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Maslinic acid modulates secreted phospholipase A_2-IIA (sPLA$_2$-IIA)-mediated inflammatory effects in macrophage foam cells formation

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Secretory phospholipase A_2-IIA (sPLA$_2$-IIA) is one of the key enzymes causing lipoprotein modification and vascular inflammation. Maslinic acid is a pentacyclic triterpene which has potential cardioprotective and anti-inflammatory properties. Recent research showed that maslinic acid interacts with sPLA$_2$-IIA and inhibits sPLA$_2$-IIA-mediated monocyte differentiation and migration. This study elucidates the potential of maslinic acid in modulating sPLA$_2$-IIA-mediated inflammatory effects in THP-1 macrophages. We showed that maslinic acid inhibits sPLA$_2$-IIA-mediated LDL modification and suppressed foam cell formation. Further analysis revealed that sPLA$_2$-IIA only induced modest LDL oxidation and that inhibitory effect of maslinic acid on sPLA$_2$-IIA-mediated foam cells formation occurred independently of its anti-oxidative properties. Interestingly, maslinic acid was also found to significantly reduce lipid accumulation observed in macrophages treated with sPLA$_2$-IIA only. Flow cytometry analysis demonstrated that the effect observed in maslinic acid might be contributed in part by suppressing sPLA$_2$-IIA-induced endocytic activity, thereby inhibiting LDL uptake. The study further showed that maslinic acid suppresses sPLA$_2$-IIA-induced up-regulation of PGE_2 levels while having no effects on COX-2 activity. Other pro-inflammatory mediators TNF-α and IL-6 were not induced in sPLA$_2$-IIA-treated THP-1 macrophages. The findings of this study showed that maslinic acid inhibit inflammatory effects induced by sPLA$_2$-IIA, including foam cells formation and PGE_2 production.

Keywords. COX-2; endocytosis; foam cells; maslinic acid; PGE_2; sPLA$_2$-IIA

1. Introduction

Atherosclerosis is currently one of the leading causes of mortality around the world. It is characterized by accumulation of fat deposits in macrophages in major arteries (Stocker and Keaney 2004). There is consensus that atherosclerosis is caused by increased oxidative stress characterized by lipid oxidation in vascular wall, which subsequently taken up by macrophages, forming foam cells. Foam cells play an important role in atherosclerosis development. It is thought to be the hallmark of early atherogenesis (Aviram 1999). Accumulation of foam cells indicates plaque build-up and causes hardening and narrowing of arteries. Atherosclerosis is threatening to the human body as it can lead to heart attack and stroke. A crucial step in the pathogenesis of atherosclerosis is believed to be the oxidative modification of LDL (Esterbauer *et al.* 1993). Oxidized LDL (oxLDL) is known to stimulate macrophages foam cell formation and inflammatory responses. The uptake of oxLDL by scavenger receptors leads to the accumulation of cholesterol within the foam cells in the atherosclerotic lesions (Itabe *et al.* 2011).

Secretory phospholipase A_2 (sPLA$_2$) enzymes play key roles in vascular inflammation and atherosclerosis (Tellis and Tselepis 2009). It is known that PLA_2 enzymes regulate the provision of arachidonic acid (AA) to the cyclooxygenase (COX) and lipoxygenase biosynthetic pathways. sPLA$_2$ hydrolyses the sn-2 ester bond in the glyceroacyl phospholipids present in lipoproteins and cell membranes, inducing structural and functional changes and forming

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lysophospholipids and non-esterified fatty acids. The products of this hydrolysis process can trigger a variety of inflammatory actions that lead to atherosclerotic plaque development. $sPLA_2$-IIA is expressed at very high levels in acute and chronic inflammation disorders such as atherosclerosis (Rosenson and Gelb 2009). Transgenic expression of human $sPLA_2$-IIA in mice results in spontaneous atherosclerosis (Ivandic *et al.* 1999) that is transferable to non-transgenic mice by transplantation of bone marrow (Tietge *et al.* 2005). Furthermore, $sPLA_2$-IIA-mediated modification of LDL phospholipids increases its susceptibility to $sPLA_2$, transforming oxLDL to a more atherogenic form (George and Johnson 2010). The effects of $sPLA_2$-IIA in causing lipoprotein modification and vascular inflammation serve as a basic concept that $sPLA_2$-IIA inhibitors may be useful for cardiovascular and inflammatory diseases (Rosenson and Hurt-Camejo 2012).

