STRUCTURAL BASIS FOR THE RECOGNITION OF OXIDIZED PHOSPHOLIPIDS IN OXIDIZED LOW DENSITY LIPOPROTEINS

BY CLASS B SCAVENGER RECEPTORS CD36 AND SR-BI

by

DETAO GAO

Submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Thesis Adviser: Dr. Eugene Podrez
     Dr. Lawrence M. Sayre
     Dr. Robert G. Salomon

Department of Chemistry

CASE WESTERN RESERVE UNIVERSITY

January 2012
We hereby approve the thesis/dissertation of

______________________________
Detao Gao

candidate for the PhD degree *.

(signed) Dr. Gregory P. Tochtrop
(chair of the committee)

Dr. Anthony J. Pearson

Dr. James D. Burgess

Dr. Charles Hoppel

Dr. Robert G. Salomon

(date) 08/02/2011

*We also certify that written approval has been obtained for any proprietary material contained therein.
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<td>AcSPC</td>
<td>1-acetyl-2-suberyl-\textit{sn}-3-phosphocholine</td>
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<td>ApoB-100</td>
<td>apolipoprotein B-100</td>
</tr>
<tr>
<td>DCC</td>
<td>N, N’-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMAP</td>
<td>dimethylaminopyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DPPA</td>
<td>1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphatidic acid sodium salt</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dihexadecanoyl-\textit{sn}-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>EIC</td>
<td>extracted ion chromatogram</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
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<td>HODA-PC</td>
<td>9-hydroxy-12-oxo-10-dodecenoic acid esters of lysoPC</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<td>$^{125}\text{I}$-NO$_2$-LDL</td>
<td>$^{125}\text{I}$-LDL oxidized by the MPO-H$_2$O$_2$-NO$_2$- system</td>
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<td>IPTG</td>
<td>isopropyl (\beta)-D-thiogalactoside</td>
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<td>KDdiA-PC</td>
<td>9-keto-10-dodecenedioic acid ester of lysoPC</td>
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<td>KODA-PC</td>
<td>9-keto-12-oxo-10-dodecenoic acid esters of lysoPC</td>
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KOdiA-PC  5-keto-6-octenedioic acid ester of lysoPC
KOOA-PC  5-keto-8-oxo-6-octenoic acid esters of lysoPC
α-LA     alpha-lactalbumin
β-LG     beta-lactoglobulin
LC-ESI-MS liquid chromatography-electrospray ionization-mass spectrometry
lysoPC   lysophosphatidylcholine
MPM      mouse peritoneal macrophages
MPO      myeloperoxidase
NBS      N-bromosuccinimide
NA       no addition
ONE      4-oxo-2-nonenal
oxLDL    oxidized low density lipoprotein
oxPC     oxidized phospholipids
PAcPC    1-palmityl-2-acetyl-sn-glycero-3-phosphocholine
PAF      platelet activating factor
PAPC     1-hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-sn-glycero-3-phosphocholine
PBS      phosphate buffered saline
PC       phosphatidylcholine
Pd/C     palladium on carbon
PdiOSPC  1-palmityl-2-(3’,6’-dioxa)-suberyl-sn-glycero-3-phosphocholine
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<tr>
<td>PDPC</td>
<td>1-palmityl-2-dodecanedioyl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PGI(_2)</td>
<td>prostaglandin I(_2)</td>
</tr>
<tr>
<td>PGPC</td>
<td>1-palmityl-2-glutaryl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PMPC</td>
<td>1-palmityl-2-maleyl-(sn)-glycero-3-phosphocholine</td>
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<tr>
<td>POVPC</td>
<td>1-palmityl-2-(5’-oxovaleryl)-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PPPC</td>
<td>1-palmityl-2-phthaly1-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PSG</td>
<td>1-palmityl-2-suberyl-(sn)-glycerol</td>
</tr>
<tr>
<td>PSPA</td>
<td>1-palmityl-2-suberyl-(sn)-glycero-3-phosphatidic acid</td>
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<tr>
<td>PSPC</td>
<td>1-palmityl-2-suberyl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PSPH</td>
<td>1-palmityl-2-suberyl-(sn)-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-hexanol</td>
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<tr>
<td>PSPP</td>
<td>1-palmityl-2-suberyl-(sn)-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-propanol</td>
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<td>PSuPC</td>
<td>1-palmityl-2-succinyl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>P6HHPC</td>
<td>1-palmityl-2-(6’-hydroxy)-hexanoyl-(sn)-glycero-3-phosphocholine</td>
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<td>P8AOPC</td>
<td>1-palmityl-2-(8’-amino-8’-oxo)-octanoyl-(sn)-glycero-3-phosphocholine</td>
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<tr>
<td>P8HOPC</td>
<td>1-palmityl-2-(8’-hydroxy)-octanoyl-(sn)-glycero-3-phosphocholine</td>
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| P9MNPC       | 1-palmityl-2-(9’-methoxyl-9’-oxo)-nonanoyl-\(sn\)-glycero-3-
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<tr>
<td>phosphocholine</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-n-butylammonium fluoride</td>
</tr>
<tr>
<td>TBDMSCl</td>
<td>tert-butyldimethylsilyl chloride</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<td>UV</td>
<td>ultraviolet</td>
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Abstract

By

DETAO GAO

Specific oxidized phospholipids (oxPC\textsubscript{CD36}) accumulate \textit{in vivo} and serve as high affinity ligands for class B scavenger receptors CD36 and SR-BI. The structural basis for the recognition of oxPC\textsubscript{CD36} by CD36 and SR-BI is poorly understood. In the present study, a series of phospholipids having various functional groups at the \textit{sn}-1, 2 and 3 positions were designed and synthesized.

The synthetic phospholipids were tested for their ability to serve as ligands for CD36 and SR-BI. We demonstrated that all three groups of oxidized phospholipids, including an intact \textit{sn}-1 hydrophobic acyl chain, a hydrophilic \textit{sn}-3 phosphocholine or phosphatidic acid group and the polar terminus of a \textit{sn}-2 acyl group, are indispensable for high affinity binding. We further found that a negatively charged carboxylate terminus on the \textit{sn}-2 acyl group suffices to generate high binding affinity to both receptors. In addition, factors such as polarity, rigidity, optimal chain length of the \textit{sn}-2 acyl and \textit{sn}-3 head group and negative charge in the \textit{sn}-3 head group of phospholipids further enhance the binding affinity. The synthetic phospholipids with a carboxylate group at the \textit{sn}-2 position cannot effectively induce platelet activation, despite their high binding affinity to CD36 receptor.
KOOA-PC and KODA-PC bind to CD36 and SR-BI with high affinity. With “γ-oxo-α-enal” groups, KOOA-PC and KODA-PC are highly electrophilic and reactive toward nucleophiles such as lysine residues and histidine residues. Thus, they may react with CD36 or SR-BI expressed on cells. The resulting covalent adducts can be analyzed with LC-ESI-MS to discover the binding sites. In Chapter 4, pilot studies were carried out. Schiff base adducts of KOOA/KODA-PC with butylamine and CD36 peptides were detected by UV and LC-MS/MS. Pure KOOA-PC and KODA-PC are not stable in aqueous solutions. The addition of inert phospholipids (e.g., lysoPC, POPC) could stabilize them and promote Schiff base formation. Two enrichment methods have been developed and are being used to explore the binding sites of oxPC_{CD36} on CD36 and SR-BI.
Chapter 1

Introduction
1.1 Oxidative stress

Oxidative stress describes an organism’s imbalance between two biochemical processes: the formation of reactive oxygen species (ROS) and the elimination of the formed ROS by antioxidants.

