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BE-I-PLA2, a novel acidic phospholipase A_2 from *Bothrops erythromelas* venom: Isolation, cloning and characterization as potent anti-platelet and inductor of prostaglandin I_2 release by endothelial cells

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ABSTRACT

A novel acidic Asp49 phospholipase A_2 was isolated from *Bothrops erythromelas* (jararaca malha-de-cascavel) snake venom by four chromatographic steps. BE-I-PLA2 present a molecular weight of 13,649.57 Da as estimated by mass spectrometry. N-terminal and four internal peptides were sequenced, covering around one-third of the complete toxin sequence. The complete BE-I-PLA2 cDNA was cloned from a *B. erythromelas* venom-gland cDNA library. The cDNA sequence possesses 457 bp and encodes a protein with significant sequence similarity to many other phospholipase A_2 from snake venoms. When tested in platelet rich plasma, the enzyme showed a potent inhibitory effect on aggregation induced by arachidonic acid and collagen, but not ADP. On the other hand, BE-I-PLA2 did not modify aggregation in washed platelet. Furthermore, no action of BE-I-PLA2 on the principal platelets receptors was observed. Chemical modification with *p*-bromophenacyl bromide abolished the enzymatic activity of BE-I-PLA2, but its anti-platelet activity was only partially inhibited. In human umbilical-cord veins endothelial cells, BE-I-PLA2 was neither apoptotic nor proliferative but stimulated endothelial cells to release prostaglandin I_2, suggesting an increase of its potential anti-platelet activity *in vivo*. Further studies are required in order to determine the exact mechanism of action of BE-I-PLA2 in the inhibition of platelet aggregation.

1. Introduction

Phospholipases A_2 (PLA_2, EC 3.1.1.4) are enzymes that catalyze the hydrolysis of the *sn*-2 fatty acyl bond of phospholipids to release free fatty acids and lysophospholipids. These enzymes have been found in mammalian tissues, arthropods and in all snake venoms. Based on their source, amino acid sequence, chain length and disulfide bond patterns, PLA_2s are divided in

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11 groups. Snake venom PLA_2s are divided into groups I and II, and most of the PLA_2s from Viperidae venom belong to class II [1]. PLA_2s from snake venoms display several biological effects, including pre or postsynaptic neurotoxicity [2,3], cardiotoxicity [4], myotoxicity [5], platelet aggregation induction or inhibition [6,7] and hypotension [8].

Actually, according to their platelet effects, snake venoms PLA_2 can be divided into three groups: class A includes the phospholipases able to induce platelet aggregation; class B: PLA_2 enzymes which inhibit platelet aggregation induced by several physiological agonist; class C: PLA_2 that presenting biphasic responses on platelets (pro- and anti-aggregating properties) [9].

Platelets and endothelium are important components of the homeostatic mechanism. The endothelium is responsible to maintain blood fluidity by producing inhibitors of aggregation platelet and blood coagulation, by modulating vascular tone and permeability, and by providing a protective envelope separating hemostatic blood components from reactive subendothelial structures. Platelets are responsible by the primary hemostasis, by forming large multicellular aggregates, thus creating a physical barrier that limits blood loss from vessels, and by accelerating coagulation cascade activation and fibrin formation [10].

In this paper we reported the purification, cloning and action in platelets and endothelial cells (HUVECs) of the first phospholipase A_2 isolated from *Bothrops erythromelas* venom, a small Viperidae snake of great epidemiological relevance to Brazilian Northeastern region.

2. Material and methods

2.1. Venom and reagents

A pool of venom was prepared by collecting and freezing drying individual samples of 32 young and adults *B. erythromelas* snakes from both sexes, inhabiting the state of Pernambuco (Brazil) and maintained in the laboratory of the UFPE. Superdex 75, Mono Q columns and Ettan MALDI-TOF mass spectrometer were from Amersham Biosciences (Uppsala, Sweden). C4 column was obtained from Vydak (The Separations Group). TSK G2000 was obtained from Tosoh Bioscience LLC (Montgomeryville, PA, USA). EDTA, 2-mercaptoethanol, human thrombin, endothelial cell growth factor (ECGF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibodies PE-conjugated-anti-CD54, CD55 and CD62E were obtained from PharMingen (BD Biosciences, USA). Arachidonic acid, ADP and collagen were obtained from Chrono-log Corporation (Havertown, PA, USA). Endothelial cell growth supplement from bovine neural tissue was purchased from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS), trypsin, RPMI 1640 medium, HAM F 12 medium, penicillin and streptomycin were purchased from Cultilab (Campinas, SP, Brazil). EIA kit 6-keto-protaglandin F1α was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Sequencing grade endoproteinase Lys-C, Glu-C and Asp-N were from Boehringer Mannheim (Germany). All other chemicals used were of the highest purity commercially available.

2.2. Purification of BE-I-PLA2

Gel filtration and ion exchange were carried out on a FPLC GE System. Reverse phase chromatographies were carried out on a HPLC Shimadzu System.

