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The Biochemical Pathway Leading to Lpa Generation Upon Blood Coagulation

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**THE BIOCHEMICAL PATHWAY LEADING TO LPA GENERATION UPON
BLOOD COAGULATION**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Alyssa Lynn Jefferson Bolen
May 2011

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DEDICATION

This dissertation is dedicated to my family:
Karen and Ken Jefferson
Andrea Jefferson
James Boler

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I am greatly thankful to my advisor, Dr. Gabor Tigyi, who has taught me so much. Through his love of science he has taught me to overcome the obstacles and disappointments and to see the positive side. He has trained me to be a leader and encouraged me to step out of the norm and push my boundaries. He taught me to follow where life leads, keep an open mind, and to be confident in my work and my choices.

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ABSTRACT

Platelet activation initiates an upsurge in 18:2 and 20:4 lysophosphatidic acid (LPA) production. The biochemical pathway responsible for LPA production during blood clotting is not fully understood. We have purified a phospholipase A₁ (PLA₁) from thrombin-activated human platelets using sequential chromatographic steps followed by fluorophosphonate-biotin affinity labeling and proteomics. We identified acyl-protein thioesterase 1 (aka. lysophospholipase A1, accession code O75608) as a novel PLA₁. Addition of this recombinant PLA₁ significantly increased the production of *sn*-2-esterified polyunsaturated LPCs and the corresponding LPAs in plasma. We next examined the regioisomeric preference of lysophospholipase D/autotaxin (ATX), which is the subsequent step in LPA production. To prevent acylmigration regioisomers of oleyl-*sn*-glycero-3-phosphocholine (LPAF) were synthesized. ATX preferred the *sn*-1 over the *sn*-2 regioisomer of LPAF. We propose the following LPA production pathway in blood: 1) Activated platelets secrete PLA₁. 2) PLA₁ generates a pool of *sn*-2 lysophospholipids. 3) These newly generated *sn*-2 lysophospholipids undergo acyl migration to yield *sn*-1 lysophospholipids, which are the preferred substrates of ATX. 4) ATX cleaves the *sn*-1 lysophospholipids to generate *sn*-1 LPA species predominant with 18:2 and 20:4 fatty acids.

TABLE OF CONTENTS

Chapter 1. General Introduction	1
1.1 Phospholipid Production.....	1
1.1.1 Lysophosphatidic Acid.....	1
1.1.2 The GPAT Pathway of LPA Synthesis	1
1.1.3 LPA Generation by MAGK	1
1.1.4 LPA Synthesis from PA	1
1.1.5 LPA Production via PLA ₂ Hydrolysis.....	3
1.1.6 LPA Production via PS-PLA ₁	3
1.1.7 LPA Production during LDL Oxidation.....	3
1.1.8 LPA Generation by Phospholipase and Lysophospholipase D Cleavage	3
1.1.9 LPA Upregulation during Blood Coagulation.....	3
1.2 LPA Degradation	5
1.2.1 LPA Degradation by Phosphatases	5
1.2.2 LPA Degradation by Lysophospholipases	5
1.2.3 LPA Degradation by Acyltransferases	5
1.3 LPA Receptor and Signaling	6
1.3.1 Plasma Membrane Receptors	6
1.3.2 Intracellular Receptor, PPAR γ	7
1.4 Roles of LPA.....	8
1.4.1 LPA in Vascular Biology	8
1.4.2 The Role of LPA in Cancer	8
1.4.3 LPA in Cell Survival and Apoptosis	9
1.5 Phospholipases and Lysophospholipases.....	9
1.5.1 Phospholipases	9
1.5.2 Phospholipase A ₁	11
1.5.3 Phospholipase A ₂	11
1.5.4 Lysophospholipase D	12
1.5.5 Lysophospholipase A-I /II.....	12

Chapter 2. The Phospholipase A₁ Activity of Lysophospholipase A-I Links Platelet Activation to LPA Production during Blood Coagulation	14
2.1 Introduction.....	14
2.2 Materials and Methods.....	16
2.2.1 Materials.....	16
2.2.2 Isolation and Activation of Human Platelets.....	16
2.2.3 Measurement of Phospholipase A ₁ Activity	16
2.2.4 Partial Purification of PLA ₁	17
2.2.5 FP-Rhodamine Labeling.....	18
2.2.6 FP-Biotin Labeling and Purification	18
2.2.7 Proteomic Analysis of FP-Biotin-Labeled Products in Partially Purified Platelet Supernatants	19
2.2.8 RNA Purification and RT-PCR.....	19
2.2.9 MGLL Activity Assay.....	20
2.2.10 Construction of Recombinant LYPLA-I/APT1 Expression Plasmid.....	20
2.2.11 Recombinant LYPLA-I/APT1 Purification.....	21
2.2.12 Monitoring LPC and LPA Production in Plasma.....	21
2.2.13 Synthesis of <i>sn</i> -2 lyso-PAF 18:1	22
2.2.14 Determination of Regioisomeric Selectivity of ATX against Two Lyso-PAF Substrates	22
2.3 Results.....	23
2.3.1 Phospholipases Released from Activated Platelets Generate Lysophospholipids but LPA Production Requires ATX from Plasma	23
2.3.2 Partial Purification of Phospholipase A ₁ from Supernatants of Activated Human Platelets	23
2.3.3 Activity Based Purification of Platelet PLA ₁	25
2.3.4 Identification of Serine Hydrolases Secreted from Thrombin Activated Human Platelets	25
2.3.5 Recombinant Human LYPLA-I/APT1 Acts Primarily as a Phospholipase A ₁ and Does Not Break Down LPA.....	28
2.3.6 LYPLA-I/APT1 Contributes to the Production of LPA during Blood Coagulation.....	31
2.3.7 Regioisomeric Preference of ATX.....	33
2.4 Discussion	36
2.5 Supplemental Information	42

Chapter 3. Quantification of <i>sn-1</i> and <i>sn-2</i> Regioisomers of LPA and LPC	46
3.1 Introduction.....	46
3.2 Method Development.....	46
3.2.1 Production of the <i>sn-2</i> Regioisomer of LPC and LPA.....	46
3.2.2 LC-MS/MS Method Development for Separation of the <i>sn-1</i> and <i>sn-2</i> Regioisomers of LPA	47
3.2.3 Stability of the <i>sn-2</i> Regioisomers of LPA and LPC	50
3.2.4 Application	56
3.2.5 Conclusions	56
List of References	59
Vita	74

LIST OF TABLES

Table 2.1	Summary of chromatographic steps.....	26
Table 2.2	LC-MS/MS candidate protein peptide sequences.....	29
Table 2.3	Kinetic parameters for ATX-mediated hydrolysis of LPAF 18:1.....	38
Table 3.1	Retention times of LPA and LPC species under varying conditions.....	53

LIST OF FIGURES

Figure 1.1	Chemical structure of <i>sn-1</i> lysophosphatidic acid 18:1.....	2
Figure 1.2	Specificity of phospholipases	10
Figure 2.1	ATX is required for LPA production in activated platelet supernatant.....	24
Figure 2.2	FP-Rhodamine binds to PLA ₁ inhibiting activity and NBD-LPS production.....	27
Figure 2.3	Human recombinant LYPLA-I/APT1 with and without a GST tag are purified for analysis.....	30
Figure 2.4	Human recombinant LYPLA-I/APT1 acts first as a PLA ₁ then also has lysophospholipase activity when incubated with NBD-PC	32
Figure 2.5	Human recombinant LYPLA-I/APT1 increases the amount of LPA 18:2 and LPA 20:4 in a time-dependent manner.....	34
Figure 2.6	Human recombinant LYPLA-I/APT1 increases the amount of LPA produced	35
Figure 2.7	Comparison of the cleavage of <i>sn-1</i> with <i>sn-2</i> LPAF 18:1 by ATX.....	37
Figure 3.1	HPLC-MS/MS chromatogram of LPA 20:0 regioisomers under varying conditions	48
Figure 3.2	HPLC-MS/MS chromatogram of LPA 18:1 and 20:0 regioisomers	49
Figure 3.3	HPLC-MS/MS chromatogram of LPA 18:2 regioisomers.....	51
Figure 3.4	HPLC-MS/MS chromatogram of separation of LPA 18:2 regioisomers	52
Figure 3.5	HPLC-MS/MS chromatogram of <i>sn-2</i> LPA 18:2 at varying time points post synthesis	54
Figure 3.6	HPLC-MS/MS chromatogram of <i>sn-2</i> LPC 18:1 at varying time points post synthesis	55
Figure 3.7	HPLC-MS/MS chromatogram of LPC 18:1 at varying time points in plasma and serum.	57
Figure 3.8	HPLC-MS/MS chromatogram of LPA 18:2 at varying time points in plasma and serum.	58

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AGPAT	1-acylglycerol 3-phosphate acyltransferase
ATP	adenosine triphosphate
ATX	autotaxin
C1P	ceramide-1-phosphate
cPLA ₂	cytosolic PLA ₂
ERK1/2	extracellular signal-regulated kinase
FP	fluorophosphonate
GPAT	glycerophosphate acyltransferase
GPCR	G protein coupled receptor
iPLA ₂	calcium independent phospholipase A ₂
LDL	low density lipoprotein
LPA	lysophosphatidic Acid
LPA ₁₋₇	LPA receptor 1-7
LPAAT	lysophosphatidic acid acyltransferases
LPAF	lyso platelet activating factor
LPC	lysophosphatidyl choline
LPLD	lysophospholipase D
LPS	lysophosphatidyl serine
LYPLA1	lysophospholipase A-I
MAGK	monoacylglycerol kinase
MMP	matrix metalloproteinases
MRM	multiple reaction monitoring
NHERF2	Na ⁺ /H ⁺ exchanger regulatory factor-2
NPP	nucleotide pyrophosphatase/phosphodiesterase
PA	phosphatidic acid
PC	phosphatidyl choline
PDZ domain	PSD-95/Disc-large/ZO-1 domain
PLA ₁	phospholipase A ₁

PLA ₂	phospholipase A ₂
PLB	phospholipase B
PLC	phospholipase C
PPAR γ	peroxisome proliferator-activated receptor gamma
PRP	platelet rich plasma
PS	phosphatidyl serine
PS-PLA ₁	phosphatidyl serine specific PLA ₁
S1P	sphingosine-1-phosphate
sPLA ₂	secretory phospholipase A ₂
TLC	thin layer chromatography
TRIP 6	thyroid receptor-interacting protein 6
VEGF	vascular endothelial growth factor

CHAPTER 1. GENERAL INTRODUCTION

1.1 PHOSPHOLIPID PRODUCTION

1.1.1 Lysophosphatidic Acid

Lysophosphatidic acid (LPA; Figure 1.1) has been studied by many chemists, biochemists, biologists, and physicians since it was first identified in 1957 as the active ingredient of a smooth muscle-stimulating substance, Darmstoff (Vogt, 1957a; b). Many advances have been made throughout the years in determining the production, breakdown, and receptors for LPA, but many aspects of LPA are still unknown.

Various species of LPA, dominated by 16-, 18-, and 20- carbon long acyl chains, are produced in cells as an intermediate to lipid synthesis and extracellularly in many biological fluids. The different species of LPA provides a unique opportunity for specificity within the receptors. For example, most LPA GPCRs prefer unsaturated species.

1.1.2 The GPAT Pathway of LPA Synthesis

LPA is generated via many different mechanisms. One pathway of LPA production is through glycerophosphate acyltransferase (GPAT). GPAT acylates glycerol 3-phosphate to generate LPA in the endoplasmic reticulum and mitochondria (Das and Hajra, 1984; Aoki, 2004; Gendaszewska-Darmach, 2008). This LPA may be quickly broken down into phosphatidic acid (PA) by monoacylglycerolphosphate acyltransferase, but some of this LPA may contribute to the intracellular LPA.

1.1.3 LPA Generation by MAGK

A lipid kinase involved in the pathway of LPA production was identified in 1962 (Pieringer and Hokin, 1962). This kinase was later identified as monoacylglycerol kinase and the phosphorylation of monoacylglycerol by this enzyme within the plasma membrane contributes to the production of LPA (Bektas *et al.*, 2005).

1.1.4 LPA Synthesis from PA

LPA is also generated via the hydrolysis of fatty acids at either the *sn-1* position or the *sn-2* position of PA by (phospholipase A₁) PLA₁ or (phospholipase A₂) PLA₂ enzymes respectively. PA-specific PLA₁ and PLA₂ enzymes have been reported in human (Billah *et al.*, 1980; Sano *et al.*, 2002; Sonoda *et al.*, 2002), horse (Billah *et al.*, 1981), and pig (Inoue and Okuyama, 1984) platelets. However this is shown to only

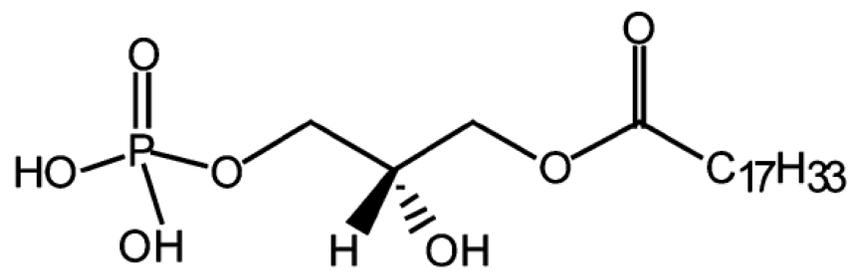


Figure 1.1 Chemical structure of *sn*-1 Lysophosphatidic acid 18:1.

produce a small portion (10%) of LPA detected in serum (Gaits *et al.*, 1997; Aoki *et al.*, 2002b; Cummings *et al.*, 2002; Sano *et al.*, 2002; Aoki, 2004).

1.1.5 LPA Production via PLA₂ Hydrolysis

LPA is also produced by a secretory or type II phospholipase A₂ (sPLA₂) (Fourcade *et al.*, 1995). LPA has also been shown to be generated in a spatially regulated fashion by the Ca²⁺-independent PLA₂ at the leading edge of migrating monocytes (Carnevale and Cathcart, 2001; Mishra *et al.*, 2008).

1.1.6 LPA Production via PS-PLA₁

Another pathway of LPA production involves a phosphatidylserine (PS)-specific phospholipase A₁ (PS-PLA₁) that can generate lysophosphatidylserine (LPS) This LPS is then converted into LPA by intracellular phospholipase D action (Nagai *et al.*, 1999, Aoki *et al.*, 2002a).

1.1.7 LPA Production during LDL Oxidation

Low density lipoprotein (LDL) often gets oxidized which produces many lipid constituents. Mildly oxidized low density lipoprotein (moxLDL) induces platelet and endothelial cell shape changes (Siess *et al.*, 1999). The pro-thrombotic and pro-atherogenic properties of the moxLDL are due to LPA.

1.1.8 LPA Generation by Phospholipase and Lysophospholipase D Cleavage

Lysophospholipase D (LPLD) activity is present in most biological fluids. LPLD-mediated production of LPA was first projected in the early 1980's in rat plasma (Tokumura *et al.*, 1986). LPLD acts on LPLs by removing the headgroup from LPLs resulting in LPA. This enzymatic pathway for LPA production will be described in greater detail in a subsequent section.

1.1.9 LPA Upregulation during Blood Coagulation

Although many pathways are responsible for LPA production, perhaps the most significant pathway is a multi-step process linked to activation of platelets (Mauco *et al.*, 1978; Schumacher *et al.*, 1979; Eichholtz *et al.*, 1993; Gaits *et al.*, 1997; Sano *et al.*, 2002; Aoki *et al.*, 2008). This multistep mechanism involves the release of unidentified PLA₁ and/or PLA₂ enzymes from activated platelets generating a new pool of lysophospholipids (LPLs), which in turn are cleaved by a lysophospholipase D also known as autotaxin (ATX) (Tokumura *et al.*, 2002; Umezu-Goto *et al.*, 2002).

Under normal circumstances, LPA levels in plasma are less than 100nM and the rank order of species is 18:2 > 18:1 > 18:0 > 16:0 > 20:4. These levels increase during blood coagulation to reach up to 10 μ M and show an increase in the unsaturated species (Baker *et al.*, 2001; Sano *et al.*, 2002; Watanabe *et al.*, 2007; Hosogaya *et al.*, 2008). The rank order of LPA species in serum is 20:4 > 18:2 > 16:0 > 18:1 > 18:0 (Sano *et al.*, 2002).

The mechanism behind the increase in LPA upon blood coagulation is still unknown. It was first proposed that platelets were the source of high levels of LPA in serum. However, the amount of LPA released from activated platelets is insufficient to explain the large amount of LPA in serum. Tigyi, *et al.* first showed that platelets secrete enzymes that act on plasma phospholipids that in turn generate LPA, which has been confirmed by other groups (Baker *et al.*, 2001; Aoki *et al.*, 2002a). Linoleoyl- (18:2) and arachidonoyl (20:4) species make up 84% of the LPA found in serum (Baker *et al.*, 2001). The difference in the abundance of molecular species between serum and plasma leads to the hypothesis that LPA present in serum is generated via a pathway distinct from that of LPA present in plasma. Furthermore, the dominating abundance of the arachidonoyl (20:4) and linoleoyl (18:2) species of LPA in serum suggests that these LPAs are derived through a PLA₁ enzymatic mechanism because these two lipids are esterified to the *sn*-2 position of plasma lipids.

Preliminary data support this hypothesis of LPA production. It has been shown that the incubation of NBD fluorescent labeled phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) with supernatant from thrombin stimulated platelets are converted into lysophospholipids by PLA₁ activity. When spiked into serum or plasma these lysophospholipids are then converted into LPA (Baker *et al.*, 2001). Previous studies have shown that lysophospholipase D (LPLD) is capable of generating LPA in plasma by preferentially acting on unsaturated lysophosphatidylcholine (Tokumura *et al.*, 1998). This suggests that thrombin activated platelets produce LPA through the cleavage of phospholipids to lysophospholipids by platelet derived PLA₁ and to a much lesser extent PLA₂ and then LPC is further cleaved by LPLD into LPA. It is well established that fatty acids esterified to the *sn*-2 position are unstable and undergo acyl migration in minutes to yield a more stable *sn*-1 regioisomer (Pluckthun and Dennis, 1982; Sano *et al.*, 2002). Thus, we predict that the arachidonoyl- and linoleoyl-LPA are first derived as a *sn*-2 regioisomers from the corresponding lysophospholipids and then rapidly undergo acyl migration to yield the *sn*-1 isomers that are stable and have been detected in serum.

1.2 LPA DEGRADATION

1.2.1 LPA Degradation by Phosphatases

Lysophospholipids are dephosphorylated by the lipid phosphate phosphohydrolase family (LPP) (Brindley and Waggoner, 1998). There are four known isoforms that have been cloned and characterized in mammals, LPP1/PAP-2a/PAP-2 α 1 (Kai *et al.*, 1997), LPP1a/PAP-2 α 2 (Leung *et al.*, 1998), LPP2/PAP-2c/PAP-2 γ (Roberts *et al.*, 1998), and LPP3/PAP-2b/PAP-2 β (Kai *et al.*, 1997). Each member of the LPP family exhibits selectivity for various phospholipid substrates. The LPP1 family prefers LPA as a substrate followed by PA, sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (C1P) (Waggoner *et al.*, 1996). LPP2/PAP-2c/PAP-2 γ prefers PA, then C1P, LPA, and S1P (Roberts *et al.*, 1998). Lastly, LPP3/PAP-2b/PAP-2 β converts PA and LPA with the same efficiency and to a much lesser extent will convert S1P and C1P. LPA degradation occurs by the removal of the phosphate group by phosphatases to yield monoacylglycerol. LPA is also degraded by a nuclear LPA phosphohydrolase which is yet unidentified (Baker and Chang, 2000).

1.2.2 LPA Degradation by Lysophospholipases

LPA can also be degraded by the hydrolysis of the acyl group from the glycerol backbone by lysophospholipase enzymes. Whereas many lysophospholipids prefer LPC substrates over LPA, LPA has been reported in rabbit neuronal nuclei to act as a competitive inhibitor of LPC lysophospholipase activity (Baker and Chang, 2000). It has also been reported that LPA is a substrate of a distinct LPA lysophospholipase activity (Baker and Chang, 2000).

A lysophospholipase with LPA lysophospholipase activity has been purified from rat brain (Thompson and Clark, 1994). It is an 80kDa enzyme and prefers 1-oleoyl and 1-stereoyl LPA species followed by 1-palmitoyl and 1-myristoyl LPA. The purified enzyme does not hydrolyze LPE, LPI, or LPS (Thompson and Clark, 1994).

1.2.3 LPA Degradation by Acyltransferases

1-acylglycerol 3-phosphate acyltransferase (AGPAT), also known as lysophosphatidic acid acyltransferases (LPAAT) catalyzes the transfer of an acyl group from acyl-CaA to LPA to form PA. Five members of the LPAAT family have been identified: LPAAT α /1-AGPAT 1 (Kume and Shimizu, 1997; Aguado and Campbell, 1998), LPAAT β /1-AGPAT 2 (Aguado and Campbell, 1998), LPAAT γ /1-AGPAT 3, LPAAT δ /1-AGPAT 4, and LPAAT ϵ /1-AGPAT 5. LPAAT α and LPAAT β have a much higher catalytic activity than the other family members (Leung, 2001) and therefore contribute most of the LPAAT activity in cells. LPAAT α shows a marked preference for LPA over the other members of the family. LPAAT α is found consistently in most

tissues, however LPAAT β is differentially expressed and elevated in many tumor tissues relating the overproduction of PA to many cancer types.

It is important to note that LPAAT esterifies the *sn*-2 position of LPA to produce PA using acyl-CoAs as substrates and has been shown to prefer arachidonoyl (20:4)-CoA (Yuki *et al.*, 2009; Koeberle *et al.*, 2010). This generates unsaturated fatty acids in the *sn*-2 position of PA, which can then receive a choline or serine headgroup and become a prime substrate for a PLA_{1/2} enzyme.

