Effect of L-amino acid oxidase from *Calloselasma rhodosthoma* snake venom on human neutrophils

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ABSTRACT

The *in vitro* effects of LAAO, an L-amino acid oxidase isolated from *Calloselasma rhodosthoma* snake venom, on isolated human neutrophil function were investigated. LAAO showed no toxicity on neutrophils. At non-cytotoxic concentrations, LAAO induced the superoxide anion production by isolated human neutrophil. This toxin, in its native form, is also able to stimulate the production of hydrogen peroxide in neutrophils, suggesting that its primary structure is essential for stimulation the cell. Moreover, the incubation of LAAO and phenol red medium did not induce the production of hydrogen peroxide. Furthermore, LAAO was able to stimulate neutrophils to release proinflammatory mediators such as IL-8 and TNF-α as well as NETs liberation. Together, the data showed that the LAAO triggers relevant proinflammatory events. Particular regions of the molecule distinct from the LAAO catalytic site may be involved in the onset of inflammatory events.

1. Introduction

Neutrophils constitute the major cellular component of the innate immune response. The initial step in the inflammatory response is adhesion and migration of neutrophils from microvasculature into the tissues and their subsequent retention within inflammatory sites, where they ingest pathogens and produce reactive oxygen species (ROS), proteinases, bactericidal proteins and cytokines which either alone or in concert may interact in up- or down- regulating the inflammatory processes (Granger and Kubes, 1994; Witko-Sarsat et al., 2000).

The activation of the oxidative metabolism, known as the respiratory burst, involves the phagocyte NADPH oxidase (an enzymatic complex composed of cytosolic proteins – p40phox, p47phox and p67phox- and membrane proteins – p22phox and gp91phox). The generation of superoxide anion via the activation of NADPH oxidase is the starting material for the production of a vast of reactive oxidants (Kim and Dinauer, 2001). Superoxide can also be

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generated through the mitochondrial electron transport chain, xanthine–xanthine oxidase, and cytochrome P450. Mitochondria generate superoxide mostly by the univalent reduction of oxygen in complexes I and III of the electron transport chain (Andreyev et al., 2005).

Neutrophils are exquisite targets of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) and chemokines such as interleukin-8 (IL-8). These cytokines amplifies several functions of neutrophils including their capacity of adhering to endothelial cells and to produce ROS; and the chemokines act as potent attractants and favor their orientated migration toward the inflammatory site. Both cytokines and chemokines may also act priming agents of neutrophils (Witko-Sarsat et al., 2000).

L-amino acid oxidases (LAAOs, EC 1.4.3.2) are flavoenzymes that catalyze the stereospecific oxidative deamination of L-amino acids to the corresponding α-ketoacid, with the production of hydrogen peroxide and ammonia via an imino acid intermediate (Curti et al., 1992). These enzymes exhibit a marked affinity for hydrophobic amino acids, including phenylalanine, tryptophan, tyrosine and, leucine.

LAAOs are found in variety different organisms such as bacteria, fungi, green algae, and snake venoms (Du and Clemetson, 2002; Zuliani et al., 2009; Guo et al., 2012). Among them, LAAOs isolate from snake venoms (SV-LAAOs which may represent 1–9% of the total protein) are the best characterized. SV-LAAOs are present in significantly high concentrations in most snake venoms and contribute to their yellowish color containing flavin as the prosthetic group (Du and Clemetson, 2002; Zuliani et al., 2009; Guo et al., 2012). LAAO has been found to contribute to the toxicity of the venom due to the production of hydrogen peroxide during the oxidation reaction.

A salient feature of SV-LAAOs is their glycosylation, a feature that was first reported by deKok and Rawitch (1969). Following the elucidation of the three-dimensional structure of LAAO from *Calloselasma rhodosthoma* (a Malayan pit viper) (Pawelek et al., 2000) the chemical nature of the glycan substituents was deduced by NMR spectroscopy demonstrating that the glycosylation is remarkably homogeneous, in contrast to most other glycoproteins (Geyer et al., 2001).

The exact biological functions of LAAOs are still unknown. It is supposed that these enzymes may be involved in allergic inflammatory response and specifically associated with mammalian endothelial cell damage (Suhr and Kim, 1996; Macheroux et al., 2001). Furthermore, LAAOs have various biological properties such as antimicrobial activity, induction of apoptosis, inhibition of platelet aggregation and anti-HIV activity. These effects are mainly associated with the production of hydrogen peroxide, since catalase activation, a H_2O_2 scavenger, inhibits the biological effects of LAAOs as well as H_2O_2 (Du and Clemetson, 2002; Zuliani et al., 2009; Guo et al., 2012).

The present study was therefore designed to address the effects of LAAO from *Calloselasma rhodosthoma* (a Malayan pit viper) on isolated human neutrophils particularly on ROS (superoxide anion and hydrogen peroxide) and cytokines production and examined the contribution of LAAO activity on hydrogen peroxide production.

2. Materials and methods

2.1. Chemicals and reagents

Crystallized *Calloselasma (Agkistrodon) rhodostoma* venom was purchased from Sigma Chem. Co. (MO, USA). MTT, RPMI-1640, L-glutamine, gentamicin, phorbol myristate acetate (PMA), Histopaque 1077, DMSO, OPD (o-1,2-phenylenediamine dihydrochloride), horseradish peroxidase and nitroblue tetrazolium (NBT) were purchased from Sigma (MO, USA). DuoSet Elisa human TNF-alpha/TNFSF1A and DuoSet Elisa human CXCL8/IL-8 were purchased from R&D Systems (Oxon, United Kingdom). Quant-iT™ Picogreen dsDNA was obtained from Invitrogen (CA, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Sao Paulo, Brazil). All salts and reagents used obtained from Merck (Darmstadt, Germany) with low endotoxin or endotoxin-free grades.