ROS includes various oxygen-containing radicals and peroxides such as hydroxyl radical (·OH), superoxide anion (·O$_2^-$), hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$), organic hydroperoxide (ROOH), alkoxy radical (·OR), hypochlorous acid (HOCl), and nitric oxide (NO$^-$), etc. A variety of exogenous and endogenous sources enhance the formation of ROS. Potential exogenous sources for ROS include ultraviolet light, ionizing radiation, and environmental toxins, whereas endogenous sources include mitochondria, peroxisomes, lipoxygenases, NADPH oxidases, and others.$^1$

On the other hand, there is a complex network of cytoprotective antioxidant metabolites and enzymes in organisms that work together to prevent the damage to cellular components like DNA, proteins, and lipids.$^2, 3$ For example, superoxide dismutases (SOD) can catalyze the dismutation of superoxide into less reactive oxygen and hydrogen peroxide, and catalase can catalyze the decomposition of hydrogen peroxide to water and oxygen.$^4$

Under normal conditions, organisms can remove or detoxify the ROS to a low level and thereby maintain balance. ROS can be beneficial because they can be used by the immune system to attack and kill pathogens. However, once this balance has been disturbed, the resulting oxidative stress could cause damage to various cellular components. In humans, oxidative stress has been found to be involved in many diseases including atherosclerosis, Parkinson’s disease, Alzheimer’s disease, and others.
1.2 Oxidized low density lipoprotein (oxLDL)

LDL is the major cholesterol-bearing lipoprotein in human serum. LDL transports cholesterol to various tissues and cells where it will be used. LDL particles contain, on average, 1600 molecules of cholesteryl ester, 600 molecules of free cholesterol, 700 molecules of phospholipid (64% phosphatidylcholine (PC), 1.5% phosphatidylethanolamine (PE), 26% sphingomyelin (SM), and 11% lyso phosphatidylcholine (LPC)), 180 molecules of triglyceride (TG), and 1 molecule of Apolipoprotein B-100 (ApoB-100). The phospholipids, free cholesterol and ApoB constitute the outer layer of LDL. The inner core of LDL contains mostly cholesteryl ester and TG.

1.2.1 The oxidation of LDL

In the LDL phospholipid shell, polyunsaturated fatty acid (PUFA) chains are major oxidative targets. PUFAs are susceptible to oxidation because of the methylene groups located between double bonds (bisallylic methylene groups). The hydrogen atoms on the bisallylic methylene groups are easily abstracted by ROS free radicals, and the resulting bisallylic radicals are stabilized by resonance delocalization throughout the conjugated system. The bisallylic radicals react with oxygen to give peroxyl radicals, which can then abstract a hydrogen atom from another bisallylic methylene group, thereby generating a new bisallylic radical and a hydroperoxide. In this way, the radical chain reaction is initiated and propagated. With further oxidation and rearrangement, various oxidized phospholipids including isoprostane phospholipids, isolevuglandin phospholipids, and others are generated (Scheme 1.1). Further oxidative fragmentation leads to the
formation of phospholipids with various truncated \( sn \)-2 acyl groups and their short-chain aldehyde counterparts such as 4-hydroxy-2-non-enal (HNE) and 4-oxo-2-non-enal (ONE) (Scheme 1.1). These aldehyde compounds have high electrophilic reactivity and can modify the amino acid residues in ApoB-100.\(^{11-14}\)

![Scheme 1.1 Generation of oxidized phospholipids and their short chain counterparts by the oxidation of phospholipids with PUFA (LH) in LDL. \( r \cdot \) represent the ROS radicals, \( L^- \), \( LO^- \) and \( LOO^- \) are alkyl, alkoxy and alkylperoxy radicals derived by hydrogen abstraction from LH.]

\[
\text{PC} = \begin{array}{c}
\text{O} \\
\text{O} \\
\text{C}_{15}\text{H}_{31} \\
\end{array}
\]

\[
\text{O} \\
\text{O} \\
\text{P} \\
\text{O} \\
\text{C}_{15}\text{H}_{31} \\
\end{array}
\]
1.2.2 Oxidized phospholipids in oxLDL contribute to the atherosclerosis in multiple ways

Watson and coworkers identified two oxidized phospholipids (1-palmitoyl-2-(5’-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC)) (Scheme 1.1) in the fatty streak lesions of cholesterol-fed rabbits. In addition, Podrez et al. found a novel class of oxidized phospholipids (oxPC CD36) (Scheme 1.1) that serve as high-affinity ligands for class B scavenger receptor CD36 in oxLDL and in atherosclerotic lesions. The elevated oxidized phospholipid/ApoB-100 ratio was reported to be closely related to obstructive coronary artery disease. PON1-null mice were shown to develop more atherosclerotic lesions than control mice, probably due to their hindered ability to remove oxidized phospholipids.

Studies on the role of oxidized phospholipids in atherosclerosis demonstrated that they act in several ways to promote atherogenesis. First, oxidized phospholipids can induce inflammatory reactions and stimulate endothelial cells to recruit and bind monocytes. The monocytes subsequently enter the subendothelial space and differentiate into macrophages. Second, oxidized phospholipids mediate the recognition and uptake of oxLDL by macrophages via class B scavenger receptor CD36, which ultimately leads to foam cell formation, which is an early and critical event in atherosclerotic plaque formation. Third, specific oxidized phospholipids were reported to prevent binding of high density lipoprotein (HDL) to class B scavenger receptor SR-BI, and to impede the SR-BI-mediated selective uptake of cholesteryl esters in hepatocytes. Thus, the oxPC_{CD36} may interfere with the reverse cholesterol transport—an
atheroprotective process in which HDLs remove excessive cholesterol from peripheral tissues and transport it back to the liver for reutilization or excretion.\textsuperscript{29} Fourth, oxidized phospholipids can induce the activation of platelets and promote atherothrombosis.\textsuperscript{30-35} These studies clearly demonstrate that the interaction between oxidized phospholipids and class B scavenger receptors is very important for the development of atherosclerosis.