Recent research has shown that phospholipase A_2 may be a potential target for maslinic acid. Maslinic acid is a pentacyclic triterpene found in a variety of natural sources. It is a common element in the diet where olive fruit and olive oil are included (Fernández-Navarro *et al.* 2010; Lozano-Mena *et al.* 2014). The antioxidant activity of maslinic acid has been reported in several studies where it was shown to reduce susceptibility of plasma to lipid peroxidation and inhibit copper sulfate ($CuSO_4$)-induced LDL oxidation while showing peroxyl radical scavenging activity and copper chelating effect (Montilla *et al.* 2003; Zamora *et al.* 2000; Allouche *et al.* 2010). Maslinic acid also shows anti-inflammatory properties by inhibiting inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression (Yap and Lim 2015). Mechanism studies further revealed that maslinic acid regulates iNOS and COX-2 expression via the NF-κB signaling pathway, including suppression of p65 translocation to the nucleus and IκBα phosphorylation (Huang *et al.* 2011). Meanwhile, the release of prostaglandin E_2 (PGE_2), a downstream enzymatic product derived from COX-2, was also reduced in primary human chondrocyte, primary rat astrocytes, and neuroblastoma type cell line in response to treatment with maslinic acid (Zamora *et al.* 2000).

Nevertheless, the mechanism on how maslinic acid regulates the NF-κB inflammatory signaling pathways which contribute to the inhibition of iNOS/COX-2 activity and NO/PGE_2 release remains unknown. Interaction with PLA_2 enzyme upstream the arachidonic acid pathway may be one of the mechanisms which explains the anti-inflammatory activity of maslinic acid. In the previous studies, it was shown that maslinic acid interacts with $sPLA_2$-IIA at the catalytic site and it inhibits $sPLA_2$-IIA-induced monocyte adhesion and migration, an immune-inflammatory process leading to atherosclerotic lesion development (Yap *et al.* 2016). Therefore, this study aimed to investigate whether maslinic acid would affect $sPLA_2$-IIA-induced lipid modification and macrophage foam cells formation. The results of this study will serve to drive further the development of maslinic acid as a potential compound for prevention and treatment of atherosclerosis and other inflammatory diseases.

2. Materials and methods

Maslinic acid was purchased from Cayman Chemicals; Lyophilized LDL (L8292) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (P1585) (Missouri, USA). Recombinant human $sPLA_2$-IIA was expressed, purified, and quantified as described (Cupillard *et al.* 1997).

2.1 *Cell culture*

THP-1 cells were cultured at 37 °C under 5% CO_2 atmosphere in RPMI 1640 (GIBCO) with 100 U/mL penicillin/streptomycin, 2 mM glutamine, 10 % fetal bovine serum and maintained at 2×10^5 cells/mL.

2.2 *LDL modification*

LDL modification was performed according to previously established protocol (Xu *et al.* 2010). Briefly, native LDL diluted in PBS to a concentration of 2 mg/mL was incubated with $CuSO_4$ (50 μM, final concentration) for 24 h at 37 °C, protected from light. For modification of LDL with $sPLA_2$-IIA, 2 mg/mL of LDL was incubated with 500 nM of $sPLA_2$-IIA for 24 h at 37 °C. In order to study the effect of maslinic acid on $CuSO_4$ and $sPLA_2$-IIA-mediated LDL oxidation, the LDL samples were incubated in the presence of a range of maslinic acid (5, 10, 20 and 50 μM). Oxidation was terminated by adding Na_2EDTA and then dialyzing against PBS. The extent of LDL oxidation was determined by measuring the TBARS value.

2.3 *TBARS assay*

Decomposition of the unstable peroxides derived from polyunsaturated fatty acids will result in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). Briefly, the incubated samples were mixed with TBA indicator solution at room temperature for 2 h. The absorbance of the supernatant was determined using microplate reader at 532 nm. The absorbance obtained from the experimental sample was compared to a standard curve obtained using known concentrations of MDA.

2.4 *Cell treatment*

THP-1 macrophages were induced by incubating THP-1 monocytes with phorbol ester PMA at a final concentration of 50 ng/mL for 48 h. Converted THP-1 cells will become adherent with flattened appearance which represents the human monocyte-derived macrophages. To evaluate the effects of modified LDL on macrophage foam cells formation, THP-1 macrophages (2×10^5 cells/mL) were incubated with either native LDL, $sPLA_2$-IIA-modified LDL and $sPLA_2$-IIA-modified LDL samples in the presence of maslinic acid (5, 10, 20 and 50 μM) for 24 h (as indicated in Section 2.2 LDL oxidation). A parallel set of experiments which determines the extent of $sPLA_2$-IIA enzyme treatment alone at inducing lipid accumulation was also performed. Briefly, THP-1 macrophages were treated with either 1 μg/mL of $sPLA_2$-IIA, or $sPLA_2$-IIA enzyme in the presence of maslinic acid (5, 10, 20 and 50 μM). The extent of lipid accumulation in macrophages was tested by Oil-Red-O (ORO) staining.

