

Dynamics of Arachidonic Acid Mobilization by Inflammatory Cells

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Keywords: Arachidonic Acid; Phospholipid Remodeling; Eicosanoid Metabolism;
Lipidomics; Mass Spectrometry.

Running Title: AA Mobilization in Inflammatory Cells

Abstract

The development of mass spectrometry-based techniques is opening new insights into the understanding of arachidonic acid (AA) metabolism. AA incorporation, remodeling and release are collectively controlled by acyltransferases, phospholipases and transacylases that exquisitely regulate the distribution of AA between the different glycerophospholipid species and its mobilization during cellular stimulation. Traditionally, studies involving phospholipid AA metabolism were conducted by using radioactive precursors and scintillation counting from thin layer chromatography separations, that provided only information about lipid classes. Today, the input of lipidomic approaches offers the possibility of characterizing and quantifying specific molecular species with great accuracy and within a biological context associated to protein and/or gene expression in a temporal frame. This review summarizes recent results applying mass spectrometry-based lipidomics approaches to the identification of AA-containing glycerophospholipids, phospholipid AA remodeling and synthesis of oxygenated metabolites.

Abbreviations: AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂α, group IVA cytosolic PLA₂; iPLA₂, Ca²⁺-independent PLA₂; sPLA₂, secreted PLA₂; CoA-IT, CoA-independent transacylase; ACS, acyl-CoA synthetase; ESI, electrospray ionization; HPLC, high performance liquid chromatography; MS, mass spectrometry; TLC, thin layer chromatography; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol.

1. Introduction

The traditional view of lipids typically acting as membrane building blocks or fuel has changed dramatically in recent years. Now, it is widely accepted that these biomolecules are centrally involved in cell signaling, and that imbalances in lipid metabolism are causative agents of a number of high-prevalence disorders, ranging from cardiovascular disease, diabetes and obesity to cancer [1-3]. Hundreds to thousands of lipid species can be identified in a given biological system at a cell or tissue levels, each of them with a particular distribution and function [4].

The significant advances in analytical techniques over the last years, especially mass spectrometry (MS) and the parallel improvement in molecular ionization, together with the possibility to use this technique as a detector in chromatographic separation, have constituted a breakthrough in lipid analysis and, therefore, a better understanding of lipid biochemistry has been possible. This has contributed to the development of lipidomics [5-7], a branch of metabolomics that pursues a thorough scientific study of lipids. Lipidomics not only aims at characterizing and analyzing lipid species in a particular state of the cell —called the *static* composition—, but also aims at integrating the understanding of lipid pathways (synthesis, remodeling or transport) with their biological roles and with gene regulation and protein expression [5, 7-11].

There are two major strategies for lipidomic analysis. The first one, called global lipidomics, pursues the analysis of the whole lipidome of biological samples, by using either direct infusion methods or previous liquid chromatography steps. On the other hand, targeted or focused lipidomics, is based on setting conditions for analysis of specific categories of lipids, thus improving sensitivity [11-14].

As lipidomics is focused on profiling the *static* composition of molecular species in biological samples, the term metabolipidomics has been coined to emphasize the dynamical aspects of lipids in cells, organs, tissues and whole organisms [15]. In this case, lipids containing stable isotopes are used to follow their metabolic fate through the various possible pathways, thus facilitating the identification of minor species with rapid turnover rates.

2. Cellular Utilization of Arachidonic Acid

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is an ω -6 essential fatty acid, obtained directly from diet or, alternatively, synthesized from linoleic acid (18:2n-6) through the successive actions of Δ 6-desaturase, elongase and Δ 5-desaturase, that occurs mainly in liver but also in other tissues (Figure 1). In western diets it is calculated that the intake of AA is 0.2-0.3 g/day, whereas the intake of linoleic acid is 10-20 g/day, indicating that the amount of AA coming from linoleic acid is higher than that coming directly from the diet [16, 17].

AA is the common precursor of the eicosanoids, a family of lipid mediators with key roles in physiology and pathophysiology, particularly in inflammatory reactions [18, 19], although the fatty acid can exert potent biological roles by itself, e.g. by inducing apoptosis [20-22]. Additionally, when present in elevated concentrations, AA can give rise to significant quantities of its 2-carbon elongation product, adrenic acid (22:4n-6). Adrenic acid is the precursor of 22-carbon *1a,1b*-dihomologue prostaglandins (dihomoprostaglandins) [23-26]. The potent biological activity of the eicosanoids

compels the cells to tightly control the levels of free AA in such a manner that availability of free AA is frequently a rate-limiting step in eicosanoid generation [27, 28].