B. erythromelas venom (150 mg) was dissolved in 1 mL of 50 mM ammonium acetate buffer, pH 5.0, and the insoluble material was removed by centrifugation. The supernatant was applied to a Superdex 75 column pre-equilibrated with the same buffer used for protein elution, at a flow rate of 0.5 mL/min. The phospholipasic activity of the eluted fractions was tested according to the methodology described by Holzer and Mackessy [11]. The more active pool of fractions was lyophilized, diluted in 50 mM Tris–HCl buffer, pH 8.0, and subsequently submitted to a Mono Q anion exchange column (50 mm × 5 mm) previously equilibrated with the same buffer. Elution was carried out isocratically with the equilibration buffer, followed by a linear gradient from 0 to 50% of equilibration buffer containing 1 M NaCl, at a 1 mL/min flow rate. The peak containing the higher phospholipasic activity was applied to a C4 column (150 mm × 46 mm) previously equilibrated with 0.1% trifluoroacetic acid (TFA). The sample was eluted with a linear gradient of 0–100% using 0.1% trifluoroacetic acid in 90% acetonitrile, at a flow rate of 0.7 mL/min. The more active peak was rechromatographed in the same column, the fractions being eluted with a linear gradient of 0–60% using 0.1% trifluoroacetic acid in 90% acetonitrile, at a flow rate of 0.7 mL/min. Protein contents were determined either by the method of Markwell *et al.* [12] or by measuring the absorbance at 280 nm. The molecular mass of purified protein was determined with a MALDI-TOF mass spectrometer.

2.3. Amino acid sequencing

Edman degradation was performed with an automatic gas-phase sequencing machine (PPSQ-23 Sequencer, Shimadzu), under the conditions recommended by the manufacturer. For sequencing, enzyme samples were reduced, alkylated and digested with trypsin, endopeptidase Asp-N or endopeptidase Glu-C at an enzyme to substrate ratio of 1:20 (w/w). Peptides were separated on a 4.6 mm × 15 mm C4 Vydac column using an acetonitrile gradient (0–90%) in 0.1% (v/v) trifluoroacetic acid for 60 min at a 0.7 mL/min flow rate.

2.4. Chemical modification

Modification of histidine residues with *p*-bromophenacylbromide (*p*-BPB) was performed as previously described by Diaz-Oreiro and Gutierrez [13]. The resulting product was applied to a TSK G2000 size exclusion column (7.5 mm × 60 cm), previously equilibrated with 50 mM ammonium bicarbonate, pH 7.0. The eluate absorbance was monitored at 220 nm and the fraction corresponding to the enzyme alkylated was collected. The reaction yield was monitored using an Ettan MALDI-TOF mass spectrometer in linear mode.

2.5. Cloning and sequencing

A 3 μL sample of a venom gland cDNA library [14] in pGEM 11 zf+ was used to transform electrocompetent DH5α cells. After

1 h of incubation in SOC medium, the cells were plated on LB/agar plates containing ampicilin for selection of the transformants. Isolated colonies were randomly picked and grown over-night in 5 mL of LB/amp in an orbital shaker at 37 °C. The plasmids were purified by alkaline lysis followed by phenol/chlorophorm/isoamilic acid extraction. The inserts were sequenced on an Applied Biosystems automated sequencer, using the Big Dye® kit, according to the manufacturers instructions. The sequences were translated and analyzed by BLASTp for identification of phospholipase A_2 clones.

2.6. Human platelet aggregation

Venous blood from healthy volunteers was collected into plastic tubes containing 3.8% sodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation at 900 rpm for 20 min at room temperature. Washed platelets (WP) were obtained by centrifugation of PRP added of 2% EDTA (EDTA/PRP 1:20) at 2500 rpm for 15 min followed by two washes with washing buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.9 mM Na_2HPO_3, 2.1 mM $MgCl_2$, 22 mM $C_6H_5Na_3O_7$, 0.055 mM Glucose, 0.053 mM BSA, pH 6.5). The pellet was suspended in 2 mL Tyrode buffer (10 mM Hepes, 134 mM NaCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM MgCl, 0.055 mM glucose, pH 7.4). The platelets were counted in Sereno-Baker 9020+AX System and adjusted for a final concentration of $3 \times 10^8\ mL^{-1}$ with Tyrode buffer. The aggregation was monitored at 37 °C for 10 min in a Chronolog aggregometer using 400 μL of platelet suspension and thrombin (1 U), arachidonic acid (0.5 mM), ADP (10 μM) or collagen (2 μg/mL) with continuous stirring. For inhibition tests, different amounts of native and alkylated BE-I-PLA2 or 200 μM acetylsalicylic acid (positive control of inhibition) were added 10 min before the addition of agonists.

