Sepharose CL-4B column (1 × 10 cm) equilibrated with 0.05 M Tris-0.5 M $(NH_4)_2SO_4$, pH 7.5 (buffer B). The elution was carried out with a 0.25 mL/min flow rate, first with buffer B, followed by a 0–60% ethylene glycol gradient in buffer B. Active fraction (FPIII) was concentrated by ultrafiltration using an Amicon YM-2 membrane and was re-chromatographed by an affinity Benzamidine-Sepharose column (1 × 10 cm) equilibrated with 0.05 M Tris - 0.5 M NaCl pH 7.4. The elution was carried out at a 1 mL/min flow rate with 0.05 M Glycine pH 3; the fractions (1 mL) were collected into tubes that contained 100 μL 1 M Tris, pH 9. Active fractions were collected, lyophilized, and dried using a Speed-Vac system. The protein concentration was determined at 280 nm. In all chromatographic steps the proteolytic activity was followed by urokinase-like amidolytic activity using chromogenic substrate S-2444 (Guerrero et al., 2001).

2.3. SDS-PAGE analysis

Polyacrylamide gel electrophoresis was carried out following the Laemmli (1970) method using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). Protein bands were visualized by staining with Coomassie Blue R-250. Molecular mass estimations were determined using commercial standard proteins.

2.4. Amidolytic activity

Amidolytic activity of Lonomin V was measured by a micromethod standardized by Guerrero et al. (2001). Briefly, in 96-well polystyrene plates, a mixture of 80 μL of the recommended buffer for substrate, 10 μL of the venom or fractions (0.1–1 mg/mL) and 10 μL of substrate 0.16 mM S-2444, were placed in each well. After incubation at 37 °C for 30 min, the absorbance at 405 nm was measured. Specific activity was calculated as mUA/min/mg.

2.5. Fibrinolytic activity

Fibrinolytic activity of Lonomin V was studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972). Briefly, fibrin plates were settled using 3-cm diameter Petri dishes with 1.5 mL of a 0.1% plasminogen-rich fibrinogen (10% plasminogen as contaminant) in 5 mM imidazol saline buffer, pH 7.4. The fibrinogen was allowed to clot by adding 75 μL of bovine thrombin (10 IU/mL, in 0.025 M $CaCl_2$). The mixture was incubated at room temperature for 30 min and then 10 μL (1–50 μg) of each sample were applied over the fibrin. After 24 h incubation at 37 °C, the diameters of the fibrin hydrolysis (lysed areas) were measured. The activity was then recorded in mm^2, which was a measurement of the lysis areas consisting of the greatest and least diameters, which could be measured at right angles.

2.6. Proteolytic activity on fibrinogen, collagen, GPIIb-IIIa and P-selectin

Degradation of fibrinogen, P-selectin, and GPIIb-IIIa was evaluated by HPLC using a Protein-Pak 300 column (Waters Delta 600, Millipore Corp, Milford, Massachusetts, USA) equilibrated with 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4. The elution was carried out at a flow rate of 0.5 mL/min with same buffer. Prior to HPLC studies, proteins were incubated with Lonomin V at a 1 μg Lonomin V:100 μg protein ratio, for 6 h at 37 °C. The samples were loaded at room temperature into a Protein-Pak 300 column. Fibrinogen chains were visualized using a 12% SDS-PAGE gel (Laemmli, 1970), incubated with Lonomin V at a 1 μg Lonomin V:100 μg fibrinogen ratio, at different times at 37 °C.

Type IV collagen was incubated with Lonomin V at a 1 μg Lonomin V:100 μg collagen ratio, at different times at 37 °C. Degradation of collagen was visualized by a 4–12% NuPAGE Bis-Tris Mini gel using the XCell *SureLock* Mini-Cell (Invitrogen Life Technologies, USA). The gel was stained with 100 mL Simply Blue Safe Stain (Invitrogen Life Technologies, USA).

2.7. Fibrinogen platelet adhesion

2.7.1. Preparation of platelet-rich plasma and washed human platelet suspensions

Blood freshly obtained from three healthy adult volunteers who had confirmed not using any drugs known to interfere with platelet function, during the previous 14 days, was collected with a ratio of 1:6 of acid citrate/dextrose solution-ACD (85 mM sodium citrate; 71 mM citric acid; 111 mM dextrose). The platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 190 *g* for 20 min. Then, it was centrifuged at 1700 *g* for 15 min at 24 °C, in presence of 0.25 μg/mL prostaglandin E-1 (platelet activation inhibitor). Afterward, the pellet of platelets was washed with Tyrode's buffer (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM Na_2HPO_4, 6 mM glucose, 0.2% human serum albumin) pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.5 IU/mL apyrase. The suspension was centrifuged at 1700 *g* for 15 min at 24 °C and the washing was repeated. The washed platelets were finally suspended in Tyrode's buffer. Platelets were counted using a Neubauer chamber and adjusted to the concentration required using Tyrode's buffer.

2.7.2. Fibrinogen platelet adhesion

To determine the effect of Lonomin V on platelet adhesion, washed platelets were used to eliminate procoagulant proteins present in plasma, which could activate the platelets, altering the assay results. Platelet adhesion was measured by the Lucena et al. (2008) method. Briefly, 100 μL of fibrinogen (2 mg/mL) was incubated in each well of 96-well polystyrene microplates (Corning, NY) 4 °C overnight. Unbound fibrinogen was then removed by plate inversion and washed three times with 200 μL of phosphate-buffered saline solution (PBS) pH 7.4. In the initial experiments, fibrinogen binding to plates was detected by the bicinchoninic acid method (Smith et al., 1985). To the platelet adhesion test, 100 μL/well (10×10^6 platelets), in the presence or absence of Lonomin V, were added to each well, and the plate was incubated under static conditions at 37 °C for 2 h. Non-adherent platelets were removed by plate inversion, and the wells were washed three times with 200 μL of PBS and rapidly supplemented with 150 μL of 5 mM *p*-nitrophenyl phosphate dissolved in 0.1 M citrate buffer pH 5.4, containing 0.1% Triton X-100. After incubation at room temperature for 1 h, the reaction was stopped and the color was developed by adding 100 μL of 2 N NaOH. The *p*-nitrophenol produced was estimated in a microplate reader (TECAN, Sunrise, Salzburg, Austria) at 405 nm. The percentage of platelet adhesion was determined assigning 100% to the number of platelets adhered without pre-treatment with Lonomin V. A substrate solution incubated with venom without platelets was used as control. As a negative control to adhesion, wells were coated with bovine serum albumin (2 mg/mL). The experiments were performed in triplicate.

