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Evaluation of pro-inflammatory events induced by *Bothrops alternatus* snake venom

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ABSTRACT

Inflammation is a major local feature of envenomation by bothropic snakes being characterized by a prominent local edema, pain, and extensive swelling. There are reports demonstrating that whole *Bothrops* snake venoms and toxins isolated from them are able to activate macrophages functions, such as phagocytosis, production of reactive oxygen, cytokines and eicosanoids, however, little is known about the effects of *Bothrops alternatus* (*B.a.*) venom on macrophages. In this work, we evaluated the proinflammatory effects of *B.a.* venom with *in vivo* and *in vitro* experiments using the Raw 264.7 cell line and mouse peritoneal macrophages. We detected that *B.a.* venom augments cell permeability (2-fold), and cellular extravasation (mainly neutrophils), increase proinflammatory cytokines IL1 (~300-fold), IL12 (~200-fold), and TNFα (~80-fold) liberation and induce the expression of enzymes related to lipid signaling, such as $cPLA_{2\alpha}$ and COX-2. Additionally, using lipidomic techniques we detected that this venom produces a release of arachidonic acid (~10 nMol/mg. Protein) and other fatty acids (16:0 and 18:1 n-9c).

Although much of these findings were described in inflammatory processes induced by other bothropic venoms, here we demonstrate that *B.a.* venom also stimulates pro-inflammatory pathways involving lipid mediators of cell signaling. In this sense, lipidomics analysis of macrophages stimulated with *B.a.* venom evidenced that the main free fatty acids are implicated in the inflammatory response, and also demonstrated that this venom, is able to activate lipid metabolism even with a low content of PLA_2.

1. Introduction

Bothrops gender has a widespread distribution in Central and South America where it is responsible for most ophidian accidents. In Argentina, it is responsible for 90% of snakebites, followed by those caused by *Crotalus* [1].

Bothrops alternatus (*B.a.*) venom is endowed with toxic activities, which include proteolytic, coagulant and hemorrhagic effects, with the development of prominent local tissue damage: bleeding, blisters, myonecrosis and dermonecrosis. Hemorrhage occurs not only locally, at the site of the bite, but also systemically. Among systemic effects, coagulation disorders, acute renal failure, acute respiratory failure, shock and sepsis are major alterations [2–4]. On the other hand, inflammation is a major local feature of envenomation by bothropic snakes being characterized by a prominent local edema, pain, and extensive swelling [4,5]. In most cases, the chosen treatment starts several hours after the snake accident; thus, the inflammatory process is well advanced at the time of initiation of the serotherapy. Commercial antivenoms do not effectively neutralize these local effects, resulting in a serious sequel in individuals bitten by *Bothrops* genus species [6]. Moreover, the geographic variability exhibited by venoms increases the diversity of pharmacological effects experimentally observed and as a consequence the inflammatory phenomena characterized by others may show great variations [7–9].

In that sense, current research in immunotoxicology is focused on the mechanism of the molecular events that take place in the innate immune response (cytokines, and chemokines release, arachidonic acid conversion into eicosanoids, stimulation of receptors by innate agonists,

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etc.), with the aim of finding new pharmacological targets that control the inflammation process in its early stage, to provide new complementary treatments to traditional immunotherapy [10–13]. In addition, its known that macrophages play a central role in a wide variety of inflammatory processes [14]. In this context, there are reports demonstrating that whole *Bothrops* snake venoms and toxins isolated from them are able to activate macrophages functions, such as phagocytosis, production of reactive oxygen, cytokines and eicosanoids [5,15]. However, little is known about the effects of *B.a.* venom on macrophages. Therefore, this study was designed to characterize the principal inflammatory events induced by *B.a.* venom in mice and the role of macrophages in *B.a.* venom-induced inflammatory events. Moreover, cytokines expression and elements involved in lipid mediators biosynthesis were also evaluated. In addition, it was performed a lipidomic analysis of macrophages stimulated by *B.a.* venom.

2. Materials and methods

2.1. Chemicals and reagents

Hema-3 stain was purchased from Biochemical Sciences (Swedesboro, NJ, USA). Turk solution was from Sigma-Aldrich, (St. Louis, MO, USA). Evans blue (EB) was from Cicarelli Reagents S.A. (Santa Fe, Argentina). SDS-PAGE and Western-Blot reagents were from Bio-Rad Laboratories, (Richmond, CA, USA). Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). Oligonucleotides were from Eurofins MWG Operon (Hamburg, Germany). The sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank). Internal standards for mass spectrometry were purchased from Avanti (Alabaster, AL) or Cayman (Ann Arbor, MI). Silicagel thin-layer chromatography plates were from Macherey-Nagel (Du¨ren, Germany). All other reagents were from Sigma-Aldrich.

2.2. Animals

Male Swiss mice (20 g) were housed in temperature-controlled rooms with a 12 h dark–light period and received water and food *ad libitum*. The research protocols and animals used were in accordance with the guidelines of the Committee for Ethics in the Use of Animals at the Facultad de Ciencias Exactas y Naturales y Agrimensura of the Universidad Nacional del Nordeste, and international law and policies. All efforts were made to minimize the number of animals used and their suffering.