In inflammatory cells, AA is generally found esterified in the *sn*-2 position of glycerophospholipids, particularly choline glycerophospholipids (PC), ethanolamine glycerophospholipids (PE), and phosphatidylinositol (PI) [28]. Cellular free AA levels are controlled by two competing reactions; on one hand, the phospholipase A₂ (PLA₂)-mediated cleavage of the *sn*-2 position of phospholipids to yield the free fatty acid and, on the other, the CoA-dependent acyltransferase-modulated acylation reactions that re-incorporate the free fatty acid into phospholipids [29, 30]. In resting unstimulated cells, the reacylation reactions dominate over the PLA₂-mediated deacylation step; thus free AA is kept at very low levels. Stimulation of the cells by receptor agonists results in the activation of intracellular PLA₂s [31-35]. Under these conditions, the rate of AA release clearly exceeds that of reincorporation into phospholipids; hence, net accumulation of free AA occurs that is followed by its conversion into various eicosanoids. Nevertheless, AA reacylation reactions are still very significant under stimulated conditions, as manifested by the fact that only a minor fraction of the free AA released by PLA₂s is converted into eicosanoids, the remainder being effectively incorporated back into phospholipids [36, 37].

The first step for AA incorporation into cellular lipids is the activation of the carboxyl group of the fatty acid by thioesterification with CoA. This reaction is catalyzed by acyl-CoA synthetases, of which there are many in cells [30]. In the context of AA metabolism it is worth mentioning the long chain acyl-CoA synthetases ACSL3, 4 and 6, as they show some selectivity towards AA [38-40]. The next step in the AA incorporation

is the esterification of the fatty acid into primarily the *sn*-2 position of glycerophospholipids, a reaction that is carried out by lysophospholipid:acyl CoA acyltransferases, in particular those of the membrane-bound O-acyltransferase family (MBOAT) [41]. Those that have been found to exhibit clear preference for AA include lysoPC:acyl-CoA acyltransferase 2 [42], lysoPC:acyl-CoA acyltransferase 3 [43-45], LysoPI:acyl-CoA acyltransferase and lysophosphatidic acid:acyl-CoA acyltransferase 3 [44, 46, 47]. In addition, the lipase CGI-58 shows lysophosphatidic:acyl-CoA acyltransferase with high preference towards AA [48]. For detailed information on the biochemistry and functioning of CoA-dependent acyl transferases, the reader is kindly referred to recent reviews on the subject [30, 49-51].

AA bound to phospholipid is also the subject of successive transacylation reactions aimed at ensuring the proper distribution of the fatty acid within the various cellular phospholipid pools [29, 30, 52-54]. This appears to be important not only for membrane homeostasis but also for the execution of appropriate cell responses during physiological and pathophysiological activation [55-59]. These transacylation reactions are catalyzed by CoA-independent transacylase (CoA-IT), an enzyme that transfers AA moieties preferentially from diacyl PC species to PE plasmalogens [29, 30] (Figure 1). The sequence of CoA-IT is yet to be described. Thus, currently the only manner to study the cell regulation of CoA-IT is by following its enzyme activity [60, 61].

Once the AA has been effectively incorporated into phospholipids the fatty acid can be eventually liberated, especially under cell stimulation conditions, by a number of PLA₂ enzymes. So far, more than 30 enzymes possessing PLA₂ activity have been described in mammals [62-64]. Attending to sequence homology criteria, the PLA₂s have

been classified into 16 groups [62-65], although based on biochemical features they can be grouped into 5 main families, namely the Ca^{2+} -dependent secreted PLA_2s (s PLA_2), Ca^{2+} -dependent cytosolic PLA_2s (c PLA_2), Ca^{2+} -independent cytosolic PLA_2s (i PLA_2), platelet-activating factor acetylhydrolases, and lysosomal PLA_2s . Of these, the first two have been repeatedly implicated in receptor-mediated AA mobilization in response to a variety of stimuli. Today, it is firmly established that the calcium-dependent cytosolic group IVA $\text{PLA}_2\alpha$ (c $\text{PLA}_2\alpha$) is the critical enzyme in stimulus-dependent AA mobilization and that, depending on cell type and stimulation conditions, a s PLA_2 may also participate by amplifying the c $\text{PLA}_2\alpha$ -regulated response [66-91]. Under some conditions, the Ca^{2+} -independent group VIA i PLA_2 may also effect the AA release [92-94] but in other conditions, it mediates phospholipid reacylation reactions by regulating the steady-state level of lysoPC [95-97], reflecting the multiplicity of functions that this enzyme appears to serve depending on cell type [34, 98]. For detailed information on the cellular regulation of AA mobilization by PLA_2 enzymes, the reader is kindly referred to the many comprehensive reviews that have been published in recent years covering different aspects of the subject [31-35, 51, 62-65, 99-104].