1.3 Class B scavenger receptors CD36 and SR-BI

Class B scavenger receptors CD36 and SR-BI are highly glycosylated, single-chain, integral plasma membrane proteins.\textsuperscript{36-38} They share 30\% sequence identity and an ability to recognize several common ligands including acetylated LDL, oxLDL, and oxidized phospholipids.\textsuperscript{28, 38} They are expressed in various cell types including macrophages, platelets, endothelial cells, and others.\textsuperscript{37, 39}

In humans, CD36 comprises 472 amino acids with a molecular weight of about 53,000 Da. However, CD36 is usually highly glycosylated and, thus, has an apparent molecular weight of 88,000 Da.\textsuperscript{40, 41} CD36 has one large extracellular domain, two transmembrane domains, and two short intracellular domains, as shown in Figure 1.1. In the extracellular domain, all six conserved cysteines are linked by three disulfide bonds,\textsuperscript{39, 42} some of the asparagine residues are glycosylated, which is necessary for CD36 trafficking to the cell membrane.\textsuperscript{43} The domain (155-183) has been found to bind oxLDL\textsuperscript{44} and apoptotic neutrophils.\textsuperscript{45} The domain (184-204) consists of mainly hydrophobic amino acid residues and may interact with the cell membrane.\textsuperscript{46} Both intracellular domains contain paired cysteines that are palmitoylated and thus may help
anchor the CD36 to the cell membrane. The intracellular C-terminus of CD36 has been reported to regulate efficient oxLDL internalization and degradation.

Figure 1.1 Topology and domains of CD36. Figure legends: G -- site of glycosylation, SS-- disulfide bond, P -- palmitoylation

CD36 has been shown to play a significant role in a number of physiological and pathological processes in vivo including atherogenesis, angiogenesis, uptake of apoptotic cells, and diabetes. CD36 deficiency in mice is associated with reduced atherosclerosis due to its function as a major macrophage scavenger receptor that mediates the uptake of oxLDL and subsequent foam cell formation. CD36 deficiency in platelets leads to protection from oxidative stress-induced platelet hyper-reactivity and the accompanying pro-thrombotic phenotype.

SR-BI comprises 509 amino acid residues. It has one large extracellular domain, two transmembrane domains, and two short intracellular domains. Like CD36, SR-BI is also highly glycosylated on the extracellular domain, and palmitoylated at the C-terminal intracellular domain. It is a major physiological receptor for high density lipoprotein (HDL), and contrary to CD36, is atheroprotective. SR-BI promotes cells’ cholesterol efflux to HDL and LDL, and selective cholesterol uptake from HDL and LDL by
cells. Additionally, it mediates the transfer of phospholipids from HDL and LDL to cells. SR-BI seems to act as a conduit on the cell membrane for the bidirectional movement of cholesterol and other lipids. SR-BI promotes selective uptake of cholesterol esters from HDL by hepatocytes and thus is a critical player in the reverse cholesterol transport pathway. SR-BI deficiency has been associated with increased atherosclerosis in mice. On the contrary, atherosclerosis decreased dramatically with hepatic over expression of SR-BI.

1.4 Studies on the structural basis for the recognition of oxidized phospholipids by CD36 and SR-BI

Podrez and coworkers earlier demonstrated that a novel family of specific oxidized phospholipids (oxPC) accumulates at sites of oxidative stress in vivo such as within atherosclerotic lesions, hyperlipidemic plasma, and plasma with low high-density lipoprotein levels. They demonstrated that one structural attribute for high binding affinity of oxPC to scavenger receptors CD36 and SR-BI is a sn-2 acyl group incorporating a terminal ω-hydroxy (or oxo)-α,β-unsaturated carbonyl. Gao et al. later reported that the above mentioned hydroxy alkenal phospholipids undergo cyclization and dehydration steps to produce furan-containing phospholipids with poor CD36 binding activity compared with their precursors. It has been recently shown that the oxidatively truncated sn-2 fatty acid moiety protrudes into the aqueous phase rendering it accessible for recognition.

Kar et al. reported that the region spanning CD36 amino acids 157-171 contains a binding domain for oxPC and oxLDL. In addition, they demonstrated that two
lysine groups in CD36 (Lys164/166) are indispensable for binding of oxPC_{CD36} to CD36 receptor. Their study suggested that electrostatic attraction between conserved positively charged lysines in CD36 and the negatively charged oxidized phospholipids is a mechanism of the recognition. Pearce et al. reported that CD36 (AA 28-93) is the binding domain for oxLDL.69 Ashraf and coworkers, using a fusion protein GST-SR-BI (144-205), demonstrated that SR-BI (AA144–205) contains the binding site of HDL, oxLDL and oxPC_{CD36}.28

1.5 Research Strategy

Although previous studies have already shed some light on the structural basis for the binding of oxPC_{CD36} to CD36 and SR-BI receptors, further systematic studies still need to be done to get a clear picture of the binding mechanism.

Podrez and coworkers demonstrated that oxPC_{CD36} species with \( \gamma \)-hydroxyl-\( \alpha \)-enoic acid at the sn-2 position show better binding activity to CD36 receptor than that with \( \gamma \)-hydroxyl-\( \alpha \)-enal at the sn-2 position.16 A key realization from this finding is that the negative carboxylate group of the \( \alpha \)-enoic acid ligands may be important for the binding. In addition, while oxPC_{CD36} with \( \gamma \)-hydroxyl-\( \alpha \)-enal groups at sn-2 position can effectively inhibit the binding of oxLDL to CD36, the \( \gamma \)-hydroxyl-\( \alpha \)-enal group alone (e.g. 4-hydroxynonenal) has no significant inhibition activity. From this finding, we realized that, besides the functional group at sn-2 position, other groups at sn-1 and/or sn-3 may also be needed for the high affinity binding to CD36 receptor.

To test the above hypotheses, we designed and synthesized a series of phospholipids having various functional groups at sn-1, 2 and 3 positions. These functional groups have
a variety of net charges, chain lengths and hydrophilicities. All the synthetic phospholipids were tested and compared for their binding activity to class B scavenger receptors CD36 and SR-BI to elucidate the structural basis for the recognition of oxidized phospholipids by these receptors.

Studies on the binding domains mentioned above mainly used two approaches to explore the binding domain. One is GST-fusion proteins that cover different regions of the receptors. The other one is site directed mutagenesis of the receptors. One disadvantage of these methods is that the tertiary structure of the receptors is damaged, and the secondary structure may also be altered to some extent. It is well known that the secondary and tertiary structures are critical for ligands binding process. Thus, these approaches may not be accurate for exploring the binding domain of oxPC$_{\text{CD36}}$ and oxLDL.

Two oxPC$_{\text{CD36}}$ phospholipids, KODA-PC and KOOA-PC (the 9-keto-12-oxo-10-dodecenolic acid and the 5-keto-8-oxo-6-octenoic acid esters of 2-lysoPC), have relatively high concentration found in vivo$^{32,70}$ and high binding affinity to CD36 and SR-BI receptors.$^{16,28}$ In addition, with a $\gamma$-oxo-$\alpha$-enal group at the sn-2 position, they are highly electrophilic reactive and can form Schiff base adducts with lysine residues and Michael adducts with histidine residues in the receptors. Thus, they can be used as probes to explore the binding site of oxidized phospholipids on CD36 and SR-BI receptors. In the present study, KOOA/KODA-PC will be used to react with intact CD36 or SR-BI receptors on cells. The resulting covalent adducts between KOOA/KODA-PC and receptors (Schiff base or Michael adducts) will be stabilized by reduction and digested with trypsin or chymotrypsin. The modified peptides will be analyzed and sequenced.
with LC-MS to find the binding site of oxidized phospholipids on both receptors. Our method uses intact receptors and will provide a direct evidence for the binding domains.
1.6 References


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Chapter 2

Chemical synthesis of a series of phospholipids with various functional groups at the $sn$-1, 2 and 3 positions*

2.1 Background

Podrez et al. reported a class of oxidized phospholipids that serve as high affinity ligands for class B scavenger receptors CD36 and SR-BI. They concluded that a phospholipid with a \( sn \)-2 acyl group that incorporates a terminal \( \gamma \)-hydroxy (or oxo)-\( \alpha \), \( \beta \)-unsaturated carbonyl is the structural characteristic required for high affinity binding. Gao et al. later reported that the above mentioned hydroxy alkenal phospholipids undergo cyclization and dehydration steps to produce furan-containing phospholipids with poor CD36 binding affinity compared with their precursors, which strengthens the above conclusion.