2.7. Action of BE-I-PLA2 in platelet receptors by flow cytometry

Initially, PRP or WP were preincubated for 30 min at 37 °C with different amounts of BE-I-PLA2. These samples (3 μL) were added to tubes containing 50 μL of phosphate-buffered saline (PBS) with saturating concentrations of FITC-conjugated anti-CD41a (GPIIb), anti-CD42b (GPIb), anti-CD62P (P-selectin), anti-CD49b (GPIa) and PE-anti-CD29 (GPIIa) or equivalent concentrations of an isotypic control IgG1. After 30 min incubation at room temperature and in the dark, samples were fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA, USA). Light scattering and fluorescence channels were set at logarithmic gain, and at least 10,000 events were collected for each sample.

2.8. Cell culture and apoptosis

Endothelial cells were obtained from human umbilical-cord veins (HUVECs) by collagenase digestion as described by Jaffe *et al.* [15]. Initially, cells were grown in RPMI 1640 medium, supplemented with FBS (10%, v/v), heparin (45 μg/mL), endothelial supplement growth factor (25 μg/mL), sodium pyruvate (1 mM), glutamine (2 mM), penicillin (100 U/mL), mercaptoethanol (50 μM) and extract of mouse brain (0.75%), at 37 °C in a humidified 5% CO_2 incubator. To exclude lipopolysaccharide interference, all experiments were performed in the presence of polymyxin B (7 μg/mL). Cells between the first and the third passage in confluent monolayer (20,000 cells/well) were incubated with BE-I-PLA2 (1 or 10 μM final concentrations) for 24 h or 48 h. Morphologic changes and cellular viability were analyzed by fluorescence microscopy after the treatment of adhered and not adhered cells with acridine orange (50 μg/mL) and ethidium bromide (50 μg/mL). In each experiment at least 200 cells were examined. HUVECs without FBS 10% were used as positive control.

2.8.1. Nitric oxide (NO) generation

After treatment of HUVECs cultured in Ham's F-12 medium with 0.3 or 3 μM BE-I-PLA2 or 5 UI/mL thrombin (positive control) for 1 h, the supernatants were collected and the oxidation products of NO, nitrite and nitrate were assayed after reduction with VCl3-saturated solution in 1 M HCl, at 90 °C. Nitric oxide generated was determined by NO-ozone chemiluminescence using a NO^{ATM} 280 NO Analyzer (Sievers, Boulder, CO) and its concentration calculated from a $NaNO_3$ standard curve using the Bag Program Software 2.2 (Sievers Instruments Inc.).

2.8.2. Prostaglandin I_2 (PGI_2) release

After treatment of HUVECs with 0.3 μM BE-I-PLA2, 1 UI/mL thrombin or 2 μg/mL LPS (positive controls) for 1 h, medium was removed and the cells cultured during 24 h in RPMI containing 10% FBS. Supernatants removed after 1 and 24 h were centrifuged for 10 min, 400 × *g* at 4 °C, in order to exclude interference of cellular debris. 6-Keto-protaglandin F1α (prostaglandin I_2 stable metabolite) levels were measured using commercial ELISA kit.

3. Results

3.1. Purification, cloning and sequencing

After four steps of purification, we obtained a homogeneous protein, with PLA_2 activity and with an estimated molecular weight of 13,649.57 Da (Fig. 1). Beside the N-terminal residues, obtained by Edman degradation, four other internal peptides were also sequenced, covering around one third of the complete toxin sequence. In order to obtain the remaining amino acids, a reverse biology strategy was employed. The open reading frame of *B. erythromelas* PLA_2 (BE-I-PLA2) is preceded by a 5′ untranslated region, followed by a 48 bp stretch encoding for a highly conserved 16 amino acid signal peptide, the mature protein and a 3′ untranslated region. All four peptide sequences obtained by Edman sequencing matched the translated cDNA sequence containing 457 bp (Fig. 2). The nucleotide sequence of BE-I-PLA2 was deposited in the GenBank under the accession number DQ359953.

According to deduced sequence, BE-I-PLA2 is composed of 122 amino acid residues which is in accordance with the molecular mass determined by MALDI-TOF (13,649.57 Da). Based on its amino acid sequence, an isoelectric point of 4.67 was calculated. Potential sites for glycosylation have not been