After the AA has been released from phospholipids under stimulation conditions, it can be metabolized into eicosanoids through four different pathways, namely cyclooxygenase, lipoxygenase, cytochrome-P450 and oxygen species-triggered reactions (Figure 2). These pathways yield a plethora of compounds, such as prostaglandins, isoprostanes, thromboxane, leukotrienes, lipoxins and epoxyeicosatrienoic acids, all of which act in a paracrine/autocrine manner through specific receptors on the plasma membrane.

For the biosynthesis of prostaglandins and tromboxane from AA, synthesis of PGH₂ via cyclooxygenase is the first step. Cyclooxygenase incorporates molecular O₂ and forms PGG₂ that is subsequently reduced to form PGH₂ by the action of the peroxidase active site. PGH₂ is the substrate of different prostaglandin and thromboxane synthases that are expressed in tissues and cells in a selective-manner and lead to the formation of PGE₂, PGD₂, PGI₂, PGF₂α and TXA₂ [105]. These compounds exert important biological functions, i.e. modulating smooth muscle tone, vascular permeability, hyperalgesia, fever and platelet aggregation [106].

Leukotrienes, eoxins, lipoxins, and hydroperoxyeicosatetraenoic acids (HPETEs) are synthesized from AA by lipoxygenase enzymes (5-, 12- and 15-lipoxygenase) that add molecular O₂ into AA stereospecifically. 5-Lipoxygenase forms 5-hydroperoxyeicosatetraenoic acid (5-HPETE) that is metabolized to LTA₄ in a second step. LTA₄ is unstable, and can rapidly be converted to LTB₄, a potent chemoattractant for polymorphonuclear leukocytes. Additionally, LTA₄ can be converted to LTC₄ which is the precursor of LTD₄ and LTE₄. These three leukotrienes constitute the slow-reacting substances of anaphylaxis that act in the allergic response [107]. Eoxins are produced in a similar manner as the leukotrienes, but by the action of 15-lipoxygenase, and are almost as potent as 5-lipoxygenase-derived leukotrienes [108]. Lipoxins are generated by transcellular biosynthesis and have anti-inflammatory and resolving roles [109].

Other metabolites, including epoxyeicosatrienoic acids (EETs) and their products from epoxy-ring hydrolysis, dihydroxyeicosatrienoic acids (DHETs), are derived from cytochrome-P450 enzymes [110]. EETs have been associated with anti-inflammatory properties in cardiovascular diseases [111].

The fourth pathway of production of eicosanoids is the reaction of AA with reactive oxygen species to yield a group of compounds known as isoprostanes. As radical-driven reactions are not stereo-selective, they generate different isomers with structures similar to those of prostaglandins. Because of their origin, isoprostanes have been used as markers of oxidative stress [112].

3. Lipidomic Studies of Cellular Arachidonate Metabolism

Traditionally, cellular studies on AA movement to and from glycerophospholipids made extensive use of radiolabeled fatty acid. After lipid extraction and chromatographic separation, quantification was carried out by scintillation counting [54-59, 66-76, 113-119]. Studies carried out in the 80's and early 90's using this general strategy demonstrated that most of the AA incorporated into cellular phospholipids resides in PC, PE or PI, that the major AA-containing phospholipid classes in many cell types, — especially those involved in innate immunity and inflammation reactions—, usually contain ether linkages at the *sn*-1 position, and that major remodeling reactions involving AA occur between PC (primarily diacyl) species and PE (primarily alkenyl) species [29, 54-56, 120-124]. However, a potential problem with the use of radioactive tracers is that sometimes, experiments are not carried out under equilibrium labeling conditions and, therefore, changes detected involve only discrete pools with higher turnover rates. Moreover, using this methodology, it is not possible to ascertain the exact molecular composition of the phospholipids to which AA is bonded.

