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A Lipidomic Perspective of the Action of Group IIA Secreted Phospholipase A_2 on Human Monocytes: Lipid Droplet Biogenesis and Activation of Cytosolic Phospholipase $A_2\alpha$

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Abstract: Phospholipase A_2s constitute a wide group of lipid-modifying enzymes which display a variety of functions in innate immune responses. In this work, we utilized mass spectrometry-based lipidomic approaches to investigate the action of Asp-49 Ca^{2+}-dependent secreted phospholipase A_2 (sPLA$_2$) (MT-III) and Lys-49 sPLA2 (MT-II), two group IIA phospholipase A_2s isolated from the venom of the snake *Bothrops asper*, on human peripheral blood monocytes. MT-III is catalytically active, whereas MT-II lacks enzyme activity. A large decrease in the fatty acid content of membrane phospholipids was detected in MT III-treated monocytes. The significant diminution of the cellular content of phospholipid-bound arachidonic acid seemed to be mediated, in part, by the activation of the endogenous group IVA cytosolic phospholipase $A_2\alpha$. MT-III triggered the formation of triacylglycerol and cholesterol enriched in palmitic, stearic, and oleic acids, but not arachidonic acid, along with an increase in lipid droplet synthesis. Additionally, it was shown that the increased availability of arachidonic acid arising from phospholipid hydrolysis promoted abundant eicosanoid synthesis. The inactive form, MT-II, failed to produce any of the effects described above. These studies provide a complete lipidomic characterization of the monocyte response to snake venom group IIA phospholipase A_2, and reveal significant connections among lipid droplet biogenesis, cell signaling and biochemical pathways that contribute to initiating the inflammatory response.

Keywords: phospholipase A_2; lipidomics; mass spectrometry; lipid signaling; inflammation; monocytes/macrophages

1. Introduction

The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids. The hydrolysis products of this reaction, free fatty acid and lysophospholipid, serve as precursors for a variety of bioactive lipid mediators with important biological roles [1]. The PLA_2s are systematically classified according to sequence homology criteria, and include 16 groups (I-XVI), most of them with several subgroups, comprising more than 30 proteins [1]. An alternative classification also exists that groups these enzymes into six major classes on the basis of biochemical similarities and/or cell regulation properties. These are the Ca^{2+}-dependent cytosolic PLA_2s, the Ca^{2+}-dependent secreted PLA_2s ($sPLA_2$), the Ca^{2+}-independent cytosolic PLA_2s, the platelet-activating factor acetyl hydrolases, the lysosomal PLA_2, and the adipose-specific PLA_2 [2].

The $sPLA_2$ family represents the largest class of PLA_2 enzymes and possesses, as a common motif, a conserved His-Asp catalytic dyad [3]. $sPLA_2$s are widely distributed in pancreatic secretions, inflammatory exudates, and also in arthropod and snake venoms. A variety of biological activities have been described for $sPLA_2$s, including digestive actions, toxic activities (neurotoxic, myotoxic, hypotensive, etc.) and immune roles. In this regard, group IIA $sPLA_2$ was defined as a pro-inflammatory PLA_2, since its gene induction and synthesis were observed after cell stimulation by endotoxin and cytokines [3–5]. In contrast, another member of the family, the group V enzyme, is described as anti-inflammatory in some models [6–8].

$sPLA_2$s have been often observed to cooperate with other PLA_2s in eliciting certain biological responses. A prominent example of this is the mobilization of arachidonic acid (AA) and attendant eicosanoid production by innate immune cells responding to inflammatory stimuli [9–12]. The Ca^{2+}-dependent cytosolic group IVA PLA_2 ($cPLA_2\alpha$) is the essential enzyme in this process [12–14]. Depending on cell type and stimulation conditions, regulatory crosstalk mechanisms exist between $cPLA_2\alpha$ and other $sPLA_2$ enzymes present in the cells—in particular, those belonging to groups IIA, V and X—which results in the amplification of the AA mobilization response [15–20].

Previous work from our laboratory has utilized advanced mass spectrometry approaches to characterize multiple aspects of PLA_2-mediated phospholipid fatty acid remodeling in cells of the innate immune system such as monocytes and macrophages [21–28]. In these studies, the activation mechanisms of multiple PLA_2 enzymes expressed by the cells were characterized [21–28]. However, no approaches were undertaken to characterize the cellular responses to exogenously added $sPLA_2$ enzymes. It has been suggested that the response of cells exposed to exogenous $sPLA_2$ is dependent upon the nature of the lipid mediator generated on the membrane where the $sPLA_2$ acts [29]. However, it is also known that some $sPLA_2$s lack catalytic activity but still exert potent biological actions [30]. This has led to the proposal that some $sPLA_2$ effects depend on protein–protein or protein–glycan interactions [3,31]. Once bound to its target(s) on the membrane, $sPLA_2$s may exert their actions via activity-independent mechanisms that affect cellular functions or trigger a cellular response [3,31,32]. Additionally, the existence of a $sPLA_2$ receptor for these enzymes has long been proposed [33]. Whether activity-based effects prevail over activity-independent effects, or both occur simultaneously, remains unclear.

Exposure of immune cells to exogenous $sPLA_2$ occurs in numerous pathological situations such as inflammatory syndromes, sepsis, autoimmune diseases, and even bite or sting envenomations [3–5]. In this work, we have used advanced mass spectrometry-based lipidomics to analyze the effect of two different group IIA $sPLA_2$, with and without catalytic activity, on human monocytes. The $sPLA_2$ enzymes utilized in this study, termed Asp-49 $sPLA_2$ (MT-III) and Lys-49 $sPLA_2$ (MT-II), were purified from the venom of the Central American snake *Bothrops asper*. MT-III is a catalytically active enzyme similar to human synovial group IIA PLA_2. MT-II is identical to MT-III, except for the replacement of Asp49 with Lys49 within the active site, which renders it catalytically inactive [34–38]. The combined use of these two PLA_2s thus constitutes an excellent tool to distinguish between the activity-dependent and -independent actions of $sPLA_2$ enzymes. Consistent with the previously described proinflammatory properties of MT-III, our data show that it promotes remarkable changes in the lipid composition of cell membranes, triggers lipid droplet biogenesis, and induces eicosanoid

synthesis. None of these actions are induced by the inactive form MT-II. These data agree with previous work demonstrating that MT-III, but not MT-II induces phospholipid hydrolysis in murine muscle cells [38].

2. Materials and Methods

2.1. Enzymes

Asp-49 sPLA2 (MT-III) and Lys-49 sPLA2 (MT-II) from Bothrops asper venom were purified by ion-exchange chromatography on CM-Sephadex C-50, using a KCl gradient from 0 to 0.75 M [36]. The complete amino acid sequence and toxicological profile of these enzymes have been previously described in detail [37]. The absence of endotoxin contamination in the batches used was demonstrated by performing the quantitative Limulus amebocyte lysate assay [39], which revealed no detectable levels of endotoxin (< 0.125 EU/mL).

2.2. Cell Culture

Human monocytes were isolated from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Written informed consent was obtained from each donor. Briefly, blood cells were diluted 1:1 with phosphate-buffered saline, layered over a cushion of Ficoll-Paque, and centrifuged at 750 g for 30 minutes. The mononuclear cellular layer was recovered and washed three times, resuspended in RPMI 1640 medium supplemented with 40 μg/mL gentamicin, and allowed to adhere in sterile dishes for 2 h at 37 °C in a humidified atmosphere of CO_2/air (1:19). Nonadherent cells were removed by washing extensively with phosphate-buffered saline, and the remaining attached monocytes were used the following day [40,41]. For experiments, subconfluent cell monolayers were incubated with serum-free medium for 1 h before the addition of sPLA2. After stimulation, the monocyte monolayers were washed twice with phosphate-buffered saline, scraped with a cell scraper, sonicated with a tip homogenizer twice for 15 s, and prepared for their further analysis by mass spectrometry, as described below. For eicosanoid determinations, supernatants were collected and prepared for mass spectrometry analysis as described below.

2.3. Cellular Staining and Fluorescence Microscopy

For these experiments, the cells were plated on coverslips on the bottom of 6-well dishes in a volume of 2 mL. The cells were fixed with 1 mL of 4% paraformaldehyde in phosphate-buffered saline containing 3% sucrose for 20 minutes. Afterward, paraformaldehyde was removed by washing the cells three times with phosphate-buffered saline, and Nile Red and 4′,6′-diamidino-2-phenylindole (DAPI) stainings were carried out by treating cells with these dyes at concentrations of 5 μg/mL and 1 μg/mL, respectively, in phosphate-buffered saline for 10 minutes. Coverslips were mounted on microscopy slides with 25 μL of a polyvinyl alcohol solution until analysis by fluorescence microscopy. Fluorescence was monitored by microscopy using a NIKON Eclipse 90i device equipped with a CCD camera (model DS-Ri1; Nikon, Tokyo, Japan). A mercury HBO excitation lamp (Osram, Munich, Germany) was used, and the fluorescence was recovered using the combination of a UV-2A (Ex 330–380; DM 400; BA 420) and a B-2A (Ex 450–490; DM 505; BA 520) filter, respectively. Images were analyzed with the software NIS-Elements (Nikon). Red and blue channels were merged with the Image-J software (version 1.52a).

