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Signaling pathways involved in zymosan phagocytosis induced by two secreted phospholipases A_2 isolated from *Bothrops asper* snake venom in macrophages

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ABSTRACT

Phagocytosis, a process involved in host defense, requires coordination of a variety of signaling reactions. MT-II, a catalytically-inactive Lys49-PLA_2, and MT-III, an active Asp49-PLA_2 isolated from *Bothrops asper* snake venom, activate phagocytosis in macrophages. In this study the signal pathways mediating zymosan phagocytosis, focusing in lipidic second messengers, were investigated. Macrophages collected from male Swiss mouse peritoneum were obtained 96 h after i.p. injection of thioglycollate. Phagocytosis was evaluated with non-opsonized zymosan in the presence or absence of specific inhibitors. Data showed that both venom PLA_2s increased phagocytosis. Zileuton, Etoricoxib, $PACOCF_3$ (5-LO, COX-2 and $iPLA_2$ inhibitors, respectively), as well as WEB2170 (PAF receptor antagonist) significantly reduced phagocytosis induced by both venom PLA_2s. However, Indomethacin (COX-1/COX-2 inhibitor) and Montelukast (CysL receptor antagonist) did not affect the toxins-induced phagocytosis. Moreover, while PACOCF3 ($iPLA_2$ inhibitor), reduced the phagocytosis induced by MT-II and MT-III, $AACOCF_3$ ($cPLA_2$ inhibitor) significantly reduced the MT-II, but not MT-III-induced phagocytosis. These data suggest the effect of both $sPLA_2$s depends on $iPLA_2$ and that the effect of MT-II depends on activation of $cPLA_2$. COX-2 and 5-LO-derived metabolites as well as PAF are involved in the signaling events required for phagocytosis induced by both venom $sPLA_2$s.

1. Introduction

Phospholipases A_2 (PLA_2; EC 3.1.1.4) are interfacial enzymes that hydrolyze the *sn*-2 fatty acyl bond of phospholipids to release free fatty acids and lysophospholipids, such as arachidonic acid and lysophosphatidic acid, respectively. PLA_2s have been classified into I–XVI groups according to several criteria such as amino acid sequence similarity, cellular function and active splice variants [1–6]. Among them, group IIA PLA_2s are secreted enzymes (14–16 kDa) and include mammalian enzymes found in inflammatory exudates and those from viperid snake venoms. Group IIA secreted PLA_2s ($sPLA_2$s) from snake venoms can be further subdivided into two subgroups: (a) the Asp49 enzymes which have an aspartic acid residue at position 49 and high catalytic activity on artificial phospholipid substrates, and (b) the Lys49 enzyme homologues, which have a lysine residue at position 49 and are devoid of hydrolytic activity [7]. The presence of an aspartic acid residue at position 49, which plays a role in calcium binding, is critical for catalytic activity [7,8]. Despite their lack of catalytic activity, the Lys49 PLA_2 myotoxins exert several toxic actions, including myotoxicity and inflammation [9].

Group IV PLA_2 includes a cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), which is a ubiquitously distributed 85 kDa enzyme. In vitro, $cPLA_2$ requires a submicromolar Ca^{2+} concentration for effective hydrolysis of its substrate, arachidonic acid-containing glycerophospholipids. The N-terminal domain is responsible for the Ca^{2+}-dependent translocation of $cPLA_2$ from the cytosol to perinuclear and endoplasmic reticular membranes, where several eicosanoid-generating enzymes, such as the two cyclooxygenases (COXs) isozymes and 5 lipoxygenase (5-LO), are co-localized [6,10,11]. $cPLA_2$ has multiple phosphorylation sites, among which the mitogen-activated protein kinase-directed site (Ser^{505}) is the most crucial for in vivo activation of $cPLA_2$. These enzymatic features of $cPLA_2$ are consistent with its role in immediate eicosanoid biosynthesis occurring within minutes of stimulation, which is usually accompanied by rapid and transient cytoplasmic Ca^{2+} mobilization and mitogen-activated protein kinase activation [12]. In addition, several lines of evidence suggest that functionally active $cPLA_2$ is required for the $sPLA_2$-mediated arachidonic acid release and prostaglandin biosynthesis in agonist-primed cells [12,13].

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Another distinctive group of PLA_2s encompasses the Ca^{2+}-independent PLA_2 ($iPLA_2$). The group VI $iPLA_2$ is the most recently identified member of the PLA_2 family. Similarly to $sPLA_2$, this $iPLA_2$ exhibits no substrate specificity for arachidonic acid-containing phospholipids. This $iPLA_2$ also shares the size, intracellular localization and elements of catalytic mechanism with $cPLA_2$ [14].

These different groups of PLA_2 have been implicated in various physiological and pathological functions including lipid digestion, release of proinflammatory mediators, cell proliferation, ischemic injury, inflammatory disease, cancer, anti-bacterial defense [12], and phagocytosis [15]. This is a process by which macrophages recognize, engulf and destroy invading pathogens, and represents the first step in triggering host defense and inflammation but also for tissue homeostasis. Phagocytosis is initiated by engagement of receptors on the surface of the phagocytes after recognition of cognate ligands on the particle [16,17]. Receptors on the plasma membrane of macrophages can be divided into opsonic or nonopsonic receptors. Among the nonopsonic receptors, there are lectin-like recognition molecules such as CD169 and CD33, also related C-type lectins such as Dectin-2, Mincle or DNGR-1 scavenger receptors and Dectin-1, which is a receptor for fungal β glucan [17]. The signal transduction pathway mediating phagocytosis is the subject of intense investigation and is known to include protein tyrosine kinases, protein kinase C (PKC), rise in Ca^{2+}, actin polymerization and membrane movements. A rapidly expanding body of evidence suggests that PLA_2s play an integral role in phagocytosis by generating essential second messengers. We have demonstrated that myotoxin III (MT-III), an Asp-49 catalytically-active PLA_2, and myotoxin II (MT-II), a catalytically-inactive Lys-49 PLA_2-like protein, isolated from *Bothrops asper* snake venom, are able to stimulate macrophages leading to production of H_2O_2 and increased phagocytosis [18]. However, the signaling pathways regulating phagocytosis induced by these toxins are unknown. The present study was therefore designed to evaluate the signaling pathways involved in phagocytosis of non-opsonized zymosan particles via β glucan receptors stimulated by these venom $sPLA_2$s on isolated macrophages, focusing our attention on the involvement of lipid mediators, mainly those derived from COX-1, COX-2, 5-LO, LTA_4 hydrolase, $cPLA_2$ and $iPLA_2$ pathways and PAF.

