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Escuela de Doctorado Universidad de Valladolid

PROGRAMA DE DOCTORADO EN
INVESTIGACIÓN BIOMÉDICA

TESIS DOCTORAL:

Phospholipid Arachidonic Acid Remodeling in Macrophages: Role of Plasmalogen Species.

Presentada por Patricia Lebrero Fernández para optar al grado de Doctora por la Universidad de Valladolid.

Dirigida por:

Dr. Jesús Balsinde Rodríguez

Dra. María Ángeles Balboa García

MINISTERIO DE
CIENCIA, INNOVACION
Y UNIVERSIDADES

CSIC
Consejo Superior de Investigaciones Científicas

Universidad de Valladolid

INSTITUTO DE BIOLOGÍA Y GENÉTICA MOLECULAR

D. Jesús Balsinde Rodríguez, doctor en Ciencias Químicas y Profesor de Investigación del Consejo Superior de Investigaciones Científicas, y Dª María Ángeles Balboa García, doctora en Biología e Investigadora Científica del Consejo Superior de Investigaciones Científicas, certifican:

Que Dª Patricia Lebrero Fernández, Graduada en Biología por la Universidad de León y Máster en Investigación Biomédica por la Universidad de Valladolid, ha realizado bajo su dirección los estudios y experimentos necesarios para la elaboración de la memoria titulada "*Phospholipid Arachidonic Acid Remodeling in Macrophages: Role of Plasmalogen Species*", que presenta para optar al Grado de Doctor por la Universidad de Valladolid.

Y para que así conste donde proceda, firman la presente:

Fdo. Jesús Balsinde Rodríguez　　　　Fdo. María Ángeles Balboa García

Valladolid, 2019

LIST OF PAPERS

This thesis is based on the following studies:

I. **Lebrero, P.**, Astudillo, A.M., Rubio, J.M., Fernández-Caballero, L., Kokotos, G., Balboa, M.A., and Balsinde, J. (2019) 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* 8:799.

II. Astudillo, A.M., Meana, C., Guijas, C., Pereira, L., **Lebrero, P.**, Balboa, M.A., and Balsinde, J. (2018) Occurrence and biological activity of palmitoleic acid isomers in phagocytic cells. *J. Lipid Res* 59:237-249.

III. Gil-de-Gómez, L., Astudillo, A.M., **Lebrero, P.**, Balboa, M.A., and Balsinde, J. (2017) Essential role for ethanolamine plasmalogen hydrolysis in bacterial lipopolysaccharide priming of macrophages for enhanced arachidonic acid release. *Front. Immunol.* 8:1251

RESUMEN

Introducción

El ácido araquidónico (*cis*-5,8,11,14-eicosatetraenoico; AA) es el precursor de los eicosanoides, una gran familia de mediadores con papeles fundamentales en las fases de iniciación y resolución de la inflamación. En las células el AA se encuentra esterificado en la posición *sn*-2 de los glicerofosfolípidos de membrana, y la participación de las fosfolipasas A_2 liberando el ácido graso constituye un paso limitante para la síntesis de eicosanoides, un proceso que también depende de los niveles de expresión y actividad de las ciclooxigenasas y lipooxigenasas que metabolizan el AA.

El AA es el principal ácido graso poliinsaturado presente en las membranas de las células del sistema inmune innato y no se encuentra distribuido de forma uniforme entre los glicerofosfolípidos, si no que existen diferencias en su distribución entre diferentes especies moleculares. Monocitos y macrófagos muestran una distribución característica del AA entre las clases de fosfolípidos, siendo los fosfolípidos de etanolamina (PE) los que más AA contienen, seguidos de los fosfolípidos de colina (PC) y del fosfatidilinositol (PI). Atendiendo a las especies moleculares dentro de las clases de fosfolípidos, los plasmalógenos de etanolamina están particularmente enriquecidos con AA. Esta distribución asimétrica del AA en las células es clave para la regulación de la síntesis de eicosanoides ya que, dependiendo de la fuente de fosfolípidos del AA, pueden producirse ciertos eicosanoides con mayor preferencia que otros. Por ejemplo, la producción de metabolitos a través de lipooxigenasas en macrófagos peritoneales de ratón estimulados con zimosán, parece estar asociada con la movilización de AA de PC, y no de PE o PI. Esto implicaría que no todas las clases de fosfolípidos que contienen AA son accesibles a las fosfolipasas A_2 que producen la liberación del ácido graso. Por lo tanto, dependiendo de la compartimentalización del AA entre los distintos fosfolípidos de membrana puede constituir el tercer paso limitante para la síntesis de eicosanoides.

La incorporación del AA en los fosfolípidos celulares tiene lugar principalmente por el reciclaje del ácido graso proveniente de la posición *sn*-2 de los glicerofosfolípidos a través del ciclo de Lands. En esta ruta, los lisofosfolípidos generados por fosfolipasas A_2 constitutivamente activas, como la fosfolipasa A_2 independiente de calcio del grupo VIA ($iPLA_2\beta$), son utilizados por una aciltransferasa dependiente de coenzima A (CoA) para incorporar el AA en los fosfolípidos. La remodelación posterior a través de reacciones de transacilación entre los fosfolípidos, distribuye el AA en las clases de fosfolípidos apropiadas.

La transacilasa independiente de CoA (CoA-IT) es la principal enzima implicada en la remodelación de AA en la mayoría de las células. Esta enzima cataliza la transferencia de AA y otros ácidos grasos poliinsaturados principalmente desde especies diacil-PC a especies liso-PE o liso-PC. La CoA-IT presenta una gran afinidad por los aceptores lisofosfolipídicos que contienen un enlace éter en la posición *sn*-1 del esqueleto de glicerol, en particular los lisoplasmalógenos de etanolamina (1-alquenil-2-lisoglicerofosfoetanolamina) y alquil-liso-PC (1-alquil-2-lisoglicerofosfo-colina). Esta circunstancia podría explicar por qué el contenido de AA en especies de PC y PE con enlace éter es generalmente más alto que en sus equivalentes diacil. Aunque la secuencia de CoA-IT todavía no se ha identificado, su actividad ha sido caracterizada en preparaciones celulares y se han descrito inhibidores farmacológicos. Dado que algunas de las fosfolipasas A_2 mejor conocidas, como la fosfolipasa A_2 citosólica del grupo IVA ($cPLA_2\alpha$) o la $iPLA_2\beta$, han mostrado actividad CoA-IT en ensayos *in vitro*, se ha propuesto que, *in vivo*, la reacción CoA-IT puede representar una función no identificada de otra fosfolipasa A_2 descrita. Basándose en características bioquímicas comunes, como la unión a membranas o la independencia de calcio, se ha propuesto como candidata la fosfolipasa A_2 citosólica del grupo IVC ($cPLA_2\gamma$). Estudios de sobreexpresión de la $cPLA_2\gamma$ han proporcionado evidencias *in vivo* de que la enzima regula la composición de ácidos grasos de los fosfolípidos, aunque no está claro si las reacciones de remodelación implicadas son dependientes o independientes de CoA.

Objetivos

En trabajos previos se ha establecido como objetivo delinear los mecanismos moleculares que participan en la movilización del AA en células fagocíticas en respuesta a estímulos de la respuesta inmune innata, para elucidar las fuentes de AA implicadas en los procesos de liberación y reacilación, estudiar las fosfolipasas A_2 que regulan el metabolismo del ácido graso, y descubrir nuevos marcadores lipídicos específicos de estímulo cuyas rutas de síntesis pueden proporcionar dianas farmacológicas.

Por ello, el objetivo los objetivos específicos planteados en esta tesis fueron los siguientes:

- Tener un mejor conocimiento de los procesos que regulan la disponibilidad celular de AA, lo cual incluye estudiar la influencia de la compartimentalización del AA y examinar la dependencia de su remodelación del contenido de plasmalógenos.

- Descubrir nuevas características reguladoras de las reacciones de transacilación independientes de CoA en la homeostasis del AA y revelar el papel de las fosfolipasas A_2 en las respuestas de remodelación.

- Estudiar el proceso del primado con LPS de macrófagos para la liberación de AA y caracterizar el papel de las especies plasmalógenas en los mecanismos implicados en la disponibilidad de AA.

Métodos experimentales

Para estudiar los procesos de remodelación del AA regulado por fosfolipasas A_2 en macrófagos respondiendo a estímulos de la respuesta inmune innata, se han empleado técnicas de estudios lipidómicos basadas en espectrometría de masas, las cuales combinan con gran eficiencia la separación e identificación de metabolitos con sensibilidad. El desarrollo de las técnicas de cromatografía líquida (LC/MS) y cromatografía de gases (GC/MS) acopladas a espectrometría de masas han sido muy útiles para esclarecer los lípidos implicado en estos procesos a nivel de especies moleculares. Además, para caracterizar el papel de las especies plasmalógenas, se han empleado líneas celulares deficientes en plasmalógenos derivadas de células RAW (generadas por Dr. R. A. Zoeller). Por otro lado, se emplearon inhibidores químicos, así como RNA de interferencia para estudios de inhibición de distintos miembros de las fosfolipasas A_2.

Resultados y discusión

Metabolismo del ácido araquidónico en macrófagos.

Los macrófagos participan en los procesos inflamatorios debido, en parte, a su capacidad para sintetizar y liberar grandes cantidades de mediadores derivados del AA, denominados eicosanoides. Mediante técnicas basadas en espectrometría de masas, se caracterizó y comparó la composición y distribución de especies de fosfolípidos en macrófagos peritoneales de ratón (RPMs) y en la línea celular murina macrofágica RAW264.7. Ambos tipos celulares presentan diferencias en el contenido y distribución de ácidos grasos entre las distintas especies de fosfolípidos, principalmente de ácidos grasos poliinsaturados (PUFAs). Las células RAW264.7 presentan un menor contenido de AA en comparación con los RPMs, especialmente en los fosfolípidos de colina (PC), mientras que el contenido de AA en fosfolípidos de etanolamina (PE) es similar. A pesar de esta diferencia, el proceso de remodelación del AA desde PC a PE presenta un perfil similar en términos cualitativos y se produce a mayor velocidad (tiempo de remodelación menor) en las células RAW264.7.

Papel de los plasmalógenos de etanolamina.

Los estudios comparativos realizados con células deficientes en plasmalógenos mostraron que no hay diferencias significativas en la composición de ácidos grasos en ausencia de especies plasmalógenas y que el contenido de AA es muy similar en todas las clases de fosfolípidos. Siendo las especies plasmalógenas de PE las que mayor contenido de AA presentan, esto puede explicarse debido a una elevación compensatoria del contenido de AA en las especies diacil-PC y PE. Además, se comprobó que la velocidad y cinética de remodelación del AA desde PC a PE es la misma en las células deficientes, por lo que la remodelación de AA no está influenciada por el contenido celular de plasmalógenos.

Para estudiar si la deficiencia de plasmalógenos puede alterar la activación de los macrófagos, se indujo la polarización a macrófagos pro-inflamatorios (M1) o anti-inflamatorios (M2). Los resultados mostraron que no hay diferencias en los niveles de expresión de marcadores asociados a cada fenotipo. Además, el perfil de eicosanoides producidos tras la estimulación con zimosán es el mismo en presencia y ausencia de plasmalógenos, detectándose únicamente productos de la ruta de las ciclooxigenasas, principalmente prostaglandina D_2 (PGD_2).

Papel de las fosfolipasas A_2 en la remodelación.

La actividad de la $cPLA_2\alpha$ es fundamental en las reacciones de desacilación del AA de los fosfolípidos de membrana, mientras que las aciltransferasas dependientes de CoA reincorporan el ácido graso y son las consiguientes reacciones de transacilación entre los fosfolípidos las que distribuyen el AA entre las distintas clases y especies moleculares, lo que explica el alto contenido de AA en los plasmalógenos de etanolamina. La principal enzima implicada es la transacilasa independiente de CoA (CoA-IT), cuya secuencia no ha sido identificada. Sé evaluó la implicación de distintas isoformas de PLA_2s, observándose que la $cPLA_2\gamma$ es la única enzima que interviene en el proceso de remodelación, ya que las células con la enzima silenciada exhibían defectos en la remodelación del AA desde PC a PE, reflejado por una disminución significativa del tiempo de remodelación.

Incremento de la liberación de AA mediante el primado con LPS.

El LPS posee la capacidad de primar las células del sistema inmune innato a través del reconocimiento por TLR4 e incrementar la liberación de AA cuando están expuestas a un segundo estímulo inflamatorio. Los resultados muestran que PC es el principal aceptor para la incorporación de AA en macrófagos activados y que el primado con LPS no influye en dicho proceso. Además, una exposición con LPS previa a la estimulación disminuye el contenido de AA en algunas especies de PE. De acuerdo con la implicación de CoA-IT en reponer AA en PE, los resultados muestran que en células sin primar disminuye el contenido de todas las especies de fosfolípidos excepto PE, mientras que al primar con LPS se produce un incremento de la liberación de AA desde PE, especialmente de las especies plasmalógenas. Estos resultados indican que el primado con LPS bloquea la ruta de la CoA-IT y, por tanto, la disminución de la remodelación de AA a las especies plasmalógenas de etanolamina es responsable del incremento de movilización del ácido graso en las células activadas.

Por otro lado, el primado con LPS disminuye la remodelación de AA desde PC a PE, lo que resulta en una reducción de la incorporación del ácido graso en las especies plasmalógenas de PE. Esta diferente compartimentalización del AA deriva en una mayor liberación de PE y un incremento de los niveles de AA libre debido a la hidrólisis de PC mediada por la cPLA$_2\alpha$. Por tanto, la disminución de la actividad de CoA-IT tras el primado con LPS constituye un riesgo debido al incremento de la producción de eicosanoides como consecuencia de una mayor liberación de AA desde PC.

En resumen, todos estos descubrimientos enfatizan la participación de los plasmalógenos en la ejecución de algunas respuestas de los macrófagos y exponen las reacciones de desacilación/reacilación como un paso crítico para el aumento de la liberación del AA en macrófagos primados con LPS. Esto podría deberse a una inhibición de la CoA-IT, que favorece la acumulación de AA en PC al bloquear la transferencia del ácido graso a PE. Además, se ha demostrado que la cPLA$_2\gamma$ es la principal enzima implicada en la remodelación de AA mediada por CoA-IT y que los plasmalógenos están implicados en este proceso.

Conclusiones

En conjunto, estos resultados proporcionan nueva información para un mejor conocimiento de los procesos de regulación de la disponibilidad celular de AA y conducen a las siguientes conclusiones principales:

1. El contenido celular de plasmalógenos no influye en los niveles o la distribución de AA en los macrófagos.
    1.1. Las reacciones de remodelación de AA mediadas por CoA-IT son independientes del contenido celular de plasmalógenos. Los enlaces de las especies molecular no influyen en la distribución del AA en células del sistema inmune innato, que parece depender principalmente de la composición del grupo polar de los fosfolípidos de membrana.

1.2. El contenido celular de plasmalógenos no influye ni en los niveles de AA ni en su distribución relativa entre las clases de fosfolípidos. Parece ser que son los niveles endógenos de AA y no de plasmalógenos lo que determina la velocidad de remodelación del AA entre los fosfolípidos.

1.3. La implicación de los plasmalógenos es diferente en algunas, pero no todas, respuestas de los macrófagos. Esto refleja algún tipo de especificidad biológica de este tipo de fosfolípidos.

1.4. Las fosfolipasas A_2 citosólicas de los grupos IVA y IVC (cPLA$_2\alpha$ and cPLA$_2\gamma$) presentan distintos papeles en la homeostasis celular del AA: la primera regula la liberación de AA, pero no su remodelación, mientras que la segunda hace lo contrario.

1.5. La cPLA$_2\gamma$ es la principal enzima implicada en la remodelación del AA en fosfolípidos.

2. La hidrólisis de los plasmalógenos de etanolamina es un paso importante en el primado de macrófagos con LPS.

2.1. El primado con LPS provoca la disminución de la incorporación de AA en los plasmalógenos de etanolamina y facilita la hidrólisis neta de dichas especies después de la estimulación con zimosán, lo cual parece deberse a una reducción de la remodelación de AA por una reducción de la activación de CoA-IT.

2.2. El bloqueo de la ruta de la CoA-IT a través del primado con LPS es macrófagos estimulados conduce a un incremento de los niveles de AA libre disponibles para la síntesis de eicosanoides, probablemente debido a un mayor acceso de la cPLA$_2\alpha$ a las reservas de AA presentes en PC.

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ABBREVIATIONS

AA: arachidonic acid

AA-DHAP-R: acyl/alkyl-dihydroxyacetone reductase

AAG3P-AT: alkyl/acyl-glycero-3-phosphate acyltransferase

ACS: acyl-CoA synthetase

ACSBG: bubblegum acyl-CoA synthetase

ACSS: short-chain acyl-CoA synthetase

ACSM: medium-chain acyl-CoA synthetase

ACSL: long-chain acyl-CoA synthetase

ACSVL: very long-chain acyl-CoA synthetase

ADHAP-S: alkyl-DHAP synthetase

AdPLA$_2$: adipose-specific phospholipase A$_2$

AGPAT: 1-acyl-glycerol-3-phosphate O-acyltransferase

AMP: adenosine monophosphate

ATP: adenosine triphosphate

BSA: bovine serum albumin

CoA: coenzyme A

CoA-AT: CoA-dependent acyltransferases

CoA-IT: CoA-independent transacylase

COX: cyclooxygenase

cPLA$_2$: cytosolic phospholipase A$_2$

CYP450: cytochrome P450

DGLA: dihomo-γ-linolenic acid

DHAP: dihydroxyacetone phosphate

DHAP-AT: dihydroxyacetone phosphate acyltransferase

DHETs: dihydroxyeicosatrienoic acids

DMEM: Dulbecco's Modified Eagle Medium

eCBs: endocannabinoids

EETs: epoxyeicosatrienoic acids

EPA: eicosapentanoic acid

ER: endoplasmic reticulum

ERK: extracellular signal–regulated kinase

FAME: fatty acid methyl ester

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPCRs: G protein-coupled receptors

GPL: glycerophospholipids

GC/MS: gas chromatography/mass spectrometry

HETE: hydroxyeicosatetraenoic acid

HHT: hydroxyheptadecatrienoic acid

HpETE: hydroperoxyeicosatetraenoic acid

IgG: immunoglobulin G

IL: interleukin

IL-1R: interleukin 1 receptor

iPLA$_2$: calcium-independent phospholipase A$_2$

IsoPs: isoprostanes

JNK: c-Jun N-terminal kinase

LC/MS: liquid chromatography/mass spectrometry

LOX: lipoxygenase

$LPLA_2$: lysosomal phospholipase A_2

LPLAT: acyl-CoA:lysophospholipid acyltransferase

LPS: bacterial lipopolysaccharide

LRR: leucine-rich-repeat

LT: leukotriene

LX: lipoxin

LysoPL: lysophospholipid

LysoPC: lysophosphatidylcholine

LysoPE: lysophosphatidylethanolamine

LysoPI: lysophsphatidylinositol

MAPK: mitogen-activated protein kinase

MBOAT: O-acyltransferase membrane-bound

NSAIDs: non-steroidal anti-inflammatory drugs

PA: phosphatidic acid

PAF: platelet-activating factor

PAF-AH PLA_2: PAF-acetylhydrolase A_2

PAMP: pathogen-associated molecular pattern

PC: phosphatidylcholine

PCR: polymerase chain reaction

PE: phosphatidylethanolamine

PGs: prostaglandins

PGE_2: prostaglandin E_2

PGHS: prostaglandin H synthase

PH: phosphohydrolase

PI: phosphatidylinositol

PL: phospholipid

PLA_2s: phospholipase A_2 enzymes

PLC: phospholipase C

PRRs: pattern recognition receptors

PS: phosphatidylserine

PUFA: polyunsaturated fatty acid

qPCR: quantitative polymerase chain reaction

RPMs: resident peritoneal macrophages

SE: standard error

Ser: serine

sn: stereospecifically numbered

SPM: specialized pro-resolving lipid mediator

sPLA_2: secreted phospholipase A_2

TLC: thin-layer chromatography

TNFα: tumor necrosis factor α

TLR: toll-like receptor

TX: thromboxane

INTRODUCTION

1.1. The inflammatory response: the immune system.

The immune system is traditionally classified in two types: innate and adaptive. The first one, also known as non-specific immunity, constitutes the early line of host defense and it is present in almost all multicellular organisms. In contrast, the adaptative immune system is antigen-specific and includes memory that makes future responses more efficient.

The innate immune system consists of cellular and biochemical mechanisms that provide a rapid response to invading pathogens, among which the inflammation process stands out. This represents the organism response to local injury caused by an external damage or aggression. It leads to the accumulation of blood cells and it is triggered to control the damage and begin the repair processes, consisting on a defense against microscopic invaders (1). Inflammation is classically characterized by the cardinal signs: redness, swelling, heat, pain and loss of function. Local damage induces an immediate and acute response started by the release of chemical mediators.

The main immune innate components are physical and chemical barriers, phagocytic cells, dendritic cells, innate lymphoid cells and circulating plasma proteins. Phagocytic cells play an essential role within the innate immune system, cleaning tissues in a non-specific way. There are two types of phagocytic cells: mononuclear and polymorphonuclear (**Figure 1**). The mononuclear phagocyte system includes a population of bone marrow-derived myeloid cells that circulate in the blood as monocytes and populate tissues as macrophages during inflammation. It has long been considered that macrophages are derived and differentiated from blood monocytes, show variable morphology and have different functions consistent with the tissue in which they arise (2,3). However, recent studies have questioned the hypothesis that tissue-resident macrophages arise from circulating monocytes indicating that they do not originate from monocytes in a steady-state but indicating that some macrophage compartments are established by fetal precursors and maintained independently of hematopoiesis (4-7).

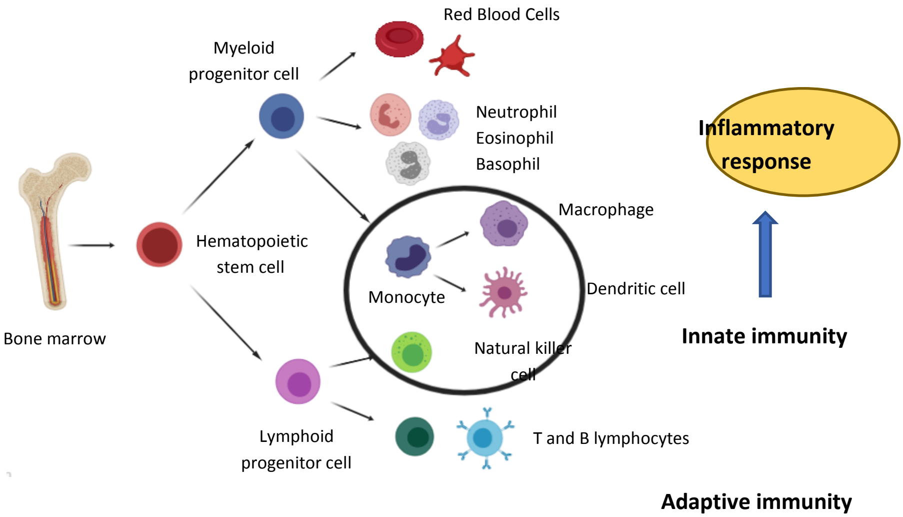

Red Blood Cells
Myeloid progenitor cell
Neutrophil
Eosinophil
Basophil
Inflammatory response
Macrophage
Hematopoietic stem cell
Monocyte
Dendritic cell
Bone marrow
Innate immunity
Natural killer cell
Lymphoid progenitor cell
T and B lymphocytes
Adaptive immunity

Figure 1. Innate and adaptive immune cell types.

1.1.1. Pathogen recognition: Toll-like receptors.

Monocytes are endowed with chemokine receptors and pattern recognition receptors (PRRs), which distinguish conserved molecular patterns of strange organisms, and can adapt to their local microenvironment and to develop into unique types of macrophages. Monocytes can exit the blood, adhere to vascular endothelial cells and migrate to tissues where they differentiate into macrophages, under inflammatory conditions. Macrophages, as resident phagocytic cells, can be involved in the tissue homeostasis via the clearance of pathogens and apoptotic cells, and they can directly recognize a wide variety of pathogens through the PRRs, also known as pathogen-associated molecular patterns (PAMPs), that induce production of inflammatory cytokines (8,9).

Several classes of PRRs, including Toll-like receptors (TLRs), are known to play essential roles in the recognition of distinct microbial components. Exposure of immune cells to the ligands of these receptors activates intracellular signaling cascades that induce the acute inflammatory response to return to normal homeostasis. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing leucine-rich-repeat (LRR) motifs in variable number, and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R).

A total of 13 mammalian TLR paralogues have been described (**Table 1**), each responsible for the recognition of different microbial structures. Based on their primary sequences, TLRs can be divided into several subfamilies, namely from TLR1 to TLR13, of which only 10 (TLR1 to TLR10) are expressed in human and 12 (TLR1, 2, 3, 4, 5, 6, 7, 8, 9 and TRL11, 12, 13) in mice. The receptors are differentially localized within the cells, so they can mediate recognition of extracellular and intracellular pathogens. Based on their localization, TLRs are classified into two subfamilies: while TLRs 1, 2, 4, 5, 6 and 10 are located on cell surface, TLRs 3, 7, 8, 9, 11, 12 and 13 are expressed almost exclusively in intracellular compartments (such as endoplasmic reticulum, endosome, lysosome or endolysosome), and their ligands require internalization to endosomes so that the signaling is possible. To signal and activate inflammatory responses, TLRs recognize structural components unique to bacteria, fungi and viruses, so that each subfamily can recognize related PAMPs. Cell surface TLRs mainly recognize microbial membrane components such as lipids, proteins and lipoproteins. TLR4 recognizes bacterial lipopolysaccharide (LPS) and TLR2 along with TLR1 or TLR6 recognizes several PAMPs, including lipoproteins, peptidoglycans and zymosan. However, intracellular TLRs identify nucleic acids derived from both bacteria and viruses and can also recognize self-nucleic acids in disease environments such as autoimmunity. In addition, non-esterified fatty acids (saturated fatty acids) can signal through TLR2 and TLR4 on macrophages and induce pro-inflammatory gene expression (10-13).

TLR2 has a wide diversity of ligand recognitions because it can recognize the ligands in association with structurally related TLRs such as TLR1 and TLR6, forming heterodimers: TLR2-TLR1 recognize Gram-negative bacteria-derived triacyl lipopeptide, and TLR2-TLR6 identify diacyl lipopeptide from mycoplasma. In association with the structurally unrelated C-type lectin family (known as dectin-1), TLR2 can also recognize zymosan (β-1,3-glucan and β-1,6-glucan). TLR4 recognizes LPS from Gram-negative bacteria and its activation is supported by another protein known as LBP (LPS-binding protein). An active component of LPS, the lipid A, forms a complex with a recognition subunit MD2 (myeloid differentiation protein-2), which interacts with TLR4 and activates signaling (14).