2.4. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of Fatty Acid Methyl Esters

Total lipids from approximately 10^7 cells were extracted according to Bligh and Dyer [42]. For separation of total phospholipids from neutral lipids, the following internal standards were added: 10 nmol of 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 10 nmol of 1,2,3-triheptadecanoylglycerol, 20 nmol of nonadecanoic acid, and 30 nmol of cholesteryl tridecanoate. Phospholipids were separated from neutral lipids by thin-layer chromatography, using *n*-

hexane/diethyl ether/acetic acid (70:30:1, *v*/*v*/*v*) as the mobile phase [43]. For separation of phospholipid classes, the following internal standards were added: 20 pmol each of 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, and 1,2-dinonadecanoyl-sn-glycero-phosphoinositol. Phospholipids were separated twice with chloroform/methanol/28% (*w*/*w*) ammonium hydroxide (60:37.5:4, *v*/*v*/*v*) as the mobile phase, using plates impregnated with boric acid [44]. The bands corresponding to the different lipid classes were scraped from the plate, and fatty acid methyl esters were obtained from the various lipid fractions by transmethylation with 0.5 M KOH in methanol for 60 minutes at 37 °C [45–49]. Analyses were carried out using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in an electron impact mode (EI, 70 eV). (Agilent Technologies, Santa Clara, CA, USA). Data acquisition was carried out both in scan and selected ion monitoring mode. Scan mode was used for compound identification, comparing with authentic fatty acid methyl ester standards, and the National Institute of Standards and Technology MS library spectra. Selected ion monitoring mode was used for quantitation, using 74 and 87 fragments for saturated, 83 for monounsaturated, 67 and 81 for diunsaturated, and 79 and 91 for polyunsaturated fatty acid methyl esters. A 37-component mixture (Supelco, Sigma-Aldrich, Madrid, Spain) was used for calibration curves.

2.5. Mass Spectrometry Analysis of Free Fatty Acids

The thin layer chromatography spots corresponding to the non-esterified free fatty acid fraction were scraped, redissolved in *n*-hexane, and analyzed separately in an Agilent 1260 Infinity high-performance liquid chromatograph coupled to an API2000 triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). The column was a Supelcosil LC-8 (150 × 3 mm, 3 μm particle size), protected with a Supelguard LC-8 (20 × 3 mm) guard cartridge (Sigma-Aldrich). The mobile phase was used on a gradient of solvent A (methanol with 0.01% ammonium hydroxide) and solvent B (water with 0.01% ammonium hydroxide). The gradient was started at 60% solvent A and 40% solvent B. The former was linearly increased to 95% at 10 minutes, and held at 95% solvent A until 18 minutes. The initial solvent mixture (60%, 40%B) was recovered at 20 minutes and the column was re-equilibrated for an additional 5 minutes before the injection of next sample. The flow rate was fixed at 400 μL/min. Non-esterified fatty acid fraction, extracted from silica plates and filtered, was re-dissolved in 100 μL of methanol/water 60:40 *v*/*v* and 90 μL were injected into the high-performance liquid chromatograph. The parameters for electrospray ionization source of mass spectrometer were set as follows: Ion spray voltage, −4500 V; CUR, 20 psi; GS1, 40 psi; GS2, 80 psi; TEM, 525 °C. The analyzer mode was set to Q1MS (DP, −70 V; EP, −10 V; FP, −300 V) performing a m/z scan between 100 and 400 with a step size of 0.1 amu. Non-esterified fatty acids were detected as $[M-H]^-$ ions using the Analyst 1.5.2 software version (Applied Biosystems, Carlsbad, CA, USA), and chromatographic peaks were quantified by comparison with peaks of authentic analytical standards.

2.6. Liquid Chromatography/Mass Spectrometry (LC/MS) Analyses of Phospholipids

This was carried out exactly as described elsewhere [21–26,50,51], using a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck), coupled on-line to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Ethanolamine-containing phospholipids (PE) and phosphatidylinositol (PI) species were detected in negative ion mode as $[M-H]^-$ ions in MS experiments. Choline-containing phospholipids (PC) species were detected in positive ion mode, as $[M+H]^+$ ions by MS. Acyl chains in PI and PE species were identified by multiple reaction monitoring MS^2 experiments on chromatographic effluent by comparison to previously published data [21–26,50,51]. For the identification of acyl chains in PC species, ionization was carried out in negative mode with the post-column addition of acetic acid at a flow rate of 100 mL/h as $[M+CH_3CO_2]^-$ adducts, and acyl chains were identified by MS^3 experiments. Quantification was carried out by

integrating the chromatographic peaks of the previously identified phospholipid species and comparing with an external calibration curve made with authentic standards.

2.7. Liquid Chromatography/Mass Spectrometry (LC/MS) Analyses of Eicosanoids

Analysis of eicosanoids by LC/MS was carried out exactly as described elsewhere [6,25,52], using an Agilent 1260 Infinity high-performance liquid chromatograph coupled to an API2000 triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). Quantification was carried out by integrating the chromatographic peaks of each species and by comparing with an external calibration curve made with analytical standards [6,25,52].

2.8. Immunoblot

Cells were lysed with 20 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 0.5% Triton X-100, 1 mM Na_3VO_4, 150 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma-Aldrich, Madrid, Spain) at 4 °C. Homogenates were then clarified by centrifugation at 13,000× *g* for 10 minutes. Protein from the supernatants was quantified according to Bradford [53], and 100 μg of protein was analyzed by immunoblot using an antibody specific for the phosphorylated form of cPLA2α at Ser505 (Cell Signaling, Danvers, MA, USA) [54,55]. The detection of immunoreactive bands was conducted by chemiluminescence (ECLTM, Amersham Biosciences, Little Chalfont, UK).

3. Results

The two group IIA sPLA2s utilized in this study are from *Bothrops asper* venom and differ in a natural mutation at position 49. Asp49-sPLA2, with catalytic activity, was named MT-III; Lys-49-sPLA2, devoid of catalytic activity, was named MT-II [34–38]. When added to human monocytes, catalytically active MT-III, at concentrations not compromising cell viability (0.4 μg/mL), promoted an extensive loss of phospholipid-bound fatty acids, as measured by GC/MS (Figure 1).

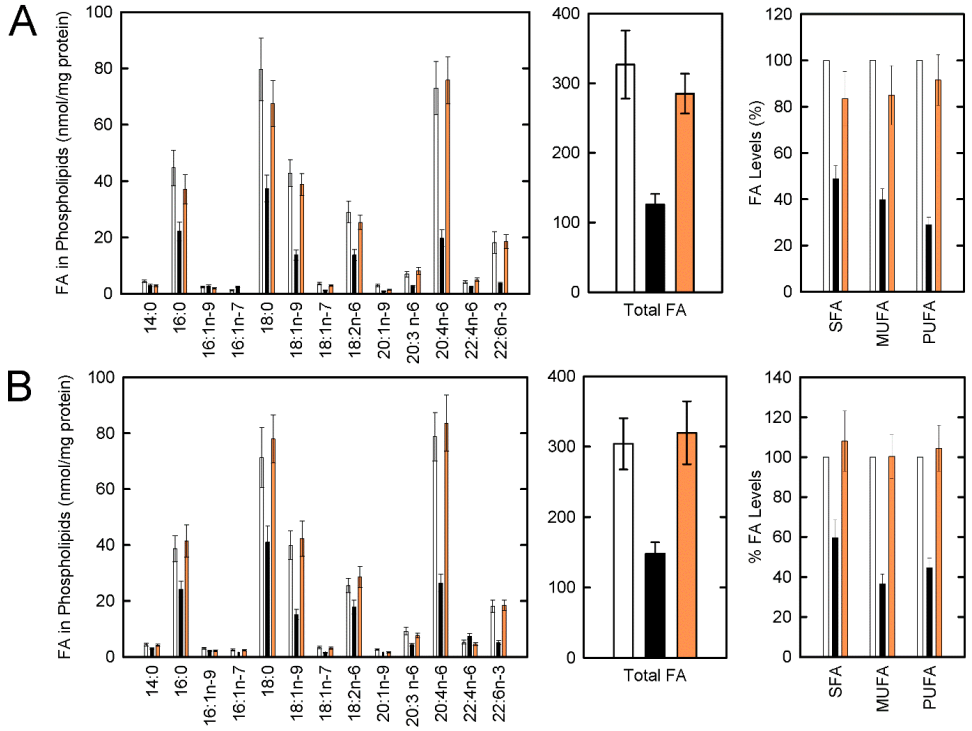


Figure 1. Phospholipid fatty acid content of human monocytes. The cells were either untreated (open bars) or treated with Asp-49 Ca^{2+}-dependent secreted phospholipase A_2 (sPLA2) (MT-III) (black bars) or Lys-49 sPLA2 (MT-II) (orange bars) for 1 h (**A**) or 6 h (**B**). Afterward, phospholipids were isolated

and their fatty acid content was measured by GC/MS. The profile of fatty acids, the total phospholipid fatty acid amount, and distribution according to the number of double bonds is given. Fatty acids are designated by their number of carbon atoms and, after a colon, their number of double bonds. To differentiate isomers, the *n*−x (*n* minus x) nomenclature is used, where *n* is the number of carbons of a given fatty acid, and x is an integer which, subtracted from *n*, gives the position of the last double bond of the molecule. The data are expressed as mean values ± standard error of three independent determinations. Fatty acid (FA); saturated fatty acid (SFA); monounsaturated fatty acid (MUFA); polyunsaturated fatty acid (PUFA).

Despite the relatively high phospholipid hydrolysis rates detected in these experiments, cell viability always remained above 90%, as assessed by the MTT assay [56–58]. The action of MT-III was prominent on all kinds of fatty acids, including saturated, monounsaturated, and polyunsaturated. Importantly, no significant differences were observed between unstimulated control cells and MT-II-treated cells at any time tested. Since there was little difference in phospholipid hydrolysis between 1 h and 6 h, a 1 h time point was chosen to be employed in all subsequent experiments. The marked decrease in cellular AA (20:4n−6) levels after treating the monocytes with MT-III, as shown in Figure 1, was striking. Given the key role of AA in inflammatory reactions as a precursor of eicosanoids, we set out to characterize further the effect of MT-III on this particular fatty acid. Figure 2 shows the profile of AA-containing glycerophospholipid species of human monocytes, as measured by LC/MS.