2. Materials and methods

2.1. Chemicals and reagents

AACOCF3, PACOCF3 were obtained from Calbiochem (USA). Etoricoxib (Arcoxia®), Rofecoxib (Vioxx®) and Montelukast (Singulair®) were from Merck Sharp Dohme, Brazil and Zileuton (Zyflo®) was from Abbott Laboratories, USA. Bestatin and WEB2170 were kindly provided by Dr. Paulo Flavio Silveira (Butantan Institute, Sao Paulo, Brazil) and Dr. Sonia Jancar (Immunology Department, Biomedical Sciences Institute, University of Sao Paulo, Sao Paulo, Brazil), respectively. Hema3 stain was obtained from Biochemical Sciences Inc. (USA). PGE_2 EIA kit monoclonal was purchased from Cayman Chemical, USA, Zymosan, RPMI1640, Indomethacin, L glutamine, penicillin G and streptomycin from Sigma, USA. All salts used were obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male Swiss mice (18–20 g) were used. These animals were housed in temperature-controlled rooms and received water and food ad libitum until used. These studies were approved by the Experimental Animals Committee of Butantan Institute (Reference n° 064/2002) in accordance with the procedures laid down by the Universities Federation for Animal Welfare.

2.3. Phospholipases A_2

The myotoxic Lys-49 PLA_2 (MT-II) and Asp-49 PLA_2 (MT-III) from *Bothrops asper* snake venom were purified according to Lomonte and Gutiérrez [19] and Kaiser et al. [20], respectively. Homogeneity was demonstrated by SDS-polyacrylamide gel electrophoresis run under reducing conditions.

2.4. Harvesting of macrophages

Thioglycollate–elicited macrophages were harvested 4 days after intraperitoneal (i.p.) injection of 1 mL of 3% thioglycollate, according to Zuliani et al. [18]. Animals were killed under halothane and exsanguinated. Then, peritoneal lavage was performed, after a gentle massage of the abdominal wall, using 3 mL of cold phosphate-buffered saline (PBS), which contained 10 U/mL heparin. The peritoneal fluid, containing thioglycollate-elicited macrophages, was collected. Total peritoneal cell counts were determined in a Neubauer's chamber, and the differential counts were performed in smears stained with Hema3. The cell population consisted of more than 95% macrophages, as determined by morphological and phagocytic criteria.

2.5. Phagocytic activity of peritoneal macrophages

Thioglycollate-elicited macrophages were plated on 13 mm diameter glass coverslips (Glass Tecnica, Brazil) in 24-well plates at a density of 2×10^5 cells per coverslip and allowed attaching for 30 min at 37 °C under a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with cold PBS. Cell monolayers were incubated with RPMI-1640 supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin and 2 mM L glutamine (control) or with MT-II or MT-III (6.3 μg/mL) diluted in RPMI, at 37 °C and 5% CO_2 for 5 and 60 min at 37 °C and 5% CO_2. After extensive wash in cold PBS the monolayers were incubated for 40 min at 37 °C and 5% CO_2 with non-opsonized zymosan, prepared as described below, and unbound particles were removed by washing with cold PBS. Cells were fixed with 2.5% glutaraldehyde for 15 min at room temperature and the coverslips were mounted in microscope slides. The extent of phagocytosis was quantified by contrast phase microscopic observation. At least 200 macrophages were counted in each determination and those containing three or more internalized particles were considered positive for phagocytosis [18]. Results were presented as the percentage of cells positive for phagocytosis.

The zymosan particles, obtained from yeast cell walls, were suspended in PBS at a concentration of 3 mg/mL in PBS, pH 7.2. After that, the zymosan suspension was sonicated for 15 min and total zymosan particles were determined in a Neubauer's chamber. The ratio of zymosan particles per macrophage was 1:10.

2.6. Pharmacological treatments

The following inhibitors were used in these studies: 1 μM Indomethacin, 1 μM Etoricoxib, 1 μM Rofecoxib, 1 μM Zileuton, 10 μM Bestatin, 1 μM Montelukast, 50 μM WEB2170, 20 μM AACOCF3, 4 μM PACOCF3 and 1 μM SB202190. These compounds or their vehicles (controls) were added to macrophage cultures 15 min (for Indomethacin, Etoricoxib, Rofecoxib, Zileuton, Montelukast and AACOCF3), 30 min (for WEB2170, PACOCF3 and SB202190) and 60 min (for Bestatin) before stimulation of cells either with MT-II or MT-III. Then, the assay was carried out as described above.

2.7. Prostaglandin E_2 (PGE_2) evaluation

Concentrations of PGE_2 in the supernatant of macrophages cultures at 5 and 60 min after incubation with MT-II or MT-III (6.3 μg/mL) or medium culture, or after macrophages submitted to phagocytosis of non-opsonized zymosan particles for 40 min at 37 °C and 5% CO_2, were measured by a specific enzymatic immunoassay (EIA) previously described by Pradelles et al. [21]. In brief, 50 μL aliquots of each extracted sample were incubated with the eicosanoids conjugated with acetylcholinesterase and the specific rabbit antiserum in 96-well microtitration plates, coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbances of the samples were recorded at 412 nm in a microplate reader, and concentrations of the eicosanoids were estimated from standard curves.

