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Phospholipase A_2 enhances the endothelial cell detachment effect of a snake venom metalloproteinase in the absence of catalysis

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ABSTRACT

Microvessel disruption leading to hemorrhage stands among the most dangerous consequences of envenomings by snakes of the family Viperidae. A PIII metalloproteinase (SVMP), balteragin, purified from the venom of the snake *Bothrops alternatus*, displays a potent hemorrhagic effect, and a moderate myotoxicity *in vivo*. Previous studies described the ability of this SVMP to induce the detachment of C2C12 myoblasts in culture, without causing cytolysis. Surprisingly, a purified acidic phospholipase A_2 (PLA_2) from the same venom was found to increase this detaching activity of the SVMP on myoblasts. Since endothelial cells are a natural target of SVMPs *in vivo*, the possibility that this synergistic effect is also observed on this cell type was explored in the present work. In addition, a first approach of the mechanism of action of this effect was studied. Results clearly confirm that the acidic PLA_2, despite lacking toxicity towards endothelial cells, significantly enhances the detaching effect of the SVMP even at a concentration as low as 1 μg/mL. Inhibition of enzymatic activity of the PLA_2 by chemical modification with *p*-bromophenacyl bromide did not affect the synergistic activity, suggesting that this effect is not dependent on phospholipase enzymatic activity and may instead be the consequence of an interaction of the PLA_2 with endothelial cell plasma membrane. To our knowledge, this is the first report of a synergistic action of a non toxic PLA_2 in enhancing the detachment of endothelial cells induced by a metalloproteinase.

1. Introduction

Viperid snake venoms are complex biological secretions able to induce drastic pathophysiological alterations as a consequence of the action of diverse toxic components. In addition to systemic effects, mostly hemorrhage, coagulopathy and cardiovascular shock, these venoms provoke a prominent local tissue pathology, characterized by skeletal muscle necrosis, hemorrhage, blistering and edema [1]. These effects of rapid onset are mediated mainly by snake venom metalloproteinases (SVMPs) and phospholipases A_2 (PLA_2s) [2], which are generally abundant constituents of viperid venoms [3]. SVMPs are capable of degrading various proteins of the basement membrane, as well as endothelial cell components involved in cell-matrix and cell–cell adhesion, often leading to microvascular damage and hemorrhage [4]. On the other hand, basic PLA_2s from viperid venoms are the main factors responsible for their ability to induce myonecrosis [5]. Furthermore, a group of catalytically inactive PLA_2 homologs, despite their inability to hydrolyze phospholipids, are capable of inducing myonecrosis [6,7]. In addition, viperid venoms also contain acidic PLA_2s which, despite being highly active catalytically, are generally devoid of toxicity [8,9]. The adaptive role of these enzymes in envenomings remains elusive.

In previous studies on the venom of *Bothrops alternatus*, an acidic-type PLA_2 [2] and a P-III SVMP [10] were purified and characterized. The PLA_2 (BaSpIIRP4) was shown to lack myotoxicity in a mouse model and, accordingly, to be devoid of cytotoxic effects on the myogenic cell line C2C12 in culture [11]. The SVMP (baltergin),

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in contrast, induced a potent hemorrhagic effect *in vivo*, and additionally, a moderate myonecrosis [10], together with an *in vitro* cell-detaching action upon the C2C12 cultures. Surprisingly, the addition of the non-toxic acidic PLA_2 to myoblasts incubated with the SVMP resulted in an increment in the detaching effect induced by the latter, thus evidencing a hitherto unknown synergistic effect between these types of venom components [11]. The potential synergisms between diverse venom components is a poorly investigated subject of high significance that should be further explored for understanding the action of snake venoms from an integrative perspective [12]. In that sense, a synergism between two types of myotoxic PLA_2s (Asp49 and Lys49) from *Bothrops asper* venom has been recently described [13,14].

Since endothelial cells are a natural target of SVMPs *in vivo*, the possibility that this synergistic effect is also observed on this cell type was explored. Results clearly confirm that the acidic PLA_2, despite lacking toxicity towards endothelial cells, significantly enhances the detaching effect of the SVMP baltergin, thus evidencing that the previously described synergistic effect may occur in other physiologically-relevant cell types in addition to myoblasts. Additionally, a first approach to study the mechanism of this synergism was performed by using a chemical inhibitor to abrogate the catalytic activity of the PLA_2. Our observations show that there was no difference with the effect triggered by the active form, suggesting that the mechanism is independent of enzymatic phospholipid hydrolysis.

2. Materials and methods

2.1. Venom and toxins

B. alternatus crude venom was supplied by the serpentarium of Corrientes, Argentina, and kept at −20 °C after vacuum drying. The SVMP (baltergin) and the acidic PLA_2 (Ba SpII RP4) were isolated from this venom as previously described [2,10]. Venom and toxins solutions were prepared immediately before the experiments. Proteins constitute 93% of the dry weight of this venom, as determined by the Biuret reaction. This value is consistent with reports that proteins comprise 90–95% of the dry weight of venoms [15].

