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A catalytically-inactive snake venom Lys49 phospholipase A_2 homolog induces expression of cyclooxygenase-2 and production of prostaglandins through selected signaling pathways in macrophages

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ABSTRACT

The effects of a snake venom Lys-49 phospholipase A_2 (PLA_2) homolog named MT-II, devoid of enzymatic activity, on the biosynthesis of prostaglandins and protein expression of cyclooxygenase-2 (COX-2) and signaling pathways involved were evaluated in mouse macrophages in culture and in peritoneal cells ex vivo. Stimulation of macrophages with MT-II leads to production of prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) and protein expression of COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1). Inhibition of cytosolic PLA_2 ($cPLA_2$), but not Ca^{2+} independent PLA_2 ($iPLA_2$) reduced release of PGD_2 and PGE_2 and expression of COX-2 induced by MT-II. Inhibition of nuclear factor κB (NF-κB) significantly reduced MT-II-induced PGE_2, but not PGD_2 production and COX-2 expression. Inhibitors of either protein kinase C (PKC), protein tyrosine kinase (PTK), or extracellular signal-regulated kinase (ERK) pathways abrogated MT-II-induced NF-κB activation and reduced COX-2 expression and PGE_2 release, whereas the p38 mitogen-activated protein kinase (MAPK) inhibitor reduced MT-II-induced COX-2 expression and PGD_2 production. Inhibition of phosphatidylinositol-3-kinase (PI3K) pathway abrogated MT-II-induced NF-κB activation, but affected neither prostaglandins production nor COX-2 expression. MT-II-induced production of PGD_2 and PGE_2 and COX-2 expression were also observed in vivo after intraperitoneal injection into mice. Collectively, our data demonstrate that a catalytically-inactive PLA_2 homolog is capable of inducing prostaglandins biosynthesis and COX-2 expression in macrophages in both in vitro and in vivo models, indicating that the enzymatic activity of PLA_2 is not necessary to trigger these effects. MT-II-activated NF-κB, $cPLA_2$ and distinct protein kinases are the principal steps involved in these cellular events.

1. Introduction

Phospholipases A_2 from snakes of the Viperidae family are classified within group IIA secretory phospholipases A_2 ($sPLA_2$s), which have similar structural features to $sPLA_2$s found in inflammatory exudates of mammals (Burke and Dennis, 2009). Two different types of $sPLA_2$s have been characterized in bothropic venoms: (1) catalytically active PLA_2s, i.e. Asp-49 PLA_2s, having the conserved residues of the catalytic network and the calcium-binding loop, and (2) catalytically-inactive variants, having Lys instead of Asp at position 49. This modification precludes an effective calcium binding and, as a consequence, enzymatic activity is abolished (Ward et al., 2002). Therefore, these PLA_2 homologs exert their actions independently of enzymatic phospholipid degradation (Lomonte et al., 2003; Lomonte and Gutiérrez, 2011). For this reason, the Lys49 PLA_2 homologs have attracted attention as models for the induction of inflammation by a catalytically independent mechanism of action. Some studies have demonstrated that myotoxin-II (MT-II), a Lys49 PLA_2 homolog isolated from *Bothrops asper* venom induces edema (Chaves et al., 1998) and pain (Chacur et al., 2003). Moreover, MT-II induces release of cytokines and eicosanoids at the local of injection (Zuliani et al., 2005a) and activates inflammatory functions of macrophages (Zuliani et al., 2005b). These cells have crucial roles during inflammation by generating prostaglandins, relevant mediators in numerous pathophysiological conditions. However, the mechanisms responsible for MT-II-induced production of prostaglandins in macrophages and the signaling pathways involved in this effect are still unknown.

$sPLA_2$s by acting associated or not with the cytosolic PLA_2 ($cPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) play key roles in numerous cellular processes by regulating release of arachidonic acid from cell

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membrane phospholipids. Once released, arachidonic acid can be metabolized by cyclooxygenases, leading to biosynthesis of distinct prostaglandins by the corresponding terminal synthases that exhibit cell- and tissue-specific distributions. Three isoforms of cyclooxygenases, designated COX-1, COX-2 and COX-3, have been described (Fitzpatrick, 2004; Warner and Mitchell, 2004). COX-1 is a constitutive isoform present in most tissues and generates prostaglandins with physiological functions. Although the COX-3 has been cloned (Kis et al., 2006), its function has yet to be studied. In turn, COX-2 is up-regulated by inflammatory cytokines and growth factors and is constitutively expressed in several tissues (Hinz and Brune, 2002). The expression of COX-2, and consequently of prostaglandins, is highly regulated by distinct signaling pathways (Murakami et al., 1999). Of the major inflammatory cells, macrophages have cyclooxygenases associated with distinct terminal synthases such as prostaglandin D and prostaglandin E synthases, being currently accepted that the microsomal prostaglandin E synthase (mPGES) is functionally linked to COX-2 (Murakami et al., 2000).

In this study, we examined the effect of, MT-II, for eliciting prostaglandin production and COX-2 protein expression in macrophages both in vitro and in vivo. The involvement of the nuclear transcription factor nuclear factor κB (NF-κB), cytosolic PLA_2 ($cPLA_2$) and Ca^{2+} independent PLA_2 ($iPLA_2$), as well as p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), protein tyrosine kinase (PTK) and protein kinase C (PKC) signaling cascades in this effect for prostaglandin D2 (PGD_2) and prostaglandin E_2 (PGE_2) biosynthesis were also investigated.