Table 1. Toll-like receptors in the innate immune response.

TLR	Ligand	Location	Expression
TLR1	Triacyl lipopeptides	Cell surface	Human/mice
TLR2	Peptidoglycan/Zymosan	Cell surface	Human/mice
TLR3	Double-stranded RNA	Intracellular compartments	Human/mice
TLR4	LPS	Cell surface	Human/mice
TLR5	Flagellin	Cell surface	Human/mice
TLR6	Diacyl lipopeptides/Zymosan	Cell surface	Human/mice
TLR7	Single-stranded RNA	Intracellular compartments	Human/mice
TLR8	Single-stranded RNA	Intracellular compartments	Human/mice
TLR9	Unmethylated CpG DNA	Intracellular compartments	Human/mice
TLR10	Not determined	Cell surface	Human
TLR11	Profilin-like protein	Intracellular compartments	Mice
TLR12	Not determined	Intracellular compartments	Mice
TLR13	Not determined	Intracellular compartments	Mice

1.1.2. Eicosanoids as mediators in the inflammatory response.

The inflammation process is promoted by the release of chemical mediators that increase vascular wall permeability permitting the migration of blood cells into the surrounding tissue. These mediators include lipids, peptides, reactive oxygen species, amino acid derivatives and enzymes. The type of chemical mediators produced depends on the cell type, the anatomical site involved, the nature of the inflammatory stimulus and the stage during the inflammatory response.

Along with other signaling mediators, bioactive lipids regulate many cell functions such as immune regulation, inflammation and maintenance of homeostasis. Some lipid mediators acting as second messengers are formed from fatty acids released from membrane phospholipids upon cellular activation and the fatty acid composition of cell membrane phospholipids influences the cell function through different mechanisms such as membrane order and lipid raft assembly. This suggests that fatty acids may play an important role in promoting or suppressing inflammatory processes. The formation and functions of these molecules relies on the prevalence of omega-6 or omega-3 polyunsaturated fatty acid (PUFA) precursors (15,16).

Eicosanoids, phospholipids and sphingolipids, endocannabinoids (eCBs) and specialized pro-resolving lipid mediators (SPMs), are the main families of bioactive lipids. Eicosanoids constitute the most distinguishable family of bioactive lipids involved in innate immunity and have multiple biological functions in the development of diseases associated with inflammatory processes. Their main role consists in amplifying or reducing inflammation, coordinating cytokine and chemokine production, leukocyte recruitment, antibody formation, cell proliferation and migration, and antigen presentation.

They are formed from the omega-3 eicosapentaenoic acid (EPA; 20:5n-3), or the omega-6 fatty acids dihomo-γ-linolenic acid (DGLA; 20:3n-6) and especially, arachidonic acid (AA; 20:4n-6). They comprise prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxins (LXs), isoprostanes (IsoPs), and epoxyeicosatrienoic acids (EETs). Eicosanoid biosynthesis is initiated by oxygen radical reactions after AA release from phospholipids under stimulation conditions. The AA acts as a substrate for enzymatic reactions catalyzed by specific oxygenases: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450); or can be metabolized by non-enzymatic reactions (**Figure 2**). In general, AA-derived eicosanoids are pro-inflammatory mediators, although it is now recognized that other eicosanoids derived from AA, such as lipoxin A_4, have anti-inflammatory effects and that prostaglandin E_2 (PGE_2), for example, act in both pro- and anti-inflammatory ways (17).

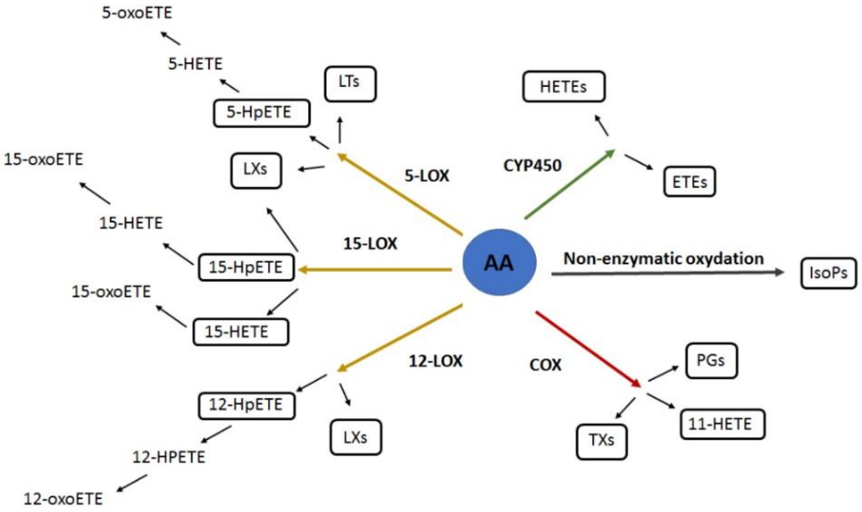

5-oxoETE
5-HETE
5-HpETE
LTs
LXs
5-LOX
15-oxoETE
15-HETE
15-HpETE
15-LOX
15-oxoETE
15-HETE
12-LOX
12-HpETE
LXs
12-HPETE
12-oxoETE
AA
CYP450
HETEs
ETEs
Non-enzymatic oxydation
IsoPs
COX
PGs
11-HETE
TXs

Figure 2. Overview of the eicosanoid synthesis pathways from arachidonic acid. AA, arachidonic acid; COX, cyclooxygenase; CYP450, cytochrome P450 enzymes; ETE, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid, IsoP, isoprostane; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane.

Biosynthesis of prostaglandins (PGs) and thromboxanes (TXs)

Prostanoid synthases are mainly expressed on innate immune cells and prostanoid receptors are expressed on both innate and adaptive immune systems. During inflammation, activated innate immune cells produce prostanoids that will act in a paracrine manner on lymphocytes and in an autocrine way, modulating their own function (18,19).

AA can be enzymatically converted to prostanoids (PGs and TXs) by COX enzymes. PGs are signaling molecules synthesized *de novo* when AA is metabolized by COX, also known as prostaglandin H synthase (PGHS), which incorporates molecular O_2 and forms the intermediate endoperoxide PGG_2. Two enzymes possessing COX activity exist: COX-1 (PGHS-1) and COX-2

(PGHS-2). COX-1 is constitutively expressed in most tissues, while COX-2 expression is transiently induced in response to a variety of cell stimuli. PGG_2 is then reduced by peroxidase activity, which reduces peroxide to hydroxyl to form PGH_2. Depending on the cell type and tissue, PGH_2 is further metabolized into PGE_2, PGD_2, PGF_{2a}, prostacyclin (PGI_2), and TXA_2. These compounds can achieve important biological functions, such as vascular permeability, muscle tone modulation, fever and platelet aggregation (20). The action of PGs and TXs is mediated through binding to G protein-coupled receptors (GPCRs), and the differential expression and distribution of COX isoforms within the inflammatory cells, will determine the profile of prostanoid production. PGE_2 is the most abundant eicosanoid and the primary metabolite of AA, and it can act locally in autocrine or paracrine ways. PGE_2 is a well-known mediator of cancer, inflammation, fever, atherosclerosis and other pathophysiological processes. Owing to that, non-steroidal anti-inflammatory drugs (NSAIDs) have been developed to inhibit COX enzymes, being their primary therapeutic effect due to reduced PGE_2 biosynthesis. Monocyte/macrophage-derived PGE_2 plays an important physiological role due to these immune cells possess high PGE_2 biosynthetic capacity (21).

Biosynthesis of leukotrienes (LTs)

Leukotrienes are synthesized from AA by the action of 5-lipoxygenase (5-LOX) enzyme, which is translocated to the nuclear envelope during cell stimulation. 5-LOX catalyzes the first enzymatic step by adding molecular O_2 to AA. Thus, 5-hydroxperoxyeicosatetraenoic (5-HpETE) is formed and metabolized to LTA_4, an unstable intermediate that can be hydrolyzed to generate LTB_4 (a potent stimulus for inflammatory leukocyte function) or LTC_4 by glutathione addition, which is subsequently converted into LTD_4 and LTE_4 (22,23). Like PGs, LTs have a short half-life and are mainly involved in localized signaling. They participate in defense reactions and pathophysiological conditions such as hypersensitivity and inflammation, and are synthesized in inflammatory cells including granulocytes, mast cells and macrophages, dendritic cells, and B lymphocytes (24,25).

Biosynthesis of lipoxins (LXs)

Lipoxins were the first eicosanoids reported with both anti-inflammatory and pro-resolving actions (26). They are short-lived eicosanoids derived from AA through the activity of 12/15-LOX, which mediate the conversion of the fatty acid to 15-hydroxyeicosatetraenoic acid (15(S)-HETE), and the following action of 5-LOX, that transforms this intermediate into LXA_4 and LXB_4. LXA_4 has anti-inflammatory protective actions in several pathophysiologic processes.

LXs are appreciated for their ability to promote resolution by attracting monocyte cells. It is also known that LXs promote efferocytosis, meaning macrophage clearance of apoptotic polymorphonuclear leukocytes and they have also been assigned anticancer and neuroprotective properties (27-29).

In addition, LOX enzymes can synthesize other products with biological activity, including HETEs, which can be reduced to oxoeicosatetraenoic acids (oxoETEs) and hepoxilins (HXA3 and HXB3), that are characterized by the presence of an epoxide group (20).

Cytochrome P450 monooxygenase pathway

The CYP monooxygenase family of enzymes catalyzes the oxidation of AA to generate eicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs). The EETs have been associated with anti-inflammatory properties in cardiovascular diseases and low levels seem to be implicated in obesity and diabetes progression (30-32).

In summary, it is clear that changes in cell activation and signaling pathways can alter the eicosanoid biosynthesis and the overall balance, producing eicosanoids with similar or opposing functions (33).

1.1.3. Arachidonic acid release upon cellular activation.

Microbial recognition by phagocyte cells triggers the production of cytokines, chemokines and lipid mediators, this resulting the induction of microbial killing. Not all microbes induce the same responses and the nature of the effector response depends on the innate immune recognition receptors involved (34).

Zymosan stimulation

Among all the stimuli that can activate immunoinflammatory cells for AA mobilization, zymosan is one of the best known and has been used for over 50 years. Zymosan is a cell wall preparation of *Saccharomyces cerevisiae*, primarily composed of glucans, mannans, mannoproteins, and chitin, compounds that have been implicated in recognition of yeast by the innate immune system (35). Like other pathogens, zymosan can be opsonized, that is, it can be covered by the opsonins present in serum, which include immunoglobulin G (IgG) and complement factors. These molecules can be recognized by membrane receptors and make phagocytes more efficient for to ingest bacteria (36).

A variety of receptors have been implicated in recognition and phagocytosis of zymosan particles. Opsonized zymosan principally binds to Fc receptors (FcR), which recognize IgG particles, and to complement 3 receptors (CR3), that recognize opsonized particles with the C3b complement and, specifically, with the C3bi fragment (37). Despite CR3 has the ability to promote phagocytosis, it is unable to trigger AA release in murine resident peritoneal macrophages (38). In contrast, the predominant receptors involved in non-opsonized zymosan recognition are the beta-glucan receptor Dectin-1 and, to a lesser extent, the Toll-like receptors TLR2/TLR6 (39-41).

AA release and the subsequent eicosanoid synthesis in zymosan-stimulated mouse peritoneal macrophages was first described decades ago (42-44), and it was demonstrated that the enzyme responsible of effecting the AA release is a phospholipase A_2 (45-47). Later it was shown that the main effector is the group IVA cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$), which is regulated by phosphorylation and the elevation of intracellular Ca^{2+} concentration. During zymosan phagocytosis, the c-Jun N-terminal kinase (JNK) phosphorylates cPLA$_2\alpha$, with the subsequent release of AA (48-50). It is also described that group V secreted phospholipase A$_2$ (sPLA$_2$-V) participates in this process (51,52).

In addition to AA release, zymosan recognition is implicated in pro-inflammatory cytokine production, activation by phospholipase C (PLC) phosphorylation and an increase in intracellular calcium (53,54).

LPS stimulation

As Gram-negative bacteria produce sepsis and septic shock, constituting a major cause or morbidity and mortality, bacterial lipopolysaccharide (LPS) plays a central role in the inflammatory response. LPS stimulates innate immunity cells via engagement of TLR4. It is known that, while LPS alone is a poor stimulus for AA release on its own, it can prime macrophages for enhanced release of AA triggered by a second inflammatory stimulus (11,55,56). In contrast to zymosan, the LPS effect on AA release is not observed during the first two hours of exposure to the stimulus, and it has been described that long incubation times are needed for AA release to occur (38,57). However, it has been described that one hour of LPS preincubation before zymosan stimulation of mouse peritoneal macrophages, is enough to trigger an enhanced AA release response (56).

1.1.4. Arachidonic acid mobilization.

Arachidonic acid (AA) is found at relatively high levels in innate immunity cells, such as monocytes, macrophages and dendritic cells. AA plays a central role in inflammatory reactions, as it is the common precursor of the eicosanoids, and free AA can exert pathophysiological functions on its own, for example, inducing apoptosis (58,59). AA is a 20-carbon fatty acid with four methylene-interrupted *cis* double bonds. It belongs to the omega-6 polyunsaturated fatty acid family (PUFAs), being its chemical designation *cis*-5,8,11,14-eicosatetraenoic acid, abbreviated as 20:4n-6 (**Figure 3**).

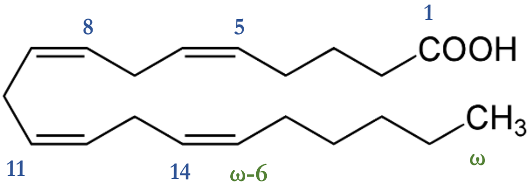


Figure 3. Arachidonic acid hairpin configuration.

AA can be obtained from food such as animal organs and meat, fish, seafood and eggs (60-63), but the major AA source is linoleic acid, an 18-carbon PUFA containing two *cis* double bonds (18:2n-6), which animals cannot synthesize. It is abundant in many nuts, fatty seeds and their derived vegetable oils (63). It is converted into AA by stepwise desaturation and chain elongation: it is first oxidized by Δ6-desaturase to γ-linolenic acid (18:3n-6), which is further elongated to dihomo-γ-linolenic acid (20:3n-6) and this is oxidized by Δ5-desaturase (64-67) (**Figure 4**).

AA exhibits high biological activity and its availability in free form constitutes a limiting factor for eicosanoid synthesis. While low levels of AA can render a protective response (68,69), the free molecule and its derivatives appear to induce an autotoxic response and cause metabolic disfunctions when produced in excessive quantities (70,71). Because of this, cells must exert an exhaustive control on free AA levels. For this purpose, it is required the action of a large group of enzymes working together to ensure low free AA levels in resting cells, so the eicosanoid synthesis and other biological processes are avoided; as well as guarantee AA availability for eicosanoid production in case of cell stimulation.

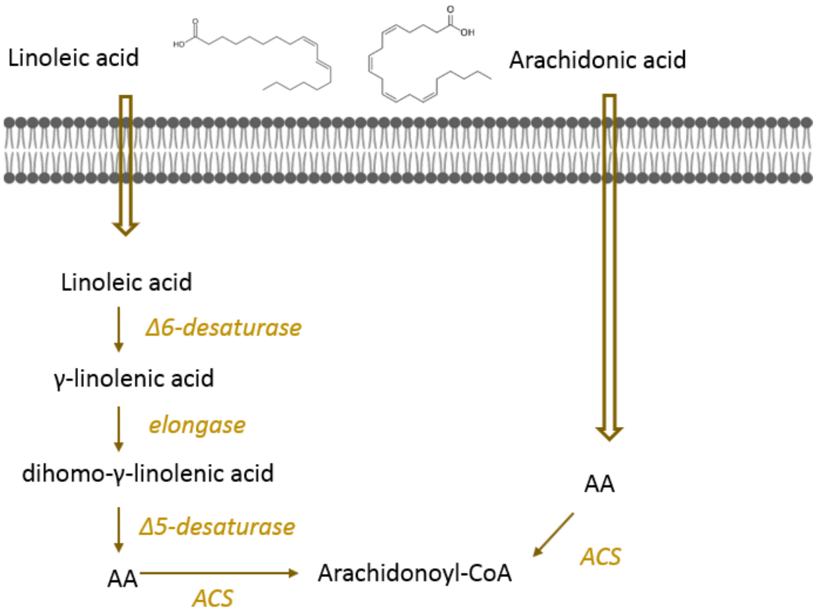

Linoleic acid
Arachidonic acid
Linoleic acid
Δ6-desaturase
γ-linolenic acid
elongase
dihomo-γ-linolenic acid
Δ5-desaturase
AA
ACS
Arachidonoyl-CoA
AA
ACS

Figure 4. Arachidonic acid sources.

Once AA is synthesized in vivo or obtained from the diet, it is incorporated into glycerophospholipids (GPLs), which are composed of a glycerol backbone esterified with two hydrophobic fatty tails at the *sn*-1 and 2 positions (stereospecifically numbered, *sn*) and a hydrophilic head-group at *sn*-3. In mammalian cells, many plasma membrane and cytoplasmic phospholipids contain AA in the *sn*-2 position of the glycerol backbone (**Figure 5**).

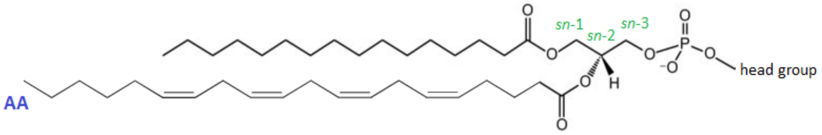

sn-1
sn-2
sn-3
head group
AA

Figure 5. Glycerophospholipid structure containing arachidonic acid in the *sn*-2 position of the glycerol backbone.

To regulate AA availability, there are selective pathways within inflammatory cells that traffic the fatty acid into glycerophospholipid pools. The levels of free AA are modulated by two opposing reactions: on one hand, phospholipid deacylation, on the other hand, reacylation back into phospholipids (PLs). The fatty acid excision from the *sn*-2 position of glycerophospholipids is carried out by phospholipase A_2 enzymes (PLA_2s), and the reincorporation into phospholipids by the coordinated action of acyl-CoA synthetases (ACS) and CoA-dependent acyltransferases (CoA-AT) (72,73). This deacylation-reacylation cycle is known as the Lands cycle (**Figure 6**).

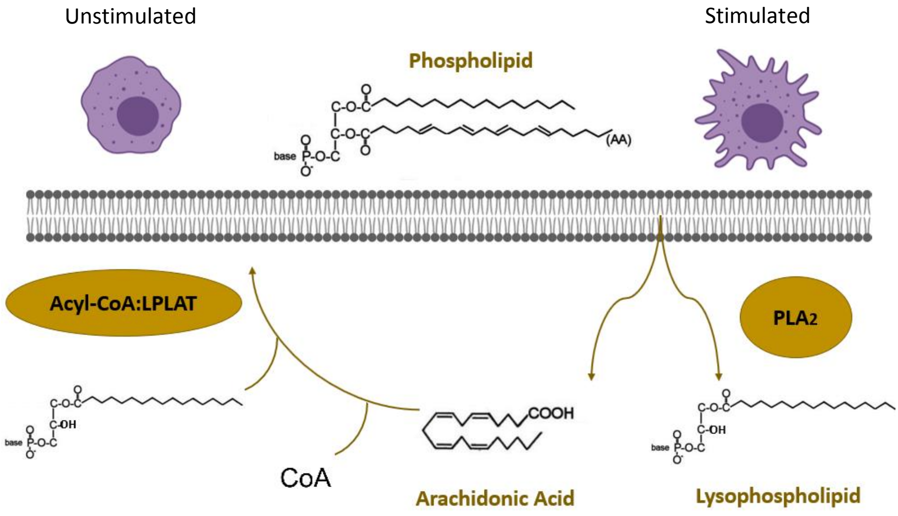


Figure 6. Phospholipid remodeling with arachidonic acid via the deacylation-reacylation cycle (Lands cycle). The PLA_2 releases the fatty acid from a phospholipid, and then the free fatty acid is incorporated into a lysophospholipid by an acyl:CoA-lysophospholipid acyl transferase (acyl-CoA:LPLAT).

Depending on the cell state, one reaction dominates over the other one. Considering this, in resting conditions, reacylation dominates and AA is mainly esterified in the *sn*-2 position of phospholipids. In this case, the free AA amount available for eicosanoid production is low. However, in stimulated cells, the deacylation reaction dominates, resulting in elevated levels of free AA and an enhanced eicosanoid synthesis (73). Even so, under activation conditions, reacylation process is still very significant, which is manifested by the fact that only a small fraction of the AA released is used for eicosanoid synthesis, being most of it reacylated back into PLs by lysophosphatidic-acyltransferases (LPLAT) (72). The enzymes that participate in AA levels control are described below.

1.2. Enzymes of arachidonic acid metabolism.

1.2.1. The Phospholipase A_2 (PLA_2) family.

The phospholipase A_2 family (PLA_2) is a heterogeneous group of lipolytic enzymes that have been classified into several subfamilies (74-79).

Based on sequence homology criteria, the most recent classification involves more than 30 enzymes included in 16 groups (80). With the increased PLA_2 diversity, it has been useful to use a simplified classification which divides the PLA_2 enzymes in 6 groups, according to biochemical and functional characteristics: secreted phospholipase A_2s ($sPLA_2$), calcium-dependent cytosolic phospholipase A_2s ($cPLA_2$), calcium-independent phospholipase A_2s ($iPLA_2$), PAF acetylhydrolases (PAF-AH PLA_2), lysosomal phospholipase A_2s ($LPLA_2$), adipose-specific phospholipase A_2s ($AdPLA_2$). Within the different groups there can be different paralogs, namely more than one homologous PLA_2 gene within a species. In that case, each PLA_2 is assigned with a subgroup letter.

The members of this family typically hydrolyze the ester bond in *sn*-2 position of glycerophospholipids, releasing a fatty acid and the corresponding lysophospholipid (**Figure 7**). By default, substrates include short fatty acid chain oxidized phospholipids, and long fatty acid chain phospholipids with sn-2 acyl chains up to 20 carbons (arachidonate) and longer. However, some of them present low or no PLA_2 activity, whereas they show phospholipase A_1, lysophospholipase, neutral lipid lipase or transacylase/acyltransferase activities(80-82).

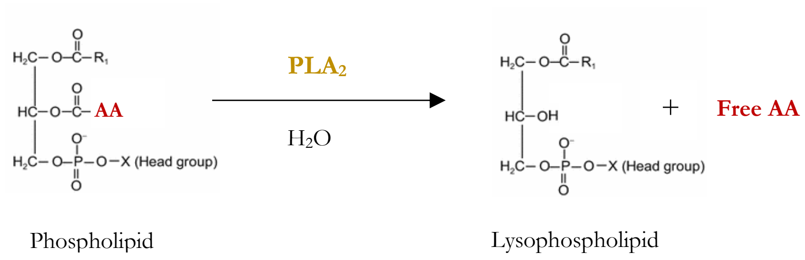


Figure 7. Hydrolysis reaction of arachidonic acid from the phospholipid *sn*-2 position catalyzed by phospholipase A_2 enzymes.

Cytosolic phospholipase A_2s ($cPLA_2$)

The $cPLA_2$s are cytosolic proteins with high molecular weight (61-114 kDa) which share a lipase common GXSXS consensus sequence and a catalytic serine (Ser) in their active site. As of today, 6 different $cPLA_2$s have been identified in mammals and they are classified within group IV of the PLA_2 family with the suffixes A-F, although we usually refer to them as α, β, γ, δ, ε and ζ (**Table 2**) (83-87).

Table 2. Group IV Cytosolic Phospholipase A_2s ($cPLA_2$s).

Subgroup	**Alternative name**	**Sources**	**Molecular mass (KDa)**
IVA	$cPLA_2α$	Human macrophage-like U937 cells/platelets/RAW264.7/rat kidney, ubiquitous	85
IVB	$cPLA_2β$	Human pancreas/liver/heart/brain, ubiquitous	100-114
IVC	$cPLA_2γ$	Human heart/skeletal muscle	61
IVD	$cPLA_2δ$	Murine placenta	91
IVE	$cPLA_2ε$	Murine heart/skeletal muscle/testis/thyroid	95
IVF	$cPLA_2ζ$	Murine thyroid/stomach	95

With the exception of the group IVC or cPLA$_2$γ, these enzymes possess a C2 domain of Ca^{2+} binding at their amino terminus, which allows them to translocate to intracellular membranes, for which they only require micromolar concentrations of Ca^{2+} (80,81,88). The first one that was discovered was the cPLA$_2$α, which uses a Ser/Asp dyad (89-94). The cPLA$_2$s β, δ, ε and ζ belong to a group of genes located on the human chromosome 15, whereas the cPLA$_2$s α and γ are in the chromosome 1 and 19, respectively (95,96). The first one that has been cloned and the one that has been more studied of all of them is the group IVA PLA$_2$ or cPLA$_2$α (87). The main reason is that it possesses high specificity for AA and an elevated capacity to release it and trigger the eicosanoid production in activated cells (78). A summary of the activity characteristics of each member of the group IV PLA$_2$ family is provided in **Table 3**.

Table 3. Group IV Cytosolic Phospholipase A$_2$s (cPLA$_2$s) activity characteristics.

Subgroup	Activation factor	Substrate	Activity
IVA (cPLA$_2$α)	Ca^{2+}, PIP$_2$, phosphorylation	PC, PE, PI high *sn*-2 AA specificity	PLA$_2$, PLA$_1$, Lyso-PLA transacylase
IVB (cPLA$_2$β)	Ca^{2+}	PC, PE no *sn*-2 specificity	PLA$_1$, PLA$_2$, Lyso-PLA transacylase
IVC (cPLA$_2$γ)		PC low *sn*-2 AA specificity	PLA$_1$, PLA$_2$, Lyso-PLA
IVD (cPLA$_2$δ)	Ca^{2+}	PC, PE	PLA$_1$, PLA$_2$, Lyso-PLA
IVE (cPLA$_2$ε)	Ca^{2+}	PC, PE	PLA$_1$, PLA$_2$, Lyso-PLA
IVF (cPLA$_2$ζ)	Ca^{2+}	PC, PE	PLA$_1$, PLA$_2$, Lyso-PLA

a) Group IVA cytosolic phospholipase A_2 (cPLA$_2\alpha$).