3. Isolation and biochemical characterization of Cr-LAAO

Calloselasma rhodostoma crude venom (30 mg) was dissolved in 1.0 mL of 0.02 M Tris–HCl buffer, pH 8.0, centrifuged at $755 \times g$ for 10 min at room temperature and the clear supernatant applied on a 70 cm $\times$ 0.9 cm Superdex G-75 column, which was previously equilibrated and then eluted with the same buffer. The samples of 1.0 mL/tube, at a flow rate of 0.75 mL/min were collected and monitored at 280 nm. The fraction I showing LAAO activity was lyophilized, diluted with 0.02 M Tris–HCl buffer, pH 8.0 and then applied on a 4.0 $\times$ 0.6 cm Q -Sepharose Fast Flow column (GE Healthcare), previously equilibrated with the same buffer. The chromatography was carried out at a flow rate of 1.0 mL/min, using a crescent concentration NaCl gradient (0–100%). To evaluate the purity degree, the fraction II that containing Cr-LAAO was submitted to 12.5% SDS-PAGE (Laemmli, 1970). The molecular weight was confirmed by matrix assisted laser desorption ionization mass spectrometry (Axima TOF/TOF Shimadzu Biotech) using sinapinic acid as ionization matrix. The analyses were operated in linear mode and the mass spectra obtained by the average of the laser pulses.

Activity of L-amino acid oxidase: This test was performed before each experiment to verify the activity of LAAO. For this, 10 µg of toxin (0.01 mL) were added to the reaction mixture containing horseradish peroxidase (50 µg/mL), 100 µM L-leucine, 10 µM 3′3′diaminobenzidine in 100 mM Tris–HCl buffer (pH 7.8) in a final volume of 1.0 mL was incubated at 37 °C for 30 min. The reaction was stopped using a solution of 10% citric acid (0.5 mL) and the absorbance was measured on a spectrophotometer at 490 nm.

Inactivation of the enzyme L-amino acid oxidase: For the inactivation of the enzyme, LAAO was submitted to a temperature of 80 °C for 30 min.

3.1. Neutrophil isolation

Peripheral blood neutrophils were obtained from self-reportedly healthy (18–40 years), and informed consent

was obtained at the time of the blood draw. All participants gave informed consent prior to their inclusion in the study and the Center of Tropical Medicine Research (Rondonia, Brazil) Research Ethics Committees (number 108/2010) approved this study. In brief, after local asepsis blood was collected in vacuum tubes containing heparin and diluted in phosphate buffered saline (PBS, 14 mM NaCl, 2 mM $NaH_2PO_4H_2O$, $7mMNa_2HPO_412H_2O$), pH 7.4. For separation of leukocytes Histopaque 1077 was added to the tubes and then the diluted blood was added carefully over the reagent. After centrifugation at $400 \times g$ for 30 min, the neutrophils were collected from the bottom of the tube, together with the erythrocytes and transferred to another tube. Lysis of red blood cells was performed using lysis buffer, homogenized and subjected to a temperature of -8 °C for 5 min, and centrifuged. Neutrophils were washed with PBS and an aliquot of isolated neutrophils was used for determining the total number of neutrophils in a Neubauer's chamber after cell staining (1:20, v/v) with Turk solution (violet crystal 0.2% in acetic acid 30%). The purity of the isolated cell population was determined by Panotic staining of cytospin preparations and by flow cytometry analysis (FACscan). The mean purity achieved by our isolation technique was 99% of neutrophils.

3.2. Cytotoxic assay

Neutrophils (2×10^5 cells/mL) were suspended in RPMI culture medium, supplemented with gentamycin (100 μg/mL), ʟ-glutamine (2 mM) and 10% fetal bovine serum. Then the cells were incubated in duplicate in 96-well plates with Cr-LAAO at concentrations of 6, 12.5, 25, 50 and 100 μg/mL or RPMI (control) for 12 h, at 37 °C in a humidified atmosphere (5% CO_2). Next 10 μL of MTT (5 mg/mL) was added and incubated for 1 h. After centrifugation at $400 \times g$ for 5 min, the supernatant was removed and added 100 μL of DMSO to dissolve the crystals formed. Subsequently, the plates were kept for 18 h at room temperature. The crystals of formazam formed were evaluated by spectrophotometer at 540 nm. The results were expressed in optical density compared to the control.

3.3. Superoxide anion production assay

In this assay the generation of superoxide was estimated by reducing nitroblue tetrazolium (NBT), a yellow liposoluble compound that becomes insoluble and blue in its reduced form (Setubal et al., 2011). In brief, neutrophils (2×10^5/100 μL) were incubated with 100 μL of RPMI containing NBT 0.1% (control) or 100 μL of different concentrations of Cr-LAAO (6, 12.5, 25, 50 and 100 μg/mL), diluted in RPMI containing 0.1% NBT, and incubated for 1 h at 37 °C in humidified atmosphere (5% CO_2). At the end of the incubation period, the vials were centrifuged for 30 s at $800 \times g$ and the cells were washed twice with warm PBS. The NBT reduced deposited inside the cells were then dissolved, first by adding 120 μL of 2 M KOH to solubilize cell membranes and then by adding 140 μL of DMSO to dissolve blue formazan with gentle shaking for 10 min at room temperature. The dissolved NBT solution was then transferred to a 96-well plate and absorbance was read on a microplate reader at 620 nm. Data were expressed as absorbance.

3.4. Determination of hydrogen peroxide (H_2O_2) production by human neutrophils

The technique used was described by Pick and Keisari (1980), adapted for microassay by Pick and Mizel (1981), and with modifications proposed by Russo et al. (1989). In brief, neutrophils (2×10^5/50 μL) were resuspended in 1.0 mL of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 0.56 mM phenol red) containing 0.05 mg/mL of horseradish peroxidase. Then the cells were incubated with Cr-LAAO at several concentrations in the native form and inactive form for 12 h at 37 °C in humidified atmosphere (5% CO_2). Cells were maintained simultaneously with or without stimulation by phorbol myristate acetate (PMA, 50 ng/mL). After this time, the reaction was stopped by addition of 1 N sodium hydroxide (10 μL). The basal production of hydrogen peroxide using Cr-LAAO at concentrations of 50 and 100 μg/mL dissolved in phenol red medium was also assessed. The absorbance was measured spectrophotometrically at 620 nm against blank constituted of phenol red medium. The data generated were compared to a standard curve conducted for each test. The results were expressed as μM of H_2O_2 produced.