2.8. Platelet aggregation

Platelet aggregation was estimated by turbidimetry using a dual-channel Chrono-log model 560 CA aggregometer (Havertown, USA). Platelet-rich plasma (PRP) was prepared by mixing fresh blood sample with trisodium citrate solution (3.8%, w/v) in a volume ratio of 9:1, followed by centrifugation at 190 *g*, 24 °C for 20 min to sediment leukocytes and erythrocytes. The platelet count was adjusted to 3.0×10^8 platelets/mL with platelet-poor plasma. Four hundred microliters of citrated PRP were pre-incubated at 37 °C with a stir bar in a silicone-treated glass cuvette. Then, 10 μL of Lonomin V or Tyrode's buffer (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM Na_2HPO_4, 6 mM glucose, 0.2% human serum albumin, pH 7.4) alone were added 4 min before addition of the platelet aggregation inducer. Aggregation was induced by adding ADP (final concentration 10 μM), collagen (final concentration 2.5 μg/mL), thrombin (final concentration 0.6 U/mL) and ristocetin (final concentration 1.25 mg/mL) and the changes in light transmittance were continuously recorded for 8 min. The maximum aggregation response obtained after addition of inducer in the absence of Lonomin V was taken as 100% aggregation. The percentage of inhibition was measured by

comparing light transmittance of venom and control. The IC_{50} value was calculated from a dose-dependent curve that was achieved from five different Lonomin V concentrations using the software program Excel. In order to verify if the effect on platelets is due to proteolytic activity of LV, serine protease inhibitors (2 mM PMSF, 10 mM benzamidine/HCl) as well as a cysteine protease inhibitor (10 mM iodoacetamide) were also used in some experiments.

2.9. Flow cytometry platelet receptors analysis

Determination of receptors, expressed on Lonomin V treated and non-treated platelets was performed by flow cytometry analyses using anti-integrins and anti-P-selectin monoclonal antibodies. Briefly, 200 μL of washed platelets suspension (1×10^6 platelets) were incubated for 30 min at 37 °C with increasing concentrations of Lonomin V of 0.2, 2, 20, 40 and 204 nM. Then, two washes were carried out with Tyrode's buffer. Then, platelets were fixed with 3.2% paraformaldehyde in PBS, for 30 min at 4 °C and washed twice with Tyrode's buffer. The platelets were resuspended in 100 μL buffer Tyrode and incubated for 30 min at 4 °C with 10 μL of the mouse fluorescein (FITC) monoclonal antibodies or Phycoerythrine (PE) conjugates (anti-P-Selectin-FITC and anti-$\alpha_{II}\beta_3$-PE) and anti-$\alpha_5\beta_1$ using a secondary antibody conjugated with FITC. Then, the platelet suspensions were washed twice with Tyrode's buffer and resuspended in 500 μL of PBS. Finally, 75 μL this suspension were mixed with 425 μL PBS to process them in the flow cytometer system (Becton Dickinson FACStar, Braintree, MA, U.S.A.) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals from 10×10^3 cells, excluding any debris, were collected to calculate mean fluorescence intensity of single cells. Results were expressed as mean platelet fluorescent intensity in arbitrary units. As a positive control to integrin-antibody binding, platelets were previously treated with PBS in absence of Lonomin V. As a negative control, non-specific immunoglobulin G (IgG) was used for each experiment. For the studies with P-selectin, 200 μL of washed platelet suspension (1×10^6 platelets) were activated with 0.4 IU/mL of bovine thrombin for 5 min at room temperature prior to Lonomin V incubation.

2.10. Platelets viability

Platelet viability was measured in presence of Lonomin V by a modification of the Han et al. (2007) method. Briefly, 100 μL platelets in Tyrode's buffer were seeded at about 10×10^6 cells in eppendorf tubes, and incubated for 30 min at 37 °C with different amounts of Lonomin V. After incubation, the platelets were centrifuged at 1100 *g* for 5 min, and the supernatant was discarded for add 100 μL Tyrode's buffer. Then, the platelets were incubated with 100 μL 0.5 mg/mL MTT for 4 h at 37 °C, followed by incubation with 150 μL DMSO at 37 °C overnight. When formazan crystals dissolved completely, 350 μL of platelets suspension was placed in 96-well plates and read on a microplate reader (TECAN, Sunrise, Salzburg, Austria) at 620 nm. The results are presented as percentages of control and means ± SEM.

2.11. Statistical analysis

Results on platelet adhesion or aggregation and flow cytometry were expressed as the mean ± standard deviation, and analyzed using the Dunnett's Multiple Comparison test using the software program Graph Pad Prism. Differences were statistically significant if p was $<$ than 0.05. All experiments were done in triplicate.

3. Results

3.1. Isolation of Lonomin V

In previous purifications, LV was obtained using ion exchange and hydrophobic chromatography on DEAE-Trisacryl and Phenyl Sepharose columns, respectively (Lucena et al., 2006, 2008). In order to improve the LV purification, this study included an additional step using a Benzamidine-Sepharose affinity column. The crude hemolymph of *L. achelous* caterpillars was fractionated first on a DEAE-Trisacryl column. Two fractions with fibrinolytic activity on a fibrin plate and amidolytic activity urokinase-like were obtained, which were named FDI and FDII. Then, FDI with the highest fibrinolytic activity was applied to the Phenyl Sepharose column, and an active fraction named FPIII was obtained. When FPIII was re-chromatographed on a Benzamidine-Sepharose column, an active fraction called Lonomin V (LV) was obtained (Fig. 1A,B &C). SDS-PAGE analysis (12% gel) of Lonomin V, under reduced conditions revealed a ~24 kDa main protein band (Fig. 1D). The purity and molecular weight was also visualized by gel filtration chromatography on a Protein-Pak 300-HPLC column (data not shown). The characteristics of the Lonomin V used in this study are shown in Table 1. Lonomin V presented a strong urokinase-like activity in comparison to crude hemolymph. In previous studies the LV was purified using a two-step procedure with approximately 1.56% yield and a 5.68 fold increase in specific fibrinolytic activity (Lucena et al., 2008). This study included a new purification step using a Benzamidine-Sepharose affinity column; obtaining 1.36% yield and 2.76 fold increase in specific activity. There was no difference in the recovery of the LV when both procedures were compared. The difference found in the purification factor could be due to the quality of the lot of crude hemolymph used in the purification.

3.2. Effect on fibrinogen, P-selectin, GPIIb-IIIa and collagen

The electrophoretic study showed that the crude hemolymph and Lonomin V degraded fibrinogen in a time-dependent manner. Complete degradation of Aα and Bβ chains was observed at 30 min of incubation, with degradation of the γ chain at 24 h, producing fragments of variable molecular weight (Fig. 2A and B).

The effect of Lonomin V on purified fibrinogen, P-selectin and GPIIb-IIIa was also visualized by HPLC on a Protein-Pak 300 column. Fibrinogen molecule was eluted with a retention time of 10.9 min. At the ratio of 1:100 (Lonomin V:Fibrinogen), the fibrinogen molecule was degraded in three main fragments with retention times of 11.6, 12.8 and 16.2 min, corresponding to 320, 308 and

71 kDa, according to the program of GPC Empower Waters (Fig. 2C). At conditions of study, the Lonomin V not produced significantly changes on P-selectin or GPIIb-IIIa molecules (data not shown).

The effect of Lonomin V on collagen molecule was observed in Fig. 2D. The control type IV collagen solution at 37 °C for 6 h revealed three main protein bands with apparent molecular masses of 190, 170 and 94 kDa. Following incubation with Lonomin V at a ratio of 1:100 (Lonomin V:Collagen), the two bands of 190 and 170 kDa gradually disappeared. The complete disappearance of these bands was observed after 4 h of incubation, producing two main fragments of 160 and 40 kDa.

