UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mass Spectrometric Analysis of Specificity and Inhibition of Group IVA Cytosolic Phospholipase A_2 on Natural Membrane Phospholipids

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by

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The Thesis of Yuan Chen is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015

DEDICATION

This thesis is dedicated to my incredible parents: Linsen Chen and Caixia Zhang. Their unconditional love has sustained me through every hard moment, and I am extremely grateful to have them in my life.

EPIGRAPH

"If you really want it, you'll push past pain.

Use it, control it.

If you really want it, then live your dreams.

You can do anything you want to, if you really want it."

--*Kobe Bryant*

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LIST OF ABBREVIATIONS

AA arachidonic acid

PLA_2 phospholipase A_2

GIVA $cPLA_2$ Group IVA cytosolic PLA_2

EPA Eicosapentaenoic acid

LC liquid chromatography

LC-MS/MS liquid chromatography-tandem mass spectrometry

MS/MS Tandem mass spectrometry

LPS lipopolysaccharide

MRM multiple reaction monitoring

NSAID non-steroidal anti-inflammatory drug

MS mass spectrometry

PC phosphatidylcholine

PE phosphatidylethanolamine

PG prostaglandin

PI phosphatidylinositol

$PI(4,5)P_2$ phosphatidylinositol 4,5-bisphosphate PLA_2 phospholipase A_2

PIP_2 Phosphatidylinositol 4,5-bisphosphate PLA_2 Phospholipase A_2

16:0 Lyso PC 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine

17:0 Lyso PC 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine

18:0 Lyso PC 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine

16:0 Lyso PA 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt)

16:0 Lyso PE 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine

16:0 Lyso PG 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)

16:0 Lyso PS 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt)

18:0 Lyso PS 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt)

Egg PC L-α-phosphatidylcholine (Egg, Chicken)

Egg PA L-α-phosphatidic acid (Egg, Chicken) (sodium salt)

Egg PE L-α-phosphatidylethanolamine (Egg, Chicken)

Egg PG L-α-phosphatidylglycerol (Egg, Chicken) (sodium salt)

Brain PS L-α-phosphatidylserine (Brain, Porcine) (sodium salt)

PAPA 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt)

PAPC 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine

PAPE 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine

PAPG 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)

PAPS 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine (sodium salt)

Brain PI(4,5)P_2 L-α-phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt)

DPPC 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine

DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

PSPC 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine

POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

PLPC	1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine
PAPC	1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
PDPC	1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
SMPC	1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine
SPPC	1-stearoyl-2-palmitoyl-sn-glycero-3-phosphocholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
SOPC	1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine
SLPC	1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine
SAPC	1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
SDPC	1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
$C_{12}E_8$	Octaethylene glycol monododecyl ether
MS	Multiple sclerosis
AD	Alzheimer's disease
PD	Parkinson's disease
TXB_2	Thromboxane B_2
PGE_2	Prostaglandin E_2
LTB_4	Leukotriene B_4

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Chapter 3 and 4, in part is currently being prepared for submission for

publication. Varnavas D. Mouchlis, Yuan Chen, Edward A. Dennis. “Mass Spectrometric Analysis of Specificity and Inhibition of Phospholipase A_2 on Natural Membrane Phosholipids”. Ms. Chen will be a co-author on this paper.

ABSTRACT OF THE THESIS

Mass Spectrometric Analysis of Specificity and Inhibition of Group IVA Cytosolic Phospholipase A_2 on Natural Membrane Phospholipids

by

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Master of Science

University of California, San Diego, 2015

Professor Edward A. Dennis, Chair

Phospholipase A_2 (PLA_2) constitutes a superfamily of enzymes that is well-studied by the scientific community. PLA_2s are membrane-associated enzymes that catalyze the hydrolysis of the ester bond at the *sn*-2 position of membrane phospholipids liberating free fatty acids, predominantly arachidonic acid (AA). The PLA_2 superfamily consists of 16 groups and numerous subgroups and they can be considered as six major types: the secreted ($sPLA_2$), the cytosolic ($cPLA_2$), the

calcium-independent ($iPLA_2$), the platelet-activating factor acetylhydrolase (PAF-AH), lysosomal PLA_2 ($LPLA_2$), and the adipose-PLA (AdPLA).

The human Group IVA $cPLA_2$ (GIVA $cPLA_2$) was cloned and sequenced from U937 cells in 1999. This enzyme contains 749 amino acids and has a molecular weight of 85.2 kDa. The crystal structure of the enzyme was resolved in 1999 and was found to contain an N-terminal C2 domain and a C-terminal catalytic domain. GIVA $cPLA_2$ is of important biological relevance and involved at the earliest stage of inflammation since through its specificity for AA at the *sn*-2 position, it is the main AA provider for the eicosanoid biosynthetic pathway. Understanding the function of the enzyme is vital to find new ways to modulate inflammatory diseases in contrast to inhibiting traditional downstream pathways.

In this study, we have designed a liquid chromatography/mass spectrometry (LC/MS) high-throughput assay to test the specificity of GIVA $cPLA_2$ toward different substrates. We tested various natural phospholipid substrates and found a preference for GIVA $cPLA_2$ for 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC). We also validated the assay for determining inhibitors constants and potency using commercially available inhibitors.

CHAPTER 1

Introduction to Phospholipase A_2

1.1 Introduction to the Development of the PLA_2 Superfamily

Phospholipase A_2 (PLA_2) enzymes are located in many tissues and cells. They constitute one of the earliest enzyme families to be studied, which were found naturally in snake venoms in the late 19th century. Therefore, they were originally purified and characterized from the cobra and rattlesnake venom.[1] The last thirty years, there has been great interest in research on PLA_2s since their products are important in many inflammatory diseases. The PLA_2 superfamily consists of different groups of enzymes which catalyze the ester bond at the *sn*-2 position of membrane phospholipids.[2]

While the number of PLA_2 enzymes has increased over the years, scientists have designed a system with Roman numerals to number the different subgroups of PLA_2s, followed by letters to differ each paralog within the same subgroup of PLA_2s.[3] Each enzyme was named and assigned to its group based on its molecular weight, homology, amino acid sequence, cellular localization, and calcium dependence.[4]

It was further realized that, in PLA_2 found in cobra and rattlesnakes, there are six disulfides in common; however, one of them is obviously located in a different place, which led to the designation of Group I for cobras and Group II for rattlesnakes.[1] The PLA_2 purified from the bee venom was named as Group III, since it was very different. The new cytosolic PLA_2 ($cPLA_2$) was then assigned as Group IV (GIV). The newly discovered secreted PLA_2 ($sPLA_2$) from macrophages was named Group V. First of all, $sPLA_2$ possesses a small molecular weight of 14kDa with a catalytic requirement for calcium and a disulfide bridges to keep the structure to be rigid. Secondly, the calcium-sensitive cytosolic PLA_2 ($cPLA_2$), which possesses a molecular weight of 85

kDa has been purified, expressed and characterized. Their structure and characteristics are very special, and they will be discussed a little bit further below. Thirdly, from some cells and tissues, many different Ca^{2+}-independent cytosolic PLA_2s ($iPLA_2$) with a molecular weight ranging from 29 kDa to 85 kDa were achieved by purification. It's worth mentioning that they have no relationship to $cPLA_2$ in structure. Fourthly, the Platelet-activating factor acetylhydrolases (PAF-AHs), also known as lipoprotein-associated PLA_2 (Lp-PLA_2), are unique among all the subfamilies of PLA_2s, due to their calcium independence and specificity for short or/and oxidized acyl groups at the *sn*-2 position of PAF,[2] which contains two subgroups named as GVII and GVIII.[1] Several newly discovered $sPLA_2$s were defined as Group XV (GXV), a new type of PLA_2 because their pattern of disulfide bonding or sequence is differed obviously from common $sPLA_2$ groups. They presented optimal enzyme activity around pH 4.5 and proved to have a marked specificity for lysosomes. Therefore, the new group was designed as Lysosomal PLA_2 ($LPLA_2$). Most recently, a novel PLA_2 was extracted from adipose tissue and termed as Group XVI (GXVI).[1] It was summarized in Table 1-1.

PLA_2s were categorized into six main types, and each type has been correlated with various lipid signaling process and diseases. The involvement of PLA_2 in many diseases has attracted the interest of the pharmaceutical industry as well as many researchers to develop potent and selective inhibitors for each group.[5]

Table 1-1. The Classification of PLA_2 superfamily. (Adapted from (1))

Type	Group	Subgroup	Molecular mass (kDa)
	GI	A, B	13-15
	GII	A, B, C, D, E, F	13-17
	GIII		15-18
	GV		14
$sPLA_2$	GIX		14
	GX		14
	GXI	A, B	12-13
	GXII	A, B	19
	GXIII		<10
	GXIV		13-19
$cPLA_2$	GIV	A(α), B(β), C(γ), D(δ), E(ε), F(ζ)	60-114
$iPLA_2$	GVI	A(β), B(γ), C(δ), D(ε), E(ζ), F(η)	84-90
PAF-AH	GVII	A(Lp-PLA_2), B(PAF-AH II)	40-45
	GVIII	A(α_1), B(α_2), β	26-40
$LPLA_2$	GXV		45
AdPLA	GXVI		18

In biological membranes, many ingredients including steroids, protein and phospholipids, are hydrophobic. Also, they are vulnerable to constitute insoluble precipitates or crystals at the interface between water and lipid. Therefore, researches on phospholipases are very challenging. PLA_2 acts on phospholipids, accumulating in aqueous solution, to build structures named micelles, liposomes, and vesicles, etc.[1] As for the PLA_2s, they always catalyze the insoluble phospholipid substrates at lipid-water interface. Because the phospholipids are accumulated molecules, the enzyme binds the substrate molecules at the interface, especially in the presence of phospholipids-detergent mixed-micelles.[6] Under the process called solubilization, the involved detergents are hydrophilic and able to create various shapes of micelles above a critical concentration. The lamellar structures will transform into mixed micelles and result in the formation of a thermodynamically stable isotropic solution.[7]

1.2 Introduction to Human GIVA Cytosolic PLA_2

The human Group IVA $cPLA_2$ was purified, cloned, and sequenced from U937 cells, and its gene was commonly expressed in human tissues. The enzyme consists of 749 amino acids and demonstrates to a protein has a molecular weight of 85 kDa. The crystal structure of the enzyme revealed an N-terminal C2 domain that was found to contribute in the translocation of the enzyme on the membrane surface in the presence of calcium, as well as a C-terminal α/β hydrolase catalytic domain that binds its phospholipids substrate.

There are three well-known forms of human Group IV Phospholipase A_2 paralogs (GIV PLA_2), Group IVA, IVB and IVC, all overexpressed in sf9 cells. The first group IV $cPLA_2$, abbreviated as GIVA PLA_2, was discovered in human source platelets in 1986 and characterized in 1991.[8] It was reported that the $cPLA_2$ cDNA contains 2880 nucleotides in total, including about 500 nucleotides for the 3'-untranslated region and around 200 nucleotides at the 5'-untranslated region. It is also worth mentioning that $cPLA_2$ prefers to act catalytically on the phospholipids containing arachidonic acid (AA) when there is free Ca^{2+} showing in stimulated cells.[2] Among all the paralogs of PLA_2, it is the only one proven to show increased activity with PIP_2, which improves its chance to be detected in cells.[1] The GIVA $cPLA_2$ plays a crucial role in the inflammation response. It has attracted much attention from researches and is considered as one of the most well-studied cytosolic enzymes, due to its preference for the AA released from the *sn*-2 position of phospholipids. AA can be then modified by cyclooxygenase enzymes (COX-1 and COX-2) to become

prostaglandins or leukotrienes by lipoxygenases enzymes. Hence, the activated PLA_2 can initiate the synthesis process of prostaglandins and leukotrienes, two potent inflammatory mediators. Lysophospholipids, likewise, as the other product of the reaction, can be utilized to produce PAF, as the inflammatory mediator.[2]

It is well known that the release of lysophospholipids and AA represents the rate-limiting steps in the whole production process of PLA_2s activation.[5] After being released from the membrane phospholipids, AA is further metabolized into lipid mediators and serves as intracellular messengers. This progress can happen in two separated cell types.[2] Upon the cell surface, the intracellular targeted protein may leave the cells and connect with specific receptors. These different lipids obtained from the PLA_2 action process are of great importance in the treatment of diseases with biological and therapeutic implications, due to their involvement in the tissue injury process that cause the serious inflammatory reactions. Therefore, PLA_2 is becoming an amazing target for the discovery and improvement of potential inhibitors, especially aiming for inflammatory or degenerative diseases.[2]

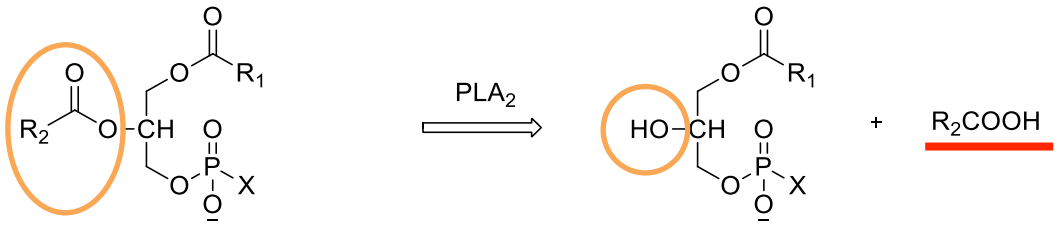

Figure 1-1. The mechanism of the reaction catalyzed by phospholipase A_2 at the *sn*-2 position. The PLA_2 superfamily of enzymes catalyzed this reaction. X represents any of a variety of polar head groups. R_1 and R_2 are both fatty acid chains.