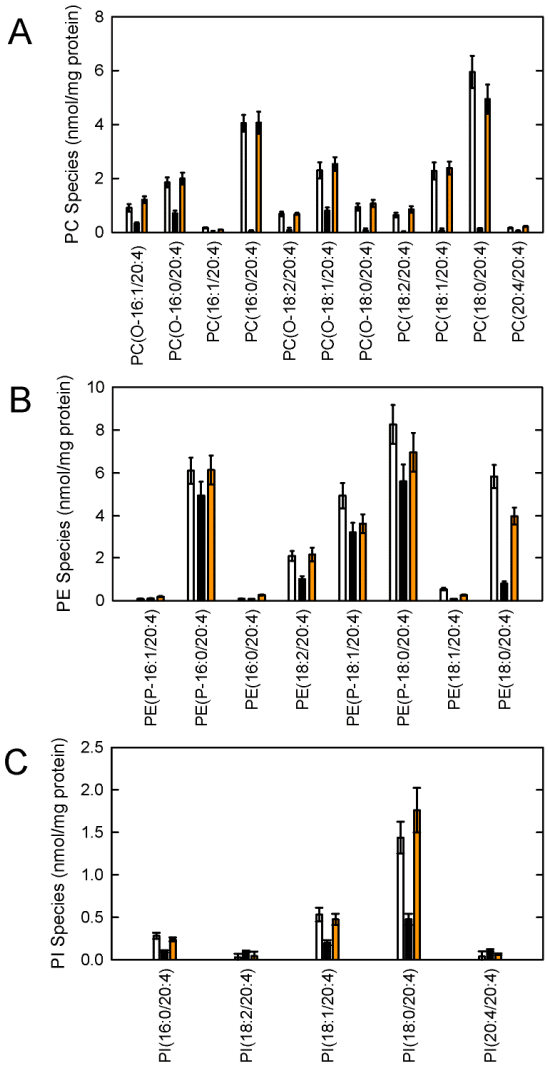


Figure 2. Arachidonic acid (AA)-containing phospholipid species of human monocytes. The cells were either untreated (open bars) or treated with MT-III (black bars) or MT-II (orange bars) for 1 h. Afterward, the distribution profile of AA between choline-containing phospholipids (PC) (**A**), ethanolamine-containing phospholipids (PE) (**B**), and phosphatidylinositol (PI) (**C**) was determined by LC/MS. Fatty chains within the different phospholipid species are designated by their numbers of carbons and double bonds. A designation of O- before the first fatty chain indicates that the sn-1 position is ether linked, whereas a *p*- designation indicates a plasmalogen form (sn-1 vinyl ether linkage). The data are expressed as mean values ± standard error of three independent determinations.

Treatment of the monocytes with MT-III, but not MT-II, resulted in a marked decrease in the total cellular content of AA-containing PC and AA-containing PI (Figure 2). Note that some of the most abundant species such as the diacyl species PC(18:0/20:4) or PC(18:1/20:4) almost disappeared after treating the cells with MT-III. Regarding PE species, it was noted that the diacyl species also experienced dramatic decreases, similar to their PC and PI counterparts; however, the plasmalogen forms were much less affected (Figure 2). Although this could indicate that plasmalogen species may not be within the reach of MT-III, it seems likely that, immediately after hydrolysis, these species were rapidly replenished with AA via CoA-dependent transacylation reactions at the expense of diacyl PC species [21,25,26,52,59–61].

Figure 3 shows the LC/MS analysis of major phospholipid species not containing AA. In many cases, fragmentation of the *m*/*z* peaks detected in MS analyses yielded fragments corresponding to several species, which made it not possible to unequivocally assign structures to these *m*/*z* peaks. Thus, the data are given in abbreviated form, indicating phospholipid class and number of carbon atoms and double bonds of the two lateral chains together. In Table S1, the fatty acid combinations detected for each *m*/*z* are shown. For example, PI (34:1) represents a mix of PI (18:0/16:1) plus PI (16:0/18:1), and PI (36:2) represents a mix of PI (18:0/18:2) and PI (18:1/18:1).

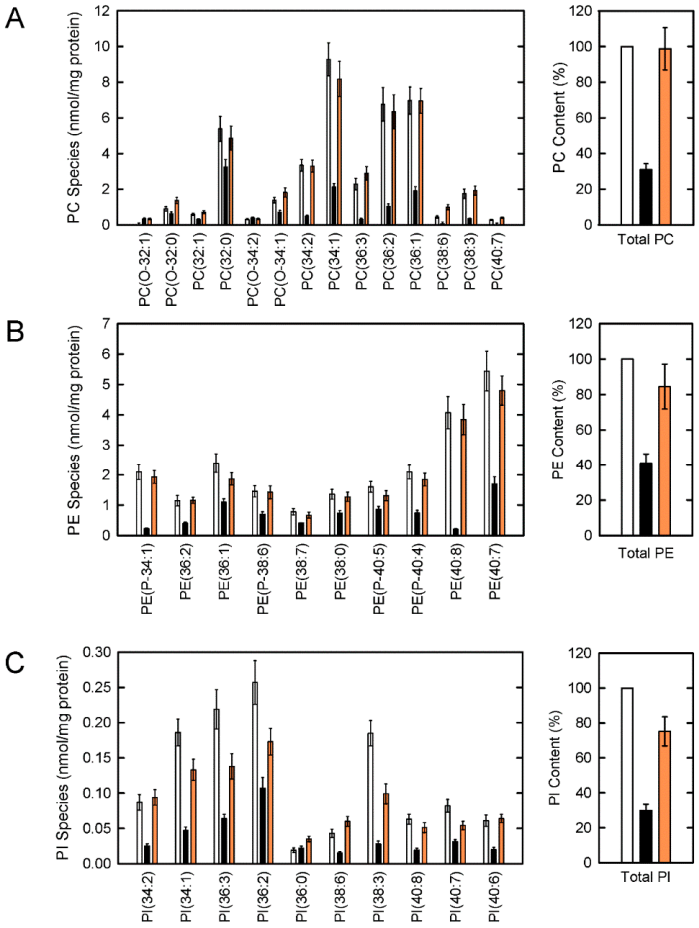


Figure 3. Phospholipid species not containing AA of human monocytes. The cells were either untreated (open bars) or treated with MT-III (black bars) or MT-II (orange bars) for 1 h. Afterward, the cellular content of PC (**A**), PE (**B**), and PI (**C**) molecular species was determined by LC/MS. Species are given in abbreviated form, indicating phospholipid class and number of carbon atoms and double bonds of the two lateral chains together. The data are expressed as mean values ± standard error of three independent determinations.

Marked reductions in all phospholipid classes were observed in the MT-III-treated monocytes. PE plasmalogen species not containing AA were hydrolyzed to much more extent that their AA-containing counterparts (*cf*. Figures 2B and 3B). Notably, some PI species such as PI (34:1), PI (36:3), PI (36:2) and PI (38:3) showed clear decreases also on stimulation with MT-II (Figure 4C). This finding represents the only positive effect observed in this study for MT-II with regard to lipid turnover. Its significance is unclear at this time but we speculate that it could constitute a ligand-like effect of MT-II related to activation of PI-dependent signaling (e.g., PI 3-kinase or intracellular Ca^{2+}-mediated pathways) via ligand binding to elements of the cell surface [31].

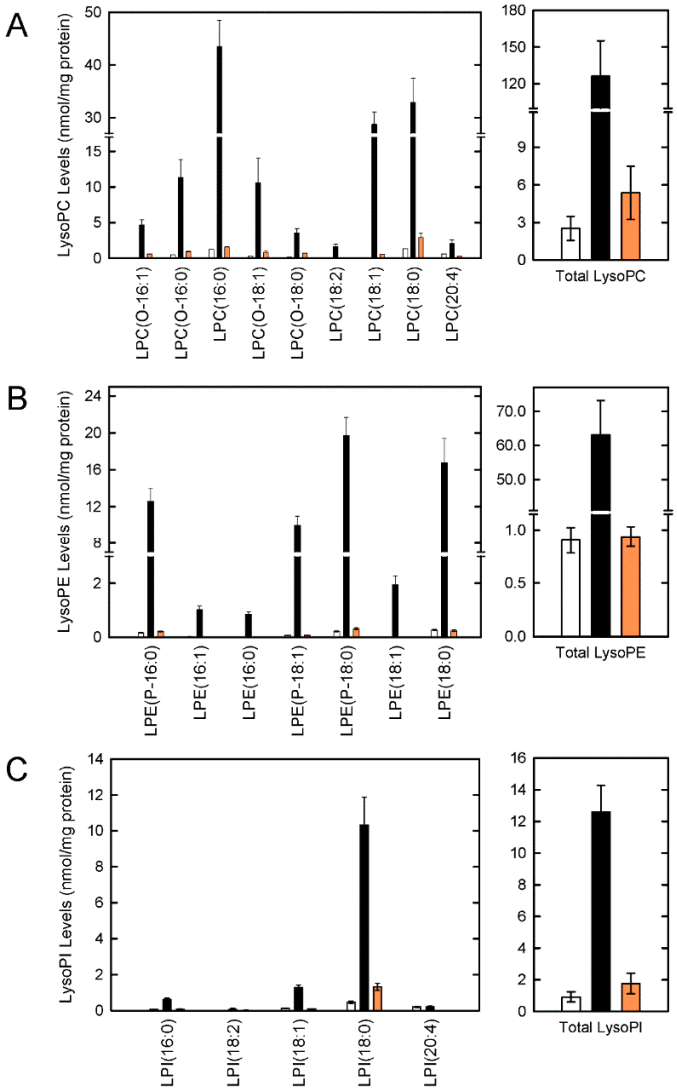


Figure 4. Lysophospholipid molecular species of human monocytes. The cells were either untreated (open bars) or treated with MT-III (black bars) or MT-II (orange bars) for 1 h. The cellular content of lysoPC (**A**), lysoPE (**B**), and lysoPI (**C**) molecular species was determined by LC/MS. Fatty chains within the different lysophospholipid species are designated by their numbers of carbons and double bonds. A designation of O- before the fatty chain indicates an sn-1 ether linkage, whereas a P- designation indicates an sn-1 vinyl ether linkage. The data are expressed as mean values ± standard error of three independent determinations.