1.3 LPA RECEPTOR AND SIGNALING

1.3.1 Plasma Membrane Receptors

LPA is a phospholipid mediator that acts through multiple targets that include G protein-coupled receptors (GPCRs) and a nuclear receptor, peroxisome proliferator-activated receptor gamma (PPAR γ) as well as enzymes. To date there are eight known LPA-specific receptors (LPA₁₋₈). LPA₁₋₃ are members of the endothelial differentiation gene (EDG) family (Tigyi and Parrill, 2003; Choi *et al.*, 2008; Mutoh and Chun, 2008; Rivera and Chun, 2008; Ishii *et al.*, 2009; Noguchi *et al.*, 2009; Peyruchaud, 2009; Tigyi, 2010). These three members share 45-56% overall amino acid identity. The transmembrane domains of the human LPA EDG family receptors share 81% homology with one another, however only 6% is conserved within the C-terminal tail. There are also five S1P-specific receptors that are included in this gene cluster of GPCRs: S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅.

LPA₁/EDG2 mRNA is highly abundant in brain tissue, and to a lesser extent in heart tissue, the gastrointestinal tract, and the reproductive system (An *et al.*, 1997; Tigyi, 2010). It has also been found in the rabbit cornea (Wang *et al.*, 2002). LPA₁/EDG2 elicits its actions through the activation of heterotrimeric G proteins which results in the inhibition of adenylyl cyclase (Hecht *et al.*, 1996), DNA synthesis, Ca²⁺ signaling, phospholipase C activation (An *et al.*, 1998), activation of the serum response element (Contos and Chun, 1998), and stimulation of Rho signaling (Fukushima *et al.*, 1998).

LPA₂ is found primarily in testis and leukocytes (An *et al.*, 1998; Tigyi, 2010). It is also found in embryonic and neonatal mouse brain tissue. It elicits its actions through G protein pathways that mediate intracellular Ca²⁺ mobilization, which activates MAP kinase and serum response element (An *et al.*, 1998; Bandoh *et al.*, 1999).

LPA₃ mRNA is mainly expressed in the human heart, prostate, testis, pancreas (Bandoh *et al.*, 1999; Im *et al.*, 2000), rat kidney and testis (Im *et al.*, 2000), and rabbit cornea (Wang *et al.*, 2002). LPA₃ is similar to LPA₂ in that it activates the MAP kinase pathway however; in addition, it also stimulates adenylyl cyclase (Bandoh *et al.*, 1999).

LPA₄/p2y9/GPR23 (Noguchi *et al.*, 2003; Yanagida *et al.*, 2007), LPA₅/GPR92 (Kotarsky *et al.*, 2006; Lee *et al.*, 2006; Williams *et al.*, 2009; Yin *et al.*, 2009), LPA₆/GPR87 (Tabata *et al.*, 2007), LPA₇/p2y5 (Pasternack *et al.*, 2008; Shimomura *et al.*, 2008), and LPA₈/p2y10 (Murakami *et al.*, 2008) are more closely related to the purinoreceptor cluster of GPCR and only share about 20% homology with the LPA EDG family receptors.

LPA₄/p2y9/GPR23 is found primarily in the ovary, uterus, and placenta (Ishii *et al.*, 2009). It has also been found in the brain and been shown to cause LPA-dependent growth cone collapse and neurite retraction through G_{12/13}-RhoA-Rho-associated kinase (ROCK) activation (Lee *et al.*, 2007). The ligand preference of LPA₄/p2y9/GPR23 is LPA 18:1>18:0>16:0>14:0>1-O-alkyl glycerophosphate>1-O-alkenyl glycerophosphate (Noguchi *et al.*, 2003).

LPA₅/GPR92 is found in the heart, placenta, dorsal root ganglia, small intestine, spleen, brain, B cells, intestinal CD8⁺ lymphocytes, and platelets (Kotarsky *et al.*, 2006). The ligand preference of LPA₅/GPR92 is 1-O-alkyl glycerophosphate 18:1>1-O-alkyl glycerophosphate 18:0>LPA 18:1>20:4=16:0=18:3>farnesyl monophosphate>farnesyl diphosphate>LPA 18:0 (Noguchi *et al.*, 2003).

LPA₆/GPR87 is typically found in brain, skeletal muscle, and the reproductive organs. LPA₇/p2y5 is found in the skin and hair. Its ligand preference is LPA 18:2>18:1>20:4>18:0>16:0>14:0 (Yanagida *et al.*, 2009). LPA₈/p2y10 has been found in the uterus, prostate, brain, lung, placenta, and skeletal muscle (Murakami *et al.*, 2008).

The various species of LPA allows for the activation of specific LPA targets. In addition to the different species, the LPA₃ and P2RY5 receptors have shown a slight preference for the *sn-2* regioisomer over the *sn-1* (Bandoh *et al.*, 2000a; Yanagida *et al.*, 2009). However, the stability of the *sn-2* regioisomer is still uncertain.

1.3.2 Intracellular Receptor, PPAR γ

PPAR γ is crucial for many biological responses. It is an active component of regulating lipid and glucose homeostasis (Evans, 2005), cell proliferation (Mueller *et al.*, 1998), apoptosis (Elstner *et al.*, 1998), and inflammation (Ricote and Glass, 2007; Tigyi, 2010). Due to these responses it plays a critical role in many diseases such as diabetes (Lehmann *et al.*, 1995), atherosclerosis (Li *et al.*, 2000), and cancer (Sarraf *et al.*, 1998). PPAR γ has been shown to only be activated by unsaturated LPA species and the S-stereoisomer.

1.4 ROLES OF LPA

1.4.1 LPA in Vascular Biology

LPA plays an important role in angiogenesis (Osborne and Stainier, 2003) arteriosclerosis, inflammation, thrombosis, and is present in atherosclerotic lesions (Siess and Tigyi, 2004). Atherosclerosis is the primary cause of heart disease and stroke underlying about 50% of deaths in developed countries (Libby, 2002; Steinberg, 2002; Zhang *et al.*; 2004). LPA has been identified as a biologically active lipid in mildly-oxidized LDL (mox-LDL), human atherosclerotic plaques, and supernatant of activated platelets (Libby, 2002; Sano *et al.*, 2002; Zhang *et al.*, 2004). It is also known that serum LPA level is significantly elevated in patients with acute myocardial infarction. More importantly, mox-LDL and the lipid-rich core of human atherosclerotic plaques have been shown to stimulate platelets through the activation of LPA receptors. Atherosclerosis begins with neointima formation via the proliferation and migration of vascular smooth muscle cells (VSMC) from the media to the intima. This results in accumulation of lipids in the arterial intima, activation of macrophages, dedifferentiation of vascular smooth muscle cells and endothelial injury (Libby, 2002). LPA has been shown to elicit neointima formation when applied topically to the arterial wall (Zhang *et al.*, 2004).

1.4.2 The Role of LPA in Cancer

LPA provides cell survival advantages in an autocrine or paracrine fashion. ATX has been found in many cancer cell types which provides a mechanism for generation of LPA. One well established instance in which LPA has a role in cancer pathology is ovarian cancer. Reports have shown that LPA levels in ovarian cancer ascites are highly elevated (Xu *et al.*, 1995; Baker *et al.*, 2002; Sutphen *et al.*, 2004) presumably due to an increase in ATX expression (Xu *et al.*, 1998; Dimova *et al.*, 2006).

Expression of LPA₂ mRNA was also shown to be upregulated in ovarian cancer cells (Goetzl *et al.*, 1999a) providing evidence of its involvement in ovarian cancer. LPA₂ supports the aggressiveness of ovarian cancer in two different ways. First, it provides a feed-forward loop by increasing the production of vascular endothelial growth factor (VEGF), urokinase (uPA), and matrix metalloproteinases (MMP) (Zebrowski *et al.*, 1999; Hu *et al.*, 2001; Huang *et al.*, 2004; So *et al.*, 2004; So *et al.*, 2005) which in turn upregulates ATX production and results in increased LPA levels (Ptaszynska *et al.*, 2008). Secondly, the LPA₂ receptor mediates chemoresistance through its C-terminal interaction of the thyroid receptor-interacting protein 6 (TRIP 6), Siva-1, and PDZ binding proteins: NHERF2 and MAGI-3 (Yamada *et al.*, 2005; Lin *et al.*, 2007; Zhang *et al.*, 2007a; Zhang *et al.*, 2007b; E *et al.*, 2009). These findings provide a basis for the importance of discovering receptor antagonists and ATX inhibitors for potential anticancer therapeutics.

1.4.3 LPA in Cell Survival and Apoptosis

Through its pro-mitotic and anti-apoptotic properties, LPA promotes cell survival. LPA inhibits the activation of the mitochondrial apoptotic pathway which is further linked to the activation of the ERK1/2 and Akt kinases (Goetzl *et al.*, 1999b; Deng *et al.*, 2003; Radeff-Huang *et al.*, 2004).

Because of its anti-apoptotic properties, LPA has been studied in a clinical role. LPA can be used to reduce the side effects of radiation and chemotherapy in non-intestinal cancer patients because it reduces the damage to the intestinal epithelial cells induced by gamma radiation and chemical agents that cause apoptosis (Deng *et al.*, 2002).

1.5 PHOSPHOLIPASES AND LYSOPHOSPHOLIPASES

1.5.1 Phospholipases

Phosphatidic acid contains two fatty acid chains and a phosphate esterified to a glycerol backbone. The hydroxyl group at the C3 position of the glycerol is esterified to phosphoric acid and is termed the *sn*-3 position. All major phosphoglycerides are derived from phosphatidate. An ester bond is formed between the phosphate group of phosphatidate and the hydroxyl group of an alcohol such as serine or choline. Phospholipases cleave phospholipids to yield lysophospholipids. Phospholipase A₁ (PLA₁) cleaves at the *sn*-1 position whereas phospholipase A₂ (PLA₂) cleaves at the *sn*-2 position. A phospholipase B (PLB) enzyme will cleave at both the *sn*-1 and *sn*-2 position and Phospholipase C (PLC) and phospholipase D (PLD) cleave on either side of the phosphate group. The specificities of the phospholipases are shown in Figure 1.2.

Phospholipases are typically serine hydrolases, which are important in blood coagulation, inflammation, and angiogenesis (Liu *et al.*, 1999). Most serine hydrolases are potently inhibited by fluorophosphonates (FP) or fluorophosphonate derivatives. However fluorophosphonates do not inhibit cysteine, aspartyl, or metalloproteases (Liu *et al.*, 1999). FP's reactivity requires that the serine hydrolase be in a catalytically active state (Leung *et al.*, 2003). This allows for much greater selectivity. Radiolabeled, rhodamine-labeled, or biotin-labeled fluorophosphonate probes are available (Patricelli *et al.*, 1999). The rhodamine and biotin-labeled probes are rapid, selective and highly sensitive. These probes have the fluorescent reactive group and the biotin tag coupled through a long alkyl chain and two amide bonds (Liu *et al.*, 1999).

FP- Rhodamine labeled probes can be used to label all active serine hydrolases within a compound sample. Typical SDS gel electrophoresis can be used to separate proteins and the fluorescent label can then be visualized using a flat-bed Infra-red scanner. The biotinylated probes can be bound to avidin or streptavidin and separated

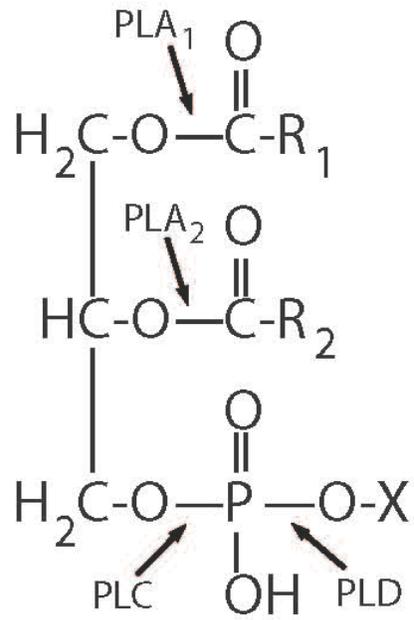


Figure 1.2 Specificity of phospholipases. Arrows indicate cleavage site of the indicated phospholipase.

from all other proteins. The biotinylated probe-bound serine hydrolase can then be digested or boiled off the avidin beads and then this highly purified product can be analyzed using LC-MS or gel electrophoresis.

1.5.2 Phospholipase A₁

PLA₁ catalyzes the cleavage of a glycerophospholipid at the *sn-1* position generating a *sn-2* lysophospholipid and a fatty acid. Whereas many PLA₂ isoforms have been purified, cloned, and characterized, little is known of PLA₁. A similar PLA₁ to the one we are studying has been described to some degree by Smith and Silver (Smith *et al.*, 1973). It has an acidic pH optimum of 4.8, but has not been biochemically identified and cloned.

To date, there are two phosphatidic acid-selective cytosolic PLA₁s named mPA-PLA₁ α and mPA-PLA₁ β which are specifically expressed in human testis (Verheij *et al.*, 1981). It was shown that mPA-PLA₁ α and mPA-PLA₁ β hydrolyzed the ester bond at the *sn-1* position of phosphatidic acid to generate LPA, but did not hydrolyze the ester bond of PC, PS, or PE (Verheij *et al.*, 1981; Aoki, 2004).

Another known type of PLA₁ is phosphatidylserine-specific PLA₁ (PS-PLA₁). PS-PLA₁ is secreted from rat platelets, but is not found in mouse or human platelets (Aoki *et al.*, 2002a; Smart *et al.*, 2004). PS-PLA₁ hydrolyzes PS, but does not hydrolyze PC, PE, PI, or PA. PS-PLA₁ is approximately 55kDa, stored intracellularly, and is secreted when the cells are activated by thrombin, collagen, or calcium ionophore A23187 (Smart *et al.*, 2004). This suggests that under physiological conditions PS-PLA₁ is secreted during rat blood coagulation.

1.5.3 Phospholipase A₂

Many forms of mammalian PLA₂s have been identified. These include group IVA Ca²⁺-dependent cytosolic PLA₂ (cPLA₂) (Leslie, 1997), group VI Ca²⁺-independent PLA₂ (iPLA₂) (Balsinde and Dennis, 1997), and secretory PLA₂ (sPLA₂) (Valentin and Lambeau, 2000).

Group IVA Ca²⁺-dependent cytosolic PLA₂ (cPLA₂) are 85kDa and are typically located in the cytosol and translocated to the nuclear envelope and endoplasmic reticulum upon activation (Schievella *et al.*, 1995; Pouliot *et al.*, 1996). cPLA₂s have been shown to play an important role in the arachidonic acid release, eicosanoid production and signal transduction (Clark *et al.*, 1995; Kramer and Sharp, 1997; Leslie, 1997).

Group VI Ca²⁺-independent PLA₂ (iPLA₂) are also 85kDa and have been shown to play a key role in apoptosis and mediating basal phospholipid remodeling (Balsinde and Dennis, 1996; Atsumi *et al.*, 1998). It has been reported that this enzyme works as a

multimeric complex of 270-350kDa through protein-protein interactions mediated by ankyrin repeats (Ackermann *et al.*, 1994; Larsson *et al.*, 1998).

Secretory PLA₂s have a molecular weight of 14-19kDa (Murakami and Kudo, 2004) and hydrolyze the ester bond at the *sn*-2 position of phosphoglycerides releasing fatty acids and lysophospholipids. To date, ten secretory phospholipase A₂s (sPLA₂s) have been identified in mammals (IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) (Schaloske and Dennis, 2006). Humans possess all except IIC (Murakami and Kudo, 2004). The mammalian family of sPLA₂s are Ca²⁺ dependent. There are many known inhibitors of sPLA₂s including Me-Indoxam which is the most potent inhibitor for most sPLA₂s (Bacha *et al.*, 2006).

1.5.4 Lysophospholipase D

Plasma lysoPLD cleaves lysophospholipids to produce LPA and was recently identified as autotaxin (ATX) (Tokumura *et al.*, 2002; Umezu-Goto *et al.*, 2002), a 125kDa glycoprotein. ATX has been shown to promote tumor cell motility, metastasis, and angiogenesis via a pertussis toxin-sensitive mechanism (Stracke *et al.*, 1997; Nam *et al.*, 2001; Lee *et al.*, 2002). LPLD/ATX is a member of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family and is also known as NPP2. NPP family-members are capable of hydrolyzing adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in order to generate nucleoside 5'-monophosphates which distinguishes them from other members of the lipase superfamily.

ATX contains a short N-terminal tail, a transmembrane domain, two cysteine-rich somatomedin B-like domains, and a C-terminal catalytic domain. ATX mRNA is widely expressed in the brain, ovary, lung, intestine, and kidney (Tice *et al.*, 2002; Mills and Moolenaar, 2003). ATX shows product feedback inhibition which is thought to contribute to maintaining plasma levels of LPA in the nanomolar range (Durgam *et al.*, 2005; van Meeteren *et al.*, 2005).

ATX has long been known as a tumor-motility factor which provides a link between ATX and cancer. ATX has numerously been shown to be upregulated in several human cancers and has been shown to enhance tumor aggressiveness (Nam *et al.*, 2000) perhaps through the generation of LPA.

1.5.5 Lysophospholipase A-I /II

As mentioned previously, lysophospholipases contribute to the breakdown of LPLs by hydrolyzing the ester bond to produce a free fatty acid and a glycerolphosphate derivative. There are large molecular mass Lysophospholipase As (LYPLAs) and small molecular mass LYPLAs. Low molecular mass LYPLAs have been reported to be found in rabbit heart (Gross and Sobel, 1983), pig gastric mucosa (Sunaga *et al.*, 1995), murine macrophage P388D₁ (Zhang and Dennis, 1988; Zhang *et al.*, 1991), rat and beef liver (de

Jong *et al.*, 1974; Sugimoto *et al.*, 1996), and human HL60 cells (Garsetti *et al.*, 1992). Many of these tissues contain two isoforms: LYPLA-I and LYPLA-II which are similar in size and have similar properties. For example, both enzymatic activities are independent of Ca^{2+} , Mg^{2+} , and EDTA (Zhang *et al.*, 1991; Garsetti *et al.*, 1992; Sunaga *et al.*, 1995; Sugimoto *et al.*, 1996). Despite their similarities, much evidence has been reported to recommend that these enzymes are two distinct proteins. For example, LYPLA-I has a much broader LPL substrate specificity than its LYPLA-II counterpart. LYPLA-I has been shown to actively cleave LPC, LPE, LPG, LPI, LPS, and the acyl analog of PAF, whereas LYPLA-II will only cleave LPE and LPC (Garsetti *et al.*, 1992; Sunaga *et al.*, 1995; Sugimoto *et al.*, 1996). LYPLA-I hydrolyzes both *sn-1* and *sn-2* regioisomers of LPC at identical rates (Wang *et al.*, 1997).

Neither enzyme has been shown to have significant PLA_1 or PLA_2 activity in rabbit heart, pig gastric mucosa, murine macrophage P388D₁, or human HL60 cells (Gross and Sobel, 1983; Zhang and Dennis, 1988; Zhang *et al.*, 1991; Garsetti *et al.*, 1992; Sunaga *et al.*, 1995). However, these enzymes were only extensively characterized using lysophospholipids substrates and human platelets have not been tested previously for activity.

It is not uncommon for many phospholipases to show lysophospholipase activity and all are typically grouped into the large molecular mass LYPLAs. Group IV Ca^{2+} -dependent cytosolic PLA_2 exhibits strong lysophospholipase activity for *sn-1* LPC but shows little activity when presented with *sn-2* LPC (Loo *et al.*, 1997). Cytosolic Ca^{2+} -independent phospholipase A₂ (iPLA₂) also exhibits lysophospholipase activity along with PLA_1 , transacylase, and PAF acetylhydrolase activity which has been shown to be dependent on substrate presentation (Wolf and Gross, 1996; Lio and Dennis, 1998). PS- PLA_1 (Higashi *et al.*, 1988; Yokoyama *et al.*, 1995; Sato *et al.*, 1997) and PA- PLA_1 (Higgs and Glomset, 1996) also exhibit lysophospholipase activity as well as phospholipase activity.

Another function of LYPLA-I/APT1 is the deacylation/depalmitoylation of G proteins, ghrelin, and other thioacylated protein substrates (Duncan and Gilman, 1998; 2002; Hirano *et al.*, 2009; Satou *et al.*, 2010). The K_m of the lysophospholipase activity is about 8 times higher than the K_m value of the thioesterase activity of the enzyme (Hirano *et al.*, 2009) indicating that LYPLA-I/APT1 has a much higher thioesterase activity than lysophospholipase activity. However, the subcellular localization of the enzyme plays an important role in the function of the enzyme.

CHAPTER 2. THE PHOSPHOLIPASE A₁ ACTIVITY OF LYSOPHOSPHOLIPASE A-I LINKS PLATELET ACTIVATION TO LPA PRODUCTION DURING BLOOD COAGULATION*

2.1 INTRODUCTION

Lysophosphatidic acid (LPA) is a multifunctional phospholipid mediator and second messenger responsible for a wide variety of cellular responses (Pages *et al.*, 2001; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Tigyi, 2010). LPA elicits its actions through cell surface G protein-coupled receptors (Pages *et al.*, 2001; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Tigyi, 2010) and through the nuclear peroxisome proliferator activating receptor γ (PPAR γ , Tsukahara *et al.*). LPA has been shown to play a role in many physiological functions and human diseases (Pages *et al.*, 2001; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Tigyi, 2010). The biochemical pathways involved in LPA production in biological fluids are not yet fully understood.