3.5. Interleukin-8 (IL-8) and tumor necrosis factor-α (TNF- α) quantifications

Neutrophils (2×10^5/50 μL) were incubated with different concentrations of Cr-LAAO for 12 h at 37 °C in humidified atmosphere (5% CO_2). After centrifugation the supernatant were used for determination of IL-8 and TNF-α levels by specific EIA, as described by Schumacher et al. (1988). Briefly, 96-well plates were coated with 100 μL of the first capture monoclonal antibody anti-IL-8 (4 μg/mL) or anti-tumor necrosis factor-α (4 μg/mL) and incubated for 18 h at 37 °C. Following this period, the plate was washed with washer buffer (PBS/Tween20). After that, 200 μL of blocking buffer, containing 5% bovine serum albumin (BSA) in PBS/Tween20, were added to the wells and the plates incubated for 1 h at 37 °C. Following this period, wells were washed and 50 μL of either samples or standard were dispensed into each well and the plates incubated for 2 h at 37 °C. After this period, the plate was washed and 100 μL of detection antibody anti IL-8 (20 ng/mL) or TNF-α (250 ng/mL) was added for 2 h at 37 °C. After incubation and washing, 100 μL of streptoavidin-peroxidase were added, followed by incubation and addition of the substrate (100 μL/mL 3,3′,5,5′-tetramethybenzidine). Finally sulfuric acid (50 μL) was added to stop the reaction. Absorbances at 540 and 450 nm were recorded and concentrations of IL-8 and TNF-α were estimated from standard curves prepared with recombinant IL-8 or TNF-α. The results were expressed as pg/mL of each cytokine.

3.6. Neutrophil extracellular traps (NETs) release

Neutrophils (2×10^5/50 μL) were incubated with different concentrations of Cr-LAAO (12.5, 25, 50 and

100 μg/mL) or RPMI (control) or PMA (500 ng/mL, positive control) for 1, 3 and 6 h at 37 °C in humidified atmosphere (5% CO_2). After centrifugation the supernatant were used for determination of NETs release accordingly to procedure described in kit Quant-iT™ Picogreen dsDNA (Invitrogen). Briefly, 50 μL of samples were incubated with 100 μL of PI (Quant-iT) and 50 μL of PE buffer in a 96-well dark plate. After 15 min incubation absorbances at 520 nm emission and 480 nm excitation were recorded and NETs release were estimated from standard curve. The results were expressed as ng/mL of DNA.

3.7. Statistical analysis

Means and S.E.M. of all data were obtained and compared by one-way ANOVA, followed by Tukey test with significance probability levels of less than 0.05.

4. Results

4.1. Isolation and biochemical characterization of Cr-LAAO

Initially, *C. rhodostoma* snake venom was submitted to a molecular exclusion chromatography on Superdex-75, which resulted in five fractions (Fig. 1A). Fraction I, the high molecular weight fraction with the LAAO activity (results not shown) was lyophilized and subjected to an ion exchange chromatography on Q-Sepharose resin (Fig. 1B), from which was obtained the fraction II that showed enzymatic activity. In SDS-PAGE, the fraction II showed a single band about 55 kDa, in the presence of reducing agents (Fig. 1C) that correspond to Cr-LAAO. Moreover, using mass spectrometry technique was obtained a main sharp peak with molecular weight of 59,311.33 Da (Fig. 1D) supporting data from SDS-PAGE.
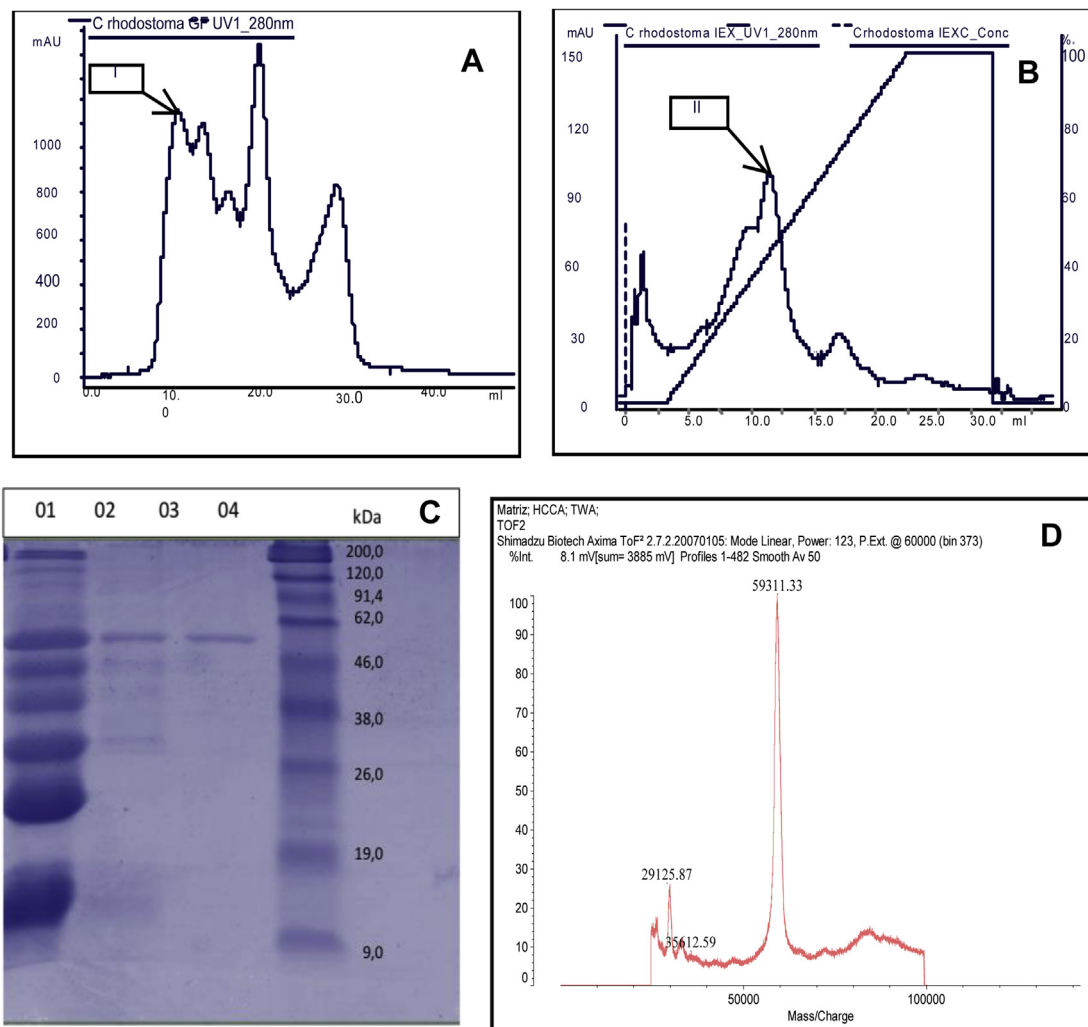

Fig. 1. Isolation and biochemical characterization of Cr-LAAO. (A) – Elution profile of *C. rhodostoma* crude venom on Sephadex G-75. (B) – Elution profile of fraction I (presence of LAAO activity) on Q –Sepharose FF. (C) – SDS-PAGE 12,5% in reducing condition of LAAO: lane 1 – *C. rhodostoma* crude venom, lane 2 – Fraction I from Sephadex G-75, lane 3 - Fraction II from Q-sepharose FF and lane 4 – Molecular weight. (E) – Mass specter of Cr-LAAO obtained by MALDI TOF/TOF spectrometer showing a main peak of 59,311.43 Da.