To complete the overall lipidomic picture of changes occurring via phospholipid deacylation in the human monocytes, the measurement of lysophospholipid species was also carried out, and the results are shown in Figure 4. We detected a large lysophospholipid production after cellular treatment with MT-III, but not with MT-II, as measured by LC/MS. Consistent with the phospholipid composition of human monocytes [62], lysoPC species were detected in greater abundance, followed by lysoPE and lysoPI.

In the next series of experiments, we assessed the metabolic fate of the free fatty acids produced upon MT-III treatment. The data shown in Figure 5 indicate that a very significant incorporation of fatty acids did occur into neutral lipid classes, i.e., cholesterol esters and triacylglycerol. Consistent with the results of Figure 1, four of the five the major fatty acids lost from phospholipids—palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n−9) and linoleic acid (18:2n−6)—were also those that were incorporated in the highest proportion in neutral lipids. Very noticeably, however, the

incorporation of AA into neutral lipids was negligible, suggesting other specific metabolic fates for this particular fatty acid.

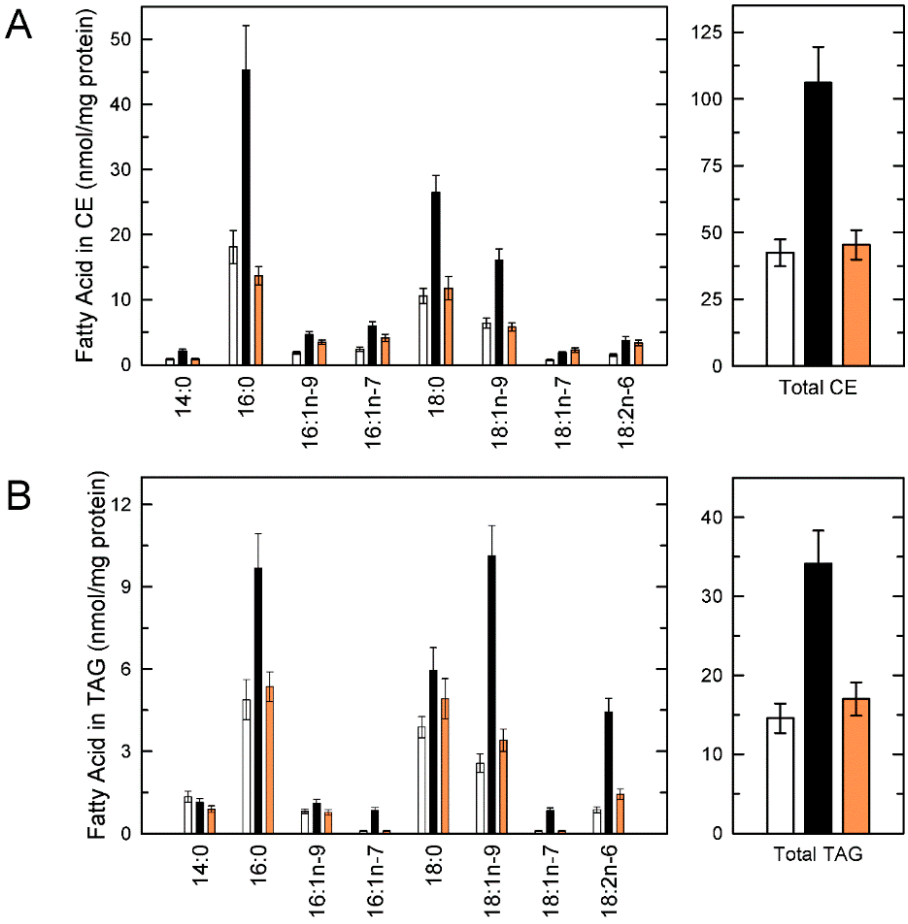


Figure 5. Fatty acid content of neutral lipids in human monocytes. The cells were either untreated (open bars) or treated with MT-III (black bars) or MT-II (orange bars) for 1 h. Afterward, cholesterol esters (CE) (**A**) and triacylglycerol (TAG) (**B**) fractions were isolated and their fatty acid content was measured by GC/MS. The profile of fatty acids, and the total CE or TAG amount are given. The data are expressed as mean values ± standard error of three independent determinations.

To investigate whether the increased synthesis of neutral lipids in the MT-III-treated cells resulted in the formation of lipid droplets, experiments were carried out to visualize these cytoplasmic organelles. Unlike human macrophages, resting human monocytes contain few lipid droplets [46]. Treatment of the cells with MT-III induced a very significant increase in the number of lipid droplets in comparison with control cells, incubated with culture medium alone (Figure 6). Mammalian cells contain five long-chain acyl-CoA synthetases, termed ACSL-1, -3, -4, -5, and -6, and human monocytes express all five of them [46]. The presence of triacsin C, a general inhibitor of long-chain acyl-CoA synthetases [63,64], quantitatively inhibited lipid droplet formation and fatty acid incorporation into neutral lipids (Figure 6). Collectively, these results suggest that lipid droplet production in MT-III-treated monocytes occurs as a consequence of increased availability of intracellular free fatty acids, which are converted into acyl-CoAs, acylated into neutral lipids, and stored in lipid droplets.

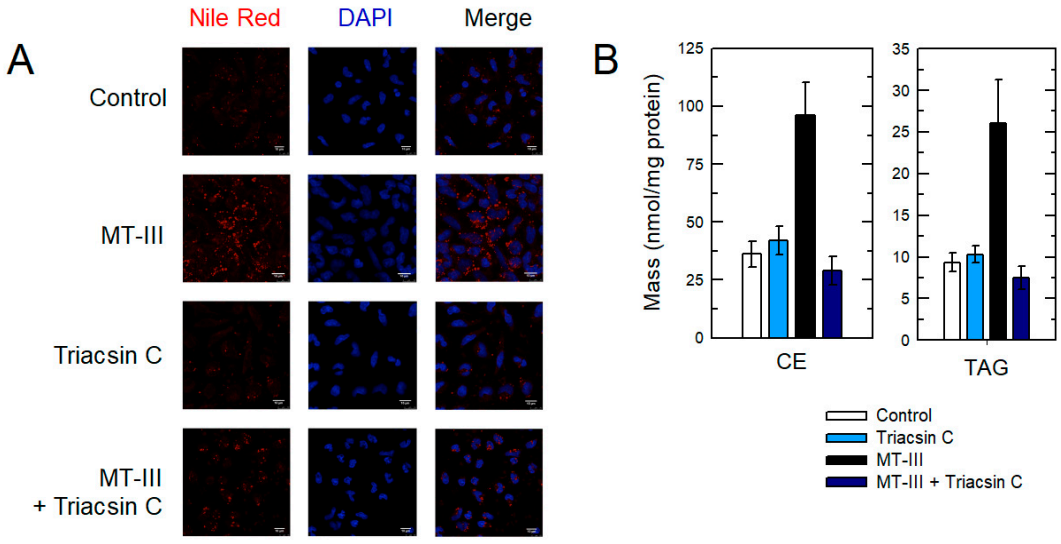


Figure 6. Lipid droplet formation in human monocytes. The cells, pretreated without or with triacsin, were exposed to MT-III as indicated. (**A**) After fixation, cells were stained with Nile Red to visualize lipid droplets (red; left panels) and DAPI to mark the nuclei (blue; central panels). Right panels show the merge. (**B**) Total fatty acid content in cholesterol esters (CE) and triacylglycerol (TAG) was analyzed in cells pretreated without (open bars and black bars) or with (light blue bars and dark blue bars) 3 μM triacsin C, and exposed to MT-III (black bars and dark blue bars) or left otherwise untreated (open bars and light blue bars). The data are expressed as mean values ± standard error of three independent determinations.

The absence of incorporation of AA into neutral lipids was unexpected, and prompted us to determine free fatty acid levels in the MT-III-treated cells. The data demonstrated the abundant presence of free AA as well as palmitic, stearic and oleic acids. Lower levels of the polyunsaturated fatty acids linoleic acid and docosahexaenoic acid were also detected (Figure 7). Importantly, only free AA levels were significantly blunted when the analyses were conducted with cells that had been pretreated with pyrrophenone prior to MT-III exposure. Pyrrophenone is a well-established inhibitor of intracellular cytosolic group IVA phospholipase $A_2\alpha$ ($cPLA_2\alpha$), and exhibits more than 1000-fold selectivity for the inhibition of $cPLA_2\alpha$ versus other types of PLA_2s, including the group IIA enzymes such as MT-III [65–68]. There was a tendency for other fatty acids—e.g., oleic acid and linoleic acid—to also decrease after pyrrophenone treatment; however, the differences failed to reach statistical significance. Collectively, these data suggest that MT-III activates $cPLA_2\alpha$; therefore, AA mobilization under these conditions would be a composite of the actions of both MT-III and $cPLA_2\alpha$. Cross-talk between $cPLA_2\alpha$ and $sPLA_2$ in AA release has often been described during the activation of innate immune cells [14–20,69–77]. Supporting our view that MT-III activates $cPLA_2\alpha$, MT-III-treated cells showed increased phosphorylation of $cPLA_2\alpha$ at Ser505, a hallmark of $cPLA_2\alpha$ activation [12,13] (Figure 7, inset).

