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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 × *g* for 10 min at room temperature and the clear supernatant applied on a 70 cm × 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 × 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