2.2. Cell culture

tEnd is an established murine endothelial cell line of capillary origin, transformed with polyoma virus [16]. Cells were routinely grown in 25 cm^2 flasks using Dulbecco's minimum essential medium (DMEM; Gibco, Argentina), supplemented with 5% fetal bovine serum (FBS; Sigma–Aldrich, USA) and penicillin-streptomycin (Invitrogen, USA), in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cell detachment assay

The detaching effect of crude venom or purified enzymes on the endothelial cell line was assessed in 96-well microplates. Cells were harvested from subconfluent monolayers after exposure to trypsin/EDTA at 37 °C and seeded at an approximate initial density of $1–2 \times 10^4$ cells/well, in growth medium (DMEM-10% FBS). When monolayers reached 80–90% confluence, variable amounts of venom or toxins were diluted in assay medium supplemented with 5% FBS and added to the cells in a total volume of 200 μL/well. 0.1% (v/v) of Tritón X-100 and culture medium were used as positive and negative controls of detachment, respectively. After 3 h of incubation, detached cells were removed by washing twice with phosphate-buffered saline (PBS, pH 7.2). The remaining adherent cells were fixed with methanol:acetic acid (3:1) and stained with 0.5% crystal violet in 20% (v/v) methanol. Finally, the dye was released from cells by the addition of ethanol:acetic acid (3:1) and absorbances were recorded at 620 nm [17]. Results were expressed as percentage, considering the absorbance of control wells incubated without venom or toxins as 100% attachment. All assays were carried out in triplicate wells.

2.4. Assessment of cytolytic effect

For the assessment of cytolysis induced by venom or toxins, cells were seeded in 96-well plates, as described above, and the release of the cytosolic enzyme lactate dehydrogenase (LDH) was used as an indicator of membrane damage [18]. Aliquots (40 μL) of the supernatant in culture wells that had been incubated with venom or toxins were collected after 3 h, and LDH activity was determined by using a UV kinetic assay kit (LDH BR CE, Spain). Cytolysis was expressed as percentage, using as 0% and 100% reference values the LDH activity of supernatants from cells exposed to medium alone, or to 0.1% (v/v) Triton X-100, respectively. All assays were carried out in triplicate wells.

2.5. Synergism between purified enzymes: concentration-dependent PLA_2 effect

Synergic activity between baltergin and PLA_2 was assayed on the endothelial cell line, by exposing cells to either individual enzymes or a combination of both. Mixtures had a fixed final concentration of baltergin (100 μg/mL) and different PLA_2 concentrations (1–250 μg/mL) in a volume of 200 μL/well. After incubation for 3 h at 37 °C, cell detachment was determined as described in Section. 2.3. Cell morphological changes were evaluated using a phase-contrast microscope (Axiovert 40, Carl Zeiss Argentina) equipped with a digital camera (Canon CCD 2272x1704, Argentina).

2.6. Synergistic effect of PLA_2: role of its catalytic activity

To further understand the synergistic mechanism, PLA_2 activity was inhibited with p-bromophenacyl bromide (p-BPB), a chemical treatment that results in the alkylation of His48, a highly conserved residue in PLA_2s which plays a key role in catalysis [19–21]. Alkylation of His48 with p-BPB eliminates enzymatic activity and reduces of the toxic and pharmacological effects of PLA_2s [22,23]. Briefly, 1 mg of enzyme was dissolved in 1 mL of 0.1 M Tris, 0.7 mM EDTA, pH 8.0 buffer. Then, 100 μL of *p*-BPB (1.5 mg/mL in ethanol; Sigma) were added and incubated at room temperature (20–25 °C) for 24 h [24]. Excess reagent and salts were eliminated by gel filtration on Sephadex G-25. Inhibition of PLA_2 catalytic activity was confirmed by indirect hemolytic activity assay [25] and phenol red assay [8]. Afterward, cells were exposed to different mixtures of baltergin (100 μg/mL) and *p*-BPB-modified PLA_2 (1, 5, 10 or 100 μg/mL), in a volume of 200 μL/well for 3 h at 37 °C. Untreated PLA_2 was used as positive control of synergism. Cell detachment was determined as described in Section. 2.3.

2.7. Immunization and purification of IgG antibodies to baltergin and PLA_2

Anti-baltergin and anti-PLA_2 sera were obtained from rabbits immunized with the corresponding purified enzymes. An initial dose of 0.1 mg, emulsified in complete Freund's adjuvant and injected intramuscularly and subcutaneously, was followed by booster doses of 0.3 mg in Freund's incomplete adjuvant, at weekly intervals. Serum antibody levels were monitored by gel immunodiffusion [26] and ELISA [27] against the corresponding antigens.

The IgG fraction from each serum was purified by affinity chromatography on Sepharose-Protein G (HiTrap Protein G HP 1 mL, GE Healthcare) using an ÄKTAprime plus (GE Healthcare) system. IgG was eluted with 0.1 M glycine, pH 2.5, immediately neutralized with 2 M Tris–HCl, pH 9.0, and dialyzed against PBS. Protein concentration was determined by the Biuret reaction, and the purity of IgG was assessed by SDS-PAGE (10% gels) under reducing or non-reducing conditions [28]. Immunological specificity was evaluated by gel immunodiffusion tests of the IgG antibodies against the purified enzymes and crude venom, demonstrating single immunoprecipitation lines with the corresponding antibodies.