1.3 Structure and Activation Mechanisms

The GIV cPLA$_2$ six group members show similarities in the calcium-dependent lipid binding C2 domains and the catalytic α/β hydrolase domains. There is also distinct disparities among them, with a novel cap region discovered just in GIVA PLA$_2$.[1] We will use the most well-studied Human GIVA PLA$_2$ to represent the structure and activation mechanisms of the whole Group IV PLA$_2$ since the knowledge of the others is still very limited. Besides, GIVA PLA$_2$ has lysophospholipase and transacylase activity; hence, it is regarded to be the most potential isoform among all the cPLA2.[8] It was developed as the central model for the inhibitors due to its high selectivity for AA at the *sn*-2 position of membrane phospholipids. What's more, its fundamental role in the mediation process of lipids, due to the increased expression after the activation of cPLA$_2$, has been correlated to the synthesis of the inflammatory mediators. The crystal structure of the intact GIVA PLA$_2$ was determined by Dessen et al.[9]

Table 1-2. Cytosolic Group IV PLA_2 members. Adapted from (10, 1)

Group	Source	Molecular mass(kDa)	Domain	Activation factor	Alternate names
IVA	Human/murine	85	C2 α/ β hydrolase Cap	Ca^{2+} PIP2 CIP Phosphory-lation	cPLA2α
IVB	Human	114	C2 α/ β hydrolase	Ca^{2+}	cPLA2β
IVC	Human	61	α/ β hydrolase		cPLA2γ
IVD	Human/murine	92-93	C2 α/ β hydrolase	Ca^{2+}	cPLA2δ
IVE	Murine	100	C2 α/ β hydrolase	Ca^{2+}	cPLA2ε
IVF	Murine	96	C2 α/ β hydrolase	Ca^{2+}	cPLA2ζ

In general, GIVA $cPLA_2$ function depends on the intracellular calcium, through the calcium-binding site located at the C2 domain, which triggers the translocation of the enzyme on the membrane surface. After the association with the membrane, the enzyme utilizes a catalytic dyad of Ser-228/Asp-549 that located within the α/β domain to catalyze the hydrolysis of its phospholipid substrate. In common, mitogen-activated protein kinases (MAPKs) phosphorylation, the ceramide-1-phosphate (C1P) and phosphatidylinositol 4,5-bis phosphate (PIP_2) were considered to be necessary for the increased activity.[1]

The $cPLA_2$ structure was characterized by the crystallography and X-Ray and demonstrated to contain two domains, both of which build an anti-parallel β sandwich with eight-strand for the N-terminal and C-terminal.[11] There is some similarity between the structure of C2 domain in the entire enzyme and the structure of a single C2 domain. Even the two topological folds are obviously different, they both

displayed that the two Ca^{2+} bind at the tip of the C2 domain. The crystal structure was damaged in high calcium concentration by soaking crystals of the protein with a difference of the electron density detected, implying that the addition of Ca^{2+} may cause a distinct conformational transition.[12] Also, when the amount of intracellular Ca^{2+} is increasing, $cPLA_2$s will bind to their goal membrane through the C2 domain. The early research stated that the C2 domain is responsible for the Ca^{2+} binding and $cPLA_2\alpha$ (GIVA $cPLA_2$) translocation, and Ca^{2+} activated $cPLA_2\alpha$.[1] Subsequently, the catalytic α/β hydrolase domain will directly connect with the phospholipid substrate at the active site, and the catalytic domain is tethered to the C2 domain by a peptide strand. The two domains are flexible to interact with each other, showing that their slight rotation is capable of optimizing the interactions between the membranes.[12] A novel cap region is conserved only in $cPLA_2\alpha$ in the catalytic domain, among all the GIV PLA_2 isoforms. There is also a lid region within the cap region, capable of protecting the modeling of the phospholipid substrate from reaching the active site.[8] However, the lid region opened, upon the binding of the phospholipid substrate, indicating that there may be a conformational change in the structure, which may lead to the relocation of the lid to promote the accessibility of the solvent in the hydrophobic area.[13] Recent work demonstrated an actual conformational change of the lid region, using the lipid substrate and inhibitors connected to the active sites. According to the phospholipid modeling, the phospholipid molecule must interact with the residues close to the cap region and get extracted from the membrane interface.[1]

1.3.1 Calcium Activation

The binding of the Ca^{2+} to the C2 domain is a critical step for membrane localization. The intracellular calcium concentration increases with the extracellular stimuli, which promotes the hydrolysis of AA. Afterward, the $cPLA_2$ reoriented from the cytosol to the membrane region.[14] The two Ca^{2+} ions can neutralize the negative charge to help with the hydrophobic interaction of the C2 domain with membranes in phospholipid substrates. The hydrogen/deuterium (H/D) Exchange Mass Spectrometry (DXMS) experiments show the binding between the C2 and catalytic domain with a low H/D exchange rate. It indicated that the binding of two different calcium ions could lead the region surrounding the anion hole at the tip area within the C2 domain to tighten up due to the reduced H/D exchange rates. It was illustrated that the effects of Ca^{2+} binding depended largely on the C2 domain, which can expose more hydrophobic residues and repel hydrophilic residues. Thus, the H-bonds have thus been stabilized because of the removal of an extra hindrance of the surface to water. This conformational change may initiate the GIVA $cPLA_2$ interfacial activation.[15]

The Ca^{2+}-bound C2 domain is likewise critical in the hydrolysis process of phospholipid by $cPLA_2$. It has been suggested that the interaction between the catalytic domain and the C2 domain is capable of rigidifying both domains. Within the same cap region, several inhibitors also exhibit the changing H/D exchange rate.[16] Thus, it further indicated the calcium binding might also play a significant role in the catalytic domain and the contact residues within the cap region.

1.3.2 PIP_2/C1P Activation

Though phosphatidylinositol-4, 5-bisphosphate ($PI(4,5)P_2$) is a minor phospholipid component in the cell membrane, it is implicated to play a regulatory role in the deformation of the cell physiology. $cPLA_2$ can also be activated by binding to PIP_2 in a manner independent of Ca^{2+}. GIVA PLA_2 has a specificity toward PI $(4,5)P_2$.[17] Lys-488, Lys-541, Lys-543, and Lys-544 are identified as the binding site and essential for PIP_2-mediated GIVA PLA_2 activation and translocation. It cannot be activated without the C2 domain, indicating that the orientation of the catalytic domain and the C2 domain is crucial for PLA_2 activity. What's more, the research showed that the activation of $cPLA_2$ by $PI(4,5)P_2$ can increase membrane penetration and catalytic efficiency with the higher rate of lipid substrate hydrolysis.[18]

A phosphorylated bioactive sphingolipid, ceramide 1-phophate (C1P), as a lipid second messenger involved in lipid signaling, was demonstrated to be a critical metabolite of ceramide.[19] By stimulating the release of AA through activation of GIVA PLA_2, C1P plays an important role in the mediation of the inflammatory response, but dependent of Ca^{2+}. It has appeared as a potent bioactive agent since they can control myriads aspects of cell physiology like mammalian inflammatory responses and cell survival.[19] It can bind to the C2 domain of GIVA PLA_2 and also require bioactive lipid to translocate to intracellular membranes in answer to an inflammatory process. The latest work showed that C1P can induce GIVA PLA_2 primarily by decreasing the dissociation constant of the enzyme to increase its residence time, regulating the association of $cPLA_2\alpha$ with micelles or vesicles.[18]

1.3.3 Phosphorylation

The stimulation of the release of AA can lead to serine phosphorylation and activation of $cPLA_2\alpha$.[20] Phosphorylation by mitogen-activated protein kinases (MAPKs), will boost the enzymatic activity of $cPLA_2$. $cPLA_2\alpha$ was originally discovered to be phosphorylated at Ser-505 and stimulated by protein kinase C (PKC) and p42-MAP.[20] Subsequently, the sites for phosphorylation were recorded at Ser-454, Ser-437, Ser-505, Ser-515 and Ser-727 in *Spodoptera frugiperda* (Sf9) insect cells by using mass spectrometry.[1]

The sf9-baculovirus expression system is proven helpful for identification of the phosphorylation sites. And the phosphorylation by protein kinases, instead of MAP, may play a regulatory role in the release of AA.[21] In the crystal structure, all the phosphorylation sites turn to be at the low electron density.[9] These four sites are identified in the cap region, implying the phosphorylation may play a crucial role in changing or adjusting the conformation of the cap. Later some of them were considered to be involved in cellular function.[1]

1.3.4 Membrane Interaction

The interfacial activation on the GIVA PLA_2 can help to demonstrate higher activity against phospholipid membrane or large phospholipid accumulation. Three mechanisms dominate the interaction between the GIVA PLA_2 and the membrane: translocation regulated by calcium, binding of second lipid messengers and phosphorylation.[1] Each of the mechanisms is capable of the increasing GIVA PLA_2 catalytic efficiency. First of all, Ca^{2+} binding helps the C2 domain to penetrate the cell

membrane. Afterward, the C2 domain can fix the $cPLA_2\alpha$ and assist the catalytic α/β domain and access the membrane.[1] The catalytic sites are required to maintain the right position of GIVA $cPLA_2$ toward the membrane, and it may be influenced by the conformational change in the C2 domain, $PI(4,5)P_2$ binding or phosphorylation, to regulate the interaction of the catalytic domain.[1] The right position is essential for the connection between GIVA PLA_2 and substrate phospholipid at the active sites. According to the phospholipid modeling, the substrate molecule must reach the residues close to the cap region after being abstracted from the interface on the membrane. Consequently, the molecules will enter or push the lid away and connect with the active sites. By using the green fluorescent protein (GFP), C2 domain is indicated sufficient and necessary for the enzyme translocation in response to Ca^{2+}.[22]

1.4 Biological Functions and Disease Implications

Biological Functions. Extra production of these lipid mediators, PAF and eicosanoids, as well as increased PLA_2 activity, may cause inflammatory diseases and neuronal injury. It is generally believed that the release of AA is the rate-limiting step in the generation of PAF and eicosanoids.[23] In the typical situation, although some AA is converted to prostaglandins, thromboxanes, and leukotrienes, there is still a lot of AA re-entering the brain phospholipids.[23] AA is also able to manage the neuronal function through diverse mechanisms directly. Another potent mediator, PAF, is the product of 1-alkyl-2-lysophospholipid generated by PLA_2 and can catalyze the reactions, too.[1]

Lysophospholipids have the potential to alter the fluidity and permeability of the membranes. The aggregation of the lysophospholipids is under the control of both re-acylation and metabolism. As a result, strict management of GIVA PLA_2 activity is essential for the maintenance of the usual levels of AA, lysophospholipids and PAF in the brain and human body.[23]

Recent research about oxidative stress is correlated with neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), spinal cord injury, and multiple sclerosis (MS). The inflammatory responses in the brain show that the mediators generated by PLA_2 and enhanced PLA_2 activity build a core role and contribute significantly. Lysophospholipids were reported to serve on second messengers for G-protein coupled receptor (GPCR) signaling and are firmly associated with a large number of cancers. Thus, there was a trend to develop novel medicines to inhibit the activity of $cPLA_2$ to prevent myriads of disease conditions, ranging from systemic and acute inflammatory states to cancer. A booming quantity of pharmacologic and biochemical inhibitors of PLA_2 are going to maintain its status in cellular signaling and physiological situations.[24]

Phospholipid Hydrolysis. There are two crucial pathways for the metabolism of AA, cyclooxygenase (COX) and 5-lipoxygenase (5-LO). COX-2 and other goal synthases convert AA and produce thromboxanes with prostaglandins. GIVA PLA_2 activity is reported to enhance accompanied with apoptosis induced by AA and its metabolites. Furthermore, the excess hydrolysis of substrate bioactive lipids will eventually result in the alteration of the membrane permeability, aggregation of phospholipid peroxide, release of free fatty acid, and apoptosis caused by the change

of cell function.[25] The subsequent process induced by the decreased ATP may lead to the reduction of phospholipid and neuronal damage associated with some neurodegenerative diseases, such as spinal cord injury and ischemia.[25]

Disease Implications. According to early data, the amino acid residues in the porcine pancreatic PLA_2 is reachable to connect with biochemical inhibitors and that the ingredients with a preference for this area may present high inhibitory effects for PLA_2. As a consequence, human GIVA PLA_2 with a low molecular weight of about 85 kDa has become a target for the structure-based design of the possible pharmacologic inhibitors, which plays a significant role in clinical research. The ideal situation is the extracellular PLA_2 activity should be suppressed by the inhibitors selectively while the other enzymes involved in cellular signal transduction are not affected.[26]

Though various gene forms of PLA_2 can be expressed and characterized in human and rat brains, not all of them are clarified. Central nervous system (CNS)-associated scientists have centered their research on the GIVA $cPLA_2$, GVIA $iPLA_2$, and GII $sPLA_2$.[27] With more and more knowledge of the structure, function and intracellular localization of PLA_2 superfamily revealed and the mechanism of gene and expression within specific PLA_2 are being elucidated. Diversified distributions exist among various types of cells. The main constituents of membrane phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin, can be induced by hydrolysis, also associated with the mitochondrial respiratory disease. The importance of the mitochondrial phospholipid metabolism is reflected in the energy conversion during postischemic reperfusion. That indicated that the disruption

of the mitochondrial respiratory chain induced by PLA_2 activity was involved in the progress of ROS.[28] Therefore, the metabolism of phospholipids was essential in mediating the inflammatory and oxidative reaction in the CNS-related diseases.

It was reported that PLA_2 is also associated with the neurodegenerative disease, therefore, the PLA_2 inhibitors will be able to deal with neurodegenerative process-associated diseases, and act as anti-inflammatory and protective agents for the related disorders. As a result, the inhibition studies on PLA_2 activity are beneficial for new drugs design for the inflammation related with the neurodegenerative disease.