2.3. Venoms

B.a. venom was obtained from seven adult specimens measuring 90–120 cm, captured in the north-east region of Argentina and kept at the Corrientes City Zoo (CEPSAN, Corrientes, Argentina). *Crotalus durissus terrificus* (*C.d.t.*) venom was also obtained from five adult specimens measuring 100–130 cm. Snakes were milked and the secretion was lyophilized; after that, it was kept frozen at −20 °C. The small amount of insoluble material was centrifuged and the clear supernatant was used for studies. The protein content was estimated using the Bradford method.

2.4. Induction of inflammatory reaction and cell counts

B.a. venom (5 μg/ml), dissolved in 1 ml sterile saline (0.15 M NaCl), was injected intraperitoneally (i.p.). Control animals received 1 ml of sterile saline alone. At selected time intervals after these injections, animals were sacrificed under halothane atmosphere and the inflammatory exudate was withdrawn after washing the cavities with 2 ml phosphate-buffered saline (PBS), pH 7.2. Aliquots of the washes were used to determine total cell counts, using a Neubauer chamber after dilution (1:20, v/v) in Turk solution (0.2% crystal violet dye in 30% acetic acid). For differential cell counts, cytospin preparations were stained with Hema3 stain. Differential cell counts were performed counting at least 100 cells, which were classified as either polymorphonuclear or mononuclear cells, based on conventional morphological criteria [10].

2.5. Evaluation of vascular permeability (VP)

Changes in VP were evaluated by quantifying extravasation of Evans blue (EB) dye into the mouse peritoneal cavity at several time intervals (15, 30, 60 and 120 min) after i.p. injection of *B.a.* (0.5 μg/animal) or sterile saline (control). EB (20 mg/kg, i.v.) was injected 20 min before each time interval, when the animals were sacrificed by overexposure to CO_2 and exsanguinated. The peritoneal cavity of each animal was then washed with 1 ml of saline solution pH 7.2 and centrifuged at 500 *g* for 5 min. The concentration of EB in the supernatant was determined by recording the absorbance at 620 nm. Results were expressed as μg EB/ml, using a standard curve (0.5–25 μg/ml) [13].

2.6. Edema forming activity

Groups of five mice (CF-1, 18–22 g) were injected in the subplantar region of the right foot with 50 μL of *B.a.* venom solution (20, 100 or 200 μg/ml). The left foot pads of the same mice were injected with 50 μL of phosphate buffered saline solution (pH 7.2) as a control. After different intervals (0–6 h), the edema at the paw was measured using a low pressure spring caliper [16]. The edema was expressed as the percentage increase of thickness of the right foot compared to that of the left one.

2.7. Cell culture

In order to obtain peritoneal macrophages, animals were killed under CO_2 atmosphere and cells were then harvested by washing peritoneal cavities with 3 mL apyrogenic phosphate buffered saline (PBS), pH 7.2. The wash volumes were centrifuged at 1296 *g* for 6 min (4 °C), supernatants were discarded and cell pellets were appropriately diluted and maintained in serum-free RPMI 1640 medium, supplemented with 2 mM L-glutamine and 40 μg/mL gentamicin sulfate at 37 °C with 5% CO_2. After that, cells (1×10^6 macrophages/well) were seeded in 6 wells culture plates and maintained in culture medium for 24 h before stimulation.

RAW 264.7 cell were purchased in ATCC. Cells were grown in plastic flasks at 37 °C in a humidified atmosphere of 5% CO_2/air, with RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10.000 UI/ml) and streptomycin (10 mg/ml). Cells were passaged weekly after treatment with trypsin (2.5 mg/ml) and 0.2% EDTA in a balanced salt solution without Ca^{2+} or Mg^{2+}. When required, cells were seeded at high density ($0.5–1.0 \times 10^6$ cells/cm^2) in 6- or 96-well microplates. When 80–90% of cell confluence is reached, the cells were stimulated with *B.a.* venom at the indicated times.

2.8. Quantitative PCR

For the detection of cytokines or mammalian $cPLA_2$, $iPLA_2$ and GX PLA_2, total RNA was extracted from cells (Raw 264.7 or peritoneal macrophages) with 6 h exposure to different doses of *B.a.* venom. Total RNA was extracted using the TRIzol reagent method (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was obtained by using the Moloney murine leukemia virus reverse transcriptase (Promega) from 2 μg of RNA, according to the manufacturer's protocol. Quantitative PCR was carried out with an ABI 7500 machine (Applied Biosystems, Carlsbad, CA) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA) and specific primers for each gene of interest. Cycling conditions were: 1 cycle at