3.3. Platelet adhesion studies

Platelets pre-treated with Lonomin V (10×10^6 platelets/nM of venom protein) reduced the adhesion to fibrinogen by 19, 20, 36, 37 and 37% with 0.2, 2, 20, 40 and 204 nM of Lonomin V, respectively (Fig. 3). Statistically significant differences with concentrations of 20, 40 and 204 nM were observed ($p < 0.01$).

3.4. Platelet aggregation studies

Anti-platelet activity of Lonomin V (LV) was also tested on platelet aggregation assays using human PRP. In the absence of LV, the ADP or collagen - inducer platelet aggregation percentage were 70 ± 2% and 76 ± 5%, respectively. Platelets pre-treated with Lonomin V reduced ADP-induced platelet aggregation by 9, 9, 44, and 56% with 2, 20, 204 and 408 nM of Lonomin V, respectively. Platelets pre-treated with Lonomin V reduced collagen-induced platelet aggregation by 2, 16, 22, 67 and 100% with 2, 20, 40, 204 and 408 nM of Lonomin V, respectively. The results showed that this molecule dose-dependently inhibited ADP and collagen - induced platelet aggregation, with IC_{50}s of 388.35 and 68.71 nM, respectively (Fig. 4A and B). Pre-incubation of platelets with 408 nM of Lonomin V, for 4 min at 37 °C, inhibited ristocetin - induced platelet aggregation by 13%, while pre-incubation of platelets with 2040 nM of Lonomin V, for 4 min at 37 °C, did not inhibit thrombin - induced platelet aggregation (data not shown). In the experiments done in the presence of protease inhibitors and ADP as agonists, the percentage of inhibition

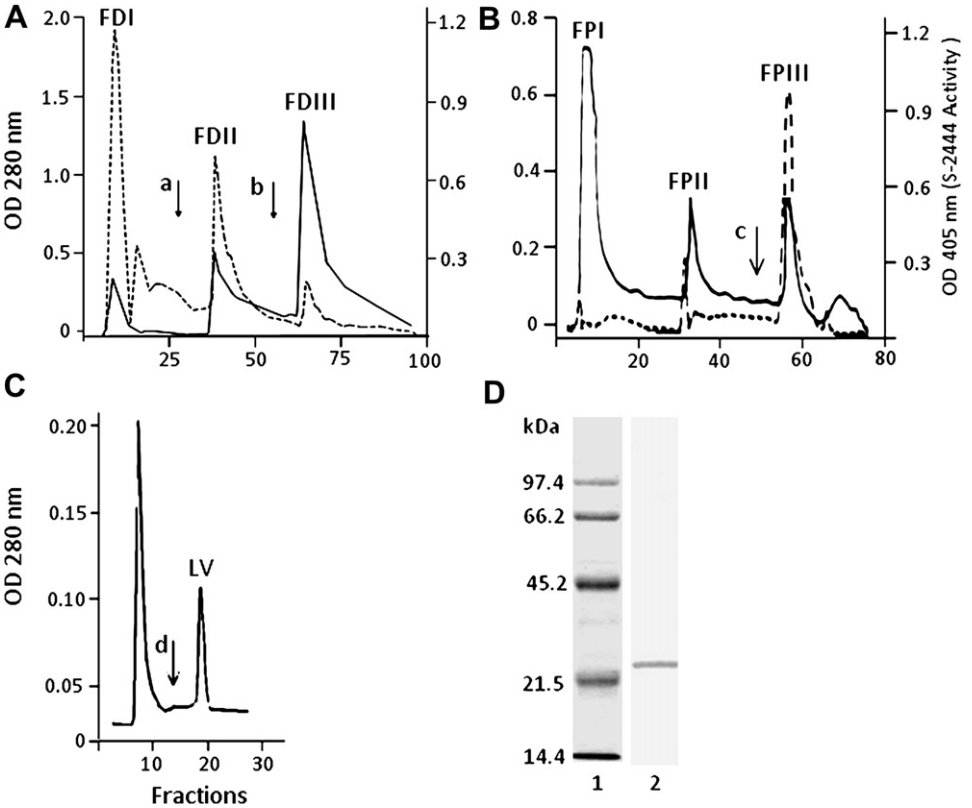

Fig. 1. Lonomin V (LV) Purification: A) Anion exchange chromatography of *Lonomia achelous* hemolymph (28.0 mg/2.5 mL) on DEAE-Trisacryl column (27 × 1.5 cm) equilibrated with Tris-HCl 0.021 M, pH 8.6 (buffer A). Elution was carried out at 1 mL/min, first with buffer A and later with same buffer + (a) 0.15 M NaCl (b) 0.5 M NaCl. B) Hydrophobic interaction chromatography of FDI fraction (1.4 mg/2 mL) on a Phenyl Sepharose CL-4B column (1 × 10 cm) equilibrated with 0.05 M Tris-0.5 M $(NH_4)_2SO_4$, pH 7.5 (buffer B). The elution was carried out at 0.25 mL/min, first with buffer B, followed by (c) 0–60% ethylene glycol gradient in buffer B. C) Affinity chromatography of FPIII (0.23 mg/mL) on Benzamidine-Sepharose column (1 × 10 cm) equilibrated with 0.05 M Tris-0.5 M NaCl pH 7.4. The elution was carried out at 1 mL/min with 0.05 M Glicine pH 3; fractions (1 mL) were neutralized with 100 μL 1 M Tris pH 9. Urokinase-like amidolytic activity (–). Absorbance at 280 nm (–). D) SDS-PAGE of Lonomin V under reduced conditions (12% gel). Lanes 1) Molecular weight markers; 2) Lonomin V (15 μg). The gel was stained with brilliant blue R-250 Coomassie.

Table 1
Characteristics of *Lonomia achelous* hemolymph venom and Lonomin V.

Samples	Total Protein (mg/mL)	Recovery of protein (%)[b]	Purification factor (fold)[c]	Fibrinolytic activity	
				Amidolytic Activity mUA/min/μg[a] (S-2444- Urokinase)	Fibrin Plate mm^2/μg
Crude hemolymph	11.00 ± 0.4	100	1.00	23.3	289
Lonomin V	0.15 ± 0.05	1.36	2.76	50.3	800

[a] Amidolytic activity determined using chromogenic substrates [11].
[b] Recovery of protein was defined as the total of mg/mL of Lonomin V obtained in the Benzamidine-Sepharose column.
[c] Purification factor was the number of times that specific fibrinolytic activity in fibrin plate increased over the crude hemolymph.

of aggregation in the presence of 204 nM of LV pre-incubated with 10 mM benzamidine and 10 mM iodoacetamide was 76 and 82%, respectively (data not shown). In contrast, the incubation of Lonomin V with 2 mM PMSF showed a decrease of ADP platelet aggregation inhibition induced by this protease.