The $cPLA_2$ can be discovered in the human and rat brain, and generously expressed in glial cells and neurons. The gene of $cPLA_2\beta$, $cPLA_2\delta$, $cPLA_2\varepsilon$ and $cPLA_2\zeta$ can form a gene bundle in mouse and human chromosome separately. It was indicated that GIVA PLA_2 is capable of suppressing estrogen-associated breast cancer cell growth and preventing intestinal tumor.[29] The studies emphasized that the $cPLA_2\alpha$ will become a promising target for therapeutic inhibitor in endocrine-related breast cancer. In animal studies, that the formation of colon cancer activated by Azoxymethane (AOM), was proved by the increasing number of tumors.[30] Moreover, plenty of evidence illustrates that the lack of GIVA PLA_2 activity can alleviate other certain kinds of carcinoma, such as lung cancer,[31] adenocarcinoma tumors, glioblastoma, and Lewis lung carcinoma.[32] The regulatory mechanisms of PLA_2 in cellular progress and different enzymatic interaction have yet to be demonstrated.

Status of $cPLA_2$ Inhibitors. There are several traditional and novel chemical inhibitors of phospholipase A_2, such as bromoenollactone, methyl arachidonyl

fluorophosphonate, fatty acid trifluoromethyl ketones, indole-based inhibitors, 1,3-disubstituted propan-2-ones and polyfluoroalkyl ketones, 2-oxomides, amide inhibitors and phytochemical based inhibitors of PLA_2.[33] Over the last two decades, numerous inhibitors have been developed, but quite a few have the potential to be applied with clinical value. Currently, there are not many possible clinical candidates to inhibit the enzymatic activity of PLA_2. Deficient efficacy or selectivity, as well as low affinity and bioactivity in the animal models, are the major obstacles to the development of PLA_2 inhibitors. For example, Varespladib (LY315920), as an inhibitor of the $sPLA_2$, is a potential anti-inflammatory drug targeting severe coronary and chest syndrome that was under active investigation by Anthera Pharmaceuticals from 2006 to 2012; however, it was stopped due to deficient efficacy.

The first potent inhibitor of $cPLA_2$ was a trifluoromethyl ketone of AA, Bristol-Mayers Squibb later developed a more complicated $cPLA_2$ inhibitors with a structure containing a ketone group likewise. One of the most well-studied structures of inhibitors is the indole-based compounds, such as Ecopladib and WAY-196025, characterized by a carboxylic acid group, including diverse simple or complex side chains. As for the carboxylic acid group, it was first presented in the inhibitor of GIVA $cPLA_2$ designed by Lehr and Genetics Institute, belonging to Wyeth. 2-Oxoamides is another critical class of $cPLA_2\alpha$ inhibitors, including AX007 and AX074, etc. They can bind in the certain active site of $cPLA_2\alpha$ and show consequent powerful anti-hyperalgesic activity. Most recently, there is another type of inhibitors targeting at another well-studied and important class of $cPLA_2\alpha$, named as 1,3-

disubstituted propan-2-ones, originally introduced by AstraZeneca, expanded upon by Lehr. Subsequently, these inhibitors have been evaluated in some *in vitro* and *in vivo* studies. Scientists are looking forward to the success of clinical trials of $cPLA_2$ inhibitors, especially those associated with inflammatory disease.[1]

1.5 Conclusions

An increasing number of the PLA_2 superfamily enzymes have appeared, leading to their properties and structures to be completely illustrated, since more than 100 years ago. As a result, the PLA_2 has become a potential therapeutic target for many diseases, bringing about a variety of inhibitors and specificity studies, which lead to a promising future for this field. We are trying to outline the more consistent and useful relationship between different groups within current classification system in the next chapters.

CHAPTER 2

Expression and Purification of Human Group IVA Cytosolic Phospholipase A_2

2.1 Introduction to $cPLA_2$

$cPLA_2\alpha$, also known as the first cytosolic PLA_2, GIVA PLA_2, was reported to be an activity by Christina Leslie and Ruth Kramer,[34] characterized in macrophage and platelet cells.[2] It was first isolated and sequenced from U937 cells in 1991,[35] and found to contain a C2 catalytic domain and a α/β hydrolase domain. It was reported to be located in the cytosolic compartment and translocated to the endoplasmic reticulum and nuclear membrane when activated.[36] The $cPLA_2\alpha$ becomes most extensively studied isoforms among $cPLA_2$s. It was reported that $cPLA_2$ can selectively cleave the arachidonic acid (AA) from natural membrane phospholipids and translocate to membrane vesicles in the presence of the change of free Ca^{2+} concentration.[35] The activity of the 85.2 kDa $cPLA_2\alpha$ was demonstrated to be controlled by various factors including its phosphorylation by MAP kinase, the binding activators including PIP_2 and C1P, and the intracellular calcium concentration. Unlike the other isoforms of $cPLA_2$, the $cPLA_2\alpha$ possesses a unique cap region with a lid region inside, at the catalytic domain. It has a particular regulatory and structural characteristic within the whole PLA_2 family, with the ability to control some other progress such as cell proliferation, platelet activation and generation of selected second messengers.[1] The gene of GIVA $cPLA_2$ can be expressed ubiquitously in almost all human tissues. Some phospholipids are capable of serving as the substrate for GIVA PLA_2, for example, phosphatidylcholine (PC) is the most well-studied substrate phospholipid for diverse PLA_2. Phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS) are also utilized in phospholipase studies.[10]

Therefore, they were always used to measure the activity of PLA_2 in diverse experiments.[37] We will discuss in depth about the selectivity of $cPLA_2$ toward each species in the next chapter. $cPLA_2$s was previously indicated to share a broad range of homology among rat, mouse and human. While $cPLA_2\alpha$ and other species share over 95% identity, other $cPLA_2$ shows around 54% and 82% homology in these species. The X-ray crystal structure of $cPLA_2\alpha$ unveiled that an N-terminal lipid-binding C2 domain attached to a C-terminal catalytic domain, whose topology is different from those of other isoforms.[9] Over the years, studies have presented that the mechanism of GIVA PLA_2 employs a conserved active site catalytic dyad consisting of a nucleophilic serine Ser-228 and an aspartic acid Asp-549.[38] This novel dyad is able to catalyze the hydrolysis of phospholipids. Besides, Arg-200 is also indispensable for the catalytic activity.[39]

2.2 Protein Expression and Purification

2.2.1 Materials

All reagents were analytical reagent grade or better.

2.2.2 Expression and Purification of GIVA $cPLA_2$

We expressed human GIVA $cPLA_2$ using the Baculovirus Expression Vector System (BEVS), initiated and patented by Summers[40]. This technology system was developed to process the infected insect cells to produce a lot of expected recombinant proteins quickly and economically.[40]

Cells Harvesting. The human GIVA $cPLA_2$ were expressed in *Spodoptera frugiperda* (sf9) insect cells using BEVS. Sf9 cells were seeded in 1 L shaker flasks containing a suspension culture of 500 mL SF900 III SFM medium and incubated at

27 ^{o}C for 24 h before infection. Then the cells were infected at density of ~ 1.5× 10^{6} cells/mL with a BEVS containing the cDNA of C-terminal 6×His-tag GIVA $cPLA_2$ at a Multiplicity of Infection (MOI) of 0.1 for 96 h. The cells were harvested by centrifugation for 5 min at 4 ^{o}C at 5,000×g, afterwards were collected and stored at -80 ^{o}C. The cell pellet was thawed on ice before purification.

Protein Expression. Sf9 cells were collected in lysis buffer (25 mM Tris-HCl, 100 mM NaCl, 1 M Urea, 2 mM ATP, 10 mM β-Mercaptoethanol, and protease inhibitors obtained from Roche). Vortex them until they were dissolved and homogeneous. Use ultrasonic homogenizer (Qsonica, Newtown, CT) to sonicate the cells for 10 seconds each followed by a 60 seconds breaks on ice between sonications, and repeated three times. Afterward, the cells were gently shaken by the rotary-shaker Nutator (BD, Sparks, MD) for 60 min followed by 10 min's cooling on ice. This cell slurry was then homogenized and broken 3 times for 10 seconds each with a 1 min break on ice. Shake the cells on the rotary-shaker for 30 min. This insoluble solution was then removed by centrifugation for 1 h at 4 ^{o}C at 13,000×g.

The clear lysate was mixed with Ni-NTA resin, which was previously equilibrated in lysis buffer (1 mL Ni-NTA agarose (*Qiagen, Valencia, CA*) was gently mixed with 4 mL lysis buffer, centrifuged for 1 min at 4 ^{o}C at 1000×g, and the supernatant was removed three times), and mixed gently on a rotary-shaker at 4 ^{o}C for 1 h. The lysate-Ni-NTA mixture was loaded on a column and washed with the wash buffer (25 mM Tris-HCl, 250 mM NaCl, 2 mM ATP, and 10 mM imidazole). Collected the flow-through passed from the column for SDS-PAGE analysis. Finally the enzyme was eluted with the elution buffer (25 mM Tris-HCl, 50 mM NaCl, and

250 mM imidazole, and 30% glycerol), with the dithiothreitol (DTT) added to each elution fragment at a final concentration of 2 mM. The concentration of the enzyme was measured using the Bradford assay. The purified GIVA PLA_2 was preserved in the protein buffer at -20°C for mass spectrometry (MS) experiments.

2.3 Results and Discussions

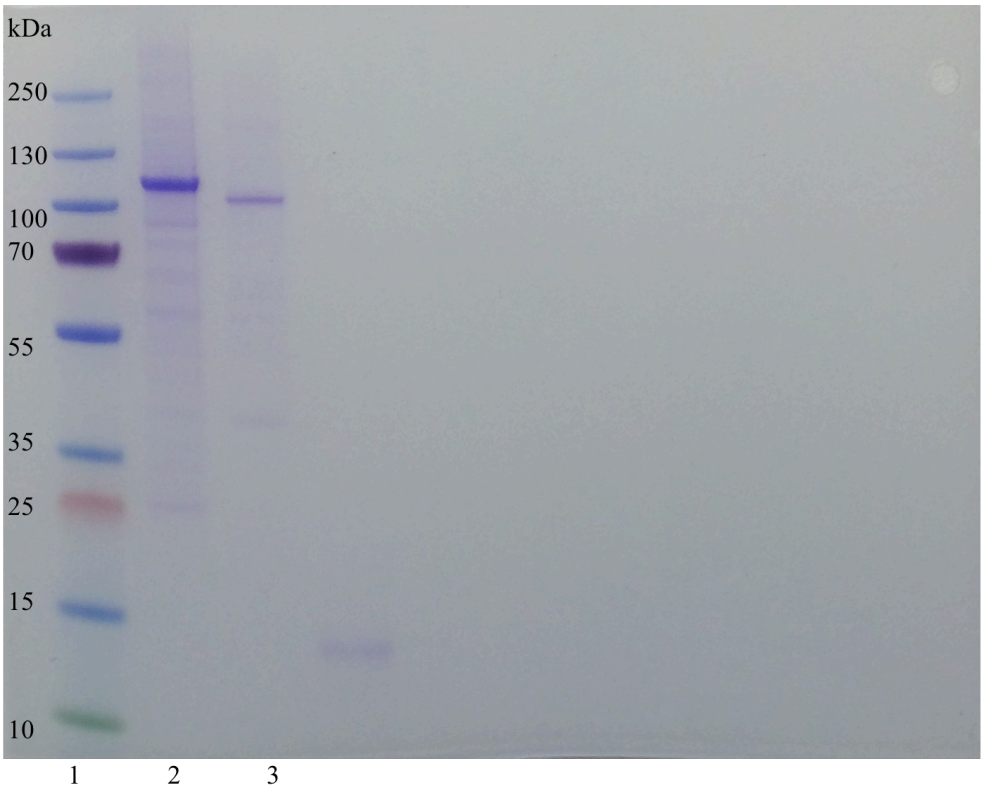

Figure 2-1. SDS-PAGE analysis of $cPLA_2$ extracted from baculovirus infected sf9 insect cells. Lane 1, protein ladder (10-250 kDa) was used as markers; Lane 2, purified human GIVA $cPLA_2$ expressed with the molecular weight about 85 kDa. *Lane 3, purified $iPLA_2$ expressed using the same procedures.

The purification was analyzed by SDS-PAGE in order to verify that the enzyme was in acceptable purify. The enzymatic activity was tested towards PAPC substrate in a mixed micelle *in vitro* assay, and the enzyme was used to perform all our group, acyl-chain specificity, and IC_{50} experiments described in chapter 3 and 4.

CHAPTER 3

Mass Spectrometry Methodology on Phospholipase A_2

3.1 Introduction to Mass Spectrometry

The molecules of phospholipids that make up the lipid bilayer of all living cells also act as the structural entities within subcellular constituents. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), coupled with Multiple Reaction Monitoring (MRM), serve as a fundamental and valuable tool for the accurate, simultaneous multiple analytes detection, and measurement of diverse phospholipids, which can be applied to large sample batches and high-throughout assays.[41] MS usually consist of three different parts: an ion source to convert gas phase molecules to ions, a mass analyzer to sort the ions using electromagnetic fields and a detector to evaluate the quantity and produce data.[42]

Conceptually, the MRM mode, allows the detection of the transitions of different species between the precursor and main fragment of a given phospholipid, to determine the composition of an element by observing its fragmentation. This targeted MS analysis with MRM increases the detection limit for phospholipids via fast and continuous monitoring of certain ions.[43] The charged molecules or fragments were generated by the ionization of various glycerophospholipids, including phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, cardiolipin and phosphatidylserine.