2.8. Statistical analyses

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

3. Results

3.1. Venom $sPLA_2$s modulates phagocytosis of non-opsonized zymosan particles

We previously showed that MT-II stimulated macrophage phagocytosis mediated by complement, Fcg, mannose and β glucan receptors, and that MT-III stimulated only macrophage phagocytosis mediated by mannose and β glucan receptors [18]. Because both toxins stimulated phagocytosis via β glucan receptors we sought to evaluate the signal transduction pathways and second messengers required for internalization of the target in adherent macrophages treated with non-cytotoxic concentrations of MT-II or MT-III or with RPMI (control). As previously shown, MT-II and MT-III increased phagocytosis at concentrations of 1.5 up to 6.3 μg/mL. Because the concentration of 6.3 μg/mL of both $sPLA_2$s caused the most pronounced effect (Fig. 1A), we used this concentration and various time intervals. The increase of phagocytosis induced by MT-II and MT-III by thioglycollate-elicited macrophages is shown in Fig. 1B. Thioglycollate-elicited macrophages incubated with RPMI showed an average of phagocytosis of non-opsonized zymosan particles of 3.7% (5 min), 8.8% (15 min), 12.3% (30 min) and 15% (60 min). Incubation of macrophages with MT-II resulted in a marked increment of phagocytosis of non-opsonized zymosan particles, which was significantly higher than control. The average percentage increment, after 5 to 60 min of incubation with the toxin, was 143% (5 min), 163% (15 min), 139% (30 min) and 132% (60 min). Moreover, incubation of macrophages with MT-III resulted in a significant increment of non-opsonized zymosan particles phagocytosis, which was higher than in controls. In the case of MT-III, the average percentage increment of phagocytosis from 5 to 60 min of incubation was 150% (5 min), 184% (15 min), 153% (30 min) and 189% (60 min).

3.2. COX-2 pathway is involved in the phagocytosis of non-opsonized zymosan induced by MT-II and MT-III in macrophages

In order to assess the role of COXs enzymes in macrophage phagocytosis and considering that COX-2 is an inducible isoform during inflammatory processes [22], we sought to determine the specificity of COX-2 modulation for phagocytosis mediated by MT-II or MT-III. As shown in Fig. 2A and B, preincubation of macrophages with the specific COX-2 inhibitors Etoricoxib and Rofecoxib resulted in a reduction of the phagocytic index, suggesting a role of COX-2 metabolites in the phagocytosis process induced by both venom $sPLA_2$s. However, as demonstrated in Fig. 2C, preincubation of macrophages with Indomethacin, a COX-1/COX-2 enzyme inhibitor, did not affect phagocytosis induced by both toxins, suggesting that COX-1 isoform does not play a role in this event.

3.3. PGE_2 is produced upon macrophage treatment with venom $sPLA_2$

In order to evaluate the participation of PGE_2 in phagocytosis mediated by MT-II and MT-III, this lipid mediator was quantified after incubation with MT-II or MT-III or RPMI (control), for periods of 5 or 60 min in the presence or absence of zymosan particles. Fig. 3A shows that macrophages incubated with RPMI alone (control) showed a release of 774 ± 6.8 pg/mL and 3778 ± 191.3 pg/mL of PGE_2 after 5 and 60 min of incubation, respectively. This release was significantly increased after stimulation of cells with MT-II, with an increment of 176% and 186% after 5 and 60 min of incubation, respectively. When cells were stimulated with MT-III, there was a significant increase in PGE_2 release (318% at 5 min and 249% at 60 min), as compared to controls. There was no significant difference between the effects of the two myotoxins in each time period studied. As shown in Fig. 3B, when macrophages were pre-incubated for 5 min with RPMI and then stimulated

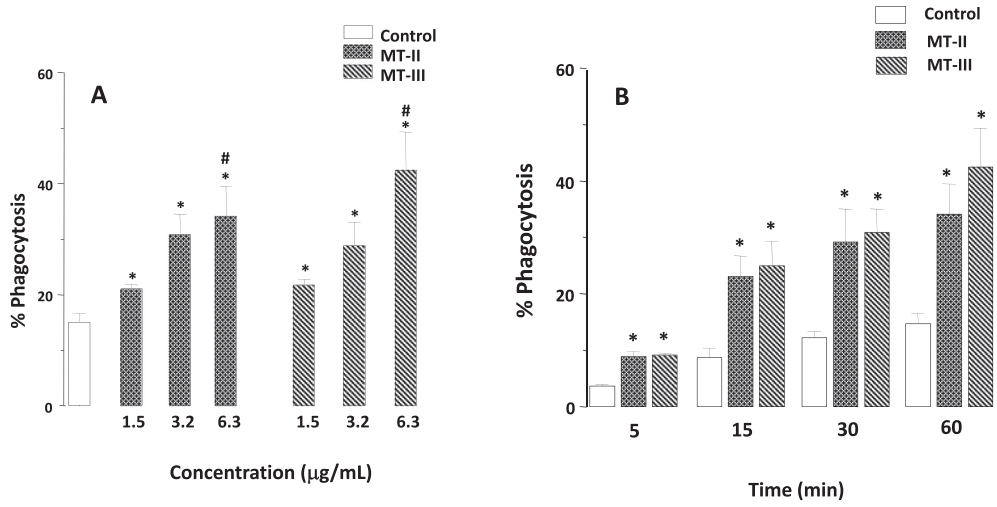

Fig. 1. Concentration response (A) and time course of MT-II and MT-III treatment (B) on the phagocytosis of non-opsonized zymosan particles. Macrophages were incubated with MT-II or MT-III (6.3 μg/mL) or RPMI (control) for 5, 15, 30 or 60 min before addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. *$p < 0.05$ compared with control (ANOVA).

