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Lipoxygenase-derived eicosanoids are involved in the inhibitory effect of *Crotalus durissus terrificus* venom or crotoxin on rat macrophage phagocytosis

S.C. Sampaio [a], T.C. Alba-Loureiro [b], P. Brigatte [a], R.G. Landgraf [c], E.C. dos Santos [d], R. Curi [b], Y. Cury [a,*]

[a] *Laboratory of Pathophysiology, Butantan Institute, Avenue Vital Brazil, 1500, 05503-900 São Paulo, Brazil*
[b] *Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Avenue Prof. Lineu Prestes, 1524, 05508-900 São Paulo, Brazil*
[c] *Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Avenue Prof. Lineu Prestes, 2415, 05508-900 São Paulo, Brazil*
[d] *Laboratory of Immunopathology, Butantan Institute, Avenue Vital Brazil, 1500, 05503-900 São Paulo, Brazil*

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Abstract

Crotalus durissus terrificus snake venom and its major toxin, crotoxin or type II PLA_2 subunit of this toxin, induce an inhibitory effect on spreading and phagocytosis in 2 h incubated macrophages. The involvement of arachidonate-derived mediators on the inhibitory action of the venom or toxins on rat peritoneal macrophage phagocytosis was presently investigated. Peritoneal cells harvested from naive rats and incubated with the venom or toxins or harvested from the peritoneal cavity of rats pre-treated with the toxins were used. Zileuton, a 5-lipoxygenase inhibitor but not indomethacin, a cyclooxygenase inhibitor, given in vivo and in vitro abolished the inhibitory effect of venom or toxins on phagocytosis. Resident peritoneal macrophages incubated with the venom or toxins showed increased levels of prostaglandin E_2 and lipoxin A_4, with no change in leukotriene B_4. These results suggest that lipoxygenase-derived eicosanoids are involved in the inhibitory effect of *C.d. terrificus* venom, crotoxin or PLA_2 on macrophage phagocytosis.

Keywords: Lipoxygenase-derived eicosanoids; Lipoxin; Macrophage; Phagocytosis; *Crotalus durissus terrificus* venom; Phospholipase A2

1. Introduction

Venom of the South American rattlesnake *Crotalus durissus terrificus* (CdtV) modulates macrophage function (Sousa-e-Silva et al., 1996; Sampaio et al., 2001). This venom stimulates the production of hydrogen peroxide and nitric oxide, antimicrobial activity and glucose and glutamine metabolism of these cells (Sampaio et al., 2001). On the other hand, the venom presents an inhibitory effect on the spreading and phagocytosis activities of 2 h incubated macrophages (Sampaio et al., 2001). This inhibitory effect of the crotalid venom is mediated by crotoxin (CTX), the main neurotoxic component of the venom (Sampaio et al., 2003). The crotoxin molecule is composed of an acidic non-toxic and non-enzymatic polypeptide named crotapotin and of a weakly toxic phospholipase A_2 (PLA_2) (Slotta and Frankel-Conrat, 1938; Bon et al., 1988). More recently, Sampaio et al. (2005) showed that the PLA_2 subunit, but not crotapotin, is

* Corresponding author. Tel.: +55 11 3726 7222; fax: +55 11 3726 1505.
E-mail address: yarac@attglobal.net (Y. Cury).

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responsible for the inhibitory action of crotoxin on macrophage function.

Type II secreted phospholipase A_2, which hydrolyses the sn-2 ester bound of membrane phospholipids, release arachidonate. Free arachidonic acid can then be converted into eicosanoids, such as prostaglandins and leukotrienes (Needleman et al., 1986; Smith, 1992). Arachidonate-derived mediators can be generated by the activation of cyclooxygenase (COX) and lipoxygenase in macrophages (Naraba et al., 1998; Takahashi et al., 2005). Macrophages/monocytes are able to release eicosanoids in response to a proinflammatory stimulus (Rouzer et al., 1980; Scott et al., 1980; Lee et al., 1992; Naraba et al., 1998; Jiang et al., 2003).