We used to process the inhibitors with the radioactive assay. However, just ^{14}C-labeled PC L-α-1-Palmitoyl-2-Arachidonyl-Phosphatidylcholine (10uCi) is available commercially, however, at a high price. PAPA, PAPE, PAPG, PAPS with ^{14}C-labeled cannot be achieved in the current market. Therefore, it was impossible to perform the radioactive assays of the PLA_2 selectivity toward each phospholipid

species. However, we can utilize MS assay to predict accurately the selectivity or specificity of each class or acyl-chain of phospholipids, which is more convenient, quick and less expensive.

The performance of reversed phase liquid chromatography involves a separation of molecules based on their interactions with a hydrophobic solvent in the presence of an aqueous buffer. The matrix always contains spherical silica beads with C18 bound to the surfaces via covalent bonds. C18 column, as the reversed phase column, offers the hydrophobic retention and methylene selectivity chromatography.[44]

3.2 Fast and High Throughput Method Development

The detection and quantification of lysophospholipids were analyzed by LC/MS with C18 column at first. Even if the data was excellent, we need to wash the machine once a week, due to the high retention of detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) which can cause the clock of the column and damage of the HPLC. What's more, the relatively low retention of RP HPLC for compounds usually demands eluents with low mixtures of organic matrix, since expulsion of eluents from the pores' spaces within C18 groups and deficient wetting always result in the low separation efficiencies and poor retention times.[45] To remedy the side effects of the old way with the C18 column, we come up with a new method and different time window with new hydrophilic interaction liquid chromatography (HILIC) columns.

Recently, the application of HILIC columns coupled with the mass spectrometry technique is gaining increasing attention as a potential analytical

approach for clinical analyses, especially in the separation of a variety of mixtures of polar chemical ingredients using a strong organic hydrophobic mobile phase combined with a hydrophilic stationary phase. In terms of the starting order of these compounds, the elution on HILIC columns performs in the opposite way as that on reversed phase columns, which means that it is able to separate the polar compounds that the C18 column cannot separate completely. Therefore, HILIC columns can efficiently separate polar hydrophilic compounds, which are not retained on reversed phase columns. The base material of HILIC columns can be either silica or polymer and may be improved with diverse types of polar functionalities. Due to its highly polar characteristic, lipids are staying in the column without producing derivatives, making the workflow faster and simpler. The separation of Lyso PC was achieved in less than 2 minutes. In HILIC column, water as the more polar solvent is very powerful in eluting polar compounds. Thus, the polarity of the mobile phase is enhanced by increasing the ratio of water in the buffer. The unbounded silica is the major ingredient encompassed in its stationery phase, and is capable of offering preferred selectivity of the polar compounds for the separation and retention time.[45]

The detection and following quantification of different species of lysophospholipids can be performed in approximately 2 minutes by either C18 or HILIC column with LC/MS in our method.

3.3 Detergent

The water-soluble enzyme, PLA_2, can perform on the water-soluble phospholipid substrates, accumulating to form a variety of structures including

micelles, liposomes, and vesicles. It was indicated that the interface is of great importance in the performance of the enzyme.[46]

Triton X-100 was a commonly used nonionic surfactant for the formation of mixed micelles. However, the presence of Triton-100 in the loading samples always reduces chromatography resolution in LC/MS and suppresses the ionization in MS analysis. For purpose of removing the Triton X-100 or avoiding sensitive detection of phospholipids by MS, a different nonionic detergent, octaethylene glycol monodocecyl ether ($C_{12}E_8$), was used in our experiments to help create micelles, in lieu of Triton X-100. $C_{12}E_8$ possesses a specific m/Z, with negligible effect on the LC/MS. The mixtures will undergo a transformation under cooling at the chain-melting temperatures of these specific phospholipids, leading to the formation of micelles.[47]

3.4 Materials and Methods

3.4.1. Reagents

All phospholipids and internal standards, including 1-palmitoyl-2-hydroxy-*sn*-glycero-3- phosphocholine (Lyso PC (16:0)), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso PC (18:0)), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt) (Lyso PS (18:0)), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine (sodium salt) (Lyso PS (16:0)), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt) (Lyso PA (16:0)), 1-palmitoyl-2- hydroxy-sn-glycero-3-phosphoethanolamine (Lyso PE (16:0)), 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), (Lyso PG (16:0)), 1-heptadecanoyl-2-

hydroxy-sn- glycero-3-phosphocholine (Lyso PC (17:0)), 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine, (PC (16:0/14:0)), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC (16:0/16:0), DPPC), 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine(PC (16:0/18:0), PSPC), 1-palmitoyl- 2- oleoyl-*sn*-glycero-3-phosphocholine (PC (16:0/18:1), POPC), 1-palmitoyl-2- linoleoyl-sn- glycerol-3-phosphocholine (PC (16:0/18:2), PLPC), 1-palmitoyl-2-arachidonoyl -*sn*-glycero -3-phosphocholine (PC (16:0/20:4), PAPC), 1-palmitoyl-2-docosahexaenoyl-sn -glycero-3- phosphocholine (PC (16:0/22:6), PDPC), 1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine (PC (18:0/14:0), SMPC), 1-stearoyl-2-palmitoyl-sn- glycero-3-phosphocholine (PC (18:0/16:0), SPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (PC (18:0/18:0), DSPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC (18:0/18:1), SOPC), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PC (18:0/18:2), SLPC), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PC (18:0/20:4), SAPC), 1-stearoyl-2-docosahexaenoyl- sn-glycero-3-phosphocholine (PC (18:0/22:6), SDPC), 1-palmitoyl-2-arachidonoyl -*sn*-glycero -3- phosphoethanolamine (PE (16:0/20:4), PAPE), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (monosodium salt) (PA (16:0/20:4), PAPA), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (PG(16:0/20:4), PAPG) and 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PS (16:0/20:4), PAPS) were obtained from Avanti Polar Lipids (Alabaster AL, USA).

Optima LC-MS grade acetonitrile (ACN), methanol (MeOH), and water were obtained from Fisher Scientific. Isopropanol (IPA) was purchased from Sigma-Aldrich. Formic acid (FA) was obtained from EMD Technologies. Dulbecco's

Phosphate Buffered Saline (DPBS) was obtained from Corning Life Science. Octaethylene glycol monododecylether ($C_{12}E_8$) was purchased from Sigma-Aldrich.

3.4.2. Preparation of Standard and Internal Standard Solution

For the preparation of standard curves, stock solutions of all lysophospholipids standards were prepared in the buffer of methanol/acetonitrile (80/20), including 16:0 Lyso PA, 16:0 Lyso PC, 16:0 Lyso PE, 16:0 Lyso PG, 16:0 Lyso PS, 18:0 Lyso PC, 18:0 Lyso PS, each of which is at a concentration of 100 μM/ml. A solution containing internal phospholipid standard 17:0 Lyso PC was prepared at 50 μM in Methanol/Acetonitrile (80/20). 300 μl of each working standard solutions for all lysophospholipids were prepared in the DP clear vials (Thermo) by serial dilution from the stock solutions to create the necessary concentrations. Then 200 μl solution was taken from the small vial, with each concentration followed by a 10μl internal standard, to the small vial with a target conical glass insert inside (Thermo). They were vortexed well. All solutions were stored at -80 °C until use.

3.4.3. $cPLA_2$ Substrate Preparation for MS Assay

A solution (100 μM) of PAPC in chloroform was dried under an argon stream. The residue was resuspended in a buffer composed of 100 mM HEPES (pH7.5), 0.09mM $CaCl_2$, and 0.4 mM $C_{12}E_8$ and gently vortexed to form mixed micelles. The mixed micelles were kept at 40°C for 1 hour and each vortex for 10 minutes. Then, 2 mM DTT was added into the mixed micelles to form the substrate solution. A 100 μL substrate solution was added into the 96-well plate (Thermo Scientific Matrix), and a cap (Cap Mats, Sepra Seal^{TM}, Thermo Scientific) covered each well to avoid

volatilization. The reaction was initiated by adding PLA_2 to the mixed micelles and incubated at 40 °C for 30 minutes. After incubation, the reaction was quenched with a 120μL quench solution (MeOH/ACN 4:1). Then internal phospholipid standards were added to the solution, and the solution of 96-well plates was used directly to LC-MS analysis for lysophospholipids.

3.4.4. UPLC-MS/MS Assay

A Shimadzu HPLC system was used, consisting of two HPLC pumps (LC-10ADvp) and a system controller (SCL-10Avp). A CTC Analytics PAL autosampler platform (Leap Technologies) and a column controller instrument (Analytical Sales & Products, Inc) were used.

Reversed-phase separation was performed on a Kinetex C18 column (5 μm, 50×2.1 mm, Phenomenex). The mobile phase consisted of (A) ACN/water (70/30 v/v) containing 10 mM ammonium acetate and (B) MeOH/ACN/H_2O (79.5/17/3.5, v/v) containing 10 mM ammonium acetate. Gradient elution was carried out for 1.6 min at a flow rate of 1.5 mL/min. All buffer used were degassed three times with argon so that the dissolved oxygen can be removed. Gradient conditions were as follows: 0.01-0.1 min, 40% B; 0.1-0.3 min, 40-100% B; 0.3-1.2 min, 100% B; 1.20-1.21 min, 100-40%B; 1.21-1.50 min, 40% B. 10 μl of each sample in triplicates was injected into the column. The temperature of the column was kept at 40 °C. All samples were kept at 4 °C throughout the analysis.

HILIC-phase separation was performed on a Kinetex HILIC column (2.6 μm, 30×2.1 mm, Phenomenex). The binary gradient consisted of (A) ACN/water (95/5;

v/v, pH=8.0) containing 25 mM ammonium acetate and (B) ACN/water (50/50; v/v, pH=7.3) containing 25 mM ammonium acetate. All buffer used were degassed three times with argon so that the dissolved oxygen can be removed. Gradient elution was carried out for 1.6 min at a flow rate of 0.8 mL/min. Gradient conditions were as follows: 0.01-0.8 min, 0% B; 0.8-1.2 min, 0-100% B; 1.2-1.5 min, 100% B; 1.50-1.51 min, 100-0%B; 10 µl of each sample in triplicates was injected into the column. The temperature of the column was kept at 40 °C. All samples were kept at 4 °C throughout the analysis.

Mass spectrometry was carried out on the ABI/Sciex 4000 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer coupled with a Turbo V ion source. Lysopholipids were detected in positive or negative electrospray ion mode according to the classes of lysopholipids. Curtain gas (CUR), nebulizer gas (GS1) and turbo-gas (GS2) were set at 10 psi, 50 psi and 20 psi, respectively. The electrospray voltage was +4.5 kV or -4.5 kV, and the turbo ion spray source temperature was 500 °C.

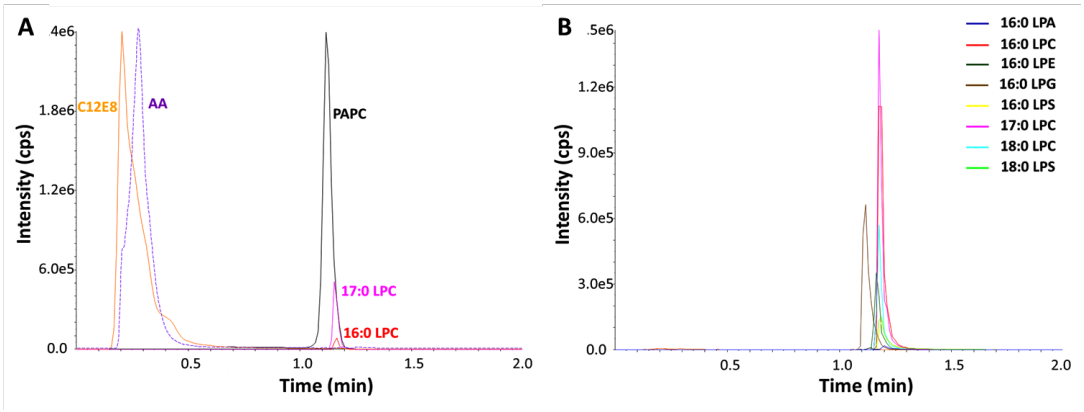

Figure 3-1. Separation of phospholipids classes using HILIC-LC/MS. (A) Multiple analysis of phospholipids and detergent $C_{12}E_8$. $C_{12}E_8$ was presented early from approximately 0.1 min to 0.7 min, PAPC about 1.0 min to 1.2 min, 16:0 LPC and 17:0 LPC from 1.1 min to 1.3 min. AA was detected and measured in negative mode (in dashed line) from about 0.1 min to 0.7 min; (B) eight classes of metabolites, lysophospholipids. 16:0 LPG was shown from 1.1 to 1.3 min, the others are all from 1.2 to 1.4 min. We kept the recording time of analytes 16:0 LPC and internal standard 17:0 LPC from 1.0 to 1.5 min, away from the detergent $C_{12}E_8$. Compared with the analysis on the C18 column, this way is much clearer for the machine. The separation in the HILIC column won't cause serious blocking problems.