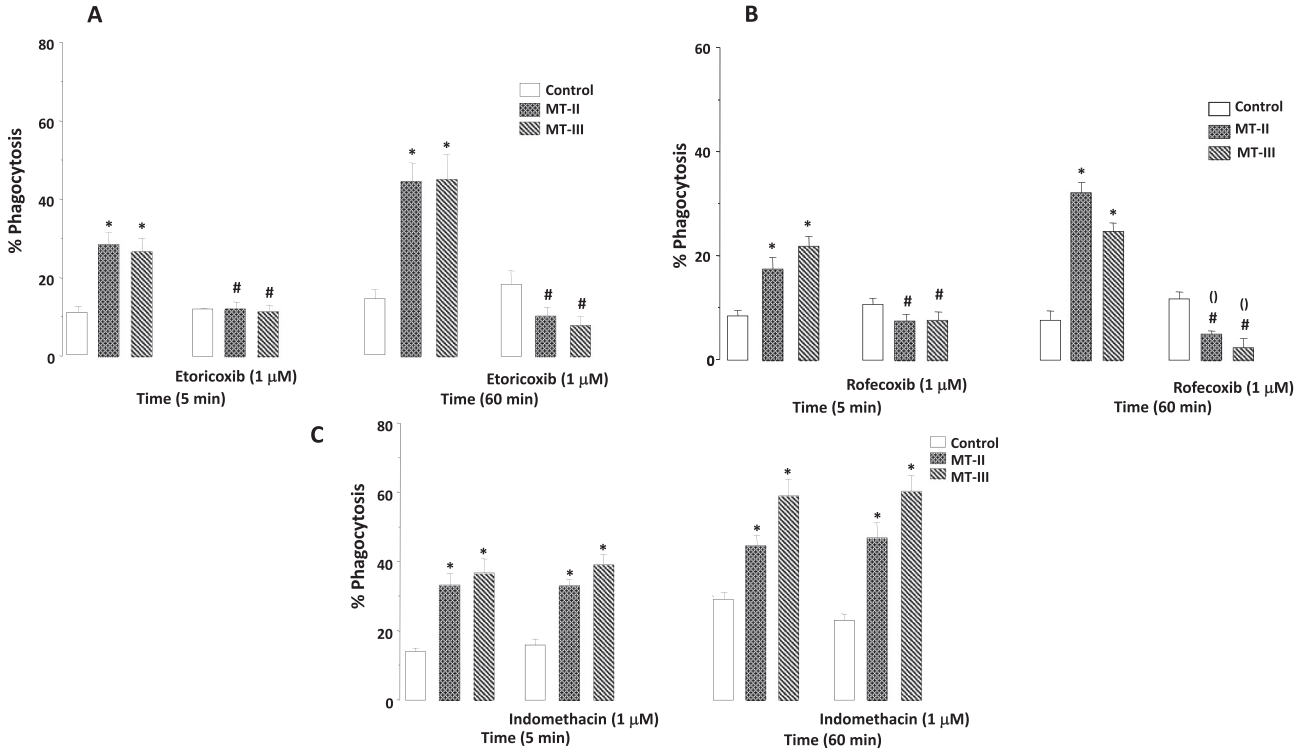

Fig. 2. Effect of a COX-1/COX-2 inhibition on phagocytosis of non-opsonized zymosan particles induced by MT-II and MT-III. Macrophages were pre-treated (15 min) with Indomethacin (1 μM) (A), or Etoricoxib (1 μM) (B) or Rofecoxib (1 μM) (C) or vehicle followed by addition of MT-II or MT-III (6.3 μg/mL) or RPMI (control) for 5 or 60 min before addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. *p < 0.05 compared with control (ANOVA).

for phagocytosis of non-opsonized zymosan particles (control) there was a release of 7711 ± 608 pg/mL of PGE_2. A significant increase of 180% was observed when the cells were pre-incubated with MT-II for 5 min before undergoing phagocytosis. When the cells were pre-incubated with MT-III, there was a significant increase of the PGE_2 release of 134% at 5 min, in relation to the control.

3.4. 5-LO pathway plays a role in phagocytosis of non-ospsonized zymosan induced by the venom sPLA$_2$s in macrophages

The participation of 5-LO enzyme on phagocytosis of IgG-opsonized microspheres was previously shown by Mancuso et al. [23]. The same pattern of response was observed here using the venom sPLA$_2$s, showing a significant reduction in phagocytosis of non-opsonized zymosan particles. Pre-incubation of macrophages with Zileuton, a 5-LO inhibitor (Fig. 4A), and Bestatin, a LTA_4 hydrolase inhibitor (Fig. 4B), significantly reduced the stimulatory effect of both MT-II and MT-III on phagocytosis. On the other hand, preincubation of macrophages with Montelukast, a cysteinyl-leukotriene receptor antagonist [23], did not modify the phagocytic index induced by both toxins, indicating that cysteinyl-leukotrienes are not involved in this process (Fig. 4C). Taken together, these data suggest that 5-LO and its major metabolite, LTB_4, are involved in the increase of non-opsonized phagocytosis induced by both venom sPLA$_2$s.