The cPLA$_2\alpha$ was simultaneously cloned in 1991 by Clark and collaborators (84) and Sharp and collaborators (87). This is the only enzyme of the PLA$_2$ family that shows marked preference for phospholipids with AA in the *sn*-2 position. The enzyme is activated in response to a variety of extracellular stimuli such as antigens, cytokines, mitogens, endotoxins, hormones and neurotransmitters, and can be regulated by different post-translational mechanisms, such as the subcellular localization, phosphorylation by mitogen-activated protein kinases (MAPKs), intracellular calcium response or the interaction with proteins and phospholipids (97). An important feature is its phosphorylation in different residues of serine (Ser^{505}, Ser^{727}, Ser^{515}), reaction that is mediated by several kinases (98-102). It has been described in various cell types that cPLA$_2\alpha$ phosphorylation in Ser^{505} increases the activity of the enzyme (103). It has been also described by using different mutants in serine that the phosphorylation of Ser^{727} modulates the activity of the enzyme, favoring the break with the p11 heterotetramer and annexin A2 and allowing its binding to the membrane for the hydrolysis of phospholipids (104). In numerous cell types it has been shown that there is an increase in the activity of cPLA$_2\alpha$ in response to agonists that can phosphorylate ERK1/2 (extracellular signal–regulated kinases), and this effect can be reversed using phosphatases. The phosphorylation of this enzyme and its translocation in response to intracellular calcium increases, act together to get a complete activation of the enzyme (105). Other studies have suggested that phosphorylation of cPLA$_2\alpha$ increases the specific activity of the enzyme but this is not enough to release AA, and additional signals are required (106).

In response to increases in the intracellular calcium concentration, the cPLA$_2\alpha$ is translocated from cytosol to the perinuclear membranes (Golgi, endoplasmic reticulum and nuclear membrane), where it facilitates the AA conversion to eicosanoids when it is colocalizing with other enzymes (107). Other localization sites have also been described, such as phagosomes during the phagocytosis of zymosan, plasma membrane and lipid droplets, where the enzyme participates in different events related to the intracellular traffic of membranes (108).

Thanks to the use of cPLA$_2\alpha$ knockout mice, it has been possible to establish the physiological and pathological roles of the enzyme. It has been implicated in arthritis, inflammatory bone resorption, autoimmune encephalomyelitis, intestinal polyposis, pulmonary fibrosis, acute respiratory distress syndrome, renal concentration, striated muscle growth, postischemic brain injury and fertility (107).

b) Group IVC cytosolic phospholipase A$_2$ (cPLA$_2\gamma$).

cPLA$_2\gamma$ (also known as group IVC PLA$_2$ or PLA$_2$G4C) was identified as a paralog of cPLA$_2\alpha$ (85,109). It contains a lipase consensus sequence, but its activity was shown to be Ca^{2+}-independent due to the absence of the C2 domain, which is involved in Ca^{2+}-dependent lipid binding and it is a highly conserved domain in other cPLA$_2$ family enzymes. In humans, this enzyme is permanently membrane-bound, as it contains a prenylation motif (CAAX box) at its C-terminus, and a putative myristoylation motif at its N-terminus (**Figure 8**) (85,109-111). The subcellular localization of cPLA$_2\gamma$ also differs from those of other cPLA$_2$ family members (112). It has been found in various membrane fractions, including the endoplasmic reticulum (ER) (85,109,113,114) and mitochondria (110). Previous studies by Asai et al. indicated that cPLA$_2\gamma$ is located in the ER but they did not find colocalization with mitochondrial markers (114). However, Tucker et al. suggested that this enzyme is located in mitochondria by immunofluorescence microscopy studies (110).

The physiological roles of cPLA$_2\gamma$ remain unclear. Since its lysophospholipase and transacylation activities can reduce the levels of toxic lysophospholipids, cPLA$_2\gamma$ could have a protective role against arrhythmia. Similarly, it is predicted that it has important functions in the heart because it is highly expressed in this tissue (85,109). Since the enzyme and its plasmalogen substrate are abundant in the myocardium (115,116), it is thought to be involved in lysophospholipid accumulation under hypoxic conditions, and lysoplasmalogens have been shown to accumulate in ischemic heart tissue (117-119).

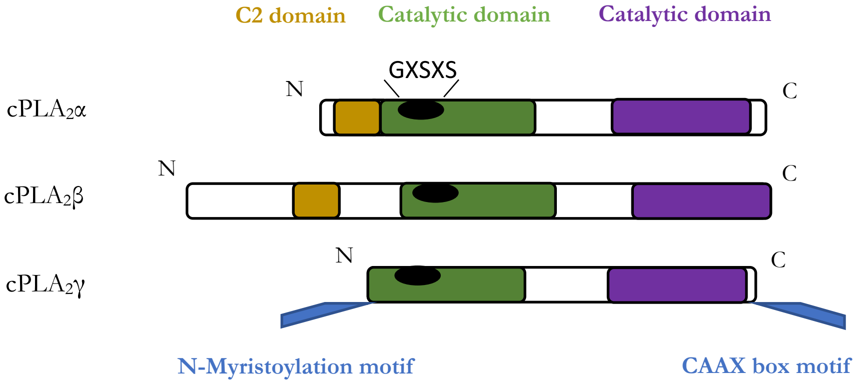


Figure 8. Schematic representation of sequence homologies among the principal members of cPLA$_2$s.

Calcium-independent phospholipase A$_2$s (iPLA$_2$s)

This group is composed by 6 different enzymes (A-F) (**Table 4**). None of them require Ca^{2+} for its catalytic activity, which is carried out by a serine (Ser) on the active site. They have a lipase motif (GXSXG), which is a common feature with cPLA$_2$s. The most studied one is group VIA iPLA$_2$ that comprises 5 variants, although only two of them have catalytic activity. Group VIA-1 was the first identified and characterized and it is classically named iPLA$_2$ (120-122). iPLA$_2$-VIA is expressed in all tissues and has no preference for any fatty acid or PL polar head. Among its multiple functions, the most important is its role in PL homeostasis, participating in the deacylation/reacylation cycle. That is why it is a key enzyme in AA distribution within PLs (123).

It has also been implicated in the release of fatty acids under oxidative stress conditions, where the role of iPLA$_2$-VIA is not based on an activity increase but in a greater susceptibility of the membrane to hydrolysis as consequence of peroxidation (124); in apoptosis by lysoPC action (125); and other processes such as secretion, cell cycle, Ca^{2+} entry, regulation of gene expression or cardiac ischemia (126).

Table 4. Group VI Calcium-independent Phospholipase A_2s (iPLA$_2$s).

Subgroup	Alternative name	Sources	Molecular mass (KDa)
GVIA - 1	iPLA$_2$	Human/murine	85
GVIA - 2	iPLA$_2\beta$	Human/murine	87
GVIB	iPLA$_2\gamma$	Human/murine	90
GVIC	iPLA$_2\delta$, NTE	Human/murine	146
GVID	iPLA$_2\varepsilon$, ADPN	Human	52
GVIE	iPLA$_2\zeta$, ATGL	Human	55
GVIF	iPLA$_2\eta$, GS2	Human	27

Secreted phospholipase A_2s (sPLA$_2$s)

Secreted phospholipase A_2s include enzymes that are secreted to the extracellular medium and, in general, they have low molecular weight (14-18 kDa), except sPLA$_2$-III (55 kDa). In addition, some of these enzymes can also act intracellularly (127-129) (**Table 5**). In addition to those identified in mammals, some of them are found in the venom of insects and reptiles. All of them have 6-8 conserved disulfide bonds, which provides a high stability in extracellular environments, and a catalytic histidine (His). Millimolar Ca^{2+} concentrations are required for their activity.

These enzymes do not show selectivity for the *sn*-2 position fatty acid in PLs, although some of them have a certain preference for anionic PLs. Those belonging to groups IIA, V and X, play a key role in eicosanoid synthesis, acting together with cPLA$_2\alpha$ in AA release (73). Their implication in atherosclerosis, neuronal and respiratory diseases, and anticoagulant properties, has also been described (130,131).

Table 5. Secreted Phospholipase A_2s (sPLA$_2$s).

Group	Sources	Molecular mass (KDa)
IA	Cobras and kraits	13-15
IB	Human/porcine pancreas	13-15
IIA	Rattlesnakes; human synovial	13-15
IIB	Gaboon viper	13-15
IIC	Rat/murine testis	15
IID	Human/murine pancreas/spleen	14-15
IIE	Human/murine brain/heart/uterus	14-15
IIF	Human/murine testis/embryo	16-17
III	Lizard/bee	15-18
	Human/murine	55
V	Human/murine heart/lung/macrophage	14
IX	Snail venom (conodipine-M)	14
X	Human spleen/thymus/leukocyte	14
XIA	Green rice shoots (PLA$_2$-I)	12.4
XIB	Green rice shoots (PLA$_2$-II)	12.9
XIIA	Human/murine	19
XIIB	Human/murine	19
XIII	Parvovirus	<10
XIV	Symbiotic fungus/bacteria	13-19

1.2.2. Acyl-CoA synthetases (ACS).

When free fatty acids are incorporated into glycerophospholipids, fatty acids require to be activated by coenzyme A (CoA). Acyl-CoA synthetases (ACSs) are ligases that form carbon-sulfur bonds (132-135). ACS enzymes are essential for de novo lipid synthesis, fatty acid catabolism and remodeling of membrane phospholipids. They catalyze a two-step reaction for the fatty acid activation (136): first, the formation of an acyl-AMP (adenosine monophosphate) intermediate by consolidation of fatty acid and adenosine triphosphate (ATP); and second, the exchange of AMP with CoA to produce activated acyl-CoA. This fatty acid activation is critical for subsequent metabolic reactions and, therefore, it is indispensable for AA incorporation into PLs.

Based on their structures and substrate specificity (as the chain length of their preferred acyl groups), ACSs can be divided into five subfamilies: short-chain (ACSS), medium-chain (ACSM), long-chain (ACSL), very long-chain (ACSVL), bubblegum (ACSBG) and another group of four enzymes that do not belong to any subfamily (ACSF).

ACSL and ACSVL are the most important in remodeling reactions, since long-chain and very-long-chain fatty acids are mainly found in glycerophospholipids, and ACSL enzymes show preference for AA. So far five ACSL isoforms have been described in mammals, named ACSL1, 3, 4, 5 and 6; and all are represented by many spliced transcript variants. Each isoform is located in multiple organelles including plasma membrane, endoplasmic reticulum, mitochondria, and cytosol (137-139).

All ACSLs have some fatty acid preferences, with ACSL4 in particular preferring AA (20:4n-6) and eicosapentaenoic acid (20:5n-3) to the other long-chain fatty acids (140,141). ACSL4 is located in peroxisomes and mitochondrial membrane (140) and the fatty acid preference of ACSL4 is important for the involvement of the enzyme in the remodeling of *sn*-2 AA in phospholipids.

1.2.3. Acyl-CoA:lysophospholipid acyltransferases (LPLAT).

Acyl-CoA:lysophospholipid acyltransferase (LPLAT) activities are widely distributed in various cells and tissues and are tightly bound to microsomal and plasma membranes. Their primary physiological role is to provide phospholipids having a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position. They play important roles in the regulation of free AA levels (142,143).

LPLATs catalyze the esterification reaction of the fatty acid, previously activated as acyl-CoA, to a hydroxyl group of glycerolipids. Within the phospholipid remodeling process, the most relevant acyltransferases are those using lysophospholipids (lysoPLs) as substrate. When inflammatory cells are activated, there is an enhanced PLA_2 activity and large amounts of lysophospholipids and AA are generated. The released AA is converted to various eicosanoids and the excess is rapidly reacylated trough sequential reactions of acyl-CoA synthetase and acyl-CoA:lysophospholipid acyltransferases. Thus, these enzymes esterify the fatty acid in the *sn*-2 position of phospholipids that has become unoccupied by PLA_2 action (**Figure 9**). The activity of these enzymes determines the level and duration of free AA.

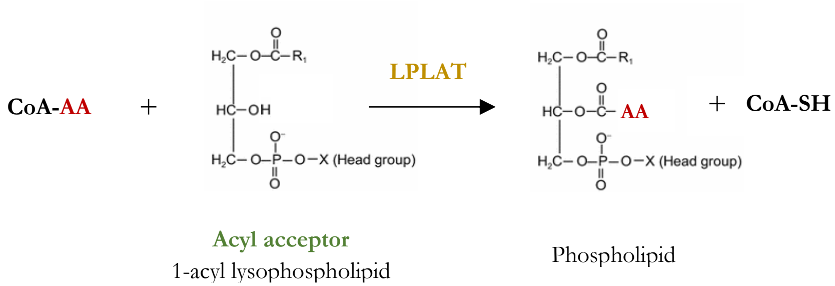


Figure 9. Acyl-CoA-dependent acyltransferase reaction for lysophospholipids.

Mammalian cells contain a large number of enzymes with LPLAT activity, located almost exclusively at the endoplasmic reticulum. They exhibit different selectivity degrees for the lysoPL acceptors and for the acyl-CoA. There are many acyltransferases that may be involved in AA recycling, specifically or as part of the general function in the phospholipid homeostatic metabolism. Two families of LPLAT enzymes have been described: the O-acyltransferase membrane-bound (MBOAT), and the 1-acyl-glycerol-3-phosphate O-acyltransferase (AGPAT) (144-146). While MBOAT members are specifically involved in the remodeling of fatty acids in the Lands cycle, those of the AGPAT family act typically in the *de novo* pathway, although some of them can also participate in remodeling reactions. Those that use lysoPLs as acceptors belong to the MBOAT family and contain several transmembrane domains and a conserved histidine (His) residue in an hydrophobic region which is thought to constitute the catalytic site (147).

Members of the AGPAT family were initially considered using lysoPA as the specific acceptor, so they were classified as acyltransferases acting in *de novo* PL biosynthesis pathway. Afterwards, it has been observed that their specificity is wider, as they can also use lysoPC and lysoPE as acceptors. They have four conserved domains (motifs I-IV) that are important for catalytic activity and the substrate binding (148,149).

1.2.4. Coenzyme A-independent transacylase (CoA-IT).

The AA transfer that takes place in the phospholipid remodeling process is carried out by CoA-IT, which transfers the fatty acid by a transacylation reaction without using CoA (150-152).

There are different factors that determine the enzymatic activity of CoA-IT, such as cell stimulation, when remodeling ratio is increased (151,153). There is also a significant difference between cells that do not proliferate, where the remodeling process can take hours (151,154,155), and proliferating cells, in which remodeling occurs in minutes (155-157). Blocking the process trough CoA-IT inhibition causes cellular apoptosis (158-161).

Although the CoA-IT activity has been studied in detail (162-167), the enzyme has not been purified and its gene sequence remains unknown, which has made it difficult to study its cellular roles in depth.

Some of the better known phospholipase A_2s, such as $cPLA_2\alpha$ or $iPLA_2\beta$, exhibit CoA-IT activity in in vitro assays (80). It was previously suggested that $cPLA_2\gamma$ may be responsible for CoA-independent transacylation, considering that membrane-bound and Ca^{2+}-independent properties are similar to those of the CoA-independent transacylation system. Previous studies confirmed that recombinant $cPLA_2\gamma$ transfers AA esterified at the *sn*-2 position of PE and PC to choline- or ethanolamine-containing lysophospholipids, including 1-O-alkyl-GPC and 1-O-alkenyl-GPE (112,168). It was also demonstrated that farnesylation is not essential for transacylation activity (169).

However, $cPLA_2\gamma$ exhibits another type of transacylation reaction: fatty acid transfer from the *sn*-1 position of LPC to the hydroxyl group at the *sn*-2 position of LPC forming PC and GPC from two molecules of LPC. This activity is termed as lysophospholipase/transacylation activity (**Figure 10**). The main difference between lysophospholipase/transacylation and CoA-independent transacylation activities is the acyl donor, the former uses LPC, whereas the latter uses PLs such as PC.

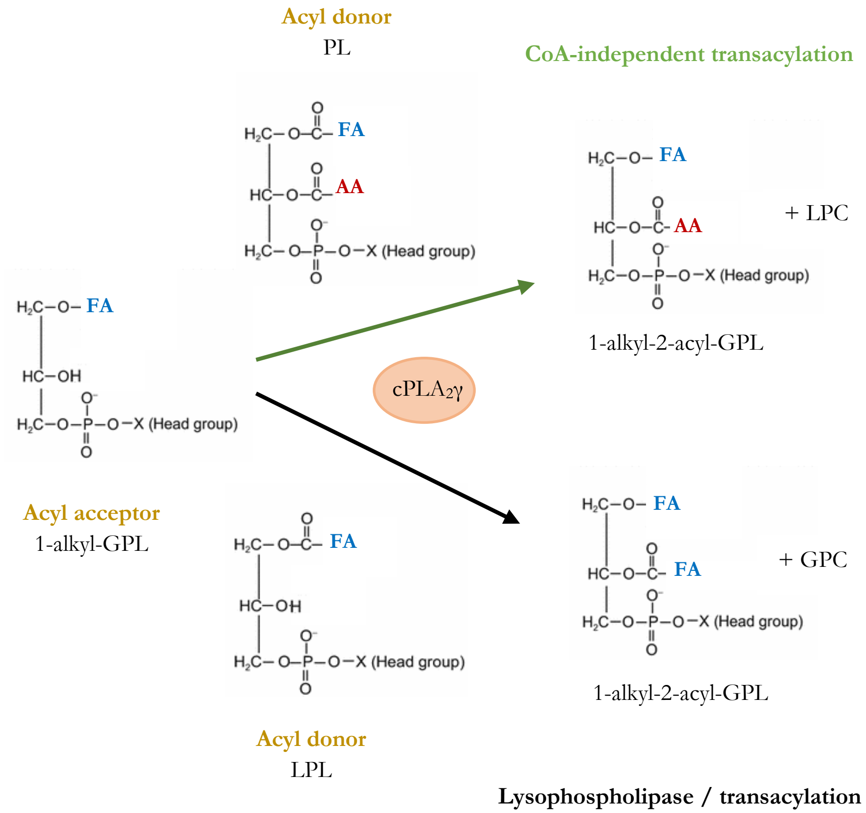

Acyl donor
PL
CoA-independent transacylation
FA
AA
O-X (Head group)
+ LPC
1-alkyl-2-acyl-GPL
cPLA2γ
Acyl acceptor
1-alkyl-GPL
Acyl donor
LPL
+ GPC
1-alkyl-2-acyl-GPL
Lysophospholipase / transacylation

Figure 10. Lysophospholipase and transacylation activity of cPLA$_2$γ.

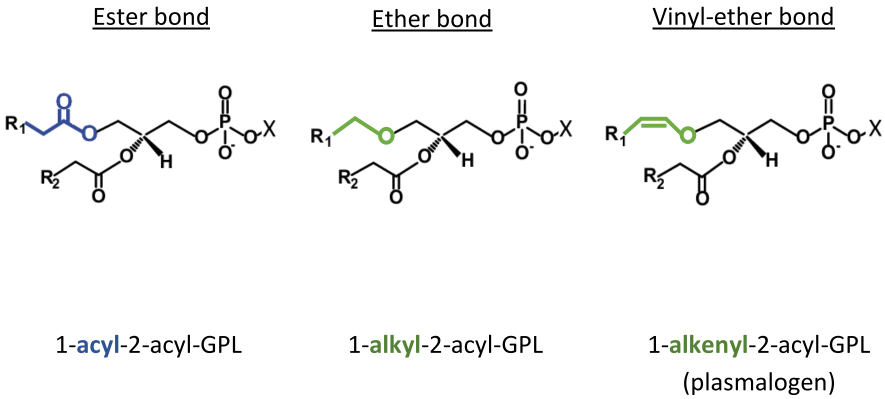
1.3. Incorporation of arachidonic acid in lipid subclasses.

Glycerophospholipids are main components of cell membranes (150). The structure comprises a glycerol backbone and a polar head. Glycerol positions are numbered in a stereospecific manner (*sn*), so that the phosphate group is located at the *sn*-3 position. According to the polar head group attached to the phosphate, there are different phospholipid classes: choline phospholipids (PC), ethanolamine phospholipids (PE), inositol phospholipids (PI), serine phospholipids (PS) and, when there is no polar head, phosphatidic acid (PA). Each phospholipid is sub-classified into the diacyl, alkyl-acyl or alkenyl-acyl type based on the chemical linkage of the fatty chain (i.e., acyl ester, ether and vinyl ether bonds) at the *sn*-1 position of glycerol. **Figure 11** shows the chemical structures of different glycerophospholipid structures depending on the *sn*-1 bond. However, at the *sn*-2 position, the acyl-chains are linked by ester bond and there is usually esterified an unsaturated fatty acid. Each sub-classified phospholipid is further grouped into distinct molecular species depending on the length and degree of unsaturation at the *sn*-1 and -2 radyl residues. Different combinations of head groups, fatty acyl chains and *sn*-1 linkages yield many different molecular species of glycerophospholipids in mammalian tissues (169).

In inflammatory cells, AA is overwhelmingly esterified in the *sn*-2 position (170), and phospholipids with ether bond in *sn*-1 position play important roles, particularly those with vinyl-ether bonds, called plasmalogen species, which contain both ethanolamine and choline polar heads (171). This is explained in **section 1.5.**

AA is mainly incorporated into the diacyl-GPC subclass in macrophages. Incorporation into ether-linked PLs occurs at a very low rate. It is thought that incorporation into diacyl-GPC occurs via acyl-CoA synthetase and acyl-CoA:1-acyl-GPC acyltransferase reactions, requiring energy for this fatty incorporation via the Lands pathway. The incorporation of exogenous AA into PI species is also very relevant, but AA in PI is not used for further remodeling reactions that transfer the fatty acid to other phospholipids.

Depending on the AA behavior and incorporation into each lipid class, cells can exhibit distinct roles and substrate specificities of acyl-CoA:lysophospholipid acyltransferases and the CoA-independent transacylation system.



1-**acyl**-2-acyl-GPL

1-**alkyl**-2-acyl-GPL

1-**alkenyl**-2-acyl-GPL (plasmalogen)

Figure 11. Representative structures of 1-acyl, 1-alkyl, or 1-alkenyl glycerophospholipids. The glycerophospholipids (GPLs) have fatty acyl side chains linked to the *sn*-1 and *sn*-2 positions of the glycerol backbone (R). Depending on the bond at the *sn*-1 position, there are different GPL subclasses. Alkenyl-acyl phospholipids are commonly referred to as plasmalogens. The polar head group (X) of ether-linked PLs is usually choline or ethanolamine.

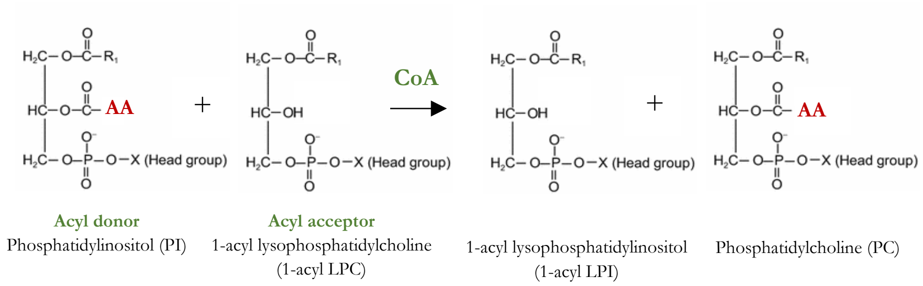
1.4. Glycerophospholipid fatty acid remodeling.

The AA deacylation/reacylation process into membrane phospholipids is not the only mechanism controlling AA mobilization in inflammatory cells. AA recently esterified to glycerophospholipids is subjected to successive remodeling reactions that exchange the fatty acid between different PLs species.

The remodeling process involves two different transacylation systems, CoA-dependent and CoA-independent (**Figure 12**). In CoA-dependent reaction, esterified fatty acids in PLs are transferred to lysophospholipids (LPLs) to form PLs in the presence of CoA, without generating free fatty acids (167,172-177). PC and PI are the main acyl donors and lyso-type glycerophospholipids, such as lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI), the principal acyl acceptors. The CoA-dependent transacylation system proceeds in the presence of physiological levels of CoA and it is mediated by reverse and forward reactions of acyl-CoA:lysophospholipid acyltransferases (178-181).

In contrast, the mechanisms of CoA-independent transacylation are not fully understood, and they are catalyzed by CoA-independent transacylases (CoA-IT); which, as indicated above, are believed to belong to the PLA_2 family. This reaction catalyzes fatty acid transfer from diacyl glycerophospholipids to a variety of LPLs without needing cofactors, and it does not require a divalent cation such as Ca^{2+}.

A) CoA-dependent transacylation reaction


CoA
AA
(Head group)
Acyl donor
Phosphatidylinositol (PI)
Acyl acceptor
1-acyl lysophosphatidylcholine
(1-acyl LPC)
1-acyl lysophosphatidylinositol
(1-acyl LPI)
Phosphatidylcholine (PC)

B) CoA-independent transacylation reaction

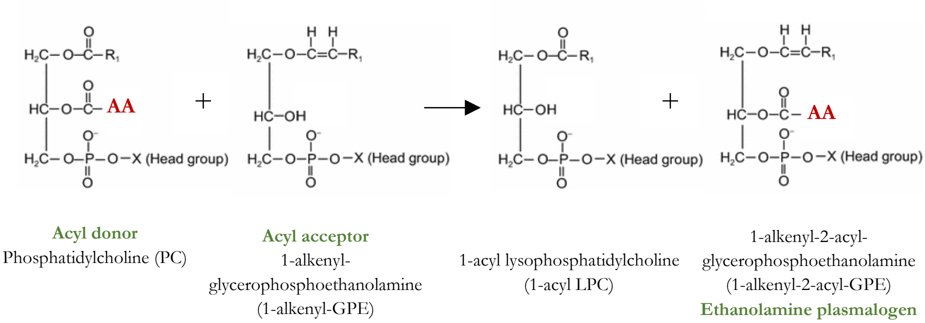

AA
(Head group)
Acyl donor
Phosphatidylcholine (PC)
Acyl acceptor
1-alkenyl-
glycerophosphoethanolamine
(1-alkenyl-GPE)
1-acyl lysophosphatidylcholine
(1-acyl LPC)
1-alkenyl-2-acyl-
glycerophosphoethanolamine
(1-alkenyl-2-acyl-GPE)
Ethanolamine plasmalogen

Figure 12. CoA-dependent and CoA-independent transacylation reactions.

Regarding donors, choline glycerophospholipids (especially diacyl-GPC) are the preferred substrate. Fatty acids esterified at the *sn*-2 but not the *sn*-1 positions of diacyl glycerophospholipids, participate in CoA-independent transacylation. In contrast, free fatty acids and acyl-CoAs are not used as acyl donors. These fatty acids are limited to C20/C22 chain-length PUFAs, with preference for AA (166,167,173,182-185).

In cells of the innate immune response, the most important consequence of the remodeling process is that, although PC is the preferred initial acceptor of exogenous AA, under equilibrium conditions AA is more abundant in PE species (186-189). (**Figure 13**).

