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12-HETE is a regulator of PGE₂ production via COX-2 expression induced by a snake venom group IIA phospholipase A₂ in isolated peritoneal macrophages

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Abstract

The snake venom miotoxin (MT)-III is a group IIA secreted phospholipase A₂ (sPLA₂) with pro-inflammatory activities. Previous studies have demonstrated that MT-III has the ability to stimulate macrophages to release inflammatory lipid mediators derived from arachidonic acid metabolism. Among them, we highlight prostaglandin (PG)E₂ produced by the cyclooxygenase (COX)-2 pathway, through activation of nuclear factor (NF)-κB. However, the mechanisms coordinating this process are not fully understood. This study investigates the regulatory mechanisms exerted by other groups of bioactive eicosanoids derived from 12-lipoxygenase (12-LO), in particular 12-hydroxyeicosatetraenoic (12-HETE), on group IIA sPLA₂-induced (i) PGE₂ release, (ii) COX-2 expression, and (iii) activation of signaling pathways p38 mitogen-activated protein kinases (p38MAPK), protein C kinase (PKC), extracellular signal-regulated kinase 1/2 (ERK1/2), and NF-κB. Stimulation of macrophages with group IIA sPLA₂ resulted in release of 12-HETE without modification of 12-LO protein levels. Pre-treatment of these cells with baicalein, a 12-LO inhibitor, decreased the sPLA₂-induced PGE₂ production, significantly reduced COX-2 expression, and inhibited sPLA₂-induced ERK; however, it did not affect p38MAPK or PKC phosphorylation. In turn, sPLA₂-induced PGE₂ release and COX-2 expression, but not NF-κB activation, was attenuated by pre-treating macrophages with PD98059 an inhibitor of ERK1/2. These results suggest that, in macrophages, group IIA sPLA₂-induced PGE₂ release and COX-2 protein expression are distinctly mediated through 12-HETE followed by ERK1/2 pathway activation, independently of NF-κB activation. These findings highlight an as yet undescribed mechanism by which 12-HETE regulates one of the distinct signaling pathways for snake venom group IIA sPLA₂-induced PGE₂ release and COX-2 expression in macrophages.

Keywords: snake venom group IIA phospholipase A₂, prostaglandin E₂, cyclooxygenase-2, 12-hydroxyeicosatetraenoic acid, 12-lipoxygenase, macrophages

1. Introduction

Secreted phospholipases A₂ (sPLA₂) are a group of lipolytic enzymes with a range of biological functions, including metabolism and production of lipid compounds, cell membrane degradation or repair and production of second messengers [1,2], both in physiological or physiopathological conditions. These enzymes are abundantly expressed in nature and have been classified into 11 distinct groups (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB), and further subdivided into secreted sPLA₂, cytosolic PLA₂ and Ca²⁺ independent PLA₂, on the basis of their localization, source, amino acid sequences, and biochemical characteristics [2,3]. Among sPLA₂s, we highlight the group IIA sPLA₂s because it has been considered clinically relevant due to its involvement in several inflammation related diseases, such as cardiovascular diseases, immune disorders, Alzheimer's disease, and cancer [4-8]. On the other hand, some sPLA₂ from the same group (IIA) are present in snake venoms and are one of the major components implicated in acute inflammatory effects caused by viperid snake venoms [9-11].

The PLA₂s hydrolyze the membrane phospholipids at the sn-2 position, generating free fatty acids such as arachidonic acid (AA). This fatty acid is a substrate for various enzymatic pathways, mainly cyclooxygenases (COX), including the isoforms COX-1 and COX-2 and lipoxygenases (LO), as 5- and 12-LO types, generating diverse lipid mediators, named eicosanoids, such as prostaglandins (PG), leukotrienes (LT) and hydroxyeicosatetraenoic acids (HETE) [12-14]. Among prostaglandins, PGE₂ is the major lipid mediator involved in pro-inflammatory events, represented by vasodilation, nociception, and increased vascular permeability processes [6,15,16]. LTB₄, in turn, is originated by 5-LO pathway and is recognized for its involvement in the recruitment of

neutrophils and potent chemotaxis effect on inflammatory response [17,18]. 12-HETE, the main product generated from AA metabolism by 12-LO and of interest in this study, has been associated with amplification of inflammatory processes [4,19,20], and is known to regulate COX-2 expression and generation of its major product, PGE₂ [21-24]. Few studies have reported that this regulatory effects exerted by 12-HETE involve activation of protein kinases, such as p38MAPK, ERK1/2, and PI3K [25-28]. Three isoforms of 12-LO have been described: platelet-type 12-LO; macrophage or leukocyte-type 12-LO; and epidermal-type 12-LO [29]. 12-HETE generated by leukocyte-type 12-LO has been demonstrated as inducing inflammatory events such as vasodilation and increased adhesion molecule expression, as well as monocyte migration and the release of inflammatory mediators, such as MCP-1/CCL2, IL-6, and TNF- α [28,30-33].

PGE₂ produced by COX-2 pathway on AA was intensely demonstrated to be involved in the local inflammatory response induced by the snake venom group IIA sPLA₂, named myotoxin-III (MT-III), isolated from *Bothrops asper* venom [34-36]. This sPLA₂ promotes an acute inflammatory response including edema, pain, and leukocyte accumulation at the site of injection by enhancing the production of inflammatory mediators, highlighting PGE₂, LTB₄ and pro-inflammatory cytokines [35-38]. Our studies have demonstrated that stimulation of isolated macrophages in culture by sPLA₂ MT-III result in COX-2-dependent release of PGE₂ and activation of distinct protein kinases, including those of the MAPK family (p38MAPK), PI3K and PKC, as well as nuclear factor kappa B (NF- κ B) [35,36,39]. These effects corroborate the demonstration that membrane cleavage products generated by group IIA sPLA₂s are important bioactive mediators involved in the release of inflammatory PGE₂, through their activation of intracellular signaling mechanisms in various cells [40-42]. Despite this knowledge, the signal transduction pathways leading to sPLA₂ MT-III-promoted protein expression of COX-2 and PGE₂ production in macrophages still need to be complemented, and the role of lipids mediators from other AA pathways as a regulatory crosstalk mechanism, cannot be ruled out. In this context, the regulatory

effect played by 12-LO or 12-HETE in a group IIA sPLA₂-induced COX-2 expression and PGE₂ release by macrophage has never been investigated. Taking into account, this study investigated the involvement of 12-LO and 12-HETE in group IIA sPLA₂-induced COX-2 protein expression and PGE₂ release in macrophages, as well as its role on signaling pathways mediated by p38MAPK, PKC or ERK1/2 in a group IIA sPLA₂-induced inflammatory effects.