2.5 *ORO staining*

The cells were fixed in 10% phosphate buffered formalin for 10 min. The cells were then washed in PBS once and then rinsed in 60% isopropanol for 15 s to facilitate the staining of neutral lipids. The cells were stained with filtered ORO working solution at 37 °C for 1 min, protected from light. The cells were de-stained with 60% isopropanol and then washed with PBS for 3 times, 3 min each. Positive-staining (red) cells which represent macrophage foam cells formation or lipid droplet accumulation were observed and images were taken using Eclipse-Ti inverted microscope with camera (Nikon Corporation, Tokyo, Japan).

2.6 *Image analysis*

The percentage of lipid accumulation in ORO-stained THP-1 macrophages (supplementary figures 1 and 2) was determined using ImageJ software according to previous established protocol (Deutsch *et al.* 2014). First, the 8-bit red-green-blue ORO-stained images were converted into binary images, which consist only of the pixels representing the lipid droplets. Then, the images were thresholded for colour saturation of the lipid droplet signal. Following binarization, the image was subjected to watershed object separation for image processing, which is used to identify borders of adjacent lipid droplets. After separation, the binary image was manually compared with the original image for consistency and correct binary conversion. Lipid droplets not separated by water-shedding were corrected using the ImageJ pencil tool. The amount and individual size of the lipid droplets in the image, displayed by ImageJ as surface area in square micrometers (μm^2), which represent the percentage of stained lipid in each image, were measured.

2.7 *Endocytic activity*

Meanwhile, the effect of $sPLA_2$-IIA enzyme on THP-1 macrophages endocytic activity was determined by flow cytometry using FITC-dextran analysis (Ibeas *et al.* 2009). THP-1 macrophages were incubated with either 1 μg/mL $sPLA_2$-IIA, or $sPLA_2$-IIA in the presence of increasing concentrations of maslinic acid (5, 10, 20 and 50 μM). After 72 h, cells were suspended in culture medium with 1 mg/mL FITC-dextran (MW 40000) and incubated at 4 °C (for background control) or at 37 °C for 30 min. Then, cells were washed with ice cold PBS containing 1% fetal bovine serum, and the FITC-dextran incorporated was analyzed by flow cytometry. The endocytic activity is expressed as the mean fluorescence intensity (MFI) index. MFI index is calculated as fluorescence of FITC-positives cells at 37 °C minus fluorescence of FITC-positive cells at 4 °C and normalized at 100 for the control non-treated cells.

2.8 *ELISA and COX-2 activity determination*

Next, the effect of maslinic acid in suppressing $sPLA_2$-IIA enzyme-induced production of inflammatory mediators TNF-α and IL-6 production, COX-2 activity and PGE_2 was determined. THP-1 macrophages (2×10^5 cells/mL) were either non-treated, treated with 1 μg/mL $sPLA_2$-IIA, or treated with $sPLA_2$-IIA along with various concentrations (5, 10, 20 and 50 μM) of maslinic acid for 24 h. After cell treatment, medium was harvested and stored at −80 °C prior to TNF-α, IL-6 and PGE_2 assay. Cells were lysed in wells by resuspension in ice cold lysis buffer prepared as described earlier (Bryant *et al.* 2011). Lysates were stored at −80 °C prior to protein determination (Bio-Rad, DC Protein Assay, Bio-Rad). ELISA was performed for TNF-α and Il-6 according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, USA). PGE_2 in medium was measured with competitive immunoassay kits according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, USA) and expressed as picograms of PGE_2/mL. COX-2 activity was assessed using COX Activity Assay Kit (Cayman Chemical, Michigan, USA).

2.9 *Statistical analysis*

Statistical analyses were performed using SPSS (version 21). One-way analysis of variance (ANOVA) and *post hoc*

Tukey's test were used to evaluate the significance difference between each treatment groups.

3. Results

3.1 *Maslinic acid inhibits sPLA$_2$-IIA-mediated macrophage foam cells formation*

It is hypothesized that sPLA$_2$-IIA enzyme might induce LDL modification which enhances lipid accumulation within macrophages. As shown in figure 1A, THP-1 macrophages incubated with sPLA$_2$-IIA-modified LDL induces significant intracellular lipid droplet accumulation as compared to cells without any treatment or cells treated with native LDL alone while incubation with increasing concentrations of maslinic acid inhibits this effect. It is suggested that maslinic acid works by inhibiting sPLA$_2$-IIA-mediated LDL oxidation, thereby resulting in lower foam cells formation. Results shown that sPLA$_2$-IIA treatment only induced modest increase in LDL oxidation (4.34 nmol MDA/mg protein) compared to native LDL (2.043 nmol MDA/mg protein) while pro-oxidizing agent $CuSO_4$ treatment (98.06 nmol MDA/mg protein) significantly enhanced LDL oxidation (supplementary figure 3). It indicates that sPLA$_2$-IIA-induced LDL modification occurred independently of their oxidation levels and that anti-oxidative effects of maslinic acid do not play a part in reducing sPLA$_2$-IIA-mediated macrophage lipid accumulation levels observed. Further study was performed to examine whether incubation of THP-1 macrophages with sPLA$_2$-IIA enzyme alone could induce lipid droplet accumulation in THP-1 macrophages. Interestingly, exogenous addition of sPLA$_2$-IIA enzyme (figure 1B) also shown to enhance lipid droplet accumulation and incubation with increasing concentrations of maslinic acid ameliorates this effect.

