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

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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

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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.