Prostanoids and leukotrienes have a potent proinflammatory activity (Ford-Hutchinson, 1985). However, increasing evidence indicates that arachidonate-derived mediators also exert antiinflammatory function, being involved in inflammatory resolution (Levy et al., 2001; Gilroy et al., 2003). A down regulatory role of arachidonate-derived mediators on macrophage function was confirmed by the observation that the level of macrophage activation is inversely correlated with the synthesis of prostaglandins, leukotrienes and lipoxins (Hsueh et al., 1982; Scott et al., 1982; Tripp et al., 1985; Calorini et al., 2000). Prostaglandins, including prostaglandin E_2 (PGE_2), inhibit macrophage phagocytosis (Oropeza-Rendon et al., 1980; Kozlov et al., 1990; Davidson et al., 1998) and, by increasing intracellular levels of cAMP, can specifically down regulate macrophage ingestion of apoptotic cells (Rossi et al., 1998). COX-2-derived cyclopentenone prostaglandins are also able to interfere with macrophage function (Azuma et al., 2001), causing suppression of inducible nitric oxide synthase activity and cytokine production (review in Gilroy et al., 2004a; Paul-Clark et al., 2004). Certain lipoxygenase-derived eicosanoids, such as lipoxin A_4 (LXA_4) and aspirin-triggered LXA_4 (endogenous 15-epimer of lipoxin A_4) exhibit antiinflammatory properties, interfering with leukocyte function (Serhan et al., 1996; Diamond et al., 1999; Munger et al., 1999; Fiorucci et al., 2003). LXA_4 has potent inhibitory effects on several inflammatory events, such as leukocyte responses to cytokines (e.g. TNF) (Pouliot and Serhan, 1999) or to microbial stimulation (Gewirtz et al., 1998), neutrophil and eosinophil migration (Clish et al., 1999), and cell surface expression of adhesion molecules (Scalia et al., 1997). LXA_4 also inhibits the modulation of proinflammatory cytokine and chemokine production by macrophages in responses to lymphokine activation (Aliberti et al., 2002).

The evidence that the PLA_2 subunit of crotoxin is involved in the inhibitory effect of crotalid venom on macrophage function led us to carry out the present study. The contribution of arachidonate-derived mediators for the inhibitory effect of the crotalid venom, crotoxin or the phospholipase A_2 subunit on macrophage phagocytosis was investigated in in vivo and in vitro experiments. The involvement of eicosanoids was examined by treatment with indomethacin, an inhibitor of cyclooxygenase, and zileuton, an inhibitor of 5-lipoxygenase. The production of prostaglandin E_2, leukotriene B_4 (LTB_4) and lipoxin A_4 by macrophages incubated in the presence of *C.d. terrificus* venom, crotoxin and PLA_2 was also determined.

2. Material and methods

2.1. Animals

Male Wistar rats, weighing 160–180 g, were used. All procedures were in accordance with the guidelines for animal experimentation, and the ethical committee for the use of animals of the Butantan Institute approved the practices (CEUAIB, protocol number 020/2000).

2.2. Venom

Lyophilised venom of *C.d. terrificus* was obtained from the Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, and stored at −20 °C. Venom was dissolved in sterile saline (0.85% w/v NaCl solution) at the moment of use.

2.3. Crotoxin and subunits

Crotoxin was purified from *C.d. terrificus* venom by anion-exchange chromatography as previous described by Faure et al. (1994) using a Mono-Q HR 5/5 column in a FPLC system (Pharmacia, Uppsala, Sweden). Fractions (1 ml/min) were eluted in a linear gradient of NaCl (0–1 M in 50 mM Tris–HCl; pH 7.0). Three pools (I–III) were obtained during the crotoxin purification process, being pool II the correspondent pure crotoxin fraction. The crotoxin subunits (crotapotin and PLA_2) were obtained using a modification of the procedure described by Faure and Bon (1988); Faure et al. (1991). First, crotoxin was dissolved in 2.0 ml of 50 mM Tris–HCl (pH 7.5), in the presence of 6 M urea, and filtered in a Millipore membrane (80 μm). Both crotapotin and PLA_2 were purified by preparative FPLC on a Mono-S HR 5/5 column. Fractions (1 ml/min) were eluted in a linear gradient of NaCl (0–0.5 M in 50 mM Tris–HCl; pH 7.5). Before pooling, the fractions containing CTX or subunits were checked for homogeneity by non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12.5%). PLA_2 activity was determined by a colorimetric assay using a synthetic chromogeneic substrate. One-hundred microliters of phosphate buffer saline (PBS) containing 12.5 or 25 μg of crotoxin or 1.5, 3, 6, or 12.5 μg of PLA_2 were added to 1.0 ml of reaction medium (10 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl) and incubated for 20 min at 37 °C, in the presence of 100 μl of chromogeneic substrate 4-nitro-3-(octanoyloxy) benzoic acid (Sigma Chem. Co, USA), 3 mM, in 5 mM acetonitrile.