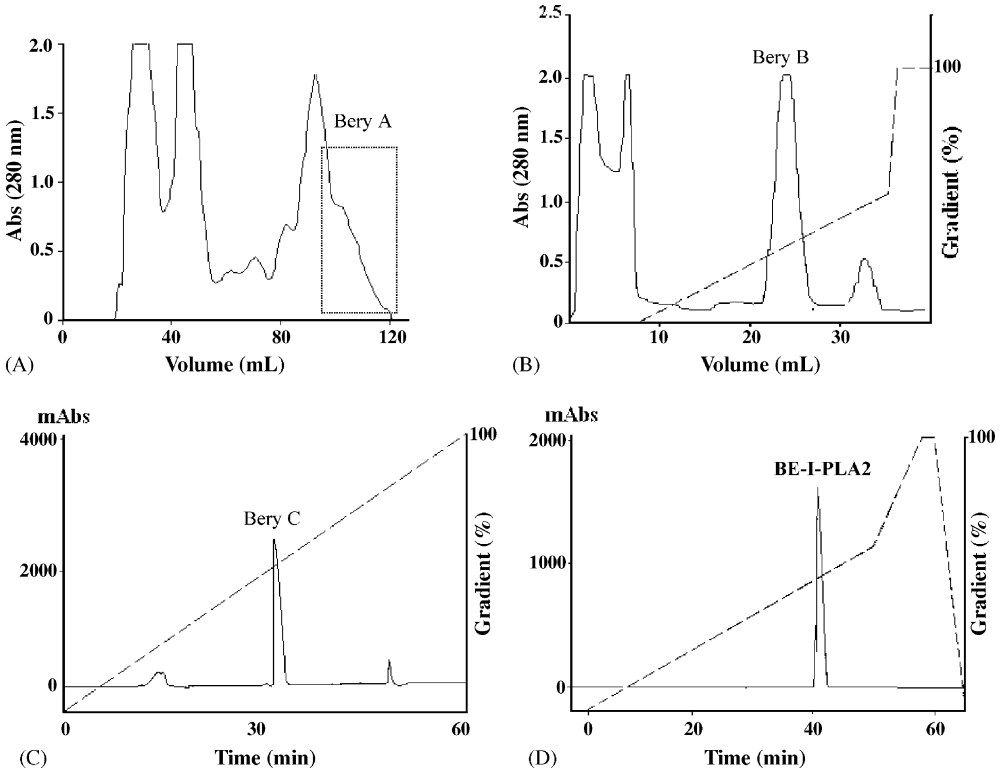

Fig. 1 – Purification of BE-I-PLA2. (A) Gel filtration chromatography of *B. erythromelas* venom (150 mg) on a Superdex 75 column equilibrated and eluted with 50 mM ammonium acetate buffer, pH 5.0, at a flow rate of 0.5 mL/min. (B) Ion-exchange chromatography of Bery A on a Mono Q column equilibrated with 50 mM Tris–HCl buffer, pH 8.0 and eluted with a gradient (0–50%) of 1 M NaCl and flow rate of 1 mL/min. (C) Reversed-phase of Bery B on a C4 column equilibrated with solvent A (0.1% TFA in water) and eluted with 0–100% gradient of solvent B [acetonitrile/solvent A, 9:1, v/v] and a flow rate of 0.7 mL/min. (D) Reversed-phase of Bery C in the same column and buffers of (C) using 0–60% gradient of solvent B and a flow rate of 0.7 mL/min. BE-I-PLA2 corresponding to purified protein.

identified into BE-I-PLA2 sequence. When compared with other PLA_2s, its sequence presented higher similarities with a phospholipase A_2 from *Bothrops pictus* (93%) of unknown function, and with a anti-platelet phospholipase A_2 from *Gloydius shedaoensis* (90%). Homologies of 82 and 77% were obtained with the platelet inhibitors BJ-PLA2 from *Bothrops jararaca* and BthA-I-PLA(2) from *Bothrops jararacussu* venom, respectively (Fig. 3).

3.2. *Chemical modification*

According to the mass spectra analysis, in which one mass envelope was observed, the alkylation procedure was successful, with no evidences of non-inactivated enzyme. When assayed for catalytic activity, the enzyme displayed no signs of residual activity, once again suggesting a fully effective alkylation (data not shown).

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1     ATGAGGACTCTCTGGATAATGGCCGTGTTGCTGGTGGGCGTCGAGGGGAGCCTGGTGCAA
       M  R  T  L  W  I  M  A  V  L  L  V  G  V  E  G  S  L  V  Q
61    TTTGAGACGTTGATCATGAAAATTGCGGGGAGAAGTGGTGTTTGGTACTACGGCTCTTAC
       F  E  T  L  I  M  K  I  A  G  R  S  G  V  W  Y  Y  G  S  Y
121   GGATGCTACTGCGGCTCGGGAGGCCAAGGCCGGCCACAGGACGCCAGCGACCGCTGCTGC
       G  C  Y  C  G  S  G  G  Q  G  R  P  Q  D  A  S  D  R  C  C
181   TTTGTGCACGACTGCTGTTATGGAAAAGTGACCGACTGCGACCCCAAAGCGGACGTCTAC
       F  V  H  D  C  C  Y  G  K  V  T  D  C  D  P  K  A  D  V  Y
241   ACCTACAGCGAGGAGAACGGGGTTGTCGTCTGCGGAGGGGACGACCCGTGCAAGAAGCAG
       T  Y  S  E  E  N  G  V  V  V  C  G  G  D  D  P  C  K  K  Q
301   ATTTGTGAGTGCGACAGGGTTGCGGCAACCTGCTTCCGAGACAATAAGGACACATACGAC
       I  C  E  C  D  R  V  A  A  T  C  F  R  D  N  K  D  T  Y  D
361   AACAAATATTGGTTTTTCCCGGCCAAAAATTGCCAGGAGGAATCAGAGCCATGCTAANTC
       N  K  Y  W  F  F  P  A  K  N  C  Q  E  E  S  E  P  C
421   TCTGCAGGCCGGGAAAAACCCCTCAAATTACACAATC
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Fig. 2 – cDNA and deduced amino acid sequences of BE-I-PLA2. The deduced sequence of BE-I-PLA2 (GenBank accession no. DQ359953) is shaded. Boxes indicate the amino acid sequences obtained by Edman degradation.