3.5. Studies of platelet receptors in presence of Lonomin V

To identify which receptors on platelets interact with Lonomin V, anti-receptor monoclonal antibodies (Mabs) binding to platelets, in presence or absence of Lonomin V, was examined by flow cytometry. Fig. 5 shows the results of

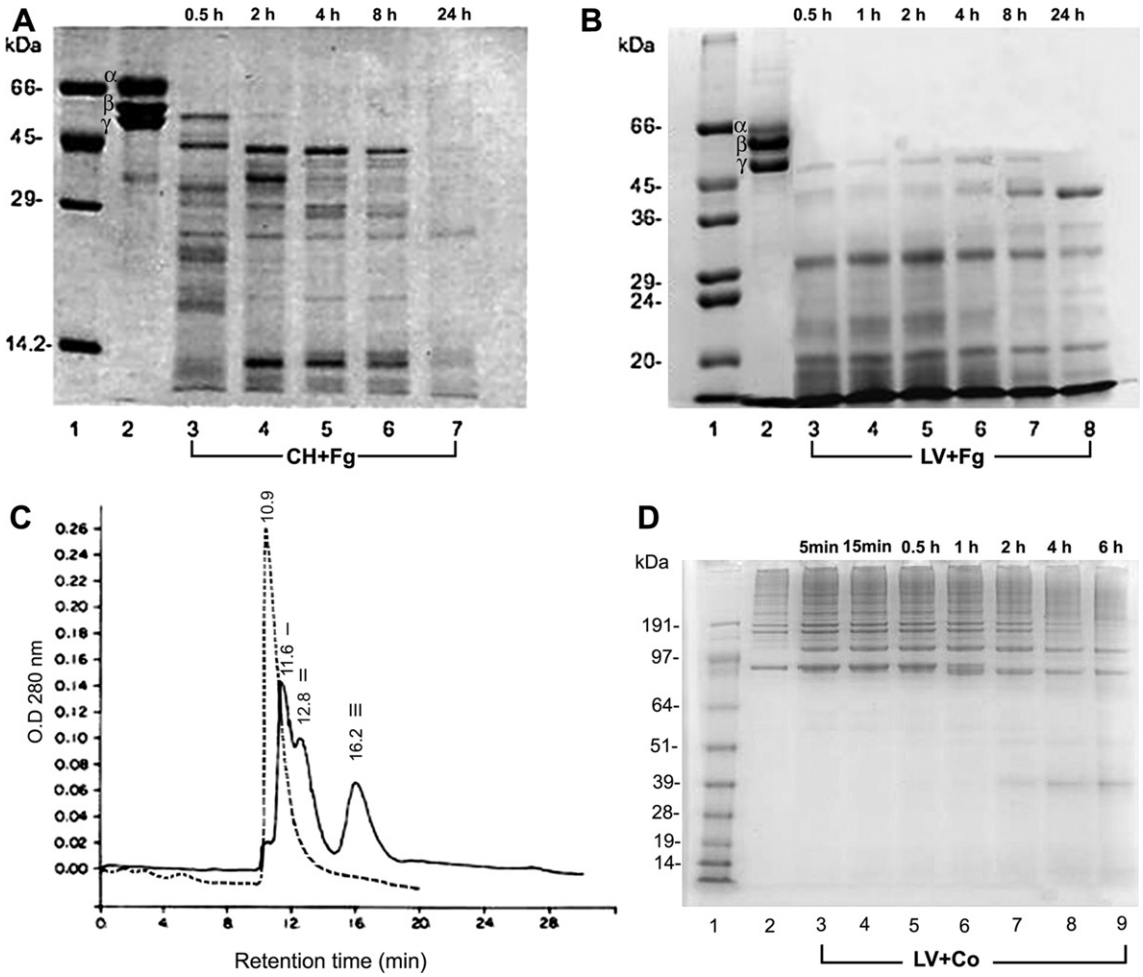

Fig. 2. Effect of crude hemolymph (CH) and Lonomin V (LV) on fibrinogen (Fg) and collagen IV (Co). Samples (15 μg) were electrophoresed under reduced conditions in the presence of SDS. A) CH and Fg were incubated at 1 μg CH:100 μg Fg ratio at different times at 37 °C. Lanes 1) Molecular weight standards; 2) Fg control; 3–7) Fg + CH at 30 min, 2, 4, 8 and 24 h. B) LV and Fg were incubated at 1 μg LV:100 μg Fg ratio at different times at 37 °C. Lanes 1) Molecular weight standards; 2) Fg control; 3–8) Fg + LV at 30 min, 1, 2, 4, 8 and 24 h, respectively. C) LV and Fg were incubated at 1 μg LV:100 μg Fg ratio for 6 h at 37 °C. Analysis by HPLC on a Protein-Pak 300 column, equilibrated with 0.05 M Tris - 0.15 M NaCl, pH 7.4, at a 0.5 mL/min flow rate. D) LV and Co were incubated at 1 μg LV:100 μg Co ratio at different times at 37 °C. Lanes 1) Molecular weight standards; 2) Co control; 3–9) Co + LV at 5, 15, 30 min, 1, 2, 4, and 6 h, respectively. These are subjective representative traces.

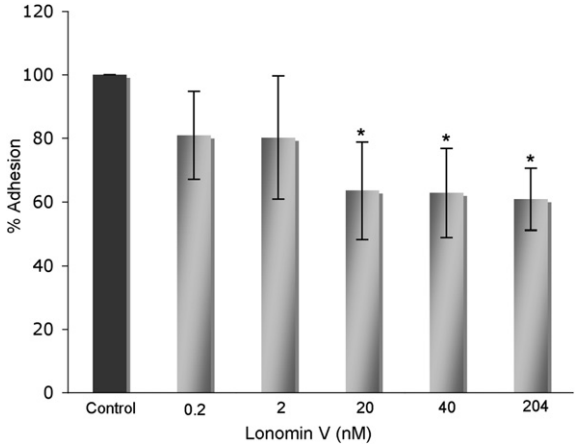

Fig. 3. Inhibition of platelet adhesion to fibrinogen by Lonomin V (LV). Platelets (10×10^6 mL) were pre-incubated with different concentrations of LV for 30 min at 37 °C, before the addition of platelets into fibrinogen coated wells. Platelets adhesion was determined using the acid phosphatase activity. Results are expressed as adhesion percentage. All experiments were conducted in triplicate and repeated at least three times. Data are presented as mean ± SD ($n = 9$).

receptor expression on platelets revealed by the fluorescence intensity. Lonomin V inhibited anti-$\alpha_{IIb}\beta_3$ integrin binding to platelets by 56, 57, 52, 54 and 58% at the concentrations of 0.2, 2, 20, 40 and 204 nM respectively (Fig. 5A). Additionally, Lonomin V inhibited anti-P-Selectin binding to platelets by 28, 37, 33, 33 and 31% at the same concentrations (Fig. 5B). In comparison with the control, a significant difference with all concentrations tested was observed with $\alpha_{IIb}\beta_3$ integrin ($p < 0.01$), while with P-selectin, a statistical significant difference was observed with 2, 20, 40 and 204 nM ($p < 0.05$). These concentrations did not present any effect on the $\alpha_5\beta_1$ integrin (data not shown).

3.6. Platelet viability in presence of Lonomin V

Platelet viability was also assayed in presence of Lonomin V at different concentrations. Platelet viability was not affected when platelets were assayed with 0.2, 2, 20, 40 and 204 nM of Lonomin V (data not shown).