2. Materials and Methods

2.1. Reagents

12-HETE and PGE₂ enzyme immunoassay (EIA) kits, rabbit polyclonal anti-murine 12-LO and COX-2, and baicalein were purchased from Cayman Chemicals (Ann Arbor, MI, USA); mouse monoclonal anti-β-actin was from Sigma Aldrich Co. (St. Louis, MO, USA); peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit antibodies were from GE Healthcare (Buckinghamshire, UK). PD98059 was purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Antibodies to phospho-ERK (p-p44/42 MAPK) and ERK (p44/42 MAPK) were from Cell Signaling Technology (Danvers, MA, USA). RPMI 1640 was purchased from Sigma-Aldrich. Ethanol absolute for analysis was obtained from Merck (Darmstadt, Germany). The salts used were purchased from Merck, GE Healthcare (Buckinghamshire, UK) and Bio-Rad (Hercules, CA, USA).

2.2. Animals

Male Swiss mice (18-20 g) were used. The animals were housed in temperature-controlled rooms, with a relative humidity of $65.3 \pm 0.9\%$ and a 12 h dark-light period, and received water and food ad libitum. The animal- and research protocols used in this study followed the Instituto Butantan, SP, Brazil's Ethical Committee guidelines for the use of animals in experiments (CEUAIB, protocol number 1323/14), and international policies on experimental animal care. All efforts were made to minimize the number of animals used and their suffering.

2.3. Phospholipase A₂

The group IIA sPLA₂ named MT-III was isolated by ion-exchange chromatography on CM-Sephadex C-25 from *Bothrops asper* venom [43]. A final purification step using high-performance liquid chromatography (HPLC) semi-preparative C8 column was performed as previously described [44]. Homogeneity was demonstrated by analytical reverse phase HPLC. The absence of endotoxin contamination in the sPLA₂ preparation was demonstrated by the quantitative Limulus amoebocyte lysate (LAL) test [45], which revealed undetectable levels of endotoxin (<0.125 EU/mL).

2.4. Collection and culture of resident peritoneal macrophages and pharmacological treatments

Resident peritoneal macrophages were harvested by washing the peritoneal cavities of the mice 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 pre-incubated in 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 gentamicin, at 37°C and 5% CO₂ atmosphere. Non-adherent cells were removed by three washes with RPMI medium. After cell adhesion, peritoneal cells, which were initially composed of 40-50% F4/80 positive cells and more than 30% CD19 positive cells, became predominant in F4/80 positive cells (more than 90% of the cells). This was demonstrated by performing flow cytometry using F4/80-fluorescein isothiocyanate (Clone A3-1, Bio-rad, Hercules, CA, USA) and CD19-phycoerythrin antibodies (Clone 1D3, BD Bioscience, San Jose, CA, USA), and a FACSCalibur flow cytometer (BD Bioscience). When appropriate, distinct set of culture cells were incubated with 40 µM of baicalein [46,47], an inhibitor of 12-LO or 50 µM of PD98059 [48], a selective inhibitor of ERK1/2, or appropriate vehicle (DMSO 0,01%) for 30 min before sPLA₂ MT-III at 0.4 µM

[38,39] or saline solution (control). After this time, the plates were centrifuged at 500 g for 6 min at 22°C and the supernatants were stored at -80°C for quantification of 12-HETE or PGE₂ by EIA kit. The cells were collected to determine the protein expression of COX-2, 12-LO, p-ERK, and ERK1/2 by western blotting or to analysis the nuclear translocation of NF-κB by electrophoretic mobility shift assay (EMSA).

2.5. Quantification of PGE₂ and 12-HETE concentrations

Concentrations of PGE₂ and 12-HETE were determined by EIA, using commercial kits from Cayman Chemicals. In brief, 50 μL aliquots of cell supernatant were incubated with the eicosanoids conjugated with acetylcholinesterase and the specific rabbit antiserum, in 96-well plates coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbance of the samples was recorded at 405 nm in a microplate reader (Labsystem Multiskan, Midland, ON, Canada), and concentrations of PGE₂ or 12-HETE estimated from standard curves.

2.6. Western blotting

COX-2 and 12-LO protein expression was detected in macrophages by western blotting. Aliquots of 1×10^6 cells were lysed with 100 μL of sample buffer (0.5 M Tris-HCl, pH 6.8, 20% SDS, 1% glycerol, 1 M β-mercaptoethanol, 0.1% bromophenol blue) and boiled for 10 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% bis-acrylamide gels overlaid with a 5% stacking gel. Proteins were then transferred to a nitrocellulose membrane (GE Healthcare) using a Mini Trans-Blot[®] from Bio-Rad. The membrane was blocked for 1 h with 5% w/v nonfat dry milk in Tris-buffered saline-Tween 20 (TTBS) (20 mM Tris, 100 mM NaCl and 0.5% Tween 20), and incubated with primary antibodies against COX-2 (1:1500) or 12-LO (1:5000) and β-actin (1:2000). To analyze the activation of ERK1/2, p38MPAK or PKC induced by sPLA₂ MT-III, the membrane was blocked for 1 h in TTBS/5% w/v BSA, and incubated with

antibodies against p-p44/42 MAPK or p44/42 MAPK or p-p38MAPK or p38MAPK or p-PKC or PKC, respectively, at 4°C, with gentle shaking, overnight. The membrane was then washed and incubated with an appropriate secondary antibody conjugated to horseradish peroxidase. Detection was performed using the enhanced chemiluminescence method (GE Healthcare). Densities of the band were determined by an LAS Imager 3000 (GE Healthcare) using the image analysis software from Quantity One[®] (GE Healthcare).