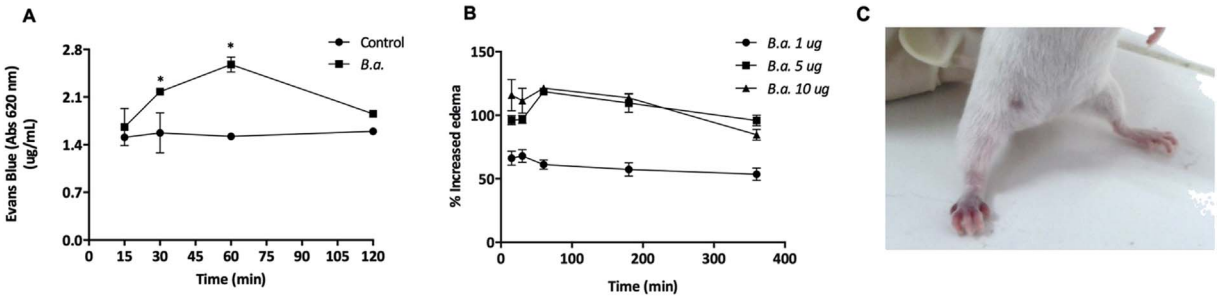

Fig. 1. *Changes in vascular permeability (VP) caused by B.a. venom.* **A.** Animals were injected with *B.a.* venom (*i.p.*; 0.5 μg/animal) or PBS (control). VP was determined by measuring extravasation of Evans blue dye as described in Materials and methods. **B.** Edema caused by action of *B.a.* venom (1, 5 or 10 μg) in mouse plantar pad. Values are mean ± SD of 4–6 animals. *p < .05 compared with the control group. **C.** Edematogenic effect of *B.a.* venom on mouse foot-pad. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

95 °C for 3 min and 40 cycles at 95 °C for 12s, 60 °C for 15s and 72 °C for 28s. The relative mRNA abundance for a given gene was calculated using the algorithm $2^{-\Delta\Delta Ct}$, with β-actin and cyclophilin A as internal standard [17]. Primers used for murine genes were as follows: β-Actin, 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCA TGT-3'; Cyclophilin, 5′-TGGAAGAGCCAAGACAGACA-3′and 5′-TGCCG GAGTCGACAATGAT-3'; Il10,5′-CGCAGCTCTAGGAGCATGTG-3′and 5′-GCTCTTACTGACTGGCATGAG-3'; Il12, 5′- TGGTTTGCCATCGTTTT GCTG -3′ and 5′-ACAGGTGAGGTTCACTGTTTCT-3'; Il13, 5′-CAGCCTC CCCGATACCAAAAT-3′ and 5′-GCGAAACAGTTGCTTTGTGTAG-3'; Il1b, 5′-GCAACTGTTCCTGAACTCAACT-3′ and 5′-ATCTTTTGGGGTCCGTCA ACT-3'; TNFα-a, 5′-ACGGCATGGATCTCAAAGAC-3′ and 5′-AGATAGC AAATCGGCTGACG-3'; Il6, 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and 5′-TTGGTCCTTAGCCACTCCTTC-3'; iNOS-1, 5′-GTTCTCAGCCCAACAA TACAAGA-3′ and 5′-GTGGACGGGTCGATGTCAC-3'; iNOS-2, 5′-ACATC GACCCGTCCACAGTAT-3′ and 5′-CAGAGGGGTAGGCTTGTCTC-3'.

2.9. COX-2 and $cPLA_{2\alpha}$ activation

COX-2 and $cPLA_{2\alpha}$ were detected in RAW 264.7 by Western blotting. Aliquots of *B.a.*-stimulated and non-stimulated cells (1.5×10^6 cells) were lysed with 100 μL sample buffer (0.5 M Tris-HCl, pH 6.8, 20% SDS, 1% glycerol, 1 M β-ME, 0.1% bromophenol blue) and boiled for 10 min. Samples were resolved by SDS-PAGE on 10% bis-acrylamide gels overlaid with a 5% stacking gel. Proteins were then transferred to nitrocellulose membrane using a Mini *Trans*-Blot (Bio-Rad Laboratories, Richmond, CA, USA). The membranes were blocked for 1 h with 5% nonfat dry milk in TBS (20 mM Tris, 100 mM NaCl, and 0.5% Tween 20) and incubated with primary antibodies against COX-2 (1:1000 dilution), $cPLA_{2\alpha}$ or 505-Ser $cPLA_{2\alpha}$ (1:000) and β-actin (1:10000) for 1 h. They were then washed and incubated with the appropriate secondary antibody conjugated to HRP. Bands were detected by the ECL method, according to the manufacturer's instructions (GE Healthcare). Band densities were quantified with a GS 700 densitometer (Bio-Rad Laboratories) using the image analysis software from Molecular Analyst (Bio-Rad Laboratories).

2.10. Gas chromatography/mass spectrometry analysis of fatty acids methyl esters (FAMEs)

A cell extract corresponding to 10^7 cells was used and, before the extraction of lipid, internal standards were added. Ten nmol of 1,2,3-triheptadecanoylglycerol was added to assess the total cellular fatty acid composition. Total lipids were extracted according to Bligh and Dyer method, and then were transmethylated with 500 μL of 0.5 M KOH in methanol for 60 min at 37 °C. To neutralize, 500 μL of 0.5 M HCl was added. Extraction of fatty acid methyl esters was made with 1 ml n -hexane twice.