The reaction was stopped by placing the tubes on ice with addition of 100 μl Triton X-100 (2.5%). The tubes were kept at room temperature for 5–10 min and absorbance determined at 425 nm. All assays were run in duplicate.

2.4. Peritoneal cell preparation

Animals were anaesthetised with ether and killed through exsanguination by sectioning the cervical vessels. The peritoneal cavity was washed with 10 ml cold phosphate-buffered saline (PBS), pH 7.4. After a gentle massage of the abdominal wall, the peritoneal fluid, containing resident macrophages, was collected. Total peritoneal cells were determined in a Neubauer's chamber and the differential counts were performed in smears stained with a panchromatic dye (Rosenfeld, 1947). For all measurements, samples of individual animals were used. The assays were always performed in duplicates.

2.5. Phagocytic activity of peritoneal macrophages

The phagocytic activity was determined as described in our previous studies (Sampaio et al., 2003, 2005). Briefly, 100 μl of cell suspension in PBS (approximately 2×10^5 of total cells) were placed onto glass coverslip and left to adhere for 20 min at room temperature. The coverslips were rinsed with PBS and incubated in RPMI 1640 medium, at 37 °C, for 2 h. The coverslips containing the adherent and spreading macrophages were then incubated with 1 ml of RPMI 1640 medium containing opsonized sheep erythrocytes, opsonized zymosan or non-opsonized *Candida albicans*, for 40 min, at 37 °C, in an atmosphere containing 5% CO_2.

2.6. Treatments

The inhibitory effect of crotalid venom, crotoxin or phospholipase A_2 on the phagocytic activity of macrophages was assessed by in vivo and in vitro experiments. For in vivo study, the venom (30 μg in 300 μl per rat) or saline, in the same volume (control), was s.c. injected 2 h before collecting peritoneal cells. The dosage of venom was the same previously used and did not cause clinical signs of crotalid envenomation (Sampaio et al., 2001, 2003, 2005). For the in vitro treatment, cover slips containing adhered macrophages (2.0×10^5) were incubated in 1 ml of RPMI 1640 medium added of phospholipase A_2 (2.85 nM), crotapotin (3.75 nM), crotoxin (3.3 nM), CdtV (0.5 μg/ml), or with no addition (control), at 37 °C, in an atmosphere of 5% CO_2. For the in vitro determination of production of prostaglandin E_2, leukotriene B_4 and lipoxin A_4, adhered macrophages (1×10^6) were incubated in 1 ml of RPMI 1640 medium added of phospholipase A_2, crotapotin, crotoxin, CdtV or with no addition (control). After 2 h, the cover slips containing spreading macrophages were washed with PBS and prepared for the phagocytosis assay. The concentration of venom or toxins was the same previously used and did not show any sign of cell toxicity as assessed by Trypan blue exclusion and flow cytometry by the exclusion of propidium iodide (Sampaio et al., 2003; de Lima et al., 2005; Sampaio et al., 2005).

The involvement of eicosanoids in the inhibitory effect of the venom or toxins on macrophage function was evaluated by in vivo and in vitro experiments. For in vivo study, indomethacin (4 mg/kg, i.v.) (Jancar et al., 1989; Tavares-de-Lima et al., 1989; Chacur et al., 2003), a cyclooxygenase inhibitor, or zileuton (100 mg/kg, p.o.) (Horizoe et al., 1998), a lipoxygenase inhibitor, were administered 30 or 60 min before injection of the venom, respectively. For the in vitro study, the cover slips containing adhered macrophages were incubated with indomethacin (1 μM) (Coquette et al., 1992; Vicente et al., 2001) for 15 min or with zileuton (50 μM) (Horizoe et al., 1998; Canetti et al., 2003) for 10 min. Afterwards, the cover slips were washed with PBS and then incubated with the venom or toxins as described above.