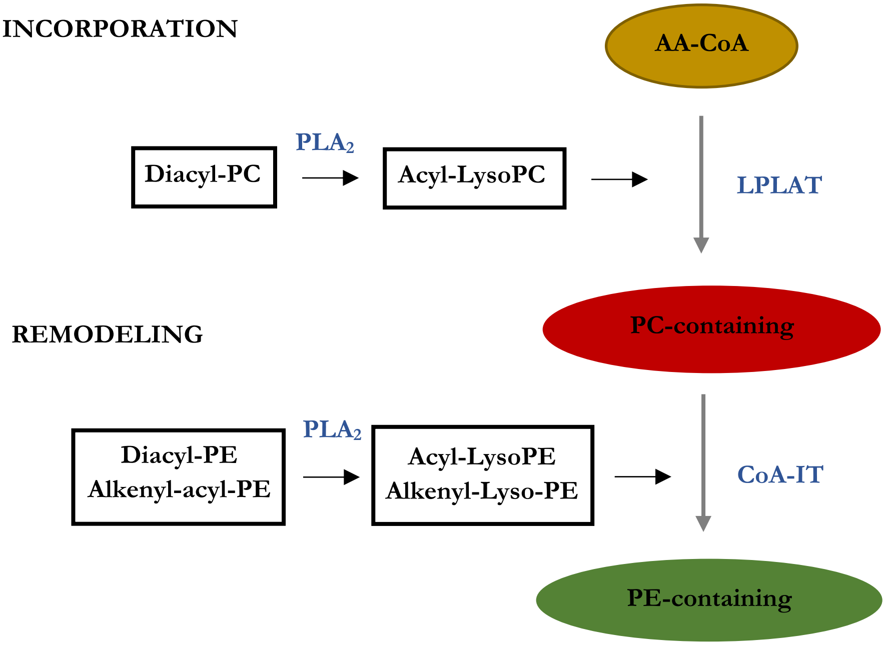

INCORPORATION
AA-CoA
Diacyl-PC
PLA$_2$
Acyl-LysoPC
LPLAT
REMODELING
PC-containing
Diacyl-PE
Alkenyl-acyl-PE
PLA$_2$
Acyl-LysoPE
Alkenyl-Lyso-PE
CoA-IT
PE-containing

Figure 13. Incorporation and mobilization of arachidonic acid in phospholipids.

The CoA-independent transacylation reaction was first described in human platelets and this activity was also detected in microsomes and membrane fractions of several mammalian cells and tissues (166,183-185,190). Transacylation reactions are indispensable for a correct distribution of AA within PL pools, placing the fatty acid in the appropriate localization, which seems to be important for the correct execution of the eicosanoid biosynthesis during stimulation conditions (73,172). This remodeling is also important for platelet-activating factor (PAF) generation (191), as it has observed that 1-alkyl-2-lyso-GPC (lysoPAF) is a typical CoA-IT activity product (162-164,192). The AA distribution into PL pools is also implicated in cell membrane homeostasis and in the control of cellular responses during both physiological and pathophysiological activation (193,194).

1.5. Essential role of plasmalogens.

In inflammatory cells, phospholipids with ether-bond in the *sn*-1 position represent an important group. Plasmalogens were first described by Feulgen and Volt in 1924 and contain both ethanolamine and choline as polar head of the glycerol backbone (171). Plasmalogen biosynthesis starts in peroxisomes. The DHAP-AT enzyme (dihydroxyacetone phosphate acyltransferase) triggers the binding of a fatty acid esterified with CoA to DHAP (dihydroxyacetone phosphate). Subsequently, the *sn*-1 ether bond is introduced by the ADHAP-S (alkyl-DHAP synthetase) by replacing the fatty acid with an alcohol. Next step is the ketone group reduction from the sn-2 position of alkyl-DHAP, resulting in the generation of 1-alkyl-*sn*-glycero-3-phosphate (1-alkyl-G3P), a reaction catalyze by AA-DHAP-R (acyl/alkyl-dihydroxyacetone reductase), an enzyme found both in peroxisomes and endoplasmic reticulum (ER). Once in the ER, the synthesis of plasmalogen species is completed: the AAG3P-AT (alkyl/acyl-glycero-3-phosphate acyltransferase) carries out an acylation, leading to the generation of 1-alkyl-2-acyl-*sn*-glycero-3-phosphate. The action of a phosphohydrolase (PH) and a ethanolamine-phosphotransferase, replaces the phosphate group by adding cytidine-diphosphate-ethanolamine (CDP-ethanolamine) to form, in presence of magnesium, 1-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine (alkyl-acyl-GPE) (195). The synthesis of plasmalogens is completed by a desaturation reaction, in which the electron transport-dependent cytochrome b5 and the 1-alkyl desaturase are involved (196). Through successive methylations of the polar head, PC plasmalogens can be synthetized from PE plasmalogens (197) (**Figure 14**).

The functions of ether-linked phospholipids have not yet been fully elucidated. The alkyl ether bond is more chemically stable than the ester bond, so it is resistant to hydrolysis by phospholipases, as well as the alkenyl ether, although it is susceptible to acid. The vinyl ether bonds of plasmalogens can protect mammalian cells against the damaging effects of reactive oxygen species, whereby they have been described as endogenous antioxidants (198).

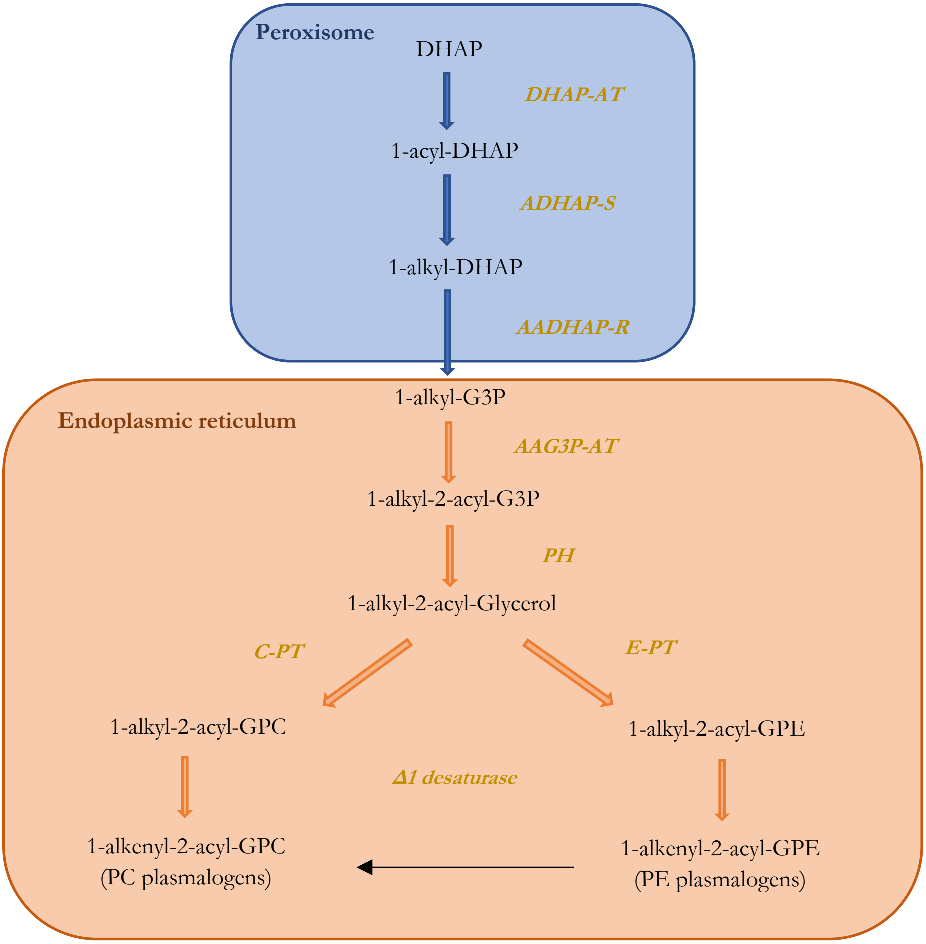

Peroxisome
DHAP
DHAP-AT
1-acyl-DHAP
ADHAP-S
1-alkyl-DHAP
AADHAP-R
Endoplasmic reticulum
1-alkyl-G3P
AAG3P-AT
1-alkyl-2-acyl-G3P
PH
1-alkyl-2-acyl-Glycerol
C-PT
E-PT
1-alkyl-2-acyl-GPC
1-alkyl-2-acyl-GPE
Δ1 desaturase
1-alkenyl-2-acyl-GPC
(PC plasmalogens)
1-alkenyl-2-acyl-GPE
(PE plasmalogens)

Figure 14. Plasmalogens synthesis pathway.

The structure and composition of plasmalogens dictates their role in cell membranes. As the lack of the carbonyl oxygen at the sn-1 position affects the hydrophilicity of the head group and allows stronger intermolecular hydrogen bonding, the formation of non-lamellar lipid structures by ethanolamine plasmalogens is favored, allowing to adopt an inverse hexagonal form (199). Their tendency to not form lipid bilayers make them into mediators in the structure and dynamics of membranes, thus diminishing surface tension and viscosity. Plasmalogens are also ubiquitously found within lipid rafts (200), and have also been found to play roles in the regulation of plasma membrane biophysical properties such as fusion tendency, fluidity, and thickness (201,202).

Previous studies with plasmalogen-deficient cells have shown that reduction in plasmalogen levels leads to altered phagocytosis of zymosan particles by macrophages, which can be attributed to changes in the plasma membrane fluidity and the formation of the lipid rafts (203).

Another striking feature of ether-linked membrane PLs is their high AA content, which suggests that one of their main functions is to serve as cellular AA stores. Large amounts of ether-linked phospholipids are detected in tissues such as the brain and heart, and in inflammatory cells such as neutrophils, macrophages, platelets, and lymphocytes. Although a high proportion of AA is found in *sn*-2 positions of ether-linked glycerophospholipids (204), arachidonoyl-CoA acyltransferase activity for 1-alkyl-GPC and 1-alkenyl-GPC is very low (205-207).

In addition, they have been associated with different pathophysiological states. For example, reduced levels of brain tissue plasmalogens have been associated with Alzheimer’s disease (208), X-linked adrenoleukodystrophy (209), and defects in central nervous system myelination (210).

AIMS

The main goals of the laboratory have been to delineate the molecular mechanisms underlying AA mobilization in phagocytic cells responding to stimuli of the innate immune response, to elucidate the sources of AA involved in the processes of release and reacylation, to study the phospholipase A_2 enzymes regulating phospholipid fatty acid metabolism, and to uncover new stimulus-specific lipid activation markers whose metabolic pathways of synthesis can provide targets for pharmacological intervention. These mechanisms have been studied by taking advantage of advanced mass spectrometry-based lipidomic approaches, which combines great efficiency in the separation and identification of metabolites with sensitivity. The development of LC/MS and GC/MS-based techniques has been useful to elucidate, at a molecular species level, the lipids involved in these processes.

Thus, the overall aim of this thesis was to characterize further the molecular mechanisms underlying arachidonic acid mobilization in phagocytic cells responding to stimuli of the innate immune response.

The specific aims were:

- To understand better the regulatory processes underlying cellular AA availability, which includes to study the influence of AA compartmentalization and to examine the dependence of AA remodeling on plasmalogen content.

- To unveil new regulatory features of CoA-independent transacylation reactions in cellular AA homeostasis and to uncover the phospholipase A_2 enzymes mediating phospholipid remodeling responses.

- To study the process of LPS priming of macrophages for AA release and to characterize further the role of plasmalogen forms in the mechanisms involved in AA availability.

MATERIALS AND METHODS

3.1. Materials.

3.1.1. Culture media.

RPMI medium 1640 with L-Glutamin (Gibco; Paisley, UK)
Dulbecco's Modified Eagle Medium (DMEM) (Lonza; Walkersville, MD, USA)
Opti-MEM medium (Lonza)
Penicillin (Gibco)
Streptomycin (Gibco)
Gentamicin (Lonza)
Fetal bovine serum (Gibco)

3.1.2. Liquid reagents and solvents.

Lipid extraction and separation

Chloroform, Optima® LC/MS grade (Fisher Scientific; Madrid, Spain)
Methanol, Optima® LC/MS grade (Fisher Scientific)
n-hexane, Optima® LC/MS grade (Fisher Scientific)
2-propanol or isopropanol, Optima® LC/MS grade (Fisher Scientific)
Diethyl ether (Scharlab; Barcelona, Spain)
Acetic acid (Scharlab)
Ammonium hydroxide 28%, Optima® LC/MS grade (Scharlab)

For other uses

Methanol, anhydrous 99.8% (Sigma-Aldrich; Madrid, Spain)
Hydrochloric acid 35% (Scharlab)
Butanol (Scharlab)
Ethanol, absolute (Scharlab)
Dimethyl sulfoxide (DMSO), extra pure (Scharlab)
Triton X-100 (Scharlab)
Liquid scintillation (Beckman Coulter; Fullerton, CA, USA)
Bradford reagent (BioRad Protein Assay Dye Reagent Concentrate)

3.1.3. Solid reagents and products.

Bovine serum albumin (BSA), fatty acid and endotoxins free (Sigma-Aldrich)
Ammonium acetate (Sigma-Aldrich)
Iodine (Scharlab)
Silicagel G thin-layer chromatography plates (Macherey-Nagel; Düren, Germany)
Spectrophotometry cuvettes (Kartell Labware; Noviglio, Italy)
Syringe filters (Merck Millipore; Darmstadt, Germany)
Tris-HCl (Scharlab)

3.1.4. Lipids.

Standards for mass spectrometry

1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (PC(17:0/17:0))
1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (PC(15:0/15:0))
1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PE(14:0/14:0))
1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylinositol (PI(16:0/16:0))
1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (PS(14:0/14:0))
1,2-dimyristoyl-*sn*-glycero-3-phosphate (PA(14:0/14:0))
1,2-diheptadecanoyl-*sn*-glycerol (1,2-DAG(17:0/17:0))
1,2,3-triheptadecanoyl-*sn*-glicerol (TAG(17:0/17:0/17:0))
Cholesteryl erucic acid (CE(22:1n-9))
Mix of 37 fatty acid methyl esters
Mix of PE species, from milk
Mix of PI species, from soy
Mix of PS species, from bovine brain

All from Avanti (Alabaster, AL, USA) and Cayman (Ann Arbor, MI, USA).

Deuterated and radioactive lipids

[5,6,8,9,11,12,14,15-^{2}H]Arachidonic acid (Sigma-Aldrich)

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (sp. act. >180 Ci/mmol)
(PerkinElmer; Boston, MA, USA)

Standards for thin-layer chromatography

Mix of PC species, from soy (Sigma-Aldrich)
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidilcholine (PC(16:0/18:1))
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidilethanolamine (PE(16:0/18:1))
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (PS (16:0/18:1))
Phosphatidylinositol Liver, bovine (PI)

All from Avanti (Alabaster, AL, USA) and Cayman (Ann Arbor, MI, USA).

3.1.5. Phospholipase A_2 inhibitors.

cPLA2α inhibitor (Pyrrophenone)[1]
sPLA2 inhibitor (GK241)[2]
iPLA2 inhibitor (FKGK18)[2]
iPLA2 inhibitor (GK436)[2]
iPLA2 inhibitor (Bromoenol lactone, BEL) (Sigma-Aldrich)

[1](Synthesized and provided by Dr. Alfonso Pérez; Department of Organic Chemistry, University of Valladolid)

[2] (Synthesized and provided by Dr. G. Kokotos; Department of Chemistry, National and Kapodistrian University of Athens)

3.1.6. Stimuli.

Arachidonic acid (AA, 20:4n-6) (Sigma-Aldrich)
Zymosan (Sigma-Aldrich)
LPS from Escherichia coli O111:B4 (Sigma-Aldrich)
Interferon-γ (InmunoTools; Friesoythe, Germany)
Interleukin-4 (InmunoTools)
Interleukin-13(InmunoTools)

3.1.7. Quantitative PCR (qPCR).

siRNA transfection, RNA extraction, cDNA synthesis and qPCR

Lipofectamine (Invitrogen)
Trizol ® Reagent (Invitrogen; Carlsbad, CA, USA)
Chloroform, molecular biology degree (Scharlab)
2-propanol, molecular biology degree (Scharlab)
Ethanol, molecular biology degree (Scharlab)
Verso cDNA synthesis kit (Fisher Scientific)
Brilliant III Ultra-Fast SYBR ® Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA)

Oligonucleotides and small interfering RNA

Quantitative PCR oligonucleotides

Gen	**PLA$_2$GIVC**
Forward sequence	5'- AGGAGCTGAAACATCGGTATGA -3'
Reverse sequence	5'- CTGCAAAGATGGGATAGGGC -3'

From: Eurofins Genomics (Ebersbeg, Germany)

Gen	**GAPDH**
Forward sequence	5'-AGGTCGGTGTGAACGGATTTG-3'
Reverse sequence	5'-TGTAGACCATGTAGTTGAGGTCA-3'

From: Eurofins Genomics

Macrophage polarization marker	Sequence (F: forward/R: reverse)
Cxcl1	F: 5'-CTGGGATTCACCTCAAGAACATC-3' R: 5'-CAGGGTCAAGGCAAGCCTC-3'
Cox2	F: 5'-TGAGCAACTATTCCAAACCAGC-3' R: 5'-GCACGTAGTCTTCGATCACTATC-3'
Il1b	F: 5'-GCAACTGTTCCTGAACTCAACT -3' R: 5'-ATCTTTTGGGGTCCGTCAACT-3'
Il6	F: 5'-TAGTCCTTCCTACCCCAATTTCC -3' R: 5'-TTGGTCCTTAGCCACTCCTTC-3'
Il12b	F: 5'-TGGTTTGCCATCGTTTTGCTG -3' R: 5'-ACAGGTGAGGTTCACTGTTTCT-3'
Nos2	F: 5'-CCAAGCCCTCACCTACTTCC-3' R: 5'-CTCTGAGGGCTGACACAAGG-3'
Tnfa	F: 5'-ACGGCATGGATCTCAAAGAC -3' R: 5'-AGATAGCAAATCGGCTGACG-3'
Fizz1	F: 5'-CCAATCCAGCTAACTATCCCTCC-3' R: 5'-ACCCAGTAGCAGTCATCCCA-3'
Arg1	F: 5'-TGGCTTGCGAGACGTAGAC -3' R: 5'-GCTCAGGTGAATCGGCCTTTT-3'
Mrc1	F: 5'-CTCTGTTCAGCTATTGGACGC-3' R: 5'-CGGAATTTCTGGGATTCAGCTTC-3'
Mrc2	F: 5'-ATCCAGGGAAACTCACACGGA-3' R: 5'-GCGCTCATCTTTGCCGTAGT-3'
Tgfb	F: 5'-CTCCCGTGGCTTCTAGTGC -3' R: 5'-GCCTTAGTTTGGACAGGATCTG-3'
Ym2	F: 5'-CAGGTCTGGCAATTCTTCTGAA-3' R: 5'-GTCTTGCTCATGTGTGTAAGTGA-3'
Cyclophilin	F: 5'-TGGAAGAGCCAAGACAGACA -3' R: 5'-TGCCGGAGTCGACAATGAT-3'

From: Eurofins Genomics

Small interfering RNA

Name	siPLA$_2$GIVC
Sequence	5’-GGAGGAGAGAGGAAGAGAA-3’

From: Eurofins Genomics

Name	siRNA Scramble
Sequence	5’-UGGUUUACAUGUCGACUAA-3’

From: Dharmacon (Lafayette, CO, USA)

3.1.8. Buffers and solutions.

Phosphate buffered saline (PBS) buffer 1X:

Composition: NaCl 136 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM
Use: cell washing and base for other buffers

Tris-Acetate-EDTA (TAE) buffer 1X:

Composition: Tris-base 40mM, acetic acid 0.1%, EDTA 1mM pH8
Use: agarose gels for DNA separation

Detergent buffer:

Composition: PBS, Triton X-100 0.1%
Use: radioactivity assays

3.1.9. Equipment.

Gas chromatography-mass spectrometry (GC/MS)

Gas chromatograph (Agilent 7890A)
Mass spectrometer (Agilent 5975C)
Column (Agilent DB23)
Analysis software: Agilent G1701EA MSD Productivity Chemstation

Liquid chromatography-mass spectrometry (LC/MS)

Automatic injector (Agilent G1329B)
Quaternary pump (Agilent G1311C)
Triple quadrupole mass spectrometer (QTRAP4500)
Gas generator (Infinity 1033, Peak Scientific)
HILIC Column (Fortis)
Silica precolumn (Supelco)
Analysis software: Analyst v.1.5.2 (AB Sciex)

Quantitative PCR (qPCR)

ABI7500 (Applied Biosystems; Carlsbad, CA, USA)
Analysis software: 7500 Software v.2.3

Sonication: Vibracell$_{\mathrm{TM}}$ 75115 (Bioblock Scientific)

Spectrophotometry: BioPhotometer Plus (Eppendorf)

RNA quantification: Nanodrop ND-1000 (Thermo Fisher)

Solvent evaporation: Concentrator 5301 (Eppendorf)

Precision scale: Explorer Pro (Ohaus)

Thin layer chromatography: Fungicrom Separating Chambers (Fungilab)

Radioactivity: LS 6500 (Beckman Coulter)

Temperature-specific reactions: Thermoblock (Grant)
Shaking water bath SW22 (Julabo)

3.1.10. Biological material.

For the experiments carried out in this work, macrophage primary cells and cell lines were used.

Part of the experiments were carried out using resident peritoneal macrophages from Swiss mice, raised at the University of Valladolid Animal House. Male and female mice with ages between 10-12 weeks were used equally, without mixing sexes in the same experiment. Mice were kept under constant temperature conditions (21-24 ºC), with a light/dark cycle of 12 h and were fed ad libitum with a special feed diet for laboratory animals and with free access to sterile water. All experimental procedures were approved by the Institutional Committee of Animal Care and Usage of the University of Valladolid and in accordance with the Spanish animal research legislation.

Macrophage RAW264.7 cells and the ether phospholipid-deficient RAW.12 and RAW.108 cells (generously provided by Dr. R. A. Zoeller) (211,212), and P388D$_1$ macrophage-like cells (MAB clone, generously provided by Dr. E.A. Dennis, University of California, San Diego) were also used to perform the studies.

3.2. Methods.

3.2.1. Cell isolation and culture.

Isolation of resident peritoneal macrophages

To obtain resident peritoneal macrophages, Swiss mice were sacrificed by exposing them to CO_2. Afterwards, abdominal skin was removed, and peritoneal lavage was carried out using 5 ml cold PBS. The resulting intraperitoneal content of two extractions, was collected and centrifuged 10 min at 300 xg. Cells were cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37ºC in a humidified atmosphere of 5% CO_2. After 3 h, non-adherent cells were washed with PBS and macrophages were used the day after the isolation.

Cell lines

Macrophage RAW264.7, RAW.12 and RAW.108 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C with 5% CO_2. Scrapers were used to detach the cells and cultured in 12-well plates at least 2 h before performing the experiments to get an 80% confluence ($5x10^5$ cells per well). For experiments, cells were placed in serum-free medium.

3.2.2. Cellular treatments, stimulation and inhibition.

Arachidonic acid treatment

When AA enrichment was necessary, cells were preincubated for 48 h with 25 μM fatty acid (complexed with bovine serum albumin at a 2:1 ratio).

Stimulation conditions

When experiments with activated macrophages were conducted, cells were incubated in serum-free medium for 1 h before stimulation. A 100 ng/ml LPS priming step was carried out for 1 h, conditions in which best priming effect on AA release happens. Afterward, yeast-derived zymosan was added for 1 or 2 h at 150 μg/ml for macrophage activation.

For zymosan preparation, particles were suspended in PBS and boiled for 60 min at 100°C, following by a 30 min centrifugation at 3220 xg and washed three times. The pellet was resuspended in 20 mg/ml PBS and froze until used. Zymosan was sonicated three times for 15 s and diluted in serum-free medium before addition to the cells. *In vitro* assays demonstrated that there was no endogenous phospholipase A_2 activity in the zymosan batches utilized (213,214)

Phospholipase A_2 inhibition

The chemical inhibitors utilized for phospholipase A_2 inhibition were: 2 μM pyrrophenone (pyrr) for cPLA$_2\alpha$, 10 μM FKGK18 and 5 μM GK436 for iPLA$_2\beta$, 5 μM bromoenol lactone (BEL) and 10 μM GK241 for sPLA$_2$ enzymes. Doses were established according to previous laboratory work.

When inhibitors were used, they were added 30 min before the addition of zymosan, and after priming for 10 min before stimulation if done.

Macrophage polarization

To polarize macrophages, the cells were treated with LPS (250 ng/ml) plus interferon-γ (500 U/ml) to generate classically macrophages (M1), or with interleukin-4 (20 ng/ml) plus interleukin-13 (20 ng/ml) to generate alternatively macrophages (M2). In both cases, incubations were carried out for 8 h.

3.2.3. Arachidonic acid mobilization: radioactivity assays.

To carry out studies of arachidonic acid mobilization, radioactive fatty acid was used ([^{3}H]AA). For all experiments, cells were cultured in 12-well plates (5×10^5 cells per well) and placed in serum-free medium 1 h before the beginning of the experiments.

Measurement of ^{3}[H]AA release

To radiolabel the cells, they were incubated overnight (16 h) with 0.25 μCi/ml [^{3}H]AA. After this period, non-incorporated AA was removed by washing the cells four times with serum-free medium containing 0.5 mg/ml free-fatty acid albumin. 1 h before the addition of the stimulus, cells were placed in serum-free medium with albumin to avoid the re-acylation of the [^{3}H]AA released, which is complexed with the albumin. Supernatants were collected, centrifuged to clear of detached cells, mixed with 3 ml liquid and measured for radioactivity by liquid scintillation counting.

Measurement of [^{3}H]AA incorporation

Cells were exposed to exogenous 0.25 μCi/ml [^{3}H]AA during the stimulation period. After washing with serum-free medium, supernatants were removed at the indicated times. Cells were scraped with 0.1% Triton X-100 for total lipid extraction and thin-layer chromatography separation was carried out.

Measurement of [^{3}H]AA remodeling

For these experiments, the cells were pulse-labeled with [^{3}H]AA (0.25 μCi/ml) for 15 min at 37°C. To remove the non-incorporated label, the cells were then washed four times with medium containing 0.5 mg/ml bovine serum albumin and placed in serum-free medium. Cells were collected in 200 μl 0.1% Triton X-100 at the corresponding periods of time. After lipid extraction, phospholipid classes were separated by thin-layer chromatography and the radioactivity incorporated into each class was determined by liquid scintillation counting. The time at which the radioactivity content of PC equals that of PE is defined the remodeling time (215).