3.2 *Inhibition of sPLA$_2$-IIA-induced endocytosis by maslinic acid*

It is proposed that sPLA$_2$-IIA might enhance uptake of modified LDL or lipid droplet formation by altering the cells' endocytic capacity. THP-1 macrophages treated with sPLA$_2$-IIA in the presence of increasing concentrations of maslinic acid were incubated with FITC-dextran, followed by flow cytometry analysis. Flow cytometric analysis demonstrated that sPLA$_2$-IIA induced specific uptake of FITC-Dextran in THP-1 macrophages (figure 2B) compared to non-treated control cells (figure 2A) while incubation with 50 μM (figure 2C) maslinic acid suppressed the effects observed. Analysis of the mean fluorescence intensity (MFI) index of endocytosis (figure 2D) showed that increasing concentrations (5, 10, 20 and 50 μM) of maslinic acid inhibit sPLA$_2$-IIA-induced endocytosis in THP-1 macrophages.

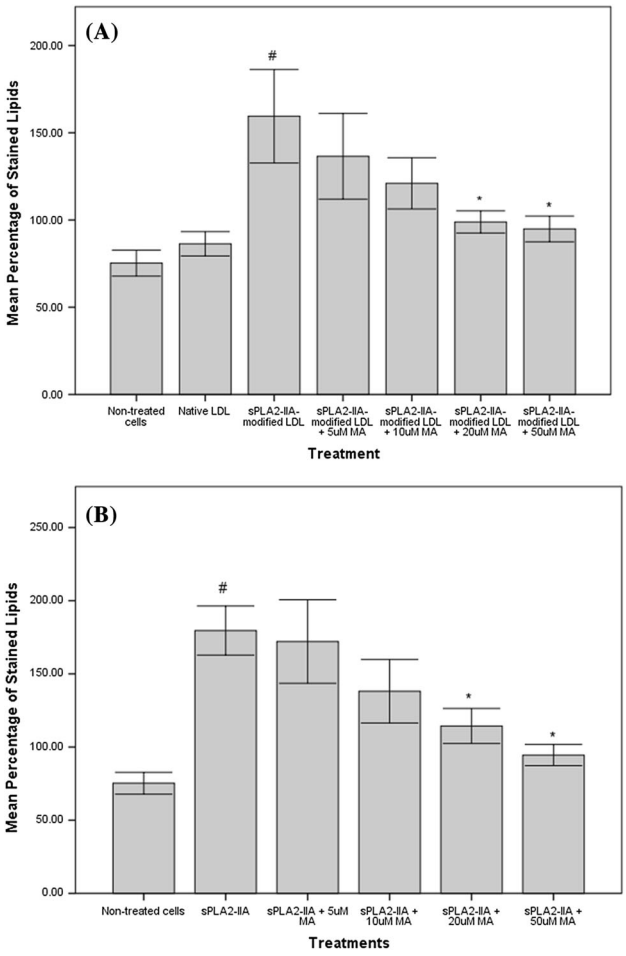

Figure 1. Effects of maslinic acid on sPLA$_2$-IIA-induced macrophage lipid accumulation. (**A**) THP-1 macrophages were either non-treated, incubated with native LDL or sPLA$_2$-IIA-modified LDL in the presence or absence of 5, 10, 20 or 50 μM maslinic acid. (**B**) THP-1 macrophages were either non-treated, incubated with sPLA$_2$-IIA enzyme alone or in the presence or absence of 5, 10, 20 or 50 μM maslinic acid. ORO staining procedure was performed to quantify the mean percentage of stained lipids. Images were taken with NIS Element AR 3.2 software and processed using Image J software. Each bar represents mean of triplicate measurements of three independent experiment ± standard deviation (n = 3). Statistical significance was determined by One-way ANOVA followed by a Tukey's test where several experimental groups were compared to the control group. # represents $p<0.05$ compared to non-treated or native LDL groups; * represents $p<0.05$ compared to sPLA$_2$-IIA-modified LDL or sPLA$_2$-IIA treatment.