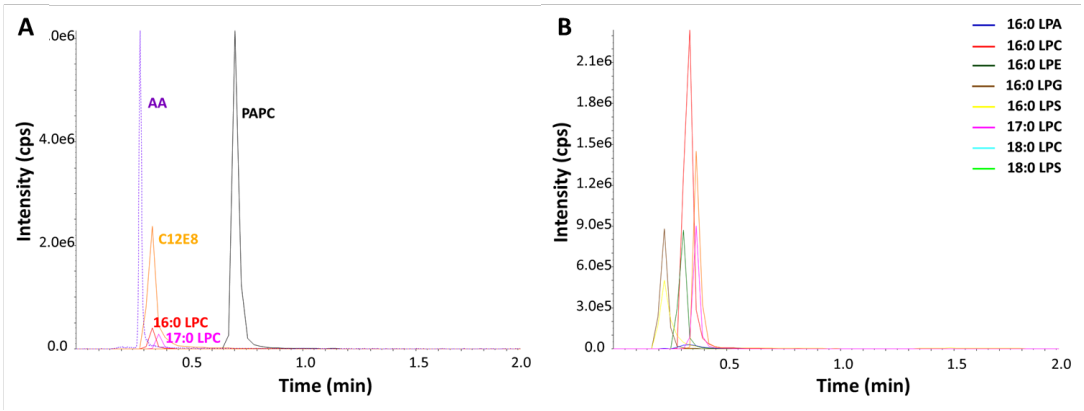

Figure 3-2. Earlier way used for separation of phospholipids classes using C18-LC/MS. (A) Multiple analysis of phospholipids and detergent $C_{12}E_8$. $C_{12}E_8$ was presented from approximately 0.3 min to 0.9 min with a little dragging in the column, PAPC about 0.6 min to 1.0 min, 16:0 LPC and 17:0 LPC from 0.3 min to 0.6 min. AA was detected and measured in negative mode (in dashed line) from about 0.3 min to 0.4 min; (B) Eight classes of metabolites, lysophospholipids. 16:0 LPG were shown from 0.2 to 0.4 min; the others were all from around 0.3 to 0.5 min. We kept the recording time of analytes Lyso PC and internal standard 17:0 LPC from 0 to 0.6 min, and the rest went to the waste.

Elution order of phospholipid classes with C18 column was: AA> $C_{12}E_8$ > Lyso Lipids > PAPC. There was an overlapping of $C_{12}E_8$ and 16:0 LPC, which caused a clocking problem for columns and a consequent signal drop. We moved to HILIC column, so we were able to solve the problem of overlapping and largely reduce the phenomenon of signal drop.

3.4.5. Data Analysis

Lysophospholipids were analyzed using scheduled multiple reaction monitoring (MRM). Mass spectrometer parameters including the declustering potentials and collision energies were optimized for each analyte. Nitrogen was employed as the collision gas. Data acquisitions were performed using Analyst software (Applied Biosystems). MultiQuant software (Applied Biosystems) was used to quantify all metabolites.

Analysis of PAPC, PAPE, PAPA, PAPG and PAPS was performed in positive mode. A crucial aspect of our method was the inclusion of 1 internal standard for analysis of 5 lysophospholipids. MRM was listed in the following table:

Table 3-1. The scheduled characteristics for MRM.

Q1	Q3	ID	CE (volts)
411.3	155	16:0 LPA	22
496.3	184.1	16:0 LPC	36
454.3	313.3	16:0 LPE	27
485.3	313.3	16:0 LPG	27
498.3	313.3	16:0 LPS	28
510.3	184.1	17:0 LPC	36
524.3	184.1	18:0 LPC	27
526.3	341.2	18:0 LPS	28
761.4	341.3	18:0 LPI (4,5)P_2	36
697.5	599.4	PAPA	23
782.5	184.1	PAPC	31
740.5	599.4	PAPE	27
788.5	599.4	PAPG	21
784.5	599.4	PAPS	28
1048	627.7	18:0/20:4 PI(4,5)P_2	35
539.4	133.1	$C_{12}E_8$	26

3.5 Results and Discussion

3.5.1. Method for Monitoring Activity of cPLA$_2$ with LC-MS Development

PAPC is used as the primary source of the substrate of cPLA$_2$. cPLA$_2$ can hydrolyze the *sn*-2 ester bond of phospholipids to release free fatty acid arachidonic acid (AA) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso PC (16:0)) products. So we employed tandem mass spectrometry to monitor the production of these species.

Usually, phosphatidylcholines (PCs) are more ionizable in positive mode of MS while free fatty acids are more ionizable in negative mode. However, the sensitivity of Lyso PC (16:0) in positive mode is much higher than that of AA in negative mode. So, we decided to determine the product Lyso PC (16:0) of enzyme

reaction in the positive mode so as to monitor $cPLA_2$ activity.

PLA_2 exhibits a large variety of cellular functions, including those which are antibacterial and antiviral. Many compounds as inhibitors need to be evaluated in their activity toward PLA_2. Thus, a high throughput method for monitoring $cPLA_2$ activity is an urgent demand. In our experiment, 96-well plate was first used for incubation of enzyme and substrate. The volume of substrate and enzyme need for the reaction is very little; 100 uL substrate and 5 uL enzyme are enough.

Second, a small dimension RPLC column (1.0 I.D.×50 mm) was used for analyzing Lyso PC (16:0). The retention time of Lyso PC (16:0) is about 1.5 min. With the washing of the PAPC retained from the column, the whole LC time is 1.6 min. It is fast enough for the evaluation of inhibitor activity on a larger scale.

MRM can be tested in the same assay on the chromatography, and present a special fragment ion that can be detected and quantified in a very complicated matrix, and the method allows for additional selectivity by monitoring the chromatographic co-elution of multiple transitions for a given analyte. This characteristic makes the MRM ideal for specific and sensitive quantitation. For Lyso PC (16:0), 496.3 and 184.1 were selected as the parent and product ion. Moreover, the extraction of Lyso PC from the reaction and quench solution was omitted. After the incubation of enzyme, as well as substrate and quenching, the solution can be loaded into the LC-MS directly and the whole process for this experiment is very simple.

It was hard to recognize individual lipids in diverse mixtures without some separation on Mass Spectrometry. Therefore, HPLC was used to deal with this

problem to amplify the advantage of the UPLC-MS/MS, identifying a variety of phospholipids by using MRM to recognize each unknown class. This LC/MS-based lipidomic analysis has an advantage over the conventional methodology of shotgun lipidome.[48]

For the quantitation of Lyso PC (16:0), an internal standard Lyso PC (17:0) was used. After the incubation of the enzyme and substrate, the internal standard Lyso PC (17:0) was added into the 96-well plate, together with the quench solution. An internal standard is used to monitor the chromatographic response and facilitate normalization, which allows for accurate quantification. This approach, also known as the isotope dilution method, works on the principle that internal standards have different precursors and/or product ion masses but are chemically and structurally related to the target analytes. 510.3 and 184.1 were selected as the parent and product ion of the internal standard Lyso PC (17:0) for the MRM mode.

As the ionization efficiency is not constant from day to day, the standard curves were prepared every time on the same day as the assays performed. We firstly placed all the species of lysophospholipids we needed (including 16:0 LPC, 18:0 LPC, 16:0 LPA, 16:0 LPE, 16:0 LPG, 16:0 LPS and 18:0 LPS) together at the original concentration of 10 μg/ml for each. We then made a serial dilution of this mixture. Before we run the assay, the same amount, 2.5 μM of internal standard 17:0 LPC, was added to each sample at different molar ratios and tested in triplicates. The determination of each peak area for the phospholipids is achieved by calculating the peak area of specific lyso PC divided by the internal standard as measured in each sample.

Analysis of different acyl-chains of PCs was performed in positive mode. 524.3 and 184.1 were selected for parent and product ion of standard Lyso PC (18:0) for MRM mode, and Lyso PC (17:0) was also used as internal standard for analysis of Lyso PC (18:0).

Phospholipids have a hydrophobic acyl chain and a hydrophilic head group. The classes of phospholipids depend on the polar head groups and the acyl chains can vary in chain length and extent of saturation. The quantitative assays on MS with MRM method need the optimization of parameters of Q1 and Q2, applied on a triple quadrupole mass spectrometer, to allow monitor of the specific ions of ions.[49]

Lyso PC (16:0) quantitation was performed using the stable isotope dilution method. A linear standard curve was generated where the ratio of analyte standard peak area to internal standard peak area was plotted versus the amount of analyte standard. The method offers good linearity for the analytes, the R^2 value is above 0.99, and the typical standard phospholipid curves are shown in Figure 3-3.

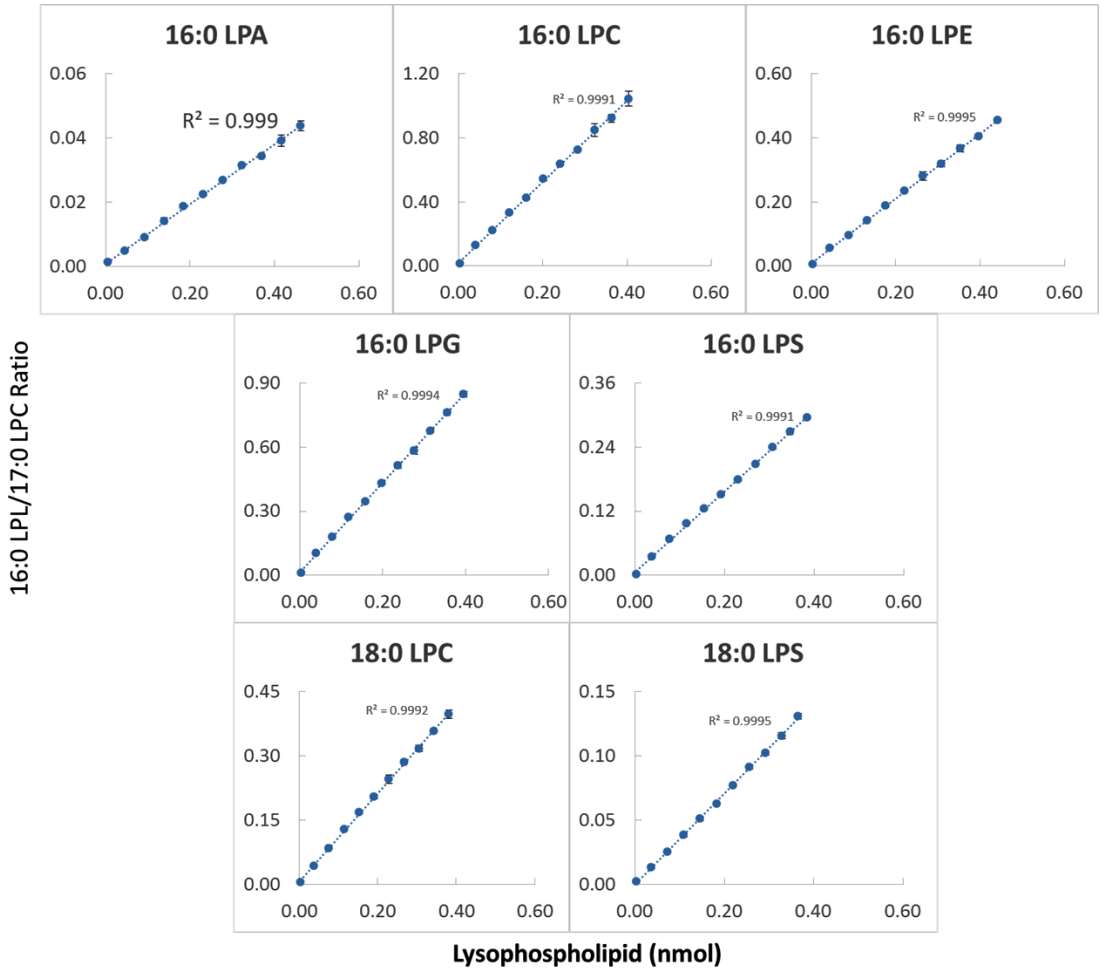

Figure 3-3. Picture of representative standard curves of seven classes of phospholipids with 17:0 LPC as the internal standard. Every point stands for the ratio of analyzed peak area of each phospholipid class to that of 17:0 LPC at a specific concentration. Standardized mixtures of phospholipids in different concentrations were added to be measured.

3.5.2. Time Course and Amount Response Studies of $cPLA_2$

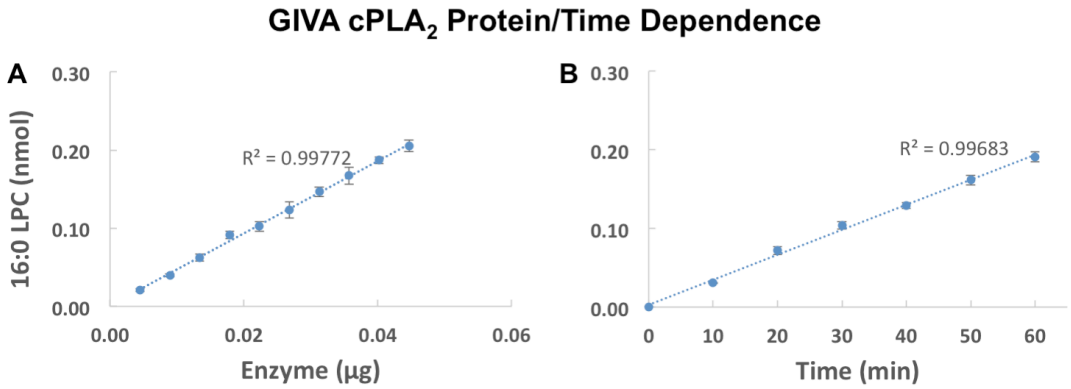

Figure 3-4. Picture of protein and time course of activity of GIVA $cPLA_2$. (A) Amount-dependent release from $cPLA_2$. The assay was carried out within 30 min; the dose of enzyme started from 1 µl per sample, to 2,3, etc. until 10 µl /sample with the concentration of 0.045 µg/µl. The amount of analyte 16:0 LPC was linearly increased with the increasing of the enzyme amount. (B) Time-dependent release of 16:0 LPC in response to $cPLA_2$. This assay was carried out using the same amount 5 µl of GIVA $cPLA_2$ in each sample in triplicates, stopped at 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min.[50] We can see the amount of 16:0 LPC almost increased linearly due to the activity of $cPLA_2$ with the negligible binding effect. Data are output as average ± standard deviation of the average. Each point represents the intensity of triplicate samples of 16:0 LPC.