2.7. Electrophoretic mobility shift assay (EMSA)

NF-κB binding capacity was assessed by EMSA. Nuclear extracts from peritoneal adherent macrophages (3×10^6 cells/well) were obtained as previously described [49], and protein concentration determined by the Bradford method [50]. NF-κB binding capacity was evaluated as previously described [51]. Briefly, end-labeled [γ -³²P] ATP oligonucleotides containing an NF-κB consensus-binding site (5'-AGTTGAGGGGACTTTCCCAGGC-3') were incubated for 20 min at room temperature with 5 μg of nuclear extract protein. DNA-protein complexes were then separated on a 5.5% non-denaturing polyacrylamide gel, using a running buffer of 45 mM Tris, 45 mM borate, and 1 mM EDTA buffer. The gels were vacuum dried (at 80°C) and subjected to autoradiography. The blots were analyzed by scanner densitometry (STORM 840, Dynamic Molecular, Sunnyvale, CA, USA), and the results obtained through densitometric analysis. The results was expressed relative to the control condition (unstimulated control).

2.8. Statistical analysis

Results were expressed as mean ± SEM. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's range test. Values of probability lower than 5% ($p < 0.05$) were considered significant.

3. Results

3.1. Group IIA sPLA₂ induces 12-HETE production but not the protein expression of 12-LO

Incubation of isolated macrophages with group IIA sPLA₂ significantly increased ($p < 0.05$) 12-HETE concentration from 30 min (78093 ± 10333 pg/mL) up to 6 h (21461 ± 11012 pg/mL) in comparison to respective controls (7170 ± 4176 pg/mL at 30 min; 2200 ± 541.5 at 6 h) (Figure 1A). Maximal release occurred at 30 min, with a 90% increase.

Since 12-LO is a key enzyme in the 12-HETE biosynthetic pathway, the effect of sPLA₂ on the expression of this protein was assessed in isolated macrophages by western blotting analysis. The expression of 12-LO did not differ ($p > 0.05$) between macrophages incubated with sPLA₂ and those incubated with saline only (Figures 1B and 1C).

3.2. 12-HETE is involved in COX-2 expression and PGE₂ production induced by group IIA sPLA₂ in macrophages

The Fig 2A and B show that pretreatment of macrophages with baicalein at 40 μ M, but not at 20 μ M, significantly reduced ($p < 0,05$) sPLA₂-induced COX-2 protein expression in comparison to cells incubated with sPLA₂ (positive control). From this result, the concentration of 40 μ M was elected for the proposed studies. Pre-treatment of macrophages with baicalein resulted in a significant reduction ($p < 0,05$) of release of PGE₂ induced by group IIA sPLA₂ (Figure 2C). Moreover, this pretreatment significantly inhibited sPLA₂-induced release of 12-HETE ($p < 0,05$) in comparison with respective cells treated with group IIA sPLA₂ (Figure 2D).

3.3 ERK1/2 is an important component in the signaling of COX-2 expression and PGE₂ production induced by group IIA sPLA₂ in macrophages

sPLA₂-induced COX-2 protein expression and PGE₂ levels, respectively, in macrophages pretreated with PD98059 or with vehicle only, were examined (Figures 3A-C). Unstimulated macrophages showed a weak basal COX-2 protein expression and PGE₂ release when pre-incubated with or without inhibitors of kinases (Figures 3A -C). In contrast, pre-incubation of cells with PD98059, an ERK1/2 activation inhibitor significantly reduced COX-2 protein expression and PGE₂ release (by 82% and 48%, respectively) induced by this venom group IIA sPLA₂, in comparison to stimulated cells. These results implicate ERK1/2 signaling protein in the COX-2 pathway induced by group IIA sPLA₂. Next, to determine whether activation of NF-κB is mediated by an ERK1/2 signaling pathway, we evaluated the translocation of NF-κB to the macrophage nucleus under sPLA₂ stimulation. Pre-treatment of resident peritoneal macrophages with PD98059 did not alter group IIA sPLA₂-induced activation of NF-κB (Figure 3D). These results suggest evidence ERK1/2 is not involved in by sPLA₂-induced activation of NF-κB. The downstream factors activated by ERK1/2 remain to be investigated.

3.4 12-HETE is involved in ERK1/2 phosphorylation induced by group IIA sPLA₂

In order to evaluate the role of the derived 12-LO mediator in the effects of group IIA sPLA₂, the effects of baicalein on sPLA₂-stimulated p38MAPK, PKC, and ERK1/2 protein kinases phosphorylation were tested. As assessed by western blotting results showed that the activation of PKC or p38MAPK kinases phosphorylation by group IIA sPLA₂ were not modified by pre-treatment with baicalein compound (Figures 4A-B). On the other hand, sPLA₂-induced ERK1/2 protein phosphorylation was significantly inhibited by baicalein, when compared with macrophages treated with vehicle and group IIA sPLA₂ ($p < 0,05$) (Figures 4C-D), but levels of ERK total protein

were not affected by baicalein (Figure 4E). To better demonstrate this last effect, the Fig. 5A and B showed that the treatment of macrophages in culture with 0.4 μ M of sPLA₂ induced significantly ERK1/2 phosphorylation from 5 up to 30 min ($p < 0.05$), indicating that group IIA sPLA₂ triggers signaling pathway mediated by ERK1/2.

4. Discussion

In our present study, we showed for the first time a group IIA sPLA₂ isolated from snake venom induced early and sustained release of 12- (S)-hydroxy-eicosatetraenoic (12-HETE) by macrophages. Bee venom (*Apis mellifera*) and snake venom (*Bungarus multicinctus*) sPLA₂s also stimulate production of 12- (S)-hydroxy(peroxy)eicosatetraenoic (12-H(P)ETE) by platelets [53], which is an active precursor of 12-HETE [29, 54], indicating that venom sPLA₂s can upregulate 12-LO pathway. In addition an association between mammalian PLA₂s from groups IIA and IV and platelet-type 12-LO during the inflammatory response has also been demonstrated by some studies [39, 55]. In the present study, our data evidenced similar active association between PLA₂ and leukocyte-type 12-LO which is in turn the isoform expressed by macrophages [55,56].