Podrez and coworkers also demonstrated that \( \gamma \)-hydroxy-\( \alpha \)-enoic acid ligands show better binding affinity to CD36 receptor than \( \gamma \)-hydroxy-\( \alpha \)-enal ligands. While the \( \alpha \)-enal group has high electrophilic reactivity, the \( \alpha \)-enoic acid group has no such reactivity. A key realization from this finding is that electrophilic reactivity is not a prerequisite for receptor binding. The carboxylic acid group of the \( \alpha \)-enoic acid ligand has a negative charge at pH 7.4, which may be important for the binding. In addition, while oxPC\(_{CD36}\) with \( \gamma \)-hydroxy-\( \alpha \)-enal groups at \( sn \)-2 position can effectively inhibit the binding of oxLDL to CD36, the \( \gamma \)-hydroxy-\( \alpha \)-enal group alone (e.g. 4-hydroxynonenal) has no significant inhibition activity. This finding suggests that, besides the functional group at \( sn \)-2 position, the other groups at \( sn \)-1 and/or \( sn \)-3 may also be needed for high affinity binding to CD36 receptor.

Previous studies have already shed some light on the structural basis for the binding of oxidized phospholipids to class B scavenger receptors. However, more systematic studies need to be done to obtain a clear picture of the binding mechanism. In this chapter,
a series of phospholipids having various functional groups at sn-1, 2 and 3 positions were designed, synthesized, and characterized. In order to check the importance of an sn-1 long hydrophobic chain, we designed a phospholipid with an acetyl group at sn-1 position, which is very short and hydrophilic, as shown in Figure 2.1A. At sn-2 position, we designed various functional groups including carboxylic acid, amide, hydroxyl, and methyl ester groups, as shown in Figure 2.1B. Among these functional groups, the carboxylic acid group has a negative charge at pH 7.4, and all others are neutral. These phospholipids were to be used to check the effect of negative charge on the binding activity. In addition, we varied the chain length and rigidity of the sn-2 chain to check their effects. At the sn-3 position, we explored various functional groups including hydroxyl, phosphate, phosphocholine and analogues with different chain lengths, as shown in Figure 2.1C. These functional groups have a variety of hydrophilicities, net charges, and chain lengths. All these factors may affect the binding activity.

All the synthetic phospholipids were tested and compared for their binding activity to class B scavenger receptors CD36 and SR-BI to elucidate the structural basis for the recognition of oxidized phospholipids by these receptors.
Figure 2.1 Phospholipids with various functional groups at the sn-1, 2 and 3 positions

2.2 Results and discussion

2.2.1 Synthesis of maleic, phthalic, succinic, glutaric acid monoesters, and acetic acid ester of lysoPC

As reported earlier,\(^5\) 1-palmityl-2-maleyl-sn-glycero-3-phosphocholine (PMPC, \(2.1\text{a}\)), 1-palmityl-2-phthalyl-sn-glycero-3-phosphocholine (PPPC, \(2.1\text{b}\)), 1-palmityl-2-succinyl-sn-glycero-3-phosphocholine (PSuPC, \(2.1\text{c}\)), 1-palmityl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC, \(2.1\text{d}\)), and 1-palmityl-2-acetyl-sn-glycero-3-phosphocholine (PAcPC, \(2.1\text{e}\)) were prepared with similar methods as shown in Scheme 2.1. The esterification of lysoPC with the corresponding anhydride under catalysis by dimethylaminopyridine (DMAP) in chloroform gives the synthetic phosphocholines (\(2.1\text{a-e}\)). Among these synthetic phospholipids, PMPC was prepared by Dr. De Lin from Dr. Sayre’s Lab.
Scheme 2.1 Synthesis of PMPC, PPPC, PSuPC, PGPC, and PAcPC

2.2.2 Synthesis of dodecanedioic, suberic, and 3, 6-dioxo-suberic acid monoesters of lysoPC

1-Palmityl-2-dodecanedioyl-sn-glycero-3-phosphocholine (PDPC, 2.5a), 1-palmityl-2-suberyl-sn-glycero-3-phosphocholine (PSPC, 2.5b), and 1-palmityl-2-(3’,6’-dioxa)-suberyl-sn-glycero-3-phosphocholine (PdiOSPC, 2.5c) were prepared by similar methods, as shown in Scheme 2.2. Among these phospholipids, PDPC and PdiOSPC were prepared by Dr. De Lin from Dr. Sayre’s lab.

In the first step, the dicarboxylic acids (2.2a-c) were protected at one end with a benzyl group to yield the dicarboxylic monobenzyl esters, as previously described. In this esterification reaction, benzyl bromide dissolved in dimethylformamide (DMF) was added dropwise to the dicarboxylic acids, which were used in excess to ensure that the mono ester was the major product. Triethylamine was used to neutralize the hydrobromic acid generated in the reaction. In the second step, the dicarboxylic monobenzyl esters were first activated by N, N’-dicyclohexylcarbodiimide (DCC), then coupled with lysoPC
with catalysis by DMAP to form the corresponding synthetic PCs (2.4a-c). In the third step, the benzyl groups of synthetic PCs (2.4a-c) were removed by hydrogenolysis with catalysis by palladium on carbon (Pd/C) to give the synthetic PCs (2.5a-c), as previously reported.

Scheme 2.2 Synthesis of PDPC, PSPC, and PdiOSPC

2.2.3 Synthesis of 6-hydroxy hexanoic and 8-hydroxy octanoic acid esters of lysoPC

1-Palmityl-2-(6'-hydroxy)-hexanoyl-sn-glycero-3-phosphocholine (P6HHPC, 2.8a) and 1-palmityl-2-(8'-hydroxy)-octanoyl-sn-glycero-3-phosphocholine (P8HOPC, 2.8b) were prepared with similar methods, as shown in Scheme 2.3. As reported earlier, the hydroxyl group of 6-hydroxyhexanoic acid and 8-hydroxyoctanoic acid were protected by treatment with tert-butyldimethylsilyl chloride (TBDMSCl) in the presence of
imidazole, which neutralized the hydrochloric acid generated during the reaction. In the second step, TBDMS ether intermediates (2.6a, b) with a carboxylate group at the other end were first activated by DCC, then coupled with lysoPC under the catalysis of DMAP to form the corresponding synthetic PCs (2.7a, b). In the third step, the desilylation was achieved by treatment with tetra-n-butylammonium fluoride (TBAF) to give the synthetic PCs with a hydroxyl group at the terminal of sn-2 position (2.8a, b).