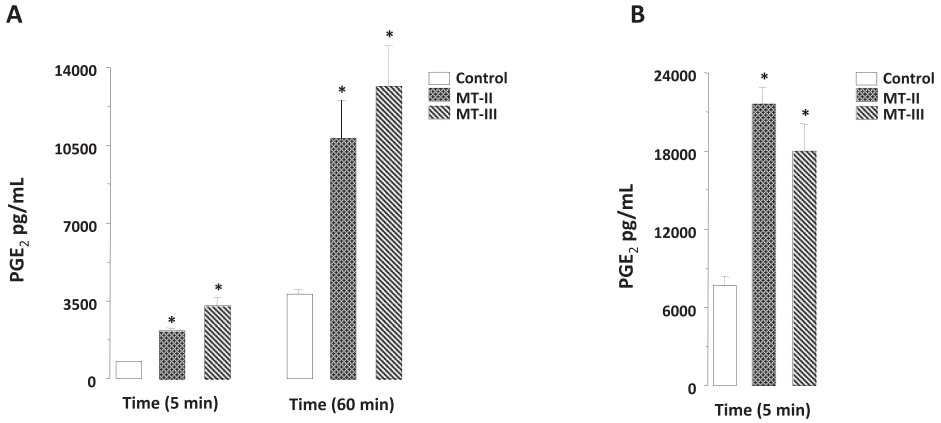

Fig. 3. PGE_2 production induced by MT-II or MT-III treatment in absence or after phagocytosis of non-opsonized zymosan particles. Macrophages were incubated with MT-II or MT-I II (6.3 μg/mL) or RPMI (control) before (A) or after phagocytosis of non-opsonized zymosan particles (B). Afterwards, the supernatant was collected and used for PGE_2 EIA quantification. Values are mean ± S.E.M. from 5 animals. *p < 0.05 compared with control (ANOVA).

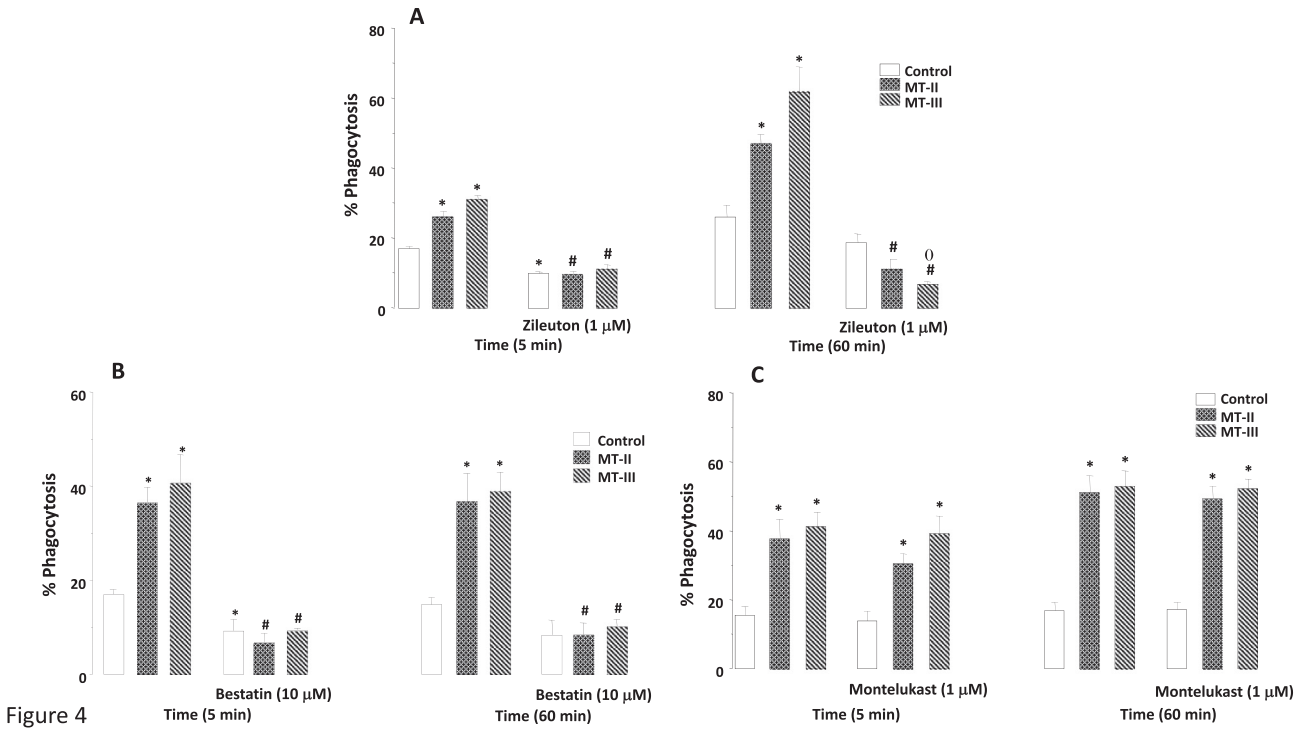

Figure 4

Fig. 4. Effect of 5-LO and LTA_4 hydrolase inhibitors or a cysteinyl-leukotriene receptor antagonist on phagocytosis of non-opsonized zymosan particles induced by MT-II and MT-III. Macrophages were treated with Zileuton (1 μM) for 15 min (A), Bestatin (10 μM) for 60 min (B) or Montelukast (1 μM) for 15 min (C), or vehicle for 15 or 60 min before incubation with MT-II or MT-III (6.3 μg/mL) or RPMI (control), for 5 or 60 min followed by addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with MT-II without Zileuton, $^{()}p < 0.05$ compared with Zileuton control (ANOVA).

3.5. PAF is involved in phagocytosis of non-ospsonized zymosan induced by MT-II and MT-III

We also evaluated the role of PAF in the signal transduction pathways in phagocytosis induced by MT-II and MT-III, by blocking the plasma membrane receptors for PAF. Preincubation of macrophages with WEB2170, a PAF receptor antagonist, for 30 min [24] before the addition of the toxins, reduced the phagocytic index, suggesting a role for PAF in macrophage phagocytosis induced by $sPLA_2$s (Fig. 5).