2.8. Selective neutralization assays

In order to selectively neutralize either baltergin or PLA_2 in the whole venom, *B. alternatus* venom was incubated with anti-baltergin or anti-PLA_2 IgGs, respectively, for 30 min at 37 °C, using an IgG:venom ratio of 25:1 (w/w). This ratio was selected after verifying that the PLA_2 activity of 1 μg of venom was completely neutralized by 25 μg of the antibodies, as assessed by an indirect hemolytic activity assay [25], and that the hemorrhagic activity of baltergin at this same ratio was abrogated, as evaluated in a mouse skin test [29]. After incubation, venom alone (50 μg/mL) or IgG:venom mixtures were added to endothelial cells, and cell detachment and cytolysis were determined as described. All experiments were performed in triplicate wells.

2.9. Statistical analysis

Data represent the mean ± standard deviation (SD) of at least three replicates. Statistical significance was tested by ANOVA followed by Tukey test, and p values lower than 0.05 were considered significant.

3. Results

3.1. Cell detachment and cytolytic effects of B. alternatus crude venom

The whole venom of *B. alternatus* induced a concentration-dependent detachment of endothelial cells, as determined by the crystal violet uptake assay (Fig. 1). Using regression analysis, the half-maximal effect of the venom was estimated at a concentration of 4.94 μg/mL. In contrast, the cytolytic activity of the venom was negligible at the same incubation time period (3 h), without significant LDH release to supernatants even at the highest concentration tested (75 μg/mL). Thus, the venom induced endothelial cell detachment without cytolysis, i.e. without plasma membrane disruption.

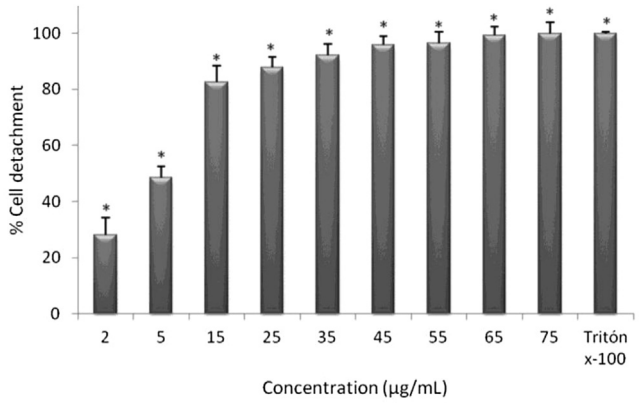

Fig. 1. Endothelial cell detachment induced by different concentrations of *Bothrops alternatus* crude venom after 3 h of incubation. Cell detachment was determined by crystal violet uptake, as described in Materials and methods. Readings from cells incubated with medium alone are considered as 0% detachment (100% attachment). Each bar represents mean ± SD of triplicate assays. *All venom concentrations resulted in statistically significant differences in comparison to untreated cells ($p < 0.05$).

3.2. Synergism between purified B. alternatus venom enzymes

Endothelial cells were exposed to different concentrations of each enzyme, independently or in combination, for 3 h, to evaluate cell detachment and cytolytic effects. Incubation with the SVMP baltergin (1–250 μg/mL) induced a concentration-dependent loss of cell adhesion, whereas incubation with the acidic PLA_2 alone (1–250 μg/mL) did not cause any detaching effect (data not shown). When both toxins where added together to the cells, the resulting detaching effect was markedly higher than that corresponding to the same amount of baltergin alone. Thus, despite the fact that the acidic PLA_2 did not induce any cell detachment *per se*, it clearly enhanced the detachment induced by the SVMP.

The degree of synergism was dependent upon PLA_2 concentration (Fig. 2). Addition of a PLA_2 amount as low as 1 μg/mL to baltergin already caused a significant enhancement of the cell detaching activity of the latter. This effect increased proportionally when using higher amounts of the PLA_2 in the mixture (5, 10 and 50 μg/mL), reaching a maximum synergy at 50 μg/mL and above (Fig. 2).

In agreement with the experiments performed with crude venom, neither of the isolated enzymes caused a significant LDH release from endothelial cells, indicating that detachment occurred in the absence of cytolysis. Morphological assessment of cell changes by phase-contrast microscopy showed that baltergin induced a gradual rounding of the cells that finally slough off the monolayer, and some evidence of shrinkage, chromatin condensation and membrane blebbing. In the case of the acidic PLA_2, cell morphology did not differ from that of control cultures, characterized by the typical spindle-shaped appearance of these cells. When the two enzymes were added together, more detachment and extended areas devoid of cells were observed (Fig. 3).

3.3. Synergistic effect of PLA_2 inactivated by p-BPB

Inhibition of catalytic activity of PLA_2 was verified by indirect hemolytic activity assay and phenol red assay; no PLA_2 activity was detected by these two methods after incubation with *p*-BPB. Cell detachment was not significantly different when catalytically active or inactive PLA_2s were used (Fig. 4). Thus, these results suggest that synergism is likely to be independent of catalytic activity of PLA_2.