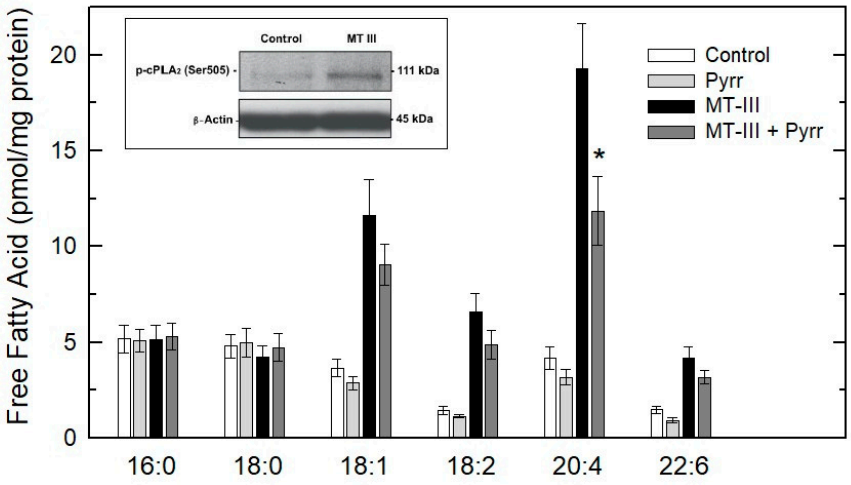


Figure 7. Free fatty acid release by human monocytes. The cells, treated without (open bars and black bars) or with (light gray bars and dark gray bars) 1 μM pyrrophenone (pyrr), were exposed to MT-III (black bars and dark gray bars) or left otherwise untreated (open bars and light gray bars). Free fatty acids were isolated and analyzed by LC/MS. The data are expressed as mean values ± standard error of three individual replicates. * $p < 0.05$, significantly different from cells not treated with pyrrophenone (Student's t-test). Inset: cell protein was separated by SDS-PAGE and the phosphorylation of cPLA2α at Ser^{505} was analyzed by immunoblot using a specific antibody. β-actin was used as a load control.

Figure 8 shows that a substantial part of the AA lost from phospholipids by the action of MT-III was metabolized to a variety of eicosanoids, mostly from the cyclooxygenase pathway. Prostaglandin E_2 and thromboxane B_2 were the major metabolites produced by the monocytes, in agreement with previous estimates [78–80]. Lower amounts of products of the lipoxygenase and cytochrome P450 pathways were also detected upon MT-III stimulation (Figure 8).

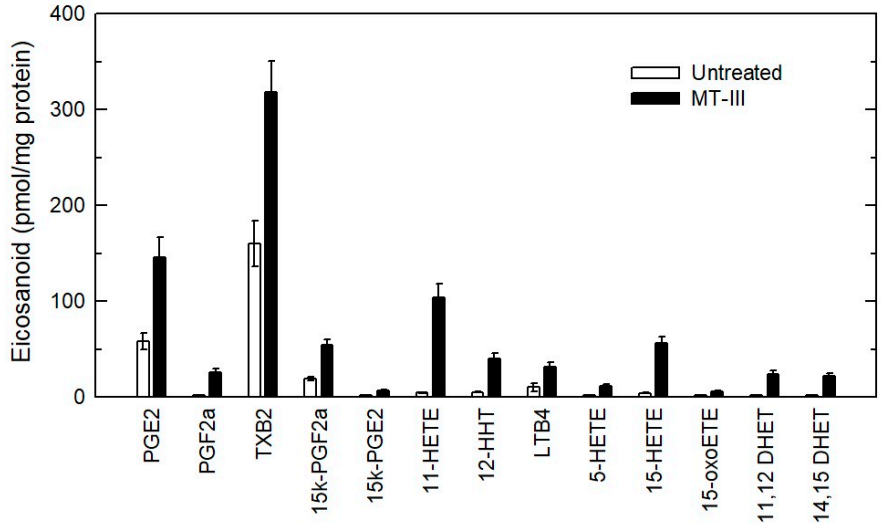


Figure 8. Eicosanoid production by stimulated monocytes. The cells were either untreated (open bars) or treated with MT-III (black bars) for 1h. Afterward, the eicosanoid content in the supernatants was analyzed by LC/MS. The data are expressed as mean values ± standard error of three individual replicates. Prostaglandin E_2 (PGE2); prostaglandin $F_{2\alpha}$ (PGF2a); thromboxane B_2 (TXB2); 15-ketoprostaglandin $F_{2\alpha}$ (15k-PGF2a); 15-ketoprostaglandin E_2 (15k-PGE2); 11-hydroxyeicosatetraenoic acid (11-HETE); 12- hydroxyheptadecatrienoic acid (12-HHT); leukotriene B_4 (LTB4); 5-

hydroxyeicosatetraenoic acid (5-HETE); 15-hydroxyeicosatetraenoic acid (15-HETE); 15-oxoeicosatetraenoic acid (15-oxoETE); 11,12-dihydroxyeicosatrienoic acid (11,12-DHET); 14,15-dihydroxyeicosatrienoic acid (14,15-DHET).

4. Discussion

This work provides a mass spectrometry-based lipidomic analysis of the actions of group IIA secreted phospholipase A_2 on human peripheral blood monocytes. Although previous work has dealt with the interactions of this class of enzymes with cell surface structures and subsequent signaling [3,31], no reports, to the best of our knowledge, have characterized the global changes in the cellular lipidome as done in this study.

Group IIA $sPLA_2$ is synthesized and secreted by a variety of cells in response to inflammatory cytokines, and is found at large amounts in fluids from inflammatory exudates. Furthermore, group IIA $sPLA_2$ enzymes are also present in the venom of scorpions, wasps and, more abundantly, snake venoms, where they behave as relevant inducers of acute inflammation reactions [81]. However, it remains to be clarified how this secreted protein acts on the outer surface of the plasma membrane of mammalian cells to activate immune cells and trigger inflammation. To shed light on this issue, we first analyzed the complete lipidomic profile of metabolites produced by the action of the enzyme. Importantly, AA, a major player in inflammation reactions, is the fatty acid showing the largest decrease in monocyte membranes after MT III exposure, followed by palmitic acid, stearic acid and oleic acid. Other polyunsaturated fatty acids such as linoleic acid, dihomo-γ-linoleic acid and docosahexaenoic acid are also released in smaller quantities and do not contribute significantly to the pool of bioactive oxygenated metabolites produced under these conditions.

We observed that several PC species disappear almost entirely from the membrane. Intriguingly, it has been suggested that membranes enriched in PC may behave as poor substrates for $sPLA_2$-IIA [32]. Several studies also indicated that the phospholipid preference may be partially explained by the number of positively charged amino acids and the lack of tryptophan in the interfacial binding site. These residues may constitute key structural determinants that permit binding and hydrolysis to whole membranes [3]. Other $sPLA_2$ family members such as the -IB and -V proteins possess tryptophan residues on their putative interfacial binding surfaces; therefore, they show an enhanced capacity to bind to PC-rich vesicles [82]. Our results, using a pathophysiologically relevant setting, raise the concept that in addition to sequence differences, the molecular composition of the membrane to which the $sPLA_2$ binds—including protein components—may influence the subsequent hydrolytic steps. Our results may also provide an appropriate experimental frame to relate the catalytic activity of various $sPLA_2$ on different classes of phospholipids with their sequences for future studies.

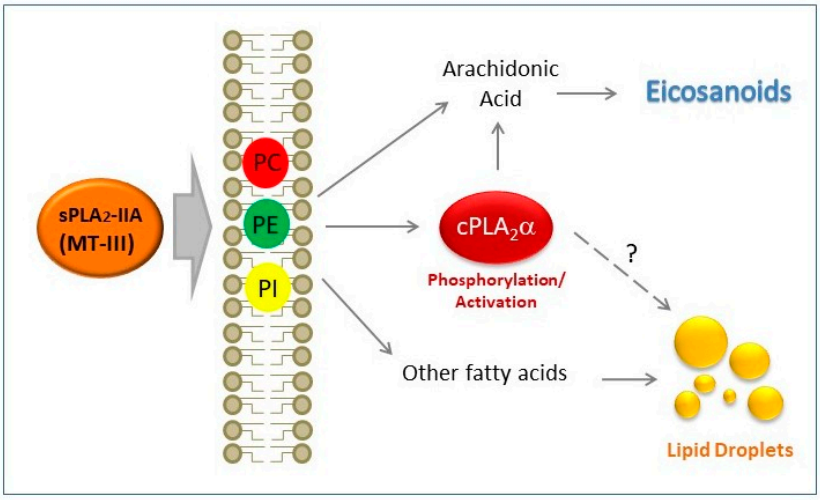
Lipid droplet biogenesis has been demonstrated to be associated with signaling events triggered by inflammation and metabolic stress [83–87]. We show here that the catalytic activity of group IIA $sPLA_2$ is required for lipid droplet formation to occur, it probably being the only factor involved, since inactive MT-II does not reproduce the effect. Our results suggest that the extensive hydrolysis of membrane phospholipids promoted by MT-III generates free fatty acids that are channeled to neutral lipids and the formation of cytoplasmic lipid droplets. In support of this view, neutral lipid formation is strongly blunted by the acyl-CoA synthetase inhibitor triacsin C, indicating that the activation of the carboxyl group of a free fatty acid is a required event. In turn, this implicates the participation of CoA-dependent acyltransferase reactions utilizing free fatty acids, not the direct transfer of fatty acids between lipids via CoA-independent transacylation reactions.

A striking feature of the present work is that, of all major fatty acids released by MT-III, AA was excluded from incorporating into neutral lipids. We have recently shown that human monocytes exposed to micromolar amounts of AA do incorporate the fatty acid into neutral lipids, implying that this pathway is fully functional in these cells [88]. This is an interesting concept because recent work has suggested a link between lipid droplets and AA metabolism in mast cells and neutrophils [89,90].