We have previously reported that group IIA sPLA₂ from *Bothrops asper* snake venom MT-III triggers inflammatory activity at the site of the injection [35,57], inducing a prominent release of PGE₂ from macrophages by up-regulating COX-2 expression, which is dependent on activation of distinct protein kinases [39]. The involvement of 12-LO as the underlying mechanism of sPLA₂-induced up-regulation of COX-2 was confirmed by inhibiting expression of COX-2 protein with baicalein [58-60]. Thus, this group IIA sPLA₂ activates downstream pathways required for up-regulation of COX-2 expression and PGE₂ production through the catalytic activity of 12-LO and release of 12-HETE, accompanied by high production of AA induced by sPLA₂ MT-III [35]. These data are in agreement with findings that 12-HETE directly or 12-LO activity are involved in COX-2 expression and PGE₂ production in pancreatic, mesangial cells and human colonic myofibroblasts [21,23,61]. Moreover, our data are in line with report that 12-H(P)ETE regulates the arachidonic

acid mobilization through up-regulation of cytosolic PLA₂ in human macrophages by group V sPLA₂, in order to amplify inflammatory mechanisms [62]. To our best knowledge, this is the first evidence for the role of 12-HETE in the regulation of snake venom group IIA sPLA₂-induced COX-2 protein expression and PGE₂ biosynthesis.

12-HETE elicits inflammatory, immunomodulatory and leukocyte chemoattraction [63,64]. In turn, it stimulates signaling pathways involving protein kinases that play regulatory roles on the route of cyclooxygenase activity and production of lipid mediators [21,23, 65-67]. The 12-HETE mediator is known to activate p38 mitogen-activated protein kinases (p38MAPK), protein kinase C (PKC), and extracellular signal-regulated kinases1/2 (ERK1/2) to up-regulate inflammatory effects in various cell types [25-28,33,61,68]. Our findings showed that inhibition of 12-LO by baicalein reduced ERK1/2 activation in cells stimulated by group IIA sPLA₂, indicating participation of ERK1/2 in the pathway triggered by 12-HETE in our experimental condition. Interestingly, baicalein did not affect sPLA₂-induced activation of p38MAPK and PKC evidencing that phosphorylation of these kinases is largely independent of 12-LO up-regulation in isolated macrophages. This finding highlights that the up-regulation of 12-LO by group IIA sPLA₂ regulates ERK1/2 phosphorylation in isolated macrophages. The lipid mediator 12-HETE is then an upstream activator of ERK1/2 upon stimulation by group IIA sPLA₂. These results add relevant knowledge about the signal pathways triggered by group IIA sPLA₂s leading to the production of PGE₂. This sequential activation of 12-HETE and ERK1/2 has also been demonstrated to mediate mucus secretion in chronic pulmonary inflammatory diseases [66] and pancreatic cancer and epidermoid carcinoma cells proliferation [25,36, 66].

In the present study, group IIA sPLA₂ was found to induce the phosphorylation of ERK1/2 and this effect is consistent with other studies showing activation of ERK as key link of inflammation, cell proliferation and lipid body formation induced by both mammalian group IIA sPLA₂[69-71] and snake venom group IIA sPLA₂ [52,72,73]. In addition, our findings showed that

group IIA sPLA₂-induced PGE₂ production and COX-2 expression, is largely dependent on ERK1/2 phosphorylation, in agreement with previous reports that have evidenced the involvement of ERK1/2 pathway in AA metabolism by sPLA₂s [52, 72, 74-76]. Surprisingly, our data showed that this downstream of activation is independent of nuclear translocation of NF-κB. Our previous study has demonstrated that production of PGE₂ and expression of COX-2 induced by sPLA₂ MT-III result from both NF-κB-linked p38MAPK and PKC and NF-κB-independent PI3K kinase pathways [39]. Considering this information we hypothesize that a transcription factor other than NF-κB is involved in the present described pathway.

Thus, the present study report that 12-LO and its product 12-HETE play an important role in the upstream cascade involved in sPLA₂-induced ERK1/2 phosphorylation carry on in COX-2 protein up-regulation and PGE₂ production, as illustrated in Figure 6. Taken together, by this study we revealed a new aspect of the effect of group IIA sPLA₂ in macrophage inflammatory functions by demonstrating that distinct arachidonic acid metabolism products may positively regulate crosswise of group IIA sPLA₂-induced-COX-2 and -PGE₂ up-regulation. Therefore, these findings bring new insights into the mechanisms of amplification of the inflammatory response induced by a snake venom group IIA sPLA₂.