Analysis of fatty acid methyl esters was carried out in an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV) equipped with an Agilent 7693 autosampler and an Agilent DB23 column (60 m length x 250 μm internal diameter x 0.15 μm film thickness) under the conditions described previously. Briefly, the oven temperature was held at 50 °C for 1 min, then increased to 175 °C at a rate of 25 °C/min, then increased to 215 °C at a rate of 1.5 °C/min, and the final ramp being reached at 235 °C at a rate of 10 °C/min. The final temperature was maintained for 5 min, and the run time was 39.67 min. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00 and the quantification of fatty acid methyl esters was achieved by integration of chromatographic peaks comparing with authentic analytical standards [17].

2.11. Statistical analysis

Results are expressed as mean ± SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey test. Differences with an associated probability (p value) of less than 5% (p < .05) were considered significant.

3. Results

3.1. B.a. venom provokes vascular permeability (VP) changes and increases cellular influx into the peritoneal cavity

Induction of the innate immune response is primarily manifested by the activation of the vascular endothelium and cell migration to the affected site. In order to evaluate this, changes in VP were determined at selected intervals after i.p. injection of *B.a.* venom (0.5 μg/animal). Fig. 1A shows the time course of the increase in VP in the peritoneal cavity. There was a significant rise in Evans blue extravasation from 30 to 60 min, with the highest values at 60 min, decreasing rapidly thereafter to reach basal levels at 120 min. This evidence is compatible with the edematogenic activity results (Fig. 1B). The lower doses showed a maximum at 30 min, (2.5 μg) or at 60minitues (5 μg). In contrast, using the highest dose (10 μg) we observed the maximum increase 3 h after the injection. This is probably because it becomes significant the participation of proteases, that break tissues and exacerbate the edema.

The increase in VP should be followed by cell migration, consequently, we next determined the time course of leukocyte influx into the peritoneal cavity after injection of *B.a.* venom. It led to a significant accumulation of leukocytes at the injection site (Fig. 2). Compared with the saline group, the number of these cells remained increased from the 1st to the 6th hour following venom injection. Differential cell counts showed that cells present in the peritoneal cavity were predominantly PMNs or Monocytes, the former were present in greater quantities. The maximum migration was observed after 3 h, and although increases in the two populations were found, only the PMN showed significant differences compared to their controls.

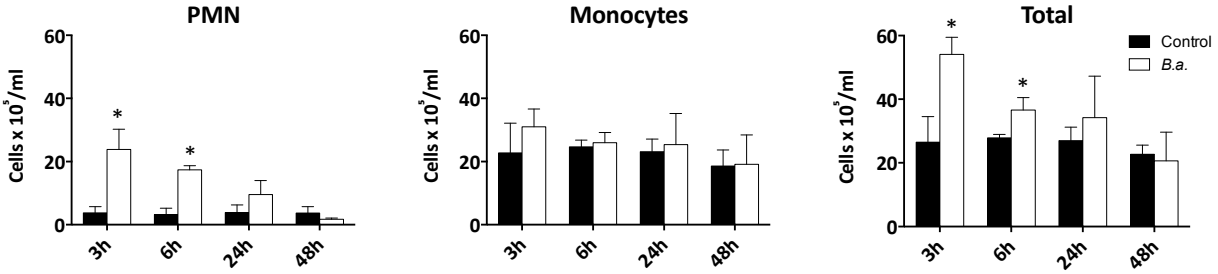

Fig. 2. *Leukocyte influx induced by B.a. venom in mouse peritoneal cavity*. Venom (5 μg/mouse) or PBS (control group) was injected *i.p.* The peritoneal cavity was then washed with PBS for cell counts at the intervals time indicated above. Each point represents the mean ± SD of 4–5 animals. *p < .05 compared with the control group.

3.2. B.a. venom induces cytokines synthesis on macrophages in the activation of the inflammatory process

Plasma exudation by vascular permeabilization, cell migration and edema at venom injection sites can be at least partially explained by the local generation of innate immune mediators, such as cytokines. Expression of mRNAs of major pro-inflammatory cytokine genes was evaluated using RAW 264.7 and mice peritoneal macrophages cell cultures. We obtained a dose dependence in the mRNAs cytokines expression using different exposition times (1, 6 and 12 h) and concentrations (50, 5 and 0.5 μg/ml) of *B.a.* Best differences were observed with 6 h of stimulation, and this are shown in Fig. 3.

Lipopolysaccharide (LPS) and *Crotalus durissus terrificus* (*C.d.t.*) venom were used as inflammation controls since their well-known pro and anti-inflammatory abilities, respectively. We obtained remarkable increases in all the proinflammatory cytokines evaluated. Thus, the venom showed greater induction of mRNA synthesis for IL1, IL12 and TNFα.

Interestingly, it also induced the expression of IL10 and IL13, which could be interpreted as a mechanism of counter-regulation of the onset of the inflammatory process.

The same study was performed on mouse peritoneal macrophages (Fig. 4). Although values on the graphs show less pronounced increases, the trend is similar to those of previous experiments. TNFα, IL1 and IL12, and, to a lesser extent, IL6 show clear increases in cells stimulated by botropic venom. In clear opposition we did not observe these effects in cells stimulated with crotalic venom (excluding TNFα).