3.3 *Maslinic acid lowers PGE$_2$ concentration in sPLA$_2$-IIA-induced treatment without affecting COX-2 activity and inflammatory cytokines TNF-α and IL-6 levels*

The activity sPLA$_2$-IIA enzyme leads to the generation of AA, an essential substrate in the production of anti-inflammatory prostaglandins like PGE$_2$ through the action of COX-2. Hence, the effect of maslinic acid in inhibiting COX-2 activity

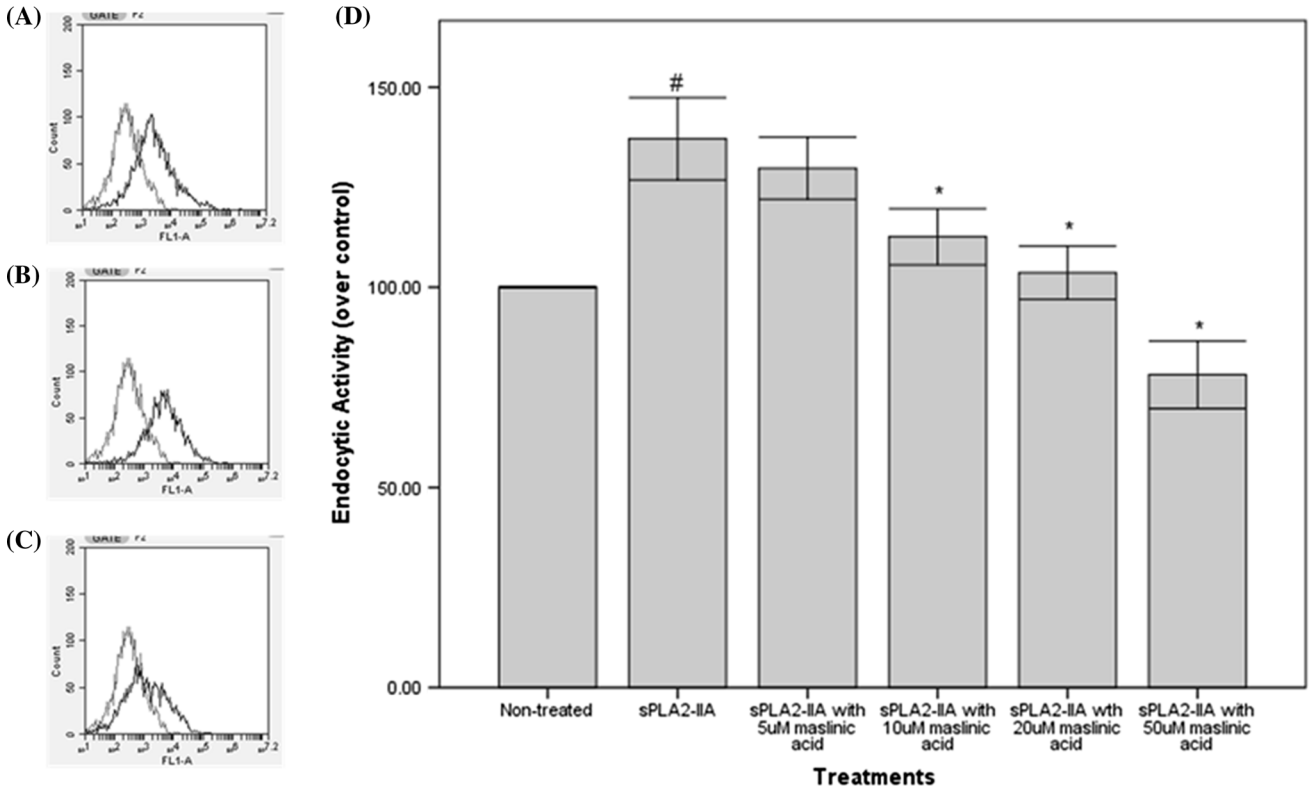

Figure 2. Effects of maslinic acid on sPLA$_2$-IIA-induced endocytic activity. THP-1 macrophages were either untreated (**A**), treated with 1 μg/mL sPLA$_2$-IIA (**B**), or 1 μg/mL sPLA$_2$-IIA in the presence of 50 μM maslinic acid for 72 h (**C**). Then, the THP-1 macrophages were incubated with FITC-dextran for 30 min and analyzed by flow cytometry. Histograms represent experiment out of three (**A**–**C**). Light curves represent unspecific endocytosis and dark curves signify endocytosis of cells cultured as indicated. Endocytic activity (**D**) is expressed as mean fluorescence intensity index ± SD normalized by setting controls to 100%. # represents $p<0.05$ compared to non-treated cells; * represents $p<0.05$ compared to sPLA$_2$-IIA-treated cells.

and PGE$_2$ production upon activation by sPLA$_2$-IIA was also investigated. COX-2 activity remained unchanged upon sPLA$_2$-IIA stimulation (figure 3) and PGE$_2$ levels in sPLA$_2$-IIA-treated THP-1 macrophages were significantly higher compared to non-treated cells while incubation with maslinic acid suppressed the effects observed (figure 4). These results suggest that sPLA$_2$-IIA enzyme activity may increase AA mobilization that is coupled to PGE$_2$ production without affecting COX-2 activity. To further investigate the anti-inflammatory effects of maslinic acid in sPLA$_2$-IIA-stimulated cells, the secretion of inflammatory cytokines TNF-α and IL-6 were measured. As shown in supplementary figure 4, both the secretion of TNF-α and IL-6 were not significantly different from non-treated cells, indicating that sPLA$_2$-IIA, in macrophages, does not affect cytokine production.