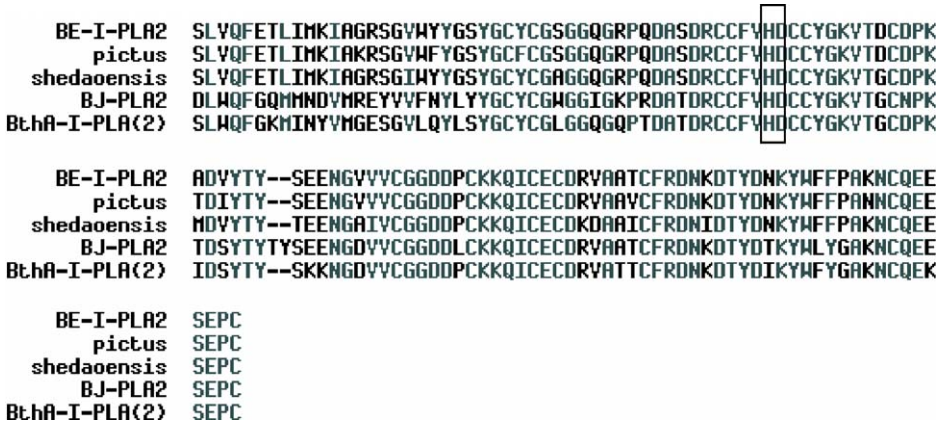

Fig. 3 – Comparative analyze of BE-I-PLA2 sequence. BE-I-PLA2 sequence was aligned with other members of the phospholipase A_2 family. Phospholipase A_2 from *Gloydius shedaoensis*, BJ-PLA2 from *Bothrops jararaca* and BthA-I-PLA(2) from *Bothrops jararacussu* venom (accession nos. AAR11860, P81243 and AAN37410, respectively) are inhibitors of platelet aggregation. Phospholipase A_2 of *Bothrops pictus* (accession no. AAF91498) has unknown function. Residues in gray indicate high consensus. Box indicates the residues His48 and Asp49 involved on active and Ca^{2+} binding sites.

3.3. *Platelet studies*

3.3.1. *Inhibition of platelet aggregation*

When tested in PRP, BE-I-PLA2 exhibited an inhibitory effect on human platelet aggregation induced by collagen and arachidonic acid, but not ADP ($n = 4$). The enzymatic effect was variable in according with the agonist (Fig. 4).

BE-I-PLA2 inhibited the platelet response to collagen in a concentration-dependent manner (Fig. 4A). In the absence of BE-I-PLA2 the aggregation percentage induced by collagen was $80 \pm 5\%$. Pre-incubation of the platelets with 0.0018, 0.009, 0.0135, 0.018, 0.09 and 0.18 μM BE-I-PLA2, for 10 min at 37 °C, resulted in reduction of the aggregation to 50 ± 4, 25 ± 2, 15 ± 4, 10 ± 2, 12 ± 2 and $9 \pm 3\%$, respectively. Fig. 4A (insert) illustrates that BE-I-PLA2 apparently did not interfere on the initial shape-change reaction induced by collagen.

Interestingly, the effect of BE-I-PLA2 on arachidonic acid-induced platelet aggregation was not dose-dependent. Fig. 4C shows the maximum aggregation percentage induced by arachidonic acid in the absence of BE-I-PLA2 ($85 \pm 5\%$ positive control) and the inhibitory effect of BE-I-PLA2 in the concentrations of 0.0018–0.18 μM. Contrasting to collagen response, pre-incubation of the platelets with 0.0018–0.009 μM BE-I-PLA2, for 10 min at 37 °C, followed by addition of 0.5 mM arachidonic acid resulted in percentages of aggregation of 75 ± 5 and $70 \pm 4\%$, respectively. On the other hand, pre-incubation with 0.0135, 0.018, 0.09 or 0.18 μM BE-I-PLA2 abolished the platelet response to agonist.

In order to explore if the inhibitory effect of BE-I-PLA2 on platelet aggregation is related to its enzymatic activity, the alkylated enzyme was tested in the same conditions of the native enzyme. Contrarily to BE-I-PLA2, no inhibitory effect on collagen or arachidonic acid-induced platelet aggregation was observed when 0.09 and 0.18 μM of alkylated enzyme were used. On the other hand, experiments using 9.0 μM of alkylated BE-I-PLA2 resulted in aggregation percentage similar to higher concentrations of native BE-I-PLA2 (12 ± 5 and 0% to collagen and arachidonic acid, respectively) (Fig. 5).

When tested in washed platelets, BE-I-PLA2 (0.18, 0.9 and 9.0 μM) was unable to inhibit the aggregation induced by collagen, thrombin or arachidonic acid. However, addition of small amounts of PRP to the washed platelets suspension restored the inhibitory effect of BE-I-PLA2 on arachidonic acid or collagen-induced aggregation (data not shown).