2. Materials and methods

2.1. Reagents

PGE_2 and PGD_2 enzyme immunoassay kits, COX activity assay kit, rabbit polyclonal anti-murine COX-2, mouse monoclonal anti-ovine COX-1 and rabbit polyclonal anti-human mPGES-1 antibodies and bromoenol lactone (BEL) were purchased from Cayman Chemical (Ann Arbor, MI, USA); mouse monoclonal anti-rat β-actin was from Sigma Aldrich Co. (St. Louis, MO, USA); peroxidase-conjugated secondary sheep anti-mouse or donkey anti-rabbit antibodies were from GE Healthcare (Buckinghamshire, UK). Arachidonyl trifluoromethyl ketone ($AACOCF_3$), H-7 dihydrochoride (H-7), Herbimycin A, 2′-amino-3′-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), 2-morpholin-4-yl-8-phenylchromen-4-one (LY294002) were purchased from Calbiochem–Novabiochem (La Jolla, CA, USA). Antibodies phospho-p38 MAPK, p38 MAPK, phospho-PI3K, PI3K, phospho-ERK, ERK, phospho-PKC were from Cell Signaling Technology (Danvers, MA), PKC was from Santa Cruz Biotechnology (Santa Cruz, CA). RPMI 1640, N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma-Aldrich. Ethanol grade p.a. was obtained from Merck (Darmstadt, Germany). The salts used were purchased from Merck, GE Healthcare and Bio-Rad (Hercules, CA).

2.2. Animals

Male Swiss mice (18–20 g) were used. These animals were housed in temperature-and humidity- contolled rooms and 12 h dark-light period, and received water and food ad libitum until used. The animals and research protocols used in this study followed the guidelines of the Ethical Committee for use of animals of Instituto Butantan, SP, Brazil (CEUAIB, protocol numbers 177/04 and 592/09) and international policies of experimental animal care. All efforts were made to minimize the number of animals used and their suffering.

2.3. Phospholipase A_2 homolog

The Lys49 PLA_2 homolog (MT-II) was isolated from *Bothrops asper* venom by ion-exchange chromatography on CM-Sephadex C-25 as described (Lomonte and Gutiérrez, 1989), followed by RP-HPLC on a C8 semi-preparative column (10 × 250 mm; Vydac) eluted at 2.0 ml/min with a 0–70% acetonitrile gradient containing 0.1% trifluoroacetic acid, during 30 min, on an Agilent 1200 instrument monitored at 215 nm. Homogeneity was assessed by analytical reverse-phase HPLC on a C4 column using a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (v/v). The absence of endotoxin contamination in the MT-II preparation was demonstrated by the quantitative *Limulus* amebocyte lysate (LAL) test (Takayama et al., 1994), which revealed undetectable levels of endotoxin (<0.125 EU/ml).

2.4. Resident peritoneal macrophages collection and culture

Resident peritoneal macrophages were harvested by washing peritoneal cavities with 2 ml of apyrogenic saline solution. Aliquots of the washes were used to count total cell numbers in a Neubauer's chamber after dilution (1:20, v/v) in Turk solution. Aliquots of either 1×10^6 or 5×10^6 cells/ml were added to 24- and 6-well polystyrene culture plates, respectively, for 3 h, with RPMI 1640 medium supplemented with 1% of L-glutamine and 100 μg/ml of garamicine, at 37 °C and 5% CO_2 atmosphere. Next, non-adherent cells were removed by vigorous washing with glutamine free RPMI 1640. The entire procedure was performed in apyrogenic conditions and all of the materials used were previously sterilized. After cell adhesion, peritoneal cells, which were, initially, composed of 40–50% of F4/80 positive cells and more than 30% of CD19 positive cells, became enriched in F4/80 positive cells (more than 90% of the cells), as demonstrated by performing flow cytometry using the F4/80-fluorescein isothiocyanate (Clone A3–1, Serotec) and CD19-phycoerythrin antibodies (Clone 1D3, BD Bioscience) and a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). In other set of assays, following cell adhesion into culture plates, MT-II (0.4 μM) was added to macrophages in culture and, at selected time intervals (1, 3, 4.5, 6 and 24 h), the plates were centrifuged at 500*g* for 6 min at 22 °C. The supernatants were stored at −80 °C and later used for determination of PGE_2 and PGD_2, whereas the cell pellets were used to determine the expression of COX-1, COX-2 and mPGES-1. Where appropriate, the following inhibitors were used: 20 μM $AACOCF_3$, an inhibitor of $cPLA_2$; 1 μM BEL, an inhibitor of $iPLA_2$; 2.5 μM TPCK, a selective inhibitor of NF-κB activation; 1 μM SB202190, inhibitor of p38 MAPK; 25 μM Ly294002 inhibitor of PI3K; 2 μM Herbimycin, an inhibitor of PTK; 20 μM H-7, inhibitor of PKC; and 25 μM PD98059, inhibitor of ERK.

2.5. Cell viability assays

Cells incubated with either inhibitors or MT-II were analyzed for viability by the tetrazolium-based (MTT) colorimetric assay and measuring lactate dehydrogenase (LDH) release. The MTT reduction assay was performed to evaluate the integrity of mitochondrial function. In brief, 2×10^5 cells/well in RPMI-1640 medium supplemented with 40 μg/ml gentamicin sulfate and 2 mM L-glutamine were plated in 96-well plates and incubated with 200 μl of selected concentrations of MT-II or inhibitors diluted in medium or with the same volume of medium alone (control) for 3, 6 and 24 h, at 37 °C in a humidified atmosphere