2.7. Extraction and quantification of eicosanoids

The production of prostaglandin E_2 and leukotriene B_4 was determined in the in vitro experiments. The plates containing adhered macrophages were incubated with the venom or toxins as described above. After 2 h, the cell-free supernatant was acidified with 1 N HCl to pH 3.4–3.6 and passed slowly through an octadecylsilyl silica column (C_{18} Sep-Pak® column, Waters® Corporation, USA), pre-washed with 10 ml of absolute ethanol and 10 ml of water. After activation of the column with 10 ml of water and 10 ml of ethanol (35%), the eicosanoids were eluted from the column with 2 ml absolute ethanol and the samples dried under a stream of nitrogen. Concentrations of PGE_2 and LTB_4 were determined by ELISA (Cayman Chemical Co., Ann Arbor, MI, USA). The sensitivity of the PGE_2 assay was of 15 pg/ml and of the LTB_4 assay was of 4 pg/ml.

The production of lipoxin A_4 was determined from cell-free supernatant acidified with 1 N HCl to pH 3.4–3.6 and passed slowly through an octadecylsilyl silica column (C_{18} Sep-Pak® column, Waters® Corporation, USA), pre-washed with 10 ml of absolute ethanol and 10 ml of water. After activation of the column with 10 ml of water, 2 ml of absolute ethanol and 2 ml of water, the eicosanoids were eluted from the column with 1 ml of water, 1 ml ether and 2 ml of methyl formate and the samples dried under a stream of nitrogen. LXA_4 concentration was determined by using ELISA kit (Neogen Corporation, USA). The sensitivity of the assay was of 20 pg/ml.

2.8. Drugs

Indomethacin was purchased from Sigma (USA) and Zileuton was from Abbott Laboratories (Zyflo®, USA).

2.9. Statistical analysis

Comparisons between groups were initially performed by analysis of variance (ANOVA). The alpha level (significance level related to the probability of rejecting a true hypothesis) was set for 0.05. Significant differences were then compared using Turkey's Honestly Significant Difference test with a significance coefficient of 0.05.

3. Results

3.1. Effect of indomethacin on venom- or toxins-induced inhibition of macrophage phagocytosis

The s.c. injection of the venom caused a marked reduction (45%) of macrophage phagocytosis of opsonized zymosan. This inhibitory effect was not modified by pre-treating the animals with indomethacin (Fig. 1(a)). In the in vitro experiments, the venom, crotoxin and phospholipase A_2 were also able to inhibit the phagocytosis of zymosan (34, 45 and 32%, respectively) by macrophages (Fig. 1(b)). This inhibitory effect was not altered by pre-incubation of the cells with indomethacin. This drug per se did not interfere with macrophage function (Fig. 1).

3.2. Effect of zileuton on venom- or toxins-induced inhibition of macrophage phagocytosis

Venom subcutaneously injected inhibited the phagocytosis of zymosan (26%), sheep erythrocytes (55%), and *C. albicans* (43%) by macrophages (Fig. 2(a), (b) or (c), respectively). The administration of zileuton abolished the inhibitory effect of CdtV on macrophage phagocytosis (Fig. 2). In in vitro experiments, pre-incubation of cells with zileuton also abolished the inhibitory effect of the venom, crotoxin and phospholipase A_2 on phagocytosis (Fig. 2(d)). Zileuton per se did not affect macrophage function (Fig. 2).