3.2.4. Lipid analyses.

Lipid extraction

Total lipids were extracted according to Bligh and Dyer (216). Cells were scraped with H_2O/methanol 1:1 (v/v), and internal standards were added. Afterwards, 3.75 volumes of chloroform/methanol 1:2 (v/v) were added, related to the volume of the initial aqueous phase. After shaking vigorously, 1.25 volumes of H_2O and 1.25 volumes of chloroform were incorporated. Samples were shacked and centrifuged for 5 min at 9300 xg at 15ºC to separate the phases. The lipids remained in the lower organic phase were collected, and a second extraction was carried out adding 2 volumes of chloroform to the aqueous phase. After repeating the process, the organic phase was combined with the previous one and evaporated by vacuum centrifugation.

Thin-layer chromatography

To separate the different classes of lipids, thin-layer chromatography was carried out using silica plates as the stationary phase, which were previously activated by heat at 70ºC for at least 2 h. The lipid extracts were dissolved in 20 μl chloroform/methanol 2:1 (v/v) and loaded on the plate next to the corresponding standards. Phospholipids were separated from neutral lipids using *n*-hexane/diethyl ether/acetic acid 70:30:1 (v/v/v) as the mobile phase, and phospholipids classes (PE, PC, PI, PS) were separated twice with chloroform/methanol/28% ammonium hydroxide 60:37.5:4 (v/v/v). Spots corresponding to the different lipid classes and sub-classes were cut out and re-extracted using 1 ml chloroform/methanol 1:1 (v/v) and 1 ml chloroform/methanol 2:1 (v/v). A 5-minute centrifugation step at 13400 xg was carried out after each solvent addition in order to collect the clean organic phase.

Fatty acids analysis by gas chromatography/mass spectrometry (GC/MS)

As fatty acids are not volatile enough to be transported by the gas phase trough the column, they cannot be studied directly by GC/MS, so a previous chemical derivatization step is required.

The derivatization step separates the fatty acids from the glycerol backbone to which they are esterified.

a) Fatty acid derivatization.

The total or separated lipid extracts were dissolved in 50 μl chloroform/methanol 2:1 (v/v) and introduced in screw threaded glass tubes. Lipid fractions were trans-methylated with 0.5 M KOH in methanol anhydrous for 60 min at 37ºC. The reaction was neutralized with one volume 0.5 M HCl and the resulting fatty acid methyl esters (FAMEs) were extracted twice by adding 2 ml n-hexane. The top organic phase was collected after vortex and centrifuge at 800 xg for 5 min at 16ºC.

b) Fatty acid methyl ester analysis.

FAMEs were obtained from the various lipid fractions by transmethylation with 0.5 M KOH in methanol for 60 min at 37 ºC (215,217-220). Analysis was carried out using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV), equipped with an Agilent 7693 autosampler and an Agilent DB23 column (60 m length x 0.25 mm internal diameter x 0.15 μm film thickness). Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00 [9, 11-14] (Agilent Technologies, Santa Clara, CA, USA).

Glycerophospholipid analysis by liquid chromatography/mass spectrometry (LC/MS)

This technique was used to determine PC, PE and PI molecular species. Lipid extracts obtained by the Bligh & Dyer method (216) and internal standards were added. The samples were redissolved in 50 μL of hexane/2-propanol/water (42:56:2, v/v/v), and 40 μL was injected into an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B autosampler (Agilent Technologies, Santa Clara, CA, USA). The column was a FORTIS HILIC (150 x 3 mm, 3 μm particle size) (Fortis Technologies, Geston, UK), protected with a Supelguard LC-Si (20 mm x 2.1mm) cartridge (Sigma-Aldrich, Madrid, Spain). The mobile phase consisted of a gradient of solvent A (hexane/2-propanol, 30:40, v/v) and solvent B (hexane/2-propanol/20 mM ammonium acetate in water, 30:40:7, v/v/v). The gradient started at 75% A from0 to 5min, then decreased from75% A to 40% A at 15min and from40% A to 5% A at 20min, was held at 5% until 40 min, and increased to 75% at 41 min. The column was then re-equilibrated by holding at 75% A for an additional 14min before the next sample injection. The flow rate through the column was fixed at 400 μL/min, and this flow entered into the electrospray ionization interface of an AB/SciexQTRAP4500 hybrid triple quadrupole mass spectrometer operated in negative ion mode (Applied Biosystems, Carlsbad, CA, USA). Source parameters were as follows: ion spray voltage, -4500V; curtain gas, 30 psi; nebulizer gas, 50 psi; desolvation gas, 60 psi; and temperature, 425 ºC. Phospholipid species were detected as $[M\text{-}H]^-$ ions except for choline phospholipids, which were detected as $[M+CH_3CO_2^-]^-$ adducts and were identified by comparison with previously published data (186,217,221-224). For the identification of each species, the following databases were used: the RCM lipid calculator (http://pharmacology.ucdenver.edu/lipidcalc) and Lipid Maps (www.lipidmaps.org). In addition, successive fragmentations were carried out in MRM (Multiple Reaction Monitoring) mode for an exact characterization of each species.

Lysophospholipid analysis by liquid chromatography/mass spectrometry (LC/MS)

For these analyses, a cell extract corresponding to 10^7 cells was used. Before lysophospholipid extraction with *n*-butanol, 200 pmol of each internal standard were added: 1-tridecanoyl-*sn*-glycero-3-phosphocholine and 1-miristoyl-*sn*-3-phosphoethanolamine. The lipids were redissolved in 100 µl methanol/H_2O 9:1 (v/v) after evaporation of the organic solvent and injected into an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B autosampler (Agilent Technologies, Santa Clara, CA, USA). The column was a FORTIS HILIC (150 x 3 mm, 3 µm particle size) (Fortis Technologies, Geston, UK), protected with a Supelguard LC-Si (20 mm x 2.1mm) cartridge (Sigma-Aldrich, Madrid, Spain).

Mobile phase was a gradient of solvent A (chloroform/methanol/water/32% ammonium hydroxide, 75:24:5:0.5, by volume) and solvent B (chloroform/methanol/water/32% ammonium hydroxide, 55:39:5.5:0.5, by volume). The gradient was started at 100% solvent A, it was decreased linearly to 50% solvent A in 2 min, it was maintained for 8 min, and finally it was decreased to 0% solvent A in 2 min. Flow rate was 0.5 ml/min, and 50 µl the lipid extract was injected.

The liquid chromatography system was coupled to an AB/SciexQTRAP4500 hybrid triple quadrupole mass spectrometer operated in negative ion mode (Applied Biosystems, Carlsbad, CA, USA). The total flow rate into the column was split, and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas was set to 8 l/min, and dry temperature was set to 365°C.

Ethanolamine and inositol lysophospholipids were detected in negative ion mode with the capillary current set at +3500 V as [M–H]− ions. Choline lysophospholipids (lysoPCs) were detected in positive ion mode as [M+H]+ ions, with the capillary current set at −4000 V. Measurements correspond to the intensity of each species divided by the intensity of the internal standards corresponding to that particular headgroup. No internal standards were available for the lysophosphatidylinositol (lysoPI) subclass, so the intensity of each species was divided by the total LPI intensity in this case. The amount of internal standard added to each sample was always identical. The relative intensity values were normalized to the measured quantity of protein present in each cell preparation.

Eicosanoid analysis by liquid chromatography/mass spectrometry (LC/MS)

Analysis of eicosanoids by LC/MS was carried out exactly as described elsewhere (222,224), using an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B Autosampler, 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 (222,224).

3.2.5. Measurement of [^{2}H]AA.

For the identification of [^{2}H]AA-containing species, LC/MS analysis was carried out by a characteristic m/z bell-shape distribution, due to the isotopic distribution of [^{2}H]AA (the m/z ratio is increased 8 units from its natural analogous since it has 8 deuterium atoms), and the corresponding [^{2}H]AA [M-H]$^-$ generated fragments (225).

Measurement of [^{2}H]AA incorporation into cellular phospholipids

Macrophages were primed or not with LPS for 1 hour before their activation with zymosan. The stimulation was carried out in the presence of exogenous [^{2}H]AA (1 μM), and levels of the deuterated fatty acid incorporated into each phospholipid specie was determined by liquid chromatography/mass spectrometry as described above (see **section 3.2.4**).

Measurement of phospholipid [^{2}H]AA remodeling

LPS-treated and untreated cells were pulse-labeled with 1 μM [^{2}H]AA for 30 min, and washed four times to remove the unincorporated fatty acid with culture medium containing 0.5 mg/ml BSA. After washing, cells were incubated with zymosan for 1 h in serum-free medium. [^{2}H]AA amount in the various phospholipid species was delineated by liquid chromatography/mass spectrometry analysis (**see section 3.2.4.**).

3.2.6. Protein quantification.

For lipidomic analysis, cellular homogenates were sonicated three times at 23% for 15 s. Protein was quantified using Braddford method (226) using a commercial kit (BioRad Protein Assay). It is a colorimetric method based on the maximum absorbance change from 465 to 595 nm that blue Coomasie G-250 undergoes when it interacts with proteins in a non-covalent way. Absorbance is measured at 595 nm to quantify the protein concentration by the Beer-Lambert Law. To calculate the sample amount of protein, a standard curve was made using different amounts of BSA, covering a range from 1 to 15 μg/ml.

3.2.7. RNA, cDNA and qPCR.

Small interfering RNA (siRNA) transfection

Small interfering RNA (siRNA) transfection was carried out to knockdown group IVC phospholipase A_2 (PLA_2G4C), also known as cytosolic phospholipase $A_2\gamma$ ($cPLA_2\gamma$). A mix of 20 nM siRNAs (5’-GGAGGAGAGAGGAAGAGAA-3’), 5 µl/ml Lipofectamine RNAiMAX and 200 µl Optimem medium, was prepared per well. After vortex, the mix was incubated for 15 min at RT to allow the complexes generation. Afterward, mix was added to wells into 12-well plates and $3x10^5$ cells dissolved in medium to a final volume of 500 µl/well were added. A scrambled siRNA was used as a negative control (5’-UGGUUUACAUGUCGACUAA-3’). Cells were maintained at normal culture conditions for 24 h, time at which the protein expression was lower. After that time, mRNA was extracted and measured by using qPCR.

RNA extraction

RNA was extracted from cells removing the culture medium and scraped with 400 µl Trizol®Reagent per well (12-well plates). Cells were maintained 5 min at room temperature (RT) and mixed with 80 µl chloroform. After 5 min at RT and a centrifuged at 12000 xg at 4°C for 15 min, the aqueous phase was extracted and mixed with 200 µl 2-propanol. After 10 min at RT, mix was centrifuged 10 min at 12000 xg at 4°C and supernatant was removed. The RNA pellet was washed twice with 400 µl 75% ethanol (v/v, with H_2O DEPC). At last, the RNA pellet was dried and resuspended in 20 µl H_2O DEPC. RNA concentration was determined by absorbance measurement at 260 nm with Nanodrop.

cDNA synthesis (RT-PCR)

The generation of complementary DNA (cDNA) from RNA templates was performed by reverse transcription and polymerase chain reaction (RT-PCR) using a verso cDNA synthesis kit. cDNA synthesis started from 1 µg RNA in a 5.5 µl final volume with H_2O DEPC. All kit reagents, shown in **Table 6**, were added until a final volume of 4.5 µl.

Table 6. Reaction mix preparation.

Reagent	Volume	Final concentration
Template (RNA)	To 5.5 µl	1 µg
5X cDNA synthesis buffer	2 µl	1X
dNTP mix	1 µl	500 µM each
RT enhancer	0.5 µl	
Verso enzyme mix	0.5 µl	
RNA primer (random hexamers/oligo dT primers)	0.5 µl	
Total volume	10 µl	

To carry out the reverse transcription, samples were heated for 30 min at 42ºC and, afterward, they were incubated 2 min at 95ºC for enzyme inactivation.

RNA quantification by Quantitative-PCR (qPCR)

To quantify gene expression levels changes, quantitative PCR (qPCR) was used. Starting from 20 ng cDNA previously synthetized, the commercial kit Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix was used. The corresponding forward and reverse primers (see **section 3.1.7.**) were added together with the kit reagents: SYBR Green, Taq polymerase and a deoxynucleotide triphosphate mix. The reactions were carried out in an ABI7500 equipment following the conditions shown in **Table 7**.

To quantify, the threshold cycle (C_T) was determined, defined as the reaction cycle in which the amplification begins to be exponential. $\Delta\Delta C_T$ algorithm was applied (227), comparing the threshold cycle of the gene of interest with the reference gene, glyceraldehyde-3-phosphate dehydrogenase.

Table 7. Temperature program for cDNA amplification by quantitative PCR.

Number of cycles	Temperature	Time
1	95ºC	3 min
40	95ºC	15 s
	60ºC	1 min
	72 ºC	28 s

3.2.8. Statistical analyses.

All experiments were carried out at least three times with biological duplicates or triplicates. Data are expressed as means ± standard error (S.E.M.). In case of comparing statistical data, analyses were carried out by Student's t-test, and differences were considered significant with $p<0.05$.

RESULTS

4.1. Differences between peritoneal macrophages and macrophage cell lines.

Macrophages play a key role in inflammation and related diseases, which is due in part to their capacity to synthesize and release large amounts of arachidonic acid (AA)-derived eicosanoid mediators. Previous studies have been delineating the molecular mechanisms underlying AA mobilization in these phagocytic cells responding to stimuli of the innate immune response (186,221-224). All studies were carried out with resident peritoneal macrophages (RPMs), which have been extensively studied as a source of oxygenated 20:4 metabolites, and the murine macrophage cell line RAW264.7, which is used as a model for primary macrophages. Thus, the first goal was to characterize and compare both types of macrophages in terms of phospholipid and fatty acid composition.

4.1.1. Lipidomic characterization of macrophages.

Using gas chromatography/mass spectrometry (GC/MS) analyses, the glycerophospholipid fatty acid composition of RPMs and RAW264.7 cells was initially evaluated. Fatty acids are designated by their number of carbon atoms, and their number of double bonds are designated after a colon. 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. **Figure 15** shows that the two cell populations differed considerably regarding the content of 20:4n-6, which was considerably higher in RPMs compared to RAW264.7. This AA deficiency was somewhat offset by increased levels of oleic acid (18:1n-9), palmitoleic acid (16:1n-7), the isomers of the latter (16:1n-x, mix of both 16:1n-9 or 16:1n-10 isomers), and mead acid (20:3n-9).

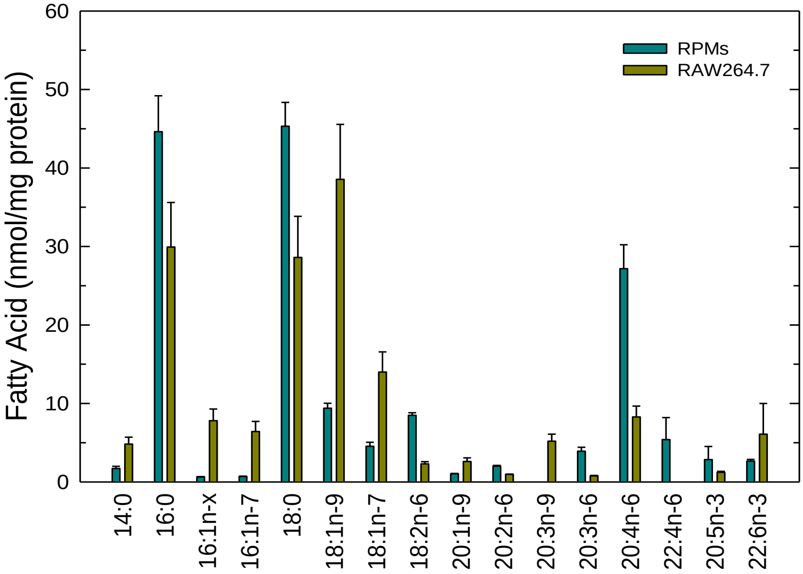


Figure 15. GC/MS analysis: total glycerophospholipid composition of RPMs versus RAW264.7 cells. 16:1n-x denotes a mix of the n-9 and n-10 isomers, which elute together.

The differences in AA content between RPMs and RAW264.7 cells may affect the relative distribution of this fatty acid among glycerophospholipid classes. The percent of AA content in each phospholipid class is shown in **Figure 16**. Although the AA content is higher in RPMs, in both cell types the ethanolamine phospholipids (PE) constitute the richest AA-containing class. While the AA content in PE is similar in percentage in both cell types, AA-containing PC species were lower in RAW264.7 cells than those observed in RPMs. Furthermore, AA content in phosphatidylinositol (PI) is higher than that of PC in RAW264.7 cells. AA percentage in phosphatidylserine (PS) was low and similar in both cell types.

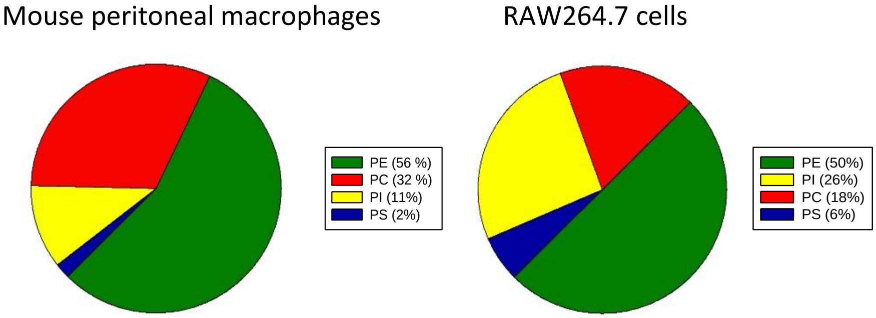


Figure 16. GC/MS analysis: AA distribution between phospholipid classes in RPMs versus RAW264.7 cells.

4.1.2. Arachidonic acid remodeling.

To study AA remodeling among the different phospholipid species and establish a comparation between RPMs and RAW264.7, cells were labeled with [^{3}H]AA for 15 min and, after removing the non-incorporated fatty acid by extensive washing, the distribution of label across the different phospholipid classes was analyzed. As shown in **Figure 17**, both cell types show qualitatively similar AA mobilization process between PC and PE species. Immediately after the labeling period, PC was the major [^{3}H]AA-containing phospholipid, and this amount underwent a decrease with time, which was paralleled by an increase of similar magnitude of AA in PE. To make comparisons between different conditions and in accordance with previous work (215), the "remodeling time" was defined as the time at which the amount of [^{3}H]AA in PC equals that in PE. Considering this, RAW264.7 cells present a fast remodeling, occurring in minutes. In contrast, remodeling in RPMs takes place in hours.

In accordance with the results of **Figure 16**, levels of labeled AA in PI were higher in RAW264.7 cells than in RPMs, and PS levels were low. Both PI and PS labels remained unchanged along the time course of the remodeling.

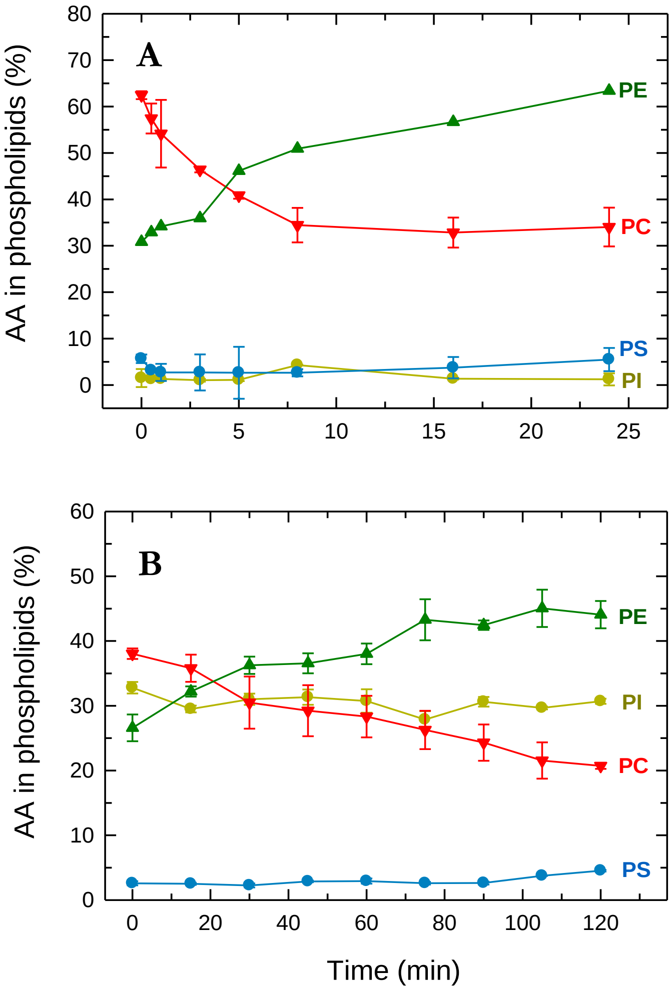


Figure 17. Phospholipid AA remodeling in RPMs **(A)** and RAW264.7 cells **(B)**. Cells were pulse-labeled with [^{3}H]AA, washed, and incubated without label for the indicated periods of time. Phospholipids were separated by thin-layer chromatography. The radioactivity incorporated into each phospholipid class was determined by liquid scintillation counting. Results are given as a percentage of the radioactivity present in phospholipids and shown as means ± S.E.M. (n=6).

4.2. Role of ethanolamine plasmalogen species.

To study the role of plasmalogen species in phospholipase A_2-regulated phospholipid AA metabolism (release and reincorporation mechanisms) in macrophages responding to stimuli of the innate immune response, studies using the plasmalogen-deficient cells lines RAW.12 and RAW.108 were carried out. These cell lines, which derive from RAW264.7 cells and were originally described by Zoeller and co-workers (211), exhibit reduced amounts of plasmalogens due to a defect in enzymes of the plasmalogen synthesis pathway. RAW.108 cells present a defect in the peroxisomal dihydroxyacetone acyltransferase (DHAP-AT); while RAW.12 cells present, in addition, a deficiency in Δ1'-desaturase activity.

AA distribution in RAW264.7 cells and plasmalogen-deficient variants

Comparing the phospholipid fatty acid composition of RAW264.7 cells and the plasmalogen-deficient variants RAW.12 and RAW.108 by GC/MS analyses (**Figure 18A**), no significant variations were detected in the fatty acid profile, including n-3 and n-6 PUFAs. The AA content was very similar in all three types and in all phospholipid classes (**Figure 18B**).

To characterize further the AA distribution between phospholipid molecular species, LC/MS was used. **Figure 19** shows that PE plasmalogen species constitute the principal pool of AA in RAW264.7 cells. Diacyl-PE species and the individual species PI(18:0/20:4), also contain high amounts of AA, unlike PC and PS species, which present lower AA content. Despite the considerable absence of ether phospholipids in the RAW264.7-variant cell lines (including ethanolamine plasmalogens and alkyl-PC species), the AA distribution among phospholipid classes is the same in all three types. As shown in **Figure 19**, this is due to a compensatory elevation of AA in diacyl-PC and PE species compared to wild type RAW264.7 cells.

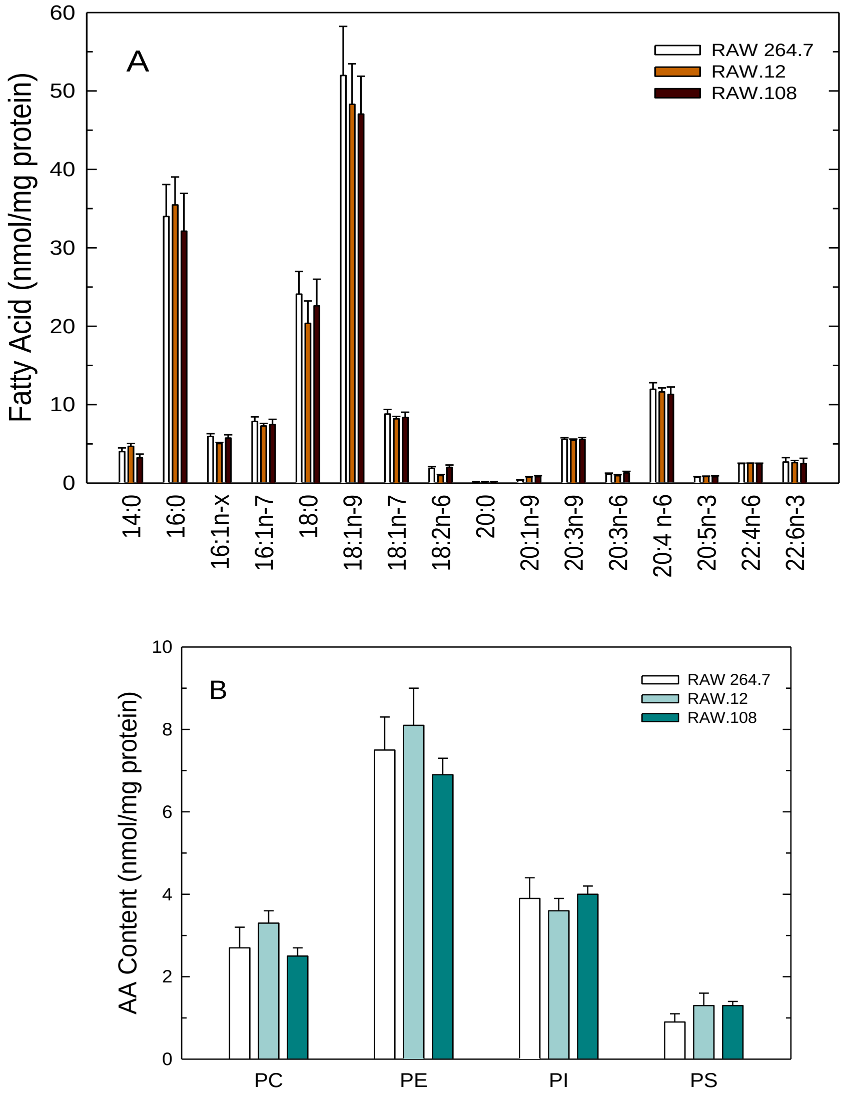


Figure 18. Phospholipid fatty acid composition of RAW264.7 cells and plasmalogen-deficient variants. **(A)** RAW264.7 cells (☐), RAW.12 (🟧), and RAW.108 (■) fatty acids profiles were determined by GC/MS. **(B)** The phospholipid classes of RAW264.7 cells (☐), RAW.12 (🟦), and RAW.108 (■) were separated and their AA content was determined by GC/MS. Results are shown as means ± S.E.M. (n=3).