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Figures

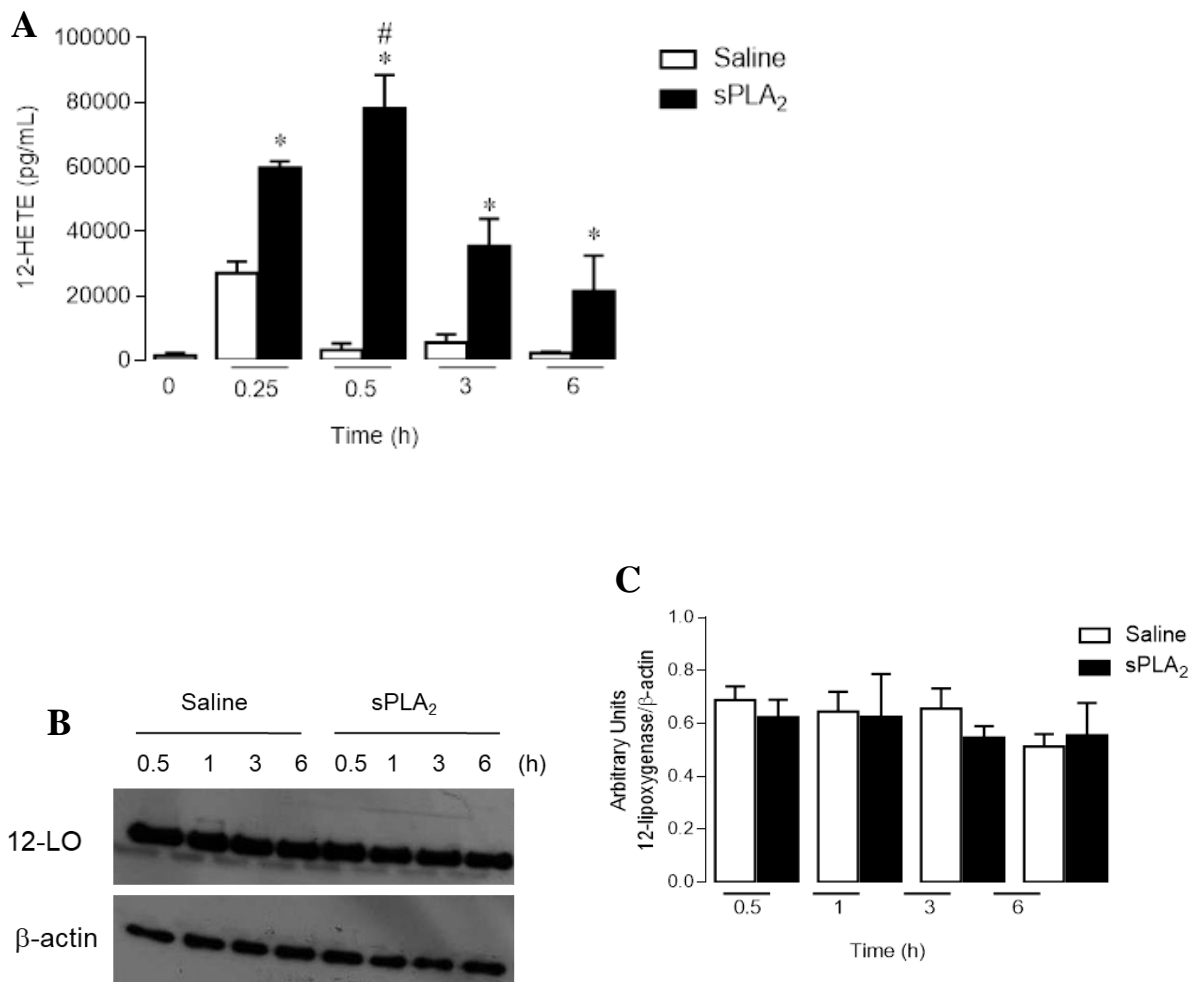


Figure 1

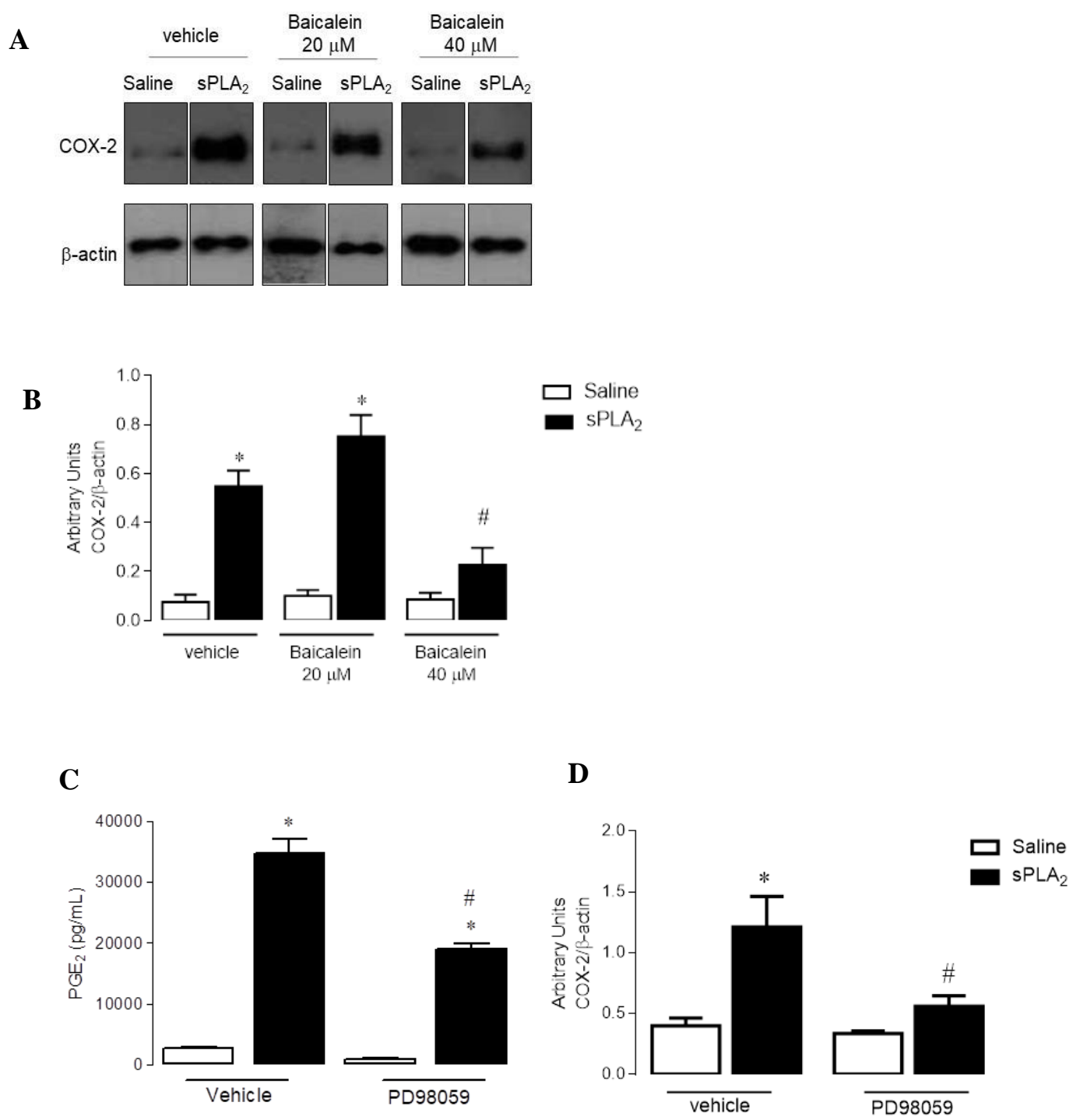


Figure 2

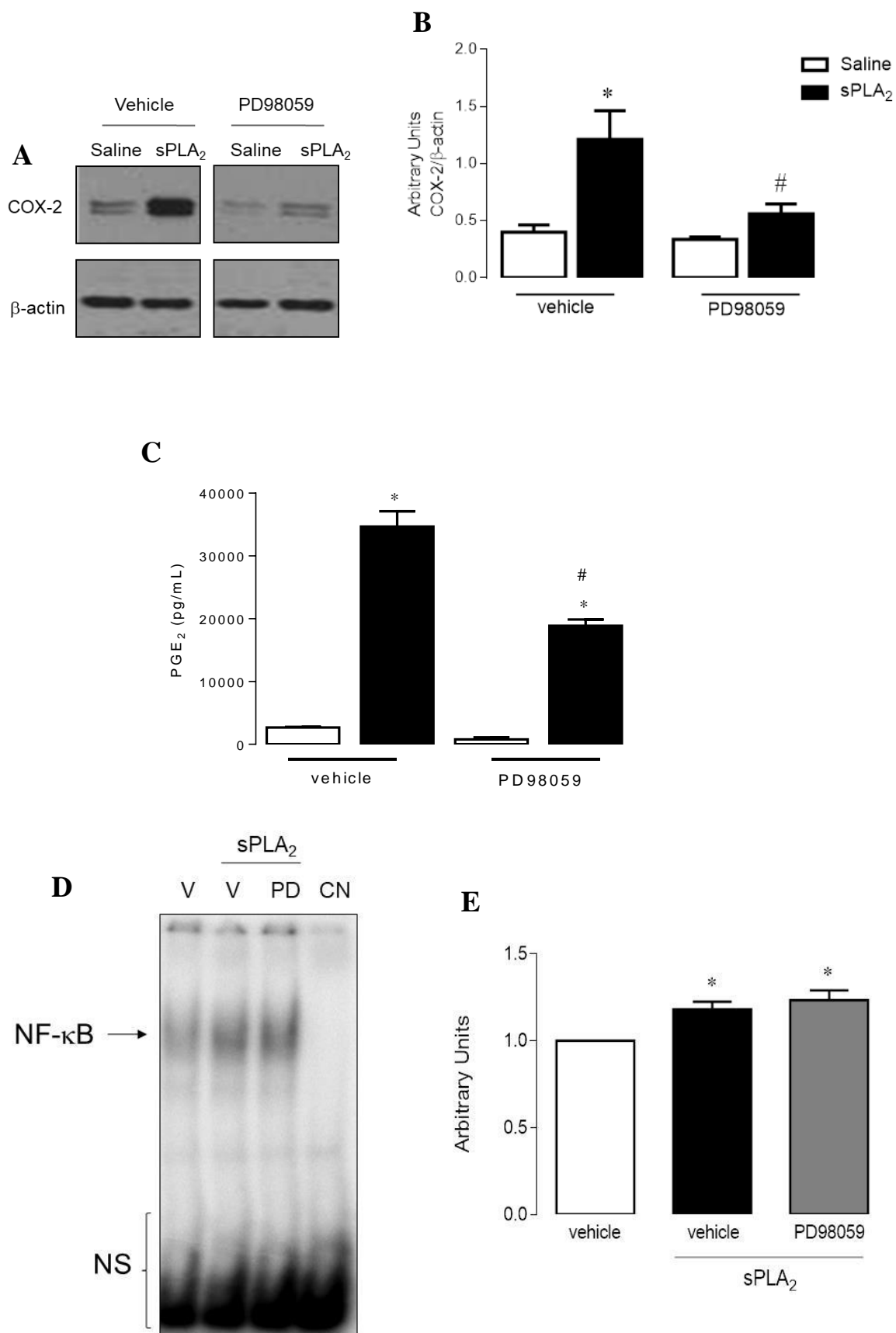


Figure 3

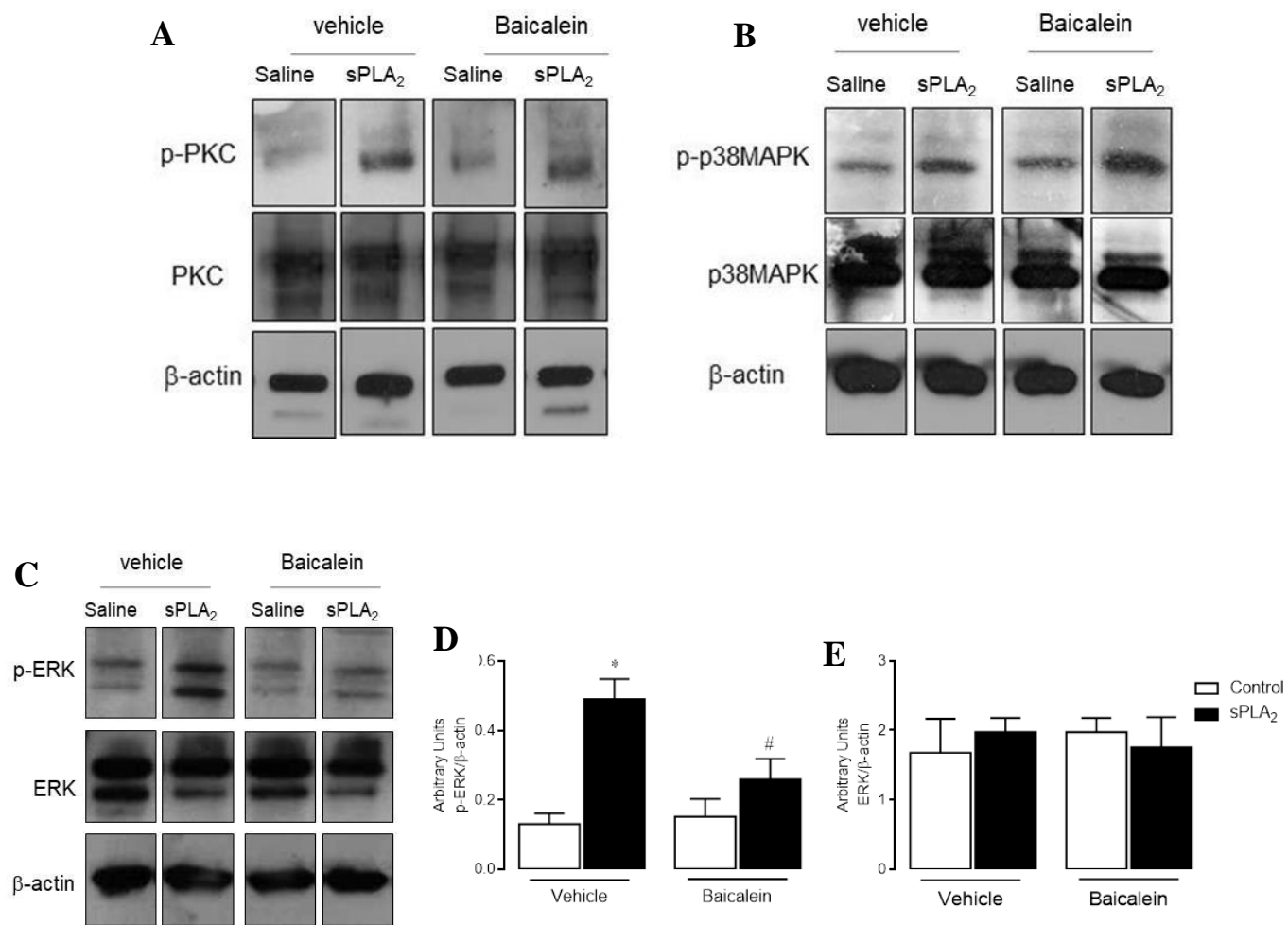


Figure 4

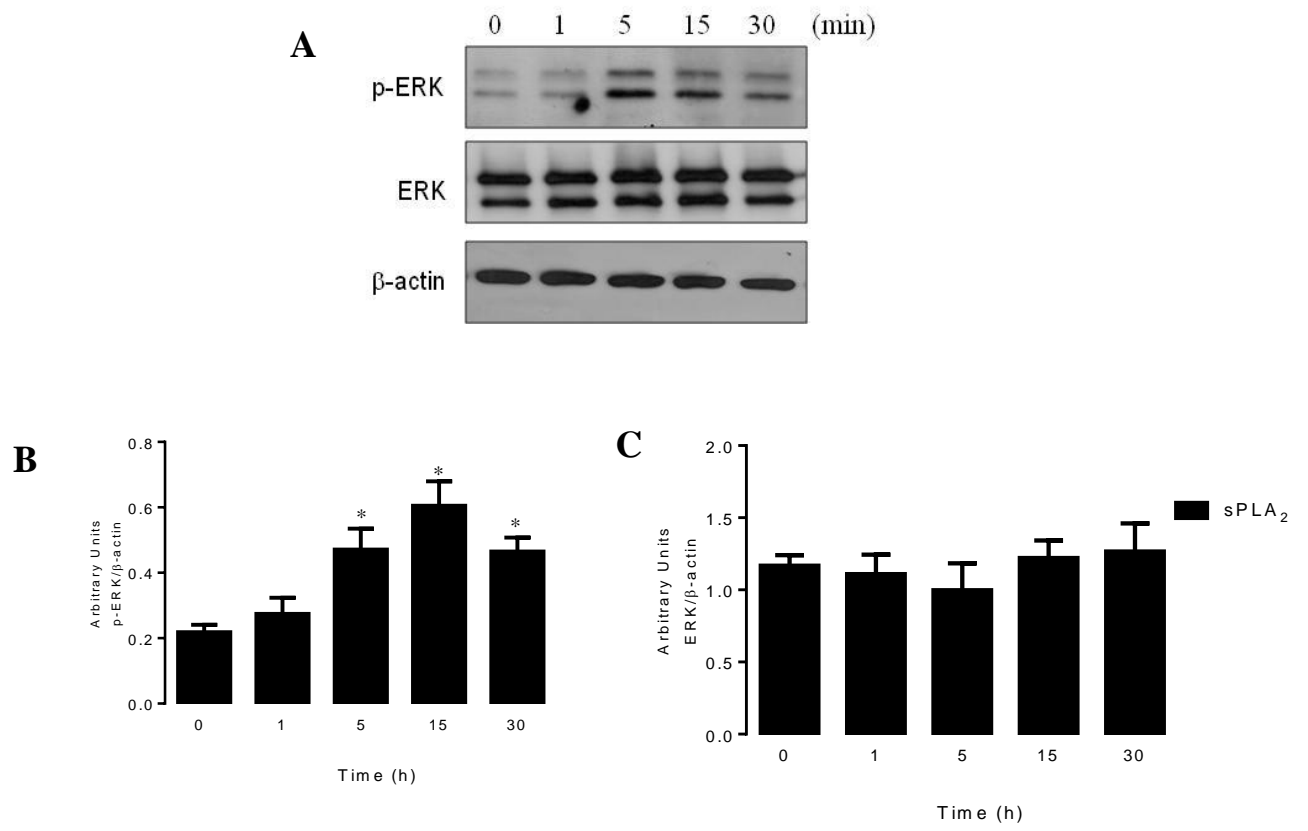


Figure 5

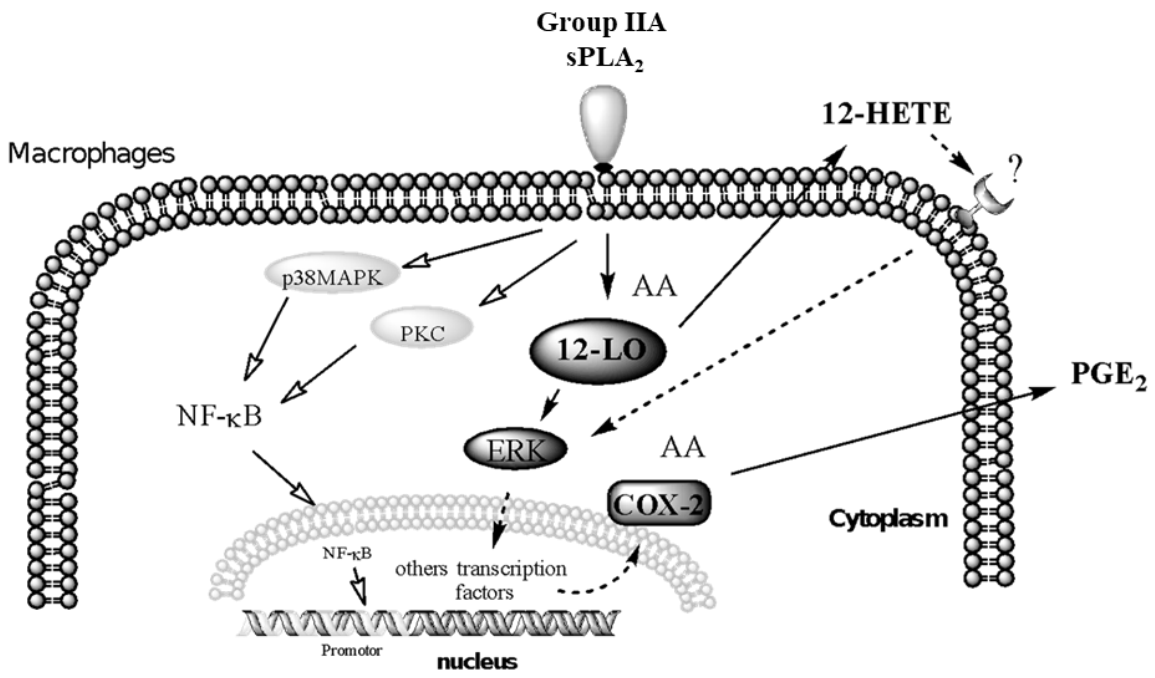


Figure 6

Legends

Fig. 1. Effect of group IIA sPLA₂ on the release of 12-HETE and protein expression of 12-LO in macrophages. Cells (1×10^6) were incubated with MT-III (0.4 μ M) or saline (control) for 15 min or 0.5, 1, 3 or 6 h. 12-HETE was quantified in culture supernatants by specific EIA (A); western blotting analysis of 12-LO and β -actin (loading control) (B); bar graph shows densitometric analysis of immunoreactive 12-LO in AU, normalized with β -actin (C). Results are expressed as mean \pm SEM from 3 to 4 experiments. * $p < 0.05$ as compared to the corresponding macrophages incubated with saline solution. # $p < 0.05$ as compared to macrophages incubated with sPLA₂ at 15 min or 1, 3, or 6 h.