The very linear protein and time courses were observed to demonstrate that such an assay condition is appropriate for measuring substrate specificity and potency of the related inhibitors of $cPLA_2$. This result also indicated that the substrates containing PC are hydrolyzed continuously at a stable rate that reflects the GIVA $cPLA_2$ specificity for the phospholipid substrate before the enzyme was consumed.[51] We also hypothesized that under the condition that the amount of enzyme is the same, it was equally likely that the enzymatic activity might depend linearly on the quantity of membrane phospholipid. All the experiments were carried out under the conditions that the enzyme is sufficient for all enzyme bonding with the interfaces.

3.5.3. Head Group and Acyl-Chain Specificity of $cPLA_2$

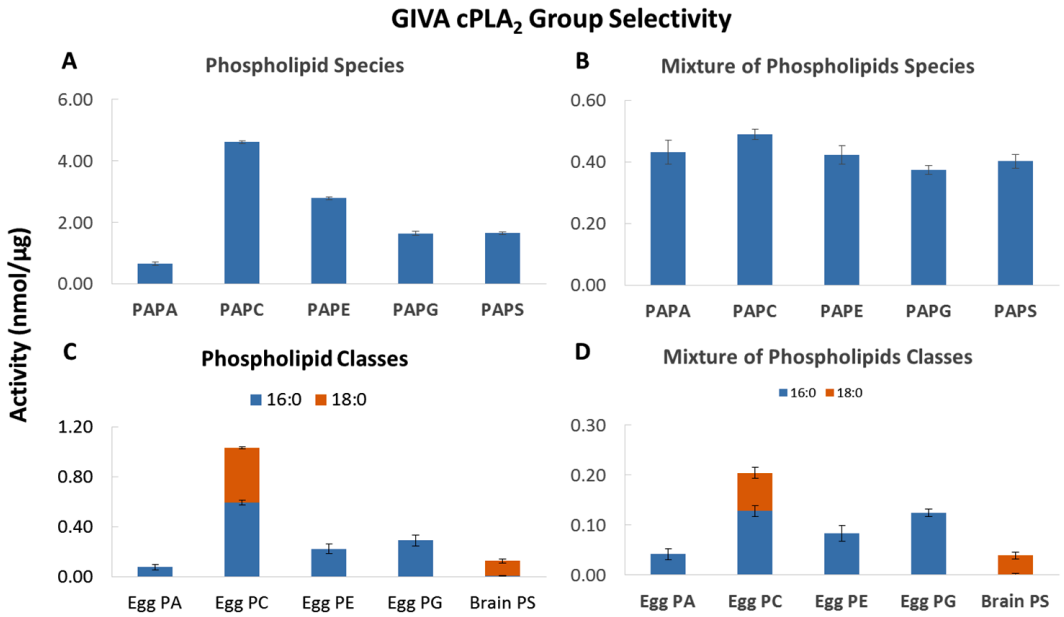

Figure 3-5. Picture of Group Specificity toward $cPLA_2$. (A) For individual phospholipid species including PA, PC, PE, PG, PS, (B) for mixed micelles including equimolar amount of PA, PC, PE, PG, PS, (C) for single phospholipid classes extracted from Egg or Brain including Egg PA, Egg PC, Egg PE, Egg PG, Brain PS (PS is just available from brain, mainly including 42% of 18:0 PC, 30% of 18:1 PC, 2% of 20:4 PC (AA) and 11% of 22:6 PC, which will produce 16:0 LPS and 18:0 LPS together; and Egg PC is a purification of egg extract mainly containing 32.7% of 16:0 PC, 1.1% of 16:1 PC, 12.3% of 18:0 PC, 32% of 18:1 PC, 17.2% of 18:2 PC, 2.7% of 20:4 PC (AA), 0.2% of 20:2 PC and 0.3% of 20:3 PC; Egg PA contains 34.2% of 16:0 PC, 1.0% of 16:1 PC, 11.5% of 18:0 PC, 31.5% of 18:1 PC, 18.5% of 18:2 PC, 2.7% of 20:4 PC (AA), 0.7% of 22:6 PC; Egg PE contains 17.3% of 16:0 PC, 0.5% of 16:1 PC, 24.2% of 18:0 PC, 18.1% of 18:1 PC, 14.0% of 18:2 PC, 16.0% of 20:4 PC (AA), 0.2% of 20:2 PC, 0.3% of 20:3 PC, 4.2% of 22:6 PC; Egg PG contains 32.9% of 16:0 PC, 0.9% of 16:1 PC, 12.2% of 18:0 PC, 30.2% of 18:1 PC, 18.7% of 18:2 PC, 3.5% of 20:4 PC (AA), 0.9% of 22:4 PC and 0.7% of 22:5 PC. They can be catalyzed to generate 16:0 LPC and 18:0 LPC (in orange) simultaneously; (D) for equimolar mixture of phospholipids classes including Egg PA, Egg PC, Egg PE, Egg PG, Brain PS. Substrate specificities of GIVA $cPLA_2$ is measured under the same assay conditions, with a concentration of 0.012 μg/μl in each sample in triplicates. The 18:0 lysophospholipids were just measured in Egg PC and Brain PS, not including the others.

The GIVA $cPLA_2$ binds to the micelles of individual (left) or mixed phospholipids (right). Substrate selectivity constants were calculated from the ratio of

the release of 16:0 LPC to 17:0 LPC after a 30-minute incubation. From Figure 3-5 (A), the enzyme activity varied from 0.65 to 4.61 nmol/μg. The rank order of preference is PAPC > PAPE> PAPG ≈ PAPS > PAPA. The largest variance in the specificity for single phospholipid species is about tenfold, implying that though the substrate containing PAPC is the best one for GIVA $cPLA_2$, $cPLA_2$ does not significantly differ between different phospholipids with different headgroups. However, from Figure 3-5 (B), the order of preference for GIVA $cPLA_2$ is all around 0.4 nmol/μg, very similar among the five species. Therefore we hypothesize that the specificity for $cPLA_2$ may largely depend on the electrostatic interaction of the phospholipid with the enzyme and the 3-D structure of the micelles.

From Figure 3-5 (C), the rank order of preference is Egg PC > Egg PG> Egg PE ≈ Brain PS > Egg PA, which is slightly different from the order in Figure 3-5 (A), and the enzyme activity varied from 0.1 to 1.0 nmol/μg. The activity is distinctly lower than those in the substrate of pure phospholipid species. Similarly, the mixture from Figure 3-5 (D) has natural unsaturated phospholipids with different head groups. The enzyme activity varied from 0.04 to 0.2 nmol /μg. And the order of specificity for $cPLA_2$ is Egg PC > Egg PG > Egg PE ≈ Brain PS > Egg PA. But taking into consideration the ratio of each individual species contained in the natural extract phospholipids, the order for (C) should be moderately changed to 16:0 PC > 18:0 PC > 16:0 PG > 16:0 PE > 18:0 PS > 16:0 PA > 16:0 PS. At the same time, the order for (D) may be altered to 16:0 PC > 16:0 PG > 16:0 PE > 18:0 PC > 16:0 PA >18:0 PS > 16:0 PS. It seems they don't seriously follow the amount of AA contained in each extract.

Since according to the fatty acid distribution of each extracts shown in the description of Figure 3-5, Egg PE has 16% of AA, much more than the others, but the figure doesn't show it exhibits more lysophospholipase activity than the others.

From the new rank orders, it was also noticed that head groups in the mixed micelles played a very different role from what was in the individual species micelles. The difference in size, shape, structure and charge of micelles formed with diverse phospholipids may give confusing outcomes.

It was indicated that the Asp-549 and Ser-229 are in the right position, but Arg-200 is too remote to contact either of them; therefore, it was hypothesized that Arg-200 plays a critical role in binding to the charged phospholipid headgroups.[1] The equilibrium of the binding interface affected several sides of the interfacial catalysis. The micelles possess a small number of anionic phospholipids, including the species studied in this chapter. It was reported that the hydrolysis occurred after GIVA PLA_2 strongly bound to the substrate interface, just on the outlayer of the hydrolyzed substrates. As the anionic AA and lysophospholipids aggregate in the micelles, an increasing amount of enzyme will bind to the interface and arrive at a steady state to the largest extent. Also, the enzyme will bind either to the surface of the substrate or in the aqueous solution. Its quantity on the surface is dependent on the phospholipid type.

In many cases, if the binding between PLA_2 and the vesicles is weak, the optimal amount of additive can be added to strengthen it.[52] The negatively charged surface is preferred for binding to the PLA_2.

Herein, we make a proposal that since the micelles in (C) and (D) are mixtures of five species, their surfaces, containing uniform mixed phospholipids, should differ from the surfaces of single-species micelles due to electrostatic interaction, and reasonably show a similar activity to that of different phospholipid species. The performance is not only limited to the GIVA $cPLA_2$ activity on the substrate of pure anionic phospholipids, but also in a relationship with the substrate of zwitterionic lipids.[52]

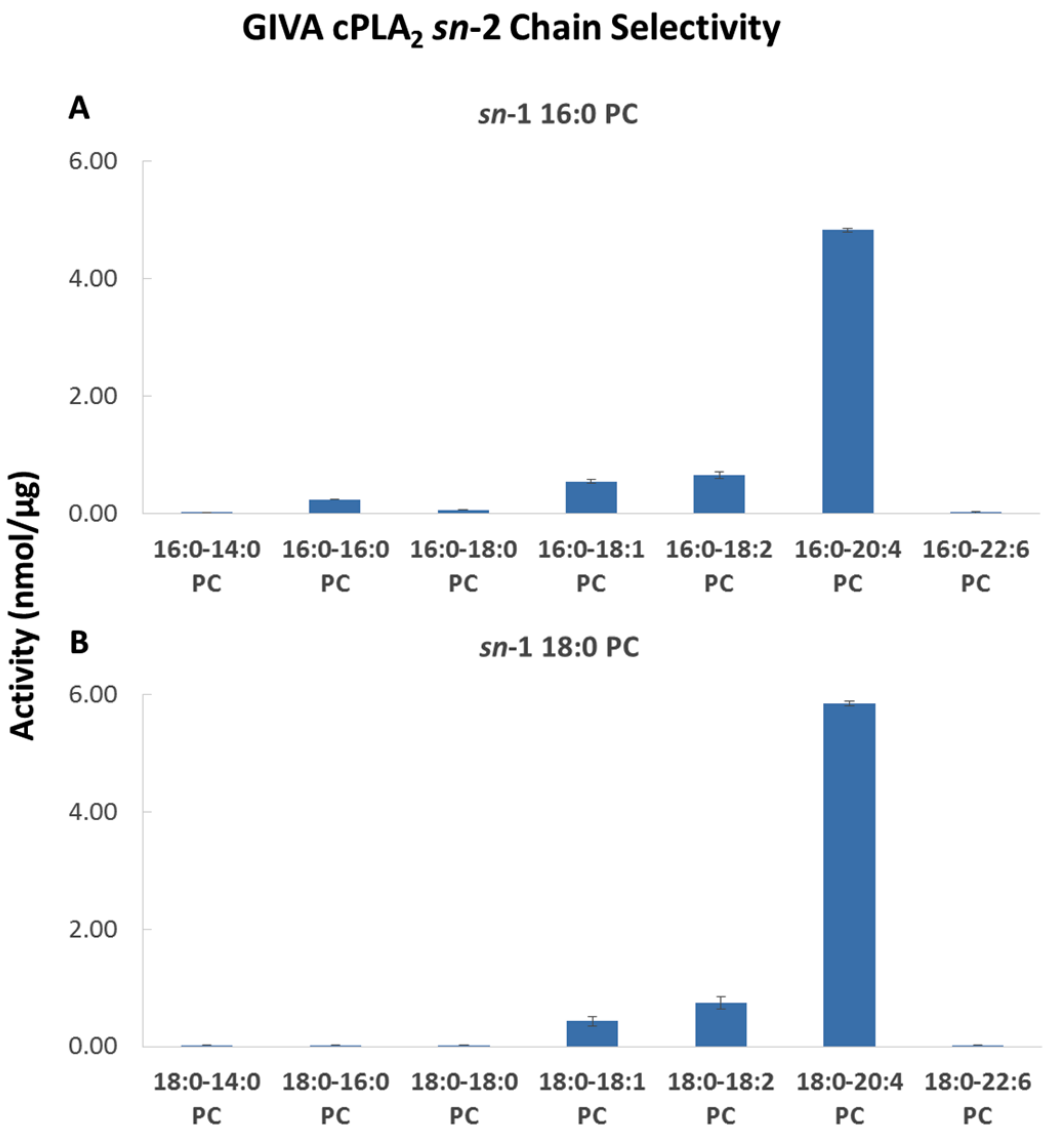

Figure 3-6. Picture of Acyl-chains of PCs Specificity toward cPLA$_2$. (A) for sn-1 16:0 PC including 16:0-14:0 PC, 16:0-16:0 PC, 16:0-18:0 PC, 16:0-18:1 PC, 16:0-18:2 PC, 16:0-20:4 PC, 16:0-22:6 PC, (B) for sn-1 18:0 PC including 18:0-14:0 PC, 18:0-16:0 PC, 18:0-18:0 PC, 18:0-18:1 PC, 18:0-18:2 PC, 18:0-20:4 PC, 18:0-22:6 PC. Both of the assays were performed within 30 min. The dose of GIVA cPLA$_2$ was kept 5 µl/sample in triplicates, with the concentration of 0.017 µg/µl each. Data are expressed as the mean ± standard deviation of the average. Each point represents the intensity of triplicates samples of 16:0 LPC.