3.4. Selective neutralization assays

In order to evaluate the synergistic action between the SVMP and the PLA_2 within the context of the whole venom, each enzyme was selectively inhibited by using specific antibodies raised in rabbits. As shown in Fig. 5, the cell detaching effect of the venom was completely inhibited by antibodies to baltergin, indicating a major role of this SVMP, and perhaps other cross-reacting SVMPs, in the activity. Antibodies to PLA_2, on the other hand, reduced the cell detaching effect of the venom by nearly 70%, in agreement with a relevant synergistic role of this non-toxic enzyme in the cell detaching activity.

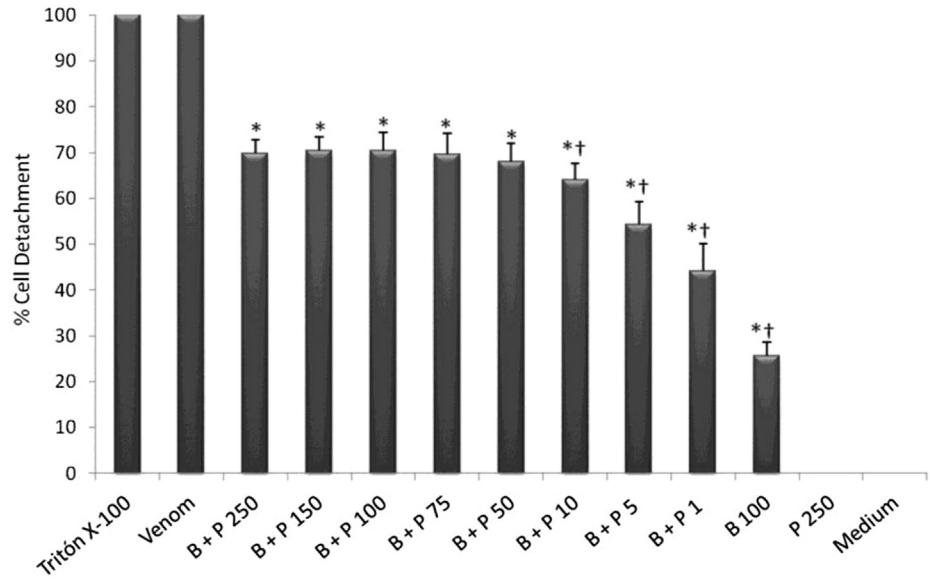

Fig. 2. Enhancement of the endothelial cell detaching effect of baltergin (SVMP) by PLA_2. Cells were incubated for 3 h with either baltergin (B: 100 μg/mL, total volume: 200 μL) or PLA_2 (P: 250 μg/mL total volume: 200 μL), or a combination of both (B: 100 μg/mL + P: 1–250 μg/mL, final concentration of each enzyme). *Bothrops alternatus* venom (100 μg/mL, total volume: 200 μL) and 0.1% Tritón X-100 were used as positive controls of detachment; culture medium was used as negative control. Cell detachment was determined by crystal violet uptake, as described in Materials and methods. Each bar represents mean ± SD of triplicate assays. (*) indicate a statistically significant ($p < 0.05$) difference in comparison to untreated cells. (†) indicate a statistically significant ($p < 0.05$) difference in comparison to synergism with equal amounts of both enzymes (100 μg/mL, each).

4. Discussion

Snake venoms constitute complex biological mixtures, as revealed by proteomic analysis [3,30]. Such combinations of toxins exerting diverse activities pose a challenge for the study of venom toxicity and mechanisms of action. A largely neglected aspect in the characterization of the toxic profiles of venoms is the evaluation of potential synergisms or antagonisms that may exist between their various components. A recent study evidenced a hitherto unknown role for acidic venom PLA_2s [11], often described as being devoid of toxicity. An acidic PLA_2 isolated from the venom of *B. alternatus* did not induce cytotoxicity in myoblasts in cell culture, or myonecrosis in mice, but was able to potentiate the cell–detachment activity of an SVMP isolated from the same venom. These observations raised