The advent of HPLC coupled to MS, or direct infusion MS, has made it routinely

possible to collect structure information, thus greatly strengthening our knowledge on the cellular dynamics of AA. [12-14, 125-140]. Moreover, by using a metabolipidomic approach (i.e. combining the power of MS with use of stable isotopes such as deuterium-labeled AA), dynamic information can also be obtained as to the differential mobilization of various cellular AA pools under defined stimulation conditions, thus allowing the understanding of lipid turnover in contrast with static measures under equilibrium conditions [15, 141].

Identification of the phospholipid acyl chains as well as their positioning in the *sn*-1 versus the *sn*-2 position of the glycerol backbone can be obtained through fragmentation experiments (ESI-MS/MS). Such assignments are usually straightforward for analyses of AA-containing PE and PI in the negative ion mode. Analysis of AA-containing PC species is usually carried out in negative mode with postcolumn addition of acetic acid, as $[M+CH_3CO_2]^-$ adducts [14, 127, 132, 136-138, 140].

Using $[^2H]_8AA$ and reverse phase-HPLC-ESI-MS, Balgoma *et al.* [142] identified the PI molecular species that initially incorporate AA in human U937 promonocyte-like cells and peripheral blood monocytes. The unusual species $PI([^2H]AA/[^2H]AA)$ was found to behave as a major but short-lived acceptor of the AA, and its route of biosynthesis was described to involve the direct acylation of both the *sn*-1 and *sn*-2 positions of PI. Likewise, similar studies on the initial incorporation of AA into PE species revealed a fundamental difference between human U937 promonocytes and peripheral blood monocytes in that the former, but not the later, incorporate large quantities into various PE molecular species at short times [61]. This difference is attributed to the higher remodeling rate of U937 cells compared to monocytes, which in

turn appears to be related to the intrinsically low AA content that these cells exhibit [61].

Rouzer *et al.* [143] compared the fatty acid remodeling of murine residential peritoneal macrophages and RAW 264.7 macrophage-like cells after exposure to yeast-derived zymosan. Using tandem MS, AA deficiency of cultured cells compared with their primary counterparts was confirmed, as was the different distribution of AA among phospholipids in AA-enriched RAW 264.7 cells and peritoneal macrophages [143]. These data are relevant in that they emphasize the potential pitfalls of using AA-deficient cells by long term culture in studies of AA metabolism. Although this kind of studies have been useful to describe biochemical pathways, they have not always produced meaningful data from a physiological point of view, i.e. when compared with primary cells, since the AA deficiency of cultured cells usually results in much lower amounts of AA mobilized –and hence of eicosanoids produced–, and probably also the preferential use of AA phospholipid pools with higher turnover rates [74, 143].

In subsequent studies, Rouzer *et al.* [144] reported that the bulk of cPLA₂α-mediated AA release in zymosan-stimulated macrophages arises from AA-containing PC species whereas AA-containing PE species show little or no changes. Similar to these studies, using reverse phase HPLC-ESI-MS, Balgoma *et al.* [145] described that all major AA-containing PC and PI species decrease in a cPLA₂α-dependent manner in zymosan-stimulated human monocytes, with only minor changes in the levels of AA-containing PE species. Taking into account previous work utilizing [³H]AA-labeled cells under equilibrium labeling conditions [55-58], it seems likely that PE molecules also serve as immediate substrates for stimulus-induced AA mobilization in monocytes/macrophages, but these are rapidly reacylated using AA from other sources, e.g. PC, via CoA-IT-

mediated reactions [144, 145]. In this regard, a previous lipidomic study with human platelets demonstrated that ethanolamine plasmalogen species constituted by far the major sources of AA mobilized after thrombin stimulation of the cells at very short times, i.e. 90 s [146]. Collectively, these studies highlight a key distinctive advantage of applying an ESI-MS lipidomics approach to the analysis of AA metabolism, that is, the possibility of identifying the specific molecular species of phospholipids that act as donors and acceptors of AA moieties during activation. Thus, conclusions can now be drawn on the molecular specificity of the phospholipid substrate and the phospholipid-hydrolyzing phospholipase(s) involved.