4.2. Effect of LAAO on human neutrophils viability

To further investigate the Cr-LAAO on neutrophils function we isolated these cells by density gradient. The purity of the isolated neutrophils obtained by density gradient was 98.5% as determined by flow cytometry using the pan-granulocyte marker CD66b (Mannoni et al., 1982) and by Panotic staining of cytospin preparations (Inserted). To test the toxicity of Cr-LAAO on isolated human neutrophils we used the MTT assay. To this end, the effect of 12 h incubation of several concentrations of Cr-LAAO was investigated. As shown in Fig. 2, incubation of LAAO at a concentration of 6–100 μg/mL did not affect human neutrophils viability in comparison with control cells incubated with culture medium alone. This finding evidenced that Cr-LAAO at this period of time and at these concentrations is not toxic to human neutrophils.

4.3. Superoxide anion (O_2^-) production by human neutrophils induced by LAAO

In order to investigate the ability of Cr-LAAO to induce superoxide anion production by human neutrophils, the cells were incubated with non-cytotoxic concentrations of Cr-LAAO or RPMI (control), in the presence of NBT. As shown in Figs. 3A and 1 h incubation of human neutrophils with different non-cytotoxic concentrations of Cr-LAAO did not induce an increase in superoxide production compared to negative control (RPMI), but there was a significant
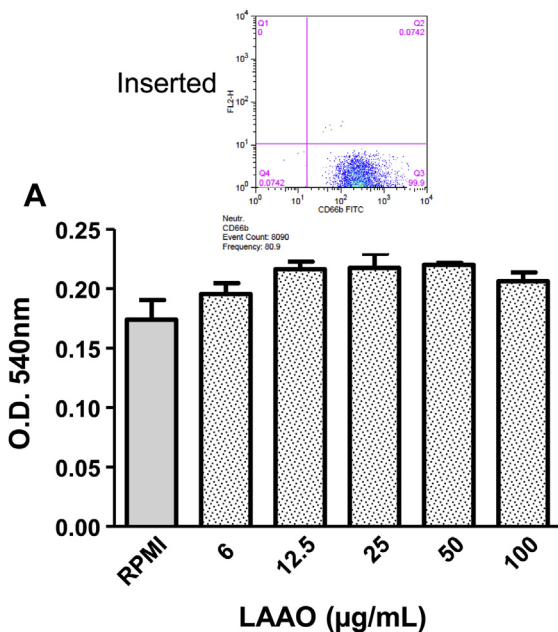
Fig. 2. Effect of Cr-LAAO on neutrophils viability. Inserted. Human neutrophils were isolated from buffy coats of healthy adult blood donors through a density gradient method and analyzed in FACScan. (A) 2×10^5 cells were incubated with different concentrations of Cr-LAAO or RPMI (control) for 12 h at 37 °C in humidified atmosphere of 5% CO_2. After cytotoxicity was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] metabolization method. Values represent the mean S.E.M. from 4 to 5 donors.
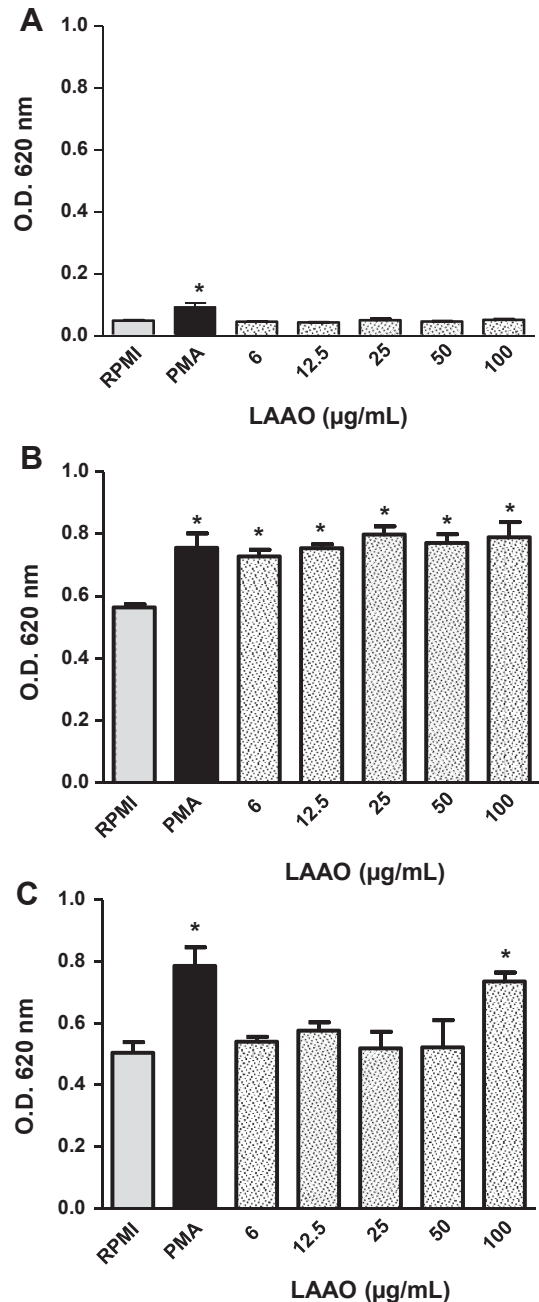
Fig. 3. Effect of Cr-LAAO on superoxide production by neutrophils. Neutrophils (2×10^5) were incubated for 1 h or 3 h or 6 h with RPMI, 0.1% NBT, 10 μL of PMA (500 ng/mL) (positive control), Cr-LAAO (6; 12.5; 25; 50 and 100 μg/mL) for the formation of formazan crystals resulting from the reduction of NBT by superoxide. The crystals were solubilized and the absorbance of the supernatant was determined at 620 nm in a spectrophotometer. Data were expressed as OD. Values are mean S.E.M. from 4 to 5 donors. *$P < 0,05$ compared with control (ANOVA).