LPA can be produced by several intra- and extracellular biochemical pathways. Intracellularly, LPA is synthesized by a glycerophosphate acyl transferase-catalyzed reaction of glycerol-3-phosphate with acyl-CoA in the endoplasmic reticulum and mitochondria (Aoki, 2004; Gendaszewska-Darmach, 2008). LPA has also been shown to be generated in a spatially regulated fashion by the Ca²⁺-independent phospholipase A₂ at the leading edge of migrating monocytes (Carnevale and Cathcart, 2001; Mishra *et al.*, 2008). Third, acylglycerol kinase phosphorylates monoacylglycerol to form LPA (Bektas *et al.*, 2005). A fourth pathway in humans, involves phosphatidylserine (PS)-specific phospholipase A₁ (PS-PLA₁) that generates lysophosphatidylserine (LPS), which in turn is converted to LPA by phospholipase D in mast cells (Nagai *et al.*, 1999; Aoki *et al.*, 2002a).

Extracellularly, LPA can be produced by a secretory phospholipase A₂ (PLA₂) (Fourcade *et al.*, 1995), oxidative modification of low density lipoprotein (Siess *et al.*, 1999), or by the action of phosphatidic acid-specific phospholipase A₁ (Aoki *et al.*, 2002a; Cummings *et al.*, 2002). However, the most important pathway is a multi-step process linked to the activation of platelets (Mauco *et al.*, 1978; Schumacher *et al.*, 1979; Eichholtz *et al.*, 1993; Gaits *et al.*, 1997; Sano *et al.*, 2002; Aoki *et al.*, 2008). This mechanism involves release of unidentified PLA₁ and/or PLA₂ enzymes from activated platelets generating a new pool of lysophospholipid (LPL) substrates, which in turn are

* Adapted by permission: Alyssa L. Bolen, Anjaparavanda P. Naren, Sunitha Yarlagadda, Sarka Beranova-Giorgianni, Li Chen, Derek Norman, Daniel L. Baker, Meng M. Rowland, Michael D. Best, Takamitsu Sano, Tamotsu Tsukahara, Karoly Liliom, Yasuyuki Igarashi, Gabor Tigyi. *The Phospholipase A₁ Activity of Lysophospholipase A-I Links Platelet Activation to LPA Production During Blood Coagulation*. Journal of Lipid Research 2011, March 9. [Epub ahead of print].

cleaved by the lysophospholipase D, autotaxin (ATX) (Tokumura *et al.*, 2002; Umezugoto *et al.*, 2002). As a result of this mechanism, plasma LPA concentration rises from a steady state concentration of approximately 100nM to serum concentrations up to 10 μ M, with a significant increase in the content of polyunsaturated acyl species (Baker *et al.*, 2001; Sano *et al.*, 2002; Hosogaya *et al.*, 2008). The role of ATX in LPA production has been clearly demonstrated by the decreased plasma LPA level in ATX knockout mice (Tanaka *et al.*, 2006; van Meeteren *et al.*, 2006).

The rank order of the acyl species of LPA in normal human plasma is 18:2 > 18:1 > 18:0 > 16:0 > 20:4. In contrast, the rank order of the LPA acyl species in serum changes to 20:4 > 18:2 > 16:0 > 18:1 > 18:0 (Sano *et al.*, 2002). The various acyl species of LPA also have differing ligand properties at target LPA GPCR (Fujiwara *et al.*, 2005). In addition to the differences in carbon chain length and degree of unsaturation, the LPA₃ and P2RY5(LPA₆) receptors also show a preference for the *sn*-2 over the *sn*-1 acyl regioisomer of LPA (Bandoh *et al.*, 2000b, Yanagida *et al.*, 2009). However, the *sn*-2 LPA regioisomer is relatively unstable. At neutral pH acyl migration begins immediately to yield the more thermodynamically stable *sn*-1 regioisomer, an equilibrium ratio of 9:1 occurs between the two forms (Pluckthun and Dennis, 1982).

The linoleoyl- (18:2) and arachidonoyl (20:4) species make up 84% of the LPA found in serum (Sano *et al.*, 2002). The mechanism behind the enrichment and increase in polyunsaturated LPA species upon blood coagulation is still unknown. Because plasma phospholipids containing 18:2 and 20:4 fatty acids are almost exclusively esterified to the *sn*-2 glycerol carbon, we hypothesized that LPA in serum enriched in these fatty acyl species must be generated by the action of a PLA₁ enzyme (Sano *et al.*, 2002). This hypothesis implies that the nascent *sn*-2 LPL generated by PLA₁ will either be rapidly cleaved by ATX and then undergo acyl migration or alternatively, undergo acyl migration prior to headgroup cleavage. Distinguishing between the latter two possibilities is challenging due to the short half-life of the *sn*-2 LPLs that precludes the use of classical biochemical separation and analytical techniques.

In the present study, we sought to identify the putative PLA₁ enzymes released from activated platelets that are responsible for the generation of polyunsaturated LPL. Starting with the supernatants from thrombin-activated human platelets, we isolated lysophospholipase A1/acyl protein thioesterase (LYPLA-I/APT1) using a series of chromatographic steps and fluorophosphonate-biotin affinity labeling-based proteomics. LYPLA-I/APT1 transcripts were subsequently shown to be abundantly expressed in platelets and in megakaryocytes. LYPLA-I/APT1 was found to possess PLA₁ activity against plasma phospholipids, did not degrade LPA, and increased LPA production when added to plasma through the production of a pool of LPL, which is further cleaved by ATX.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Fluorescently labeled 1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-*sn*-glycero-3-phosphoserine (NBD-PS 18:1-12:0), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-*sn*-glycero-3-phosphocholine (NBD-PC 18:1-12:0), dioleoyl phosphatidylserine (PS), linolenoyl phosphatidylcholine (PC), oleoyl lysophosphatidic acid (LPA), LPC 17:0, and LPA 17:0 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 1-Oleoyl-*sn*-glycero-3-phosphocholine (LPAF 18:1) was purchased from Bachem (Torrance, CA). Fluorophosphonate-Rhodamine (FP-RH) and fluorophosphonate-biotin (FP-Biotin) were a gift from Dr. Ben Cravatt (Scripps Institute, La Jolla CA). Freshly expired apheresis platelets were provided by The Regional Medical Center (Memphis, TN), Methodist University Hospital (Memphis, TN), or purchased from Key Biologics, Inc. (Memphis, TN). PGE₁ and thrombin were obtained from Sigma-Aldrich (St. Louis, MO). Trizol and Superscript III OneStep RT-PCR was purchased from Invitrogen (Carlsbad, CA).

2.2.2 Isolation and Activation of Human Platelets

The procedures detailed below were reviewed and approved by the Institutional Review Board of the University of Tennessee Health Science Center. Small-scale batches of platelet rich plasma (PRP) were prepared by adding 3.6mL acidic citrate dextrose (ACD, 0.8% citric acid, 2.2% sodium-citrate, 2.45% glucose) to 20mL of cubital venous blood drawn from a volunteer donor and centrifuged at 180 x g for 15min. The top layer of platelet rich plasma (~10mL) was transferred to a new tube. For large scale purifications, units of freshly expired apheresis platelets in ACD were obtained (approximately 60mL per unit). Then 10mL of the PRP from the small scale purification or 10mL of the apheresis platelets were diluted with 34mL Buffer A (138mM NaCl, 3.3mM NaH₂PO₄, 2.9mM KCl, 1mM MgCl₂, 20mM HEPES, 1mg/mL glucose, pH 7.5), and 1μM PGE₁ was added. The diluted PRP was centrifuged at 1400 x g at room temperature for 15min. The supernatant was discarded and the pellet containing the platelets was reconstituted in 2mL buffer A and 2mM Ca²⁺ was added. The platelets were activated using 1U thrombin per 1mL sample at 37°C for 20min and the aggregate formed was centrifuged at 9,300 x g for 5min to yield supernatant 1 (Sup1). The Sup1 was then centrifuged again at 100,000 x g for 45min to remove platelet microvesicles. This preparation was designated as supernatant 2 (Sup2).

2.2.3 Measurement of Phospholipase A₁ Activity

PLA₁ activity was measured by determining the amount of lysophosphatidylcholine (LPC) or lysophosphatidylserine (LPS) generated after incubation of sample with phosphatidyl choline (PC) or phosphatidyl serine (PS). NBD-

PC or NBD-PS (45ng to 2 μ g) was incubated in 10mM Tris (pH 7.5) or Sup2 (pH 7.5) at 37°C for 1min to 1 hour with or without the presence of an equal weight of BSA in water. For quantification, 900 ng, 90 ng, and 9 ng of the fluorescent substrates were incubated without enzyme for construction of a standard curve by plotting fluorescence intensity as a function of substrate mass. Water-saturated butanol (BuOH) was added (30-120 μ l) to stop the reaction and extract the lipids. Samples (10 μ l) were spotted on Silica Gel 60 thin layer chromatography (TLC) plates. The plates were then developed with solvent A consisting of chloroform:methanol:ammonium hydroxide (V/V/V, 6:4:1) for NBD-PS and solvent B consisting of chloroform:methanol:28% ammonium hydroxide:water (V/V/V/V, 50:40:8:2) for NBD-PC. The products were visualized using a Fotodyne imager (Hartland, WI) and quantified by the TotalLab100 software. The amount of the product generated was determined by interpolation from the standard curve. The Rf values for the various lipids were as follows: NBD-PC: 0.54, NBD-LPC: 0.38, NBD-FA: 0.77, NBD-PS: 0.45, NBD-LPS: 0.33, NBD-FA: 0.67.

For unlabeled substrates, 10 μ g PC, PS, or LPA was digested at 37°C for 1 hour in the presence of 10 μ g BSA. LPC 17:0 and LPA 17:0 (500-2000 ng in DMSO) internal standards were added prior to addition of 60 μ l BuOH. The samples were vortexed for 1min and the BuOH phase was isolated by centrifugation at 14000 x g for 1 min and dried under argon. The lipid extract was reconstituted in 30 μ l methanol:acetonitrile:Isopropanol:water (V/V/V/V, 1:1:1:1). LPC and LPA concentrations were determined by liquid chromatography-mass spectrometry (LC-MS/MS) using an Applied Biosystems Sciex ABI 4000 QTRAP tandem mass spectrometer (Foster City, CA) equipped with a TurboionsprayTM interface, a Shimadazu LC-10ADvp HPLC pump (Columbia, MD) with a Leap HTS PAL autosampler (Carrboro, NC). Samples (10 μ l) were injected onto a Tosoh TSK-ODS-100Z silica column (150 mm x 2mm; 5- μ m particle size) with a solvent consisting of methanol/water (V/V, 95:5), 5mM ammonium formate using an isocratic flow rate of 0.22mL/min (Tokumura *et al.*, 2009). The spectra were processed using Analyst software, version 1.5. The molecular species of LPC and LPA were analyzed by multiple reaction monitoring (MRM) in positive ion mode for LPC and negative ion mode for LPA. The Q3 (product ion) was set at *m/z* 184.0 for LPC and 153.0 for LPA (glycerophosphate moiety) (Tokumura *et al.*, 2009). Q1 was set for the neutral molecular ion for all LPLs. Quantification was done by calculating the ratio of peak area to that of the appropriate LPC/LPA 17:0 internal standard.

2.2.4 Partial Purification of PLA₁

PLA₁ was partially purified using sequential chromatography on an AKTA FPLC system (GE Biosciences, Piscataway, NJ) by loading 330mg (75mg/ml) Sup2 to a Q Sepharose Fast Flow ion exchange chromatography column (GE Biosciences, 2 cm x 10 cm). The column was eluted with a NaCl gradient (0-1M) at a 1mL/min flow rate over 20min with buffer C (3.3mM NaH₂PO₄, 2.9mM KCl, 1mM MgCl₂·6H₂O, 20mM HEPES, pH 7.5). The PLA₁ active fractions were combined and 5mL (~30mg) was loaded onto a Butyl-Sepharose hydrophobic interaction chromatography column (GE

Biosciences, 0.7cm x 2.5cm, 20mg/mL medium). The column was eluted using a gradient from 1.7M to 0M (NH₄)SO₄ over 20min in 0.05M Na₂HPO₄ buffer (pH 7.6). The active fractions were combined and 5mL (~2.5mg) was loaded onto a HiTrap Blue affinity chromatography column (GE Biosciences, 0.7cm x 2.5cm). The column was eluted by increasing (NH₄)SO₄ from 0 to 1.7M over 20min in 0.05M Na₂HPO₄ buffer (pH 7.6). 3mL Fractions were collected at each step. 100µl of each fraction was used for PLA₁ activity measurement using 1µg NBD-PS substrate at 37°C for 2 hours. 120µl water-saturated BuOH was added to stop the reaction and extract the lipids. The samples were vortexed and centrifuged for 1min at 14,000 x g. 10µl of the BuOH phase was spotted to a Silica Gel 60 TLC plate. The plate was developed with solvent A and the products were visualized. The fractions with PLA₁ activity from each chromatographic step were combined, activity was determined as described, and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL) following the manufacturer's protocol.

2.2.5 FP-Rhodamine Labeling

An amount of 50µg protein of the PLA₁ active Butyl-Sepharose pooled fractions (~100µl) and 0.9µg NBD-PS substrate was incubated at 37°C for 3 hours with 0.03, 0.1, 0.3, 1, 3, 10, and 30µM FP-Rh. Then 60µl water-saturated BuOH was used to stop the reaction and extract the lipid products. After mixing, the samples were centrifuged for 1min at 14000 x g. Then 10µl of the BuOH phase was spotted to a Silica Gel 60 TLC plate and developed with solvent A. The products were visualized using a Photodyne imager and quantified using standards run alongside the samples on the same TLC plate.

2.2.6 FP-Biotin Labeling and Purification

The activity-based proteomic probe, FP-Biotin, was used to label all serine hydrolases in the PLA₁-active HiTrap Blue fractions. An active pool of HiTrap Blue chromatography fractions was concentrated using an Amicon concentrator (Millipore, Billerica, MA) with a 5kDa molecular weight cutoff. An amount of 1mg protein (1mg/mL) was incubated for 2 hours with a final concentration of 5µM FP-Biotin. Streptavidin beads (Pierce, Rockford, IL) were then pre-washed three-times with binding buffer (0.1M sodium phosphate, 0.15M NaCl, pH 7.0) followed by centrifugation (3000 x g for 1min) and the supernatant was discarded. The protein complex was added to 50µl resin and incubated with mixing for 1hr at room temperature. Streptavidin-bound FP-Biotinylated proteins were washed with binding buffer in the presence of 4M urea, 0.1% SDS (W/V), and 0.2% TritonX-100 (V/V) (pH 7.2) and centrifuged for 1min at 3000 x g. The supernatant was removed and the wash procedure was repeated four times. The beads were reconstituted in 1mL 50mM TRIS-HCl (pH 7.2) centrifuged for 4min at 10000 x g and the supernatant was decanted. 200µl 50mM TRIS-HCl (pH 7.2) was added to reconstitute the final pellet.

2.2.7 Proteomic Analysis of FP-Biotin-Labeled Products in Partially Purified Platelet Supernatants

The bead-bound FP-Biotin-labeled proteins were first reduced with 5mM dithiothreitol for 30min at room temperature and alkylated with 10mM iodoacetamide at room temperature in the dark for 30min. The sample was centrifuged for 4min at 10000 x rpm and 100µl of supernatant was removed. A total of 300µl 50mM TRIS (pH 7.2) buffer and 1.5µl of 0.5µg/µl trypsin (sequencing grade, Sigma-Aldrich, St. Louis, MO) was added and incubated at 37°C for 12 hours. The sample was centrifuged for 4min at 10000 x rpm and the supernatant containing the peptide digest was transferred to a new tube; formic acid was added to a final concentration of 5% (V/V). The volume of the peptide sample was reduced to 40µl in a vacuum centrifuge. The peptides were desalted with a Zip Tip C18 microcolumn (Millipore, Billerica, MA) using the procedure recommended by the manufacturer. Peptides were eluted using 4µl of 50% acetonitrile/0.1% trifluoroacetic acid (V/V). Four microliters of water/acetic acid (0.5%) were added to the sample. LC-MS/MS experiments were performed on an LTQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA) coupled to a nanoflow LC system (Dionex, Sunnyvale, CA). The peptide sample was injected onto a fused-silica capillary column/spray needle (15 cm length, 75µm ID; New Objective, Woburn, MA) packed in-house with C18 stationary phase (Michrom Bioresources, Auburn, CA). The peptides were separated with a 90 min-gradient from 0% to 90% of mobile phase B. The composition of mobile phase B was 90% MeOH/10%water/0.05% HCOOH; the composition of mobile phase A was 2% MeOH/98% water/0.05%HCOOH. Mass spectrometric data acquisition was performed in the data-dependent mode; one cycle encompassed a full-range MS scan followed by 7 MS/MS scans on the most abundant ions from the MS scan. The LC-MS/MS data were used to search the SwissProt protein sequence database (subset of human proteins), using the program Bioworks/Sequest (Thermo Scientific).

2.2.8 RNA Purification and RT-PCR

A total of 100ml of venous blood was drawn into 20mL ACD, 1 µM PGE₁. The blood was centrifuged at 180 x g and the platelet rich plasma was filtered through a PL6T leukocyte reduction filter (Pall, Inc., Port Washington, NY). Buffer A (68mL) and 12mL ACD was added to the sample and centrifuged at 1400 x g. The supernatant was removed, the platelet pellet was resuspended in 1mL Trizol (Invitrogen) and RNA was extracted following the manufacturer's protocol. Two gene- and species-specific primers for each candidate protein were designed. Each primer was designed to produce a product between 250 and 350 basepairs-long and span an intron of more than 1000 base pairs so that DNA contamination would easily be identifiable by the size of the product. The following primers were used: LYPLA-I/APT1 1: 5'-GCAGAAGCCTT TGCAGGTAT - 3', forward 5'-ATTGCCATTCTTCACTTCTTGAT-3', reverse; LYPLA-I/APT1 2: 5'-ACTCAGTTGCTGGCTTCCAC-3', forward 5'-TGCTTGACATCCATCATTTC-3', reverse; MGLL 1: 5'-GCTGGACCTGCTGGTGT-3', forward 5'-

TGTTGCAGATTCAGGATTGG-3', reverse; MGLL 2: 5'-AAGGGCCTACCTGCTCAT-3', forward 5'-TGGCTGTCCTTTGAGAGACC-3', reverse; PLA1A 1: 5'-CCAATGATGTGGATTGAGGA-3', forward 5'-GAAGCCATCCACACAGACAC-3', reverse; PLA1A 2: 5'-GCTGTGGGCAGCTAGTAGAAC-3', forward 5'-CCAATGATGTGGA TTGAGGA-3', reverse; LCAT 1: 5'--3', forward 5'--3', reverse; LCAT 2: 5'-GCTCCTCAATGTGCTCTTCC-3', forward 5'-CGGTAGCACATCCAGTTCAC-3', reverse. Reverse-transcription polymerase chain reaction was done using the Superscript III kit (Invitrogen). 30 cycles were performed using 400ng RNA template. The PCR products were separated on 2% agarose gels and visualized with ethidium bromide staining.

2.2.9 MGLL Activity Assay

Human recombinant MGLL was purchased from Abnova Corporation (Jhongli, Taiwan) and 0.5µg enzyme was incubated with NBD-PS (0.9µg) for 3 hours at 37°C in 20µl 10mM TRIS (pH 7.5). Then 30µl water-saturated BuOH was added to extract the lipids and 10µl of the extract was spotted on a TLC plate. The plates were then developed with solvent A consisting of chloroform:methanol:ammonium hydroxide (V/V/V, 6:4:1). The products were visualized using a Fotodyne imager (Hartland, WI) and quantified by the TotalLab100 software. The amount of the product generated was determined by interpolation from the standard curve.

Recombinant monoglyceride lipase (MGLL) was purchased from Abnova Corporation (Jhongli, Taiwan) and tested for activity using an assay kit from Cayman Chemicals (Ann Arbor, Michigan) that utilizes the hydrolysis of the thioester bond of arachidonoyl-1-thio-glycerol to produce a free thiol that reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Recombinant MGLL (0.3µg or 0.5µg) or 100µl PLA₁-active Blue-Sepharose fraction was added in 150µl total volume with assay buffer (10mM TRIS-HCl, 1mM EDTA, pH 7.2), to a 2.7mM ethanolic solution of arachidonoyl-1-thio-glycerol and incubated at room temperature for 5 min. The absorbance was read at 415nm using a BioTek microplate reader.

2.2.10 Construction of Recombinant LYPLA-I/APT1 Expression Plasmid

Human full length LYPLA-I/APT1 (Accession # AF081281) was PCR-amplified. The PCR products were cleaned using the Qiagen PCR purification kit and eluted in 20µl DNA grade water. The DNA was used for ligation independent cloning (LIC) using the Ek/LIC cloning kit from Novagen (EMD, Gibbstown, NJ). Primers used for LIC cloning were: LYPLA-I/APT1-full-length-LIC Forward: GACGACGACAAGATGTGCGGCAA TAACATGTCAACC and LYPLA-I/APT1-full-length-LIC Reverse: GAGGAGAAG CCCGGTTCAATCAATTGGAGGTAGGAG. PCR-purified DNA (14.6µl) was taken into a sterile centrifuge tube and 2µl each of 10-times concentrated T4 DNA polymerase buffer and 25mM dATP were added. Dithiothreitol 1µl, 100mM and 0.4µl of T4 DNA polymerase were added to start the reaction with gentle stirring at 22°C for 30min. The

reaction was stopped by incubation at 75°C for 20min. This T4 DNA polymerase-treated insert can be annealed into any Ek/LIC vectors including the pET-41 vector used here. For the ligation reaction, 1µl Ek/LIC vector (pET-41) and 2µl of T4 DNA polymerase-treated insert were used and incubated at 22°C for 5min. Then, 1µl of 25mM EDTA was added for 5min at 22°C. The ligated product was transformed into Novablue cells (EMD biosciences, Gibbstown, NJ). Three transformants were picked, and plasmid DNA was isolated and sequenced. The sequences were confirmed to be that of LYPLA-I/APT1.