These studies showed that AA recently incorporated into neutral lipids of lipid droplets may be mobilized under activation conditions, thus providing an alternative source of free fatty acid. Therefore, our finding that AA does not incorporate into neutral lipids during exposure of the monocytes to sPLA$_2$ clearly suggests that the pathway described in mast cells and neutrophils is not operative, and the fatty acid is used to fulfill other important cellular functions. The most immediate is the direct channeling of AA to the production of proinflammatory eicosanoids that help establish a strong inflammatory reaction. Consistent with this view, we have detected abundant production of proinflammatory mediators, especially those arising from the cyclooxygenase pathway. We did not detect significant formation of putatively anti-inflammatory eicosanoids such as lipoxins or *n*−3 fatty acid derivatives.

In previous work we showed that lipid droplet formation by cells exposed to various stimulants, including MT-III, is blunted by the cPLA$_2\alpha$ selective inhibitor pyrrophenone [46,58,91]. In this work, we show that pyrrophenone significantly inhibits the accumulation of free AA in the supernatants of MT-III-treated cells. Moreover, MT-III-treated cells demonstrate increased phosphorylation of cPLA$_2\alpha$ at Ser505. Collectively, the data are suggestive of the possibility that crosstalk exists between cPLA$_2\alpha$ and MT-IIII. As a matter of fact, evidence has accumulated to suggest that the high AA specificity of cPLA$_2\alpha$ and the lack of fatty acid selectivity in sPLA$_2$s can be combined to achieve specific cellular responses [1,3,14]. Since MT-III causes extensive phospholipid hydrolysis, we speculate that the ensuing membrane disruption may favor Ca^{2+} fluxes that activate intracellular enzymes such as cPLA$_2\alpha$. A scenario such as this has even been proposed for catalytically inactive sPLA$_2$s, acting via receptor-like mechanisms [3,31]. However, in our studies, inactive MT-II does not induce phospholipid hydrolysis; thus, cPLA$_2\alpha$ has no active participation in the signaling mediated by this sPLA$_2$.

Collectively, our results highlight important actions of catalytically active group IIA sPLA$_2$ on the surface of innate immune cells. These actions may trigger different cellular responses, depending on the lipid mediator released (Scheme 1). Clearly, further research will be needed to define the role of MT-III in supplying AA for eicosanoid biosynthesis, the mechanism of crosstalk between MT-III and intracellular cPLA$_2\alpha$, and a possible role for the latter in regulating lipid droplet biogenesis, as suggested elsewhere [87,91,92].



Scheme 1. Lipid mediators and cellular responses triggered by catalytically active group IIA sPLA$_2$ (MT-III) on human monocytes.

5. Conclusions

A complex network of chemical mediators including cytokines or eicosanoids characterizes the inflammation process triggered in many diseases or envenomations, involving the hydrolytic action

of sPLA2s. Regardless of their catalytic activity, it has been demonstrated that PLA2s isolated from snake venoms (myotoxins) induce a marked local inflammatory reaction. This is characterized by an early increase in plasma extravasation, edema and a conspicuous infiltration of leukocytes followed by hyperalgesia. All these processes are the result of local and/or systemic concerted action of cytokines such as interleukin-1β, interleukin-6, tumor necrosis factor-α or interferon-γ. Despite this, lipid profiling of the changes induced by these sPLA2s on circulating blood cells had not been documented. This study provides an in depth lipidomic profiling of the monocyte response to the direct action of a group IIA sPLA2, i.e., MT-III. The data reveal significant connections among lipid droplets biogenesis, cellular signaling, and biochemical pathways that contribute to initiating the inflammatory response.

Supplementary Materials: The following are available online at www.mdpi.com/2218-273X/10/6/891/s1, Table S1: Fatty acid compositions of phospholipid species not containing AA in human monocytes.

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Abbreviation

AA	arachidonic acid
CE	cholesterol esters
cPLA2α	group IVA cytosolic phospholipase A2α
GC/MS	gas chromatography coupled to mass spectrometry
LC/MS	liquid chromatography coupled to mass spectrometry
PC	choline-containing phospholipids
PE	ethanolamine-containing phospholipids
PI	phosphatidylinositol
sPLA2	secreted phospholipase A2
TAG	triacylglycerol

References

1. Dennis, E.A.; Cao, J.; Hsu, Y.H.; Magrioti, V.; Kokotos, G. Phospholipase A_2 enzymes: Physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* **2011**, *111*, 6130–6185.
2. Murakami, M. Novel functions of phospholipase A_2s: Overview. *Biochim. Biophys. Acta* 2019, *1864*, 763–765.
3. Lambeau, G.; Gelb, M.H. Biochemistry and physiology of mammalian secreted phospholipases A_2. *Annu. Rev. Biochem.* 2008, *77*, 495–520.
4. Triggiani, M.; Granata, F.; Giannattasio, G.; Marone, G. Secretory phospholipases A_2 in inflammatory and allergic diseases: Not just enzymes. *J. Allergy Clin. Immunol.* 2005, *116*, 1000–1006.
5. Rosenson, R.S.; Gelb, M.H. Secretory Phospholipase A_2: A multifaceted family of proatherogenic enzymes. *Curr. Cardiol. Rep.* 2009, *11*, 445–451.

6. Rubio, J.M.; Rodríguez, J.P.; Gil-de-Gómez, L.; Guijas, C.; Balboa, M.A.; Balsinde, J. Group V secreted phospholipase A_2 is upregulated by IL-4 in human macrophages and mediates phagocytosis via hydrolysis of ethanolamine phospholipids. *J. Immunol.* 2015, *194*, 3327–3339.
7. Balestrieri, B.; Maekawa, A.; Xing, W.; Gelb, M.H.; Katz, H.R.; Arm, J.P. Group V secretory phospholipase A_2 modulates phagosome maturation and regulates the innate immune response against Candida albicans. *J. Immunol.* 2009, *182*, 4891–4898.
8. Boilard, E.; Lai, Y.; Larabee, K.; Balestrieri, B.; Ghomashchi, F.; Fujioka, D.; Gobezie, R.; Coblyn, J.S.; Weinblatt, M.E.; Massarotti, E.M.; et al. A novel anti-inflammatory role for secretory phospholipase A_2 in immune complex-mediated arthritis. *EMBO Mol. Med.* 2010, *2*, 172–187.
9. Dennis, E.A.; Norris, P.C. Eicosanoid storm in infection and inflammation. *Nat. Rev. Immunol.* 2015, *15*, 511–523.
10. Astudillo, A.M.; Balgoma, D.; Balboa, M.A.; Balsinde, J. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta* 2012, *1821*, 249–256.
11. Astudillo, A.M.; Balboa, M.A.; Balsinde, J. Selectivity of phospholipid hydrolysis by phospholipase A_2 enzymes in activated cells leading to polyunsaturated fatty acid mobilization. *Biochim. Biophys. Acta* 2019, *1864*, 772–783.
12. Pérez-Chacón, G.; Astudillo, A.M.; Balgoma, D.; Balboa, M.A.; Balsinde, J. Control of free arachidonic acid levels by phospholipases A_2 and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 2009, *1791*, 1103–1113.
13. Leslie, C.C. Cytosolic phospholipase A_2: Physiological function and role in disease. *J. Lipid Res.* 2015, *56*, 1386–1402.
14. Balsinde, J.; Winstead, M.V.; Dennis, E.A. Phospholipase A_2 regulation of arachidonic acid mobilization. *FEBS Lett.* 2002, *531*, 2–6.
15. Han, W.K.; Sapirstein, A.; Huang, C.C.; Alessandrini, A.; Bonventre, J.V. Cross-talk between cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and secretory phospholipase A_2 ($sPLA_2$) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: $sPLA_2$ regulates $cPLA_2\alpha$ activity that is responsible for the arachidonic acid release. *J. Biol. Chem.* 2003; *278*; 24153–24163.
16. Marshall, J.; Krump, E.; Lindsay, T.; Downey, G.; Ford, D.A.; Zhu, P.; Walker, P.; Rubin, B. Involvement of cytosolic phospholipase A_2 and secretory phospholipase A_2 in arachidonic acid release from human neutrophils. *J. Immunol.* 2000, *164*, 2084–2091.
17. Degousee, N.; Ghomashchi, F.; Stefanski, E.; Singer, A.G.; Smart, B.P.; Borregaard, N.; Reithmeier, R.; Lindsay, T.F.; Lichtenberger, C.; Reinisch, W.; et al. Groups IV, V, and X phospholipases A_2s in human neutrophils: Role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. *J. Biol. Chem.* 2002, *277*, 5061–5073.
18. Ruipérez, V.; Casas, J.; Balboa, M.A.; Balsinde, J. Group V phospholipase A_2-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. *J. Immunol.* 2007, *179*, 631–638.
19. Ruipérez, V.; Astudillo, M.A.; Balboa, M.A.; Balsinde, J. Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A_2s. *J. Immunol.* 2009, *182*, 3877–3883.
20. Balboa, M.A.; Pérez, R.; Balsinde, J. Amplification mechanisms of inflammation: Paracrine stimulation of arachidonic acid mobilization by secreted phospholipase A2 is regulated by cytosolic phospholipase A_2-derived hydroperoxyeicosatetraenoic acid. *J. Immunol.* 2003, *171*, 989–994.
21. Balgoma, D.; Astudillo, A.M.; Pérez-Chacón, G.; Montero, O.; Balboa, M.A.; Balsinde, J. Markers of monocyte activation revealed by lipidomic profiling of arachidonic acid-containing phospholipids. *J. Immunol.* 2010, *184*, 3857–3865.
22. Astudillo, A.M.; Pérez-Chacón, G.; Meana, C.; Balgoma, D.; Pol, A.; del Pozo, M.A.; Balboa, M.A.; Balsinde, J. Altered arachidonate distribution in macrophages from caveolin-1 null mice leading to reduced eicosanoid synthesis. *J. Biol. Chem.* 2011, *286*, 35299–35307.
23. Valdearcos, M.; Esquinas, E.; Meana, C.; Gil-de-Gómez, L.; Guijas, C.; Balsinde, J.; Balboa, M.A. Subcellular localization and role of lipin-1 in human macrophages. *J. Immunol.* 2011, *186*, 6004–6013.
24. Gil-de-Gómez, L.; Astudillo, A.M.; Meana, C.; Rubio, J.M.; Guijas, C.; Balboa, M.A.; Balsinde, J. A phosphatidylinositol species acutely generated by activated macrophages regulates innate immune responses. *J. Immunol.* 2013, *190*, 5169–5177.