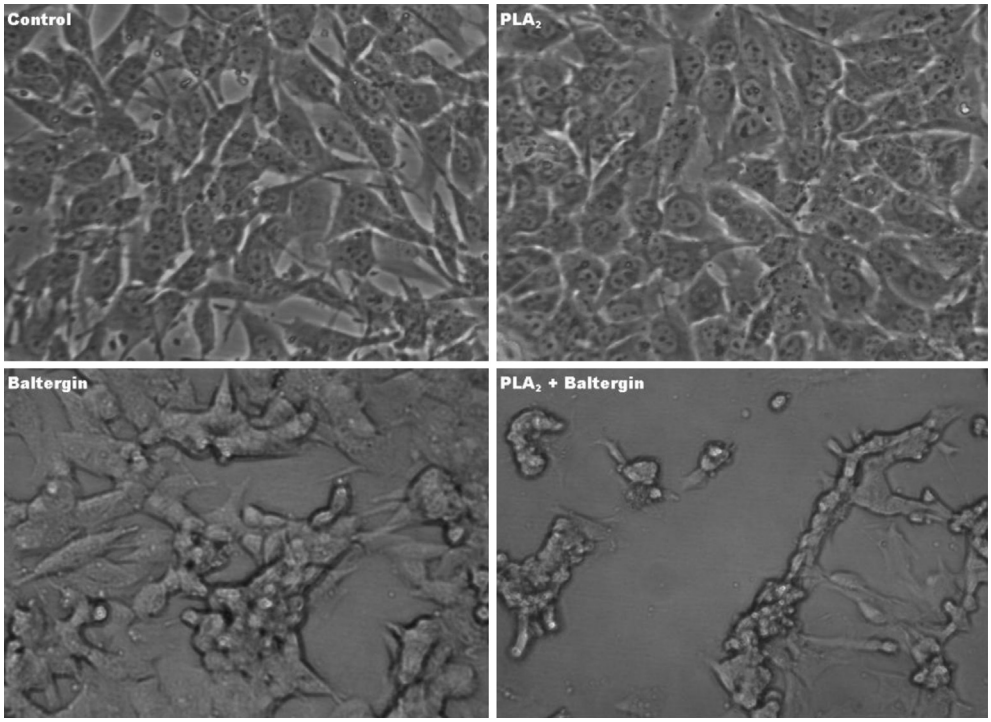

Fig. 3. Morphological changes in endothelial cell cultures exposed for 3 h to medium alone (Control), Baltergin SVMP (100 μg/mL), PLA_2 (100 μg/mL), or a mixture of both enzymes (PLA_2+ Baltergin: 100 μg/mL, final concentration each), under phase contrast microscopy (X400).

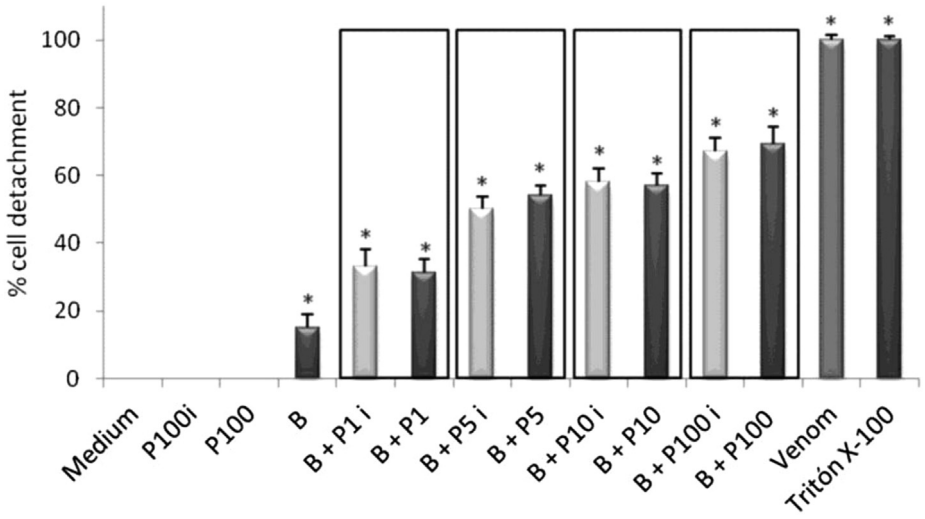

Fig. 4. Synergistic effect is independent of PLA_2 catalytic activity. Cells were exposed for 3 h with either Baltergin (B: 100 μg/mL, total volume: 200 μL) or a combination of Baltergin and catalytic active PLA_2 (B + P, Baltergin 100 μg/mL and PLA_2 1–100 μg/mL, total volume: 200 μL) or Baltergin and catalytic inactive PLA_2 (B + Pi: Baltergin 100 μg/mL and PLA_2i 1–100 μg/mL, total volume: 200 μL). *Bothrops alternatus* venom (100 μg/mL, total volume: 200 μL) and 0.1% Tritón X-100 were used as positive controls of detachment; culture medium was used as negative control. Cell detachment was determined by crystal violet uptake, as described in Materials and methods. Each bar represents mean ± SD of triplicate assays. (*) indicate a statistically significant ($p < 0.05$) difference in comparison to untreated cells.

the possibility that venom acidic PLA_2s, and perhaps other components as well, may contribute to overall venom effects by playing a synergistic role with some toxins. The present observations confirm and extend these findings by demonstrating that the same phenomenon occurs when these enzymes are tested on an endothelial cell line, a natural target of SVMPs. *B. alternatus* venom exerted a potent detaching effect on cultured endothelial cells. Results evidenced a rapid cell rounding and loss of adhesion from culture substrate, without involving a cytolytic effect, as indicated by the lack of release of cytosolic LDH to supernatants in cells exposed to the venom. Endothelial cell detachment in the absence of cytolysis had also been observed in earlier studies with BaH1, a hemorrhagic SVMP purified from the venom of *B. asper* [31,32], but possible synergisms contributing to this activity have not been analyzed. Using the same endothelial cell model, the present work evaluated the combined effect of a hemorrhagic SVMP and an acidic, non-toxic PLA_2, at different concentrations, both isolated from the venom of *B. alternatus*. These two enzymes belong to abundant protein families in this venom, since proteomic analysis indicates that it comprises mostly acidic proteins, with predominance of P-III SVMPs, which constitute 50% of identified proteins. In addition, acidic PLA_2s represent 9% of the venom [30].