3.6. Intracellular PLA_2s contribute to phagocytosis of non-opsonized zymosan by macrophages induced by MT-III and MT-II

Because MT-II and MT-III are $sPLA_2$s and the literature shows a cross talk between different groups of PLA_2s in cellular processes, we further determine whether these $sPLA_2$s were able to recruit $cPLA_2$ and $iPLA_2$ for phagocytosis in our experimental model. Fig. 6A shows that an $iPLA_2$ inhibitor, PACOCF3, reduced the phagocytic index induced by MT-II and MT-III. As shown in Fig. 6B, preincubation of macrophages

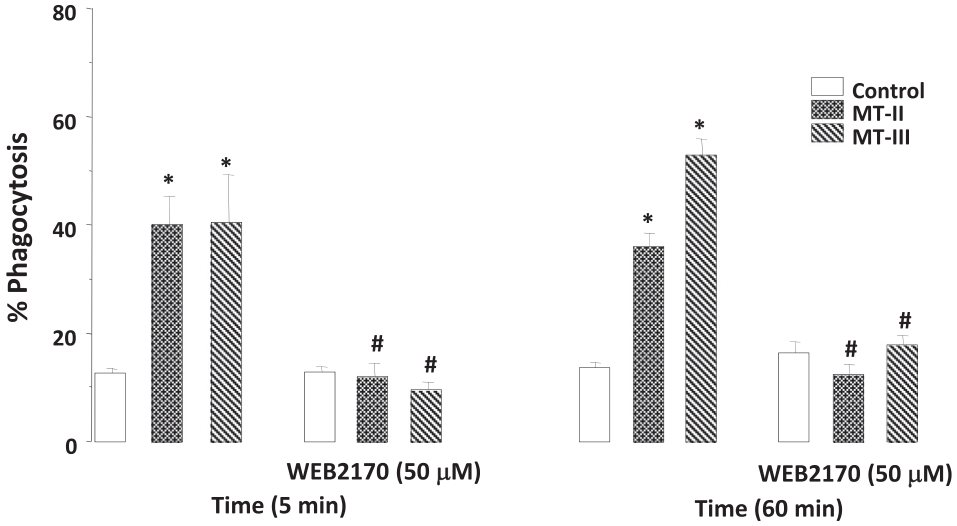

Fig. 5. Effect of a PAF receptor antagonist on phagocytosis of non-opsonized zymosan particles induced by MT-II and MT-III. Macrophages were treated with WEB2170 (50 μM) or vehicle for 30 min before incubation with MT-II or MT-III (6.3 μg/mL) or RPMI (control) followed by addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with MT-II without WEB2170 (ANOVA).

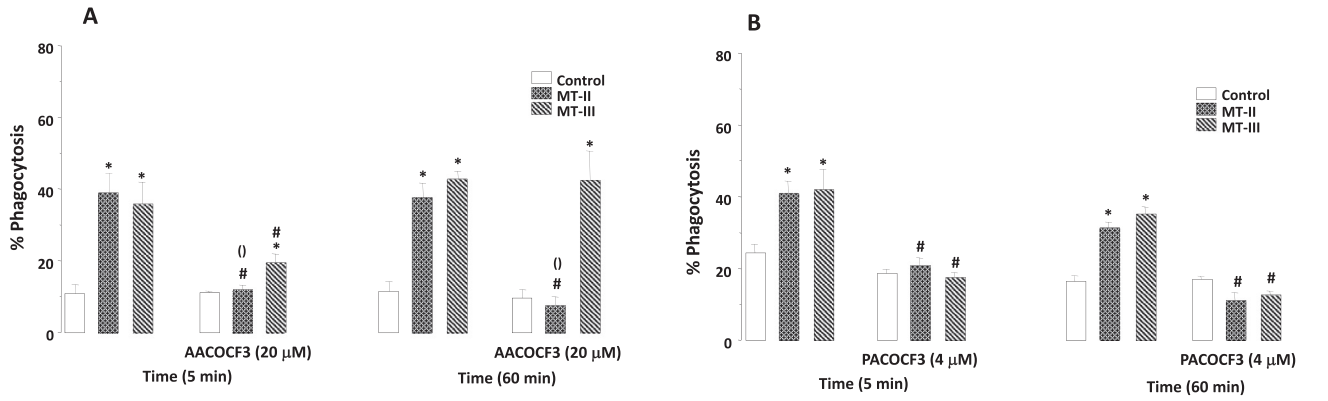

Fig. 6. Effect of $cPLA_2$ and $iPLA_2$ inhibitors on phagocytosis of non-opsonized zymosan particles induced by $sPLA_2$s. Macrophages were treated with AACOCF3 (20 μM) or PACOCF3 (4 μM) or vehicle for 15 and 30 min, respectively, before incubation with MT-II or MT-III (6.3 μg/mL) or RPMI (control), followed by addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. *$p < 0.05$ compared with control, #$p < 0.05$ compared with MT-II without AACOCF3 or PACOCF3, ()$p < 0.05$ compared with MT-II incubated with $AACOCF_3$ (ANOVA).

with $cPLA_2$ inhibitor, AACOCF3, reduced phagocytosis by about 70% when macrophages were stimulated with MT-II for 5 min and by about 80% for 60 min, but did not affect the stimulatory effect of MT-III. These data suggest that intracellular PLA_2s participate in signaling pathways involved in MT-II and MT-III-induced phagocytosis and that $cPLA_2$ amplifies MT-II-induced phagocytosis by macrophages.

3.7. p38MAPK signaling has a role in the phagocytosis of non-opsonized zymosan induced by MT-II and MT-III

To investigate the participation of p38MAPK on phagocytosis of non-opsonized zymosan stimulated by MT-II and MT-III, we preincubated macrophages with SB202190, a p38MAPK inhibitor. This preincubation significantly reduced the stimulatory effect of MT-II in phagocytosis at 60 min of incubation and significantly reduced the stimulatory effect of MT-III at 5 and 60 min of incubation (Fig. 7). These data suggest that p38MAPK participates in signaling pathways involved in phagocytosis by both myotoxins.