Scheme 2.3 The synthetic route for P6HHPC and P8HOPC

2.2.4 Synthesis of phospholipids with various functional groups at the sn-3 position

1-Palmityl-2-suberyl-sn-glycerol (PSG, 2.14), 1-acetyl-2-suberyl-sn-3-phosphocholine (AcSPC, 2.16a), 1-palmityl-2-suberyl-sn-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-propanol (PSPP, 2.16b), 1-palmityl-2-suberyl-sn-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-hexanol (PSPH, 2.16c), and 1-palmityl-2-suberyl-
sn-glycero-3-phosphatidic acid (PSPA, 2.18) were prepared using similar methods, as shown in Scheme 2.4.

In the first step, 1,2-O-isopropylidene glycerol was treated with acyl chlorides in methylene chloride in the presence of triethylamine to give monoester intermediates 2.9a, b. Subsequent removal of the isopropylidene protecting group in 80% acetic acid delivered 1-O-acyl glycerol intermediates 2.10a and b. These were then treated with TBDMSI in DMF in the presence of imidazole to selectively protect the hydroxyl group at the position 3, leading to the formation of 1-acyl-3-(tert-butyldimethylsilyl)-glycerol 2.11a, b. In the next step, 2.11a and 2.11b were treated with 7-benzyloxy carbonylheptanoyl chloride in the presence of excess triethylamine, which was used to neutralize the newly formed HCl during the reaction. The resulting 1,2-O-acyl-3-O-TBDMS ether intermediates 2.12a and 2.12b were deprotected by N-bromosuccinimide (NBS) in DMSO/THF/H2O solution to give the 1,2-di-O-acyl-sn-glycerols 2.13a and 2.13b. The hydrogenolysis of 2.13b with catalysis by palladium on carbon removed the benzyl group at the sn-2 position and produced PSG (2.14). 2.13a and 2.13b were treated with phosphorus oxychloride in the presence of triethylamine, and then N,N,N-trimethylamino-alkanol was added, followed by hydrolysis in water to give the synthetic phospholipid precursors 2.15a-c. Debenzylation of 2.15a-c by hydrogen provided the synthetic phospholipids 2.16a-c. 2.13b was treated with phosphorus oxychloride in the presence of triethylamine, followed by the hydrolysis in water to give the synthetic phospholipid precursor 2.17. Debenzylation of 2.17 by hydrogen provided the synthetic phospholipid 2.18.
Scheme 2.4 The synthetic route for PSG, AcSPC, PSPP, PSPH, and PSPA
2.2.5 Synthesis of 9-methoxyl-9-oxo-nonanoic and 8-amino-8-oxo-octanoic acid esters of lysoPC

The preparation of 1-palmityl-2-(9'-methoxyl-9'-oxo)-nonanoyl-sn-glycero-3-phosphocholine (P9MNPC, 2.19) and 1-palmityl-2-(8'-amino-8'-oxo)-octanoyl-sn-glycero-3-phosphocholine (P8AOPC, 2.20) is presented in Scheme 2.5. In the synthesis of P9MNPC, the methyl monoester of azelaic acid was first activated by DCC and then coupled with lysoPC with catalysis by DMAP to form the synthetic PC 2.19. In the synthesis of P8AOPC, suberyl chloride was first treated with 3 molar equivalents of DMAP in chloroform, and then coupled with lysoPC, followed by the addition of ammonia.

Scheme 2.5 The synthetic routes to P9MNPC and P8AOPC
2.3 Conclusion

A series of phospholipids with various functional groups at the \( sn-1, 2 \) and 3 positions were systematically prepared. Next, these phospholipids were tested for their binding activity to class B scavenger receptors CD36 and SR-BI. The results of these binding studies are reported in the ensuing chapter. The synthetic phospholipids helped to elucidate the structural basis for the recognition of oxidized phospholipids by scavenger receptors CD36 and SR-BI.

2.4 Experimental procedures

General methods

\(^1\)H NMR spectra were recorded on Varian Gemini spectrometers (200 MHz) and on a Varian Inova AS400 spectrometer (400 MHz). \(^{13}\)C NMR spectra were recorded on a Varian Gemini spectrometer (50 MHz) or on a Varian Inova AS400 spectrometer (100 MHz). All high-resolution mass spectra were recorded on a Kratos AEI MS25 RFA high resolution mass spectrometer at 20 eV. Unless otherwise stated, the solvents and reagents were of commercially available analytical grade quality, and all chemicals were obtained from Sigma-Aldrich, Fisher Scientific or Acros Organics.

1-Palmityl-2-maleyl-\( sn \)-glycero-3-phosphocholine (PMPC, 2.1a)

Maleic anhydride (49 mg, 0.5 mmol) and DMAP (6 mg, 0.05 mmol) were added to a solution of lysoPC (25 mg, 0.05 mmol) in 3 ml dry chloroform under argon. The mixture was stirred at 30 °C overnight, and then the solvent was removed under reduced pressure. The residue was applied to a 0.5 mm silica gel thin layer chromatography (TLC)
plate, which was eluted with CHCl$_3$/MeOH/H$_2$O (65/35/7). The major band ($R_f = 0.15$) was extracted with CHCl$_3$/MeOH/H$_2$O (1/2/0.8). The extract was filtered and then washed with the Bligh/Dyer method.$^{11}$ The resulting product solution was dried with anhydrous Na$_2$SO$_4$, and the solvent was evaporated producing a white solid (10 mg, 33%).

$^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 0.89 (t, $J = 6.8$ Hz, 3H), 1.20-1.36 (24 H), 1.59 (m, 2H), 2.33 (t, $J = 7.6$ Hz, 2H), 3.22 (s, 9 H), 3.63 (m, 2H), 4.06 (m, 2H), 4.20-4.30 (3H), 4.40 (dd, $J = 12.4$, 4.0 Hz, 1H), 5.31 (m, 1H), 6.29 (d, $J = 12$ Hz, 1H), 6.38 (d, $J = 12$ Hz, 1H).

HRMS (FAB): m/z calcd for C$_{28}$H$_{53}$NO$_{10}$P (MH$^+$) 594.3407, found 594.3406.

1-Palmityl-2-phthalyl-sn-glycero-3-phosphocholine (PPPC, 2.1b)

A procedure analogous to that described above for 2.1a was used to get 2.1b (60%).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.87 (t, $J = 6.8$ Hz, 3H), 1.1-1.35 (24H), 1.45-1.55 (2H), 2.15-2.35 (2H), 3.23 (s, 9H), 3.79 (br, 2H), 4.10-4.50 (6H), 5.43 (br, 1H), 7.35-7.82 (4H).

HRMS (FAB): m/z calcd for C$_{32}$H$_{55}$NO$_{10}$P (MH$^+$) 644.3563, found 644.3569.

1-Palmityl-2-succinyl-sn-glycero-3-phosphocholine (PSuPC, 2.1c)

A procedure analogous to that described above for 2.1a was used to get 2.1c (67%).