3.3. B.a. venom-stimulated macrophages activate the inflammatory process via cPLA$_{2\alpha}$ and COX-2 activation

In order to evaluate the possible implications of lipid signaling pathways in the activation of the inflammatory process, we next determined whether *B.a.* venom produces up-regulation of intracellular or secretory PLA$_2$s that could be involved in the synthesis of second messengers from membrane phospholipids (Major PLA$_2$s belonging to the growing superfamily of mammalian PLA$_2$s). Fig. 5 shows an increase in Group IVA cPLA$_{2\alpha}$ mRNA synthesis, in RAW 264.7 cells with 6 h of exposure to *B.a.* venom. Notably, an increment in the phosphorylation of the enzyme was confirmed by Western blott in the same cells exposed to *B.a.* venom for 1 and 6 h (Fig. 5B).

Since the well-known specificity of cPLA$_{2\alpha}$ for AA-containing phospholipid species [18] and the increased activity detected on this enzyme, significant amounts of AA should be released from the membrane (see below). Consequently, this fatty acid should serve as a COX substrate for the production of inflammatory eicosanoids. When RAW 264.7 cells were exposed to the action of *B.a.* venom for 1 and 6 h, COX-2 enzyme showed an evident expression (Fig. 5C). Together, the simultaneous activation of these two enzymes undoubtedly shows a concerted action for the synthesis of eicosanoids.

3.4. Fatty acids released by B.a. venom phospholipases A$_2$

In order to investigate whether *B.a.* venom PLA$_2$s exert a direct action on the phospholipids membrane to release fatty acids and activate the inflammatory process, a lipidomic analysis was developed with RAW 264.7 macrophages. These cells were exposed to the action of the venom for 12 h. After this period, cells were scraped and total lipids were extracted as indicated in Materials and Methods. Later, lipids were separated into classes (Phospholipids, Diacylglycerides, Free Fatty Acids, Triacylglycerides and Cholesterol Esters) by thin layer chromatography (TLC). Then, fatty acids belonging to the phospholipids fraction were converted to methyl esters to be analyzed by Gas chromatography/mass spectrometry (GC-MS).

Fig. 6 shows that venom-stimulated macrophages resulted in a moderate reduction of cellular fatty acids, particularly those present in membrane phospholipids. RAW 264.7 cell showed statistically significant decreases in stearic, oleic, and particularly in arachidonic acid. Simultaneously, a marked increase in the Free Fatty Acid (FFA) fraction was detected by TLC, which demonstrates an evident action of the venom-PLAs on the macrophagic cell membrane (Data not shown).

4. Discussion

It is widely known that bothropic accidents involve the development of a large local inflammatory response, which is not well neutralized by the conventional serum therapy [13], thus, further studies to better understand the local effects caused by snakes of *Bothrops* genus are needed.

By using mice experimental model, we found that *B.a.* i.p. injection caused an increase of vascular permeability which was followed by edema formation beginning at 30 min with consequently leukocyte migration. These data suggest the implication of vasoactive mediators of rapid release induced by *B.a.* snake venom. In this context, our data agree with previous findings on edematogenic activities and vascular permeability promoted by snake venoms of the *Bothrops* genus, in murine experimental models [4,19–21]. The ability of *B.a.* venom to induce leukocyte influx at the injection site is similar to that observed with *B. jararaca* venom [22]. Our results show that *B.a.* venom provoked PMN cell migration, 3 h after inoculation. Similarly, Carneiro and collaborators obtained the same result, although with a lower amount of *B. jararaca* venom [22]. This can possibly be explained due to proteomic similarities between them. *B. jararaca* venom proteome [23,24], shows a high amount of metalloproteinases (51.1%), and a low proportion of phospholipases (3.2%) likewise, to that obtained for *B.a.* venom (43.1% and 7.8% respectively) [25].

Additionally, the above phenomena can be explained at least partially, by the local generation of cytokines and chemokines. We herein evaluated the effects of *B.a.* venom on macrophages, a relevant immunocompetent tissue cell implicated in triggering inflammatory response [26]. Our results demonstrated that *B.a.* venom induced a conspicuous elevation of IL1, IL6, IL12 and TNFα in Raw 264.7 cells and peritoneal macrophages. These data clearly show the dose-