2.2.11 Recombinant LYPLA-I/APT1 Purification

GST-LYPLA-I/APT1 was purified from transformed BL21-DE3 *E. coli* bacteria. Protein expression was induced using 200µM isopropyl β-D thiogalactopyranoside. Bacteria were pelleted by centrifugation and resuspended in lysis buffer (50mM TRIS base, 1mM EDTA, and 10% sucrose containing the protease inhibitors; aprotinin 1µg/mL, leupeptin 1µg/mL, pepstatin 1µg/mL, and PMSF 500µM). Lysozyme (1mg/mL) was added to the resuspended bacteria and allowed to mix at 4°C for 30min. NP40 was added to the suspension (0.2% final concentration) and allowed to mix at 4°C for 30min. The suspension was cleared by centrifugation at 14000 x g for 30min at 4°C. Glutathione beads (ThermoFisher Scientific; 2mL of 50% slurry) were added to the cleared supernatant and allowed to mix for 2 hours at 4°C. Glutathione beads were retrieved by centrifugation and washed with 0.2% Triton-X-100 (V/V) in phosphate buffered saline (PBS) three-times, followed by a wash with PBS plus protease inhibitors. A 20µL sample of the protein bound to the glutathione beads was eluted using Laemmli sample buffer and run on a 4-15% gel to visualize the quantity and purity of the isolated protein. Protein was eluted from the beads using 20mM reduced glutathione (pH 7.8) and dialyzed against an appropriate buffer and protein and stored in small aliquots at -80°C.

The GST- tag was removed by thrombin cleavage using 1 mg of GST-LYPLA-I/APT1 mixed with 2.5 units of biotinylated thrombin (Novagen, Darmstadt, Germany) in 20mM TRIS-HCl (pH 8.4), 150mM NaCl, 2.5mM CaCl₂ buffer at 20°C for 16h. After cleavage, the biotinylated thrombin was removed using streptavidin-agarose (Novagen, Darmstadt, Germany) using a ratio of 16:1 settled resin per unit of enzyme.

Recombinant protein was tested for PLA₁ activity by incubating 0.5µg of LYPLA-I/APT1 or 0.05µg GST-LYPLA-I/APT1 with 0.9µg BSA and 0.9µg NBD-PS in 20µl of 10mM TRIS-HCl (pH 7.6) for 15min at 37°C. Water-saturated BuOH (30µl) was added to stop the reaction and extract the lipids after centrifugation. Samples (10µl) of the BuOH phase were spotted on Silica Gel 60 TLC plates which were developed with solvent A. The products were visualized using a Fotodyne imager.

2.2.12 Monitoring LPC and LPA Production in Plasma

Blood (5ml) was drawn into a heparinized or non-treated Vacutainer tubes. Blood was centrifuged at 14000 x g for 1min and the PRP was transferred to a new tube. The

PRP from the tube with no additive was allowed to clot to generate serum. An amount of 10µg recombinant LYPLA-I/APT1 enzyme was added to 1mL activated plasma and incubated at 37°C for 24 hours alongside 1mL activated plasma without addition of the enzyme. The internal standards 17:0 LPA and 17:0 LPC (20ng each) were added to 200µl of the fresh PRP or to 200µl of the serum samples after 24 h incubation. A total of 200µl of citrate phosphate buffer (30mM citric acid and 40mM Na₂HPO₄, pH 4.0), 400µl methanol, and 200µl chloroform were added to the samples and centrifuged at 14000 x g for 1min. The chloroform layer was transferred to a new tube, dried under argon gas and reconstituted in 30µl methanol:acetonitrile:isopropanol:water (1:1:1:1). LPA 18:2 and LPA 20:4 were quantified by LC-MS/MS/MS as described above.

2.2.13 Synthesis of *sn*-2 lyso-PAF 18:1

Synthesis and characterization of *sn*-2 lyso-PAF 18:1 is described in the accompanying Supplemental information.

2.2.14 Determination of Regioisomeric Selectivity of ATX Against Two Lyso-PAF Substrates

Serial dilutions of either *sn*-1 or *sn*-2 LysoPAF 18:1 (2 mM – 31.25 µM, in 20µl assay buffer, 50mM TRIS, pH7.4, 5mM CaCl₂, 1mg/mL BSA) were added to the wells in a 96 well, half area plate. A total of 20µl of either 10nM ATX (final concentration) or assay buffer were also added to the wells along with 20µl Amplex Red cocktail (10µM Amplex Red, 0.1 Unit/mL choline oxidase (CO), and 1 unit/mL horse radish peroxidase (HRP), final concentration (Amplex Red, Invitrogen, Carlsbad CA; CO, MP Biomedical, Solon OH; HRP, ThermoFisher Scientific, Waltham, MA). The plate was incubated at 37°C for 6 hours and was read at excitation/emission wavelengths of 530/590nm every 2 minutes with a BioTek Synergy 2 plate reader (Winooski, VT). A linear segment of time vs concentration of product was plotted for each substrate concentration to determine initial reaction velocity. In order to calculate product concentration, a linear segment of absorbance values of serial dilutions of resorufin (2000 - 0.0004768µM) was used to establish a calibration curve of resorufin fluorescence. Resorufin and product concentration are directly proportional in the Amplex Red assay, thus experimental absorbance values were then interpolated on the calibration curve to determine product concentration. Substrate concentration vs initial velocity was then plotted using GraphPad Prism[®] version 5.0a for Mac OS X, and a rectangular hyperbolic curve fit was used to define kinetic parameters.

2.3 RESULTS

2.3.1 Phospholipases Released from Activated Platelets Generate Lysophospholipids but LPA Production Requires ATX from Plasma

Platelet activation triggers an upsurge in production of LPA molecular species predominantly with 18:2 and 20:4 fatty acids (Mauco *et al.*, 1978; Schumacher *et al.*, 1979; Eichholtz *et al.*, 1993; Gaits *et al.*, 1997; Baker *et al.*, 2001; Sano *et al.*, 2002; Aoki *et al.*, 2008). Using biosynthetic labeling of platelet phospholipids it has been shown that only trace amounts of ^{32}P -LPA were found in the supernatant of activated platelets (Sano *et al.*, 2002). To extend this observation concerning the lack of LPA production by platelets we tested the hypothesis that platelets secrete phospholipase(s) that *de novo* generate a pool of LPL that in turn serve as substrates for ATX constitutively present in plasma (Tokumura *et al.*, 1986; Tokumura *et al.*, 1998; Tokumura *et al.*, 2000). The first question we raised in pursuing this hypothesis was whether platelet activation by thrombin leads to the release of PLA₁ detectable in the supernatant. To determine this PC18:2 (Figure 2.1A), PS18:1 (Figure 2.1B), NBD-PC (Figure 2.1C), or NBD-PS (Figure 2.1D) were incubated with Sup2 in the presence or absence of ATX. We found significant LPC generation in Sup2 regardless of whether we used PC 18:2 or NBD-PC as substrate. However, very little LPA was produced. Using the fluorescent NBD-PC substrate LPA production was below the level of detection of our assay; however, using mass spectrometry we detected a $0.028 \pm 0.06\text{ng}/\mu\text{g}/\text{h}$ rate of LPA production. When PS 18:1 was used for substrate we observed the production of LPS 18:1 and a higher rate of LPA production amounting to $4.2 \pm 0.8\text{ng}/\mu\text{g}/\text{h}$. However, using NBD-PS we still were unable to detect LPA production. In contrast, when recombinant human ATX was added, a 9.5 fold increase in the amount of LPA generated from LPC and a 2 fold increase in the amount of LPA generated from LPS with a concomitant decrease in the LPC or LPS was observed. When NBD-PC or NBD-PS analogs were used, essentially the same findings were noted validating the applicability of these fluorescent analogs for monitoring LPL production. The finding of high rates of LPC and LPS production in the Sup2 suggest that phospholipases are secreted from platelets, but lysophospholipase D is not present in sufficient quantity to provide for substantial LPA production. The results with NBD phospholipids indicate that substantial amounts of PLA₁ activity are present in Sup2 because the predominant product formed was NBD-LPC and NBD-LPS with the fluorescent label in the *sn*-2 position. We hypothesized that phospholipase(s) A₁ are secreted upon activation of platelets and we next attempted to purify these enzymes.

2.3.2 Partial Purification of Phospholipase A₁ from Supernatants of Activated Human Platelets

To further substantiate the hypothesis that a novel PLA₁ is secreted from platelets we partially purified this enzyme from Sup2. Sup2 from human platelets was prepared and applied to Q Sepharose Fast Flow ion exchange chromatography using an AKTA FPLC system. Fractions (3mL) were collected and each fraction was assayed for PLA₁.

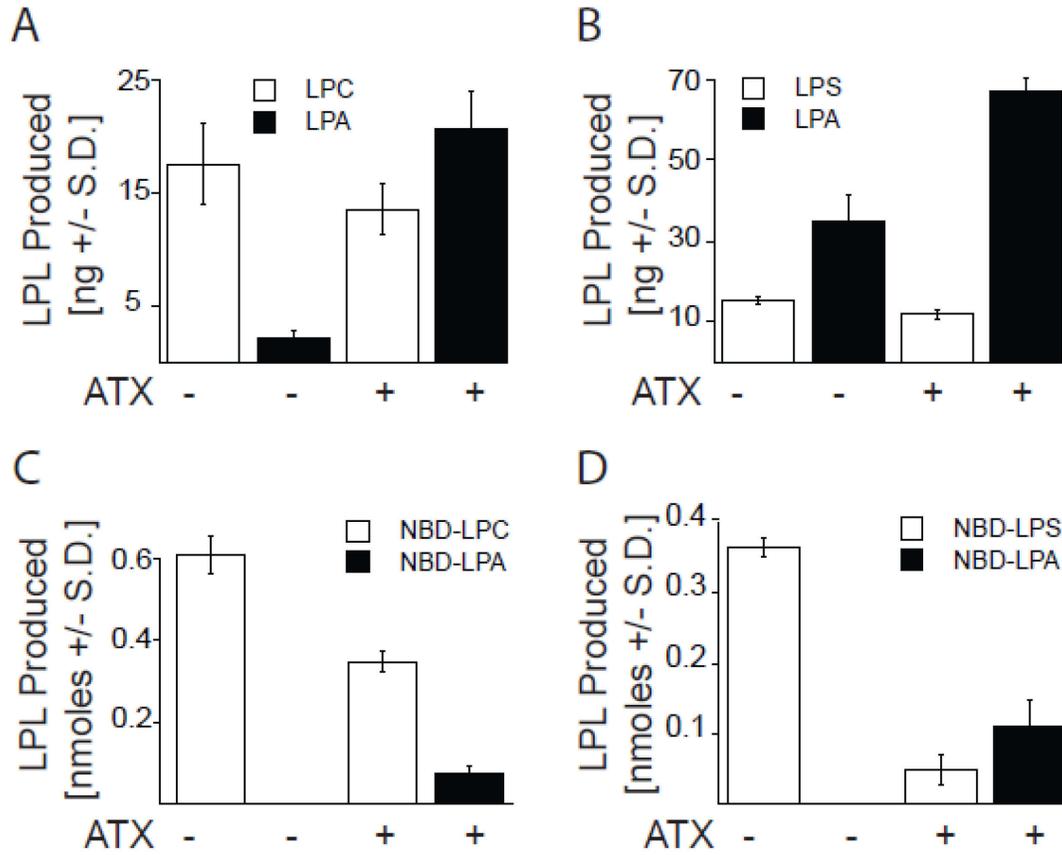


Figure 2.1 ATX is required for LPA production in activated platelet supernatant. Supernatant from thrombin-activated human platelets was incubated in the absence or presence of recombinant human ATX with 10 μ g PC 18:2 (panel A), 10 μ g PS 18:1 (panel B), 1 μ g NBD-PC 18:1/12:0 (panel C), or 1 μ g NBD-PS 18:1/12:0 (panel D) supplemented with 250 μ M BSA and 135mM NaCl in the presence or absence of 200nM ATX at 37 $^{\circ}$ C for 1 hour. Generation of the corresponding LPC and LPA species was quantified using LC/MS (panels A & B) or TLC (panels C & D). In the absence of added ATX very little LPA was produced. In sharp contrast, LPC was abundantly generated by a PLA activity. Addition of ATX leads to a 9.5-fold increase in the amount of LPA generated from LPC and a 2-fold increase in the amount of LPA generated from LPS with a concomitant decrease in the LPC or LPS. When the unnatural NBD-PC/PS analogs were used, essentially the same findings were noted validating the usability of these fluorescent analogs for monitoring LPA production. Bars are the mean of 3 independent determinations \pm S.D.

activity using NBD-PS as a substrate. Most PLA₁ activity with low PLA₂ activity contamination was found in the last portion of the flow-through fractions and no PLA₁ activity was detected in the bound fractions. The active fractions were combined and concentrated using a 5kDa Amicon concentrator to exchange the buffer and load onto a Butyl-Sepharose column. The resulting active fractions were then loaded onto a HiTrap Blue affinity chromatography column to eliminate albumin from the sample and the PLA₁ was recovered from the flow through and concentrated as above. The specific PLA₁ activity present in the various pooled fractions is shown in Table 2.1. The three chromatographic steps resulted in a 1500-fold purification of PLA₁ activity

2.3.3 Activity Based Purification of Platelet PLA₁

Even though the multistep chromatographic purification yielded a 1500-fold enrichment of PLA₁ activity the sample contained multiple protein bands when examined using SDS-PAGE. Further chromatographic purification led to loss of activity necessitating a change in the purification strategy. Most phospholipases contain a serine in the catalytic site which selectively reacts with fluorophosphonate (FP) (Leung *et al.*, 2003). Most serine hydrolases are potently inhibited by FP or its derivatives. However, FPs do not inhibit cysteine, aspartyl, or metalloproteases (Liu *et al.*, 1999). Rhodamine- and biotin-labeled FP probes are rapid, selective, and are highly sensitive affinity labels for serine hydrolases. In these probes the fluorescent reactive group or the biotin tag is coupled through a long alkyl chain and two amide bonds (Liu *et al.*, 1999).

We first determined if the PLA₁ activity present in platelets reacted with a FP probe. A Butyl Sepharose fraction was incubated with incremental amounts of FP-RH and NBD-PS substrate. An increase in concentrations of FP-RH produced a decrease in NBD-LPS production presumably due to a competition between FP-RH and NBD-PS for the catalytic site of PLA₁ (Figure 2.2A). This experiment provided evidence that PLA₁ was a serine hydrolase and reacted with FP probes. FP-RH optimally inactivated PLA₁ at 5 μ M and 2h of incubation (data not shown).

2.3.4 Identification of Serine Hydrolases Secreted from Thrombin Activated Human Platelets

Since we determined that PLA₁ reacted with the FP probes, we then labeled the partially purified PLA₁ obtained after the HiTrap Blue chromatography step with FP-biotin. The FP probe allowed us to selectively label all serine hydrolases present in our sample and the biotin allowed us to isolate the labeled proteins using streptavidin beads. We analyzed samples from three different purifications. Tandem mass spectrometry (MS/MS) data were obtained for the tryptic peptides present in the digest. The MS/MS data, which are diagnostic of the peptide sequences, were used to identify the proteins of interest through protein database searches. Based on LC-MS/MS data, four relevant candidate proteins were identified: lecithin-cholesterol acyltransferase (LCAT), phosphatidylserine specific phospholipase A₁ (PS-PLA₁), monoglyceride lipase (MGLL),

Table 2.1 Summary of chromatographic steps.

Step	$\mu\text{g protein}/\mu\text{l}$	Specific activity (units*/ μg)	Purification (fold)
Platelet supernatant (Sup2)	75.3 +/- 6.2	0.10 +/- 0.03	1
Q sepharose	16.2 +/- 1.7	1.6 +/- 0.4	16
Butyl sepharose	0.08 +/- 0.02	89.2 +/- 4.4	872
HiTrap blue	0.03 +/- 0.01	159.5 +/- 27.6	1560

*Units defined in nanomoles of LPS formed/h at 37°C.

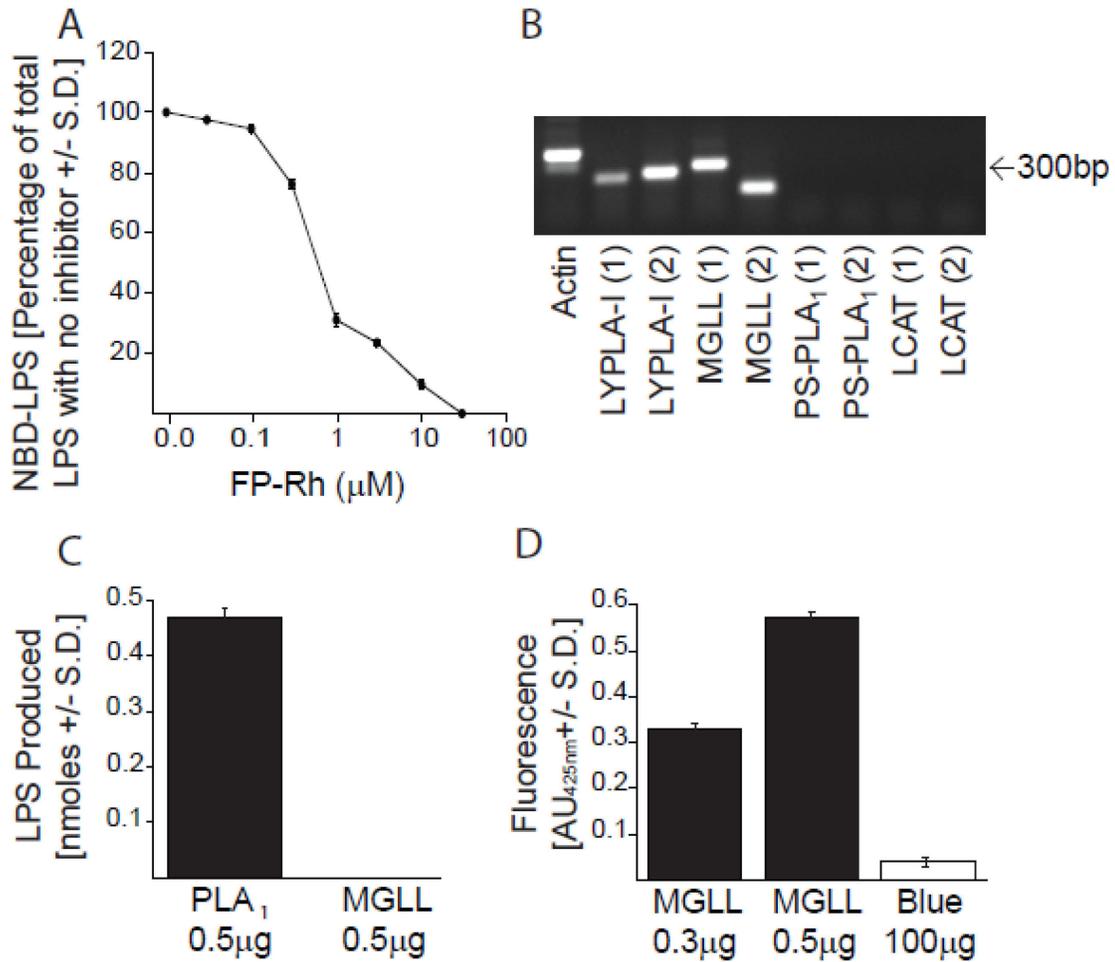


Figure 2.2 FP-Rhodamine binds to PLA₁ inhibiting activity and NBD-LPS production. Incremental amounts of FP-Rhodamine were incubated with NBD-PS and a phospholipase A1 active Butyl Sepharose fraction (panel A). The production of NBD-LPS decrease with increasing amounts of FP-RH. Expression of serine hydrolase transcripts in human platelets (panel B). RT-PCR for LYPLA-I/APT1, MGLL, PS-PLA₁ and LCAT was performed using two different gene-specific primer pairs (denoted 1 & 2) using mRNA isolated from human purified platelets. Only LYPLA-I/APT1 and MGLL amplification yielded PCR products of the expected size indicating the expression of these enzymes in human platelets. These results have been confirmed with platelets from 5 other donors of both sexes. MGLL does not possess PLA activity (panels C & D). When incubated with NBD-PS no LPS was formed thereby indicating MGLL does not possess PLA activity (panel C). Human recombinant MGLL was shown to have MGL activity to prove functionality (panel D). Bars are the mean of 3 independent determinations \pm S.D.

and lysophospholipase A-I also known as acyl protein thioesterase 1 (LYPLA-I/APT1) (Table 2.2).

Because we used platelet rich plasma as a starting material, plasma protein contamination in our sample was a possibility, hence it was important to determine whether the proteins identified in the sample were expressed in platelets. To this end, mRNA was isolated from human platelets and RT-PCR for LYPLA-I/APT1, MGLL, PLA1A and LCAT was performed using two different gene-specific primer pairs (denoted 1 & 2, Figure 2.2B). The eight primer sets were validated to amplify the appropriate transcripts derived from selected tissues. Only two of the four lipases identified by LC-MS/MS, namely LYPLA-I/APT1 and MGLL, yielded PCR products of the expected size indicating the expression of these enzymes in human platelets.

We hypothesized that either MGLL, LYPLA-I/APT1, or both might play a role in LPA production during blood coagulation. In order to determine whether any of these proteins is involved in LPA production we needed to first establish if either enzyme possessed PLA₁ enzymatic activity. Human recombinant MGLL was tested for PLA₁ activity (Figure 2.2C). No cleavage of NBD-PS or generation of NBD-LPDS product was detected suggesting that it does not function as a PLA₁. Using as much as 0.5µg recombinant MGLL in a MGL activity assay (Figure 2.2D) we verified that the enzyme was active even though it lacked PLA₁ activity. Furthermore, using the Blue-Agarose fraction rich in PLA₁ activity we were unable to detect MGLL activity using this same assay. These results suggested that MGLL does not have PLA₁ activity and therefore is unlikely to contribute to the production of LPA during blood coagulation.