3.3.2. *Platelet receptors*

Pre-incubation of PRP or washed platelets with BE-I-PLA2 (0.18, 0.9 and 9.0 μM) did not result in any significant difference in the fluorescence intensity of CD41a, CD42b, CD62P, CD49b and CD29 when compared to control.

3.4. *Endothelial cells studies*

3.4.1. *Apoptosis and proliferation tests*

BE-I-PLA2 was unable to induce detachment and apoptosis of HUVECs cultured in medium supplemented with either 1 or 10% FBS. Furthermore, this protease neither induced cell proliferation nor increased the proliferative effect of the ECGF.

3.4.2. *NO generation and PGI_2 release*

BE-I-PLA2 significantly increased the release of PGI_2 by HUVECs, but not NO. After 1 h of incubation with BE-I-PLA2 (0.3 μM), thrombin (5 U/mL) or LPS (2 μg/mL), the concentrations of 6-keto-protaglandin F1α on HUVECs supernatants were 582 ± 108, 3746 ± 284 and 497 ± 51 pg/mL, respectively, levels increased when compared to control (203 ± 13) (Fig. 6A). 6-Keto-protaglandin F1α levels on supernatants of 24 h also reveled increase PGI_2 levels for 0.3 μM BE-I-PLA2 ($13{,}012 \pm 1125$) and 2 μg/mL LPS ($35{,}148 \pm 3085$), contrarily to 5 U/mL thrombin (6249 ± 396) and to control (4540 ± 787) (Fig. 6B).

4. Discussion

Snake venoms from the Viperidae family contain a number of active components on platelets what includes inhibitors and

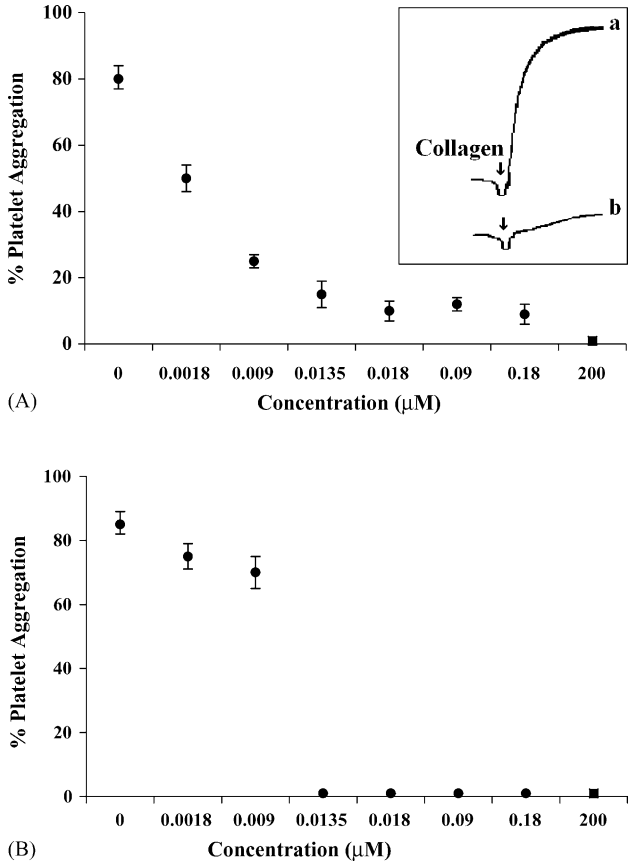

Fig. 4 – Inhibitory effects of BE-I-PLA2 on human platelet aggregation. Plasma rich platelet was pre-incubated at 37 °C for 10 min with different concentrations of BE-I-PLA2 or (■) 200 μM of acetylsalicylic acid (positive control of inhibition) followed by addition of 2 μg/mL collagen (A) or 0.5 mM arachidonic acid (B). Inserted figure in (A) correspond to plasma rich platelet pre-incubated at 37 °C for 10 min in the absence (a) or presence (b) of 0.18 μM BE-I-PLA2 followed by addition of 2 μg/mL collagen. Arrows indicate that BE-I-PLA2 apparently did not interfere on the initial shape-change reaction induced by collagen. Results are the mean values ± S.E.M. of five independent experiments performed in triplicate.