4. Discussion

Platelets play a fundamental task in hemostasis and blood clotting in a wounded vessel. Alternatively, abnormal platelet activation and arterial thrombus formation are implicated in the pathogenesis of many cardiovascular diseases including atherosclerosis. Platelet activation, shape change, and aggregation are motivated by receptor-mediated responses to wound-associated signals, including ADP, collagen, and thrombin (Wei et al., 2009; Angiolillo et al., 2010).

The physiological secretions from some invertebrates have toxic effects on mammalian blood coagulation and fibrinolytic systems including those substances that affect platelet functions. Venoms from arthropods contain potent activators and inhibitors of platelet function (Arocha-Piñango et al., 1999). Lonomin V is the first component isolated from *L. achelous* venom possessing anti-platelet effect on adhesion and aggregation functions. Lonomin V has the ability to degrade fibrinogen and fibrin as well as factor XIII (Guerrero et al., 1997, 1999).

Studies with other components isolated from invertebrates demonstrated the presence of anti-platelet activities. Iris, a serine protease inhibitor (serpin) isolated from tick, *Ixodes ricinus*, inhibits primary hemostasis measured through closure time using PFA-100, as a reliable indication of the platelet adhesion time (Leboulle et al., 2002; Prevot et al., 2006). Another common platelet inhibitor is apyrase (ATP/ADP diphosphohydrolase), isolated from saliva of *Aedes aegypti*, *Anopheles*, and *Drosophila melanogaster* among others (Champagne, 2005). Song et al.

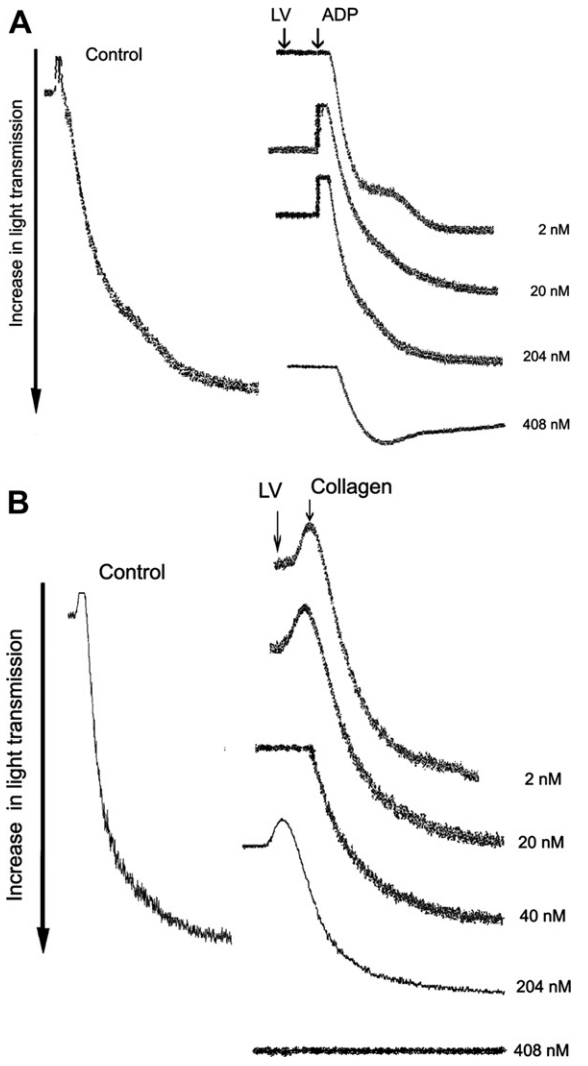

Fig. 4. Inhibition of ADP or collagen- inducer platelet aggregation by Lonomin V (LV). Various concentrations of LV were pre-incubated with PRP (3×10^8 platelets/mL), for 4 min at 37 °C prior to the addition of ADP (10 μM) (A) and collagen (2.5 μg/mL). (B). The percentage of inhibition was calculated by comparing light transmittance obtained in presence of LV against the control sample.

(2005) demonstrated that and active peptide from the venom of scorpion, inhibited platelet aggregation induced by thrombin and ADP both in *ex vivo* and *in vitro*.

Studies on platelets, done with the venom of *Lonomia obliqua* caterpillar distributed in Brazil, demonstrated no platelet aggregation (Donato et al., 1998). Berger et al. (2010a) showed platelet hypoaggregation following *Lonomia oblicua* envenomation in rats. This phenomenon was caused by intravascular generation of nitric oxide and fibrin:fibrinogen degradation products rather than a direct action of *L. oblicua* venom. Nonetheless, in a different study reported by Berger et al., showed that *L. obliqua* venom was able to induce aggregation and adhesion of human platelets *in vitro,* probably caused by the presence of a PLA_2 (Berger et al., 2010b). *L. obliqua* prothrombin activator protease (LOPAP), an activator present in caterpillar bristles, had no effect on the aggregation of washed human platelets induced by several agonists, suggesting that it might not impair platelet function *in vivo* (Chudzinski-Tavassi et al., 2001; Carrijo-Carvalho and Chudzinski-Tavassi, 2007; Waisman et al., 2009).

A group of toxins that are known to affect platelet function comprise the phospholipases A_2 and snake venom metalloproteinases (SVMPs) (Kamiguti, 2005). Some SVMPs were reported to interfere with platelet function through either inhibiting aggregation or inducing aggregation. For examples, Jararhagin from *Bothrops jararaca* venom inhibits platelet aggregation by preventing ligands from binding to β1 integrins, involving either proteolysis dependent or independent mechanisms (Kamiguti et al., 1996). Mocarhagin, a cobra venom metalloproteinase, inhibits ristocetin-induced platelet aggregation through cleaving and inactivating GPIb (Ward et al., 1996). Ohagin, another cobra venom metalloproteinase, inhibited washed platelet aggregation induced by several agonists such as ADP, TMVA (mucetin) and stejnulxin (Guo et al., 2007). Furthermore, SVMPs seem to be selective for the platelets and collagen interactions as well as with von Willebrand factor, suggesting that they cause either proteolysis of collagen and von Willebrand factor and/or interfere with their respective receptors ($\alpha_2\beta_1$ integrin and/or GPVI and GPIb) (Lu et al., 2005; Wijeyewickrema et al., 2005).