increase in O^{2-} production, when these cells were incubated with a positive control (PMA). Already after 3 h incubation of human neutrophils with non-cytotoxic concentrations of Cr-LAAO, as shown in Fig. 3B, there was a significant increase in O^{2-} production at all concentrations used, as compared to control (RPMI). Moreover, after 6 h

incubation of human neutrophils with non-cytotoxic concentrations of Cr-LAAO, there was only a significant increase in superoxide anion production, at the concentration of 100 μg/mL of Cr-LAAO, as compared to control (RPMI) (Fig. 3C). Our results reveal that Cr-LAAO activates human neutrophils to produce superoxide anion.

4.4. Hydrogen peroxide (H_2O_2) production by human neutrophils stimulated by Cr-LAAO

A growing body of evidence indicates that the biological effects of LAAOs such as antimicrobial activity, induction of apoptosis, inhibition of platelet aggregation and anti-HIV activity are attributed to the production of hydrogen peroxide, since addition of catalase and other H_2O_2 scavenger, inhibits the biological effects of LAAOs as well as H_2O_2 (Du and Clemetson, 2002; Zuliani et al., 2009). To this end to verify the ability of Cr-LAAO to induce the production of hydrogen peroxide by human neutrophils, the cells were incubated with the toxin in the native and inactive form in non-cytotoxic concentrations of Cr-LAAO or phenol red solution (negative control) or PMA (positive control). As shown in Fig. 4A incubation of neutrophils at concentrations of 50 up to 100 μg/mL in native form resulted in a significant increase in hydrogen peroxide production. Moreover, when cells were incubated with Cr-LAAO in inactive form, the stimulatory effect of the toxin was abrogated. Fig. 4B show the basal H_2O_2 production by 50 and 100 μg/mL of Cr-LAAO in native form in phenol red solution (negative control) without neutrophils. These findings evidenced the ability of Cr-LAAO to stimulate human neutrophils to produce hydrogen peroxide is related to enzymatic activity of this protein.
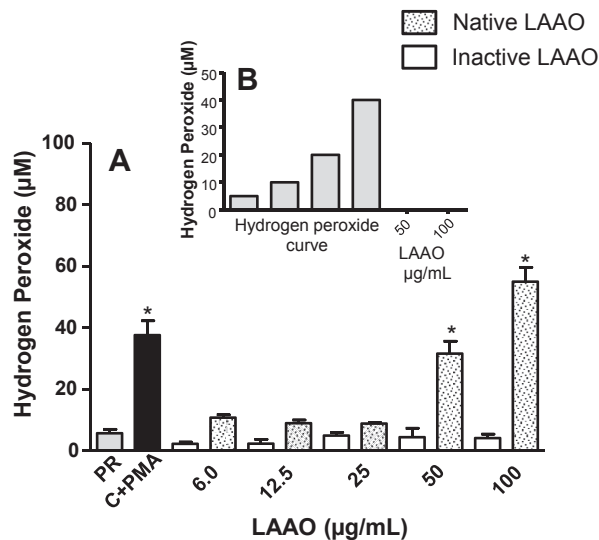
4.5. Release of cytokines (IL-8 and TNF-α) by human neutrophils induced by LAAO

To assess the ability of Cr-LAAO to activate human neutrophils and stimulate pro-inflammatory cytokine release such as IL-8 and TNF-α the cells were incubated with non-cytotoxic concentrations of Cr-LAAO or RPMI (negative control) or PMA (positive control). As shown in Fig. 5A and 100 μg/mL of Cr-LAAO induced a significant release of TNF-α by human neutrophils compared to both controls. Fig. 5B show that after 12 h incubation of neutrophils with 50 and 100 μg/mL of Cr-LAAO induced a significant release of IL-8 by human neutrophils. Our results reveal that Cr-LAAO activated human neutrophils and induced the release of IL-8 and TNF-α.

4.6. Release of NETs by human neutrophils induced by LAAO

In order to investigate the ability of Cr-LAAO to induce the liberation of NETs by human neutrophils, the cells were
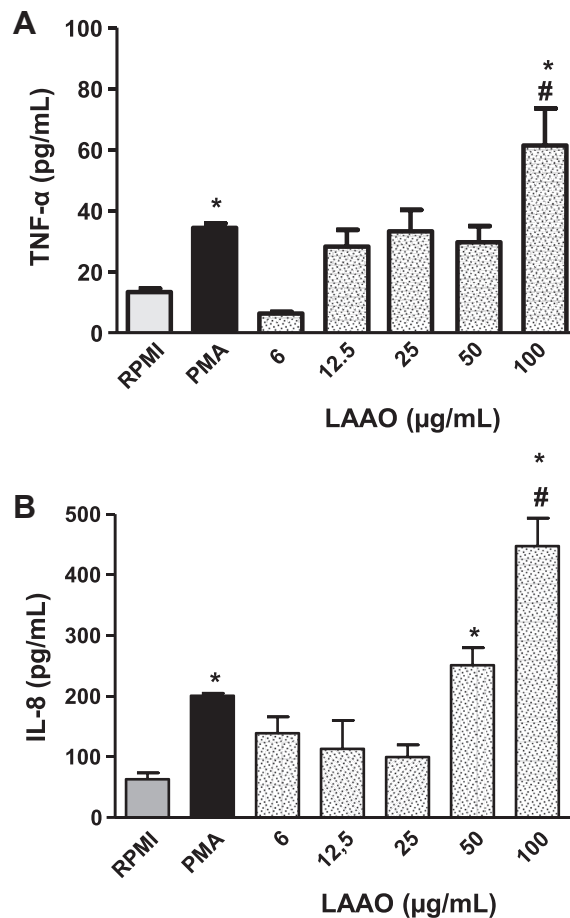

Fig. 4. Effect of Cr-LAAO on the hydrogen peroxide production. Neutrophils (2×10^5) were incubated with non-cytotoxic concentrations of native or inactive Cr-LAAO for 12 h in phenol red solution and PMA (50 ng/mL) (A). Basal production of hydrogen peroxide by the native enzyme at of concentrations 50 and 100 μg/mL dissolved in phenol red solution (B). The absorbance was measured by spectrophotometer at 620 nm. The results were expressed as μmoles of H_2O_2 produced and represent the mean ± SEM of four donors. *$p < 0.05$ compared to control (ANOVA).