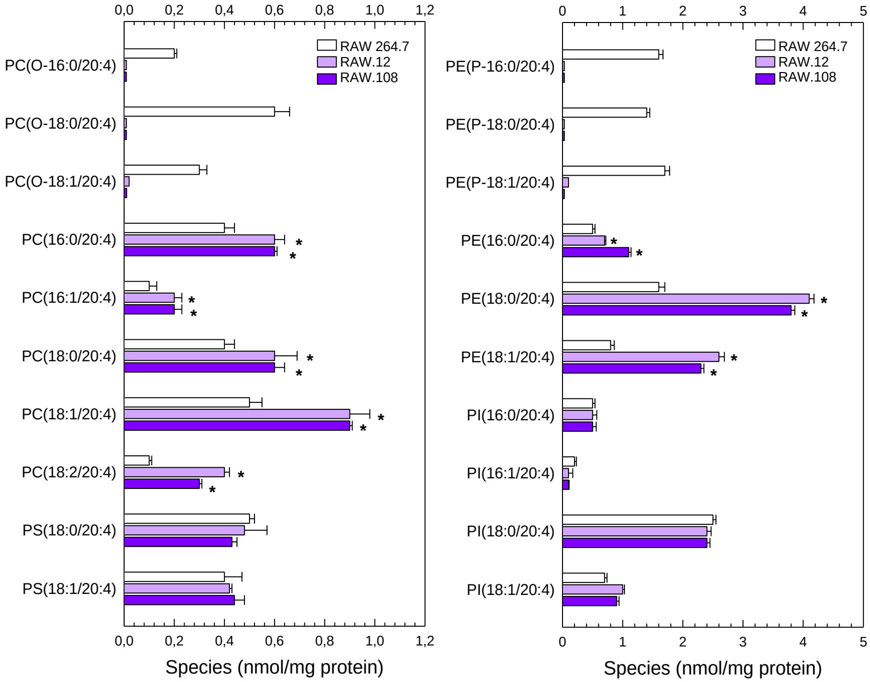


Figure 19. AA-containing species in RAW264.7 cells and plasmalogen-deficient variants. Profile of AA-containing PC, PS (left panel), PE and PI (right panel) determined by LC/MS for RAW264.7 cells (☐), RAW.12 (▨), and RAW.108 (■) fatty acids profiles were determined by GC/MS. Results are shown as means ± S.E.M. (n=3). *Significantly different ($p < 0.05$) from the corresponding species of RAW264.7 cells.

In inflammatory cells, AA mobilization is controlled by the deacylation/reacylation cycle and the successive remodeling reactions mediated by CoA-IT. Through this process, the fatty acid is exchanged among different phospholipid pools, and it is transferred primarily from AA-containing species to lysophospholipid acceptors, which are usually the ethanolamine lysoplasmalogens. It is thought that, in mammalian cells, plasmalogen enrichment with AA occurs mainly via CoA-IT-mediated reactions (72,169,172,228). The rate of AA remodeling from PC to PE was previously examined (**Figure 17B**). Data revealed that the remodeling time and kinetics of RAW264.7 cells compared to that of the plasmalogen-deficient variants RAW.12 and RAW.108, were the same (**Figure 20**). For comparative purposes, remodeling experiments were carried out using RPMs and another murine macrophage-like cell line, P388D$_1$. The remodeling time of the latter cells was found to be similar to that of RAW264.7 cells and their variants, but all were considerably lower than that of RPMs. According to these results, phospholipid AA remodeling from PC to PE is not influenced by the cellular plasmalogen content.

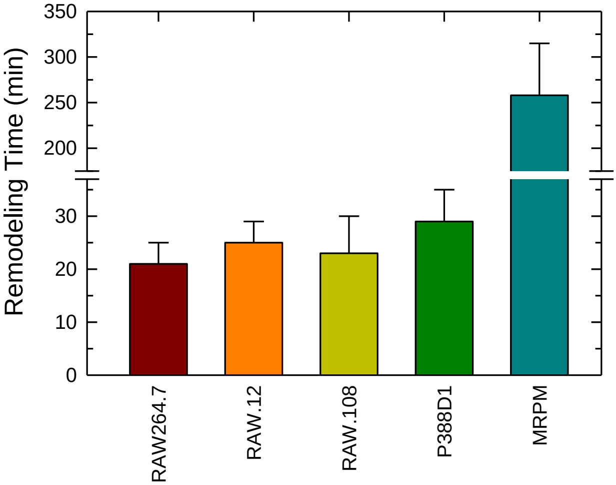


Figure 20. Phospholipid AA remodeling times for different cell types. AA remodeling was analyzed, and the remodeling time was determined. Results are shown as means ± S.E.M. (n=3).

Studies utilizing AA-enriched cells

According to the above results (**Figures 15 and 16**) and to other studies (229,230), cell lines exhibit diminished levels of PUFAs compared to normal cells. Specifically, RAW264.7 cells contain 2-3-fold less AA than RPMs (219). This AA deficiency in RAW264.7 cells could cause faster remodeling compared to normal macrophages. To test this hypothesis, cell lines were enriched with AA to reach the levels of the fatty acid present in RPMs. Culturing the cells in media supplemented with 25 μM AA (complexed with BSA at a 2:1 ratio) for 48 h, increased AA content by about 2-3 fold (219). After the incubation period, the AA distribution between phospholipid classes was studied by measuring all the AA-containing phospholipid classes by GC/MS (**Figure 21A**). AA incorporation was similar in plasmalogen-deficient cell lines compared to RAW264.7 cells. The AA distribution profile agrees with that shown in **Figure 16B**: 50% of cellular AA was distributed in PE and PI constituted the second richest AA phospholipid, followed by PC. To study how this AA enrichment affects the remodeling process, cells were labeled with [^{3}H]AA and radioactivity levels in PC and PE were measured by liquid scintillation counting. **Figure 21B** shows that the remodeling time increased to approximately 2 h (equivalent to that of RPMs) when cells were enriched with AA, compared to untreated cells (**Figure 20**), which means that the AA remodeling process from PC to PE was slowed. These results are consistent in both RAW264.7 cells and the plasmalogen-deficient RAW.12 and RAW.108.

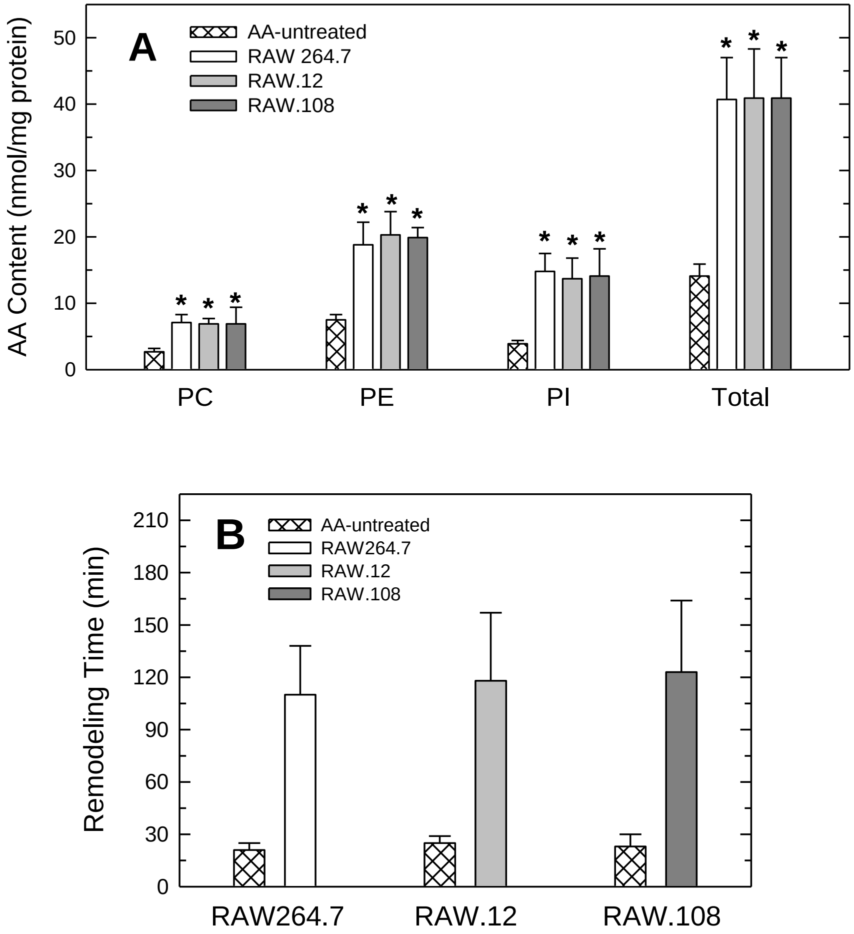


Figure 21. Phospholipid AA remodeling in AA-enriched cells. RAW264.7 cells (□), RAW.12 cells (▒), and RAW.108 cells (■) were incubated with 25 µM exogenous AA for 48 h. **(A)** Cellular AA content was analyzed by GC/MS. AA content of untreated RAW264.7 cells (▩) is shown. **(B)** AA remodeling was analyzed by radioactivity analyses. The time at which PC content equals that of PE (remodeling time) was determined. Results are shown as means ± S.E.M. (n=3). *Significantly different ($p<0.05$) from AA-untreated cells.

Role of plasmalogens in functional macrophage responses

It has been described that macrophages could go through different activation processes depending on the stimuli received (231,232). Macrophage activation can be divided in three main modes: innate, classic, and alternative. Based on this differential activation, specific set of genes are expressed which may be involved in the release of pro- or anti-inflammatory products. The innate activation is mediated by ligation of receptors, such as TLRs, and it is associated with antimicrobial activity and proinflammatory cytokine production. The classic activation (M1) is associated with high microbicidal activity, proinflammatory cytokine, and reactive oxygen species production; and it can be induced by LPS and IFN-γ, which induces TNF-α production). The alternative activation (M2) occurs when cells are cultured with interleukin-4 (IL-4) and interleukin-13 (IL-13), and it is associated with tissue repair and tumor progression (233).

To study whether the deficiency of plasmalogens could alter the different macrophage activations, cells were treated with LPS/IFN-γ or IL-4/IL-13 to induce polarization to pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, respectively. **Figure 22** shows changes in the gene expression levels of markers associated to each phenotype, assessed by qPCR. There were no differences in the expression levels of any of the genes between RAW264.7 cells and the plasmalogen-deficient variants.

Previous studies of the laboratory established a key role for ethanolamine plasmalogens in regulating the phagocytic activity (203), and in **section 4.4.** below, the importance of plasmalogens in the execution of LPS-primed responses is described. To extend the studies to other functional responses of macrophages, analysis of eicosanoids produced by zymosan-stimulated RAW264.7 cells and their plasmalogen-deficient variants, were carried out. **Figure 23** shows the profile of eicosanoids produced which was similar, both qualitatively and quantitatively, to that previously reported by Buczynski et al. (234). Cells were stimulated with 150 μg/ml zymosan for 8 h. Afterward, extracellular media was removed and analyzed for eicosanoid level by LC/MS. Only products of the cyclooxygenase pathway were detected and PGD_2 was the main eicosanoid found. By comparing the different cell types, the eicosanoid production was the same with or without plasmalogens.

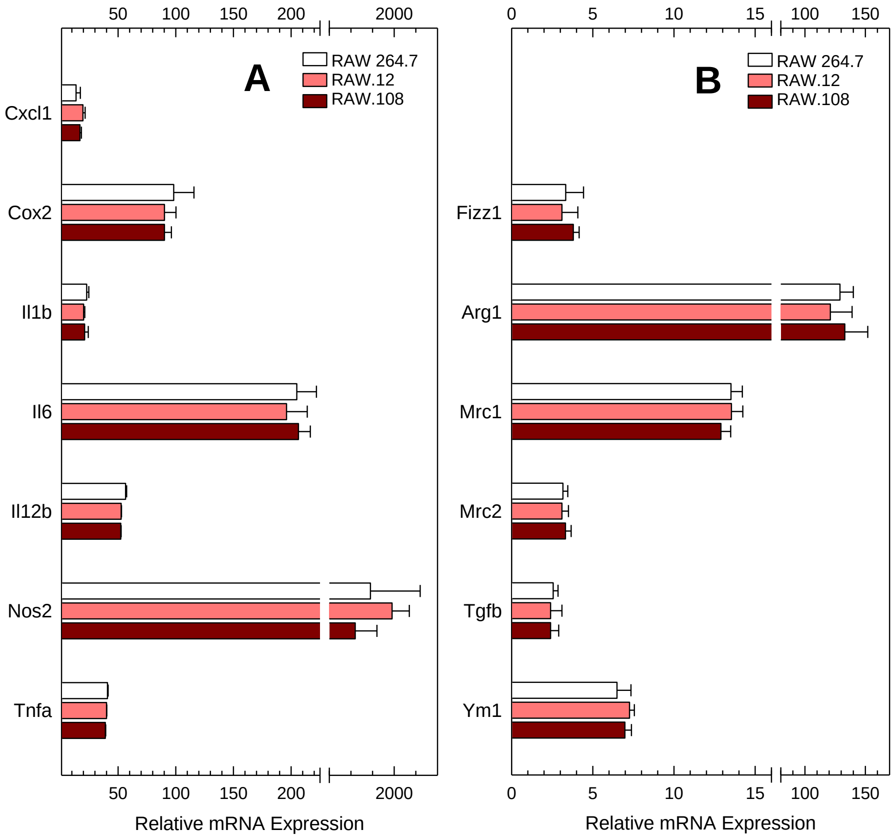


Figure 22. Macrophage polarization through the classic pro-inflammatory (M1) and the alternative anti-inflammatory (M2) pathways. RAW264.7 cells (□) and their plasmalogen-deficient variants RAW.12 (▮) and RAW.108 (▮) were treated for 8 h with LPS (250 ng/ml) plus IFN-γ (500 U/ml) **(A)** or with IL-4 (20 ng/ml) plus IL-13 (20 ng/ml) **(B)** to generate classically (M1) or alternatively (M2) polarized macrophages, respectively. The expression of the indicated markers was measured by qPCR. To normalize the data, cyclophilin A was used. A representative experiment is shown, and the data are expressed as means ± S.E.M. of three individual replicates.

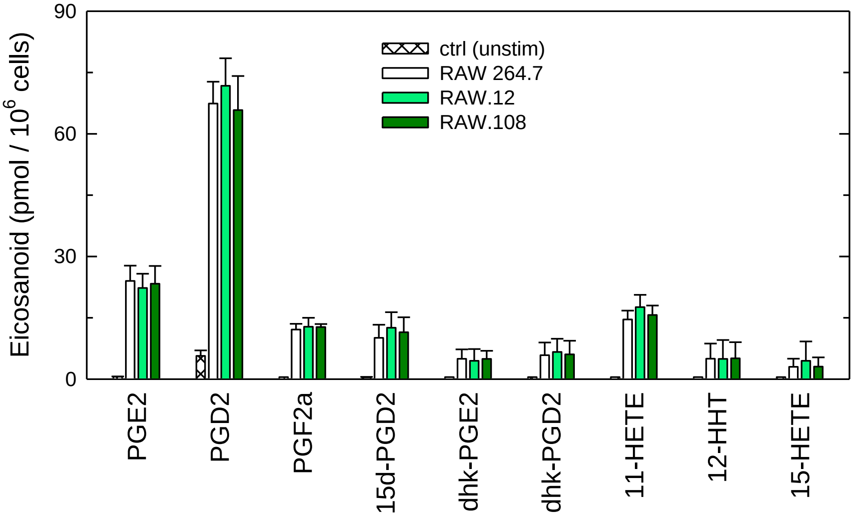


Figure 23. Eicosanoid production by RAW264.7 cells (□) and plasmalogen-deficient variants RAW.12 (🟩) and RAW.108 (■). Cells were treated with zymosan and eicosanoid levels were analyzed from extracellular media by LC/MS. Production by untreated cells (▨) is shown. Results are shown as means ± S.E.M. (n=3).

PGE2: prostaglandin E_2
PGD2: prostaglandin D_2
PGF2a: prostaglandin $F_{2\alpha}$
15d-PGD2: 15-deoxy-$\Delta^{12,14}$-prostaglandin D_2
dhk-PGE2: 13,14-dihydro-15-keto-prostaglandin E_2
dhk-PGD2: 13,14-dihydro-15-keto-prostaglandin D_2
11-HETE: 11-hydroxyeicosatetraenoic acid
12-HHT: 12-hydroxyheptadecatrienoic acid
15-HETE: 15-hydroxyeicosatetraenoic acid

4.3. A role for phospholipase A_2s in phospholipid remodeling.

In phospholipid deacylation reactions, AA is excised from the *sn*-2 position by phospholipase A_2 enzymes, of which group IVA cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) is the critical one. In stimulated cells, the enhanced activity of the enzyme results in elevated levels of free AA, which become available for eicosanoid synthesis.

In the Lands pathway, the 2-lysophospholipids generated by constitutively active phospholipase A_2 enzymes are utilized by CoA-dependent acyltransferases to incorporate AA into phospholipids (80,126,235,236). The subsequent transacylation reactions among phospholipids place the AA in the appropriate phospholipid pools, leading to an asymmetric distribution of AA between different classes and molecular species, thereby, explaining the high content of AA in ethanolamine plasmalogens. The major enzyme involved in phospholipid AA remodeling is CoA-independent transacylase (CoA-IT), which cleaves the *sn*-2 position of phospholipids but does not produce a free fatty acid. The gene sequence of CoA-IT has not been identified yet, but its activity has been characterized in broken cell preparations (237) and pharmacological inhibitors have been identified (238,239).

There are some PLA$_2$s proposed to present this CoA-IT function, such as cPLA$_2\alpha$ or iPLA$_2\beta$, which exhibit CoA-IT activity in in vitro assays (80). Another enzyme suggested as a candidate is the group IVC cytosolic phospholipase A_2 (cPLA$_2\gamma$), based on biochemical commonalities such as membrane-bound, Ca^{2+}-independent and measurable CoA-independent transacylation activity in vitro (169).

Studies were performed to unveil novel regulatory features of CoA-IT-mediated phospholipid remodeling responses in macrophages. To evaluate the involvement of PLA$_2$ isoforms, selective PLA$_2$ inhibitors were used: pyrrophenone for cPLA$_2\alpha$ (at least 3 orders of magnitude more potent for cPLA$_2\alpha$ than for iPLA$_2\beta$ or sPLA$_2$ enzymes) (240); FKGK18 for iPLA$_2\beta$ (at least 200 and 400 times more potent for iPLA$_2\beta$ than for cPLA$_2\alpha$ and sPLA$_2$s, respectively) (241); GK436, also for iPLA$_2\beta$ (at least 1000-fold more potent for iPLA$_2$β than for cPLA$_2$α, and no appreciable effect on sPLA$_2$ enzymes) (242); and GK241 for sPLA$_2$ (inhibits IIA and V forms, with no appreciable inhibition against cPLA$_2$α, iPLA$_2$β, or any other sPLA$_2$ form) (243). For comparative purposes with previous data (212,244), bromoenol lactone (BEL), an irreversible inhibitor of calcium-independent phospholipase A$_2$s with little or no effect on calcium-dependent enzymes (245,246), was also used. **Figure 24** shows that neither of these inhibitors had any effect on the remodeling time of RAW264.7 cells, suggesting that cPLA$_2$α, iPLA$_2$β and sPLA$_2$-IIA/V are not involved in phospholipid AA remodeling.

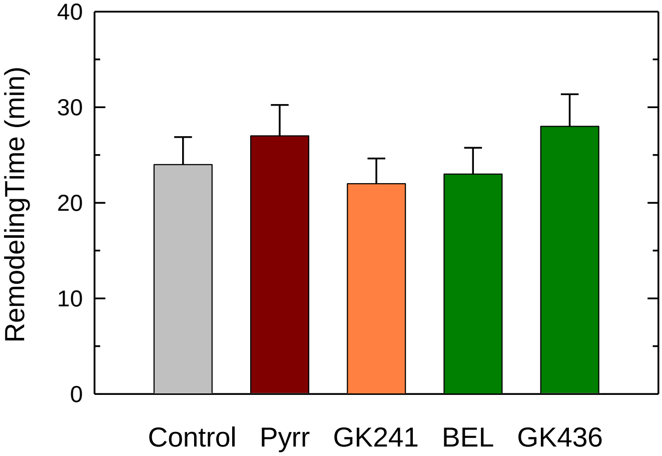


Figure 24. Phospholipase A$_2$ role in phospholipid AA remodeling. RAW264.7 were pulse-labeled with [3H]AA, washed and incubated without label in the absence or presence of the following inhibitors: 2 μM pyrrophenone (pyrr), 10 μM GK241, 5 μM BEL, 5 μM GK436. The radioactivity incorporated into each phospholipid class was measured by scintillation counting and the remodeling time was determined. Results are shown as means ± S.E.M. (n=3).

To address the possibility that cPLA$_2$γ could be involved in phospholipid AA remodeling, conditions were established to achieve silencing of cPLA$_2$γ by siRNA technology. Since no reliable antibodies against murine cPLA$_2$γ could be found, siRNA knockdown was measured by qPCR, obtaining as much as a 70-75% decrease in cPLA$_2$γ mRNA levels (**Figure 25**).

To study the contribution of cPLA$_2\gamma$ in transacylation reactions, phospholipid AA remodeling was analyzed in cPLA$_2\gamma$-silenced RAW264.7 cells. **Figure 25** shows that, even though the enzyme absence is not absolute, cells exhibit defects in AA remodeling from PC to PE, reflected by a significant increase of their remodeling time.

In a different set of experiments, RAW264.7 cells were stimulated with zymosan in the presence of the PLA$_2$s inhibitors and cPLA$_2\gamma$ silencing. As shown in **Figure 26A**, cells deficient in cPLA$_2\gamma$ released similar amounts of AA as control cells, indicating that the enzyme is not a regulator of the AA release response. Regarding to the inhibition of other PLA$_2$ enzymes (**Figure 26B**), the role of cPLA$_2\alpha$ but not of iPLA$_2\beta$ or sPLA$_2$ in AA release, was confirmed, as only inhibition of cPLA$_2\alpha$ results in blockade of the response. These results evidence the separated roles of both cytosolic phospholipase A$_2$s in cellular AA homeostasis: cPLA$_2\alpha$ regulates AA release but not phospholipid AA remodeling, while cPLA$_2\gamma$ does the opposite.

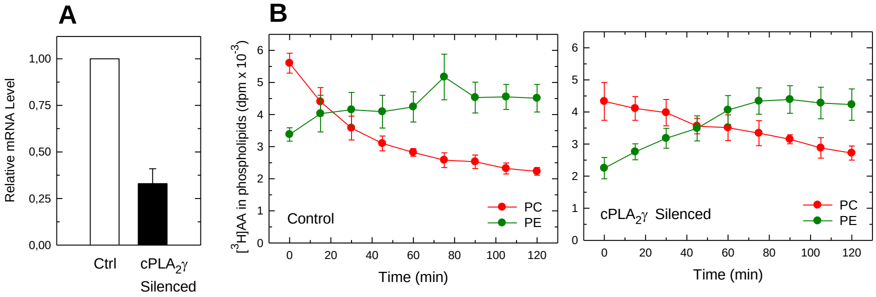


Figure 25. Phospholipid AA remodeling in RAW264.7 cells. The cells were treated with a scrambled siRNA (control) or siRNA targeting cPLA$_2\gamma$ (cPLA$_2\gamma$ silenced). Results are shown as means ± S.E.M. (n=5). *Remodeling time of cPLA$_2\gamma$-silenced cells significantly different from that of control cells ($p < 0.05$).

B

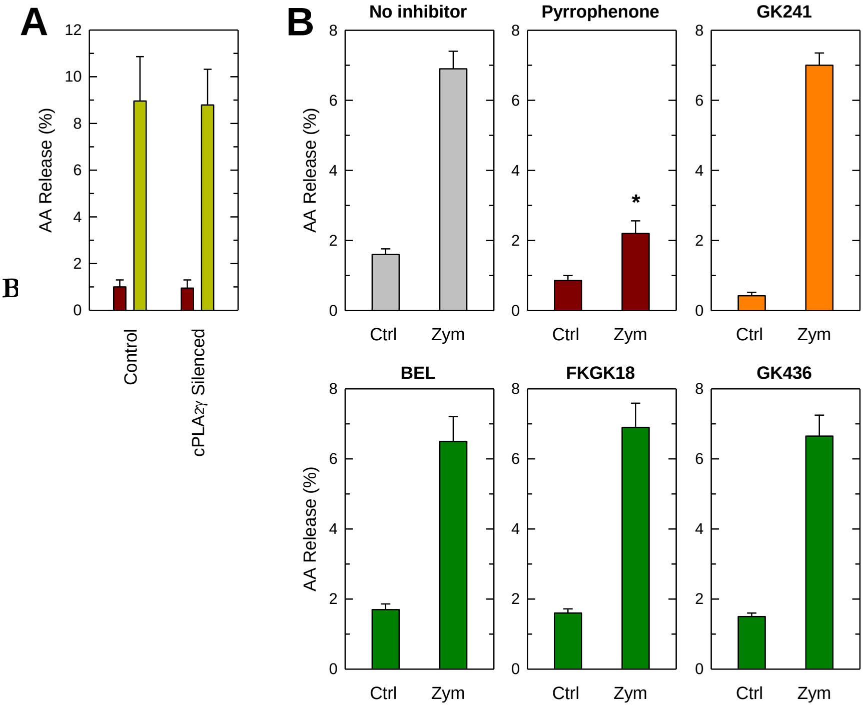


Figure 26. Stimulated AA mobilization in RAW264.7 cells. **(A)** Cells were labeled with [^{3}H]AA, treated with a scrambled siRNA (control) or siRNA cPLA$_2$γ (cPLA$_2$γ silenced), and then untreated (🟥) or treated with 150 μg/ml zymosan (🟩) for 1 h. **(B)** [^{3}H]AA-labeled cells were preincubated with either 2 μM pyrrophenone, 10 μM GK241, 5 μM BEL, 10 μM FKGK18, 5 μM GK436, or neither (no inhibitor) for 30 min. Afterward, they were untreated (Ctrl) or treated with 150 μg/ml zymosan for 1 h. Results are shown as means ± S.E.M. (n=3). *Significantly different ($p < 0.05$) from zymosan-stimulated cells in the absence of inhibitors.

4.4. LPS priming of macrophages for enhanced AA release.

LPS plays a central role in the inflammatory response, stimulating innate immune cells via TLR4 engagement. While LPS is a poor stimulus for AA release on its own, previous studies have demonstrated that pretreatment of phagocytic cells with LPS increases AA mobilization in response to a second stimulus (35,55,247). For these previous studies, radioactively labeled AA was utilized, but radioactive precursors do not always distribute homogeneously among the various phospholipid pools, thus, the subsequent AA release may lead to inaccurate estimation of the contribution of phospholipid sources with higher turnover rates (222). Considering this, gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) were used to obtain accurate estimates of AA phospholipid sources.

In accordance with previous studies (55,56,247), both in RPMs (**Figure 27A**) and RAW264.7 cells (**Figure 27B**), no appreciable amounts of AA were released in response to LPS alone. However, AA mobilization was remarkably stimulated upon macrophage activation with zymosan. A previous priming step increased the AA mobilization response when cells were stimulated with zymosan (**Figure 28A**). 1 ng/ml LPS preincubation is enough to enhanced AA release, with a maximum at 10-100 ng/ml. Zymosan concentration was established within a broad range of zymosan concentrations, being at 1 mg/ml when maximum AA release occurs (**Figure 28B**). LPS priming was observed over all range. At least a 60-minute LPS preincubation period is required to enhanced AA release (**Figure 28C**).