Fig. 2. Effect of baicalein on COX-2 expression, PGE₂, and 12-HETE production induced by group IIA sPLA₂ in isolated macrophages. Resident peritoneal macrophages (1×10^6 cells) were pretreated for 30 min with baicalein (40 μ M) and then incubated for 3 h with MT-III (0.4 μ M). Western blotting analysis of COX-2 and β -actin (loading control) of cells treated with MT-III (A); bar graph exhibits densitometric analysis of immunoreactive COX-2 in AU, normalized with β -actin (B); PGE₂ and 12-HETE were quantified in culture supernatants by specific EIA (C, D). Results are expressed as mean \pm SEM from 3 to 4 experiments. * $p < 0.05$ as compared with macrophages incubated with saline solution. # $p < 0.05$ as compared with macrophages incubated with sPLA₂ and vehicle.

Fig. 3. Effect of PD98059 on COX-2 protein expression, PGE₂ release, and NF- κ B activation induced by group IIA sPLA₂ in isolated macrophages. MT-III activates NF- κ B in cultured macrophages. (A,C) Nuclear extracts were prepared and assayed for κ B probe activity with ³²P-labeled double-stranded oligonucleotide κ B using EMSA. (B, D) Densitometric analysis of NF- κ B

band intensities. Results are expressed as mean \pm SEM from 3 experiments. * $p < 0.05$ as compared with control value. NS=Non-specific band; V=Vehicle; NC=Negative control.

Fig. 4. Effect of baicalein on p-PKC, p-p38 MAPK, and p-ERK protein expression induced by group IIA sPLA₂ in macrophages. Resident peritoneal macrophages (1×10^6 cells) were pretreated for 30 min with baicalein (40 μ M) or vehicle (control). They were then incubated for 30 min with MT-III (0.4 μ M) and western blotting of p-PKC and PKC (A); p-p38MAPK and p38MAPK (B); p-ERK and ERK (C), and β -actin (loading control) of cells treated with MT-III. Bar graph shows densitometric analysis of immunoreactive bands of p-ERK and ERK in AU, both normalized with β -actin (D and E). Results are expressed as mean \pm SEM from 3 experiments. * $p < 0.05$ as compared with macrophages incubated with saline. # $p < 0.05$ as compared with macrophages incubated with sPLA₂ and vehicle.

Fig. 5. ERK phosphorylation induced by group IIA sPLA₂ in isolated macrophages. Resident peritoneal macrophages (1×10^6 cells) were incubated for 1, 5, 15, or 30 min with MT-III (0.4 μ M). Western blot analysis of p-ERK and ERK and β -actin (loading control) (A); densitometric analysis of immunoreactive bands of p-ERK and ERK in AU, both normalized with β -actin (D and E). Results are expressed as mean \pm SEM from 3 experiments. * $p < 0.05$ as compared with macrophages incubated for zero time.

Fig. 6. Schematic model for mechanisms involved in group IIA sPLA₂-induced COX-2 expression and PGE₂ release in macrophages. MT-III induces AA release from macrophage membrane. In the 12-LO-shuttling pathway, the released AA is used for production and release of 12-HETE. The possible association of 12-HETE with specific receptors also activates the ERK1/2 cascade, thus stimulating induction of COX-2 protein expression and PGE₂ release, independently of NF- κ B

activation. On the other hand, 12-HETE is not associated with alternative cascades sPLA₂-stimulated p38MAPK and PKC, both involved in NF-κB activation and COX-2 protein expression and PGE₂ production.