4. Discussion

Previous studies have established the immuno-modulatory function of sPLA$_2$-IIA in mediating monocyte recruitment and differentiation (Ibeas *et al.* 2009). Recruitment of immune cells to the atherosclerotic lesions, where they transform into macrophages or dendritic cells amplifies the inflammatory response in that area. Macrophages accumulated in the sub-endothelial intima layer may ingest oxidized lipoproteins and transformed into lipid-rich foam cells, thereby contributing to the physical bulk of developing plaques (Hilgendorf *et al.* 2015). We showed that treatment with maslinic acid significantly reduces foam cell formation in THP-1 macrophages incubated with sPLA$_2$-IIA-modified LDL. Given that oxidative modification of LDL is recognized to be important for the sub-endothelial retention of LDL, causing LDL aggregation and foam cell formation, we hypothesized that the anti-oxidative effects of maslinic acid might contribute to the reduced sPLA$_2$-IIA-induced LDL oxidation, thereby suppressing foam cells formation. Nevertheless, sPLA$_2$-IIA only showed modest LDL oxidation compared to that of $CuSO_4$, a common oxidizing agent, indicating that sPLA$_2$-IIA did not alter oxidation modification in its effect in inducing foam cells formation. Although sPLA$_2$-IIA has been shown to cause minimal oxidative modification of LDL in vitro, studies indicated that this enzyme can cause oxidative modification of LDL by the

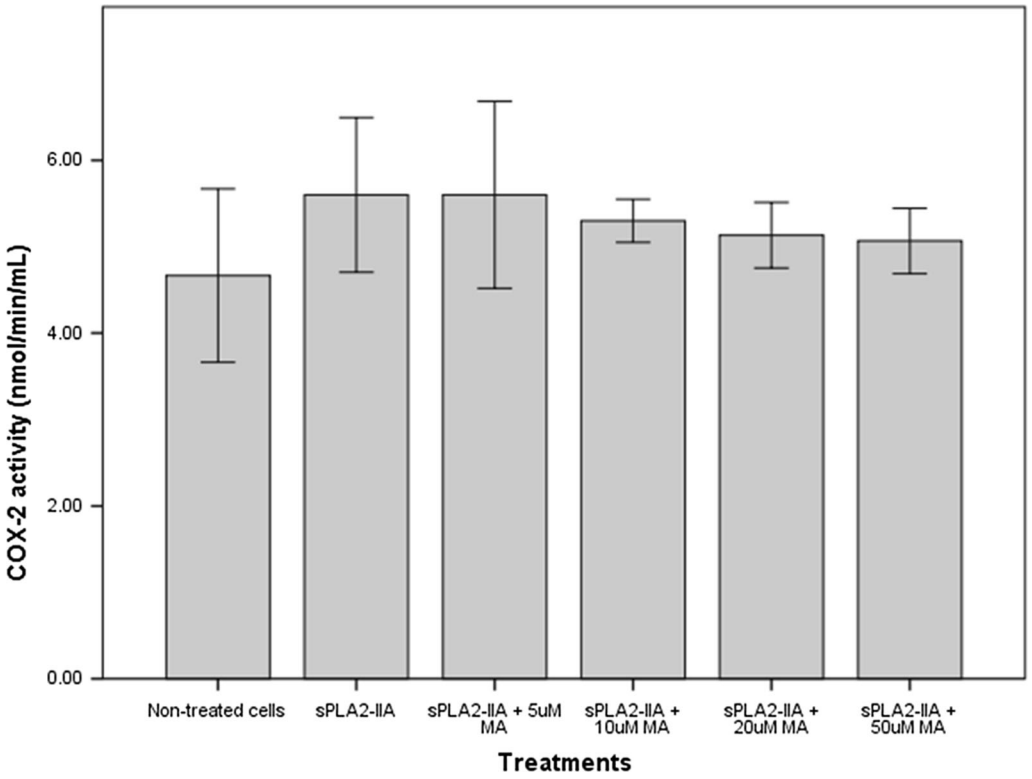

Figure 3. Effects of maslinic acid on $sPLA_2$-IIA-induced COX-2 activity. THP-1 macrophages were either non-treated, treated with 1 μg/mL $sPLA_2$-IIA, or 1 μg/mL $sPLA_2$-IIA in the presence of 5, 10, 20, and 50 μM maslinic acid for 24 h. Cell lysates were harvested and COX-2 activity was measured using COX activity assay kit. Each bar represents mean of triplicate measurements of three independent experiment ± standard deviation (n=3). Statistical significance was determined by One-way ANOVA followed by a Tukey's test where several experimental groups were compared to the control group.

cooperative action with 12/15-LOX that is present in the atherogenic lesions in vivo (Sparrow *et al.* 1988; Yla-Herttuala *et al.* 1990; Neuzil *et al.* 1998). Considering that maslinic acid is known for its copper-chelating effects, its effect on the synergistic action of oxidation and $sPLA_2$ enzymes in LDL modification deserves further examination.