In the above-mentioned studies with human monocytes [145], the levels of the two minor species PI(20:4/20:4) and PC(20:4/20:4) were found to increase in zymosan-activated cells compared with resting cells, and a third species not present in resting cells, PE(16:1/20:4), appeared under stimulation conditions [145]. Analysis of the pathways involved in the synthesis of these three lipids indicated that PI(20:4/20:4) and PC(20:4/20:4) were produced in a deacylation/reacylation pathway via cPLA₂α and acyl-CoA synthetase-dependent reactions, whereas PE(16:1/20:4) was generated via a CoA-independent transacylation reaction [145]. The finding that certain AA-containing species that are detected at low levels or not detected at all under resting conditions, significantly increase in activated cells via selected biosynthetic pathways suggests that they may be regarded as lipid markers of particular activation states of the cells.

Lipidomic profiling of AA-containing phospholipids may also be useful to define cell-specific differences. Figure 3 compares the distribution of AA-containing phospholipids in human peripheral blood monocytes [145], human monocyte-derived

macrophages [147], and mouse peritoneal macrophages [148], all obtained by reverse phase HPLC-ESI-MS under identical experimental conditions. Although the overall distribution of AA between glycerophospholipids is similar in the three cell types, some potentially useful differences are apparent. For example, the plasmalogen species PC(P-16:0/20:4) is present at low levels in human monocytes and mouse macrophages but is by far the major AA-containing PC species of human macrophages. Similarly, PC(16:0/20:4) is present in trace amounts in human monocytes and macrophages but is one of the most abundant AA-containing PC species of mouse macrophages (Figure 3). As for human monocytes, note the conspicuous absence of the plasmalogen species PE(P-18:1/20:4) and PE(P-18:2/20:4), which is counteracted by the high level of the related species PE(P-18:0/20:4), compared to human and mouse macrophages.

Although not focusing directly on AA-containing phospholipids, other lipidomic studies looking for biomarkers in various macrophage cell types, have led to the identification of unusual glycerophospholipids such as ether-linked PI species, PE species containing very long fatty acyl chains, or phospholipids containing threonine as the polar headgroup [149]. Very recently, combining HPLC-ion trap MS and HPLC-triple quadrupole MS, Yang *et al.* [150] described changes in glycerophospholipid species in response to different levels of oxidative stress induced by H₂O₂ in EA.hy926 cells. In these studies, the authors identified 7 lysophospholipid correlating with cPLA₂α activation under these conditions, which could potentially be regarded as biomarkers of oxidative stress conditions [150].

More ambitious lipidomic analyses have aimed at establishing the dynamics of lipid metabolism and regulation during inflammatory stimulation. Dennis *et al.* [151]

carried out the first quantitative approach for the characterization of the whole lipidome of an inflammatory cell. Dynamic quantitative MS analyses were carried out, together with transcriptional measurements of genes involved in lipid metabolism in RAW 264.7 macrophage-like cells stimulated with Kdo₂-lipid A, or treated with the pharmacological inhibitor compactin. This work focused on 400 lipid species from all major lipid classes, including fatty acids, acyl-CoAs, eicosanoids, glycerophospholipids, sphingolipids, sterols and glycerolipids [151]. In cells stimulated with the specific TLR4 agonist Kdo₂-lipid A, a good correlation was observed between expression of genes coding for enzymes of lipid metabolism and changes of the corresponding metabolites. However a potential shortcoming is that this information could be misleading if the regulation of enzyme levels is post-transcriptional, as is the case of e.g. cPLA₂α [151]. Increases in cholesterol, lanosterol and desmosterol were also documented, as well as increases in sphingolipid content, consistent with induction of the *de novo* biosynthesis of sphingolipids. Finally, significant changes in phosphatidic acid and PI species were detected, with increases of saturated and monounsaturated phosphatidic acid species, and decreases of polyunsaturated species such as PI(38:4) –probably PI(18:0/20:4)– at 24h, raising the possibility that lipid molecules involved in signaling still act beyond 24 h stimulation [151].