We next tested whether LYPLA-I/APT1 functioned as a PLA₁. Human recombinant GST-LYPLA-I/APT1 was expressed and purified from transformed *E.coli*. Coomassie Blue stained SDS-PAGE showed that the purified GST-tagged LYPLA-I/APT1 and the LYPLA-I/APT1 with the GST-tag removed yielded a single protein band with the expected 25 KDa size (Figure 2.3A). PLA₁ activity assays using 1µg NBD-PC in 20µl 10mM TRIS (pH 7.9) at 37°C for 10min performed on GST-tagged LYPLA-I/APT1 and thrombin-cleaved LYPLA-I/APT showed no detectable PLA₁ activity with GST-tagged enzyme (Figure 2.3B). In contrast, the LYPLA-I/APT1 with the tag removed showed activity when incubated with NBD-PC indicating that the GST tag inhibits activity of the enzyme and that LYPLA-I/APT1 possesses phospholipase A₁ activity.

2.3.5 Recombinant Human LYPLA-I/APT1 Acts Primarily as a Phospholipase A₁ and Does Not Break Down LPA

LYPLA-I/APT1 has two known functions to date. As an acyl-protein thioesterase it has been shown to deacylate G proteins and other thioacylated protein substrates (Duncan and Gilman, 1998). As a lysophospholipase it cleaves the remaining fatty acid from either carbon of the glycerol backbone of lysophosphatidylcholine or

Table 2.2 LC-MS/MS Candidate protein peptide sequences.

Protein name	Entry name	Accession code	Theoretical molecular weight (kDa)	Peptide sequence*	Occurrence of peptide found in samples
Lecithin-cholesterol acyltransferase	LCAT_HUMAN	P04180	67	DLLAGLPAPGVEVYC*L	3
				YGVGLPTPR	
				TYSVEYLDSSK	2
				SSGLVSNAPGVQIR	4
				LDKPDVVNWM#C*YR	3
				ITTTSPWMFPSR	3
				TYIYDHGFPYTDPVGV	2
Lysophospholipase A-I	LYPA1_HUMAN	O75608	25	YEDGDDTVATR	
				LAGVTALSC*WLPLR	3
				ASFPQGPIGGANR	5
				TYEGMMHSSC*QQEMM	5
				DVK	
Phosphatidyl-serine specific PLA ₁ Monoglyceride Lipase	PLA1A_HUMAN	Q53H76	55	TLVNPANVTFK	6
				FC*TALLPVNDR	1
				C*ADFQSANLFEGTDLK	1
MGLL_HUMAN	Q99685	59	LTVPFLLLQGSADR	1	

* C* indicates carbamidomethylated cysteine. M# indicates oxidized methionine.

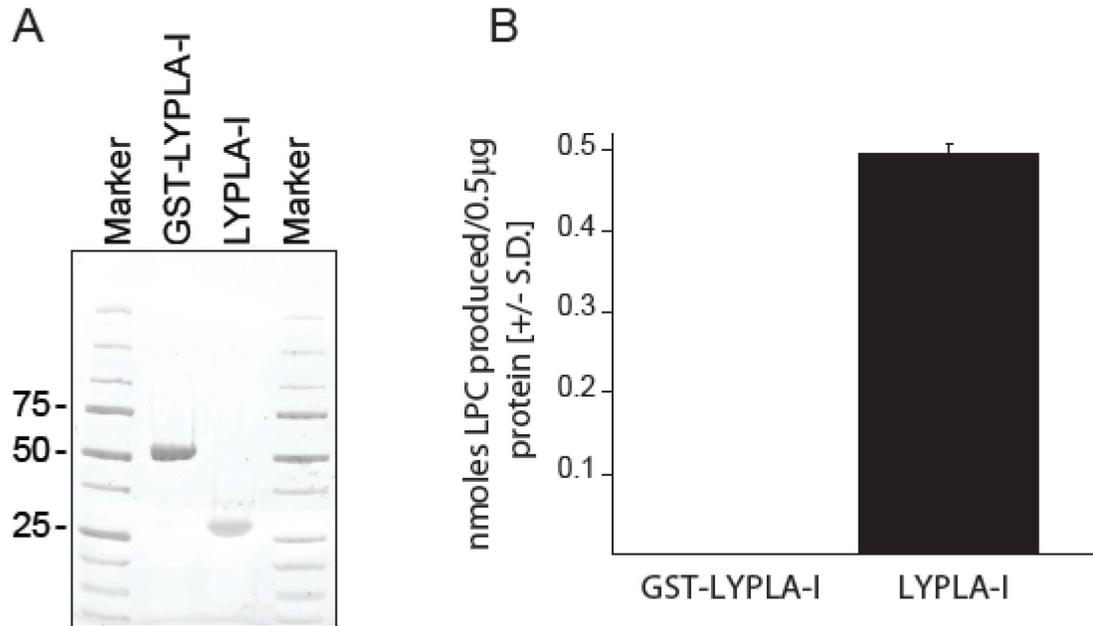


Figure 2.3 Human recombinant LYPLA-I/APT1 with and without a GST tag are purified for analysis. Coomassie Blue stained SDS-PAGE gels show the protein profiles of the GST tagged LYPLA-I/APT1 protein and the thrombin-cleaved LYPLA-I/APT1 protein (5µg each). Human recombinant LYPLA-I/APT1 must be unlabeled for functionality (panel B). PLA activity assay was performed on both GST tagged LYPLA-I/APT1 and thrombin-cleaved untagged LYPLA-I/APT1 (0.5µg each). No activity was found when the GST tag was present. The thrombin-cleaved version of LYPLA-I/APT1 has abundant PLA1 activity when incubated with NBD-PC in the presence of BSA. Bars are the mean of 3 independent determinations \pm S.D.

lysophosphatidylserine. Because this protein has lysophospholipase activity, it was challenging to separate the PLA₁ activity from the lysophospholipase activity as the product of the PLA₁ activity is also the substrate of the lysophospholipase activity. Using unlabeled substrate to measure PLA₁ activity of the recombinant enzyme was not feasible, as we were unable to distinguish how much of the product was being cleaved by the lysophospholipase activity precluding the determination of the specific PLA₁ catalytic activity. In order to establish a timeline for the dual activities of the same enzyme we used NBD-PC, which allowed us to independently monitor the generation of NBD-LPC (product of PLA₁ cleavage) and NBD-FA (product of LYPLA-I/APT1 cleavage) products for both enzymatic activities and at least initially monitor reaction preference. NBD-PC, NBD-LPC, and NBD-FA were quantified as percentage of total substrate and product (Figure 2.4A). Eleven percent of added NBD-PC was converted to NBD-LPC within 1min indicating the robust PLA₁ activity of the enzyme. In contrast, NBD-FA production reached a comparable amount, only after 5min of incubation, signifying further that the initial and preferred product of LYPLA-I/APT1 was NBD-LPC. NBD-LPC reached a maximum steady-state level of 25% after 5min, whereas NBD-FA generation continued steadily with the availability of NBD-LPC substrate for the LYPLA-I/APT1 activity of the enzyme. NBD-FA product could also be generated by PLA₂ cleavage of NBD-PC; however, this product was not seen initially making such catalytic activity unlikely. Along with the generation of NBD-LPC and NBD-FA there was a continuous decrease in the NBD-PC substrate. This time course is consistent with the hypothesis that human recombinant LYPLA-I/APT1 acts preferentially as a PLA₁ and only secondarily as a LYPLA-I/APT1.

The hypothesis that LYPLA-I/APT1 would supply LPL to ATX and thereby contribute to LPA production can only be sustained if LYPLA-I/APT1 would not cleave LPA and degrade it. To test this hypothesis recombinant LYPLA-I/APT1 was incubated with LPA 18:1 for up to 1 hour and the amount of LPA degraded was quantified by LC-MS/MS (Figure 2.4B). The amount of LPA did not decrease during the 1-h-long incubation indicating that LYPLA-I/APT1 is not a substrate for LYPLA-I/APT1 and therefore, it does not contribute to the break-down of LPA.

2.3.6 LYPLA-I/APT1 Contributes to the Production of LPA during Blood Coagulation

The predominance of 18:2 and 20:4 LPA species in serum indicate that PLA₁ cleavage must be involved in this biochemical pathway because these fatty acids are overwhelmingly esterified to the *sn*-2 carbon of phospholipids. ATX has low, but constitutive activity for *sn*-1 LPC present in plasma indicated by the low concentration of LPA and the rather slow increase in LPA in heparinized plasma *ex vivo* (Figure 2.5A & 2.5B) (Sano *et al.*, 2002). Therefore, ATX alone cannot account for the upsurge in LPA production subsequent to activation of blood clotting. We hypothesized that LYPLA-I/APT1 release from activated platelets is, at least in part, responsible for the increase in

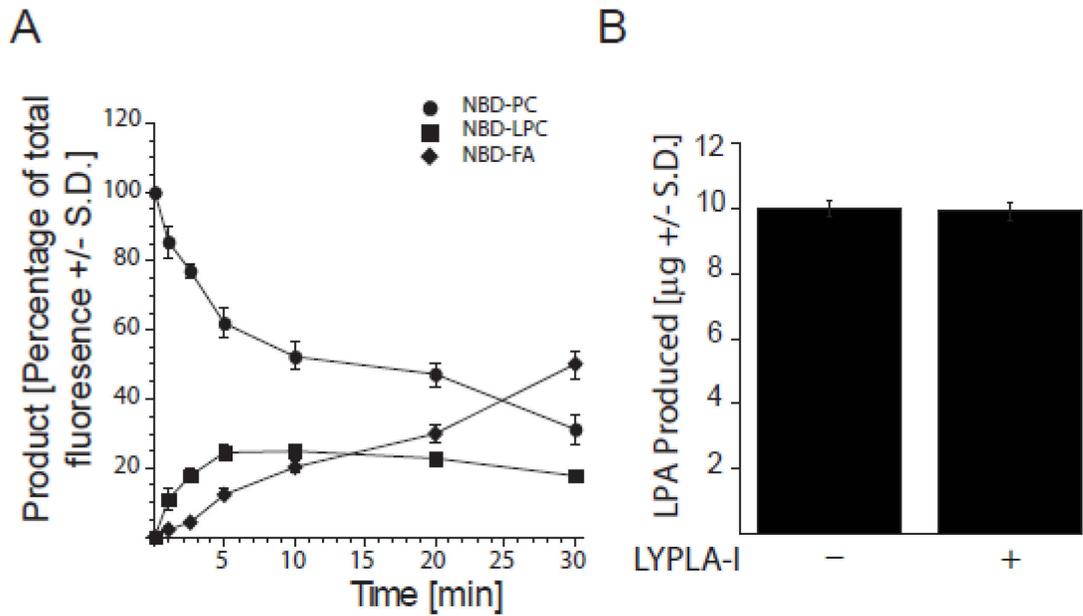


Figure 2.4 Human recombinant LYPLA-I/APT1 acts first as a PLA₁ then also has lysophospholipase activity when incubated with NBD-PC. Purified human recombinant LYPLA-I/APT1 was incubated with NBD-PC and BSA for 0, 1, 2.5, 5, 10, 20, and 30min (panel A). NBD-LPC is generated immediately indicating PLA₁ activity of the enzyme. After the initial generation of NBD-LPC, NBD-FA is generated indicating a lysophospholipase activity of the enzyme with a simultaneous decrease in the starting substrate. Human recombinant LYPLA-I/APT1 does not cleave LPA and therefore does not contribute to the break-down of LPA (panel B). LYPLA-I/APT1 was incubated with LPA 18:1 in the presence of BSA for 1 hour. The amount of LPA did not decrease during incubation indicating that LPA is not a substrate for LYPLA-I/APT1. Bars are the mean of 3 independent determinations \pm S.D.

LPA production by supplying a newly formed pool of LPL to ATX upon the activation of platelets. To examine this, heparinized plasma and human activated plasma generated by physiological means in venous blood were incubated with or without exogenously added human recombinant LYPLA-I/APT1 for 24 hours and the production of polyunsaturated LPA species was quantified using LC-MS/MS. Upon addition of LYPLA-I/APT1, LPA 18:2 (Figure 2.5A) and LPA 20:4 (Figure 2.5B) began increasing in a time-dependent manner in physiologically-activated plasma. By the end of the 24-h incubation, addition of LYPLA-I/APT1 to activated plasma significantly increased the amount of LPA 18:2 and 20:4. The generation of these LPA species in activated plasma without the addition of LYPLA-I/APT1 also increased but to a much lower extent compared to the LYPLA-I/APT1 spiked samples. It is important to note that these samples contain endogenous LYPLA-I that also contributes to the production of LPC. There was no significant increase in either LPA species when platelet activation was prevented by heparin and the removal of plasma from the blood cells within 5min of blood collection. However, there was a small increase at 24h with the addition of LYPLA-I/APT1 to heparinized plasma suggesting the enzyme could utilize physiological substrates. We also determined that heparin (18.75 U/mL) used for anticoagulation of blood inhibited 90% of the activity of ATX although it did not inhibit the phospholipase A₁ activity of LYPLA-I/APT1 (data not shown).

To extend these observations we tested the effect of exogenous LYPLA-I/APT1 addition on LPA 18:2 and LPA 20:4 production in a small sample of human donors from both sexes. Activated plasma was incubated with or without 10µg/mL human recombinant LYPLA-I/APT1 for 24 hours and the amount of LPC 18:2, LPC 20:4, LPA 18:2 and LPA 20:4 was quantified using LC-MS/MS. LPC 18:2 (Figure 2.6C) increased significantly by 1.5-fold with the addition of LYPLA-I/APT1 in contrast to a 1.3-fold increase without LYPLA-I/APT1. Similarly, LPC 20:4 (Figure 2.6D) increased 2.8-fold with the addition of LYPLA-I/APT1 and 2.5-fold without LYPLA-I/APT1. In the same samples, LPA 18:2 (Figure 2.6A) increased 3.5-fold over 24h in comparison to 2.6-fold without LYPLA-I/APT1. LPA 20:4 (Figure 2.6B) increased 4.6-fold without the addition of LYPLA-I/APT1 relative to the level found at the beginning of the incubation and 5.8-fold with the addition of LYPLA-I/APT1. The addition of LYPLA-I/APT1 significantly increased ($p < 0.05$ compared to time 0 analyzed by one-way ANOVA) the production of polyunsaturated LPC and LPA indicating that this enzyme contributes to the production of LPA during blood coagulation.

2.3.7 Regioisomeric Preference of ATX

To further understand the mechanism of LPA production upon blood coagulation we wanted to determine the regioisomeric preference of ATX. We have determined that LYPLA-I/APT1 cleaves phospholipids to yield *sn*-2 LPLs. These LPLs can either undergo acyl migration and then be further cleaved by ATX to yield *sn*-1 LPA or the *sn*-2 LPLs can first be cleaved by ATX yielding *sn*-2 LPA that then undergoes acyl migration

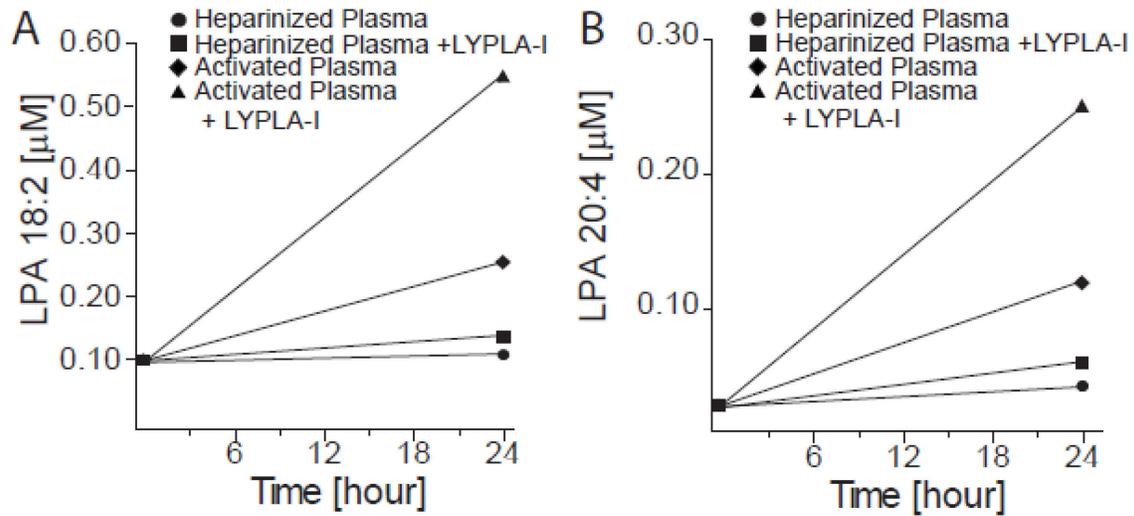


Figure 2.5 Human recombinant LYPLA-I/APT1 increases the amount of LPA 18:2 and LPA 20:4 in a time-dependent manner. Physiologically activated plasma and plasma with heparin additive were incubated with or without human recombinant LYPLA-I/APT1 for 24 hours. LPA 18:2 and LPA 20:4 in activated plasma began increasing after 3 hours and increased greatly at 24 hours. In contrast, in heparinized plasma LPA 18:2 and LPA 20:4 showed no increase except in the sample with LYPLA-I/APT1 at 24 hours.

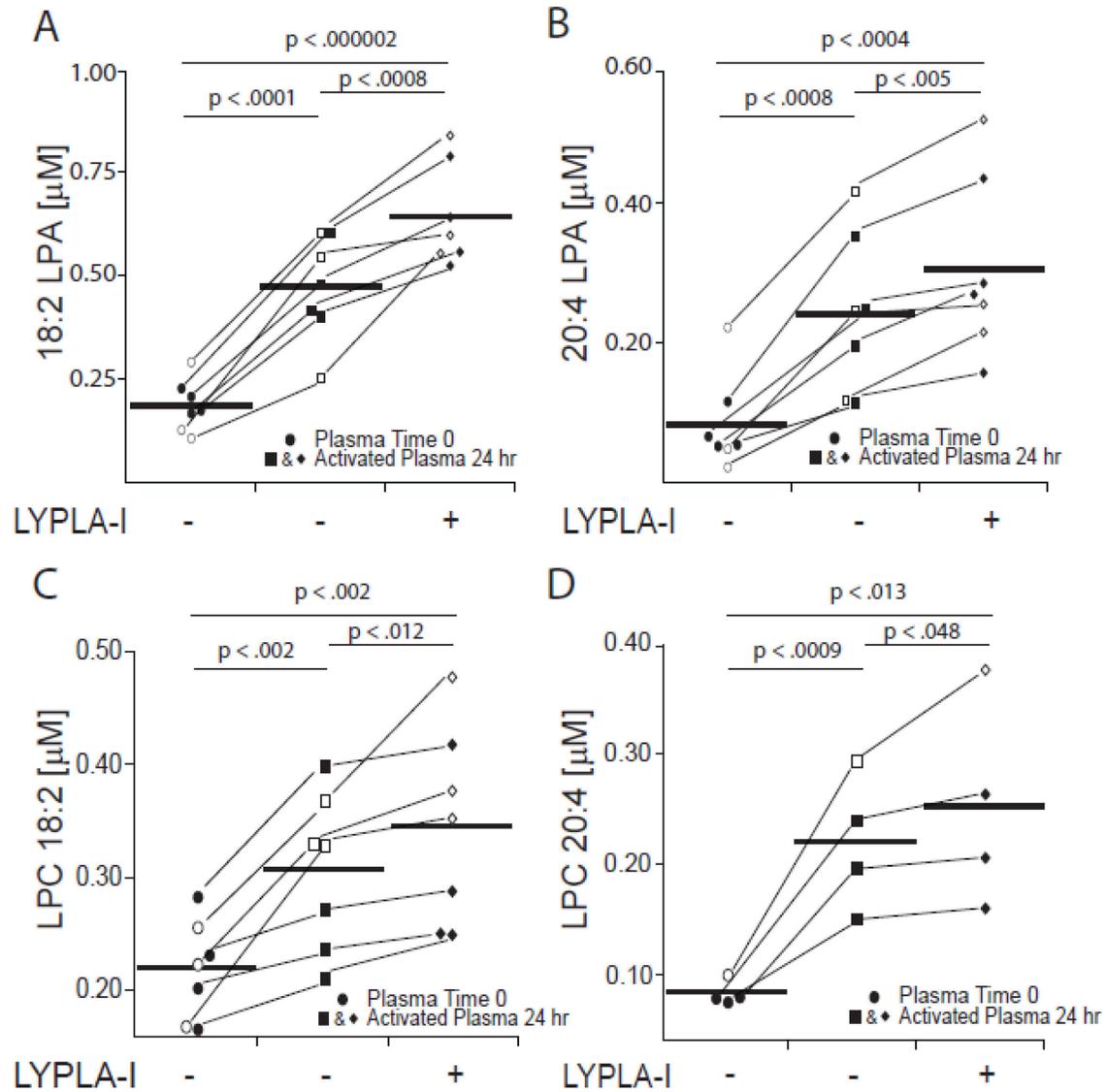


Figure 2.6 Human recombinant LYPLA-I/APT1 increases the amount of LPA produced. The addition of LYPLA-I/APT1 increases the amount of LPA 18:2 (panel A), LPA 20:4 (panel B), LPC 18:2 (panel C), and LPC 20:4 (panel D) produced in physiologically activated plasma. Physiologically activated plasma was incubated with or without human recombinant LYPLA-I/APT1 for 24 hours. LPA 18:2 and LPA 20:4 increased in 24 hours in comparison to the amount present in plasma immediately after blood draw. Production of LPA and LPC substantially increased with the addition of LYPLA-I/APT1 showing that LYPLA-I/APT1 plays a role in the production of LPA during blood coagulation. These results are from 3 female donors and 4 male donors (panel A, B & C) or 1 female donor and 3 male donors (panel D). Open symbols represent female donors and closed symbols represent male donors ($*p < 0.05$ 24 hour activated plasma with LYPLA-I vs. without LYPLA-I analyzed by paired t-test).

to form *sn-1* LPA. Since the *sn-2* regioisomer of LPC is relatively unstable ($T_{1/2} \sim 30$ min at pH 8.0), especially during incubation at 37°C (Pluckthun and Dennis, 1982) (acyl migration generates a 9:1 excess of the *sn-1* form), we were unable to use the natural substrate for ATX to experimentally determine substrate preference. In order to prevent acyl migration we used LPAF, an analogue of LPC in which the fatty acid is ether linked to the glycerol backbone. Using an Amplex Red, fluorescence-based choline release assay we determined the K_m and V_{max} of the *sn-1* regioisomer to be $96 \pm 10 \mu\text{M}$ and $0.11 \pm 0.003 \mu\text{M} \cdot \text{min}^{-1}$, respectively. In contrast, the K_m and V_{max} of the *sn-2* regioisomer were $51 \pm 4 \mu\text{M}$ and $0.03 \pm 0.004 \mu\text{M} \cdot \text{min}^{-1}$, respectively (Figure 2.7). The k_{cat} value for the *sn-1* regioisomer was $11 \pm 0.03 \text{min}^{-1}$ whereas for the *sn-2* regioisomer it was $3 \pm 0.14 \text{min}^{-1}$. K_m and k_{cat} values help to assess the efficiency of the enzymes against a given substrate: however, these values should not be used alone when considering the kinetic parameters of an enzyme. To better compare the enzyme efficiency for the two substrates the ratio k_{cat}/K_m (specificity constant) can be used. This provides a measure of catalytic efficiency and allows for direct comparison of the efficiency of an enzyme towards different substrates. The k_{cat}/K_m value for the *sn-1* and *sn-2* regioisomers of LPAF 18:1 were $0.12 \pm 0.01 \mu\text{M}^{-1} \cdot \text{min}^{-1}$ and $0.05 \pm 0.01 \mu\text{M}^{-1} \cdot \text{min}^{-1}$ respectively (Table 2.3). Thus, the k_{cat}/K_m of the *sn-1* regioisomer was 2.3 times higher than that of the *sn-2* regioisomer indicating that ATX catalyzes the *sn-1* regioisomer of LPAF 18:1 more efficiently than the *sn-2* regioisomer.