3.3. Induction of eicosanoid synthesis by macrophages incubated with venom, crotoxin or crotoxin subunits

Two hours after incubation of macrophages with venom or toxins, cell-free incubation medium presented elevated

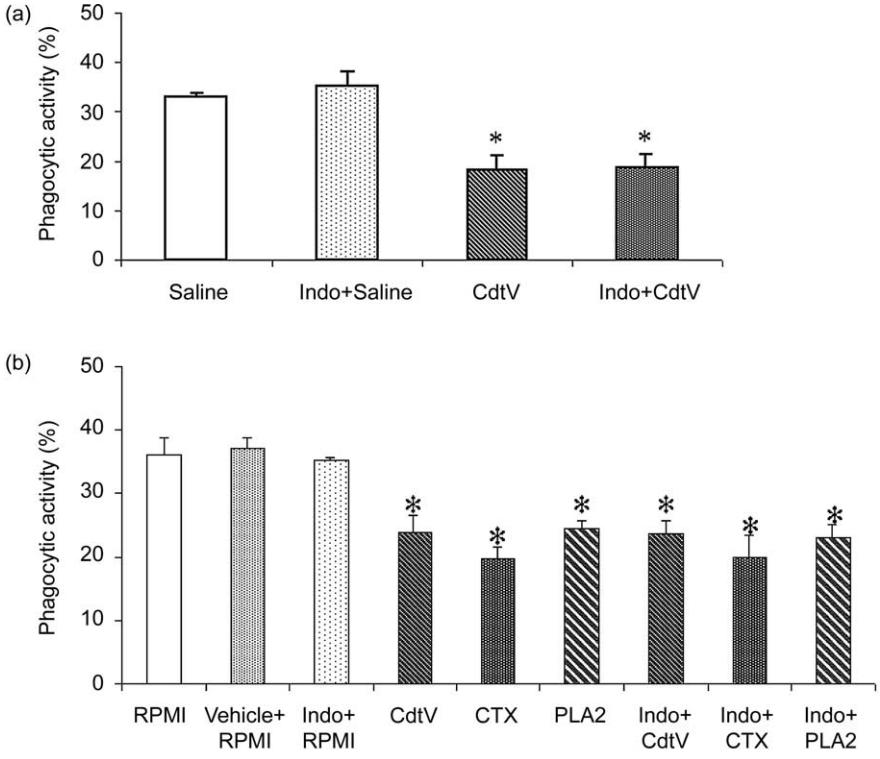

Fig. 1. Effect of indomethacin on venom- or toxins-induced inhibition of macrophage phagocytosis. The phagocytic activity was determined in (a) peritoneal macrophages collected 2 h after s.c. injection of *C.d. terrificus* venom (CdtV, 30 μg/rat) or saline (control group) and (b) peritoneal cells collected from naive rats and incubated in RPMI 1640 medium added with venom (CdtV, 0.5 μg/mL), crotoxin (CTX, 3.3 nM), crotalid phospholipase A_2 (PLA_2, 2.85 nM), or with no addition (control), at 37 °C for 2 h. In in vivo studies, indomethacin (Indo, 4 mg/kg, i.v.) was injected 30 min before venom administration. In in vitro studies, macrophages were incubated with indomethacin (1 μM) or vehicle, for 15 min, washed with PBS and then incubated with venom or toxins. Particles of zymosan opsonized with serum of untreated rats were used as phagocytic stimulus. The results are expressed as means ± SEM of five rats per group. $*P < 0.05$ significantly different from the control group.