The application of MS techniques for eicosanoid quantitation has supposed a significant breakthrough in this field of research, since traditional analyses, based in enzyme-linked immunosorbent assays (ELISA) allow the analysis of only one eicosanoid per assay, thus prohibiting great-scale assays. By using a lipidomic approach, Norris *et al.* [152] analyzed the eicosanoid profile together with protein and gene expression of

enzymes of eicosanoid metabolism when different types of macrophages –murine residential peritoneal macrophages, bone marrow-derived macrophages, thioglycollate elicited macrophages and RAW 264.7 macrophage-like cells– were exposed to Kdo₂-lipid A. Over 140 species were detected, including AA-derived metabolites generated by COX, LOX and CYP450 pathways (Figure 2) and those derived from non-enzymatic pathways. Lower levels of ω-3 fatty acid-derived protectins and resolvins were also detected. In addition, adrenic acid-derived dihomoprostaglandins [23-25] were detected in all macrophage types, suggesting that these compounds may possess biological significance. Overall, the data confirmed that although there are potentially many factors that affect eicosanoid production such PLA₂ activation, the enzymes of the AA reacylation pathway (Figure 1), and terminal synthases, the delayed phase of prostaglandin production by all macrophage types was strikingly dependent on the maximal level of COX-2 expression [152].

Andreyev *et al.* [153] focused their lipidomic analyses on the different subcellular localization of lipid species in RAW 264.7 cells, analyzing a total of 229 species in various compartments such as endoplasmic reticulum, mitochondria, nuclei, plasmalemma and cytoplasm, and addressing the changes occurring upon activation with Kdo₂-lipid A. Cell activation led to remodeling of the lipidome in all subcellular compartments, apparently in an organelle-specific manner. Changes detected included increases in phosphatidic acid in the endoplasmic reticulum –suggestive of phospholipase D activation in that compartment– or increases in highly unsaturated cardiolipins and oxidized sterols in mitochondria –suggestive of oxidative stress in this organelle–. In addition, ether-linked phospholipids increased in plasma membrane but decreased in the

endoplasmic reticulum under stimulation conditions, especially the ether species PE(P-38:4/O-38:5), which probably contains AA [153].

4. Concluding Remarks and Future Perspectives

The robustness and sensitivity of mass spectrometry converts this technique in the preferred tool for lipidomic analyses, which focuses on the complete characterization of lipid species in combination with gene/protein expression under different conditions or aiming at the understanding of turnover and lipid pathways. In this regard, lipidomic approaches, specifically the use of ESI-MS/MS with or without a previous HPLC separation and alternative use of deuterated compounds, permit the thorough characterization of cellular AA dynamics with regard to incorporation into and remodeling between different phospholipid classes, and the liberation of the fatty acid and oxygenation to form eicosanoids. Current analysis and characterization of these processes in the context of lipidomics has helped to understand new regulatory features of AA pathways of physiological and pathophysiological relevance. The study of AA regulation along with data from proteomics and genomics will enable the in-depth knowledge of the role of AA in different cell types and patients. This holistic point of view will put AA trafficking and metabolism in the context of systems biology approaches [154].

Acknowledgements

Work in our laboratory was supported by the Spanish Ministry of Science and Innovation

(Grants BFU2010-18826 and SAF2010-18831). CIBERDEM is an initiative of *Instituto de Salud Carlos III*.

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Figure Legends

Figure 1. AA incorporation in glycerophospholipids. AA is obtained directly from diet or synthesized from linoleic acid. Afterward, AA is incorporated into the *sn*-2 position of glycerophospholipids and later subjected to remodeling processes via CoA-IT-dependent reactions. ACS: acyl:CoA synthetase; LPCAT: lysophosphatidylcholine:acyl-CoA acyltransferase; PLA₂: phospholipase A₂; CoA-IT: independent-CoA transacylase.

Figure 2. The four pathways for AA metabolism into eicosanoids four different pathways; cyclooxygenase, lipoxygenase, cytochrome-P450 and non-enzymatic oxidation reactions.

Figure 3. AA-containing PC, PE and PI species in human monocytes (blue), human monocyte-derived macrophages (red) and mouse peritoneal macrophages (green). Data represent means of three independent determinations (S.E.M. < 15% for all determinations), and are given as a percent with respect to the total amount of AA-containing phospholipids. For further details see text. Shorthand notation of glycerophospholipids (abscissa) follows the guidelines proposed by Fahy *et al.* [155].