Fig. 5. Release of TNF-α and IL-8 by neutrophils induced by Cr-LAAO. Neutrophils (2×10^5) were incubated with non-cytotoxic concentrations of LAAO for 12 h. The concentrations of TNF-α (A) and IL-8 (B) in the supernatant were quantified by specific EIA. The results were expressed as pg/mL of TNF-α or IL-8 and represent the mean ± SEM of four donors. *$p < 0.05$ compared to control. #$p < 0.05$ compared to PMA (positive control) (ANOVA).

incubated with non-cytotoxic concentrations of Cr-LAAO or RPMI (control) or PMA (positive control). As shown in Figs. 6A and 1 h incubation of human neutrophils with different non-cytotoxic concentrations of Cr-LAAO induced an increase in NETs liberation compared to negative control (RPMI) and to positive control (PMA). Moreover after 3 h incubation of human neutrophils with non-cytotoxic concentrations of Cr-LAAO, as shown in Fig. 6B, there was a return to a basal level in NETs liberation at all concentrations used, as compared to controls (RPMI and PMA) until 6 h (data not shown). These findings evidenced the ability of Cr-LAAO to stimulate human neutrophils to induce NETs liberation.

5. Discussion

LAAOs are enzymes widely distributed in many different organisms such as *Corynebacterium* bacteria (Coudert, 1975), Proteus (Duerre and Chakrabarty, 1975),

Fig. 6. Release of NETs by neutrophils induced by Cr-LAAO. Neutrophils (2×10^5) were incubated with non-cytotoxic concentrations of LAAO or RPMI (control) or PMA (500 ng/mL, positive control) for 1 (A) and 3 h (B) at 37 °C in humidified atmosphere (5% CO_2). The DNA concentrations of NETs were quantified by Quant-iT™ Picogreen dsDNA kit. The results were expressed as ng/mL of DNA in the supernatant and represent the mean ± SEM of four donors. *$p < 0.05$ compared to control (ANOVA).

Neurospora crassa fungi (Niedermann and Lerch, 1990), *Chlamydomonas rheinhardtii* green algae (Vallon et al., 1993), fish (Murakawa et al., 2001), snails (Obara et al., 1992) and sea slugs (Butzke et al., 2005; Yang et al., 2005) and plants, which are involved in nitrogen utilization as an energy source, and venoms from a variety of snakes which are the most studied (Du and Clemetson, 2002; Guo et al., 2012).

Venoms comprise a complex mixture of proteins with pharmacological activities which are capable of affecting various biological systems (Chippaux and Goyffon, 1998). Among proteins found in snake venoms are the LAAOs. These toxins are found in large quantities in *Calloselasma rhodostoma* snake venom, and represent 30% of crude venom (Ponnudurai et al., 1994). Because it is a labile toxin (Coles et al., 1977) the enzymatic activity of the enzyme was measured prior to all experiments.

First of all, the enzyme was isolated from crude venom as described by Ponnudurai et al. (1994) with modifications. The homogeneity and purity grade of this enzyme was confirmed by SDS-PAGE that showed a single band in reducing condition of about 55 kDa. Moreover, the molecular weight of Cr-LAAO obtained by mass-spectrometry about 59,300 Da was compatible with that is described in the literature. Structurally, the Cr-LAAO is constituted by three domains: the FAD-binding domain, substrate binding domain and the helical domain that contributes to the formation of the channel pathway through which the substrates are conduced to the active sites (Du and Clemetson, 2002). Also are present two sites of glycosylation at Asn127 and Asn361, being the carbohydrate moiety constituted by a bis-sialylated, biantenary core-fucosylated dodecasaccharide (Geyer et al., 2001). The same author shows that, when the deglycosylation procedures are conducted under nondenaturing conditions, it is possible to observe several levels of deglycosylation. It is possible to observe different levels of deglycosylation and where it has the lowest molecular weight represents a complete removal of glycan moiety. These features are in agreement with the results obtained in our laboratory, where, after deglycosylation procedure in the same condition and using mass spectrometry, it was possible to observe two peaks representing these proteins (53,331.29 Da and 53,133.37 Da).

Despite LAAOs importance there is still limited understanding of the mechanism of action of this toxin on human neutrophils. Since this cell type is a key component of the innate immunity and it is the initial response to infection we decided to evaluate, in this study, the effects of LAAO isolated from *Calloselasma rhodostoma* on isolated human neutrophils starting evaluating the effect of Cr-LAAO on this cell viability. Data showed that the toxin did not affect the viability of neutrophils indicating their low toxicity on this cell type. The effect of LAAO on leukocytes viability was not demonstrated until now. Moreover the literature shows the toxic effect of the enzyme in bacteria first described by Skarnes (1970) and later by several authors Stiles et al. (1991), Stábeli et al. (2004) and Izidoro et al. (2006). This effect seems to be related to hydrogen peroxide, secondary product formed during the chemical reaction catalyzed by LAAO.
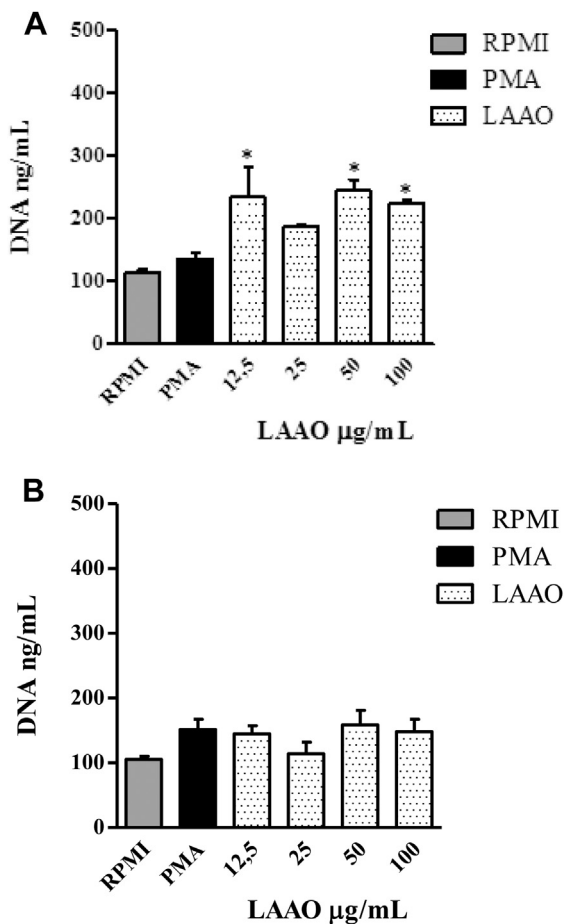
It is known that neutrophils are the first leukocyte type recruited to sites of tissue damage or infection and play a central role in the inflammatory response. These cells are functional leukocytes that have the ability to adhere and migrate, degranulate and release of inflammatory mediators such as cytokines and reactive oxygen species (ROS) and ingest particles by phagocytosis (Witko-Sarsat et al., 2000).