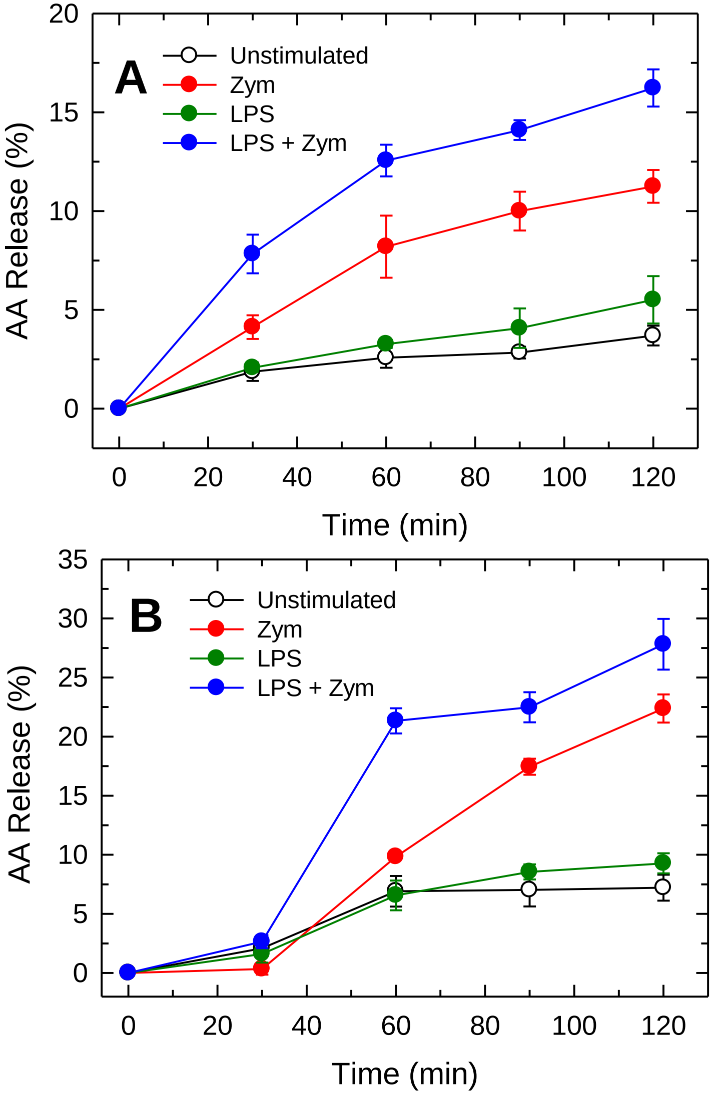


Figure 27. LPS priming or RPMs **(A)** and RAW264.7 cells **(B)** for enhanced AA release in response to zymosan. Kinetics of AA release by control (unstimulated, ○), LPS-primed unstimulated (100 ng/ml LPS for 1 h prior to t = 0, ●), unprimed zymosan-stimulated (1 mg/ml, ●), and LPS-primed zymosan-stimulated (100 ng/ml LPS for 1 h prior to t = 0 followed by 1 mg/ml zymosan for the indicated times, ●). Results are shown as means ± S.E.M. (n=3).

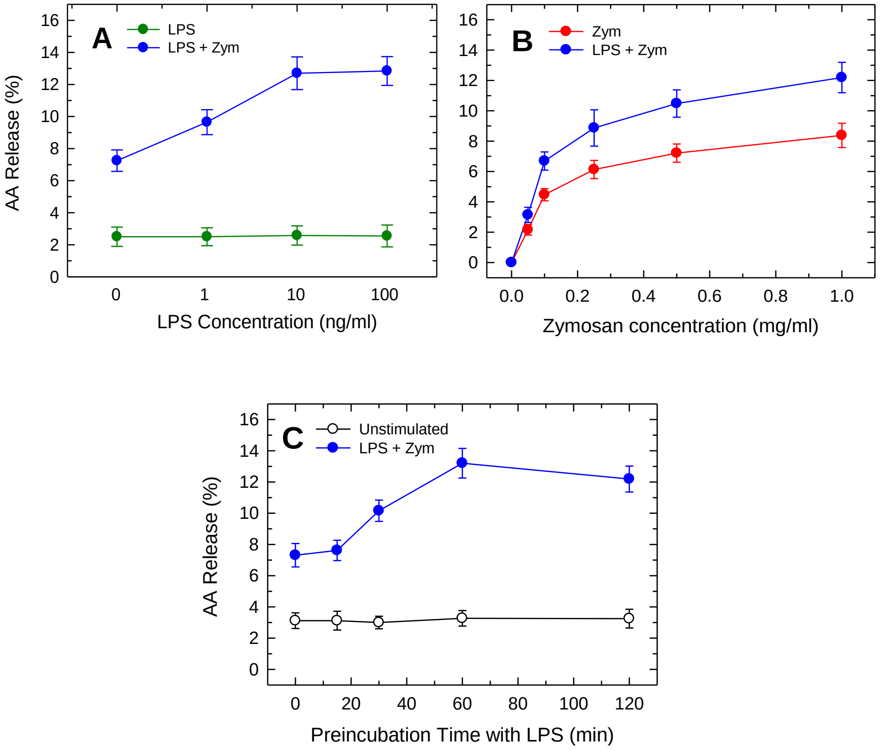


Figure 28. LPS priming of mouse peritoneal macrophages. **(A)** Effect of LPS concentration. Cells were primed with the indicated LPS concentration for 1 h and the stimulated (●) or not (●) with 1 mg/ml zymosan for 2 h. **(B)** effect of zymosan concentration. Cells were either primed (●) with 100 ng/ml LPS or not (●) for 1 h before the addition of the indicated amounts of zymosan for 2 h. **(C)** Effect of preincubation times with LPS. Cells were preincubated with 100 ng/ml LPS for the indicated times and, afterward, they were untreated (○) or treated (●) with 1 mg/ml zymosan for 2 h. The amount of AA released is expressed as % mass of fatty acid initially present in cells. Results are shown as means ± S.E.M. (n=3).

4.4.1. Phospholipid sources for AA mobilization during LPS priming.

To determine the phospholipid sources for AA mobilization in activated macrophages, liquid chromatography/mass spectrometry analyses (LC/MS) were carried out. Fatty chains within phospholipids are designated by their number of carbon atoms, and their number of double bonds are designated after a colon. A designation of O- before the first fatty acid chain indicates that the *sn*-1 position is ether-linked, whereas a P- designation indicates a plasmalogen form (*sn*-1 vinyl ether linkage) (248). **Figure 29** shows the profile of AA-containing phospholipids in unstimulated macrophages, which is consistent with previous results (221-223,249). In resting cells (□), the major AA-containing species were the ethanolamine plasmalogen species, diacyl PC species, and PI (18:0/20:4). When cells were treated with LPS, there was no change in the content or species distribution of AA (🟩). Stimulation with zymosan without previous LPS treatment, produced a marked decrease in the content of cellular AA-containing phospholipids (🟥). All major AA-containing PC species plus PI (18:0/20:4) contributed to this release, but no PE species significantly changed its AA content. Striking changes were appreciated when the LPS-primed cells stimulated with zymosan (🟦) were analyzed. The more noticeable decrease of AA was from PC and PI species, but decreases were also appreciated in PE, resulting primarily from plasmalogen species.

Once the phospholipid sources of AA were analyzed, it was necessary to determine the profile of lysophospholipids formed in activated cells after zymosan treatment to complete the study of changes occurring via phospholipid deacylation reactions. Analyses of both LPS-primed and unprimed cells (**Figure 30**) showed significant increases in several lysoPC and lysoPI species, and their levels were further increased in the LPS-primed cells. Significant increases of ethanolamine lysophospholipids were observed, but only when cells were primed with LPS before zymosan stimulation, and only the plasmalogen forms (labeled in green in the abscissa) were involved.

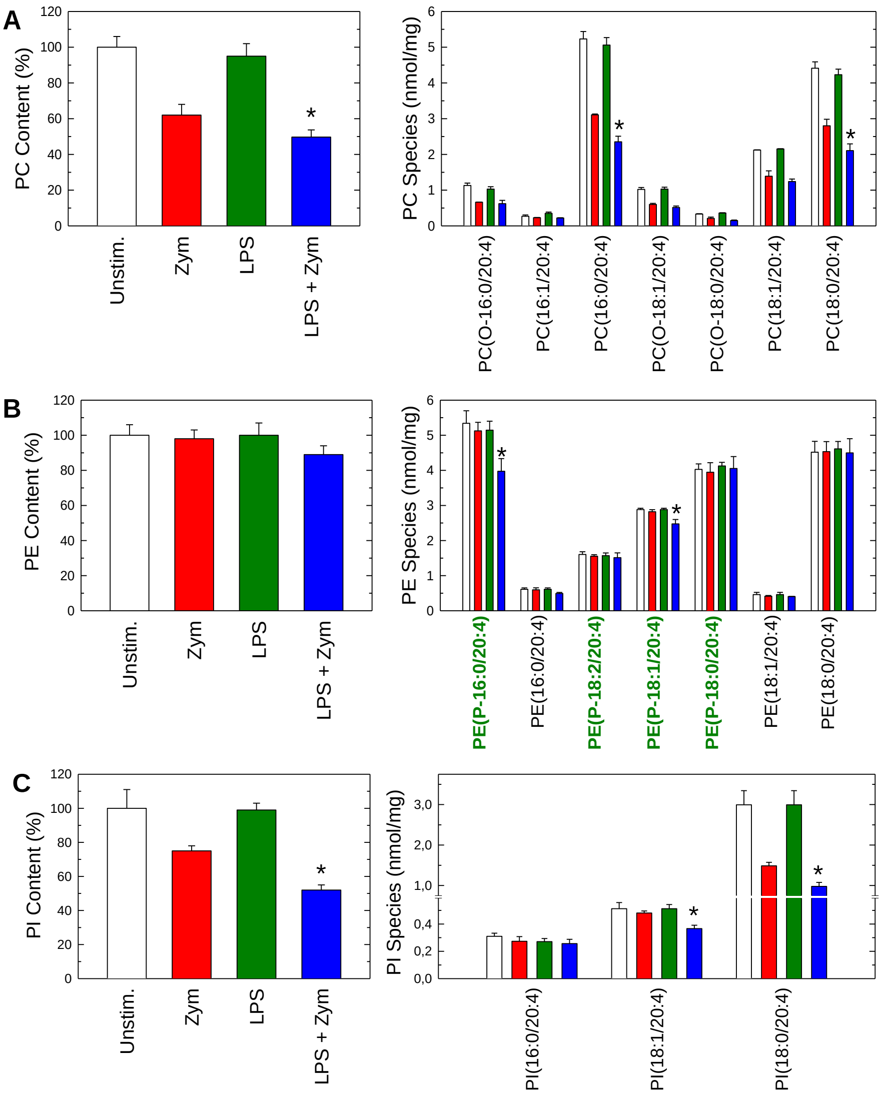


Figure 29. AA-containing phospholipid species in macrophages. Cells were untreated (☐/🟥) or treated (🟩/🟦) with 100 ng/ml LPS for 1 h. Afterward, they were stimulated (🟥/🟦) or not (☐/🟩) with 1 mg/ml zymosan for 2 h. The cellular content of AA-containing PC **(A)**, PE **(B**, where ethanolamine plasmalogen species are labeled in green in the abscissa**)** or PI **(C)** molecular species, was determined by liquid chromatography/mass spectrometry. Results are shown as means ± S.E.M. (n=4). *Incubations with zymosan but without LPS were significantly different when ($p<0.05$).

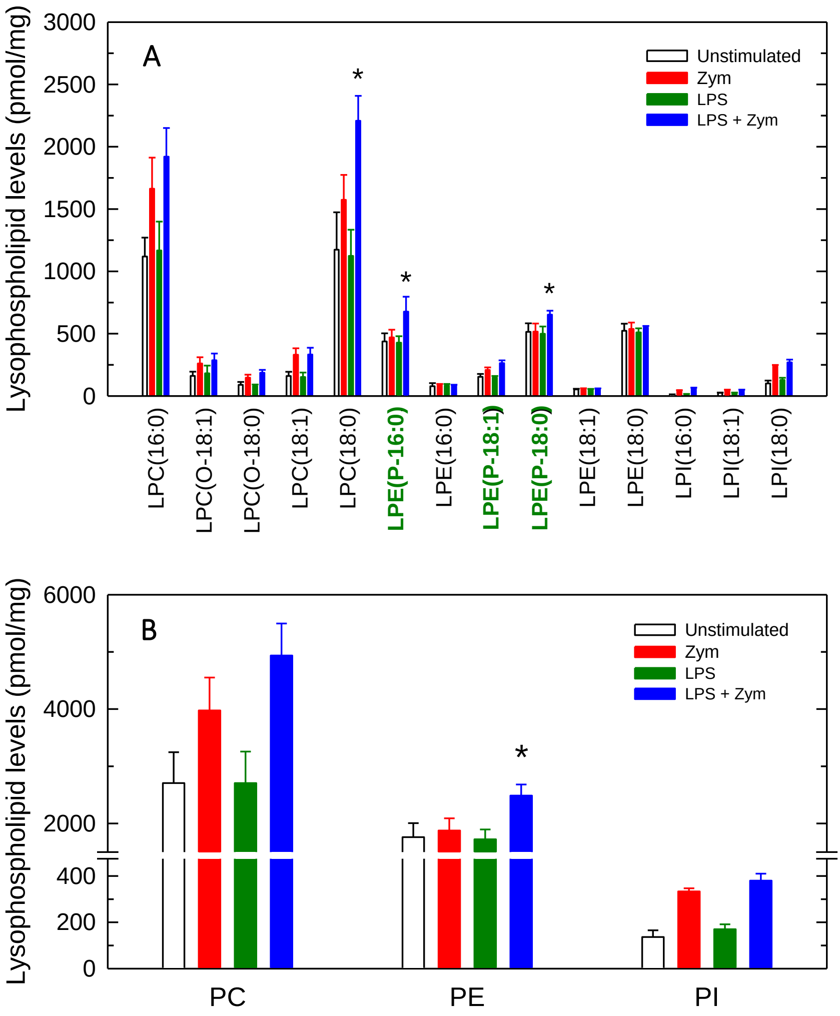


Figure 30. Lysophospholipid molecular species generated by activated macrophages. Cells were untreated (☐/🟥) or treated (🟩/🟦) with 100 ng/ml LPS for 1 h. Afterward, they were stimulated (🟥/🟦) or not (☐/🟩) with 1 mg/ml zymosan for 2 h. The cellular content of lysophospholipid molecular species **(A**, where ethanolamine plasmalogen species are labeled in green in the abscissa**)** or total lysophospholipids formed by class **(B)** was determined by liquid chromatography/mass spectrometry. Results are shown as means ± S.E.M. (n=4). *Incubations with zymosan but without LPS were significantly different when ($p < 0.05$).

4.4.2. AA reacylation and remodeling into phospholipids after LPS priming.

AA release from phospholipids in activated cells results from the phospholipase A_2 activity. To regulate AA availability and modulate free fatty acid levels, AA is reacylated back into phospholipids by CoA-dependent acyltransferases. Keeping this in mind, once phospholipid sources for AA release have been analyzed, it is important to evaluate if LPS priming has any effect on the reacylation pathway. For this purpose, metabolipidomic approaches by LC/MS were carried out. Using [^{2}H]AA, cells were incubated at the time they were stimulated with zymosan, thus labeling the initial acceptors involved in CoA-dependent acylation. The species that contained [^{2}H]AA after the stimulation were analyzed. **Figure 31** shows that most of the [^{2}H]AA was incorporated into PC species, and minor amounts were incorporated into PI species. In addition, the profile of [^{2}H]AA incorporation into PC and PI was the same both in LPS-primed or unprimed cells. No PE species incorporated significant amounts of label.

To provide a correct distribution of AA among membrane phospholipids, transacylation reactions catalyzed by CoA-independent transacylase (CoA-IT) are necessary in addition to the deacylation/reacylation cycle. As indicated above, this enzyme transfers AA between phospholipids primarily from diacyl-PC species to ethanolamine plasmalogens. This pathway was studied labeling cells with [^{2}H]AA for 30 min. As shown in **Figure 32**, most of the fatty acid was present into PC and lesser amounts into PI, but no PE species contained measurable amounts of [^{2}H]AA (□). Once cells were labeled, they were extensively washed to remove the non-esterified [^{2}H]AA. After 1 h of zymosan stimulation (🟥) the movement of [^{2}H]AA from PC to PE species was measured. A low but significant amount of label was found in the plasmalogen species PE(P-16:0/[^{2}H]AA). The ether phospholipid species PC(O-16:0/[^{2}H]AA) also accumulated label, as it is a preferred acceptor for CoA-IT-dependent transacylation reactions, like plasmalogen species. When cells were primed with LPS (🟦), neither of these species present [^{2}H]AA, which indicates that the CoA-IT pathway is blunted by LPS priming.

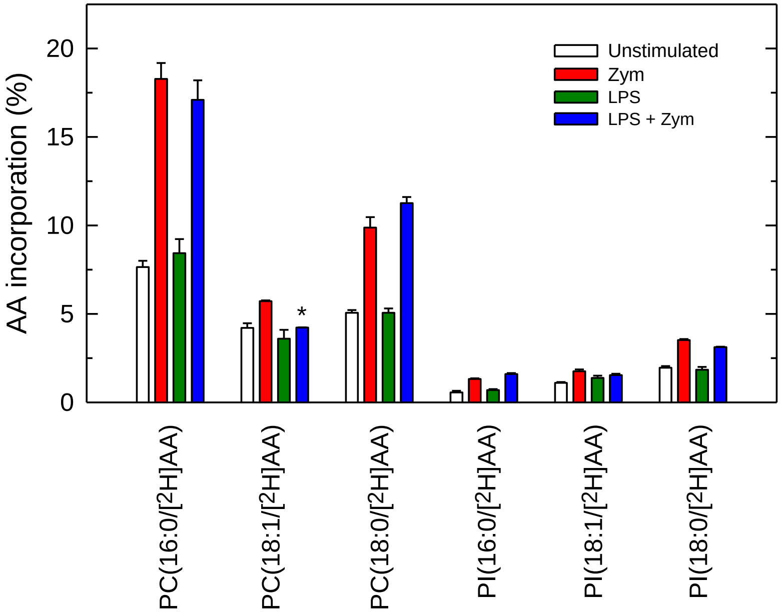


Figure 31. [^{2}H]AA incorporation into phospholipid molecular species. Cells were untreated (□/🟥) or treated (🟩/🟦) with 100 ng/ml LPS for 1 h. Afterward, they were incubated with 1 μM [^{2}H]AA at the time that they were treated without (□/🟩) or with 1 mg/ml zymosan (🟥/🟦) for 2 h. The [^{2}H]AA incorporated into different phospholipid molecular species was determined by liquid chromatography/mass spectrometry, and it is expressed as % mass of fatty acid initially added to the cells. Results are shown as means ± S.E.M. (n=3). *Incubations with zymosan but without LPS were significantly different when ($p < 0.05$).

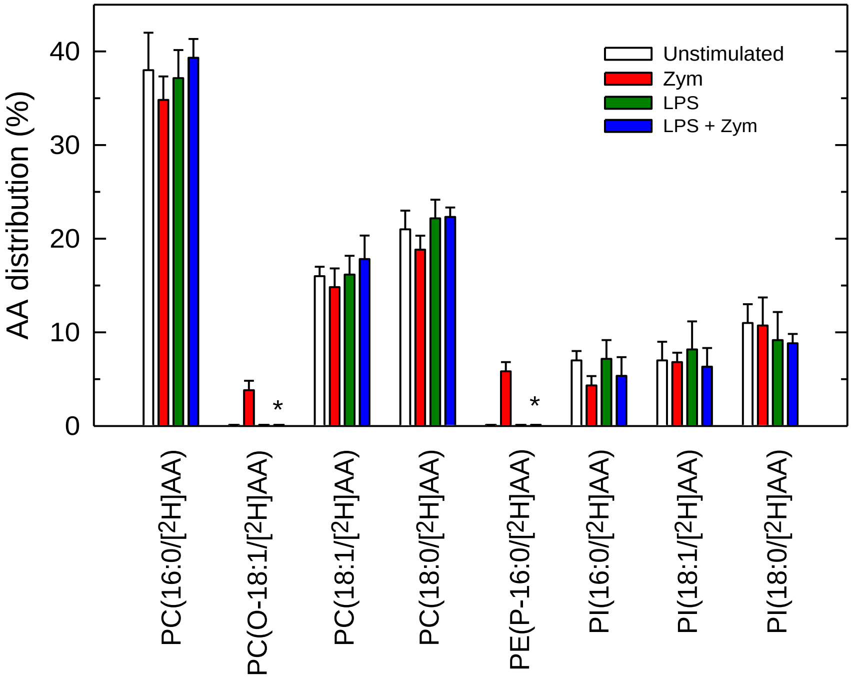


Figure 32. Phospholipid AA remodeling in macrophages. Cells were untreated (□/🟥) or treated (🟩/🟦) with 100 ng/ml LPS for 1 h. Afterward, they were incubated with 1 μM [^{2}H]AA for 30 min. After washing to remove the non-esterified [^{2}H]AA, cells were treated without (□/🟩) or with 1 mg/ml zymosan (🟥/🟦). The distribution of [^{2}H]AA among phospholipids species was determined by liquid chromatography/mass spectrometry, and it is expressed as % mass of esterified fatty acid. Results are shown as means ± S.E.M. (n=3). *Incubations with zymosan but without LPS were significantly different when ($p < 0.05$).

4.4.3. Importance of plasmalogen content for AA mobilization.

To assess the role of plasmalogen species in maintaining AA homeostasis in activated macrophages, AA release was first measured under stimulation conditions. As shown in **Figure 33**, unprimed RAW264.7 cells released abundant AA when stimulated by zymosan, and the plasmalogen-deficient variant RAW.12 released less AA than the parent strain. However, when the cells were primed with LPS before zymosan stimulation, AA release was greatly increased in RAW264.7 but not in RAW.12 cells, where it was the same as with zymosan alone. According to the lipid characterization of RAW.12 cells shown in **Figure 19**, the distribution of AA between phospholipid classes was preserved compared to RAW264.7, but there are no plasmalogen species. Thus, the data provide evidence that AA deacylation from plasmalogen species is important for LPS priming of macrophages for an increased AA release upon a second stimulus.

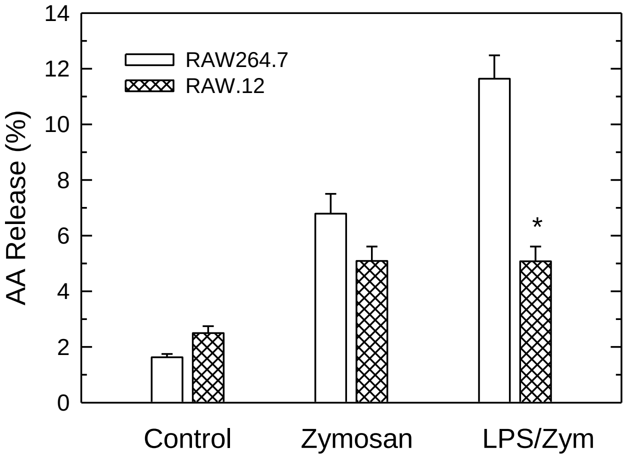


Figure 33. AA release by RAW264.7 (□) cells and plasmalogen-deficient variant RAW.12 cells (▨). Cells were untreated (control) or treated with 1 ng/ml LPS for 1 h. Afterward, they were stimulated with 150 µg/ml zymosan for 2 h. Results are shown as means ± S.E.M. (n=3). *Significant difference ($p < 0.05$) between RAW.12 versus RAW264.7.

AA mobilization in activated cells was also assessed by mass analysis. Also, to confirm the role of cPLA$_2\alpha$ in macrophage AA release under stimulation conditions, experiments with its inhibitor pyrrophenone were carried out. AA-containing phospholipids were analyzed by GC/MS. As shown in **Figure 34**, the decreases in the AA content of PC and PI species in LPS-primed macrophages, were prevented if the zymosan incubations included the inhibitor, which confirmed the role of cPLA$_2\alpha$ in AA release.

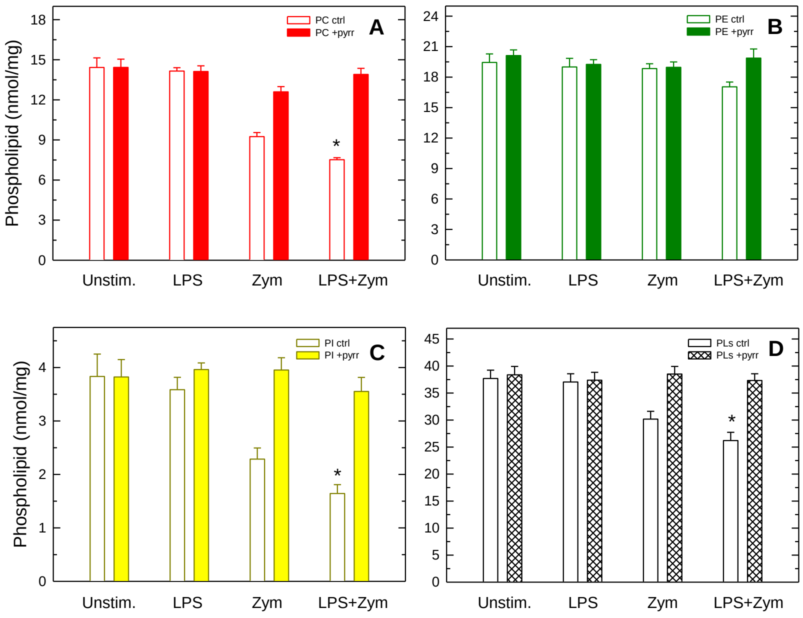


Figure 34. Effect of pyrrophenone on phospholipid deacylation. Macrophages were either untreated or treated with 100 ng/ml LPS for 1 h. Afterward, they were treated (color bars) or not (open bars) with 1 μM pyrrophenone for 10 min. Finally, cells were stimulated with 1 mg/ml zymosan for 2 h. AA-containing PC **(A)**, PE **(B)**, PI **(C)**, and total AA-containing PLs **(D)** was determined by GC/MS. Results are shown as means ± S.E.M. (n=3). *Significantly different ($p < 0.05$) from incubations with zymosan but without.