$^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 0.89 (t, $J = 6.8$ Hz, 3H), 1.25-1.35 (24H), 1.58 (m, 2H), 2.32 (t, $J = 7.2$ Hz, 2H), 2.48 (m, 2H), 2.58 (m, 2H), 3.23 (s, 9H), 3.66 (m, 2H), 4.01 (m, 2H), 4.22 (dd, $J = 12.0$, 6.4 Hz, 1H), 4.28 (m, 2H), 4.35 (dd, $J = 12.0$, 4.0 Hz, 1H), 5.23 (m, 1H). $^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 14.48, 23.74, 25.95, 30.21, 30.45, 30.49, 30.65, 30.77, 30.80, 33.08, 34.80, 54.65, 60.60, 63.47, 64.94, 67.41, 71.90, 174.39, 174.98.

HRMS (FAB): m/z calcd for C$_{28}$H$_{55}$NO$_{10}$P (MH$^+$) 596.3563, found 596.3554.
1-Palmityl-2-glutaroyl-\(sn\)-glycero-3-phosphocholine (PGPC, 2.1d)

A procedure analogous to that described above for 2.1a was used to get 2.1d (41%).
\(^1\)H NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 0.89 (t, \(J = 6.8\) Hz, 3H), 1.25-1.35 (24H), 1.59 (m, 2H), 1.89 (m, 2H), 2.32 (t, \(J = 7.6\) Hz, 2H), 2.36 (t, \(J = 7.2\) Hz, 2H), 2.44 (t, \(J = 7.2\) Hz, 2H), 3.23 (s, 9H), 3.65 (m, 2H), 4.01 (m, 2H), 4.20 (dd, \(J = 12.0, 6.8\) Hz, 1H), 4.27 (m, 2H), 4.38 (dd, \(J = 12.0, 3.6\) Hz, 1H), 5.24 (m, 1H). HRMS (FAB): m/z calcd for C\(_{29}\)H\(_{57}\)O\(_{10}\)P (MH\(^+\)) 610.3720, found 610.3695.

1-Palmityl-2-acetyl-\(sn\)-glycero-3-phosphocholine (PAcPC, 2.1e)

A procedure analogous to that described above for 2.1a was used to get 2.1e (75%).
\(^1\)H NMR (CD\(_3\)OD, 400 MHz): \(\delta\) 0.89 (t, \(J = 6.8\) Hz, 3H), 1.25-1.37 (24H), 1.60 (m, 2H), 2.08 (s, 3H), 2.33 (t, \(J = 8.0\) Hz, 2H), 3.23 (s, 9H), 3.63 (m, 2H), 4.01 (m, 2H), 4.20 (dd, \(J = 11.6, 6.8\) Hz, 1H), 4.27 (m, 2H), 4.39 (dd, \(J = 12.0, 3.6\) Hz, 1H), 5.22 (m, 1H). HRMS (FAB): m/z calcd for C\(_{26}\)H\(_{53}\)O\(_{8}\)P (MH\(^+\)) 538.3508, found 538.3526.

12-Benzyloxy-12-oxododecanoic acid (2.3a)

Dodecanedioic acid (1.0 g, 4.3 mmol) and triethylamine (Et\(_3\)N) (1.24 ml, 8.6 mmol) were dissolved in DMF (50 ml) and cooled to 0 °C. To this solution, benzyl bromide (0.34 g, 2 mmol) dissolved in DMF (50 ml) was added over a 1 h period. The reaction was stirred for an additional 4 h at 0 °C and allowed to slowly warm to room temperature overnight. The solvent was removed under reduced pressure. The obtained oily residue was dissolved in aqueous 1 N HCl saturated with NaCl (80 ml) and extracted with ethyl acetate (3 \(\times\) 50 ml). The extracts were combined and concentrated under reduced pressure.
The residue was purified by flash chromatography on a silica gel column, which was eluted with hexane / ethyl acetate (2/1) to afford the monoester as white solid (980 mg, 70%). \(^1\)H NMR (CDCl\(_3\), 200 MHz): δ 1.22-1.38 (12H), 1.55-1.63 (4H), 2.30-2.42 (4H), 5.12 (s, 2H), 7.30-7.38 (5H); This spectrum agrees with that reported previously.\(^{12}\)

8-Benzylxy-8-oxooctanoic acid (2.3b)

A procedure analogous to that described above for 2.3a was used to get 2.3b (65%). \(^1\)H NMR (CDCl\(_3\), 400 MHz): δ 1.30-1.40 (4H), 1.58-1.69 (4H), 2.30-2.39 (4H), 5.12 (s, 2H), 7.30-7.40 (5H).

3, 6-dioxa-suberic acid monobenzyl ester (2.3c)

A procedure analogous to that described above for 2.3a was used to get 2.3c (51%). 1H NMR (CDCl\(_3\), 400 MHz): δ 3.78 (s, 4H), 4.18 (s, 2H), 4.21 (s, 2H), 5.20 (s, 2H), 7.30-7.40 (5H). This spectrum agrees with that reported previously.\(^6\)

1-Palmityl-2-(12’-benzyloxy-12’-oxo)-dodecanoyl-sn-glycero-3-phosphocholine (2.4a)

The obtained monoester 12-benzyloxy-12-oxododecanoic acid 2.3a (64 mg, 0.2 mmol), 2-lysoPC (26 mg, 0.053 mmol), and DMAP (5.5 mg, 0.045 mmol) in dry CH\(_2\)Cl\(_2\) (4 ml) were stirred at room temperature. Then DCC (28 mg, 0.14 mmol) in CH\(_2\)Cl\(_2\) (0.4 ml) was added to the solution. The resulting mixture was stirred at room temperature for 24 h. The solution was then filtered, and the solvent was removed with a rotary evaporator. The obtained residue was applied to a 0.5 mm silica gel TLC plate, which was eluted with CHCl\(_3\)/MeOH/H\(_2\)O (65/35/7). The major band was extracted with
CHCl₃/MeOH/H₂O (1/2/0.8). The extract was filtered and then washed with the
Bligh/Dyer method. The resulting product solution was dried with anhydrous Na₂SO₄,
and the solvent was evaporated producing a white solid (30 mg, 71%). ¹H NMR (CDCl₃,
400 MHz): δ 0.83 (t, J = 6.8 Hz, 3H), 1.18-1.30 (36H), 1.48-1.60 (6H), 2.20-2.26 (4H),
2.30 (t, J = 7.6 Hz, 2H), 3.34 (s, 9H), 3.79 (m, 2H), 3.82-3.92 (2H), 4.05-4.12 (1H), 4.26
(m, 2H), 4.32-4.38 (1H), 5.06 (s, 2H), 5.12-5.20 (m, 1H), 7.22-7.34 (5 H).

1-Palmityl-2-(8’-benzyloxy-8’-oxo)octanoyl-sn-glycero-3-phosphocholine (2.4b)

A procedure analogous to that described above for 2.4a was used to get 2.4b (45%).
¹H NMR (CD₂OD, 400 MHz): δ 0.87 (t, J = 6.8 Hz, 3H), 1.23-1.36 (28H), 1.55-1.65
(6H), 2.28-2.40 (6H), 3.22 (s, 9 H), 3.65 (m, 2 H), 3.98 (m, 2H), 4.18 (dd, J = 12.0, 6.8
Hz, 1H), 4.25 (m, 2H), 4.41 (dd, J = 12.0, 3.6 Hz, 1H), 5.12 (s, 2H), 5.23 (m, 1H), 7.30-
7.37 (5H).