2.4 DISCUSSION

We have identified LYPLA-I/APT1 as an enzyme with PLA_1 activity that is released from human platelets during blood clotting that contributes to the generation of polyunsaturated species of LPA abundant in serum. Since the early reports of Schumacher *et al.* and Mauco *et al.* (Mauco *et al.*, 1978; Schumacher *et al.*, 1979) in the late seventies, it has been known that platelet activation and LPA production are coupled, however, the precise mechanism remained unknown (Eichholtz *et al.*, 1993; Gaits *et al.*, 1997; Sano *et al.*, 2002; Aoki *et al.*, 2008). Previously we have shown that only trace amounts of LPA are generated in activated platelets, far less than that could account for the rise in LPA concentration from the nanomolar range in plasma to the several micromolar level found in serum (Sano *et al.*, 2002). The attention of the field has since been focused on the hypothesis that activated platelets release phospholipases, which generate a new pool of LPLs that are subsequently converted to LPA by ATX (Aoki *et al.*, 2002b; Sano *et al.*, 2002). Because more than eighty percent of LPA in serum is composed of the polyunsaturated 18:2 and 20:4 acyl species, we proposed that the LPL pool accessed by the constitutive ATX in plasma is likely to be generated by PLA_1 enzyme(s) derived from activated platelets (Sano *et al.*, 2002). In pursuit of this hypothesis in the present study we report on the identification of one such PLA_1 enzyme previously known as LYPLA-I/APT1, which contributes to the increase in LPA production during blood coagulation. Even though our results suggest that this PLA_1 is a major contributor to the increase in LPA production, it may not be the only contributing phospholipase released by activated human platelets.

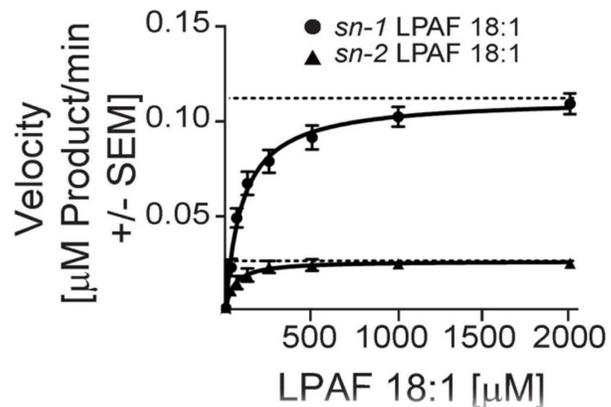


Figure 2.7 Comparison of the cleavage of *sn-1* with *sn-2* LPAF 18:1 by ATX. A linear segment of time vs. amount of product was plotted for each substrate concentration (31.25µM – 2mM) to determine initial velocity. Substrate concentration vs. initial velocity was plotted. All absorbance values were converted to µM resorufin produced to report as actual product. Bars are the mean of 3 independent determinations ± SEM.

Table 2.3 Kinetic parameters for ATX-mediated hydrolysis of LPAF 18:1.

LysoPAF	K_m (μM)	V_{max} ($\mu\text{M}\cdot\text{min}^{-1}$)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu\text{M}^{-1}\cdot\text{min}^{-1}$)
<i>sn-1</i>	96 +/- 10	0.11 +/- 0.003	11 +/- 0.03	0.12 +/- 0.01
<i>sn-2</i>	51 +/- 4	0.025 +/- 0.004	3.0 +/- 0.1	0.050 +/- 0.01

n=3, average values +/- standard error.

Previous studies have used fluorescent phospholipids or [³²P]-orthophosphate for biosynthetic labeling of the substrates to monitor platelet-derived phospholipases (Sano *et al.*, 2002). We developed LC-MS/MS techniques to monitor the sequential cleavage of PC/PS to LPC/LPS to LPA without the use of unnatural, fluorescently labeled substrates. Sano *et al.* previously showed that when fluorescently labeled PC, PE, or PS was incubated with supernatant from thrombin-stimulated platelets no LPA production could be detected. However, when plasma or serum was added LPA generation commenced suggesting that elements of the biochemical pathway were missing from the platelet supernatant (Sano *et al.*, 2002). Using our newly developed method we were able to directly demonstrate that ATX addition to platelet supernatant significantly increases LPA production indicating that there is *de novo* generation of ATX substrates within the platelet supernatant. Using *sn*-2 NBD-labeled PC and PS, we found overwhelming hydrolytic activity generating NBD-LPC/LPS consistent with the abundant presence of PLA₁ activity in the platelet supernatant. Under these conditions we found much less NBD-FA generation from either NBD-labeled phospholipid substrate, indicating that PLA₂ activity was lower than corresponding PLA₁ activity toward these substrates. In the absence of exogenously added recombinant ATX, although very little, more LPA was generated from PS than from PC. This may indicate that some LPLD enzymes are present in the supernatant that prefer LPS over LPC or that trace amounts of contaminating ATX from plasma was present in the supernatant. It is also possible that the NBD-labeled lipids are poor substrates for ATX, but are good substrates for LYPLA-I. Lastly, it is known that LYPLA-I/APT1 is a multifunctional enzyme and cleaves LPC as a lysophospholipase as well as PC as a phospholipase. Thus, the lysophospholipase activity could have consumed LPC more readily than LPS, diminishing the former substrate from ATX, thereby decreasing the amount of LPA produced.

Pamuklar and colleagues (Pamuklar *et al.*, 2009) examined the interaction of ATX with β3 integrin expressed on platelets and proposed that this mechanism could localize and augment LPA production at the surface of platelets. We did not detect substantial LPA production by activated purified human platelets in the absence of added ATX (Figure 2.1). Thus, it appears that little ATX is associated with non-activated circulating human platelets and activation of β3 integrins is required for capturing ATX to the platelet surface. Whether LPA receptors expressed in platelets activate β3 integrins thereby promoting ATX binding remains to be demonstrated. Such feed-forward mechanism amplifying LPA production at the platelet surface and aggregation via LPA receptors could play a role in thrombosis and hemostasis.

In the present study using three sequential chromatographic steps we were unable to purify the PLA₁ activity to homogeneity. We pursued an affinity-labeling strategy using a fluorophosphonate probe, which selectively reacts with serine residues in the catalytic site of serine hydrolases to which most lipases belong (Leung *et al.*, 2003). Using the FP-Biotin-labeled serine hydrolases isolated from the partially-purified PLA₁ fraction, we applied LC-MS/MS in combination with database searches to identify these enzymes. We searched for proteins that were known lipases among the FP-Biotin-labeled proteins. Most lipase domain members have characteristic surface loops (β9 loops) and a lid domain (Aoki *et al.*, 2002a). These loops are part of the active site and help determine

substrate recognition. These domains helped to identify our protein of interest. We also ignored all proteins that were too large to fit the size of the PLA₁ estimated by gel filtration (25KDa and 50KDa - data not shown) and excluded any unknown peptide sequences. Using these exclusion criteria, we identified four proteins that were potential candidates for a PLA₁. We further reasoned that if the PLA₁ is released from platelets then it should be synthesized in platelets. Therefore, we looked for RNA transcripts of the four lipases in platelets. Using this criterion we were able to eliminate LCAT and PS-PLA₁, leaving MGLL and LYPLA-I/APT1 as the two remaining candidates.

We were also able to eliminate MGLL as a potential candidate protein because the recombinant enzyme lacked PLA₁ activity. We next cloned and expressed the human LYPLA-I/APT1 and unexpectedly found that the GST-tag interfered with enzymatic activity. We do not know whether the GST tag may have interfered with protein folding or may have affected the ability of the substrate to bind to the enzyme. Despite this interference, once the GST tag was removed, we determined that the recombinant LYPLA-I/APT1 had PLA₁ activity against NBD-labeled and natural PC or PS. Our attempts to determine the K_m value of LYPLA-I/APT1 with PC or PS substrates were unsuccessful because the product of PLA₁ cleavage is also the substrate of the lysophospholipase activity of the enzyme. LYPLA-I/APT1 has been shown to cleave fatty acids esterified to either the *sn-1* or the *sn-2* carbon of LPC (Wang *et al.*, 1997). However, we determined that LPA was not cleaved by LYPLA-I/APT1 indicating that this product escapes further modification by this multifunctional enzyme.

Another function of LYPLA-I/APT1 is the deacylation/depalmitoylation of G proteins, ghrelin, and other thioacylated protein substrates (Duncan and Gilman, 1998; 2002; Hirano *et al.*, 2009; Satou *et al.*, 2010). Deacylation/depalmitoylation contributes to the regulation of lipid modifications and reverses the process of thioacylation of proteins involved in signal transduction. It has been shown that the K_m of the lysophospholipase activity is about 8-times higher than the K_m value of the thioesterase activity of the enzyme. The V_{max} is also about 17 times lower for the lysophospholipase activity than the thioesterase activity (Hirano *et al.*, 2009). Based on these data it has been proposed that LYPLA-I/APT1 has a much higher thioesterase activity than lysophospholipase activity. However, *in vivo* the activity of this enzyme is likely to be affected by the availability and presentation of the different substrates.

In order to make a distinction between substrate preference for PC and LPC of LYPLA-I/APT1, we used NBD-PC substrate and followed the time course of NBD-LPC production generated by the PLA₁ activity and NBD-FA production generated through lysophospholipase activity. We observed a considerable delay in the formation of NBD-FA relative to NBD-LPC, the latter being continuously generated. For this reason, we suggest that LYPLA-I/APT1 functions preferentially as a PLA₁ and only after the build-up of LPL concentration presumably via acyl migration it begins to cleave this substrate as a lyso-PLA₁.

Without a knockout animal model or specific inhibitors of LYPLA-I/APT1 we were limited to study the effect of the enzyme on *ex vivo* LPA production in activated

plasma. Following the addition of LYPLA-I/APT1 to heparinized plasma, we detected only a very small increase in polyunsaturated LPA production at 24h suggesting that the enzyme can utilize physiological substrates. However, this increase was confounded because heparin inhibited the activity of ATX but not the PLA₁ activity of LYPLA-I/APT1. LPA production in activated plasma began to increase after 3 hours and continued up to 24 hours (the last time point tested). Spiking LYPLA-I/APT1 into activated non-anticoagulated plasma increased LPA 18:2 and 20:4 production by 5- and 4-fold, respectively. This augmentation of LPA production indicates that LYPLA-I/APT1 recognizes and cleaves plasma phospholipids and contributes to the generation of LPA during blood coagulation. Production of LPA by this pathway is limited by the amount of LYPLA-I/APT1 released from platelets and the rate with which ATX generates LPA relative to the rate with which LYPLA-I/APT1 degrades LPL.

We considered that LYPLA-I/APT1 generates a new pool of *sn*-2 LPL, which can potentially undergo acyl migration before or after headgroup cleavage by ATX. The *sn*-2 regioisomer of LPC is relatively unstable and undergoes acyl migration to the *sn*-1 position at neutral pH at 37°C. Due to the several minute-long half-life of the *sn*-2 regioisomer (Pluckthun and Dennis, 1982) we were unable to obtain biochemical proof whether the nascent LPA generated is of the *sn*-2 or *sn*-1 regioisomer because the extraction and HPLC separation of the regioisomers takes longer than 30 minutes. To obtain indirect insight into the role of acyl migration on ATX cleavage, we synthesized the *sn*-2 regioisomer of LPAF and compared its cleavage with the *sn*-1 regioisomer. ATX cleaved the *sn*-1 LPAF with a higher k_{cat}/K_m than its *sn*-2 counterpart. Based on this finding it seems logical to propose that acyl migration occurs predominantly at the LPL stage of this biochemical pathway. However, only direct measurement of the nascent LPL and LPA regioisomers can help settle this question. An additional factor that must be taken into consideration is the short half-life of LPA in blood *in vivo*. LPA appears to be continuously produced and simultaneously broken down in blood on the minute scale (Albers *et al.*, 2010). This might create a situation that may enrich the abundance of *sn*-2 regioisomer in blood relative to the *sn*-1 regioisomer because the LPA generated is broken down before or at a similar rate with which acyl migration occurs.

Based on the results of this study, we hypothesize that LPA production during blood coagulation occurs via the following steps: 1) Upon activation platelets release LYPLA-I/APT1. 2) LYPLA-I/APT1 cleaves phospholipids to generate a new pool of *sn*-2 LPL. 3) These newly generated *sn*-2 LPLs undergo acyl migration within minutes to produce a pool of *sn*-1 LPLs. 4) ATX or other lysophospholipase D enzymes cleave the *sn*-1 LPLs to generate an upsurge of *sn*-1 LPA enriched in polyunsaturated FA. This hypothesis needs further testing and the availability of LYPLA-I/APT1 knockout animals and rapid methods for the detection of LPA and LPA regioisomers will accelerate the progress toward a better understanding of LPA production in biological fluids.

2.5 SUPPLEMENTAL INFORMATION

Synthesis and characterization of *sn*-2 lyso-PAF 18:1.

2-Oleyl-*sn*-glycero-3-phosphocholine (**1**) was synthesized. The synthetic scheme began with 3-*O*-(4-Methoxybenzyl)-*sn*-glycerol (**2**), which contains the appropriate stereochemistry for the subsequent target product and was synthesized as previously described (Perly *et al.*, 1984; Rowland and Best, 2009). First, the primary alcohol of diol **2** was selectively protected through reaction with *tert*-butyldiphenyl chlorosilane to produce compound **3**. Next, ether bond formation using oleyl triflate was performed to afford fully protected glycerol **4**, followed by deprotection of the *p*-methoxybenzyl (PMB) group to provide the free alcohol of **5**. Phosphoramidite chemistry was then used to install the choline phosphodiester of **7**, at which time it was found that the cyanoethyl protecting group on the phosphate was deprotected in situ. When followed by mass spectrometry, this was found to happen during the process of purifying compound **7**. The yield for the 2-step production of **7** is somewhat low, likely due to the challenges associated with forming mixed phosphodiesters and the purification of the lyso-lipid product. Finally, the removal of the silyl protecting group of **7** yielded LPC analog **1**. It is worth mentioning that a similar approach was also pursued that involved a glycerol precursor with opposite stereochemistry, initial removal of the silyl group, and deprotection of the PMB in the final step. However, this alternative route led significant challenges in purifying the final product, and was thus abandoned in favor of the route described above. LPC analog **1** has previously been reported in enantiomerically pure (Hirth *et al.*, 1983) as well as racemic form (Huang *et al.*, 2006) using different synthetic routes.

3-*O*-(4-Methoxybenzyl)-1-*O*-(*tert*-Butyldiphenylsilyl)-*sn*-glycerol (**3**) Diol **2** was synthesized from *S*-glycerol acetonide (purchased from AK Scientific, Inc.) according to a known procedure (Perly *et al.*, 1984; Rowland and Best, 2009). Diol **2** (0.430g, 2.026mmol) was then dissolved in dry *N,N*-dimethylformamide (20ml), to which was added *tert*-butyldiphenyl chlorosilane (0.525ml, 2.026mmol), followed by imidazole (0.359g, 5.270mmol). The reaction mixture was next allowed to stir at room temperature overnight. The solvent was then concentrated under reduced pressure and the resulting residue was dissolved in chloroform (100ml) and washed with water (2 x 50ml). The organic layer was then dried with magnesium sulfate and the solvent evaporated to yield the crude product. Purification via column chromatography with silica gel and gradient elution with 15–35% ethyl acetate/hexanes yielded **3** as a colorless oil (0.73g, 80%). The product matched previous characterizations (Greimel *et al.*, 2008). ¹H NMR (300 MHz, CDCl₃): δ 7.62–7.65 (d, *J*=9.0 Hz, 4H), 7.34–7.42 (m, 6H), 7.20–7.25 (m, 2H), 6.84–6.87 (d, *J*=9.0 Hz, 2H), 4.45 (s, 2H), 3.88–3.92 (m, 1H), 3.79 (s, 3H), 3.69–3.71 (d, *J*=6.0 Hz, 2H), 3.50–3.54 (m, 2H), 2.47 (d, *J*=6.0 Hz, 1H), 1.06 (s, 9H); MALDI-HRMS [M+Na]⁺ calcd: 473.2119, found: 473.2093.

1-*O*-(*tert*-Butyldiphenylsilyl)-2-oleyl-3-*O*-(4-Methoxybenzyl)-*sn*-glycerol (**4**) The procedure for synthesis of **4** was modified from similar ether-tail forming reactions (Jiang

et al., 2006). Compound **3** (0.660g, 1.465mmol) was dissolved in dry dichloromethane (40ml), to which was added 1,8-Bis(dimethylamino)naphthalene (proton sponge, 1.10g, 5.15mmol) and oleyl triflate (2.900g, 7.240mmol), which was prepared from a known procedure (Heyes *et al.*, 2002). The solution was next heated to reflux and allowed to stir overnight. The solvent was then removed under reduced pressure to yield the crude product **4**, which was purified by column chromatography with silica gel and gradient elution with 5–10% acetone/hexanes to yield **4** as a yellowish oil (0.714 g, 71%). ¹H NMR (300 MHz, CDCl₃): δ 7.66–7.75 (m, 4H), 7.32–7.40 (m, 6H), 7.22–7.30 (m, 2H), 6.83–6.91 (m, 2H), 5.35–5.41 (m, 2H), 4.47–4.52 (m, 2H), 3.79 (s, 3H), 3.38–3.70 (m, 7H), 2.04 (m, 4H), 1.58 (m, 2H), 1.30–1.36 (m, 22H), 1.03–1.06 (m, 9H), 0.90–0.94 (m, 3H); ¹³C NMR (100.6 MHz, CDCl₃): δ 159.14, 135.99, 135.64, 133.57, 129.90, 129.48, 129.21, 129.13, 127.66, 127.43, 113.69, 113.59, 79.48, 73.02, 71.38, 69.71, 63.50, 55.16, 32.70, 31.99, 30.18, 29.78, 29.72, 29.58, 29.33, 26.20, 22.76, 19.25, 14.21; MALDI-HRMS [M+Na]⁺ calcd: 723.4779, found: 723.4759.