DISCUSSION

5.1. Arachidonic acid metabolism in macrophages as innate immune cells.

The availability of mass spectrometry-based lipid profiling techniques provides a reliable method to characterize the glycerophospholipid composition and distribution of the species within cells and to identify species that change in response to cellular functions. This technology was used in this work to compare both mouse peritoneal macrophages (RPMs) and the macrophage-like cell line RAW264.7, as a model for subsequent studies. Here, in accordance with previous data (229), it has been demonstrated that RAW264.7 cells and RPMs show differences in phospholipid fatty acid content and the distribution of fatty acids among glycerophospholipid species. The major difference exists in the content of PUFAs in the membranes of the two cell types, with a markedly lower AA content in RAW264.7 cells as compared to RPMs, although the richest AA-containing class is PE and it is similar in both cell types. Despite AA-containing PC species is lower in RAW264.7 cells, the AA phospholipid remodeling process from PC to PE presents a similar profile, in qualitative terms, as in RPMs, although the remodeling time is much lower in RAW264.7 cells. This suggests that RAW264.7 cells incorporate AA into pools which are not as metabolically stable as those in RPMs.

5.2. Role of ethanolamine plasmalogens.

AA is thought to incorporate into PE molecular species via transacylation reactions regulated by CoA-IT, which transfers AA primarily from diacyl PC species to ether-linked species, particularly the ethanolamine plasmalogens, which are strikingly enriched in AA in innate immune cells. This is thought to be due to the fact that PE plasmalogens are major acceptors for fatty acid transfer reactions (72,169,172,228). The AA-enrichment of ether phospholipids suggests that they play key role in AA homeostasis. As shown in results, receptor stimulation of AA mobilization in plasmalogen-deficient cells is similar to that of normal cells and, to date, none of the major AA-releasing PLA_2s have been found to exhibit any preference for substrates containing ether bonds (80). In cells of the innate immune system, phospholipid AA remodeling is necessary to distribute the fatty acid within the appropriate cellular pools for its subsequent mobilization by PLA_2 enzymes. Attending to the distribution of AA among phospholipid classes, it was found that the amount of fatty acid in PE, PC and PI species was maintained in the plasmalogen-deficient cells (**Figure 18**), which is due to a compensatory elevation of AA levels in diacyl species (**Figure 19**). Results showed no difference between plasmalogen-deficient and normal cells, thus suggesting that plasmalogens do not influence phospholipid AA remodeling. These data suggest that it is not the nature of the *sn*-1 bond (acyl, alkyl or alkenyl) but the substituent at the *sn*-3 position (ethanolamine, choline or inositol), that determines the AA distribution. Additionally, another fact that supports the concept that there is a *sn*-3 substituent specificity, is that AA transacylation reactions involve only ethanolamine- and choline-containing phospholipids.

Considering that the nature of the substituent at *sn*-1 does not influence the AA transacylation reaction, CoA-IT, the enzyme catalyzing these reactions may use 1-acyl-PE just as well as 1-alkenyl-PE (72,169,172,228). This could explain why the plasmalogen-deficient variants are able to compensate their deficiency by accumulating the AA into diacyl phospholipids. Since the population of lipids within cell membrane is very dynamic due to remodeling reactions, even an exact composition and concentrations of lipid species present in resting cells is continuously changing. Thus, despite their molecular species composition being so different, both plasmalogen-deficient and normal cells mobilize AA similarly, hence supporting the idea that cells can carry out reactions with many different phospholipid compositions rather than a single composition (250).

Results of the enrichment of plasmalogens with AA (**Figure 21**) raise the possibility that this might not be related to regulatory features of AA homeostasis and eicosanoid metabolism but associated to biophysical effects and to different biological responses by the interaction of the phospholipid with other membrane components.

AA-containing ethanolamine plasmalogens are usually found as components of lipid rafts, specific membrane microdomains whose relative plasmalogen content may affect key properties such as fluidity, packing, thickness, density, and tendency to fusion. It is reported that the vinyl ether linkage at the *sn*-1 position of plasmalogen diminishes the distance between the carbons of the *sn*-1 and *sn*-2 chains by allowing their proximal regions to become parallel (251,252). The vinyl ether bond increases the thickness, membrane packing and density, which results in decreased membrane fluidity. Due to their tendency to form non-bilayer structures, it has been found that ethanolamine plasmalogens facilitate membrane fusion and fission processes by reducing surface tension. Thus, plasmalogens influence the biological behavior of membrane rafts in transmembrane signaling and membrane transport (202,253,254).

Previous laboratory results (203) showed that plasmalogen deficiency in macrophages is associated with reduced phagocytosis. Importantly, phagocytosis reduction was significantly reversed when plasmalogen-deficient cells were exposed to lysoplasmenylethanolamine (lysoPlsEtn). AA-containing ethanolamine plasmalogens are the major species found in primary macrophages and RAW264.7 cells. This cell line also contains ethanolamine plasmalogens carrying oleic acid, dihomo-γ-linolenic acid, and several omega-3 fatty acids, although most of the lipid formed from exogenous lysoPlsEtn contained AA. The marked AA preference for acylation of lysoPlsEtn in these cells might be a consequence of an elevated CoA-IT activity. Thus, exposure to lysoPlsEtn restored cellular plasmalogen levels, especially the fraction containing AA and it was accompanied by an increase in the number and size of lipid raft domains. These results suggest that membrane plasmalogens influence the plasma membrane characteristics such as the formation of microdomains that are necessary for efficient signal transduction leading to optimal phagocytosis by macrophages.

In addition, previous laboratory results (224) also had determined that the increased expression of $sPLA_2$-V in interleukin-4-treated macrophages is linked to increased levels of cellular ethanolamine lysophospholipids (lysoPE), which are necessary to support the elevated phagocytic response during zymosan stimulation. Overexpression of the enzyme produces a significant increase of the phagocytic capacity and the addition of exogenous lysoPE fully restores phagocytosis in $sPLA_2$-V-defient cells.

Increasing cellular levels of AA showed that CoA-mediated phospholipid AA remodeling rates were decreased. This raises the possibility that an AA-containing phospholipid that is only present in AA-enriched cells may act to regulate CoA-IT-mediated transacylation reactions by affecting substrate availability and/or by directly affecting the enzyme. Related to this, it was previously described a short-lived AA-containing phospholipid produced by activated cells, namely 1,2-diarachidonoyl-glycerophosphoinositol, which is able to regulate macrophage responses to innate immune stimuli (221).

The generation of lipid mediators during the innate immune response is controlled by phospholipase A_2 enzymes; thus, the type of lipid mediators generated will be determined by the phospholipid substrates that these enzymes use. Macrophages express the two major cytosolic PLA_2 forms: $cPLA_2\alpha$ and $iPLA_2\beta$ (73,80,255). Both enzymes act on different phospholipid substrates during macrophage stimulation. $cPLA_2\alpha$ manifests a marked selectivity for phospholipids that contain AA at the *sn*-2 position, so it is the rate-limiting provider of free AA for eicosanoid synthesis; while $iPLA_2\beta$ appears to specifically target phospholipids containing palmitic acid in the *sn*-1 position, generating lysoPC(16:0), which is the main acceptor for AA incorporation (222). This hydrolytic pathway operating on phospholipid pools that do not contain AA, serves to generate palmitate-containing lysoPC, which can be used to reincorporate part of the free AA. AA mobilization involves the participation of $cPLA_2\alpha$ and CoA-independent transacylation reactions that remodel the various AA pools. AA is mainly released from PC and PI, but also from PE species, where the amount of AA is restored by the action of transacylation mechanisms that use PC species as donors. The loss of AA from PC during macrophage activation is due to the $cPLA_2\alpha$-mediated hydrolysis and the AA transfer to PE molecules. Therefore, cellular compartmentalization is essential in the phospholipid fatty acid composition of the membrane to which the enzyme translocates during cell activation.

When CoA-IT is inhibited, cellular levels of AA-containing PE species decrease, and AA-containing PC species are increased. It was previously demonstrated (222) that the inhibition of AA transfer from PC to PE leads to a strong increase in lipoxygenase metabolite formation (such as 5-, 12-, and 15-HpETEs) in response to zymosan, which is consistent with the fact that AA-containing PC molecules are the major precursors of lipoxygenase metabolites (72,228). This can be explained because blunting Co-IT-mediated transacylation favors the $cPLA_2\alpha$-mediated hydrolysis of AA-containing PC species over the AA transfer from PC to PE. Thus, the formation of eicosanoid products of the lipoxygenase pathway can be limited by AA transfer from PC to PE species by Co-IT inhibition. Therefore, it would be interesting to thoroughly explore whether the synthesis of different eicosanoid molecules is regulated by the cPLA$_2$α-mediated AA hydrolysis on different phospholipid pools due to a different accessibility of enzyme to such pools, and if it can modify the progression of the inflammatory response.

Phospholipid AA remodeling is a key aspect in eicosanoid regulation because the nature and the amount of eicosanoids produced under activation conditions may depend on the composition and subcellular localization of the phospholipid pool where the AA-hydrolyzing phospholipase A$_2$ acts. In vitro studies suggest that cPLA$_2$α does not show preference for the *sn*-3 position headgroup, whereas recent studies proposed a minor preference for PC and PE species (256). As discussed in preceding paragraphs, during stimulation conditions AA is hydrolyzed from PE and the pools are replenished with AA from PC via CoA-IT-mediated transacylation reactions. As the production of lipoxygenase metabolites increased under these inhibitory conditions, it was suggested that the AA transfer among phospholipid classes regulate the production of specific eicosanoids. On this matter, results of the present work established that the eicosanoid production under stimulation conditions is not influenced by the plasmalogen deficiency (**Figure 23**).

Moreover, cPLA$_2$α has been implicated in the selective production of lipid mediators during macrophage polarization to pro-inflammatory (M1) or anti-inflammatory (M2) states. It has been recently found (257) that both types of macrophages generate pro-resolving lipid mediators in a similar manner under stimulation conditions, while, upon cell stimulation, M2 macrophages mobilize higher amounts of AA by increasing cPLA$_2$α activation, and generate more 5-lipoxygenase products, particularly LTC$_4$. Other studies (258) have also emphasized the different lipid mediator profiles of macrophages under inflammatory conditions, depending on the polarization state. These differences may be due to the compartmentalized regulation of AA-metabolizing enzymes participating in the synthesis of these lipid mediators. Preliminary studies in this work, have shown that the plasmalogen deficiency does not alter the different polarization states (**Figure 22**).

The continuous generation of lysophospholipids by PLA$_2$ enzymes is a key step in phospholipid AA remodeling, since they act as fatty acid acceptors for the subsequent CoA-IT-mediated transacylation reaction. Macrophage activation by receptor-directed agonists, such as zymosan, results in an increase of lysophospholipid levels compared to unstimulated cells

(45,46,222,224). For this work, experiments were carried out to investigate if the activation state of the cells, with this elevation of cellular lysophospholipid levels, presented any influence on their phospholipid remodeling rate. These experiments were carried out also in exogenous AA-treated cells, and both wild type and plasmalogen-deficient variants were used. There was no effect of cell activation on phospholipid AA remodeling under any of these conditions. The remodeling time of zymosan-activated cells was the same as that of resting cells, either in wild-type cells, plasmalogen-deficient cells, or exogenous AA-preloaded cells. Thus, another outcome of this work is that phospholipid AA remodeling rate is independent of the activation state of the cells, at least in RAW264.7 cells. This was unexpected since zymosan-activated macrophages increase their intracellular lysophospholipid content as a consequence of cPLA$_2$α activation and it is exactly the lysophospholipid availability which initiates phospholipid AA remodeling in cells. In contrast with previous results in phorbol ester-stimulated platelets (259), tumor necrosis factor α-stimulated neutrophils (153), and antigen-stimulated mast cells (151), this lack of an effect of cell stimulation on CoA-IT-mediated phospholipid remodeling, is consistent with work in neutrophils on the regulation of platelet-activating factor synthesis via transacylation reactions, where it was found that CoA-IT activity did not increase as a consequence of cell activation but that regulation succeed at the level of increased substrate availability (260).

5.3. A role for phospholipase A$_2$s in phospholipid remodeling.

CoA-IT is not a PLA$_2$ enzyme in a strict sense because, although it cleaves the *sn*-2 position of a phospholipid, it does not generate a free fatty acid as a product. Some well characterized PLA$_2$s, such as cPLA$_2$α or iPLA$_2$β, exhibit Ca^{2+}-independent transacylase activity in vitro (80), but so far no studies utilizing inhibitors of these characterized PLA$_2$s, had abolished CoA-IT activity (261). More recent studies have considered the possibility that CoA-IT activity may belong to group IVC PLA$_2$ (cPLA$_2$γ) (112,168,169). It is not known if this enzyme is responsible of AA transfer from PC to PE in cells since, despite of being Ca^{2+}-independent, lysophospholipase activity of cPLA$_2$γ is significantly higher than its CoA-independent transacylation activity.

Several lipid signaling enzymes, including mammalian Mg^{2+}-dependent phosphatidate phosphatases (262), platelet-activating factor acyltransferases (263), and lysophospholipid acyltransferases (264), have been cloned over the last decade. The determination of their sequences has enabled the applications of genetic approaches to unravel their pathophysiological roles. At present, CoA-IT is one major enzyme of lipid signaling whose sequence is not yet identified, being the only approach to study it the use of activity assays (215,223).

CoA-IT has been defined as a membrane-bound, calcium-independent enzyme. Yamashita and coworkers suggested that CoA-independent transacylation reactions are catalyzed by (an) enzyme(s) of the PLA_2 family and proposed $cPLA_2\gamma$ (group IVC cytosolic phospholipase A_2) as a possible candidate (112,168,169). They also found that the $cPLA_2\gamma$-catalyzed transacylase reaction works better when lysoPC is used as AA donor instead of AA-containing diacyl-PC. Furthermore, Stewart et al. (265) noted that the enzyme has higher lysophospholipase activity than phospholipase A_2 activity. However, conditions of *in vitro* specificity assays are not necessarily the same as *in vivo*, due to the compartmentalization of substrates and products in the latter situation, and because of the presence of competing enzymes that may drastically change the specificities reported.

To experimentally test the proposed involvement of $cPLA_2\gamma$ in regulating phospholipid AA remodeling, siRNA technology was used to specifically knock down cellular expression levels of $cPLA_2\gamma$. Results of these experiments indicate that $cPLA_2\gamma$-deficient cells transfer AA from PC to PE significantly more slowly than control cells (**Figure 27**). This finding is significant because it makes now possible to apply molecular approaches, such as overexpression or deletion, to study the cellular and molecular regulation of phospholipid AA remodeling reactions. It is necessary to expand the knowledge because, despite of these findings, it is not possible to indicate if $cPLA_2\gamma$ participates in the CoA-independent transacylation reaction by directly catalyzing the fatty acid transacylation, by providing the lysophospholipid acceptors that initiate the reaction, or by acting at both levels. Also, $cPLA_2\gamma$ inhibition by siRNA slows but does not eliminate phospholipid AA remodeling, so it is possible that additional enzymes operate as CoA-IT in cells. Additional work should be conducted to understand these mechanisms and if the enzyme is constitutively active or it is activated by agonist stimulation (114).

Moreover, previous studies have demonstrated that cPLA$_2$γ overexpression increases PUFAs in PE species, suggesting that this enzyme can modulate the phospholipid composition of the cells (114). Other studies established that cPLA$_2$γ is involved in monocyte/macrophage differentiation and macrophage polarization, as its inhibition led to M1 polarization, while M2 polarization was suppressed (266). Since the differentiation of monocytes into macrophages and the types of macrophages is important to develop inflammatory diseases, cPLA$_2$γ may be a good candidate for inflammatory disease therapies.

5.4. LPS priming of macrophages for enhanced AA release.

LPS promotes an inflammatory response by stimulating cells of innate immunity through TLR4 recognition (11). When macrophages are stimulated with agonists, they release large quantities of AA. LPS possesses the capacity to prime the cells for an increased released of AA when they are exposed to a second inflammatory stimulus, but this mechanism is not fully understood.

To study this response, cPLA$_2$α activation has been characterized, as this is the key enzyme mediating the AA deacylation step (156,244,267,268). Previous studies have demonstrated that cPLA$_2$α-mediated response could be intensified by the participation of an sPLA$_2$ enzyme in some conditions (73,255), and also that LPS priming enhances the basal phosphorylation state of cPLA$_2$α in macrophages and neutrophils (106,247,269).

However, the increase in enzyme activity is low, so the elevation of basal cPLA$_2$α phosphorylation is not enough to explain the enhanced AA mobilization. Considering that, in addition to the phospholipase A$_2$-mediated deacylation step, other factors should be taken into consideration to explain it, as the mechanisms regulating the AA reacylation and its subsequent re-distribution among phospholipids. To further understand these processes, several genes encoding for lysophospholipid acyltransferases have been cloned (264), revealing that the remodeling cycle contributes to the generation of membrane glycerophospholipid diversity due to a polar headgroup selectivity of the enzymes. Lysophosphatidylcholine acyltransferase 3 (LPCAT3) is the major enzyme controlling AA incorporation in stimulated cells, which limits free fatty acid availability for eicosanoid production by acylating acyl-PC (270-

272). Results in the present work agree with this finding in that PC is the major acceptor for AA incorporation in activated macrophages. In addition, results showed that LPS priming does not influence AA incorporation, as there were no differences between different phospholipid species neither in primed nor not primed macrophages.

PE constitutes a major reservoir of AA in unstimulated macrophages, but it is not a significant acceptor for direct fatty acid reacylation reactions (72,73,172,273). Consistent with this, in this work it was clearly appreciated a decreased content of AA in some PE species in the LPS-primed zymosan stimulated cells. Moreover, when metabolipidomic studies with deuterated AA were carried out to analyze direct incorporation of the fatty acid into phospholipids, there was appreciated no significant incorporation into PE species (**Figure 32**).

In the present study, AA mobilization was measured by mass in activated cells and no changes were observed in the AA content of PE molecular species, which is thought to be due to this remodeling process. This could be an explanation for the false impression that PE species do not contribute to zymosan-stimulated AA release, since their contribution is exposed when CoA-IT inhibitors are used (222). The cellular amount of PE was not declined during cellular stimulation due to the fast transfer of AA from PC to lysoPE by CoA-IT.

In accordance with this involvement of CoA-IT in replenishing PE species with AA, results showed that there were no changes in the mass amounts of PE in unprimed zymosan-stimulated macrophages, whereas all other phospholipid species experienced decreases. However, in LPS-primed cells it was observed an abundant release of AA from PE, particularly the plasmalogen species but not the diacyl forms. The elevated levels of lysoplasmenyl PE species observed in LPS-primed zymosan-stimulated cells, confirm these findings (**Figure 31**). These results confirm the involvement of CoA-IT in stimulated macrophages and strongly indicate that the CoA-IT pathway is blunted by LPS priming. Thus, suggesting that diminished recycling of AA into ethanolamine plasmalogens is responsible for the enhanced AA mobilization response of the LPS-primed zymosan-activated cells, and that alterations in CoA-IT-mediated remodeling are central to LPS priming. Moreover, they are relevant to uncover a role for LPS priming in innate immunity and inflammation processes, since LPS-primed macrophages appear to remodel AA from PC to PE more slowly compared with unprimed cells, which results in significantly reduced incorporation of AA into PE plasmalogens and therefore, lower levels of these species. This different compartmentalization of the fatty acid results in higher losses of AA from PE and higher free AA levels due to cPLA$_2\alpha$-mediated hydrolysis of PC. Thus, the subsequent diminution of CoA-IT activity after LPS priming constitutes a risk because of the intensified production of eicosanoids as a consequence of the increased release of free AA from PC (274).

Further, LPS priming of macrophages is associated with an increased hydrolysis of ethanolamine plasmalogens by cPLA$_2\alpha$, which is not counteracted by CoA-IT, as this enzyme activity is reduced during LPS priming. Preliminary cellular assays did not detect significant decreases of CoA-IT activity in the LPS-primed cells. That is why it is not clear whether LPS priming affects CoA-IT-driven remodeling reactions by acting directly on the enzyme itself, or by altering an unidentified upstream effector that could decrease the availability of the enzyme to its substrates. This possibility is similar to that described for the activity of CoA-IT in platelet-activating factor synthesis by human neutrophils, which is suggested to be regulated by substrate availability rather than by an increased (260). Moreover, macrophage priming with LPS is strikingly sensitive to transcription and translation inhibitors, indicating the involvement of rapidly turning-over proteins (55).

In summary, all these findings underline that plasmalogens participate in the execution of some responses of macrophages but not in others, thus reflecting some kind of biological specificity of these phospholipids. These works have exposed plasmalogen deacylation/reacylation reactions as critical biochemical events in LPS priming of macrophages for enhanced AA release. This could be due to a CoA-IT inhibition effect of LPS priming, which favors the AA accumulation on PC species by blocking the fatty acid transfer to PE species. Furthermore, it has been revealed the cPLA$_2\gamma$ implication as a major enzyme in CoA-IT-mediated phospholipid AA remodeling, and the role of plasmalogens in this process. All these findings are summarized in **Figure 35**.

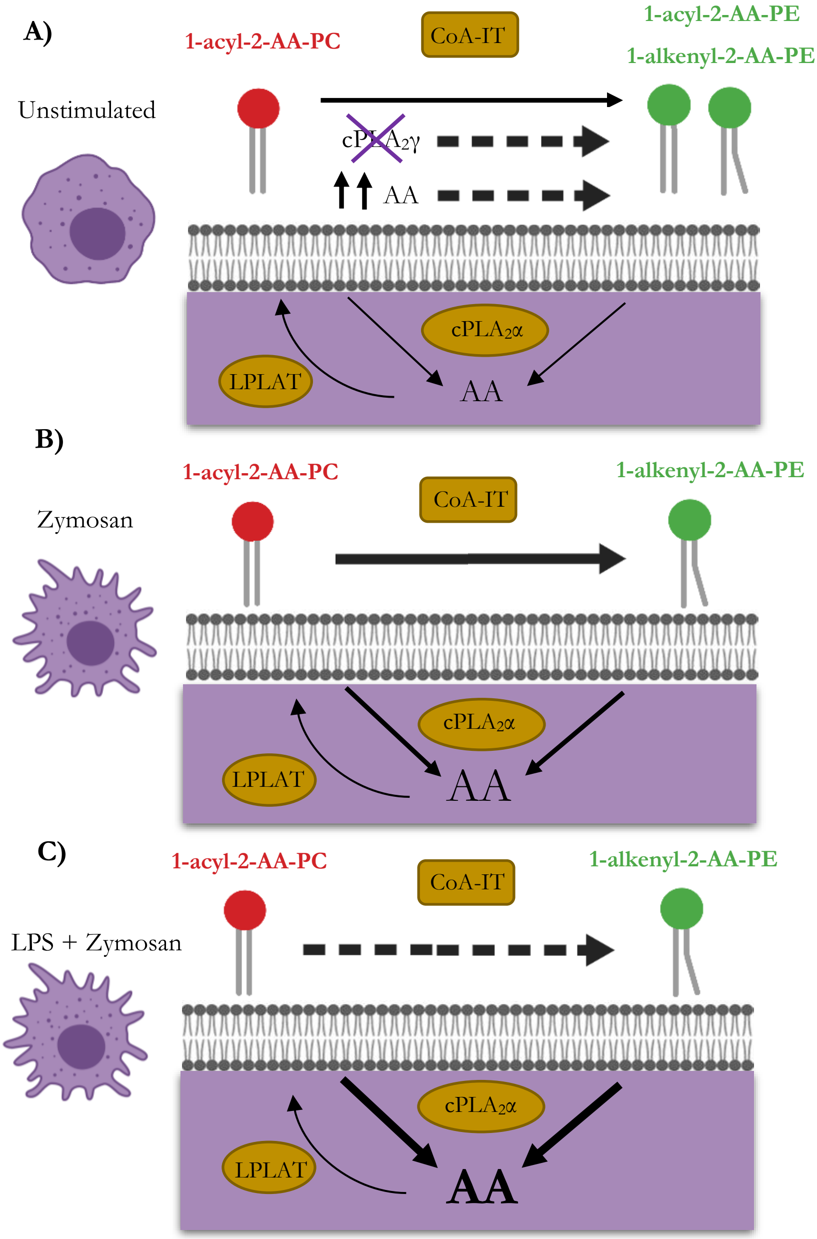

A)
Unstimulated
1-acyl-2-AA-PC
CoA-IT
1-acyl-2-AA-PE
1-alkenyl-2-AA-PE
cPLA2γ
AA
LPLAT
cPLA2α
AA
B)
Zymosan
1-acyl-2-AA-PC
CoA-IT
1-alkenyl-2-AA-PE
LPLAT
cPLA2α
AA
C)
LPS + Zymosan
1-acyl-2-AA-PC
CoA-IT
1-alkenyl-2-AA-PE
LPLAT
cPLA2α
AA

Figure 35. Interaction between CoA-dependent acyltransferases, CoA-independent transacylase, and phospholipase A_2 in regulating phospholipid AA remodeling and AA release in unstimulated **(A)**, zymosan-stimulated **(B)** or LPS-primed zymosan-stimulated **(C)** macrophages. AA, arachidonic acid; CoA-IT, CoA-independent transacylase; LPLAT, lysophospholipid:acyl-CoA acyltransferase; cPLA$_2$α, cytosolic phospholipase A_2 α.

CONCLUDING REMARKS

Taken together, the results of this work provide new information for a better understanding of the regulatory processes underlying cellular AA availability, and lead to the following major conclusions:

1. Cellular plasmalogen content does not influence AA levels or distribution in macrophages.

    1.1. CoA-IT-mediated phospholipid AA remodeling reactions are independent of the plasmalogen content of cells. Specific molecular species linkages do not influence AA distribution in innate immune cells, which appears to depend primarily on headgroup composition of membrane phospholipids.

    1.2. Cellular plasmalogen content influences neither cellular AA levels nor the relative distribution of the fatty acid among phospholipid classes. It seems that it is the endogenous cellular level of AA, not the plasmalogen status, which determines the rate of remodeling of AA among phospholipids.

    1.3. There is a differential involvement of plasmalogens in some, but not all, responses of macrophages. Hence, this reflects some sort of biological specificity of this kind of phospholipids.

    1.4. Group IVA and IVC cytosolic family members (cPLA$_2\alpha$ and cPLA$_2\gamma$) play separate roles in cellular AA homeostasis: the former regulates AA release but not phospholipid AA remodeling, while the latter does the opposite.

    1.5. Group IVC cytosolic phospholipase A$_2$ (cPLA$_2\gamma$) is a major enzyme involved in phospholipid AA remodeling.

2. Ethanolamine plasmalogen hydrolysis is an important step in bacterial lipopolysaccharide priming of macrophages.

    2.1. LPS priming causes a marked decrease in the entry of AA into the ethanolamine plasmalogens and facilitates the net hydrolysis of ethanolamine plasmalogen species after zymosan stimulation, which seems to be due to a reduction in phospholipid AA remodeling because of a diminished activation of CoA-IT.

    2.2. Blunting of the CoA-IT pathway by LPS priming in stimulated macrophages leads to an increase of free AA available for eicosanoid synthesis, probably due to the enhanced access of cPLA$_2$α to AA moieties present in PC pools.

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