1-Palmityl-2-(3’, 6’-dioxa-8’-benzyloxy)-suberyl-sn-glycero-3-phosphocholine (2.4c)

A procedure analogous to that described above for 2.4a was used to get 2.4c (61%).
¹H NMR (CDCl₃, 400 MHz): δ 0.85 (t, J = 6.6 Hz, 3H), 1.18-1.32 (26 H), 1.54 (m, 2H),
2.25 (t, J = 7.8 Hz, 2H), 3.31 (s, 9 H), 3.68-3.80 (6 H), 3.94-4.36 (10 H), 5.15 (s, 2H),
5.28 (m, 1H), 7.28-7.38 (5 H).

1-Palmityl-2-dodecanedioyl-sn-glycero-3-phosphocholine (PDPC, 2.5a)

1-Palmityl-2-(12’-benzyloxy-12’-oxo)-dodecanoyl-sn-glycero-3-phosphocholine
(2.4a) (30 mg, 0.031 mmol) in 5 ml of CHCl₃/MeOH/H₂O (1/2/0.8) was hydrogenated
over 10% Pd/C (10 mg) at atmospheric pressure for 3 h. The catalyst was removed by filtration, and the filtrate was concentrated. The obtained residue was applied to a 0.5 mm silica gel TLC plate, which was eluted with CHCl₃/MeOH/H₂O/CH₃COOH (90/26/4/1). The major band \((R_f = 0.16)\) was extracted with CHCl₃/MeOH/H₂O (1/2/0.8). The extract was filtered and then washed with the Bligh/Dyer method. The resulting product solution was dried with anhydrous Na₂SO₄, and the solvent was evaporated producing a white solid (14.9 mg, 68%). \(^1\)H NMR (CD₃OD, 400 MHz): \(\delta \) 0.89 (t, \(J = 6.8\) Hz, 3H), 1.25-1.37 (36 H), 1.55-1.64 (6H), 2.27 (t, \(J = 7.6\) Hz, 2H), 2.29-2.36 (4H), 3.22 (s, 9 H), 3.64 (m, 2 H), 4.00 (m, 2H), 4.16 (dd, \(J = 11.6, 6.8\) Hz, 1H), 4.27 (m, 2H), 4.42 (dd, \(J = 12.0, 3.2\) Hz, 1H), 5.24 (m, 1H). HRMS (FAB): m/z calcd for C₃₆H₇₁NO₁₀P (MH⁺) 708.4816, found 708.4833.

1-Palmityl-2-suberyl-sn-glycero-3-phosphocholine (PSPC, 2.5b)

A procedure analogous to that described above for 2.5a was used to get 2.5b (71%). \(^1\)H NMR (CD₃OD, 400 MHz): \(\delta \) 0.89 (t, \(J = 6.8\) Hz, 3H), 1.25-1.33 (24H), 1.33-1.38 (m, 2H), 1.55-1.65 (6H), 2.25 (t, \(J = 7.2\) Hz, 2H), 2.32 (t, \(J = 7.6\) Hz, 2H), 2.35 (t, \(J = 7.2\) Hz, 2H), 3.23 (s, 9H), 3.65 (m, 2H), 4.01 (m, 2H), 4.20 (dd, \(J = 12.0, 6.8\) Hz, 1H), 4.27 (m, 2H), 4.39 (dd, \(J = 12.0, 3.2\) Hz, 1H), 5.23 (m, 1H). \(^{13}\)C NMR (CDCl₃, 100 MHz): \(\delta \) 14.35, 22.92, 24.70, 25.10, 28.26, 28.49, 29.40, 29.56, 29.59, 29.77, 29.89, 29.93, 32.15, 34.18, 34.30, 54.50, 59.59, 62.97, 64.14, 66.44, 70.93, 173.47, 173.76. HRMS (FAB): m/z calcd for C₃₂H₆₃NO₁₀P (MH⁺) 652.4189, found 652.4183.
1-Palmityl-2-(3’, 6’-dioxa)-suberyl-sn-glycero-3-phosphocholine (PdiOSPC, 2.5c)

A procedure analogous to that described above for 2.5a was used to get 2.5c (68%).

$^1$H NMR (CDCl$_3$, 200 MHz): $\delta$ 0.87 (t, $J = 7.0$ Hz, 3H), 1.18-1.36 (24 H), 1.58 (m, 2H), 2.28 (t, $J = 7.2$ Hz, 2H), 3.31 (s, 9 H), 3.60-3.85 (6 H), 3.92-4.40 (10 H), 5.33 (m, 1H).

HRMS (FAB): calcd for C$_{30}$H$_{59}$NO$_{12}$P (MH$^+$) 656.3775, found 656.3767.

6-(tert-Butyldimethyl)silyloxy-hexanoic acid (2.6a)

6-Hydroxyhexanoic acid (528 mg, 4 mmol) and imidazole (653 mg, 9.6 mmol) were dissolved in 10ml DMF, to which was added TBDMSCl (660 mg, 4.4 mmol) at 0 °C with stirring. After 30 min, the reaction temperature rose to room temperature and continued to react overnight at room temperature. Then 20 ml ethyl ether and 20 ml saturated NaCl solution were added, and the resulting solution was acidified with H$_3$PO$_4$ to pH 3. The ethyl ether layer was separated and concentrated under reduced pressure. The obtained pale yellow oil was purified by flash chromatography on silica gel (acetone/hexane, 1/4, TLC: R$_f$ = 0.3) to afford the 6-(tert-butyldimethylsilyloxy)-hexanoic acid (2.6a) (440 mg, 45%).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.04 (s, 6H), 0.89 (s, 9H), 1.39 (m, 2H), 1.53 (m, 2H), 1.66 (p, $J = 7.6$ Hz, 2H), 2.36 (t, $J = 7.6$ Hz, 2H), 3.61 (t, $J = 6.4$ Hz, 2H). The $^1$H NMR spectrum is identical with that reported previously.$^{13}$

8-(tert-Butyldimethyl)silyloxy-octanoic acid (2.6b)

A procedure analogous to that described above for 2.6a was used to get 2.6b (57%).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.05 (s, 6H), 0.87 (s, 9H), 1.21-1.32 (6H), 1.45 (m, 2H), 1.57 (m, 2H), 2.29 (t, $J = 7.4$ Hz, 2H), 3.55 (t, $J = 6.6$ Hz, 2H).
1-Palmityl-2-(6’-tert-butyldimethylsilyloxy)-hexanoyl-sn-glycero-3-phosphocholine (2.7a)