As a consequence, cPLA$_2$ exhibits similar preferences for *sn*-2 AA between the two parallel experiments including 16:0 PC and 18:0 PC at the *sn*-1 position,

respectively. The enzyme activity for Figure 3-6 (A) varied from 0.01 to 4.83 nmol/μg, and 0.01 to 5.86 nmol/μg for Figure 3-6(B). Thus, it seems the number of carbons on *sn*-1 chain doesn't play a significant role in affecting the specificity toward GIVA $cPLA_2$.

The rank order for *sn*-2 acyl chains was 20:4 > 18:2 > 18:1 > 16:1 for both assays. At the same time, $cPLA_2$ doesn't exhibit pronounced selectivity toward acyl chains of 16:0 PC, 18:0 PC, 14:0 PC and 22:6 PC. Also worth mentioning is that the performance of 16:0-20:4 PC (PAPC) in this assay is in perfect coincidence with the activity of PAPC under another assay condition. Activity with these substrates increased 1 to 2 times as the number of double bonds in the *sn*-2 position increased from one (oleate) to two (linoleate). The $cPLA_2$ exhibits a distinct preference for AA (20:4) substrates. Apparently, the specificity for arachidonylate substrates may occur since AA is the most unsaturated fatty acid present at the *sn*-2 position of the phospholipid substrate that has thus been applied to activity in recent years.

Since GIVA $cPLA_2$ shows little preference for the binding at the *sn*-1 position, the capability of the enzyme to discriminate various chains including C20 and C22, based on various molecular masses of fatty acids is distinct, though it seems possible that phospholipids containing a sn-2 fatty acid chain with 20 carbons varied a lot from the conformation of those containing 22-carbon or other number fatty acid chains because of the double bonds.[53] The results indicated a prominent selectivity toward C20 over the other amount of carbons including C22, C20, and C18.[53] We didn't include the eicosapentaenoic acid (EPA), a 20-carbon carboxylic acid with 5 double bonds in our experiment, because it wasn't available to purchase.

More importantly, the similar trend of the increase in the two parallel experiments may imply that the GIVA $cPLA_2$ prefer unsaturated acyl chains with double bonds at the *sn*-2 position partially due to the conformation change of the acyl chains. However, it was still unclear as to why there was little activity observed from docosahexaenoic acid (DHA) chains, 16:0-22:6 PC or 18:0-22:6 PC. So we currently sustained that the specificity selectively acts within a range number of double bonds on *sn*-2 chains, actively from one to four double bonds, with four as the best. Of course, there is another explanation for the absent activity of the DHA chains, that the 22 carbons are too enough for the $cPLA_2$ to show interest in, which also means the two extra carbons can keep the specificity of $cPLA_2$ toward this chain away.

3.5.4 Discussion about The Effect of Detergent on the Analysis

Triton X-100 is a broadly used nonionic surfactant for forming mixed micelles. However, the presence of Triton X-100 in the loading samples severely suppressed ionization in the mass spectrometry (MS) analysis and decreased chromatographic resolution in LC-MS. Thus, Triton X-100 must be removed or avoided due to sensitive detection of phospholipids by MS.

Another nonionic detergent, octaethylene glycol monododecyl ether ($C_{12}E_8$), was used in our experiment, instead of Triton X-100, to form micelles. $C_{12}E_8$ has a specific m/Z, and its effect on the LC-MS, might be ignored. The Critical micelle concentration (CMC) of Triton X-100 is around 0.3 mM, though the CMC value may vary with the calcium in the assays to some extent.[54] The CMC for $C_{12}E_8$ is about 0.08 mM. The assay conditions are all carried out above the CMC. It is questionable that if the performance of detergent is neutral or more like an inhibitor if it has an affinity for

the PLA_2. However, we're sure that the activity on a substrate with $C_{12}E_8$ is higher that that with Triton X-100 through our testing experiment.

3.6 Acknowledgements

Chapter 3, in part is currently being prepared for submission for publication. Varnavas D. Mouchlis, Yuan Chen, Edward A. Dennis. "Mass Spectrometric Analysis of Specificity and Inhibition of Phospholipase A_2 on Natural Membrane Phosholipids". Ms. Chen will be a co-author on this paper.

CHAPTER 4

Inhibition Studies on GIVA Phospholipase A_2 Using Liquid Chromatography Mass Spectrometry

4.1 Introduction to the Assay on GIVA $cPLA_2$ Inhibitors

4.1.1 Introduction to the IC50 Assay

An increasing number of assays in evaluating the performance of inhibitors are directed toward a series of existing PLA_2. The IC50 value represents the concentration needed to inhibit the biological function by 50%.[55] It must be stressed that it is meaningless to compare the activity of different inhibitors if different assays, methods or substrates are applied. The molecular dynamics (MD) simulation at the docking site of the compounds has exhibited consistent interactions of PLA_2 with the inhibitors, followed by the proved stability of the docking structure. Hundreds of the inhibitors for $cPLA_2$ have been developed since the GIVA PLA_2 is considered to act as the most central PLA_2 in inflammatory diseases. The first inhibitor of $cPLA_2\alpha$ was arachidonoyl trifluoromethyl ketone (AATFK), shown to bind firmly and slowly to the enzyme, a process that is reversible. Its activity was almost four orders of magnitude more powerful toward $cPLA_2$ than for $sPLA_2$.[1]

4.1.2 Introduction to the Novel GIVA $cPLA_2$ Inhibitors

Among the diverse PLA_2s, human cytosolic PLA_2 is known as Group IVA $cPLA_2$ since it was referred to as $cPLA_2$. Animals processed with a lack of $cPLA_2$ have shown a distinct reduction in the synthesis of eicosanoids, post-ischemic brain damage, and allergic symptoms. GIVA PLA_2 is of great importance in causing inflammation; as a result, the search for inhibitors of this enzyme sparked a significant amount of interest in the studies on inflammatory diseases. Over the past several years, a variety of selective inhibitors of GIVA PLA_2 have been developed, including pyrrolidine

derivatives by Shionogi, indole-based inhibitors by Wyeth, and 2-oxoamide chemical compounds by the Kokotos and Dennis Groups.[1]

4.1.3 Pyrrophenone

Seno's Group identified a potent and novel inhibitor of human GIVA PLA_2, named pyrrophenone, based on the chemical structure of pyrrolidine[56], with its IC50 value varying from 1 to 20 nM.[57] It can potently inhibit the release of AA and the formation of thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and PAF in human blood.[1] Under the same assay conditions, its inhibitory activity is two to three orders of magnitude more powerful than that of arachidonyl trifluoromethyl ketone ($AACOCF_6$). Furthermore, it gives reversible inhibition of GIVA PLA_2 without showing the slow-binding inhibitory activity observed for $AACOCF_6$.

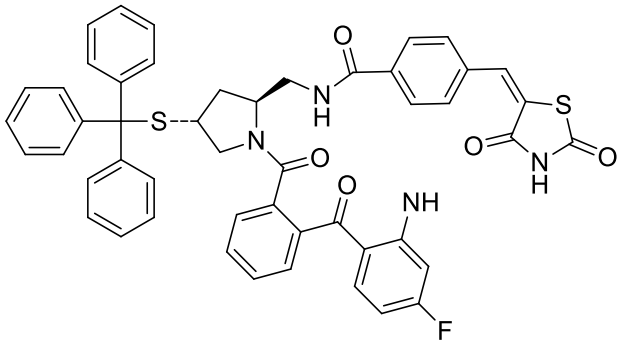

Figure 4-1. Picture of the molecular structure of pyrrophenone.[57]

4.1.4. AX074

It was reported that the inhibition of $cPLA_2\alpha$ and the relationship between the structure and activity of 2-oxoamides presents an achieved optimal distance between

the polar carbonyl group and the 2-oxoamide part.[58] Long chain 2-oxoamides form a class of potent GIVA $cPLA_2$ inhibitors that show powerful anti-inflammatory and analgesic activity *in vivo*.[59] AX074, as a 2-oxomide inhibitor, exhibits the most potent inhibition and highest binding affinity toward $cPLA_2$. In our experiment, we use AX074 as one of our inhibitor models to validate its IC50 and make a comparison between the radioactive method and mass spectrometry assay.

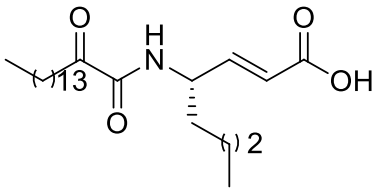

Figure 4-2. Picture of the molecular structure of the 2-Oxoamide Inhibitor AX074.[59]

Kokotos and Dennis Labs have designed these 2-oxoamides, one of the most important classes of GIVA $cPLA_2$ inhibitors.[60] The 2-oxoamides inhibitors were developed as phospholipid substrate analogues based on the electrophilic and non-cleavable 2-oxoamide moiety. It was indicated that 2-oxoamides that take advantages of δ-amino or γ-acids, including a free carboxyl group, possess the preference for GIVA $cPLA_2$ without affecting the $iPLA_2$, such as GVIA intracellular Ca^{2+}-independent $iPLA_2$. They've also shown that 2-oxoamides are more powerful when a long 2-oxoacyl residue is incorporated into the recent publication.[60b] These 2-oxoamides presented systemic anti-inflammatory, analgesic or antihyperalgesic characteristics in animal models.[60b] From the docking complex of these inhibitors, the mechanisms of the interactions of chosen inhibitors became clear.[59] It was reported that the 2-oxoamides with a free COOH group could increase the inhibition of AA release one more fold, which means selectivity plays a critical role against $cPLA_2$. The

IC50 data measured on radioactive assay and mass spectrometric assay will be discussed in detail in this chapter.

4.2 Materials and Methods

4.2.1. Reagents

L-α-1-Palmitoyl-2-Arachidonyl-[Arachidonyl-1-^{14}C]-Phosphatidylcholine (10 μCi) for substrate on radioactive assays was achieved from PerkinElmer (Waltham MA, USA).

The phospholipids and internal standards, including 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso PC (17:0)), 1-palmitoyl-2-arachidonoyl -*sn*-glycero-3-phosphocholine (PC (16:0/20:4), PAPC), and L-α-phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (Brain PI(4,5)P_2) were purchased from Avanti Polar Lipids(Alabaster AL, USA).

Optima LC-MS grade acetonitrile (ACN), methanol (MeOH), and water were obtained from Fisher Scientific. Isopropanol (IPA) was purchased from Sigma-Aldrich. Formic acid (FA) was obtained from EMD Technologies. Dulbecco's Phosphate Buffered Saline (DPBS) was obtained from Corning Life Science. Octaethylene glycol monododecylether ($C_{12}E_8$) was obtained from Sigma-Aldrich. The inhibitor pyrrophenone was purchased from Cayman Chemical (Ann Arbor MI, USA), and the AX074 was synthesized from Kokotos Lab.

4.2.2 GIVA cPLA$_2$ Radioactive Activity Assays

Pure GIVA cPLA$_2$ was expressed and verified as described in Chapter 2. The solution of lipid in chloroform was dried under an argon stream.[60a] It contained 1.53

μL of L-α-1-Palmitoyl-2-Arachidonyl-Phosphatidylcholine,[Arachidonyl-1-^{14}C]-, 10μCi (370kBq), 0.9 μL 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PC (16:0/20:4), PAPC), 1.65 μL Brain PI(4,5)P_2 every assay tube.

The residue was resuspended in a 2ml buffer consisting of 100mM HEPES at PH 7.5, 0.09 mM $CaCl_2$ and 0.4 mM $C_{12}E_8$ out of the whole buffer and was gently vortex to create mixed micelles. The micelles were kept at 40 °C for 1 hour and vortex every 10 min until one hour. Then combined the micelles with the remaining buffer together to offer the final volume of 500 μL upon the addition of the enzyme and the inhibitor. 2 mM DTT were then added to the mixed micelles to form the substrate solution. Dilute the inhibitor with DMSO in the original solution (100%) of 5mM from 80% to 0.0005%. In brief, the final mixed-micelle substrate is composing of 100 mM HEPES (pH 7.5), 90 μM $CaCl_2$, 97 μM PAPC in total, 2 mM DTT, 400 μM $C_{12}E_8$ and 3 μM BPI(4,5)P_2 per tube of 500 μL. GIVA PLA_2 enzyme was added to initiate the reaction and incubated at 40 °C for 30 min. 5ml DMSO was contained in each reaction with a different amount of inhibitor concentration added as described before. The reaction was then quenched, extracted and analyzed using silica, 2-propanol, heptane and sulfuric acid as described in the modified Dole assay.[61]

4.2.3 GIVA cPLA$_2$ LC/MS Activity Assays

In order to develop a novel assay for the overwhelming amount of inhibitor testing, we've developed a high-throughput LC/MS methodology using MRM to detect and analyze all the simultaneous analytes precisely.

As was described in last chapter, we also use LC/MS on the measurement of IC50 of the $cPLA_2$ Inhibitors in the same way. A solution (100 μM) of PAPC in chloroform was dried under an argon stream. The residue was resuspended in a buffer composed of 100 mM HEPES (pH7.5), 0.09mM $CaCl_2$, and 0.4 mM $C_{12}E_8$ and gently vortexed to form mixed micelles. The mixed micelles were kept at 40°C for 1 hour and each vortex for 10 minutes. Then, 2 mM DTT was added to the mixed micelles to form the substrate solution. A 100 μL substrate solution was added into the 96-well plate (Thermo Scientific Matrix), and a cap (Cap Mats, Sepra Seal™, Thermo Scientific) covered each well to avoid volatilization. The reaction was initiated by adding PLA_2 to the mixed micelles and incubated at 40 °C for 30 minutes. After incubation, the reaction was quenched with a 120μL quench solution (MeOH/ACN 4:1). Then internal phospholipid standards were added to the solution, and the solution of 96-well plates was used directly to LC-MS analysis for lysophospholipids.