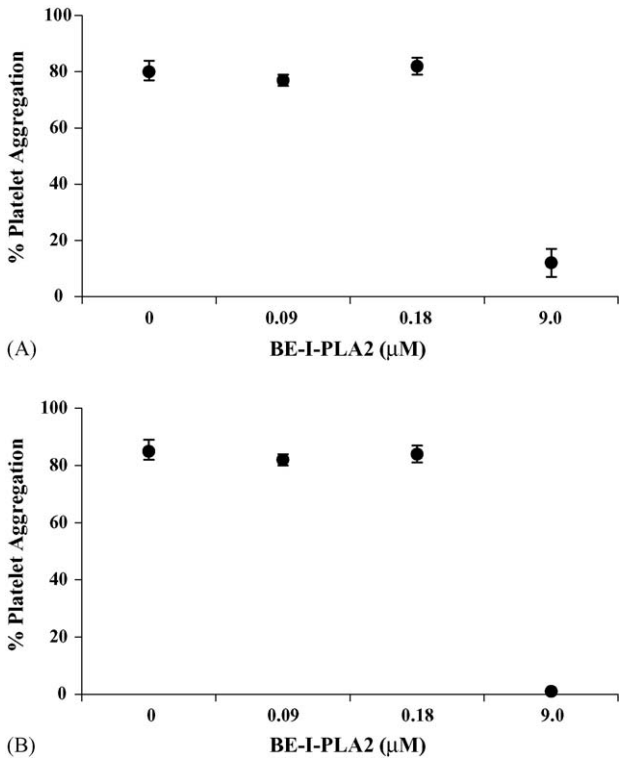

Fig. 5 – Inhibitory effects of alkylated BE-I-PLA2 on human platelet aggregation. Plasma rich platelet was pre-incubated at 37 °C for 10 min with different concentrations of alkylated BE-I-PLA2 followed by addition of 2 μg/mL collagen (A) or 0.5 mM arachidonic acid (B). Results are the mean values ± S.E.M. of four independent experiments performed in triplicate.

inductors of platelet aggregation. Several platelet inhibitors have been purified and characterized as metalloproteinases, disintegrins, C-type lectins and phospholipases A_2. Despite the several reports about PLA_2 with inhibitory action on platelet aggregation, the mechanism by which these enzymes alters the platelet function has not been well explored.

In this study we isolated a new antiplatelet acidic PLA_2 from *B. erythromelas* venom by a four-step sequential purification procedure. The primary sequence analysis of the purified enzyme, named BE-I-PLA2, showed that it contains all the PLA_2 conserved residues involved in Ca^{2+} binding in its active site and it is a catalytically active Asp49 enzyme. Functionally, BE-I-PLA2 showed to be a potent platelet aggregation inhibitor in PRP, failing to induce platelet aggregation in all doses tested. None inhibition by BE-I-PLA2 was observed in washed platelet, have been its effect restored by addition of PRP upon platelets. At first, these results suggest the dependence of plasma cofactor(s). In fact, Yuan *et al.* [16] demonstrated that the inhibition of platelet aggregation by some PLA_2 from snake and bee venoms, as well as by pancreatic PLA_2 is dependent of lipoprotein fraction present in the plasma, principally the high-density lipoprotein (HDL). Lipoproteins when hydrolyzed by a subset of secreted PLA_2 generate lysophosphatidylcholine (LysoPC) able to inhibit the platelet aggregation induced principally by collagen, but also by thrombin, ionophore, ADP, adrenaline, arachidonic acid and tromboxane A_2 [16–18]. Lyso-PC inhibit all platelet responses, including shape change, serotonin release, TXA_2 generation and platelet aggregation via stimulation of the adenylyl cyclase, followed by intracellular accumulation of the soluble second messenger cAMP, that regulates a myriad of cell processes [19].

Despite the dependence of plasma cofactor(s), our results provide some lines of evidence indicating that the enzymatic generation of lysoPC cannot be the unique action mechanism of BE-I-PLA2 upon platelets. First, in contrast to LysoPC that indistinctly affect platelet response to several agonist, BE-I-PLA2 was not able to inhibit the platelet aggregation induced by ADP. Second, BE-I-PLA2 apparently did not interfere on the initial shape-change reaction induced by collagen. Third, modification of active site of BE-I-PLA2 abolished its catalytic activity but only inhibited partially the anti-platelet activity.