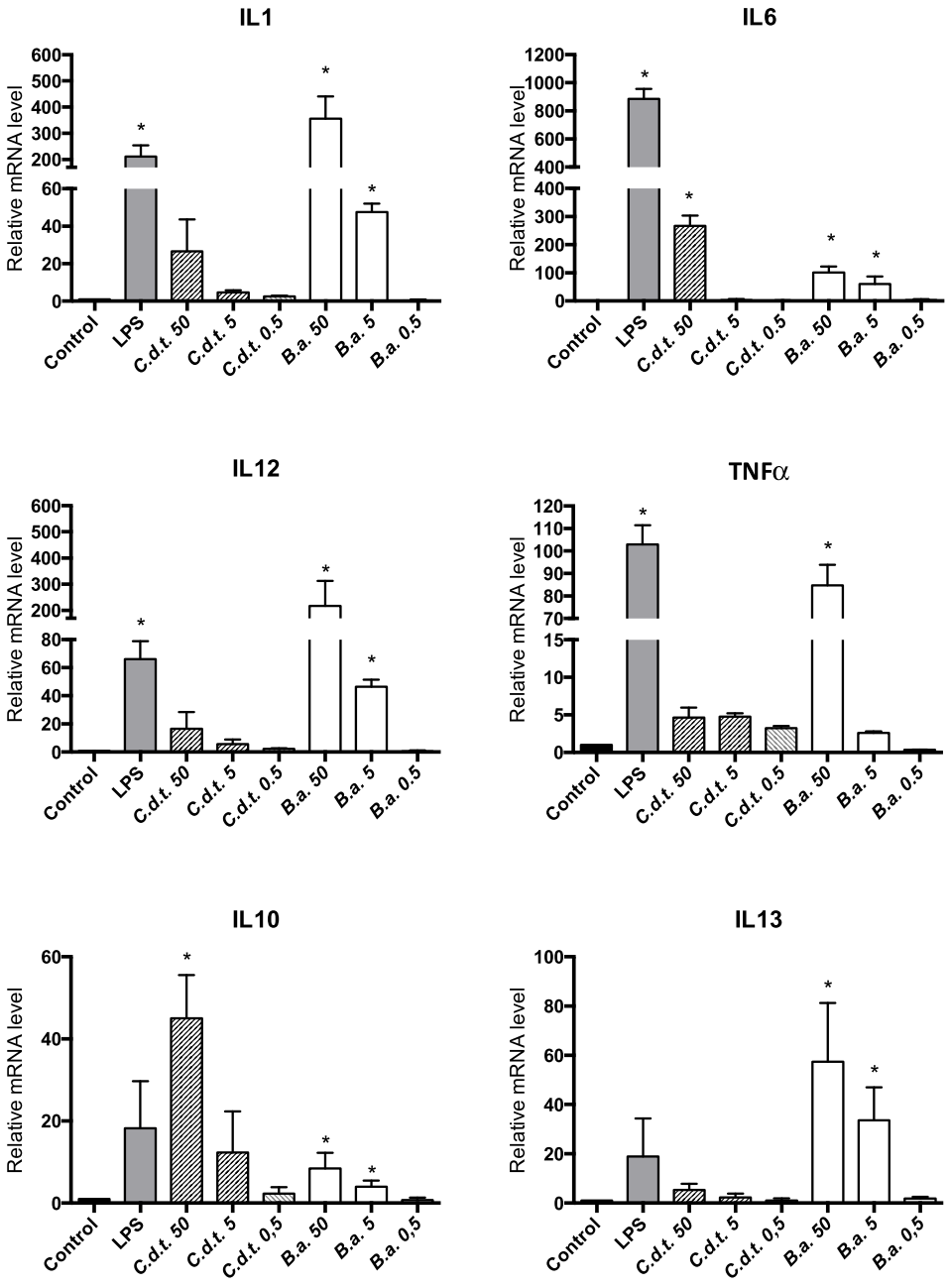

Fig. 3. *Pro-inflammatory mRNA cytokine expression induced by B.a. venom.* Raw 264.7 cells were incubated with *B.a.* venom (0.5, 5, 50 μg/ml), LPS (grey bars), *C.d.t.* venom (striped bars) or PBS (control). Total mRNA was collected after at 6 h of exposure and cytokines mRNA were quantified by RT-qPCR. Values represent the mean ± SD from 3 independent experiments * $p < .05$ compared with the control group.

dependent effect of *B.a.* venom and its evident pro-inflammatory profile. In contrast, crotalic venom exhibited reduced expression capacity of these cytokines, particularly TNFα and IL12. Up to date, there are no studies showing this differential behavior that easily explains what is clinically observed in snakebite accidents caused by both venoms or in the pathophysiological effects that they exhibit in experimental animals [10,12,13,27–33].

Following the initiation of an uncontrolled pro-inflammatory event, usually, counteracting signals may be triggered to limit the activation of the process [34]. To evaluate this, anti-inflammatory cytokines were quantified and we observed that *B.a.* venom is capable of inducing IL13 and slight amounts of IL10. Considering that both cytokines negatively modulate the inflammatory process, our results confirm the proinflammatory effect induced by *B.a.* venom and suggest that IL13 and IL10 may have a role in the control of the inflammatory response initiated by *B.a.* venom. Moreover, since the different immunosuppressive abilities that both cytokines possess, these results would allow us to speculate that the anti-inflammatory capacity of the crotalic venom is focused on IL10.

To confirm the data obtained in RAW 264.7 cell model, we evaluated the production of cytokines in mouse peritoneal macrophages. However, while significant differences were obtained, it can be noted that cytokines elevations are not so pronounced as those obtained using the RAW 264.7 cell model. This is in agreement with previous studies since it was shown that these cells have lectins receptors that bind those present in venoms, and secrete large amounts of cytokines [35]. In addition, proteomic studies performed on *B.a.* venom demonstrated the presence of type C lectin (1.7%) [25].