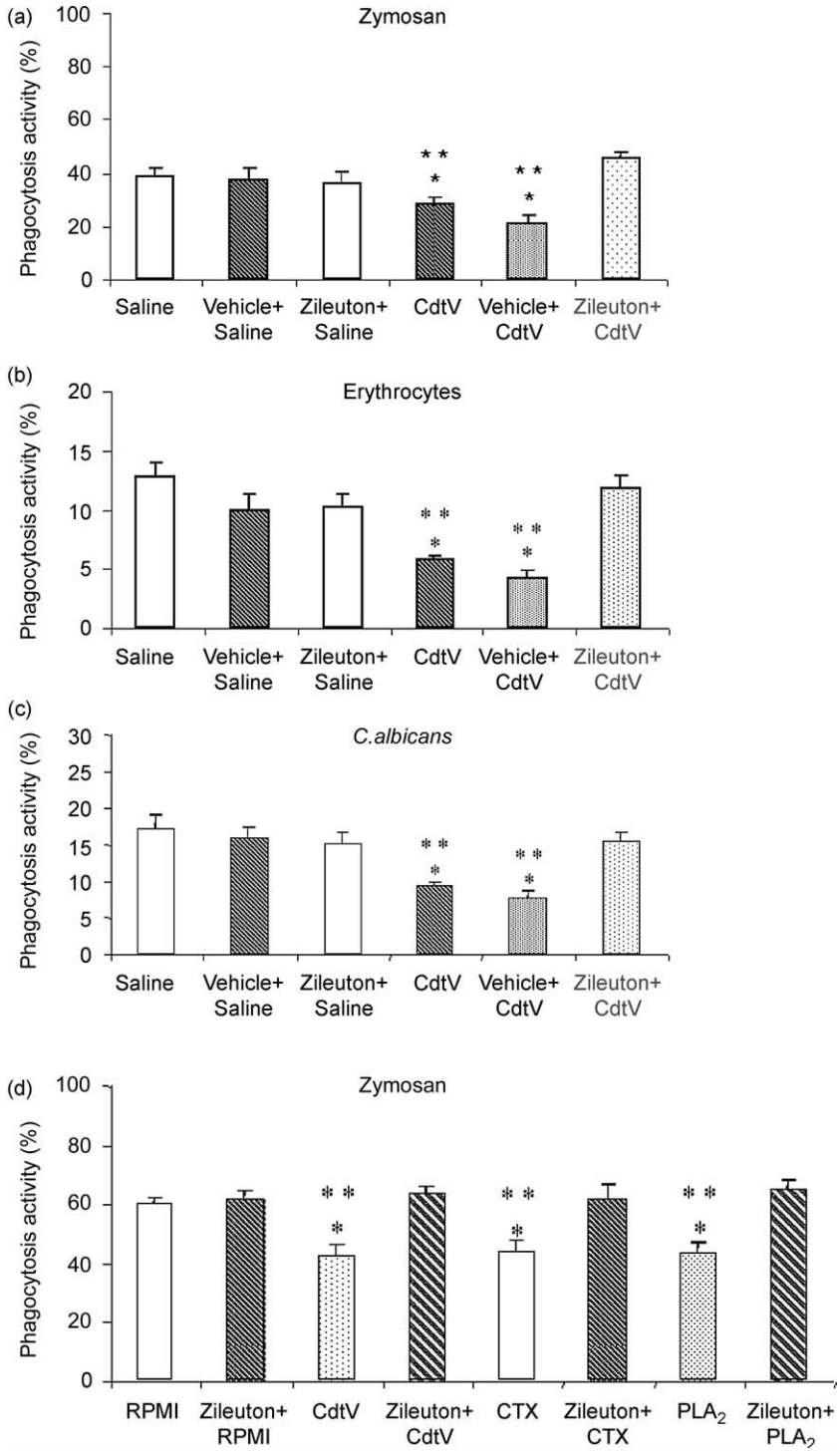

Fig. 2. Effect of zileuton on venom- or toxins-induced inhibition of macrophage phagocytosis (in vivo and in vitro studies). For in vivo studies (a,b,c), peritoneal macrophages were collected 2 h after s.c. injection of *C.d. terrificus* venom (CdtV, 30 μg/rat) or saline (control group). Zileuton (100 mg/kg, p.o.) or vehicle was injected 60 min before venom administration. For in vitro treatment (d), peritoneal cells were collected from naive rats and incubated in RPMI 1640 medium added with venom (CdtV, 0.5 μg/mL), crotoxin (CTX, 3.3 nM), crotalid phospholipase A_2 (PLA_2, 2.85 nM), or with no addition (control), at 37 °C, for 2 h. The cells were incubated in the presence of zileuton (50 μM), for 10 min. After this period, the cells were washed with PBS and then incubated with venom or toxins. Particles of zymosan opsonised with