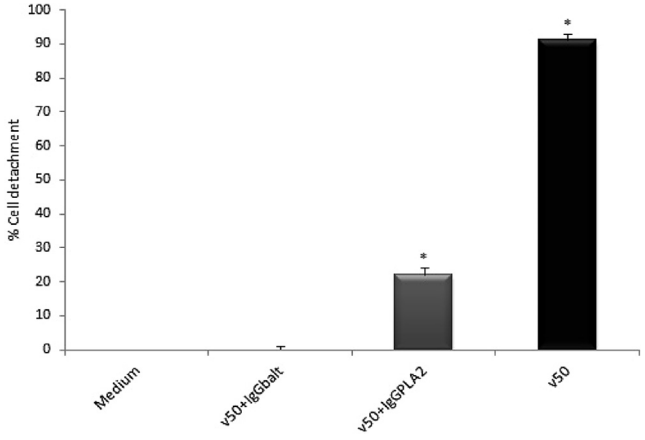

Fig. 5. Endothelial cell detaching activity of *Bothrops alternatus* venom after selective neutralization of either SVMP or PLA_2 with their corresponding specific antibodies. Cells were exposed for 3 h to venom alone (50 μg/mL; V50) or venom that was previously incubated with anti-baltergin IgG (IgGbalt) or anti-PLA_2 IgG (IgGPLA$_2$) at an antibody:venom ratio of 25:1 (w/w). Cell detachment was determined by crystal violet cell uptake assay, as described in Materials and methods. Readings from cells incubated with medium alone were considered as 0% detachment (100% attachment). Each bar represents mean ± SD of triplicate assays. Asterisks indicate a statistically significant ($p < 0.05$) difference in comparison to controls.

The purified SVMP, baltergin, reproduced the cell detaching effect of the complete venom, albeit with a lower potency, in support of the notion of a synergistic action with the acidic PLA_2 and possibly with other SVMPs, occurring in the context of the whole venom. It is known that other metalloproteinases are present in *B. alternatus* venom, *e.g.* alternagin, a P-III metalloproteinase [33], and BaG, a dimeric metalloproteinase [34], which probably contribute to the effect detected by incubating cells with the crude venom. The purified acidic PLA_2, on the other hand, did not cause detachment or morphological alterations of endothelial cells, but clearly enhanced the detaching activity of the SVMP when added together to the cultures. Thus, this phenomenon described using the myogenic cell line C2C12 as a target [11] also occurs in the case of the endothelial cell model used in this study, indicating that it may involve a mechanism common to many cell types, rather than a particular one. Moreover, in this work different concentrations of PLA_2 were tested evidencing that the synergic action occurs even at a concentration as low as 1 μg/mL. The synergistic effect of the acidic PLA_2 in enhancing cell detachment was further confirmed by performing selective neutralization experiments within the context of the whole venom. Results showed that antibodies raised against the P-III SVMP abrogated the cell detaching activity of the venom, demonstrating that baltergin, and possibly other cross-reactive venom SVMPs, are largely responsible for this venom activity. Noteworthy, the observation that antibodies specific to the acidic PLA_2 caused a major reduction of cell detachment by the venom further indicates that this PLA_2 contributes to the effect of endothelial cells in the context of the whole venom, thus evidencing the synergistic effect previously described in myoblasts. To the best of

our knowledge, this is the first report of an enhancing action of a PLA_2 on the detachment of endothelial cells induced by a metalloproteinase. Owing to the concomitant presence of acidic PLA_2s and SVMPs in many viperid venoms, these findings may have more general implications for the action of these venoms.

The mechanism of this enhancing effect on cell detachment is at present unknown and intriguing, since PLA_2s are devoid of a direct activity on extracellular matrix components, the key substrates for the action of SVMPs. A first approach to elucidate the possible molecular mechanisms that underlie this novel type of synergism between a PLA_2 and a SVMP was developed to verify whether the synergism persists when PLA_2 catalytic activity is inhibited. Observations with *p*-BPB-inhibited PLA_2 strongly suggest that the synergistic effect is not dependent on phospholipase enzymatic activity. Moreover, this effect proved to be dose-dependent, as observed with the active form of the enzyme, since the synergism occurred even when using trace amounts of the catalytically-inactive PLA_2 (1 μg/mL). Previous works demonstrated that there is no clear relationship between the extent of enzymatic activity (lipid hydrolysis) and pharmacological action of PLA_2s. For instance, a catalytically inactive Lys49-PLA_2s, still displays toxicity *in vivo* [35].

Considering that the attachment of cells to their substrate depends on the interaction between extracellular matrix proteins and integral plasma membrane proteins, which are embedded in the phospholipid bilayer, one possibility to explain our findings is that the PLA_2 might interact with lipidic molecules of the membrane bilayer, by a mechanism independent of catalysis. Such interaction, in turn, may alter membrane biophysical properties, either facilitating the contact between SVMPs and endothelial cells or affecting the strength of the binding of endothelial cell integrins with extracellular matrix proteins. Alternatively, the effect may depend on interactions of PLA_2 with integrins in endothelial cells, thus affecting their binding to matrix proteins. It is noteworthy that endothelial cells express high levels of integrin $\alpha v\beta 3$, and PLA_2s are known to interact with this integrin [36–38]. Future studies are necessary to reveal the precise mechanism of PLA_2 facilitation of SVMP-induced endothelial cell detachment.