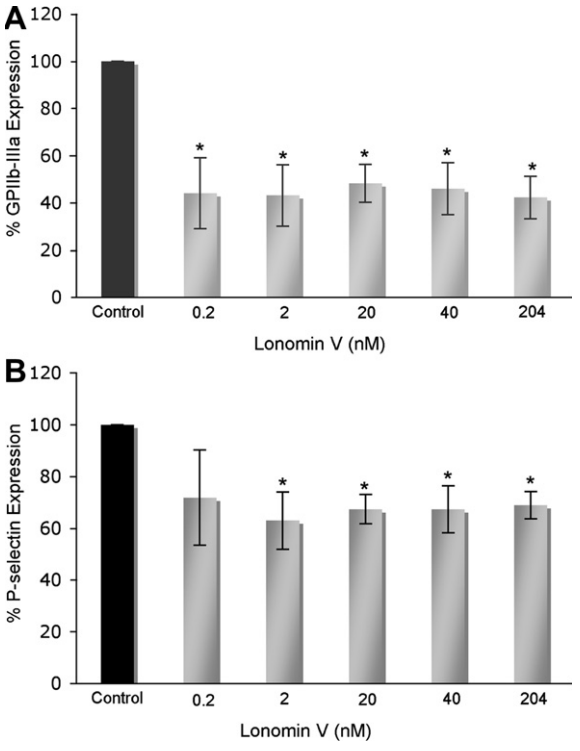

Fig. 5. Flow cytometry analysis of GPIIb/IIIa and P-selectin platelets receptors. Platelets (1×10^6 platelets) were treated with Lonomin V at various concentrations for 30 min at 37 °C, then fixed with 3.2% paraformaldehyde and analyzed by flow cytometry using and anti-$\alpha_{IIb}\beta_3$-PE (A) anti-P-selectin-FITC. (B). Fluorescence signals from 10×10^3 cells, excluding any debris, were collected to calculate mean fluorescence intensity of single cells. Results were expressed as the percentage of platelet fluorescent expression. As a positive control to receptor-antibody binding, platelets were previously treated with PBS in absence of Lonomin V. P-selectin studies were carried out with activated platelets (0.4 IU/mL thrombin/5 min prior to Lonomin V incubation).

Lonomin V similar to some SVMPs, could inhibit platelet adhesion to fibrinogen and aggregation by degrading some integrins involved in adhesion to fibrinogen such as $\alpha_v\beta_3$ and the adhesive proteins such as fibrinogen and collagen. It is noteworthy that there could be degradation of the fibrinogen bound to the plate by LV, which may indirectly affect platelet adhesion. Lonomin V inhibited platelet-rich plasma aggregation caused by several agonists, such as ADP and collagen. It is very important to note that the Lonomin V has no toxic effects on platelets at the tested concentrations, suggesting that the venom's activity on some receptors or adhesive proteins could be responsible for the observed results.

The inhibitory effect of Lonomin V on collagen-induced aggregation was more potent than ADP - induced aggregation, which may be due to a proteolysis of collagen by Lonomin V, as showed in Fig. 2D. Platelet-collagen interactions lead to platelet aggregation as well as adhesion, and purified collagen is a potent stimulator of platelet aggregation *in vivo* and *in vitro* conditions. Like von Willebrand factor binding, this allows an initial adhesion event to be amplified by recruitment of additional platelets (Nieswandt and Offermanns, 2004). Lonomin V also degrades primarily fibrinogen Aα and Bβ chains and γ chains only after 24 h (Fig. 2B). Furthermore, it was previously demonstrated that Lonomin V degraded fibronectin molecule impairing its adhesion functions (Lucena et al., 2006, 2008). In our study, the mechanism of aggregation inhibition could be in part attributed to proteolysis of receptors involved in this process; in addition, to the degradation of adhesive proteins such as fibrinogen, collagen and fibronectin. Highlighting the fact that in the aggregation assays in presence of serine protease inhibitors, only PMSF partially inhibited the effects of LV, which makes us believe that perhaps the proteolytic activity of LV could be due in part to the inhibitory effect of this protease against aggregation, without excluding the participation of other mechanisms independent of the enzymatic activity that could be affecting platelet function.

Thrombin is a very potent platelet agonist that activates platelets at extremely low concentrations. Thrombin-mediated platelet activation contributes to pathological

thrombosis through the formation of an occlusive platelet-rich thrombus. PAR-1 is the main thrombin receptor on human platelets (Angiolillo et al., 2010). Otherwise, Von Willebrand factor (VWF) is a large multimeric adhesive glycoprotein, with complex roles in thrombosis and hemostasis, present in circulating blood and in secretory granules of endothelial cells and platelets. The antibiotic ristocetin promotes the interaction of VWF and GPIb and is able to induce platelet aggregation (Papi et al., 2010). In this study, at the concentrations tested, there was no effect of LV on platelet aggregation induced by thrombin and ristocetin. Probably, the LV had no effect on the receptors and/or proteins involved in the platelet aggregation induced by these agonists.

These activities of Lonomin V were similar to the activities reported for Atroxlysin-I, a 23 kDa metalloproteinase isolated from the venom of the Peruvian *Bothrops atrox,* which hydrolyzed the Aα-chains of fibrinogen in a dose and time-dependent manner. This protein also cleaved plasma fibronectin and other extracellular matrix proteins such as collagen type I and also inhibited collagen and ADP-induced aggregation (Sánchez et al., 2010). A novel recombinant fibrinogenase named rFII possesses both fibronectin and type IV collagen cleaving activities. In addition, rFII cleaved preferentially the Aα-chains of fibrinogen, followed by the Bβ chains, and finally the γ chains were affected. Furthermore, rFII was capable of suppressing ADP-induced platelet aggregation (Jiang et al., 2009).

We analyzed the possible effect of Lonomin V on the principal receptors of fibrinogen and fibronectin, the integrins $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ (important receptors involved in platelet aggregation and adhesion). Lonomin V probably affects some step in the platelet signaling pathway that eventually affects the expression of the integrin $\alpha_{IIb}\beta_3$. Stimulation of G protein-coupled receptors by thromboxane A2 (TXA2), thrombin and ADP leads to the stimulation of various signaling pathways involving the G proteins G_q, $G_{i/z}$ and $G_{12/13}$ (Nieswandt and Offermanns, 2004). Integrin $\alpha_{IIb}\beta_3$ is the most abundant platelet membrane receptor implicated as the receptor for a variety of ligands such as fibrinogen, fibronectin and von Willebrand factor, and the binding of these ligands to this glycoprotein promotes platelet aggregation. Although many integrins can bind fibronectin, the $\alpha_5\beta_1$ integrin is the major fibronectin receptor on most cells. This integrin mediates cellular responses to fibronectin substrates as adhesion, migration, assembly of extracellular matrix, and signal transduction (Akiyama, 1996). In primary hemostasis, the receptor $\alpha_5\beta_1$ has the capacity to mediate firm platelet adhesion at sites of vascular injury (Nieswandt and Offermanns, 2004). In this study, at the concentrations tested, there was no effect of LV on $\alpha_5\beta_1$ integrin. A possible explanation could be that the $\alpha_5\beta_1$ integrin is not susceptible to LV degradation.