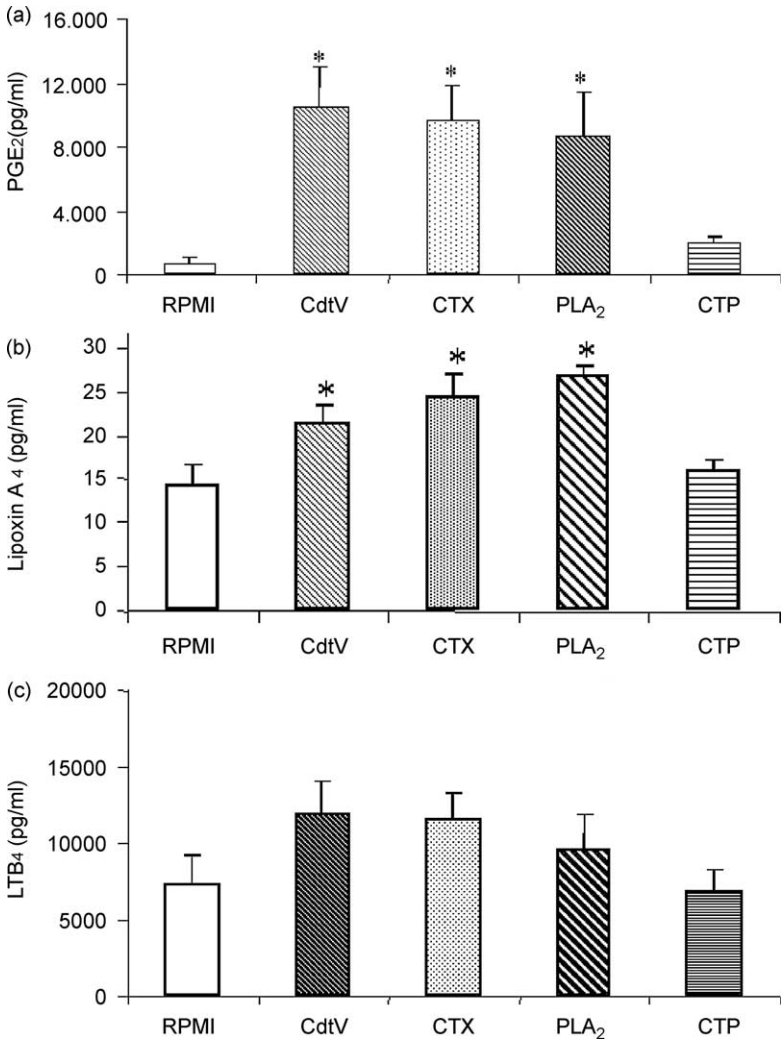

Fig. 3. Induction of eicosanoid synthesis by macrophages incubated with venom, crotoxin, phospholipase A_2 or crotapotin. Peritoneal cells were collected from naive rats and incubated in RPMI 1640 medium added with venom (CdtV, 0.5 μg/mL), crotoxin (CTX, 4.1 nM), crotapotin (CTP, 5 nM), crotalid phospholipase A_2 (PLA_2, 3.5 nM), or with no addition (control), at 37 °C, for 2 h. The levels of prostaglandin E_2 (PGF_2) (a), lipoxin A_4 (b) and leukotriene B_4 (LTB_4) (c) were determined by ELISA, in cell-free supernatant, as described in Material and methods. Values are expressed as the mean ± SEM of five animals per group. *$P < 0.05$, significantly different from the control group.

levels of PGE_2 (CdtV: 160%, CTX: 146%, PLA_2: 130%) and LXA_4 (CdtV: 50%, CTX: 69%, PLA_2: 87%) as compared to controls (medium with no addition of venom or toxins) (Fig. 3(a) and (b)). The venom or toxins did not significantly affect the production of LTB_4 as compared to controls (Fig. 3(c)). The effect of crotapotin, the other subunit of crotoxin, was also evaluated. This polypeptide however did not induce significant change of eicosanoid production (Fig. 3).

4. Discussion

The data presented herein corroborate our previous observation that *C.d. terrificus* snake venom, crotoxin and phospholipase A_2 have an inhibitory effect on phagocytosis by 2 h-incubation macrophage. This inhibitory action occurs regardless the type of receptor involved in the phagocytosis process—Fc- (opsonized sheep erythrocytes), C3b- (opsonized zymosan) or mannose- (non-opsonized particles of

serum of untreated rats (a,d), sheep erythrocytes opsonised with rabbit antiserum (b), or particles of *C. albicans* (c) were used as phagocytic stimulus. The results are expressed as means ± SEM of five animals per group. *$P < 0.05$, significantly different from the control group; **$P <$ 0.05, significantly different from Zileuton + CdtV; or Zileuton + CTX; or Zileuton + PLA_2 groups.