25. Gil-de-Gómez, L.; Astudillo, A.M.; Guijas, C.; Magrioti, V.; Kokotos, G.; Balboa, M.A.; Balsinde, J. Cytosolic group IVA and calcium-independent group VIA phospholipase A_2s act on distinct phospholipid pools in zymosan-stimulated mouse peritoneal macrophages. *J. Immunol.* 2014, *192*, 752–762.
26. Gil-de-Gómez, L.; Astudillo, A.M.; Lebrero, P.; Balboa, M.A.; Balsinde, J. Essential role for ethanolamine plasmalogen hydrolysis in bacterial lipopolysaccharide priming of macrophages for enhanced arachidonic acid release. *Front. Immunol.* 2017, *8*, 1251.
27. Rubio, J.M.; Astudillo, A.M.; Casas, J.; Balboa, M.A.; Balsinde, J. Regulation of phagocytosis in macrophages by membrane ethanolamine plasmalogens. *Front. Immunol.* 2018, *9*, 1723.
28. Monge, P.; Garrido, A.; Rubio, J.M.; Magrioti, V.; Kokotos, G.; Balboa, M.A.; Balsinde, J. The contribution of cytosolic group IVA and calcium-independent group VIA phospholipase A_2s to adrenic acid mobilization in murine macrophages. *Biomolecules* 2020, *10*, 542.
29. Dore, E.; Boilard, E. Roles of secreted phospholipase A_2 group IIA in inflammation and host defense. *Biochim. Biophys. Acta* 2019, *1864*, 789–802.
30. Mora-Obando, D.; Fernández, J.; Montecucco, C.; Gutiérrez, J.M.; Lomonte, B. Synergism between basic Asp49 and Lys49 phospholipase A_2 myotoxins of viperid snake venom in vitro and in vivo. *PLoS ONE* 2014, *9*, e109846.
31. Birts, C.N.; Barton, C.H.; Wilton, D.C. Catalytic and non-catalytic functions of human IIA phospholipase A_2. *Trends Biochem. Sci.* 2010 *35*, 28–35.
32. Koduri, R.S.; Baker, S.F.; Snitko, Y.; Han, S.K.; Cho, W.; Wilton, D.C.; Gelb, M.H. Action of human group IIA secreted phospholipase A_2 on cell membranes. Vesicle but not heparinoid binding determines rate of fatty acid release by exogenously added enzyme. *J. Biol. Chem.* 1999, *273*, 32142–32153.
33. Valentin, E.; Lambeau, G. Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. *Biochim. Biophys. Acta* 2000, *1488*, 59–70.
34. Lomonte, B.; Angulo, Y.; Calderón, L. An overview of lysine-49 phospholipase A_2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon* 2003, *42*, 885–901.
35. Gutiérrez, J.M.; Lomonte, B. Phospholipases A_2: Unveiling the secrets of a functionally versatile group of snake venom toxins. *Toxicon* 2013, *62*, 27–39.
36. Mora-Obando, D.; Díaz, C.; Angulo, Y.; Gutiérrez, J.M.; Lomonte, B. Role of enzymatic activity in muscle damage and cytotoxicity induced by Bothrops asper Asp49 phospholipase A_2 myotoxins: Are there additional effector mechanisms involved? *Peer J.* 2014, *2*, e569.
37. Kaiser, I.I.; Gutiérrez, J.M.; Plummer, D.; Aird, S.D.; Odell, G.V. The amino acid sequence of a myotoxic phospholipase from the venom of Bothrops asper. *Arch. Biochem. Biophys.* 1990, *278*, 319–325.
38. Fernández, J.; Caccin, P.; Koster, G.; Lomonte, B.; Gutiérrez, J.M.; Montecucco, C.; Postle, A.D. Muscle phospholipid hydrolysis by Bothrops asper Asp49 and Lys49 phospholipase A_2 myotoxins – distinct mechanisms of action. *FEBS J.* 2013, *280*, 3878–3886.
39. Takayama, K.; Mitchell, D.H.; Din, Z.Z.; Mukerjee, P.; Li, C.; Coleman, D.L. Monomeric Re lipopolysaccharide from Escherichia coli is more active than the aggregated form in the Limulus amebocyte lysate assay and in inducing Egr-1 mRNA in murine peritoneal macrophages. *J. Biol. Chem.* 1994, *269*, 2241–2244.
40. Pérez-Chacón, G.; Astudillo, A.M.; Ruipérez, V.; Balboa, M.A.; Balsinde, J. Signaling role for lysophospholipid acyltransferase 3 in receptor-regulated arachidonic acid reacylation reactions in human monocytes. *J. Immunol.* 2010, *184*, 1071–1078.
41. Casas, J.; Meana, C.; Esquinas, E.; Valdearcos, M.; Pindado, J.; Balsinde, J.; Balboa, M.A. Requirement of JNK-mediated phosphorylation for translocation of group IVA phospholipase A_2 to phagosomes in human macrophages. *J. Immunol.* 2009, *183*, 2767–2774.
42. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, *37*, 911–917.
43. Diez, E.; Balsinde, J.; Aracil, M.; Schüller, A. Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim. Biophys. Acta* 1987, *921*, 82–89.
44. Fine, J.B.; Sprecher, H. Unidimensional thin-layer chromatography of phospholipids on boric acid-impregnated plates. *J. Lipid Res.* 1982, *23*, 660–663.
45. Astudillo, A.M.; Pérez-Chacón, G.; Balgoma, D.; Gil-de-Gómez, L.; Ruipérez, V.; Guijas, C.; Balboa, M.A.; Balsinde, J. Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells. *Biochim. Biophys. Acta* 2011, *1811*, 97–103.

46. Guijas, C.; Pérez-Chacón, G.; Astudillo, A.M.; Rubio, J.M.; Gil-de-Gómez, L.; Balboa, M.A.; Balsinde, J. Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A_2-dependent synthesis of lipid droplets in human monocytes. *J. Lipid Res.* 2012, *53*, 2343–2354.
47. Guijas, C.; Meana, C.; Astudillo, A.M.; Balboa, M.A.; Balsinde, J. Foamy monocytes are enriched in cis-7-hexadecenoic fatty acid (16:1n-9), a possible biomarker for early detection of cardiovascular disease. *Cell Chem. Biol.* 2016, *23*, 689–699.
48. Rodríguez, J.P.; Guijas, C.; Astudillo, A.M.; Rubio, J.M.; Balboa, M.A.; Balsinde, J. Sequestration of 9-hydroxystearic acid in FAHFA (fatty acid esters of hydroxy fatty acids) as a protective mechanism for colon carcinoma cells to avoid apoptotic cell death. *Cancers* 2019, *11*, 524.
49. Astudillo, A.M.; Meana, C.; Guijas, C.; Pereira, L.; Lebrero, R.; Balboa, M.A.; Balsinde, J. Occurrence and biological activity of palmitoleic acid isomers in phagocytic cells. *J. Lipid Res.* 2018, *59*, 237–249.
50. Balgoma, D.; Montero, O.; Balboa, M.A.; Balsinde, J. Calcium-independent phospholipase A_2-mediated formation of 1,2-diarachidonoyl-glycerophosphoinositol in monocytes. *FEBS J.* 2008, *275*, 6180–6191.
51. Guijas, C.; Astudillo, A.M.; Gil-de-Gómez, L.; Rubio, J.M.; Balboa, M.A.; Balsinde, J. Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages: Comparison with arachidonic acid. *Biochim. Biophys. Acta* 2012, *1821*, 1386–1393.
52. Lebrero, P.; Astudillo, A.M.; Rubio, J.M.; Fernández-Caballero, J.; Kokotos, G.; Balboa, M.A.; Balsinde, J. Cellular plasmalogen content does not influence arachidonic acid levels or distribution in macrophages: A role for cytosolic phospholipase $A_2\gamma$ in phospholipid remodeling. *Cells* 2019, *8*, 799.
53. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248–254.
54. Pindado, J.; Balsinde, J.; Balboa, M.A. TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase A_2/cyclooxygenase-2 pathway. *J. Immunol.* 2007, *179*, 4821–4828.
55. Valdearcos, M.; Esquinas, E.; Meana, C.; Peña, L.; Gil-de-Gómez, L.; Balsinde, J.; Balboa, M.A. Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *J. Biol. Chem.* 2012, *287*, 10894–10904.
56. Leiguez, E.; Zuliani, J.P.; Cianciarullo, A.M.; Fernandes, C.M.; Gutiérrez, J.M.; Teixeira, C. A group IIA-secreted phospholipase A_2 from snake venom induces lipid body formation in macrophages: The roles of intracellular phospholipases A_2 and distinct signaling pathways. *J. Leukoc. Biol.* 2011, *90*, 155–166.
57. Zuliani, J.P.; Gutiérrez, J.M.; Casais e Silva, L.L.; Sampaio, S.C.; Lomonte, B.; Teixeira, C.F.P. Activation of cellular functions in macrophages by venom secretory Asp-49 and Lys-49 phospholipases A_2. *Toxicon* 2005, *46*, 523–532.
58. Leiguez, E.; Giannotti, K.C.; Moreira, V.; Matsubara, M.H.; Gutiérrez, J.M.; Lomonte, B.; Rodríguez, J.P.; Balsinde, J.; Teixeira, C. Critical role of TLR2 and MyD88 for functional response of macrophages to a group IIA-secreted phospholipase A_2 from snake venom. *PLoS ONE* 2014, *9*, e93741.
59. Rouzer, C.A.; Ivanova, P.T.; Byrne, M.O.; Milne, S.B.; Brown, H.A.; Marnett, L.J. Lipid profiling reveals glycerophospholipid remodeling in zymosan-stimulated macrophages. *Biochemistry* 2007, *46*, 6026–6042.
60. Chilton, F.H.; Fonteh, A.N.; Surette, M.E.; Triggiani, M.; Winkler, J.D. Control of arachidonate levels within inflammatory cells. *Biochim. Biophys. Acta* 1996, *1299*, 1–15.
61. Yamashita, A.; Hayashi, Y.; Matsumoto, N.; Nemoto-Sasaki, Y.; Koizumi, T.; Inagaki, Y.; Oka, S.; Tanikawa, T.; Sugiura, T. Coenzyme-A-independent transacylation system; possible involvement of phospholipase A_2 in transacylation. *Biology* 2017, *6*, 23.
62. Kennett, F.F.; Schenkein, H.A.; Ellis, T.M.; Rutherford, R.B. Phospholipid composition of human monocytes and alterations occurring due to culture and stimulation by C3b. *Biochim. Biophys. Acta* 1984, *804*, 301–307.
63. Hartman, E.J.; Omura, S.; Laposata, M. Triacsin C: A differential inhibitor of arachidonoyl-CoA synthetase and nonspecific long chain acyl-CoA synthetase. *Prostaglandins* 1989, *37*, 655–671.
64. Coleman, R.A.; Lewin, T.M.; Van Horn, C.G.; González-Baró, M.R. Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? *J. Nutr.* 2002, *132*, 2123–2126.
65. Ono, T.; Yamada, K.; Chikazawa, Y.; Ueno, M.; Nakamoto, S.; Okuno, T.; Seno, K. Characterization of a novel inhibitor of cytosolic phospholipase $A_2\alpha$, pyrrophenone. *Biochem. J.* 2002, *363*, 727–735.
66. Balboa, M.A.; Balsinde, J. Involvement of calcium-independent phospholipase A_2 in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J. Biol. Chem.* 2002, *277*, 40384–40389.