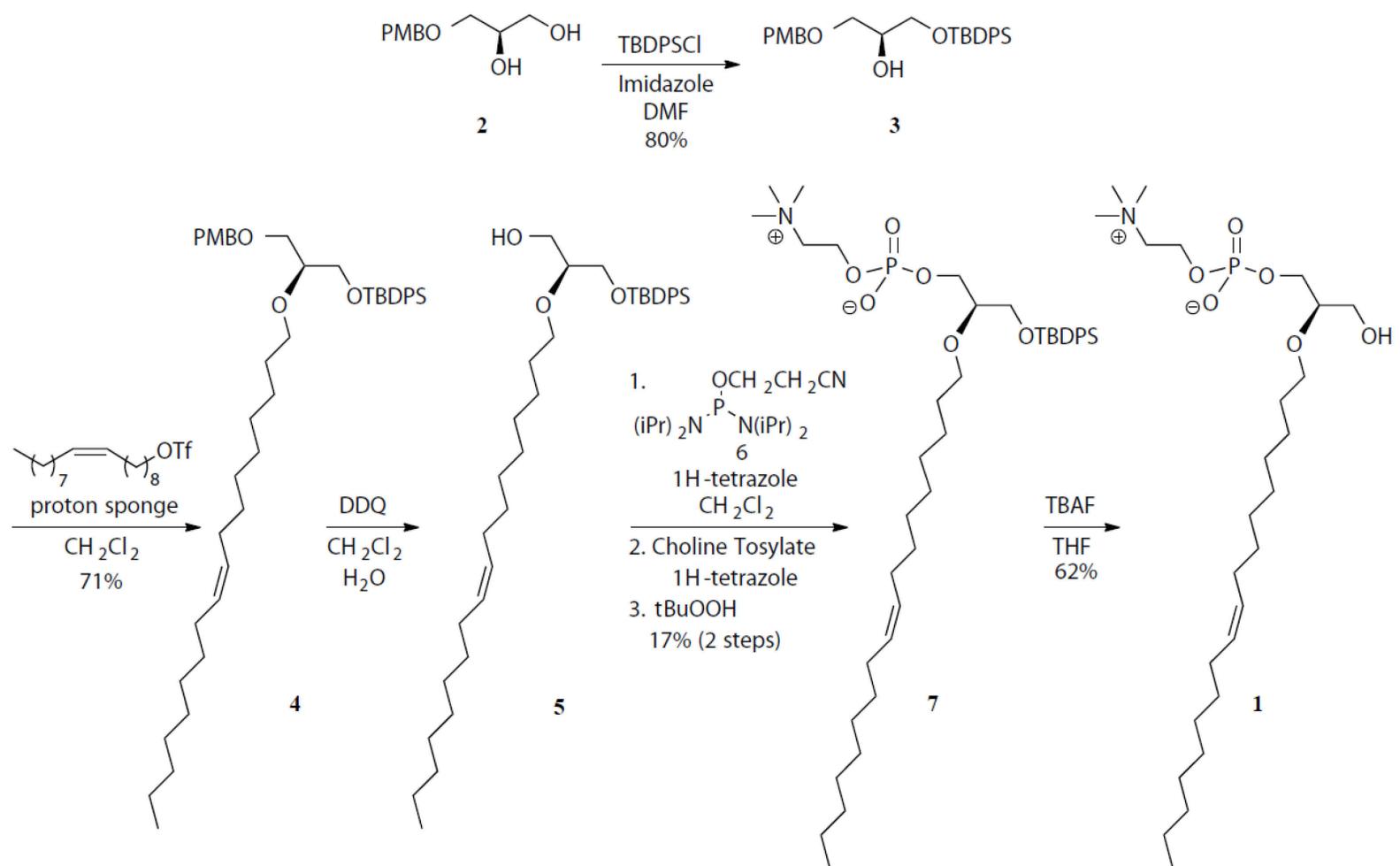
1-O-(tert-Butyldiphenylsilyl)-2-oleyl-sn-glycerol (5) Compound **4** (0.325g, 0.463mmol) was combined with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ, 0.315g, 1.389mmol) in dichloromethane (10ml) and water (1ml). The reaction mixture was then stirred at rt. After 10h, saturated sodium bicarbonate (100ml) was added to quench the reaction, and the resulting solution was then extracted twice with chloroform (2 x 100ml). The organic layers were then combined and dried with magnesium sulfate and the solvent removed by rotary evaporation to yield the crude product, which was carried on to the next step without further purification.

1-O-(tert-Butyldiphenylsilyl)-2-oleoyl-sn-glycero-3-phosphocholine (7) Crude compound **5** was combined with bis(*N,N*-diisopropylamino) cyanoethyl phosphine (**6**, 0.205g, 0.606mmol), and 1*H*-tetrazole (0.740ml of a 0.45M solution in acetonitrile, 0.333mmol) in dichloromethane (10ml), and the solution was allowed to stir at rt for 1h. To this stirred solution, choline tosylate (0.334g, 1.212mmol), and 1*H*-tetrazole (2.690ml of a 0.45M solution in acetonitrile, 1.212mmol) were added, and the solution was allowed to stir at rt for another 12h, after which *tert*-butylhydroperoxide (0.470ml, 4.848mmol) was added. After 1h, the reaction was quenched by adding 50ml of saturated sodium thiosulfate aqueous solution. Next, the resulting solution was extracted with methanol/methylene chloride (v/v 1:4, 2 x 80ml), and the organic layers were combined and dried with magnesium sulfate. The solvent was then removed under reduced pressure, and the resulting residue was purified by column chromatography with silica gel through gradient elution of 5–30% methanol/dichloromethane to yield **7** as a colorless oil (0.060 g, 17 % yield over 2 steps). ¹H NMR (300 MHz, CDCl₃): δ 7.65–7.69 (m, 4H), 7.26–7.38 (m, 6H), 5.32–5.35 (m, 2H), 4.14–4.18 (m, 2H), 3.89–3.91 (m, 2H), 3.49–3.74 (m, 5H), 3.28–3.30 (m, 2H), 3.22 (s, 9H), 1.99–2.01 (m, 4H), 1.47–1.49 (m, 2H), 1.19–1.23 (m, 22H), 1.02 (s, 9H), 0.86 (t, *J*=6.0 Hz, 3H); ³¹P NMR: δ –0.59; MALDI-HRMS [M-H]⁺ calcd: 746.4939, found: 746.4946.

2-Oleyl-sn-glycero-3-phosphocholine (1) Compound **7** (0.060g, 0.080mmol) and tetrabutylammonium fluoride trihydrate (TBAF, 0.126g, 0.40mmol) were dissolved in tetrahydrofuran (10ml), and the reaction was allowed to stir at room temperature

overnight, at which point the solvent was removed under reduced pressure. Purification of the resulting residue by column chromatography with 3 g of silica gel and a gradient eluant of 10–50% methanol/chloroform yielded **7**, but also contained TBAF as a contaminant. This mixture was then dissolved in water and stirred with Chelex-100 resin, sodium form, for 3h. The solution was then loaded directly onto a C18 reverse phase column and eluted with a gradient of water/methanol mixtures to yield **7** as an off-white paste (0.025g, 62%). The product matched previous characterizations.(Hirth *et al.*, 1983; Huang *et al.*, 2006) ^1H NMR (300 MHz, $\text{CD}_3\text{OD}/\text{CDCl}_3$, v/v 1:2): δ 5.33-5.36 (m, 2H), 4.4-4.28 (m, 2H), 4.14 (m, 1H), 3.96 (t, $J=6.0$ Hz, 2H), 3.49-3.74 (m, 5H), 3.34-3.36 (m, 2H), 3.21 (s, 9H), 1.99-2.04 (m, 4H), 1.52-1.59 (m, 2H), 1.25-1.32 (m, 22H), 0.86 (t, $J=6.0$ Hz, 3H); ^{31}P NMR: δ 4.01; MALDI-HRMS $[\text{M}-\text{H}]^+$ calcd: 508.3762, found: 508.3768.

Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies (Atlanta, GA). NMR spectra were obtained using a Varian Mercury 300 spectrometer. Mass spectra were obtained with a Voyager DE MALDI-TOF spectrometer. Synthesis of 2-Oleyl-*sn*-glycero-3-phosphocholine (**1**) is shown in Scheme 2.1.



Scheme 2.1 Synthesis of 2-oleyl-sn-glycero-3-phosphocholine.

CHAPTER 3. QUANTIFICATION OF *SN-1* AND *SN-2* REGIOISOMERS OF LPA AND LPC

3.1 INTRODUCTION

We hypothesized that the production of *sn-1* LPA during blood coagulation proceeds through the following steps: 1) Secretion of PLA₁ from activated platelets. 2) Generation of *sn-2* LPLs by PLA₁. 3) Acyl migration of *sn-2* LPLs to yield the *sn-1* regioisomer of LPLs. 4. Cleavage of *sn-1* lysophospholipids by LPLD to yield *sn-1* LPA. The predominance of 18:2 and 20:4 LPA species in serum indicate that PLA₁ cleavage must be involved in this biochemical pathway because these fatty acids are only present in the *sn-2* position of phospholipids. In order to understand LPA production in biological fluids we sought to develop a method for the separation and quantification of the *sn-1* and *sn-2* regioisomers of LPC and LPA. Using such a method one would be able to analyze human blood samples at various time points throughout the coagulation process and accurately determine a time course of the production of *sn-2* and subsequently *sn-1* lysophospholipids. We have developed an HPLC method coupled with MS/MS that separated the regioisomers of both LPC and LPA. However, the duration of the extraction and separation of the regioisomers (~40 min) was longer than the very short half-life (~10 min) of the *sn-2* regioisomer in biological samples (Pluckthun and Dennis, 1982). Consequently, were unable to reliably resolve any changes in the composition of the *sn-1* and *sn-2* regioisomers in human plasma or blood samples using this method.

3.2 METHOD DEVELOPMENT

3.2.1 Production of the *sn-2* Regioisomer of LPC and LPA

sn-2 LPA 18:2 standard was synthesized from dilinoleoyl-phosphatidic acid (18:2 PA) using *Rhizomucor miehei* lipase specific for fatty acid esters in positions 1 and 3 on the glycerol backbone (Sigma-Aldrich, St. Louis, MO). 400 µg PA 18:2 was incubated at 37° C for 1h in 100µl reaction buffer (10mM TRIS, pH 7.8, 150mM NaCl, and 2mM CaCl₂ final concentration) with 500µl lipase (10 units) and an equal volume of diethyl ether. The aqueous phase was then transferred to a new glass tube and 200µl water-saturated butanol was added. The sample was vortexed and centrifuged for 1min at 13000 x g, and the butanol layer was isolated and dried under a stream of argon gas.

sn-2 LPC 18:1 was synthesized from PC 16:0-18:1 using *Rhizopus arrhizus miehei* lipase. 400 µg PC 18:1 was incubated at 37° C for 1h in 100µl reaction buffer (10mM TRIS, pH 7.8, 10mM TritonX-100 final concentration) with 10µg lipase (0.1 units). The product was extracted using the same procedure described above for *sn-2* LPA.

3.2.2 LC-MS/MS Method Development for Separation of the *sn-1* and *sn-2* Regioisomers of LPA

We used a Tosoh TSK-ODS-100Z column (250mm x 2 mm; silica with 5- μ m particle size) HPLC column to separate *sn-1* and *sn-2* regioisomers of LPA. The HPLC column was connected to a Shimadazu LC-10ADvp pump (Columbia, MD) and interfaced to an Applied Biosystems Sciex (Foster City, CA) API 4000 tandem mass spectrometer equipped with a TurboionsprayTM interface. The typical ion source parameters used were: Capillary 3.5 kV, declustering potential (DP) 32 V, focusing potential (FP) 130 V, entrance potential (EP) -12 V, collision energy (CE) 29 eV, collision cell exit potential (CXP) 10 V, deflector -348 V, channel electron multiplier (CEM) 2200 V, and source temperature 325°C. The data was processed by Analyst software, version 1.3. Various species of LPA were analyzed by multiple reaction monitoring (MRM) in negative ion mode with Q₃ (product ion) set at *m/z* 153 (glycerophosphate moiety). LPC species were analyzed in MRM positive ion mode with Q₃ set at *m/z* 184 (glycerophosphocholine). Q₁ was set for the deprotonated molecular ion for all lysophospholipids (Tokumura, 2009).

Since commercially available *sn-1* LPA contains some *sn-2* regioisomers, we started method development by separating *sn-1* and *sn-2* regioisomers present in 5ng LPA 20:0 and 18:1 purchased from Avanti Polar Lipids (Alabaster, AL). Our goal was to obtain baseline separation for multiple LPA species. Initially we used a 150 mm Tosoh TSK-ODS-100Z column (2 mm diameter; 5- μ m particle size) and methanol/water (95/5, v/v) containing 5mM ammonium formate as the mobile phase with an isocratic flow rate of 0.22 ml/min. LPA 20:0 was reconstituted in the mobile phase for injection. The separation of the regioisomers of LPA 20:0 was incomplete (Figure 3.1A). The *sn-1* peak retention time was 5.6 min and the *sn-2* peak retention time was 5.3 min. To improve the separation, LPA was reconstituted in acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v) for injection. The separation improved slightly but baseline separation could not be achieved (Figure 3.1B). From this point on all samples were injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v) solvent.

Next, we adjusted the concentration of water in the mobile phase because increasing water concentration slows elution time and improved the separation of the regioisomers. We increased water to 7.5% V/V that improved peak separation of the LPA 20:0 *sn-1* and *sn-2* regioisomers to 0.7 min (Figure 3.1C). To further increase separation we changed the mobile phase to methanol/water (90/10, v/v) containing 5mM ammonium formate. The peak separation of the LPA 20:0 regioisomers increased to 1.28 min, but the separation of the LPA 18:1 regioisomers remained 0.60 min and poor (Figure 3.2). To increase the separation of all major acyl species we adjusted the column temperature from 40 °C to room temperature, which further improved the separation (data not shown).

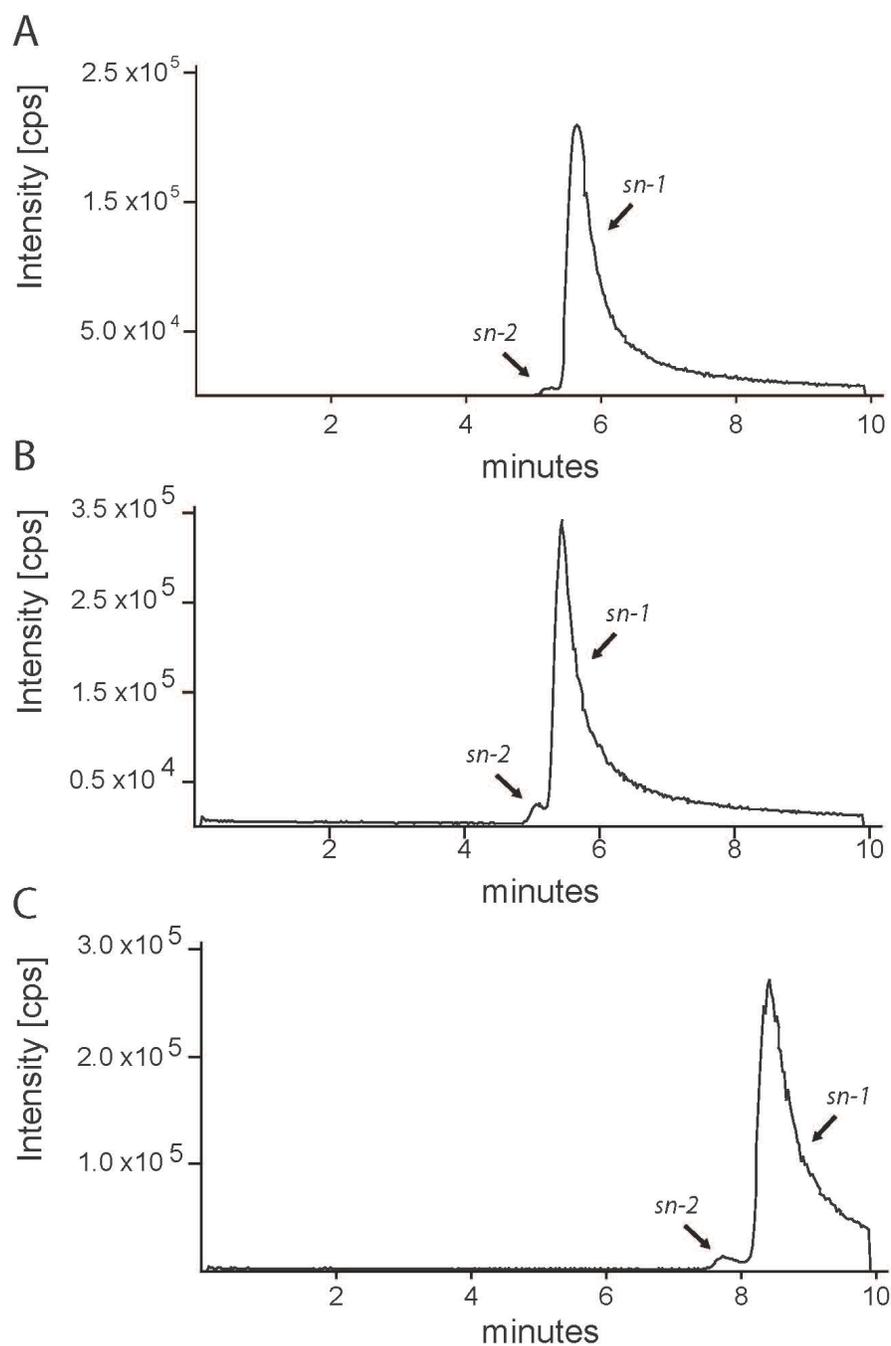


Figure 3.1 HPLC-MS/MS chromatogram of LPA 20:0 regioisomers under varying conditions. LPA 20:0 injected in methanol/water (95/5, V/V), 5mM ammonium formate solvent and mobile phase (panel A). LPA 20:0 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (95/5, V/V), 5mM ammonium formate mobile phase (panel B). LPA 20:0 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (92.5/7.5, V/V), 5mM ammonium formate mobile phase (panel C).

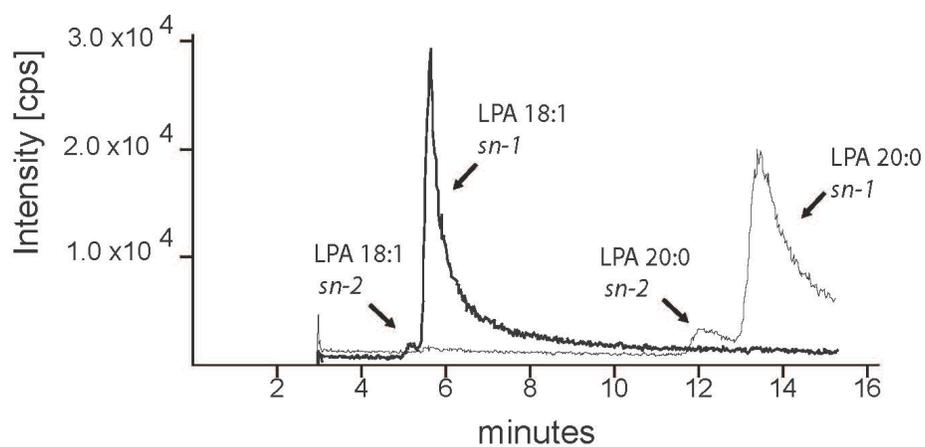


Figure 3.2 HPLC-MS/MS chromatogram of LPA 18:1 and 20:0 regioisomers. LPA 18:1 and 20:0 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (90/10, V/V), 5mM ammonium formate mobile phase.

To validate the method for the most abundant polyunsaturated LPA species we synthesized LPA 18:2 regioisomers and injected nominally equal amounts of the *sn-1* and *sn-2* regioisomers. In the commercially available LPA 18:2 (Avanti, Alabaster, AL) the peak retention times were 8.3 and at 9.2 min for the *sn-2* and *sn-1* regioisomers, respectively (Figure 3.3A). The synthetic *sn-2* LPA when injected alone showed a peak retention time at 8.4 min (Figure 3.3B). The mixture of the two regioisomers showed a peak retention time of 8.1 min for the *sn-2* and 9.2 min for the *sn-1* providing validation of the identity of the peaks detected in the commercial LPA 18:2 preparation (Figure 3.3C). Because of the large tail of the *sn-2* peak, small amounts of *sn-1* LPA could not be resolved. Because of this we decided to further improve the separation of the regioisomers by increasing the water concentration to 20% and found that this resulted in a separation of the two peaks by 3.4 min (Figure 3.4). The retention times of the various LPA and LPC species under varying conditions are summarized in Table 3.1.

3.2.3 Stability of the *sn-2* Regioisomers of LPA and LPC

Based on studies conducted with LPC, it has been widely accepted that the *sn-2* regioisomers of lysophospholipids are highly unstable and that acyl migration towards a 9:1 equilibrium ratio of *sn-1* to *sn-2* occurs rapidly in aqueous buffers in a pH dependent manner (Pluckthun and Dennis 1982). Pluckthun and Dennis showed that the rate of acyl migration increases as pH increases. They also showed that acyl migration is reduced at pH 4.0 to 5.0 but again increases as pH decreases from 4.0 to 1.0. Pluckthun and Dennis used ^{31}P NMR to show that upon synthesis of *sn-2* LPC 16:0 acyl migration begins to take place immediately and within 60 minutes more than 60% of the *sn-2* LPC 16:0 has migrated to the *sn-1* position (Pluckthun and Dennis 1982). In order to gain confidence in our method of quantification of the *sn-1* and *sn-2* regioisomers of LPC and LPA, it was imperative that we determine the stability of the *sn-2* regioisomer under the conditions used in our method. To achieve this, *sn-2* LPA 18:2 was synthesized as described above and divided into two samples. The first sample was reconstituted in isopropanol/water/methanol/acetonitrile (1/1/1/1, V/V/V/V) and immediately analyzed by LC-MS/MS with a mobile phase of methanol/water (90/10, v/v) 5mM ammonium formate (Time 0, Figure 3.5A). This sample was then left at room temperature and reanalyzed by LC-MS/MS at 1 hour and 24 hours later (1 hour at pH 4.0, Figure 3.5B; 24 hour at pH 4.0, Figure 3.5C). It was determined that the *sn-2* regioisomer was stable at pH 4.0 for at least 24 hours of incubation and no migration occurred throughout the time and conditions of the mass spec method for analysis. We also tested the stability of the *sn-2* regioisomer of LPC 18:1. *sn-2* 18:1 LPC was prepared from 16:0-18:1 PC as previously described. The sample was reconstituted with 20 μ l isopropanol/water/methanol/acetonitrile (1/1/1/1, V/V/V/V) and immediately analyzed using LC-MS/MS (Figure 3.6A). The sample was left at room temperature for 24 hours and reanalyzed (Figure 3.6B). *sn-1* and *sn-2* 18:1 LPC was also analyzed to validate retention times (Figure 3.6C). Based on these experiments we can conclude that the *sn-2* regioisomer of LPC 18:1 is stable under the conditions used for analysis.