4. Discussion

The inflammatory $sPLA_2$s are recognized as important players of the phagocytosis [25], which is a crucial process in the innate immunity, both by facilitating the killing and removal of pathogens and by priming the adaptive immune response. Therefore, identifying the signaling pathways that regulate GIIA sPLA2-induced phagocytosis is important to the knowledge on the roles played by this family of enzymes in inflammation and the defense response of immune cells.

We have previously reported that two GIIA $sPLA_2$s isolated from *Bothrops asper* snake venom, MT-II and MT-III, induced phagocytosis by macrophages via complement, Fcγ, mannose and β glucan receptors [18]. In the present study we extended our exploration of the mechanisms by which both secreted snake venom PLA_2s increase non-opsonized zymosan phagocytosis via β glucan by macrophages. We found that MT-II, a catalytically-inactive $sPLA_2$ homologue, and MT-III, an active enzyme, induced a marked increment in macrophage phagocytosis of non-opsonized zymosan particles when pre-incubated for 5 min, reaching maximum levels with 60 min of pre-incubation. Since

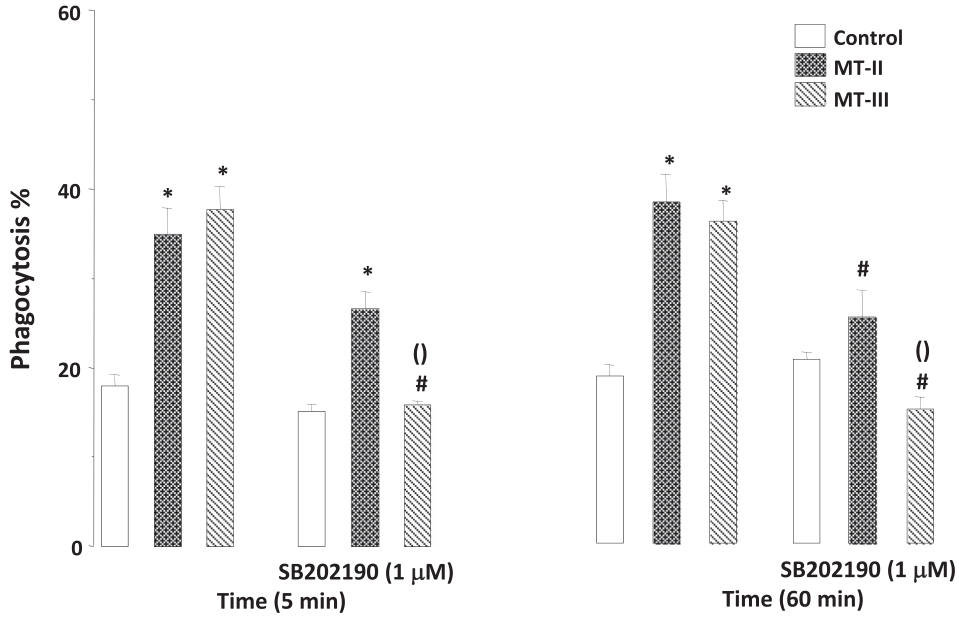

Fig. 7. Effect of a p38MAPK inhibitor on phagocytosis of non-opsonized zymosan particles induced by $sPLA_2$s. Macrophages were treated with SB202190 (1 μM) or vehicle for 30 min before incubation with MT-II or MT-III (6.3 μg/mL) or RPMI (control) in the presence or absence of followed by addition of non-opsonized zymosan particles. After 40 min, phagocytosis was evaluated by phase-contrast microscopy. Values are mean ± S.E.M. from 5 animals. *$p < 0.05$ compared with control, #$p < 0.05$ compared with MT-II without SB202190.

MT-II is devoid of enzymatic activity, the increase in the phagocytic activity by MT-II is clearly not due to toxin-mediated phospholipid hydrolysis. Thus, molecular regions distinct from the catalytic network may be involved in this effect. It has been shown that a stretch of residues, located at the C-terminus of the molecule and involving cationic and hydrophobic amino acids, are responsible for the myotoxic and cytotoxic effects of this $sPLA_2$ homologue [26,27]. Whether this molecular region is also responsible for the increment in phagocytosis by macrophages needs to be further investigated.

Prostaglandins are lipid mediators generated by sequential metabolism of arachidonic acid by COXs, mainly COX-1 and -2, two isoenzymes found in macrophages. By using pharmacological approach, we found that COX-2, but not COX-1, is involved in the increase of zymosan phagocytosis induced by both venom $sPLA_2$s. These data suggest that prostanoids derived from COX-2 pathway are involved in the stimulatory effect of toxins in zymosan phagocytosis and also indicate that MT-II and MT-III are able to induce COX-2 expression. In addition, our results demonstrated an early and marked release of PGE_2 by macrophages after stimulation of these cells with MT-II and MT-III, and under phagocytic stimulus. Because COX-2 is present in phagosome membrane [11], it is possible that the release of PGE_2 via COX-2 pathway, induced by both the venom $sPLA_2$s, contributes to the signal transduction of phagocytosis in macrophages.

The release of PGE_2 induced by MT-II strongly suggests that enzymatic activity does not play a role in this effect. It might be that MT-II is able to activate endogenous group IV PLA_2 or group VI PLA_2 and, probably, to induce the synthesis of eicosanoids that contribute to the phagocytic process. Our data are in accordance with Girotti et al. [11] who showed that COX-2 appearance in the phagosome is correlated with the time course of PGE_2 production, suggesting that the phagosome membrane may serve as a site for $cPLA_2$ or $sPLA_2$-mediated release of arachidonic acid for prostanoid production through the COX-2 pathway. Along this line, Schaloske et al. [28] demonstrated that in $P388D_1$ cells (a macrophage cell line) COX-2 is upregulated after stimulation with zymosan.