67. Ghomashchi, F.; Stewart, A.; Hefner, Y.; Ramanadham, S.; Turk, J.; Leslie, C.C.; Gelb, M.H. A pyrrolidine-based specific inhibitor of cytosolic phospholipase $A_2\alpha$ blocks arachidonic acid release in a variety of mammalian cells. *Biochim. Biophys. Acta* 2001, *1513*, 160–166.
68. Balboa, M.A.; Sáez, Y.; Balsinde, J. Calcium-independent phospholipase A_2 is required for lysozyme secretion in U937 promonocytes. *J. Immunol.* 2003, *170*, 5276–5280.
69. Balestrieri, B.; Hsu, V.W.; Gilbert, H.; Leslie, C.C.; Han, W.K.; Bonventre, J.V.; Arm, J.P. Group V secretory phospholipase A_2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. *J. Biol. Chem.* 2006, *281*, 6691–6698.
70. Casas, J.; Gijón, M.A.; Vigo, A.G.; Crespo, M.S.; Balsinde, J.; Balboa, M.A. Phosphatidylinositol 4,5-bisphosphate anchors cytosolic group IVA phospholipase A_2 to perinuclear membranes and decreases its calcium requirement for translocation in live cells. *Mol. Biol. Cell* 2006, *17*, 155–162.
71. Satake, Y.; Diaz, B.L.; Balestrieri, B.; Lam, B.K.; Kanaoka, Y.; Grusby, M.J.; Arm, J.P. Role of group V phospholipase A_2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* 2004, *279*, 16488–16494.
72. Balboa, M.A.; Shirai, Y.; Gaietta, G.; Ellisman, M.H.; Balsinde, J.; Dennis, E.A. Localization of group V phospholipase A_2 in caveolin-enriched granules in activated P388D1 macrophage-like cells. *J. Biol. Chem.* 2003, *278*, 48059–48065.
73. Balsinde, J.; Shinohara, H.; Lefkowitz, L.J.; Johnson, C.A.; Balboa, M.A.; Dennis, E.A. Group V phospholipase A_2-dependent induction of cyclooxygenase-2 in macrophages. *J. Biol. Chem.* 1999, *274*, 25967–25970.
74. Balsinde, J.; Balboa, M.A.; Yedgar, S.; Dennis, E.A. Group V phospholipase A_2-mediated oleic acid mobilization in lipopolysaccharide-stimulated $P388D_1$ macrophages. *J. Biol. Chem.* 2000, *275*, 4783–4786.
75. Balboa, M.A.; Balsinde, J.; Dillon, D.A.; Carman, G.M.; Dennis, E.A. Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *J. Biol. Chem.* 1999, *274*, 522–526.
76. Balsinde, J.; Balboa, M.A.; Dennis, E.A. Inflammatory activation of arachidonic acid signaling in murine $P388D_1$ macrophages via sphingomyelin synthesis. *J. Biol. Chem.* 1997, *272*, 20373–20377.
77. Balsinde, J.; Balboa, M.A.; Insel, P.A.; Dennis, E.A. Differential regulation of phospholipase D and phospholipase A_2 by protein kinase C in $P388D_1$ macrophages. *Biochem. J.* 1997, *321*, 805–809.
78. Hoffman, T.; Lizzio, E.F.; Suissa, J.; Rotrosen, D.; Sullivan, J.A.; Mandell, G.L.; Bonvini, E. Dual stimulation of phospholipase activity in human monocytes. Role of calcium-dependent and calcium-independent pathways in arachidonic acid release and eicosanoid formation. *J. Immunol.* 1988, *140*, 3912–3918.
79. Hoffman, T.; Brando, C.; Lizzio, E.F.; Lee, Y.L.; Hansen, M.; Tripathi. A.K.; Taplits. M.; Puri, J.; Bonvini, E.; Abrahamsen, T.G.; Carter, C.S.; Reid, G.K.; et al. Calcium-dependent eicosanoid metabolism by concanavalin A-stimulated human monocytes in vitro. Synergism with phorbol ester indicates separate regulation of leukotriene B_4 synthesis and release. *J. Immunol.* 1991, *146*, 692–700.
80. Penglis, P.S.; Cleland, L.G.; Demasi, M.; Caughey, G.E.; James, M.J. Differential regulation of prostaglandin E_2 and thromboxane A_2 production in human monocytes: Implications for the use of cyclooxygenase inhibitors. *J. Immunol.* 2000, *165*, 1605–1611.
81. Teixeira, C.; Cury, Y.; Moreira, V.; Picolo, G.; Chaves, F. Inflammation induced by Bothrops asper venom. *Toxicon* 2009, *54*, 67–76.
82. Han, S.K.; Kim, K.P.; Koduri, R.; Bittova, L.; Muñoz, N.M.; Leff, A.R.; Wilton, D.C.; Gelb, M.H.; Cho, W. Roles of Trp31 in high membrane binding and proinflammatory activity of human group V phospholipase A_2. *J. Biol. Chem.* 1999, *274*, 11881–11888.
83. Melo, R.C.N.; Weller, P.F. Lipid droplets in leukocytes: Organelles linked to inflammatory responses. *Exp. Cell Res.* 2016, *340*, 193–197.
84. Cruz, A.L.S.; Barreto, E.A.; Fazolini, N.P.B.; Viola, J.P.B.; Bozza, P.T. Lipid droplets: Platforms with multiple functions in cancer hallmarks. *Cell Death Dis.* 2020, *11*, 105.
85. Jarc, E.; Petan, T. A twist of FATe: Lipid droplets and inflammatory lipid mediators. *Biochimie* 2020, *169*, 69–87.
86. Guijas, C.; Rodríguez, J.P.; Rubio, J.M.; Balboa, M.A.; Balsinde, J. Phospholipase A_2 regulation of lipid droplet formation. *Biochim. Biophys. Acta* 2014, *1841*, 1661–1671.
87. Chapman, K.D.; Aziz, M.; Dyer, J.M.; Mullen, R.T. Mechanisms of lipid droplet biogenesis. *Biochem. J.* 2019, *476*, 1929–1942.

88. Guijas, C.; Bermúdez, M.A.; Meana, C.; Astudillo, A.M.; Pereira, L.; Fernández-Caballero, L.; Balboa, M.A.; Balsinde, J. Neutral lipids are not a source of arachidonic acid for lipid mediator signaling in human foamy monocytes. *Cells* 2019, *8*, 941.
89. Dichlberger, A.; Schlager, S.; Maaninka, K.; Schneider, W.J.; Kovanen, P.T. Adipose triglyceride lipase regulates eicosanoid production in activated human mast cells. *J. Lipid Res.* 2014, *55*, 2471–2478.
90. Schlager, S.; Goeritzer, M.; Jandl, K.; Frei, R.; Vujic, N.; Kolb, D.; Strohmaier, H.; Juliane, J.; Dorow, J.; Eichmann, T.O.; Rosenberger A.; et al. Adipose triglyceride lipase acts on neutrophil lipid droplets to regulate substrate availability for lipid mediator synthesis. *J. Leukoc. Biol.* 2015, *98*, 837–850.
91. Gubern, A.; Casas, J.; Barceló-Torns, M.; Barneda, D.; de la Rosa, X.; Masgrau, R.; Picatoste, F.; Balsinde, J.; Balboa, M.A.; Claro, E. Group IVA phospholipase A_2 is necessary for the biogenesis of lipid droplets. *J. Biol. Chem.* 2008, *283*, 27369–27382.
92. Gubern, A.; Barceló-Torns, M.; Barneda, D.; López, J.M.; Masgrau, R.; Picatoste, F.; Chalfant, C.E.; Balsinde, J.; Balboa, M.A.; Claro, E. JNK and ceramide kinase govern the biogenesis of lipid droplets through activation of group IVA phospholipase A_2. *J. Biol. Chem.* 2009, *284*, 32359–32369.