Similarly to what is observed in several pathologies, such as diabetes, atherosclerosis, metabolic syndrome, and cancer [36], bothropic envenomation engages lipid signaling [13]. The first evidence that snake venoms interfere with cellular lipid metabolism comes from the ability of their toxins to increase the cytoplasmic synthesis of small organelles called lipid droplets (LD), as we previously demonstrated [37–39]. This increase in cytoplasmic lipids may account for three reasons. First, an increase of *de novo* biosynthesis of FAs; second, the

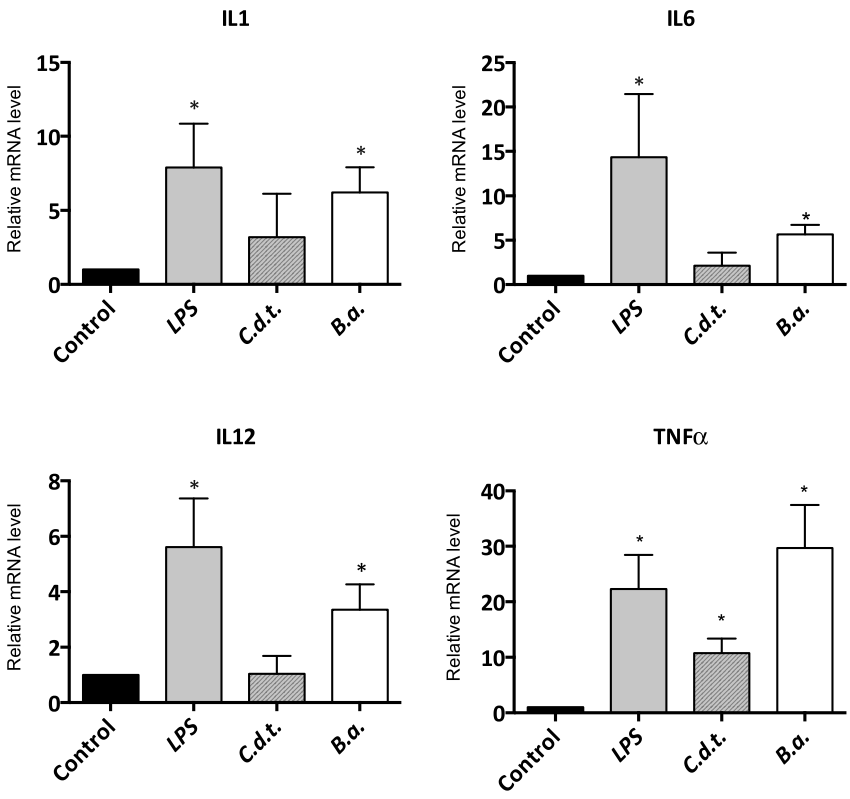

Fig. 4. Expression of cytokines mRNA induced by *B.a.* venom in mice peritoneal macrophages. Values represent the mean ± SD from 3 independent experiments. *p < .05 compared with the control group.

induction of cellular PLA_2s that hydrolyze FA esterified to membrane phospholipids, in response to the action of PRRs (pattern recognition receptors) or finally, bothropic PLA_2s contained in the venom, act directly on the membrane and release FAs from phospholipids which are then re-esterified into LDs. In order to evaluate the first hypothesis, we measured the expression of mRNAs of the inducible enzymes that regulate the rate of fatty acid synthesis, and no changes were detected in both, RAW 264.7 and peritoneal macrophages (data not shown). In contrast, we herein detected a moderate increase in $cPLA_2$ mRNA expression and also in its activity (measured by phosphorylation in Ser 505). Moreover, we found marked differences in the expression of COX-2 with different exposure times to venom. Considering the capacity of free fatty acids to activate cyclooxygenase pathway, this led us to think that FAs should be releasing from the plasma membrane, serve as substrates for the eicosanoids synthesis and in turn, stimulate the COX-2 synthesis as previously demonstrated [40,41].

To study the direct action of venom on the phospholipids cell membrane, the lipidomic fatty acid profile of mouse peritoneal