We have also examined the contribution of leukotrienes in the signal transduction of phagocytosis of non-opsonized zymosan particles induced by $sPLA_2$s. Our observations that Zileuton and Bestatin blocked phagocytosis suggest that LTB_4 is an important lipid mediator for the effect of MT-II and MT-III. It is very likely that LTB_4 is the 5-LOX product responsible for the stimulatory effect of the toxins on phagocytosis by macrophages, since it is the principal 5-LOX product in leukocytes, and since inhibition of LTB_4 synthesis reduced phagocytosis. On the other hand, no role for cysteinyl-leukotrienes was found in our experimental condition. In accordance with our data, genetic or pharmacologic inhibition/antagonism of leukotrienes impaired, while exogenous addition of leukotrienes augmented FcγR-mediated phagocytosis by macrophages and neutrophils [29–31].

Another important lipid mediator released from phospholipids is PAF. This mediator is generated in macrophages and participates in IL-6 and prostanoid release, reactive oxygen intermediates production and phagocytosis [32]. Our results showing that WEB2170 reduced the stimulatory effect of both toxins in phagocytosis indicate a role for PAF in the signaling events required for stimulation of phagocytosis of non-opsonized zymosan particles induced by both venom $sPLA_2$s. These results are in agreement with Ichinose et al. [32] who showed that PAF increased phagocytosis of latex beads dose-dependently, and its effect was reduced in the presence of PAF receptor antagonists.

Considering that a cross talk between $sPLA_2$s and intracellular PLA_2s has been reported [12,13] and that the intracellular $cPLA_2$ and $iPLA_2$ are activated and believed to participate in the phagocytic process [25,33,34] we further investigated the contribution of these intracellular PLA_2s in the effects of both venom $sPLA_2$s. By using pharmacological interference, we demonstrated that $iPLA_2$ plays a role in the signaling events required for phagocytosis of non-opsonized zymosan induced by MT-II and MT-III. On the other hand, our data showing that the inhibitor of $cPLA_2$, AACOCF3, abrogated the stimulatory effect of MT-II and partially the effect of MT-III suggest that $cPLA_2$ is important mainly for the effect of MT-II on phagocytosis of non-opsonized zymosan particles, but has little contribution to the action of MT-III. Furthermore, our findings that SB202190, a p38MAPK specific inhibitor, reduced the stimulatory effect of both toxins in phagocytosis, demonstrate an important role for p38MAPK in the signaling events required for phagocytosis stimulated by both myotoxins and give support to participation of $cPLA_2$ in the effect induced by the venom $sPLA_2$s. Our findings are in accordance with Girotti et al. [11] report evidencing that the translocation of $cPLA_2$ occurs from the cytosol to the membrane, in a calcium dependent manner, in macrophages during phagocytosis of particles of non-opsonized zymosan.

Taken together, these data demonstrate a subtle difference in the process of signal transduction involved in the stimulatory effect of the two venom $sPLA_2$s. The mechanism by which the studied $sPLA_2$s, particularly MT-II, activate $cPLA_2$ was not elucidated and deserves further study. Evidence from the literature clearly show that calcium is an essential cofactor that stimulates $cPLA_2$ binding to membranes and catalytic activity [12,35]. Thus, it can be assumed that the myotoxin can mobilize calcium from intracellular stores and/or facilitate its entry into cells and thus activate $cPLA_2$. This hypothesis requires further investigations. On the other hand, it is known that the engagement of receptors coupled to G-proteins, as by PAF and eicosanoids, culminates with the mobilization of calcium from intracellular stores. Thus, the lipid mediators produced by the two myotoxins, while acting in their receptors coupled G-protein might amplify the activation of $cPLA_2$ by a mechanism of positive feedback.

Finally, although literature is controversial about the existence of PLA_2 receptors in mouse macrophages, contribution of PLA_2 receptors in our experimental model cannot be ruled out. Hanasaki [36] pointed that the PLA_2R is expressed in alveolar type II epithelial cells and in a subset of splenic lymphocytes, but not in the peritoneal macrophages where the mannose receptor is abundantly expressed. According to Murakami et al. [37] the M-type $sPLA_2$ receptor (PLA_2R1), which binds to several group I/II/V/X $sPLA_2$s with distinct affinities is so far, the best known $sPLA_2$ binding protein. These authors pointed that PLA_2R1 appears as a key protein expressed in human kidney, and more specifically in highly specific and differentiated epithelial cells called podocytes.

In conclusion, we have shown that the two $sPLA_2$s, MT-II an MT-III, amplify the phagocytic process of non-opsonized zymosan particles mediated by β glucan receptors in macrophages. Based on data presented here, we propose that the uptake of non-opsonized zymosan particles induced by these enzymes involves particular intracellular signaling pathways and molecules in macrophages. The initiating events are novel and demonstrate that mediators like PAF and others produced by COX-2 and by 5-LOX pathways and the signaling protein p38MAPK, are relevant for the stimulatory effect induced by MT-II and MT-III in phagocytosis. Moreover, the stimulatory effect of these toxins depends on intracellular PLA_2s, like $iPLA_2$. The participation of $cPLA_2$ is more pronounced for the action of MT-II than for MT-III. This may explain the involvement of arachidonic acid derivatives, in phagocytosis of zymosan particles stimulated by the MT-II, devoid of enzymatic activity. On the other hand, p38MAPK appears to be important for the action of MT-III, but not for MT-II. The signaling pathways, as evidenced, should interact with each other for the final effect of the venom $sPLA_2$s. In addition, the data presented here further demonstrate the complexity of phagocytic mechanisms present in macrophages and the first demonstration of the stimulatory effect of venom $sPLA_2$ in this cell function.

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