In conclusion, we have demonstrated a synergistic effect between a non-toxic PLA_2 and a hemorrhagic metalloproteinase from *B. alternatus* venom on the endothelial cell detachment. Moreover, it was shown that such synergism occurs at low concentrations of PLA_2, and that its catalytic activity is not required for facilitating detachment. These results highlight the relevance of studying the potential interactions between different venom components, which are overlooked when isolated proteins are used, thus contributing to the understanding of the actions of these complex toxic mixtures.

Conflict of interest statement

The authors declare that there are no conflicts of interest regarding this manuscript.

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Transparency document

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References

[1] O. Acosta de Pérez, P. Koscinczuk, P. Teibler, M. Sánchez Negrette, R. Ruíz, G. Bogarín, Actividades hemorrágica y edematizante y alteraciones histológicas en almohadilla plantar del ratón inducidas por venenos de serpientes de los géneros *Bothrops* y *Crotalus* de Argentina, Toxicon 36 (1998) 1165–1172.
[2] M.E. García Denegri, O.C. Acosta, S. Huancahuire-Vega, D. Martins-de-Souza, S. Marangoni, S.L. Marunak, et al., Isolation and functional characterization of a new acidic PLA_2 Ba SpII RP4 of the *Bothrops alternatus* snake venom from Argentina, Toxicon 56 (2010) 64–74.
[3] J.J. Calvete, Proteomic tools against the neglected pathology of snake bite envenoming, Expert Rev. Proteomics 8 (2011) 739–758.
[4] J.M. Gutiérrez, A. Rucavado, T. Escalante, C. Díaz, Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage, Toxicon 45 (2005) 997–1011.
[5] B. Lomonte, J.M. Gutiérrez, Phospholipases A_2 from Viperidae snake venoms: how do they induce skeletal muscle damage? Acta Chim. Slov. 58 (2011) 647–659.
[6] B. Lomonte, J. Rangel, Snake venom Lys49 myotoxins: from phospholipases A_2 to non-enzymatic membrane disruptors, Toxicon 60 (2012) 520–530.
[7] J. Fernández, P. Caccin, G. Koster, B. Lomonte, J.M. Gutiérrez, C. Montecucco, et al., Muscle phospholipid hydrolysis by *Bothrops asper* Asp49 and Lys49 phospholipase a myotoxins - distinct mechanisms of action, Febs J. 16 (2013) 3878–3886.
[8] J. Fernández, J.M. Gutiérrez, Y. Angulo, L. Sanz, P. Juarez, J.J. Calvete, et al., Isolation of an acidic phospholipase A_2 from the venom of the snake *Bothrops asper* of Costa Rica: biochemical and toxicological characterization, Biochimie 92 (2010) 273–283.
[9] M. Van der Laat, J. Fernández, J. Durban, E. Villalobos, E. Camacho, J.J. Calvete, et al., Amino acid sequence and biological characterization of $BlatPLA_2$, a nontoxic acidic phospholipase A_2 from the venom of the arboreal snake *Bothriechis lateralis* from Costa Rica, Toxicon 73 (2013) 71–80.
[10] C.C. Gay, L.C. Leiva, S. Marunak, P. Teibler, O. Acosta de Perez, Proteolytic, edematogenic and myotoxic activities of a hemorrhagic metalloproteinase isolated from *Bothrops alternatus* venom, Toxicon 46 (2005) 546–554.
[11] S. Bustillo, C.C. Gay, M.E. García Denegri, L.A. Ponce-Soto, E. Bal de Kier Joffe, O. Acosta, et al., Synergism between baltergin metalloproteinase and Ba SPII RP4 PLA_2 from *Bothrops alternatus* venom on skeletal muscle (C2C12) cells, Toxicon 59 (2012) 338–343.
[12] J.M. Gutiérrez, A. Rucavado, T. Escalante, B. Lomonte, Y. Angulo, J.W. Fox, Tissue pathology induced by snake venoms: how to understand a complex pattern of alterations from a systems biology perspective? Toxicon 55 (2010) 166–170.
[13] D. Mora-Obando, J. Fernandez, C. Montecucco, J.M. Gutierrez, B. Lomonte, Synergism between basic Asp49 and Lys49 phospholipase A2 myotoxins of viperid snake venom in vitro and in vivo, PLoS One 9 (2014) e109846.
[14] M. Cintra-Francischinelli, P. Pizzo, L. Rodrigues-Simioni, L.A. Ponce-Soto, O. Rossetto, B. Lomonte, et al., Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of acceptors, Cell Mol. Life Sci. 66 (2009) 1718–1728.
[15] A.L. Bieber, Metal and nonprotein constituents in snake venoms, in: C.-Y. Lee (Ed.), Snake Venoms, Springer, Berlin Heidelberg, 1979, pp. 295–306.
[16] F. Bussolino, M. De Rossi, A. Sica, F. Colotta, J.M. Wang, E. Bocchietto, et al., Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines, J. Immunol. 147 (1991) 2122–2129.
[17] S. Bustillo, H. Lucero, L.C. Leiva, O. Acosta, E.B. Kier Joffé, J.O. Gorodner, Cytotoxicity and morphological analysis of cell death induced by *Bothrops* venoms from the northeast of Argentina, J. Venom. Anim. Toxins Incl. Trop. Dis. 15 (2009) 28–42.
[18] B. Lomonte, A. Tarkowski, L.A. Hanson, Broad cytolytic specificity of myotoxin II, a lysine-49 phospholipase A_2 of *Bothrops asper* snake venom, Toxicon 32 (1994) 1359–1369.
[19] H.M. Verheij, J.J. Volwerk, E.H. Jansen, W.C. Puyk, B.W. Dijkstra, J. Drenth, et al., Methylation of histidine-48 in pancreatic phospholipase A2. Role of histidine and calcium ion in the catalytic mechanism, Biochemistry 19 (1980) 743–750.
[20] H.M. Verheij, A.J. Slotboom, G.H. de Haas, Structure and function of phospholipase A2, Rev. physiology, Biochem. Pharmacol. 91 (1981) 91–203.
[21] D.L. Scott, A. Achari, J.C. Vidal, P.B. Sigler, Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A2 from the venom of Agkistridon piscivorus piscivorus, J. Biol. Chem. 267 (1992) 22645–22657.
[22] S.H. Andriao-Escarso, A.M. Soares, V.M. Rodrigues, Y. Angulo, C. Diaz, B. Lomonte, et al., Myotoxic phospholipases A(2) in bothrops snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*, Biochimie 82 (2000) 755–763.
[23] A.M. Soares, J.R. Giglio, Chemical modifications of phospholipases A2 from snake venoms: effects on catalytic and pharmacological properties, Toxicon 42 (2003) 855–868.