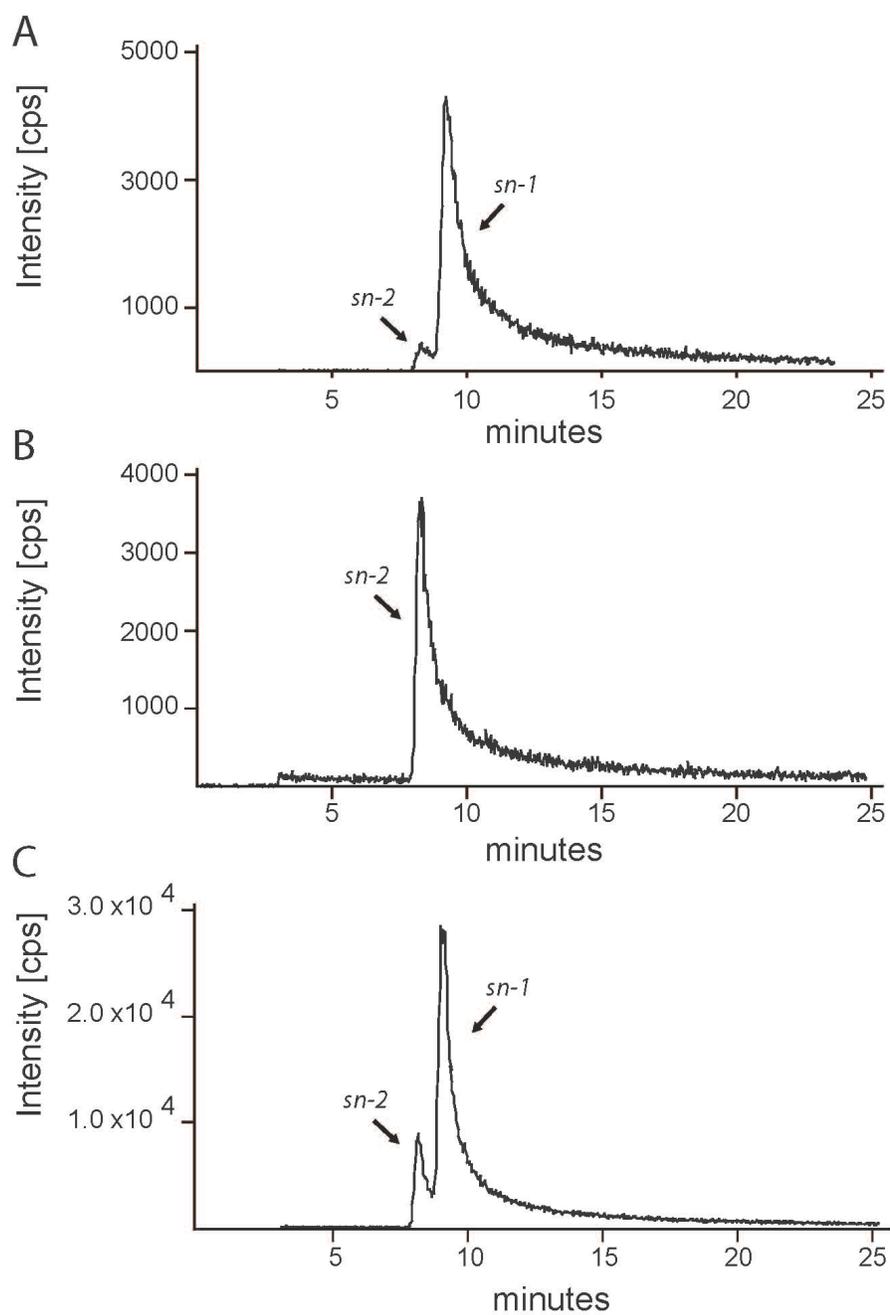


Figure 3.3 HPLC-MS/MS chromatogram of LPA 18:2 regioisomers. *sn-1* LPA 18:2 (panel A), *sn-2* LPA 18:2 (panel B), and *sn-1* and *sn-2* LPA 18:2 (panel C) injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (90/10, V/V), 5mM ammonium formate mobile phase.

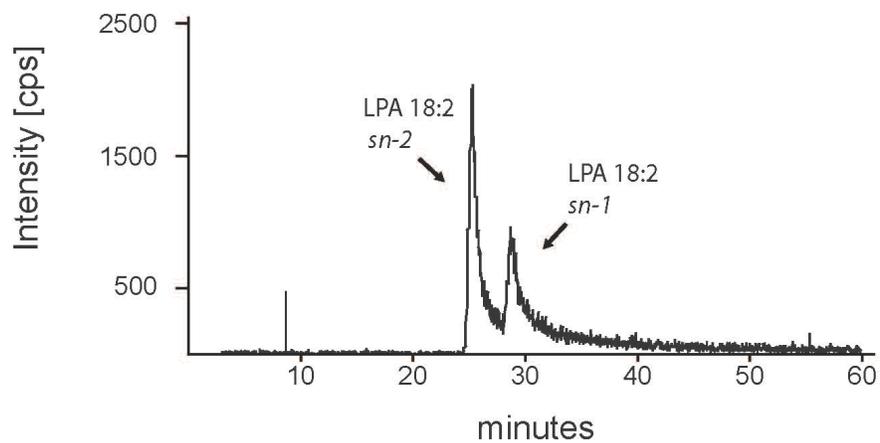


Figure 3.4 HPLC-MS/MS chromatogram of separation of LPA 18:2 regioisomers. *sn-1* and *sn-2* LPA 18:2 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (80/20, V/V), 5mM ammonium formate mobile phase.

Table 3.1 Retention times of LPA and LPC species under varying conditions.

LPA/LPC species	Solvent	Ratio of methanol/water (V/V) in Mobile Phase*	<i>sn-1</i> retention time (min)	<i>sn-2</i> retention time (min)	Peak separation (min)
LPA 20:0	Methanol/water 95/5 (V/V) 5mM ammonium formate	95/5	5.6	5.3	0.3
LPA 20:0	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	95/5	5.5	5.1	0.4
LPA 20:0	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	92.5/7.5	8.4	7.7	0.7
LPA 20:0	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	90/10	13.4	12.1	1.3
LPA 18:2	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	90/10	9.2	8.3	0.9
LPA 18:2	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	80/20	28.7	25.3	3.4
LPC 18:1	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	90/10	39.2	33.3	5.9
LPC 18:1	acetonitrile/methanol/isopropanol/water (1/1/1/1, v/v)	80/20	61.1	51.1	10.0

*Mobile phase includes 5mM ammonium formate.

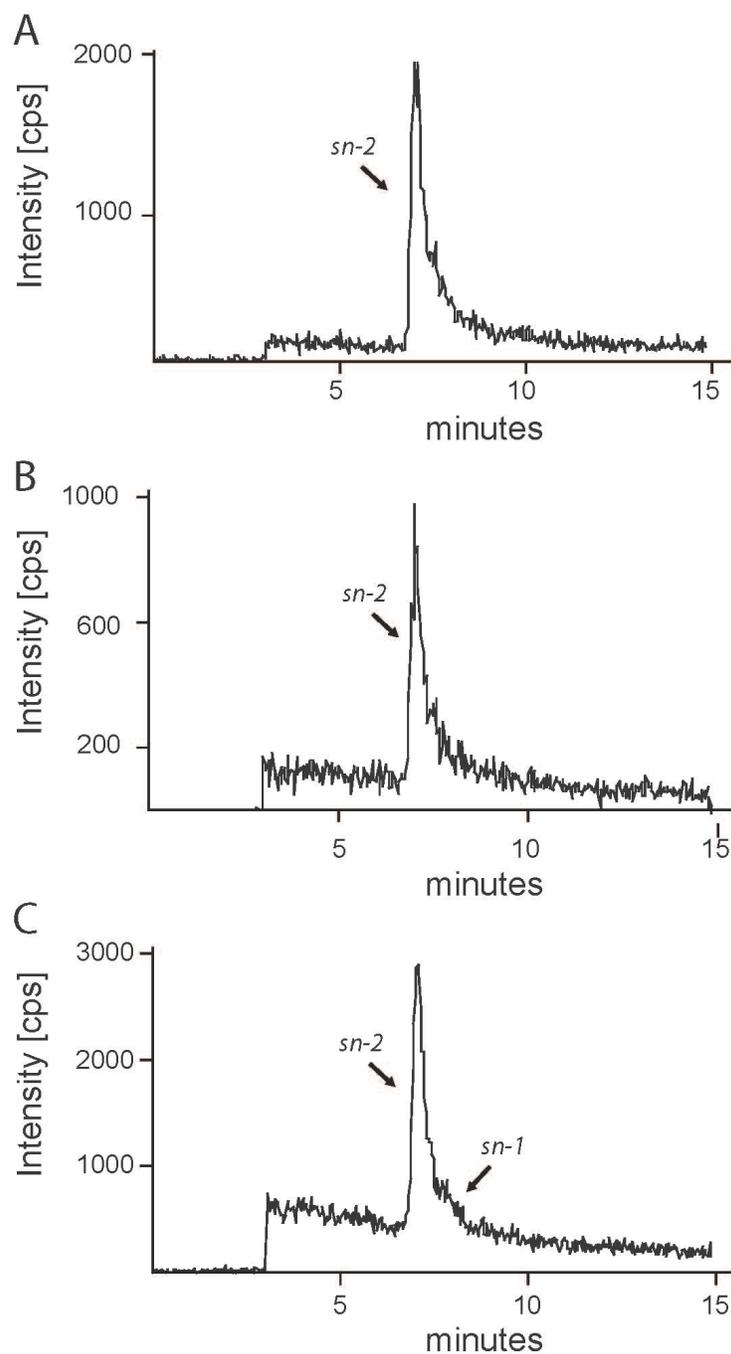


Figure 3.5 HPLC-MS/MS chromatogram of *sn-2* LPA 18:2 at varying time points post synthesis. *sn-2* LPA 18:2 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (90/10, V/V), 5mM ammonium formate mobile phase at time 0 (panel A), 1 hour at pH 4.0 (panel B), and 24 hours at pH 4.0 (panel C).

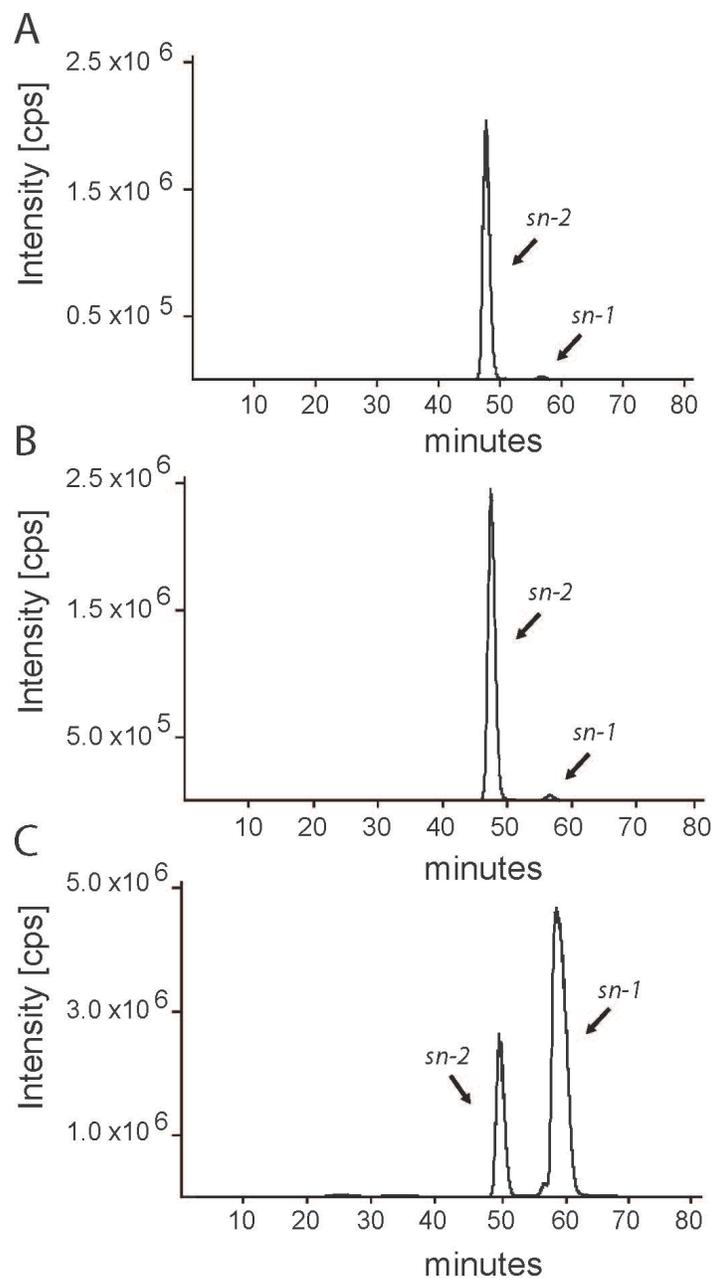


Figure 3.6 HPLC-MS/MS chromatogram of *sn-2* LPC 18:1 at varying time points post synthesis. *sn-2* LPC 18:1 injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (80/20, V/V), 5mM ammonium formate mobile phase at time 0 (panel A), 24 hours at pH 4.0 (panel B), time 0 *sn-1* and *sn-2* LPC 18:1 (panel C).

3.2.4 Application

In order to quantify the various regioisomers at different time points in human serum, 5ml blood was drawn into a heparinized or non-treated Vacutainer tubes. Blood was centrifuged at 14000 x g for 1min and the PRP was transferred to a new tube. The PRP from the tube with no additive was allowed to clot to generate serum. 1 ml aliquots of plasma and serum incubated for 1 hour and 24 hours at 37°C were spiked with 50ng LPA 17:0 internal standard. LPA and LPC were extracted as described by Bollinger, *et al.* (Bollinger, Li *et al.* 2010). High yield extraction of lipids from biological samples is difficult. Bollinger *et al.* have recently developed a method of extraction that has been shown to be cleaner and higher yield than previous methods. Regioisomers of LPA 18:2 and LPC 18:1 and 18:2 were analyzed using the appropriate m/z ratios of the regioisomers at the proper retention times. To minimize run time a mobile phase of methanol/water (90/10, v/v) 5mM ammonium formate was used for analysis of LPC species. The ratio of *sn-1* to *sn-2* LPC 18:1 and 18:2 was 9:1 in all plasma and serum samples (Figure 3.7A, B, and C). We expected this ratio in plasma however; we expected an immediate but short lived spike in the *sn-2* regioisomers in serum incubated for 1 hour. The same 9:1 ratio was found for the *sn-1* and *sn-2* regioisomers of LPA 18:2 in all samples (Figure 3.8A, B, and C).

3.2.5 Conclusions

We have developed a HPLC/MS/MS method for the detection and quantification of LPA and LPC *sn-1* and *sn-2* regioisomers. Using this method we will be able to further progress toward a more complete analysis of LPA production in biological fluids. We were unable to observe a change in the composition of the regioisomers in human plasma or serum using the extraction method described by Bollinger *et al.* (Bollinger, Li *et al.* 2010). Even though this method was designed to limit the acidification of the sample, it is possible that the extraction method encouraged acyl migration towards equilibrium with the minimal acidic nature of the extraction. This extraction method is also quite long (~1 hour to complete) which may have allowed time for acyl migration. In order to observe the *sn-2* regioisomer production and migration a much more rapid method of lipid extraction from biological samples must be developed in which the samples are at a constant pH range of 4.0 to 5.0 to minimize any pH dependent acyl migration.

Using this method in conjunction with a rapid lipid extraction into a slightly acidic solvent (pH 4.0-5.0), one can potentially analyze the composition of all species of *sn-1* and *sn-2* LPC and LPA at different time points during blood coagulation and determine changes in the regioisomeric composition of LPA production. The evaluation of this mechanism is fundamental to understanding the role the different regioisomers of lysophospholipids play in regulation of enzymatic activity, ligand binding, and receptor activation. These studies will require the development of a rapid lipid extraction method preferentially shorter than the time it takes for acyl migration. Development of such method will be subject of future investigations that are beyond the scope of the present thesis.

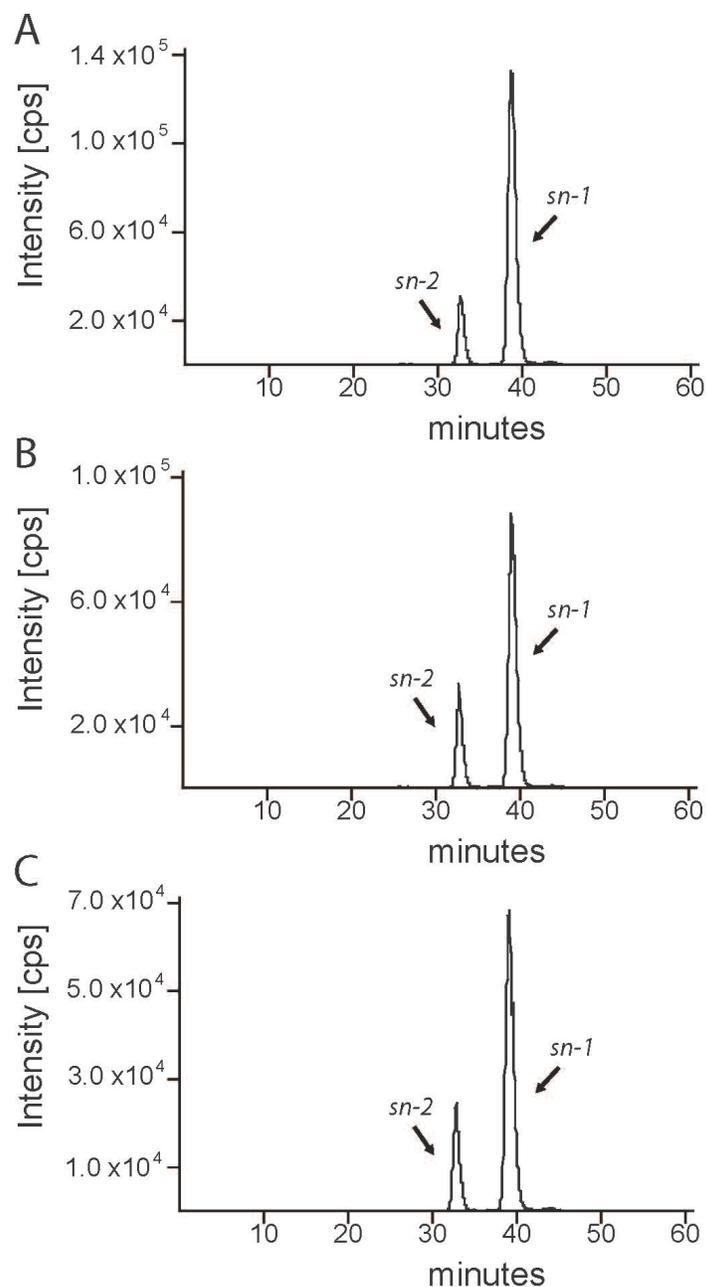


Figure 3.7 HPLC-MS/MS chromatogram of LPC 18:1 at varying time points in plasma and serum. Lipid extractions from plasma at time 0 (panel A), serum at 1 hour (panel B), and serum at 24 hours (panel C) were injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (90/10, V/V), 5mM ammonium formate mobile phase. The ratio of the *sn-1* to *sn-2* regioisomers was 9:1 for all three time points.

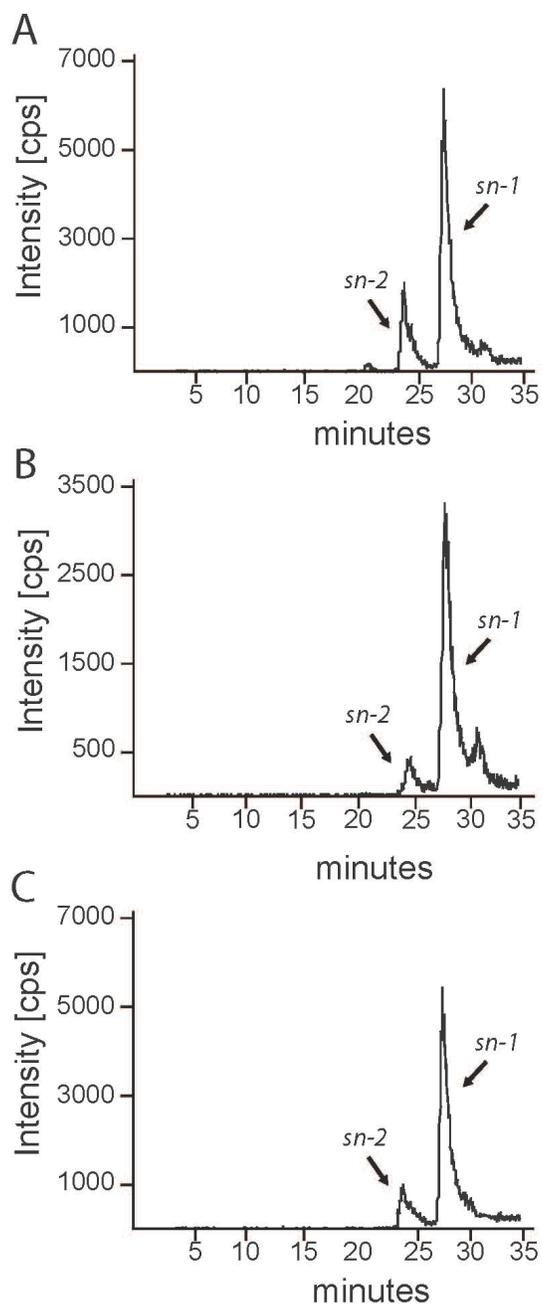


Figure 3.8 HPLC-MS/MS chromatogram of LPA 18:2 at varying time points in plasma and serum. Lipid extractions from plasma at time 0 (panel A), serum at 1 hour (panel B), and serum at 24 hours (panel C) were injected in acetonitrile/methanol/isopropanol/water (1/1/1/1, V/V/V/V) solvent and methanol/water (80/20, V/V), 5mM ammonium formate mobile phase.

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