Interestingly, our findings also demonstrated that incubation of THP-1 macrophages with $sPLA_2$-IIA enzyme alone is capable of causing intracellular lipid droplet accumulation in THP-1 macrophages. Conventional $sPLA_2$ enzymes (Group IIA, V and X) have been shown to modify extracellular non-cellular lipid components such as lipoproteins, lung surfactant, microparticles and produce lipid mediators from cellular membranes (Murakami *et al.* 2010). It is possible that $sPLA_2$-IIA hydrolyzes phosphatidylcholine (PC) in cell membranes to release lyso-PC (LPC) and free fatty acids, thereby facilitating the accumulation of lipids in macrophages (Ghesquiere *et al.* 2005). Although $sPLA_2$-IIA enzyme is 20 times less reactive compared to other enzymes in the $sPLA_2$ family such as $sPLA_2$-V and $sPLA_2$-X in hydrolyzing PC-rich vesicles and PC-rich cellular plasma membranes, some studies showed that $sPLA_2$-IIA could hydrolyze lipoprotein-bound PC to certain extent (Hurt-Camejo *et al.* 1997; Pruzanski *et al.* 1998). Considering the expression level of $sPLA_2$-IIA is considerably higher than those of other $sPLA_2$s and it is the only $sPLA_2$ isoform detected in the circulation of mammals, it is plausible that $sPLA_2$-IIA participates in releasing oxidized phospholipids in vivo. This study is the first to report on the capability of enzyme $sPLA_2$-IIA at inducing lipid accumulation in THP-1 macrophages.

Macrophages are known to take up modified LDL via receptors or fluid-phase endocytosis. The main pathway for excessive lipid accumulation occurs through CD36, scavenger receptor-A (SR-A), oxidized LDL receptor and several other receptors (Kunjathoor *et al.* 2002; Mehta 2004). Meanwhile non-modified native LDL can also enter macrophages by macro- and micropinocytosis (Kruth *et al.* 2005). Our results showed that incubation of THP-1 macrophages with $sPLA_2$-IIA enhanced THP-1 macrophage endocytic capacity. Previous studies have shown that $sPLA_2$-IIA stimulate activation of macrophages, similar to that observed with the effects stimulated by combination of PMA and IL-4 (Ibeas *et al.* 2009). It is proposed that $sPLA_2$-IIA may induce a dendritic cell-like phenotype, acting as an amplifier of the inflammatory responses and enabling these