[24] C. Diaz-Oreiro, J.M. Gutierrez, Chemical modification of histidine and lysine residues of myotoxic phospholipases A2 isolated from Bothrops asper and Bothrops godmani snake venoms: effects on enzymatic and pharmacological properties, Toxicon 35 (1997) 241–252.
[25] J.M. Gutiérrez, C. Avila, E. Rojas, L. Cerdas, An alternative in vitro method for testing the potency of the polyvalent antivenom produced in Costa Rica, Toxicon 26 (1988) 411–413.
[26] O. Ouchterlony, Antigen-antibody reactions in gels, Acta Path Microbiol. Scand. 26 (1949) 507–515.
[27] C. Chavez-Olortegui, E. Kalapothakis, A.M. Ferreira, A.P. Ferreira, C.R. Diniz, Neutralizing capacity of antibodies elicited by a non-toxic protein purified from the venom of the scorpion *Tityus serrulatus*, Toxicon 35 (1997) 213–221.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[29] H. Kondo, S. Kondo, H. Ikezawa, R. Murata, Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom, Jpn. J. Med. Sci. Biol. 13 (1960) 43–52.
[30] M. Ohler, D. Georgieva, J. Seifert, M. von Bergen, R.K. Arni, N. Genov, et al., The venomics of *Bothrops alternatus* is a pool of acidic proteins with predominant hemorrhagic and coagulopathic activities, J. Proteome Res. 9 (2010) 2422–2437.
[31] B. Lomonte, J.M. Gutiérrez, G. Borkow, M. Ovadia, A. Tarkowski, L.A. Hanson, Activity of hemorrhagic metalloproteinase BaH-1 and myotoxin II from *Bothrops asper* snake venom on capillary endothelial cells *in vitro*, Toxicon 32 (1994) 505–510.
[32] G. Borkow, J.M. Gutiérrez, M. Ovadia, In vitro activity of BaH1, the main hemorrhagic toxin of *Bothrops asper* snake venom on bovine endothelial cells, Toxicon 33 (1995) 1387–1391.
[33] D.H. Souza, M.R. Iemma, L.L. Ferreira, J.P. Faria, M.L. Oliva, R.B. Zingali, et al., The disintegrin-like domain of the snake venom metalloprotease alternagin inhibits α2β1 integrin-mediated cell adhesion, Arch. Biochem. Biophys. 384 (2000) 341–350.
[34] M.R. Cominetti, J.U. Ribeiro, J.W. Fox, H.S. Selistre-de-Araujo, BaG, a new dimeric metalloproteinase/disintegrin from the *Bothrops alternatus* snake venom that interacts with α5β1 integrin, Arch. Biochem. Biophys. 416 (2003) 171–179.
[35] S.E. Gasanov, R.K. Dagda, E.D. Rael, Snake venom cytotoxins, phospholipase as, and Zn-dependent metalloproteinases: mechanisms of action and pharmacological relevance, J. Clin. Toxicol. 4 (2014) 1000181.
[36] B.P. Eliceiri, D.A. Cheresh, The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development, J. Clin. Invest 103 (1999) 1227–1230.
[37] J. Saegusa, N. Akakura, C.Y. Wu, C. Hoogland, Z. Ma, K.S. Lam, et al., Pro-inflammatory secretory phospholipase A2 type IIA binds to integrins alphavbeta3 and alpha4beta1 and induces proliferation of monocytic cells in an integrin-dependent manner, J. Biol. Chem. 283 (2008) 26107–26115.
[38] A. Bazaa, J. Luis, N. Srairi-Abid, O. Kallech-Ziri, R. Kessentini-Zouari, C. Defilles, et al., MVL-PLA2, a phospholipase A2 from Macrovipera lebetina transmediterranea venom, inhibits tumor cells adhesion and migration, Matrix Biol. J. Int. Soc. Matrix Biol. 28 (2009) 188–193.