The oxidative burst results in the sequential production of cytotoxic reactive oxygen intermediates through initially superoxide anion radical (O^{2-}) generation followed by hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). The enzyme responsible for superoxide anion production is a multicomponent NADPH oxidase or burst respiratory oxidase (Babior, 1999). In neutrophils the formation of hypochlorous acid (HOCl) seems to be important for their microbicidal activity. HOCl is formed enzymatically from H_2O_2 by myeloperoxidase (MPO) in the presence of Cl^- ions (Anderson et al., 1997).

Taking this into account, we conducted experiments in order to verify the effect of Cr-LAAO on the superoxide anion production, an important ROS. After 1 h incubation the toxin could not stimulate neutrophil to produce superoxide anion compared with negative control. However, after 3 h, Cr-LAAO significantly stimulated the neutrophils to produce superoxide anion in all concentrations used compared with negative control, however there was no difference when compared with PMA (a positive control). This effect remained significant up to 6 h only at the high concentration (100 μg/mL). The mechanism by which the toxin stimulates the generation of superoxide anion was not clarified.

It is therefore possible that Cr-LAAO activates NADPH oxidase by a direct mechanism or indirectly through the activation of signaling pathways such as PKC, and that culminate in the activation of NADPH oxidase. Moreover, reactive oxygen species may also be generated by mechanisms independent of NADPH oxidase, via mitochondria. The production of superoxide and hydrogen peroxide in mitochondria can be stimulated by activation of mitochondrial electron transport chain, xanthine–xanthine oxidase, cytochrome P450 and small conductance calcium-activated potassium channels (Andreyev et al., 2005; Fay et al., 2006). It is noteworthy that Cr-LAAO may act through distinct mechanisms.

After superoxide anion production the hydrogen peroxide is formed. This can occur spontaneously or via superoxide dismutase (SOD) catalytic action. Since hydrogen peroxide is also a ROS produced by activated cells and is involved in phagocytic destruction of pathogens, its release was measured by neutrophils incubated with LAAO. Thus, the toxin was used in two conditions, in an enzymatically native form and inactive form.

Initially, we conducted experiments to verify the Cr-LAAO enzymatic activity on phenol red solution. The results showed that the native Cr-LAAO at concentrations of 100 and 50 μg/mL did not induce the peroxide hydrogen production as a consequence of an intrinsic catalytic manner. However, when Cr-LAAO in native form was incubated with isolated human neutrophils, there was a large production of hydrogen peroxide, indicating that the enzyme is able to stimulate neutrophils to produce hydrogen peroxide. The mechanism by which Cr-LAAO stimulates the production of hydrogen peroxide was not clear.

Another factor that must be taken into consideration is the interaction of glycan structure of the toxin to the cell surface. The peculiar feature of LAAOs isolated from snake venoms is its glycosylation. Some carbohydrates such as fucose, mannose, galactose, N-acetyl glicosamida and sialic acid have been identified in this enzyme, making a total of 5.4% of the protein (De Kok and Rawitch, 1969; Solis et al., 1999; Ali et al., 2000). Studies with *Crotalus adamanteus* snake venom showed the presence of glycosylation sites on the surface of LAAO (Wellner and Meister, 1960). Furthermore, studies with *Crotalus atrox* snake venom in eukaryotic cells have shown that blocking glycosylation inhibits the residual activity of the enzyme (Torrii et al., 2000). Ande et al. (2006) showed that glycans motifs of LAAO from *Calloselasma rhodostoma* interact with structures of cell surface and induce apoptotic cell death as a consequence of hydrogen peroxide production.

Literature shows that the glycan motifs in the molecule of LAAO related to the interaction of the enzyme with the host cell can increase the concentration of hydrogen peroxide and thus act as innate immune defense (Hughes, 2010). In fact, studies based on fluorescent staining suggested that LAAO anchor on cell surface leading to generation of high concentrations of hydrogen peroxide (Suhr and Kim, 1996, 1999).

In order to verify a possible intrinsic interference of Cr-LAAO in hydrogen peroxide production by neutrophils experiments were conducted with Cr-LAAO in an inactive form and with phenol red solution which did not contain amino acids. The results showed that inactiveCr-LAAO was unable to stimulate neutrophils to produce hydrogen peroxide. Thus, it is possible that the native structure of the enzyme is required for interaction with the cell. Studies have shown that the carbohydrates and glycoproteins portion confer important specific and biological roles as immunogenicity (Dowing et al., 2007), protection against proteolytic attack (Porto et al., 2007) and maintaining the conformation the protein in a biologically active form (Delorme et al., 1992; Wyss et al., 1995).

The ability of neutrophils to respond rapidly at the infection site is essential to host defense. Thus, it is reasonable to believe that the ability of the neutrophil to reside in a state intermediate to circulating quiescence and complete activation is optimal for plasticity. To that end, neutrophil priming is a reversible process that can enhance cell functions and limit the potential for indiscriminate host tissue damage. The original descriptions of neutrophil priming indicated that a primary agonist, typically at substimulatory concentration, enhances superoxide production triggered by a second stimulus. Neutrophils can be primed by numerous host factors and processes, including growth-factors, chemotactic factors, leukotrienes, ROS, adherence, cellular contact, cytokines and chemokines (Sheppard et al., 2005).