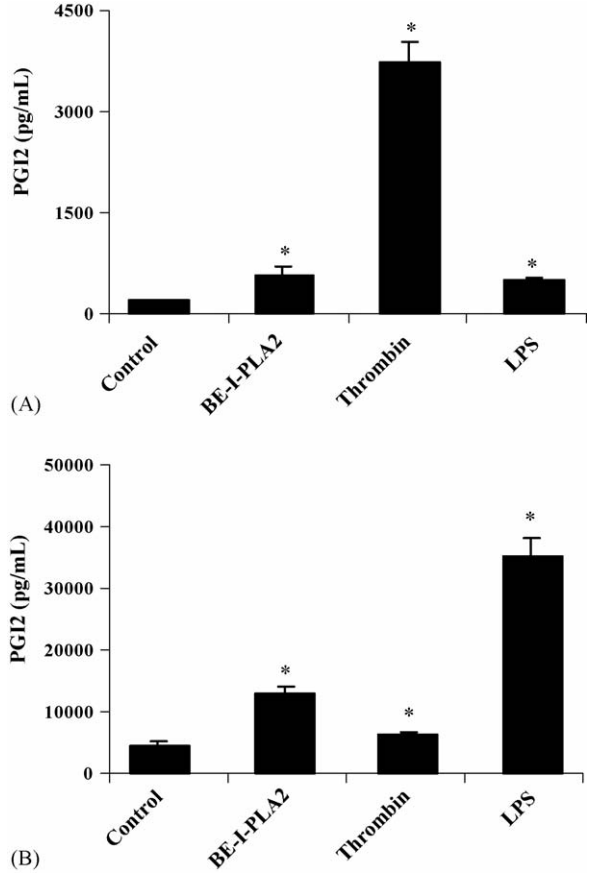

Fig. 6 – BE-I-PLA2 effect on PGI_2 release and expression by HUVECs. After treatment with 0.3 μM BE-I-PLA2 (5 μg/mL), 1 UI/mL thrombin or 2 μg/mL LPS for 1 h at 37 °C, supernatants were removed and the cells cultured during 24 h in RPMI containing 10% FBS. 6-Keto-protaglandin F1α (prostaglandin I_2 stable metabolite) levels were measured in 1 h (A) and 24 h (B) supernatants using commercial ELISA kits. Results are the mean values ± S.E.M. of four independent experiments, performed in triplicate. $p < 0.005$ by student's *T*-test.

Finally, although the collagen-induced platelet aggregation has been inhibited by BE-I-PLA2 of concentration-dependent manner, correlation dose-effect was not observed when arachidonic acid was used as agonist.

In an attempt to understand the mechanism by which BE-I-PLA2 inhibits platelet aggregation, we studied the actions of the enzyme on some important platelet receptors. Platelet membrane glycoproteins are essential for initiating platelet adhesion and aggregation. The adhesion of platelets to collagen has been reported to involve predominantly α2β1 integrin (GPIa-GPIIa). GPIb-IX-V complex is the receptor for von Willebrand factor and GPIIbIIIa is specific to fibrinogen. Adhesion of platelets to von Willebrand or collagen activates platelets, leading to Ca^{2+}-dependent activation of GPIIb-IIIa receptor that binds to fibrinogen, hence mediating platelet aggregation [20]. Our studies showed that BE-I-PLA2, or the products generated by lipoproteins hydrolysis, were not able to bind or to degrade GPIb-IX-V, GPIIbIIIa or α2β1, indicating that the pathway triggered by the enzyme for platelet inhibition is not related with these receptors. Since aggregation induced by collagen and arachidonic acid were affected by BE-I-PLA2, one action of this enzyme on TXA_2 receptors could be speculated. However, the lack of inhibitory effect aggregation platelet induced by ADP, also dependent from generation of TXA_2, undermines this hypothesis. Thus, the exact mechanism of antiplatelet activity of BE-I-PLA2 still remains to be identified.

Healthy endothelium is a metabolically active interface between the blood and extravascular tissues. Endothelial cells produce a variety of factors involved in the control of vascular tone, platelet activation and cell growth, the most important being nitric oxide (NO) and prostacyclin (PGI_2) [21]. Despite the known hypotensive effect and platelet actions exerted by some PLA_2s from snake venoms, the effects of these enzymes in endothelial cells have not been described.

Our studies demonstrated that BE-I-PLA2 in concentrations up to 10 μM did not induce detectable direct cytotoxicity or apoptotic effect on human umbilical vein nor is able to induce specific cell proliferation. In addition, BE-I-PLA2 (0.3–3 μM) did not interfere with NO release. On the other hand, 0.3 μM of this enzyme significantly increases the PGI_2 production by endothelial cells. Interestingly, a profile of PGI_2 release similar to one obtained with LPS was observed in 24 h cell culture supernatants. It is known that additionally to increase in COX activity, LPS also induce the COX-2 expression, constitutive and inducible cyclo-oxygenases, respectively, responsible by conversion of arachidonic acid to prostaglandins and consequently PGI_2 production [22]. Since HUVEC treated for 1 h with BE-I-PLA2 were able to continue releasing a high quantity of PGI_2, showing the same in the absence of stimuli (supernatants of 24 h), it is possible to suppose a modulation of COX-2 expression by BE-I-PLA2.

Huang [23,24], studying the action of phospholipases A_2 from *Vipera russelli* venom on blood pressure, suggested that the hypotensive effect of these PLA_2s is related to the increase in plasma PGI_2 and TXA_2 levels. Nevertheless, recent report indicates that PGI_2 and NO display not only a crucial role in maintaining thromboresistance and tone of the vascular wall, but also that PGI_2 is essentially a platelet suppressant and NO a regulator of vascular tone [25]. Thus, a possible explanation is that the increased PGI_2 release by endothelial cells could be involved with a amplification of the inhibitory effect of BE-I-PLA2 in platelet aggregation *in vivo*.

In summary, in this report we described the purification, characterization and complete sequencing of BE-I-PLA2, the first phospholipase A_2 from *B. erythromelas* venom. BE-I-PLA2 is a novel acidic Asp49-PLA_2, potent inhibitor of platelet aggregation induced by collagen and arachidonic acid, presenting no binding to/interference with the principal platelet receptors. BE-I-PLA2 ability to stimulate endothelial cells to PGI_2 release suggests an increase of its potential anti-platelet *in vivo*. Further studies are required in order to determine the exact mechanism of action of BE-I-PLA2 in the inhibition of platelet aggregation.

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