C. albicans) receptors. The results also suggest that the inhibitory action of the crotalid venom, crotoxin or PLA_2 on macrophage phagocytosis is mediated by eicosanoids derived from the activity of 5-lyipoxigenase. The involvement of lypoxigenase-derived mediators was observed in cells harvested from the peritoneal cavity of rats pre-treated with the venom or toxins and in macrophages obtained from naive animals and incubated with the venom or toxins. Based on these results, we can suggest that the venom, crotoxin and PLA_2 induce the synthesis of leukotrienes by macrophages. Therefore, the regulation of macrophage function by *C.d. terrificus* venom or toxins occurs by autacoid regulation of production of lipoxygenase-derived mediators.

The venom and toxins increased the production of both PGE_2 and LXA_4. In spite of the marked increase in PGE_2 levels, this prostaglandin seems not to be involved in the inhibitory action of the venom or toxins, since indomethacin did not have a significant effect on macrophage phagocytosis inhibition. On the other hand, despite the fact that zileuton was able to abolish the inhibitory action of the venom or toxins, an increase in LTB_4 levels was not detected.

Calorini et al. (2000) demonstrated that mouse peritoneal resident macrophages secrete lipoxins A_4 and LTB_4 in appreciable quantities. 12-lipoxygenase converts LTA_4 into lipoxins (Serhan and Sheppard, 1990; Romano and Serhan, 1992). Macrophages present high 12-lipoxygenase activity, indicating that, in these cells, a 5-LO/12-LO cooperation may exist for the synthesis of lipoxins (Calorini et al., 2000). The increase in LXA_4 levels induced by the crotalid venom and toxins supports the proposition that such enzymatic cooperation does occur and indicates that the venom and toxins lead to leukotriene-derived lipoxin A_4 synthesis.

The contribution of lipoxin A_4 to the inhibitory effect of the venom and toxins on macrophage function was not presently demonstrated. However, lipoxin A_4 enhances phagocytosis of apoptotic cells by macrophages with no effect on other types of phagocytosis (Knight et al., 1993) and does not cause inflammatory response (Godson et al., 2000; Levy et al., 2001) The stimulatory effect of LXA_4 on macrophage phagocytosis of apoptotic cells is modulated by an increase in actin rearrangement and in the activity of small GTPases of the Rho family (Maderna et al., 2002). Imunocytochemical and western blotting experiments showed that the crotalid venom, crotoxin or PLA_2 induce, during macrophage phagocytosis, actin reorganisation. However, there is an inhibition of tyrosine phosphorylation and in the activity of Rac and RhoA GTPases. Lipoxygenase-derived products mediate these effects of the venom and crotoxin on actin assembly, tyrosine phosphorylation, and GTPases activity (Sampaio, et al., unpublished observation).

In addition to the production of leukotrienes and lipoxins, the lipoxygenase pathway can activate specific intacellular pathways involved in the control of macrophage function. Huang et al. (1999) demonstrated a physiological role of 12/15-lipoxygenase in providing activating ligands for the nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) in macrophages. PPAR-γ is a negative regulator of monocyte function and macrophage activation (Ricote et al., 1998; Jiang et al., 1998). Therefore, a contribution of PPAR-γ to the inhibitory action of the venom or toxins on macrophage phagocytosis must be considered.

Sampaio et al. (2005) showed that the PLA_2 subunit is the active component of crotoxin for the inhibitory effect of this toxin on macrophage phagocytosis. *C.d. terrificus* venom-PLA_2 is classified as type II $sPLA_2$. Gilroy et al. (2004b) recently demonstrated the involvement of $sPLA_2$ in the synthesis of LXA_4. These authors showed that distinct phospholipase A_2 isoforms are involved in the checkpoint control of inflammation and during resolution there is an induction of $sPLA_2$ (types IIa and V), which mediates the release of PAF and lipoxin A_4.

In conclusion, the present results indicate that *C.d. terrificus* venom, crotoxin and PLA_2 subunit of this toxin induce the synthesis of eicosanoids by macrophages and suggest that lipoxygenase-derived eicosanoids are involved in the inhibitory effect of the venom or toxins on macrophage phagocytosis.

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