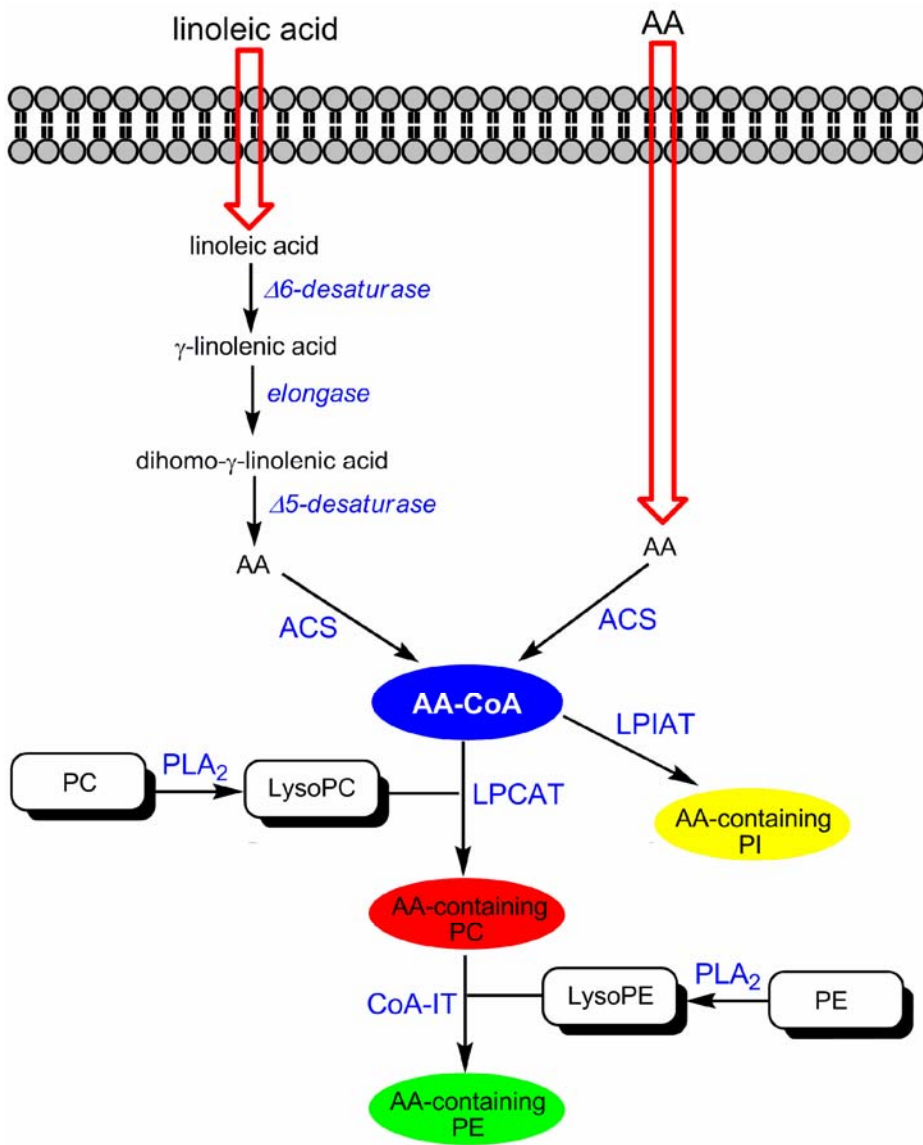


Figure 1

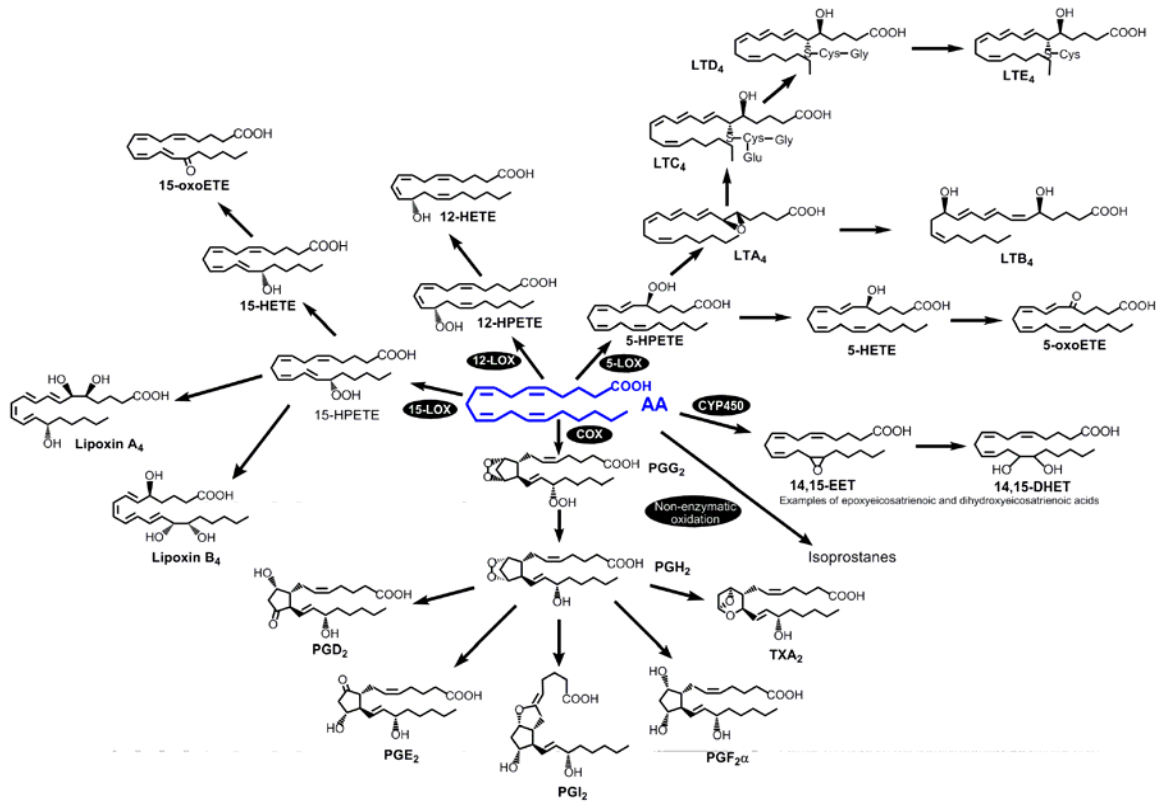