Thereby, among the various cytokines that can prime neutrophils are the cytokine TNF-α. This important mediator serves, among other actions, to guide the circulating

cells migrate to inflammatory tissue, by stimulating the expression of adhesion molecules and production of other cytokines such as IL-1 (Ebnet and Vestweber, 1999). This study thus evaluated the presence of TNF-α in the culture supernatant of isolated human neutrophils incubated with Cr-LAAO. Data showed that the toxin induced the production of TNF-α, suggesting that LAAO has the ability to stimulate neutrophils.

Moreover TNF-α participates in many other inflammatory events such as degranulation, production of reactive oxygen intermediates and chemotaxis of leukocytes (Thommesen et al., 1998). Considering the data obtained demonstrating the Cr-LAAO stimulated production of superoxide anion and hydrogen peroxide by neutrophils, it can be suggest that Cr-LAAO exert an indirect effect on TNF-α production by neutrophils favoring ROS production. In addition, TNF-α is considered a key regulator of the cytokines production (Maini et al., 1995) to induce production of IL-1 and IL-6. This cytokine also increases the release of lipid mediators such asprostaglandins and platelet-activating factor (Vassalli, 1992; Patial and Parameswaran, 2010).

Since the literature shows that TNF-α is a cytokine that it is related to edema formation (Thommesen et al., 1998) and LAAO induces edema *in vivo* (Ali et al., 2000; Izidoro et al., 2006; Stábeli et al., 2004; Tan and Choy, 1994) is possible to suggest that TNF-α found in this study may contribute to edematogenic effect induced by Cr-LAAO.

Moreover, the LAAO biological effect can also be credited to the hydrogen peroxide produced during the enzymatic reaction (Li et al., 1994; Suhr and Kim, 1996; Tan and Choy, 1994; Torrii et al., 1997). In addition to TNF, another important inflammatory mediator released after neutrophils activation is the chemokine IL-8 (Gainet et al., 1998). It is considered a potent neutrophil chemoattractant and activator and it is involved in inflammatory diseases such as psoriasis, rheumatoid arthritis, and lung diseases (Yoshimura et al., 1987; Baggiolini et al., 1994). It is expressed in response to various stimuli such as cytokines eg, IL-1β and TNF-α. IL-8 is also able to up-regulate the expression of integrins leading to diapedesis and then stimulate the release of superoxide anion (Gesser et al., 1995; Gainet et al., 1998).

To complement the studies of the effect of Cr-LAAO on neutrophil function the IL-8 production was assessed. Results showed that the Cr-LAAO induce the release of this chemokine. There is no data in the literature so far showing the effect of Cr-LAAO on the production of IL-8 and TNF-α on isolated human neutrophil which is the first description. Since the literature shows that TNF-α induce the expression of IL-8 (Deforge et al., 1992) and LAAO induces TNF-α is possible to suggest that TNF-α found in this study may contribute to signaling to induce the IL-8 expression induced by Cr-LAAO.

Neutrophils are phagocytic cells that can incorporate microorganisms and kill them via antimicrobial proteins, proteolytic enzymes, and ROS (Clifford and Repine, 1982; Borregaard and Cowland, 1997). In addition, an alternative extracellular killing mechanism has been described when the phagocytic capacity is exhausted (Brinkmann et al., 2004; Urban et al., 2006). Activated neutrophils form extracellular fibers, called neutrophil extracellular traps (NETs), composed of loosened chromatin and granule proteins such as neutrophil elastase, cathepsin G and lactotransferrin (Urban et al., 2009), molecules that efficiently kill microorganisms and contribute to an antimicrobial environment (Urban et al., 2006), that are used to immobilize the pathogens and, thereby, prevent microbial spreading (Brinkmann et al., 2004; Urban et al., 2006).

NET formation requires the production of ROS. The requirement for ROS in NET formation was shown pharmacologically and, more relevantly, by testing the neutrophils of patients with immune deficiencies. Interestingly, when neutrophils of CGD patients are treated with H_2O_2, the cells produce NETs, showing that the pathway can be rescued downstream of phagocyte NADPH oxidase – phox (Fuchs et al., 2007). Moreover, NETs in granulocytes can be induced in response to various gram-positive and -negative bacteria as well as fungi and parasites but also by sterile mediators as IL-8, TNF-α and phorbol myristate acetate (PMA) (von Köckritz-Blickwede and Nizet, 2009).

In order tom understand the effect of Cr-LAAO on neutrophil function the NETs liberation was assessed. Results showed that the Cr-LAAO induce the liberation of NETs. There is no data in the literature so far showing the effect of Cr-LAAO on the NETs liberation from isolated human neutrophil which is the first description. Since Cr-LAAO induce the both TNF-α and IL-8 release as well as ROS production and the literature shows that both cytokines and ROS induce NETs liberation is possible to suggest that TNF-α and IL-8 and ROS found in this study may contribute to NETs liberation induced by Cr-LAAO.

Taken together the data obtained showed that Cr-LAAO does not affect the viability of human neutrophils. Cr-LAAO stimulates cells to produce ROS such as superoxide anion and hydrogen peroxide. Cr-LAAO, in its native form, is also able to stimulate the production of hydrogen peroxide in neutrophils, suggesting that its primary structure is essential for stimulation the cell. Moreover, Cr- LAAO induces the release of proinflammatory mediators IL-8 and TNF-α and NETs isolation. It is noteworthy that this is the first description of the stimulatory effect of LAAO on neutrophils function. Finally, since LAAO from *Calloselasmarhodostoma* venom comprises 30% of crude venom (Ponnudurai et al., 1994) is possible that this toxin plays an important role in proinflammatory activity that characterizing this envenomation.

Authorship

J.P.Z. and A.S.P. designed the study; A.S.P., S.S.S., C.V.X., N.M.N. and F.L.S performed the experiments; W.L.P. and O.B.C. supervised the flow cytometer studies; A.M.K., S.D.S, L.A.C, R.G.S and A.M.S. performed and supervised the biochemical procedures; J.P.Z., A.S.P. A.M.S collected and analyzed the data; J.P.Z and R.G.S provided reagents; J.P.Z. and A.M.S. wrote the manuscript. All of the authors discussed the results and implications and commented on the manuscript at all stages.

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Conflict of interest

There is no conflict of interest statement.

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