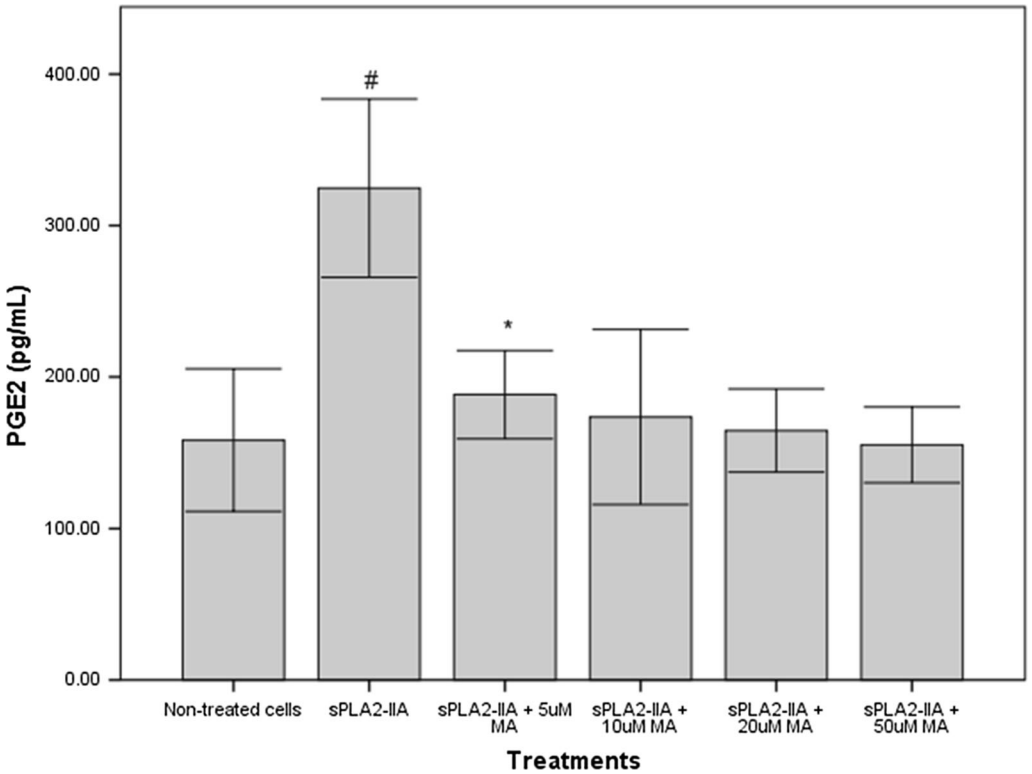

Figure 4. Effects of maslinic acid on $sPLA_2$-IIA-induced PGE_2 production. THP-1 macrophages were either non-treated, treated with 1 μg/mL $sPLA_2$-IIA, or 1 μg/mL $sPLA_2$-IIA in the presence of 5, 10, 20, and 50 μM maslinic acid for 24 h. PGE_2 levels in medium was quantified by ELISA. Each bar represents mean of triplicate measurements of three independent experiment ± standard deviation (n = 3). Statistical significance was determined by One-way ANOVA followed by a Tukey's test where several experimental groups were compared to the control group. # represents $p<0.05$ compared to non-treated cells; * represents $p<0.05$ compared to $sPLA_2$-IIA-treated cells.

cells to present antigens and support an immune response. Together with prior studies, our study confirmed that apart from contributing to LDL modification, $sPLA_2$-IIA itself also promotes phenotypic modifications including their endocytic capability, on THP-1 macrophages. We hypothesized that increased endocytic capacity observed might represent a potential mechanism which facilitates $sPLA_2$-IIA-induced macrophage lipid accumulation as observed in the current study.

Hydrolytic activity of $sPLA_2$ enzymes can induce potent release of AA leading to COX-dependent prostaglandin formation, as well as marked production of LPC while aberrant expression of $sPLA_2$-IIA serve as a positive regulator of cytokine-mediated inflammation. Exogenous addition of $sPLA_2$-IIA to cultured rheumatoid arthritis synovial cells has been shown to enhance PGE_2 production and up-regulation of the inducible cyclooxygenase, COX-2 expression (Bidgood *et al.* 2000). In this study, we showed that maslinic acid inhibit $sPLA_2$-IIA-induced PGE_2 production but did not alter COX-2 activity. Studies have shown that in the presence of $sPLA_2$-IIA alone, provision of AA to COX-2 by $cPLA_2$-α is the rate-limiting step in PGE_2 production. In contrast, in the presence of TNF, PGE_2 production is limited by the amount of COX-2 (Bryant *et al.* 2011). Thus the rate limiting step in the pathway may be either $cPLA_2$-α or COX-2 depending on the cellular context. Meanwhile, we also showed that maslinic acid does not affect TNF-α and IL-6 cytokine secretion levels in THP-1 macrophages treated with $sPLA_2$-IIA alone. Other studies have shown that TNF-α and IL-6 is enhanced only upon LPS stimulation (Curfs *et al.* 2008).

The findings in our current study showed that both $sPLA_2$-IIA-modified LDL and $sPLA_2$-IIA enzyme alone are capable of producing foam cells effect in THP-1 macrophages. $sPLA_2$ are 'secreted' enzymes that act on extracellular substrates and they exert their effects mainly by hydrolyzing cellular and non-cellular phospholipids in the presence of millimolar concentrations of Ca^{2+} or by binding to transmembrane ligands or soluble receptors (Murakami *et al.* 2010). Our previous study showed that maslinic acid inhibits the access of catalytic Ca^{2+} required for $sPLA_2$-IIA enzymatic reaction and inhibits its binding to membrane phospholipid by interacting with the interfacial residues of the enzyme (Yap *et al.* 2016). Other studies showed that maslinic acid binds to transmembrane domains and competes with cholesterol for the hydrogen-bonded ester carbonyl groups, disturbing the

localization and the physiological function of membrane-related proteins (Prades *et al.* 2011). Hence, there are possibilities that maslinic acid targets $sPLA_2$-IIA enzymatic activity itself and or act as inhibitor that block the direct binding of $sPLA_2$-IIA to membrane ligands, inhibiting the action of $sPLA_2$-IIA on LDL and THP-1 macrophages cellular membrane phospholipids, thereby suppressing $sPLA_2$-IIA-induced foam cell formation.

5. Conclusion

This study showed that maslinic acid suppressed $sPLA_2$-IIA-induced macrophage lipid accumulation and PGE_2 secretion without affecting COX-2 activity and inflammatory cytokines TNF-α and IL-6 production in THP-1 macrophages. Nevertheless, its mechanism of action is not fully understood. Further investigation into the regulatory effects of maslinic acid in $sPLA_2$-IIA-induced macrophage lipid metabolism remains to be determined.

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