Figure 2

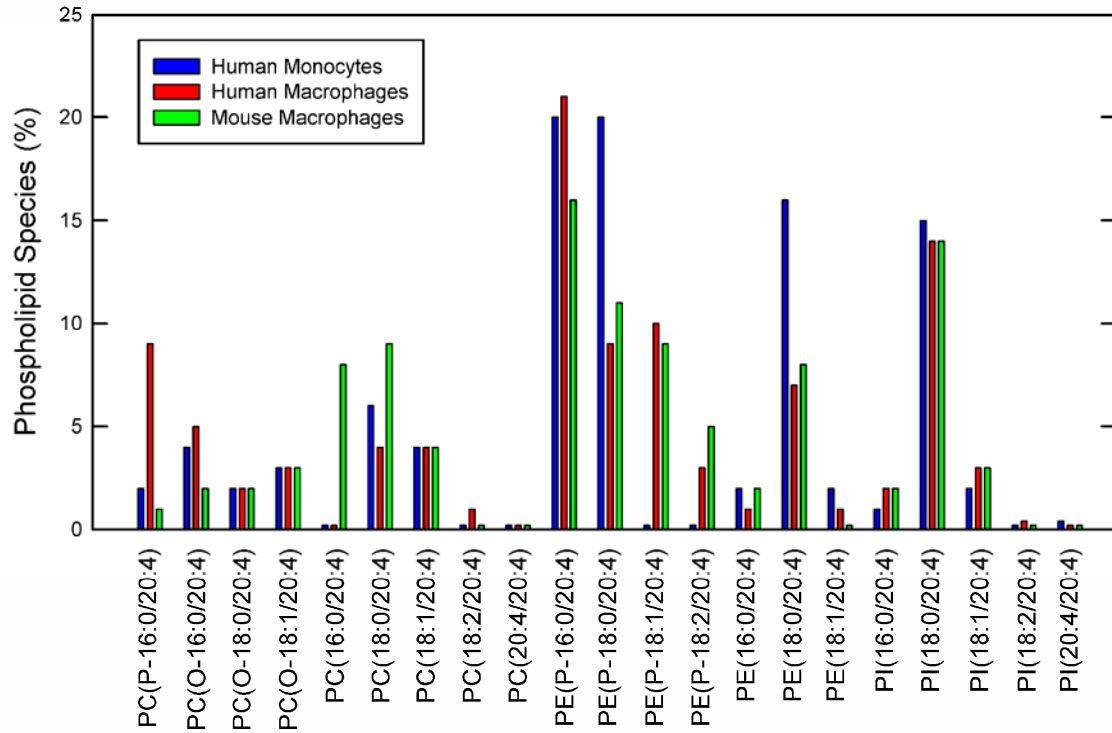


Figure 3