It is interesting to note the differences observed between the dose-dependent inhibition of platelet aggregation and the dose-independent inhibition of the GPIIb-IIIa and P-selectin expression found in the flow cytometry assays. In flow cytometry, morphological studies with washed platelets were done in order to evaluate if their respective antibodies recognized their receptors after treating the platelets with LV. It is important to take note that the percentage of inhibition was similar when tested with the different concentrations of LV. This may be because the reaction of Lonomin V with P-selectin and GPIIb-IIa receptors could be due to a saturated reaction, taking into account that there are a limited number of these receptors on platelet membranes (Nieswandt and Offermanns, 2004; Palomo et al., 2008). The studies carried out by HPLC-exclusion molecular chromatographic with GPIIb-IIIa and P-selectin pre-incubated with LV demonstrated that there was no significant morphological change in these receptors (data not shown). The decrease expression of these receptors is probably due to that LV is affecting a cell signaling pathway that is blocking the expression at the membrane level. On the other hand, it is surprising that 204 nM of LV completely inhibited platelet aggregation in presence of collagen, but only caused a 58% reduction in GPIIb-IIIa expression. However, platelet aggregation is a functional assay in which not only platelet receptors participate but also adhesive proteins present in plasma such as fibrinogen, fibronectin and collagen. Perhaps the dose-dependent inhibition of platelet aggregation in the presence of LV is a result of a synergistic effect of LV against different platelet receptors like the ADP and collagen receptors, including the adhesive proteins like fibrinogen and collagen. If we related the flow cytometry results with the adhesion assays, it is important to keep in mind that in the adhesion studies the platelets are not active and not in continuous movement, conditions required for aggregation. Therefore, it is possible that the dose-dependent inhibition effect observed in the adhesion assay not only affects receptors such as $\alpha_v\beta_3$, but could also be interacting with the fibrinogen coated to the plate. New studies at the cellular level will be carried out in order to determine specifically which receptors on platelet membranes and signaling pathway may be targeted by LV.

Furthermore, Lonomin V inhibited anti-P-selectin binding on platelet membranes that were previously activated with thrombin, indicating a possible inhibitory effect on the first events that happen in platelet adhesion to the activated endothelium mediated by this receptor. P-selectin, found together with von Willebrand factor in platelet α granules or endothelial cell Weibel-Palade bodies, is rapidly expressed on the cell surface in response to cell activation. P-selectin on platelets is crucial for the interaction of activated platelets with leukocyte P-selectin glycoprotein ligand-1 (PSGL-1). In various diseases and conditions, excessive platelet activation can lead to thrombosis, and P-selectin is found to be elevated in many cardiovascular disorders, such as unstable angina and peripheral arterial occlusive diseases (Geng, 2003; Gachet, 2008; Mestas and Ley, 2008; Van Gils et al., 2009; Semple and Freedman, 2010). Until now, the evolution of this protein has been ignored as a target of anti-adhesive molecules present in venoms.

The amount of LV associated with hemorrhagic complications in human has not been determined, considering that humans are always envenomed with the whole venom and not with a specific toxin. Future studies in animal models will be tested in order to determine

whether the concentrations of LV responsible for the anti-platelet activity *in vitro* can be relevant *in vivo*.

Arocha Piñango and coworkers suggested that the hemorrhagic syndrome resulting from contact with *L. achelous* caterpillar is primarily caused by activation of fibrinolysis and a mild disseminated intravascular coagulation (Arocha-Piñango et al., 2000; Arocha-Piñango and Guerrero, 2003). The intense fibrinolytic activity present in patients envenomated by *L. achelous* is the consequence of the plasmin-like (lonomin II) and factor XIII proteolytic/urokinase-like enzyme (lonomin V), both of which might play the most important role in hemorrhagic manifestations (Arocha-Piñango et al., 2000). In platelets, the blockage or proteolysis of integrin and/or extracellular matrix proteins inhibiting adhesion and aggregation by LV can contribute to the hemorrhagic syndrome commonly found in patients exposed to the *L. achelous* caterpillar. Considering the fundamental importance that platelets play with their respective receptors and adhesive proteins such as fibrinogen and collagen in primary and secondary hemostasis and the role that involves these cells in physiopathological processes like thrombosis and atherosclerosis, it can be deduced that the anti-platelet activity of Lonomin V shows to be a unique and potentially useful tool for investigating cell-matrix and cell–cell interactions and for the development of antithrombotic agents in terms of their anti-adhesive activities. Therefore, further studies of Lonomin V will be focused on its application as a tool for studying the interactions of integrin-ligands and for developing therapeutic agents for thrombosis and other integrin-related diseases.

Conflict of interest

The authors declare that there are no conflicts of interest concerned with this work.

Funding

This work was funded by a grant from the Science and Technology Fund (FONACIT) programs (PG-2005000400 grant), and by the Venezuelan Institute of Scientific Research (IVIC), Caracas, Venezuela.

Ethical statement

All the authors approve the material submitted for publication; each author has participated adequately in the experimental work to take public responsibility for the content. The material has no been previously reported and it is not under consideration for publication elsewhere. Authors transfer the copyright of the article to the publisher.

Acknowledgments

Financial support was obtained from grants by the Science and Technology Fund (FONACIT) programs (PG-2005000400 grant), Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela. We thank the Dr. Jose Cardier for collaboration in flow cytometry studies and the Carlos Ibarra for chromatographic technical assistance.

References

Akiyama, S.K., 1996. Integrins in cell adhesion and signaling. Hum. Cell. 3, 181–186.

Angiolillo, D.J., Ueno, M., Goto, S., 2010. Basic Principles of platelet Biology and Clinical implications. Circ. J. 74, 597–607.

Arocha-Piñango, C.L., Guerrero, B., 2003. Hemorrhagic syndrome induced by caterpillars. Clinical and experimental studies. Invest. Clin. 44, 155–163.

Arocha-Piñango, C.L., Marchi, R., Carvajal, Z., Guerrero, B., 1999. Invertebrate compounds acting on the hemostatic mechanism. Blood Coagul. Fibrinolysis 10, 43–68.

Arocha-Piñango, C.L., Marval, E., Guerrero, B., 2000. *Lonomia* genus caterpillar toxins: Biochemical aspects. Biochimie 82, 937–942.

Berger, M., Reck, J., Terra, R.M.S., Pinto, A.F.M., Termignoni, C., Guimarães, J.A., 2010a. *Lonomia obliqua* caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. Toxicon 55, 33–44.

Berger, M., Reck Jr., J., Terra, R.M.S., Beys da Silva, W.O., Santi, L., Pinto, A.F.M., Vainstein, M.H., Termignoni, C., Guimarães, J.A., 2010b. *Lonomia obliqua* venomous secretion induces human platelet adhesion and aggregation. J. Thromb. Thrombolysis 30, 300–310.

Bradley, A., Loscalzo, M., Loscalzo, J., 2007. The role of platelets in fibrinolysis. In: Michelson, A. (Ed.), Platelets. Academic Press, Elsevier, San Diego California, USA, pp. 415–430.

Carrijo-Carvalho, L.C., Chudzinski-Tavassi, A.M., 2007. The venom of the *Lonomia* caterpillar: an overview. Toxicon 49, 741–757.

Champagne, D., 2005. Antihemostatic molecules from saliva of blood-Feeding arthropods. Pathophysiol Haemost Thromb. 34, 221–227.

Chudzinski-Tavassi, A.M., Schattner, M., Fritzen, M., Pozner, R.G., Reis, C.V., Lourenco, D., Lazzari, M.A., 2001. Effects of lopap on human endothelial cells and platelets. Haemostasis 31, 257–265.

Donato, J.L., Moreno, R.A., Hyslop, S., Duarte, A., Antunes, E., le Bonniec, B. F., Rendu, F., de Nucci, G., 1998. *Lonomia obliqua* caterpillar spicules trigger human blood coagulation via activation of factor X and prothrombin. Thromb. Haemost 79, 539–542.