A Shimadzu HPLC system was used, consisting of two HPLC pumps (LC-10ADvp) and a system controller (SCL-10Avp). A CTC Analytics PAL autosampler platform (Leap Technologies) and a column controller instrument (Analytical Sales & Products, Inc) were used.

Reversed-phase separation was performed on a Kinetex C18 column (5 μm, 50×2.1 mm, Phenomenex). The mobile phase consisted of (A) ACN/water (70/30 v/v) containing 10 mM ammonium acetate and (B) MeOH/ACN/H_2O (79.5/17/3.5, v/v) containing 10 mM ammonium acetate. Gradient elution was carried out for 1.6 min at a flow rate of 1.5 mL/min. All buffer used were degassed three times with argon so that the dissolved oxygen can be removed. Gradient conditions were as follows: 0.01-

0.1 min, 40% B; 0.1-0.3 min, 40-100% B; 0.3-1.2 min, 100% B; 1.20-1.21 min, 100-40%B; 1.21-1.50 min, 40% B. 10 µl of each sample in triplicates was injected into the column. The temperature of the column was kept at 40 °C. All samples were kept at 4 °C throughout the analysis.

HILIC-phase separation was performed on a Kinetex HILIC column (2.6 µm, 30×2.1 mm, Phenomenex). The binary gradient consisted of (A) ACN/water (95/5; v/v, pH=8.0) containing 25 mM ammonium acetate and (B) ACN/water (50/50; v/v, pH=7.3) containing 25 mM ammonium acetate. All buffer used were degassed three times with argon so that the dissolved oxygen can be removed. Gradient elution was carried out for 1.6 min at a flow rate of 0.8 mL/min. Gradient conditions were as follows: 0.01-0.8 min, 0% B; 0.8-1.2 min, 0-100% B; 1.2-1.5 min, 100% B; 1.50-1.51 min, 100-0%B; 10 µl of each sample in triplicates was injected into the column. The temperature of the column was kept at 40 °C. All samples were kept at 4 °C throughout the analysis.

Mass spectrometry was carried out on the ABI/Sciex 4000 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer coupled with a Turbo V ion source. Lysopholipids were detected in positive or negative electrospray ion mode according to the classes of lysopholipids. Curtain gas (CUR), nebulizer gas (GS1) and turbo-gas (GS2) were set at 10 psi, 50 psi and 20 psi, respectively. The electrospray voltage was +4.5 kV or -4.5 kV, and the turbo ion spray source temperature was 500 °C.

4.2.4. Data Analysis

QuantaSmart™ software with Tri-Carb 2810TR Low Activity Liquid Scintillation Analyzer (PerkinElmer) was used to quantify the samples on radioactive assays.

Lysophospholipids on MS assays were analyzed using scheduled multiple reaction monitoring (MRM). Nitrogen was employed as the collision gas. Data acquisitions were performed using Analyst software (Applied Biosystems). MultiQuant software (Applied Biosystems) was used to quantify all metabolites.

Analysis of Lyso PC were performed in positive mode. For quantitation of Lyso PC (16:0), an internal standard Lyso PC (17:0) was used. After incubation of enzyme and substrate, the internal standard Lyso PC (17:0) was added into the mixed micelles at first. An internal standard is used to monitor the chromatographic response, and for normalization purpose which allows for accurate quantification. 510.3 and 510.3 and 184.1 were selected for parent and product ion of internal standard Lyso PC (17:0) for MRM mode.

All of our experiments were operated at least twice to ensure reproducibility and the results are shown as the average ± S.D. The data in triplicate samples measured by both our radioactive and LC/MS methods, have been found to fluctuate within a range of 10%. GraphPad Prism software was utilized to analyze all the data on inhibitors dilution. The data of inhibitory activity is represented as $X_I(50)$ values, is a measure of a concentration of the inhibitor on a 2D surface in micelles that can give 50% inhibition. The surface concentration (log mole fraction) is analyzed as the mole fraction of inhibitor in the total micelle substrate surface including the detergent, inhibitor, and phospholipid. The lines of each inhibitor present their dose-dependent

relationships through the data in a smoothed curve.[59]

We examined the inhibitors from two systems[62]: GIVA $cPLA_2$ bound with pyrrophenone and GIVA $cPLA_2$ bound with 2-oxoamide. These compounds aim at different functionalities of $cPLA_2$ and are very different in structures. Thus it was better to test both of the inhibitors on our different systems, to ensure that we've established a novel and reliable LC/MS method.

GIVA $cPLA_2$ selectively hydrolyzes phospholipids containing AA, hence the AA is an attractive metabolites indicator of $cPLA_2$ activity in the research of anti-inflammatory medicine.[62] The value of $X_I(50)$ was calculated when the percent inhibition was greater than 90%, to estimate their ability to inhibit the enzymatic activity. Data for AX074 and pyrrophenone were included in figure below.

The reason why we apply $X_I(50)$ to evaluate the inhibitory capacity of these compounds, instead of the IC50, is that $cPLA_2\alpha$ is just active on the surface of phospholipid including phospholipid vesicles, micelles and cell membranes. Therefore, the 2-oxoamides may accumulate in the substrate phospholipids or some transition condition that help them bind to the active site within the enzyme.[58]

4.3 Results and Discussion

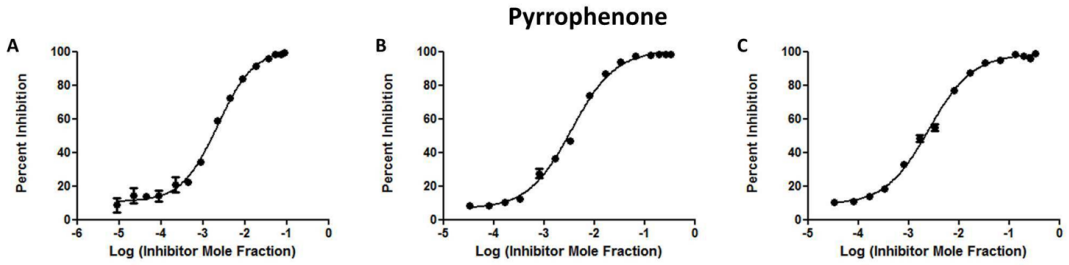

Figure 4-3. Picture of response curve for inhibitor Pyrrophenone of GIVA $cPLA_2$. (A) on Radioactive Assay with the calculated $X_I(50) = 0.0022 \pm 0.0002$, (B) on MS Assay on C18 column with the calculated $X_I(50) = 0.0036 \pm 0.0003$, (C) on MS Assay on HILIC column with the calculated $X_I(50) = 0.0025 \pm 0.0002$. They were both measured in micelles containing 400 μM $C_{12}E_8$ and 100 μM PAPC. All inhibition curves were obtained by GraphPad Prism using the nonlinear regression (one-site binding model hyperbola) to evaluate the $X_I(50)$.

Pyrrophenone can inhibit the AA release powerfully and shows to be dependent on the amount of inhibitor with original reported IC50 value of 0.024 μM.[57] In terms of different substrates, the $X_I(50)$ value (mole fraction) will be varied. Previously, pyrrophenone presented similar curves for GIVA $cPLA_2$ under three different experiment conditions. The $X_I(50)$ value is 0.00041 for the chromogenic assay, 0.00026 for the assay using PC substrate with Triton as detergent, and 0.0011 for the assay using PC and DOG.[57]

The result of our study for pyrrophenone with PC/$C_{12}E_8$ was promising, not only because the three experiments measured different metabolites (^{14}C labeled AA was measured in radioactive assay; 16:0 LPC was measured in MS assay and both of them are metabolites of the $cPLA_2$ hydrolysis reaction), the $X_I(50)$ values ranged from 0.0022 to 0.0036, and changed within a factor of 2. It is also worth mentioning that the $X_I(50)$s for radioactive 0.0022 and for MS with HILIC column 0.0025 are very similar, which means that our development of the methodology is very stable.

However, our new $X_I(50)$ value is still a little different from the reported value of 0.00026 for the PC/Triton assay, which means that the detergent may play an important role in affecting the interaction and conformation change between the enzyme, inhibitor and phospholipid substrates. As discussed before, Triton X-100 was used as a nonionic surfactant to help create the micelles, but it always suppressed the ionization and decreased chromatography resolution in the LC/MS assays. However, the effect would be negligible with another non-charged detergent $C_{12}E_8$. A substrate with $C_{12}E_8$, under radioactive assay conditions, can boom the enzyme activity two to three times more than that with Triton X-100. It was implied that these detergents might activate cells to accept the drugs and increase the medicine adsorption, largely due to several theories that the drugs experienced solubilization, tending to associate highly with the cytosolic phospholipid and further lose the membrane packing density. The free energy of binding of detergents in the membrane may increase with the number of ethoxyl groups. [63]

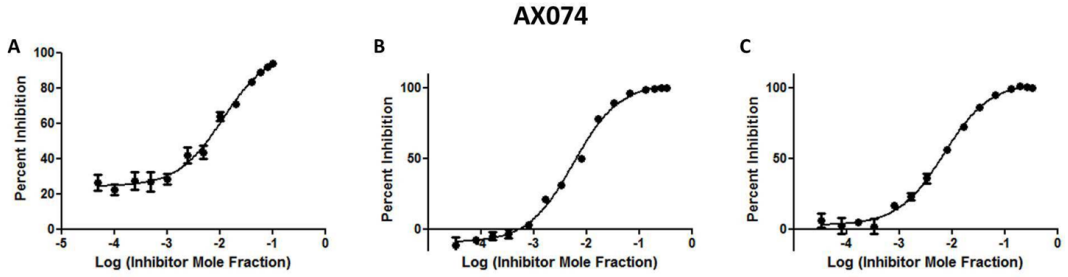

Figure 4-4. Picture of the response curve for 2-oxoamide inhibitor AX074 of GIVA cPLA$_2$. (A) on Radioactive Assay with the calculated $X_I(50) = 0.011 \pm 0.002$, (B) on MS Assay with C18 column with the calculated $X_I(50) = 0.0060 \pm 0.001$, (C) on MS Assay with HILIC column with the calculated $X_I(50) = 0.0070 \pm 0.001$. They were both measured in micelles containing 400 μM $C_{12}E_8$ and 100 μM PAPC. All inhibition curves were obtained by GraphPad Prism using the non-linear regression (one-site binding model hyperbola) to evaluate the X_I (50).

The results of our studies for AX074 with PC/$C_{12}E_8$ as the detergent was good, too. The $X_I(50)$ values ranged from 0.006 to 0.011, still changed by a factor of 2. The reported $X_I(50)$ value with PC/Triton was 0.003.[59]

The inhibitors, as chemical compounds, used to aggregate or separate into membranes or micelles, are in great need of kinetic evaluation while the concentration is determined in unit of mole fractions.[24] In kinetic studies, the measurement of enzymatic activity relies on the non-substrate phospholipids, according to the theory of "surface-dilution kinetics". Therefore, the inhibition, specificity, and specific activity of each PLA_2 subgroup or group should utilize a specific form of substrate and assay conditions.[1]

Enzymes binding onto the membrane are capable of accessing both the bilayer and monolayer of the substrate phospholipids, and the hydrophilic phase is of great importance to the catalysis during the interaction with phospholipids. The binding on the surface will assist in activating the enzyme via conformational change, followed by catalysis with the release of lysophospholipids. The phospholipids can diffuse into the active site by avoiding the hydrophobic area with the help of PLA_2.[64] The proteins turn out to possess one more tryptophan on the interfacial surface while they bind tightly to the surfaces of zwitterions. Research on the tryptophan analogs discovered that there was a balance between the hydrophobicity and disadvantageous entropy for membrane rebuilding, result in the insertion of residues onto bilayer occurred just in the glycerol area. Generally, aromatic residues selectively located in the area of the phospholipid headgroup. It was also hypothesized that the orientation of the enzyme is mainly caused by electrostatic interaction. Others factors including desolvation, may

certainly play a significant role, too. The strong connection between the interface and membrane is hereby allowed and result in the diffusion of substrate phospholipid to the active sites.[64]

Therefore the electrostatic interaction of $C_{12}E_8$ differs a lot from those of Triton and other kinds of detergent, due to the special properties of each detergent.

4.4 Conclusions

In order to replace the traditional radioactive assay due to its low efficiency as well as limited and expensive radioactive sources, we've developed the MS assay with column C18 first as interim methodology, then improved this method with HILIC phase column as final way for our high throughput assay. Both data sets of the two inhibitors are very similar (varied within a factor of 2), which means the novel LC/MS assay is a very fast and potential way to measure and evaluate the inhibitory ability of the inhibitors. Our future plan is to test the released AA with the AA-d8 as an internal standard in negative mode and measure the inhibitor in mixed micelles including PAPA, PAPC, PAPE, PAPG and PAPS, in lieu of only PAPC in negative mode. Through this new way, we will better simulate the natural membrane and present more natural and authentic IC50 values of the inhibitors. Our data is proved to help improve the efficiency in inhibitors screening; for example, we can just perform radioactive assays on 9 compounds in triplicates during a 30-minutes experiment, but with the LC/MS assay, we can do at most 27 compounds or concentrations of the same inhibitors, at the same time, within one experiment.

What's more, we can avoid any precautions for radioactive reagents and reduce our materials expenses due to the high price of the C-14 labeled PAPC.

Therefore, this method may have the potential to be employed to common enzyme classes in the future.

4.5 Acknowledgements

Chapter 4, in part is currently being prepared for submission for publication. Varnavas D. Mouchlis, Yuan Chen, Edward A. Dennis. “Mass Spectrometric Analysis of Specificity and Inhibition of Phospholipase A_2 on Natural Membrane Phosholipids”. Ms. Chen will be a co-author on this paper.

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