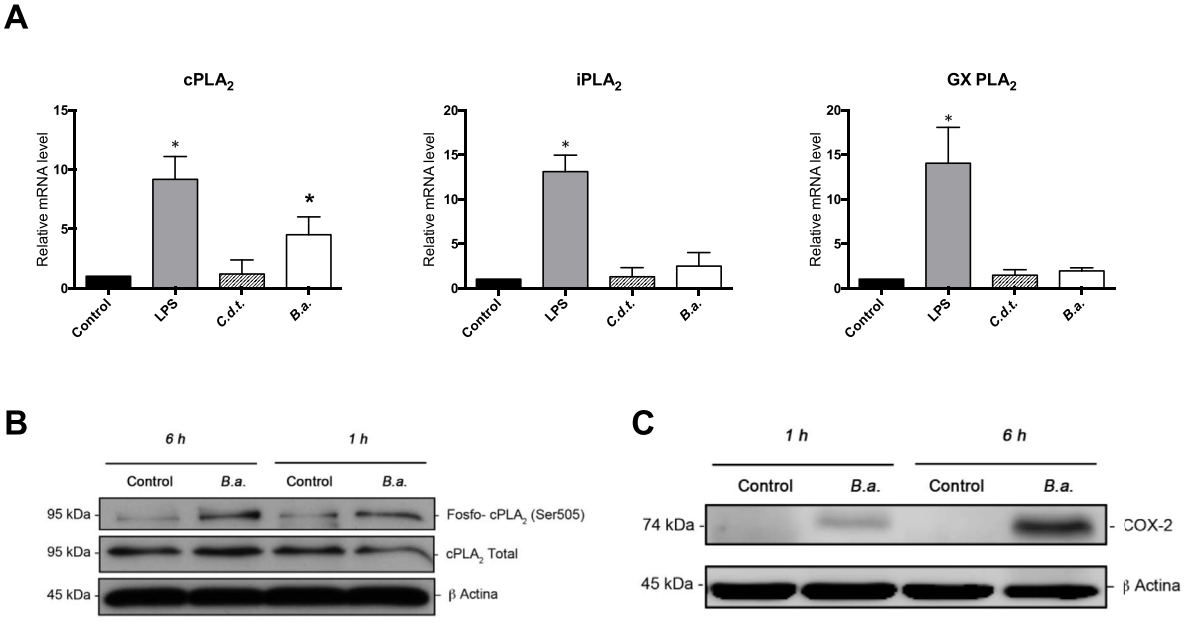

Fig. 5. A. Expression of cellular PLA_2 mRNA induced by *B.a.* venom in Raw 264.7 cells. Cells were incubated for 6 h with venoms (5 μg/ml), LPS or PBS (control), and mammalian $cPLA_2$, $iPLA_2$ and GX PLA_2 were determined by quantitative PCR. Values represent the mean ± SD from 3 independent experiments performed in triplicate. *p < .05. **B.** Expression of total and phosphorylated $cPLA_2$ after 1 and 6 h exposure to *B.a.* venom. **C.** Expression of COX-2 after 1 and 6 h exposure to *B.a.* venom. Result are representive from 3 independent experiments.

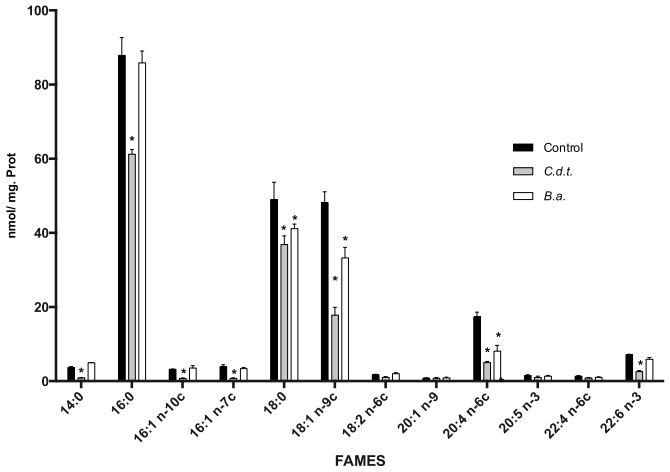

Fig. 6. Lipidomic fatty acid profile of venom-stimulated Raw 264.7 cells, determined by GC-MS. Cells were scraped and fatty acids esterified into phospholipids were released and analyzed. Values represent the mean ± SD from 3 independent experiments. *p < .05 compared with the control group.

macrophages were determined by GC-MS. Moderate diminutions were observed in stearic, oleic, and particularly arachidonic acid. Decreases in those FAs indicate an action of the ophidian PLA_2s since $cPLA_{2\alpha}$ has no specificity for these FAs containing-PLs [18]. Other venoms such as *C.d.t.*, produce much more strong decreases in the cellular FA content. This is in direct relation to the high PLA_2 content of the venom (almost 50%) [42], compared to *B.a.* venom (7.8%). In addition, it is known that Lys-49 PLA_2 of this venom does not hydrolyze PLs [25]. Given the more abrupt variations found for AA, it is possible that the hydrolysis of this fatty acid is a consequence of a synergistic action of $cPLA_{2\alpha}$ and venom PLA_2s. The release of AA surely implies the production of inflammatory eicosanoids, but in addition, those other FAs can be released into the extracellular space and act as agonists of inflammatory receptors that trigger an aggravation of the inflammatory process [39].

Taken together, results presented here show that the alterations caused by *B.a.* venom (both macro and microscopic), observed through changes in vascular permeability, edema and cellular extravasation, are based on the stimulation of specific molecular signaling pathways. These involve cytokines and key enzymes such as $cPLA_{2\alpha}$ and COX-2. Although much of these findings were described in inflammatory processes induced by other bothropic venoms, here we demonstrate that *B.a.* venom also stimulates pro-inflammatory pathways involving lipid mediators of cell signaling. In this sense, lipidomics analysis of macrophages stimulated with *B.a.* venom evidenced that the main free fatty acids are implicated in the inflammatory response, and also demonstrated that this venom, is able to activate lipid metabolism even with a low content of PLA_2. Thus, deeper lipidomic studies, poorly developed up to now in ophidian toxicology, could reveal novel pharmacological targets for the treatment of bothropic intoxications.

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Transparency document

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Appendix A. Supplementary data

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