Gachet, C., 2008. P2 receptors, platelets function and pharmacological implications. Thromb. Haemost 99, 466–472.

Geng, J.G., 2003. Interaction of vascular endothelial cells with leukocytes, platelets and cancer cells in inflammation, thrombosis and cancer growth and metastasis. Acta Pharmacol. Sin 12, 1297–1300.

Guerrero, B., Arocha-Piñango, C.L., Alves-Pinto, M., Muller, C.A., Gil San Juan, A., Amorim, S., Perales, J., 2001. Thrombolytic effect of Lonomin V in a rabbit jugular vein thrombosis model. Blood Coagul. Fibrinolysis 12, 521–529.

Guerrero, B., Arocha-Piñango, C.L., Gil, A., 1997. Degradation of human factor XIII by Lonomin V, a purified fraction of *Lonomia achelous* caterpillar venom. Thromb. Res. 87, 171–181.

Guerrero, B., Perales, J., Gil, A., Arocha-Piñango, C.L., 1999. Effect on platelet FXIII and partial characterization of Lonomin V, a proteolytic enzyme from *Lonomia achelous* Caterpillars. Thromb. Res. 93, 243–252.

Guo, X., Zeng, L., Lee, W.H., Zhang, Y., Jin, Y., 2007. Isolation and cloning of a metalloproteinase from king cobra snake venom. Toxicon 49, 954–965.

Han, Y.-P., Lu, X.-Y., Wang, X.-F., Xu, J., 2007. Isolation and characterization of a novel P-II class snake venoms metalloproteinase from *Trimeresurus stejnegeri*. Toxicon 49, 889–898.

Jiang, W., Ma, T., Su, X., Qiu, P., Yan, G., 2009. Enzymatic activities and functional characterization of a novel recombinant snake venom proteinase from *Agkistrodon Acutus*. Biochimie 91, 277–287.

Kamiguti, A., 2005. Platelets as targets of snake venom metalloproteinases. Toxicon 45, 1041–1049.

Kamiguti, A.S., Hay, C.R., Zuzel, M., 1996. Inhibition of collagen-induced platelet aggregation as the result of cleavage of alpha 2 beta 1-integrin by the snake venom metalloproteinase jararhagin. Biochem. J. 320, 635–641.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leboulle, G., Crippa, M., Decrem, Y., Mejri, N., Brossard, M., Bollen, A., Godfroid, E., 2002. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. J. Biol. Chem. 277, 10083–10089.

Lu, Q., Clemetson, J.M., Clemetson, K.J., 2005. Snake venoms and hemostasis. J. Thromb. Haemost 3, 1791–1799.

Lucena, S., Guerrero, B., Salazar, A.M., Gil, A., Arocha-Piñango, C.L., 2006. Degradation of extracellular matrix proteins (Fibronectin, Vitronectin and laminin) by serine-proteinases isolated from *Lonomia achelous* caterpillar hemolymph. Blood Coagul. Fibrinolysis 17, 427–435.

Lucena, S., Salazar, A.M., Gil, A., Arocha-Piñango, C.L., Guerrero, B., 2008. The action of Lonomin V (Lonomia achelous) on fibronectin functional properties. Thromb. Res. 121, 653–661.

Marsh, N.A., Arocha-Piñango, C.L., 1972. Evaluation of the fibrin plate method for stimating plasminogen activators. Thromb. Diath Haemorrh 28, 75–88.

Mc Michael, M., 2005. Primary hemostasis. J. Vet. Emerg. Crit. Care 1, 1–8.

Mestas, J., Ley, K., 2008. Monocyte-Endothelial cell interactions in the development of atherosclerosis. Trends Cardiovasc. Med. 18, 228–232.

Nieswandt, B., Offermanns, S., 2004. Pharmacology of platelet adhesion and aggregation. Handb Exp. Pharmacol. 165, 437–471.

Papi, M., Maulucci, G., De Spirito, M., Missori, M., Arcovito, G., Lancellotti, S., Di Stasio, E., De Cristofaro, R., Arcovitto, A., 2010. Ristocetin-induced self-aggregation of von Willebrand factor. Eur. Biophys. J. 39, 1597–1603.

Palomo, I., Toro, C., Alarcón, M., 2008. The role of platelets in the pathophysiology of atherosclerosis (Review). Mol. Med. Rep. 1, 179–184.

Prevot, P.-P., Adam, B., Zouaoui, K., Brossard, M., Lins, L., Cauchie, P., Brasseur, R., Vanhaeverbeek, M., Vanhamme, L., Godfroid, E., 2006. Anti-haemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. J. Biol. Chem. 281, 26361–26369.

Ruggeri, Z., Mendolicchio, L., 2007. Adhesion mechanisms in platelet function. Circ. Res. 100, 1673–1685.

Sánchez, E.F., Schneider, F.S., Yarleque, A., Borges, M.H., Richardson, M., Figueiredo, S.G., Evangelista, K.S., Eble, J.A., 2010. The novel metalloproteinase atrolysin-I from Peruvian *Bothrops atrox* (Jergón) snake venom acts both on blood vessel ECM and platelet. Arch. Biochem. Biophys. 496, 9–20.

Semple, J.W., Freedman, J., 2010. Platelets and innate immunity. Cell Mol. Life Sci. 67, 499–511.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Song, Y., Tang, X., Xi, Chen, Gao, B., Gao, E., Bai, L., Lv, X., 2005. Effects on scorpion venom bioactive polypolypeptides on platelet aggregation and thrombosis and plasma 6-keto-PGF_1 α and TXB_2 in rabbits and rats. Toxicon 46, 230–235.

Van Gils, J.M., Zwaginga, J.J., Hordijk, P.L., 2009. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. J. Leukoc. Biol. 85, 195–204.

Varga-Szabo, D., Pleines, I., Nieswandt, B., 2008. Cell adhesion mechanisms in platelets. Arterioscler Thromb. Vasc. Biol. 28, 403–412.

Waisman, K., Chudzinski-Tavassi, A.M., Carrijo-Carvalho, L.C., Pacheco, M.T., Farsky, S.H., 2009. Lopap: a non-inflammatory and cytoprotective molecule in neutrophils and endothelial cells. Toxicon 53, 652–659.

Ward, C.M., Andrews, R.K., Smith, A.I., Berndt, M.C., 1996. Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ibalpha. Identification of the sulfated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ibalpha as a binding site for von Willebrand factor and alpha-thrombin. Biochemistry 35, 4929–4938.

Wei, A.H., Schoenwaelder, S.M., Andrews, R.K., Jackson, S.P., 2009. New insights into the haemostatic function of platelets. Br. J. Haematol. 147, 415–430.

Wijeyewickrema, L., Berndt, M., Andrews, R., 2005. Snake venom probes of platelet adhesion receptors and their ligands. Toxicon 45, 1051–1061.

Yeh, C.H., Peng, H.C., Yih, J.B., Huang, T.F., 1998. A new short chain RGD-containing disintegrin, accutin, inhibits the common pathway of human platelet aggregation. Biochim. Biophys. Acta, 493–504.