The obtained compound 2.6a (103 mg, 0.42 mmol), lysoPC (30 mg, 0.06 mmol), and DMAP (6 mg, 0.048 mmol) were dissolved in dry CHCl₃ (3 ml) and stirred at room temperature under the protection of argon. DCC (139 mg, 0.67 mmol) was then added to the solution. The resulting mixture was stirred at room temperature for 48 h. The solution was then filtered, and the solvent was removed with a rotary evaporator. The obtained residue was applied to a 0.5 mm silica gel TLC plate, which was eluted with CHCl₃/MeOH/CH₃COOH/H₂O (50/15/14/3). The product band (Rₛ = 0.6) was extracted with CHCl₃/MeOH/H₂O (1/2/0.8). The extract was filtered and then washed with the Bligh/Dyer method. The resulting product solution was dried with anhydrous Na₂SO₄, and the solvent was evaporated producing a white solid (17.4 mg, 40%). ¹H NMR (CD₃OD, 400 MHz): δ 0.00 (s, 6H), 0.81-0.87 (12H), 1.20-1.30 (24 H), 1.34 (m, 2H), 1.44-1.62 (6H), 2.26 (t, J = 7.2 Hz, 2H), 2.29 (t, J = 7.4 Hz, 2H), 3.17 (s, 9 H), 3.56-3.61 (4H), 3.94 (t, 2H), 4.12 (dd, J = 12.0, 6.8 Hz, 1H), 4.22 (m, 2H), 4.35 (dd, J = 12.0, 3.6 Hz, 1H), 5.18 (m, 1H).

1-Palmityl-2-(6’-hydroxyhexanoyl)-sn-glycero-3-phosphocholine (P6HHPC, 2.8a)

The obtained 2.7a (17.4 mg) was dissolved in 5 ml THF. To the mixture, 100µl TBAF (1 M in THF) was added. The resulting mixture was stirred at room temperature. Five hours later, the solvent was removed with a rotary evaporator. The obtained residue was applied to a 0.5 mm silica gel TLC plate, which was eluted with CHCl₃/MeOH/CH₃COOH/H₂O (50/15/14/3). The product band (Rₛ = 0.4) was extracted
with CHCl₃/MeOH/H₂O (1/2/0.8). The extract was filtered and then washed with the Bligh/Dyer method. The resulting product solution was dried with anhydrous Na₂SO₄, and the solvent was evaporated producing a white solid (10.9 mg, 75%). ¹H NMR (CD₃OD, 400 MHz): δ 0.90 (t, J = 6.8 Hz, 3H), 1.25-1.35 (24 H), 1.40 (m, 2H), 1.50-1.70 (6H), 2.32 (t, J = 7.2 Hz, 2H), 2.36 (t, J = 7.2 Hz, 2H), 3.22 (s, 9 H), 3.54 (t, J = 6.8 Hz, 2H), 3.64 (m, 2H), 4.0 (m, 2H), 4.16 (dd, J = 12.0, 6.4 Hz, 1H), 4.27 (m, 2H), 4.40 (dd, J = 12.0, 3.6 Hz, 1H), 5.23 (m, 1H). HRMS (FAB): m/z calcd for C₃₀H₆₁NO₉P (MH⁺) 610.4084, found 610.4097.

1-Palmityl-2-(8'-hydroxy)-octanoyl-sn-glycero-3-phosphocholine (P8HOPC, 2.8b)

2.6b (99 mg, 0.36 mmol), lysoPC (30 mg, 0.06 mmol), and DMAP (6 mg, 0.048 mmol) were dissolved in dry CHCl₃ (3 ml) and stirred at room temperature under the protection of argon. DCC (70 mg, 0.34 mmol) was then added to the solution. The resulting mixture was stirred at room temperature for 48 h. The solution was then filtered, and the solvent was removed with a rotary evaporator. The obtained residue was applied to a 0.5 mm silica gel TLC plate, which was eluted with CHCl₃/MeOH/CH₃COOH/H₂O (50/15/14/3). The product band (Rᶠ = 0.42) was extracted with CHCl₃/MeOH/H₂O (1/2/0.8). The extract was filtered and then washed with the Bligh/Dyer method. The resulting product solution was dried with anhydrous Na₂SO₄, and the solvent was evaporated producing a white solid (28.2 mg). 14.1 mg of the obtained white solid was dissolved in 5 ml THF. To the mixture, 100 µl TBAF (1 M in THF) was added. The resulting mixture was stirred at room temperature. Five hours later, the solvent was removed with a rotary evaporator. The obtained residue was applied to a 0.5 mm silica
gel TLC plate, which was eluted with CHCl\textsubscript{3}/MeOH/CH\textsubscript{3}COOH/H\textsubscript{2}O (50/15/14/3). The product band (R\textsubscript{f} = 0.4) was extracted with CHCl\textsubscript{3}/MeOH/H\textsubscript{2}O (1/2/0.8). The extract was filtered and then washed by Bligh/Dyer method. The resulting product solution was dried with anhydrous Na\textsubscript{2}SO\textsubscript{4}, and the solvent were evaporated producing a white solid 2.8b (9.5 mg, 50%). \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 400 MHz): \( \delta \) 0.90 (t, \( J = 6.8 \) Hz, 3H), 1.25-1.39 (30 H), 1.53 (m, 2H), 1.55-1.65 (4H), 2.32 (t, \( J = 7.6 \) Hz, 2H), 2.35 (t, \( J = 7.6 \) Hz, 2H), 3.22 (s, 9 H), 3.54 (t, \( J = 6.8 \) Hz, 2H), 3.64 (m, 2H), 3.99 (m, 2H), 4.16 (dd, \( J = 12.0, 6.8 \) Hz, 1H), 4.27 (m, 2H), 4.41 (dd, \( J = 12.0, 3.2 \) Hz, 1H), 5.23 (m, 1H). HRMS (FAB): m/z calcd for C\textsubscript{32}H\textsubscript{65}NO\textsubscript{9}P (MH\textsuperscript{+}) 638.4397, found 638.4398.

1, 2-O-Isopropylidene glycerol acetate (2.9a)

1, 2-O-isopropylideneglycerol (3.0 g, 23 mmol) and triethylamine (3.0 g, 30 mmol) were dissolved in 20 ml methylene chloride. Acetyl chloride (2.4 g, 30 mmol, in 10 ml methylene chloride) was added dropwise at room temperature within 30 min. The mixture was stirred at room temperature for 5 h and then filtered to remove the precipitate. The filtrate was washed with water 3 times and concentrated under reduced pressure to remove the solvent, to afford 3.5 g of colorless oil. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 400 MHz): \( \delta \) 1.32 (s, 3 H), 1.37 (s, 3 H), 2.05 (s, 3 H), 3.73 (m, 1 H), 4.04-4.20 (3 H), 4.30 (m, 1 H). The \textsuperscript{1}H NMR spectrum is identical with that reported previously. The crude product was used directly in the next step without further purification.
1, 2-O-Isopropylidene glycerol palmitate (2.9b)

A procedure analogous to that described above for 2.9a was used to get 2.9b. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.88 (t, $J = 6.8$ Hz, 3H), 1.20-1.37 (24H), 1.37 (s, 3H), 1.44 (s, 3H), 1.63 (m, 2H), 2.34 (t, $J = 7.2$ Hz, 2H), 3.74 (dd, $J = 8.4$, 6.0 Hz, 1H), 4.05-4.12 (2H), 4.16 (dd, $J = 11.6$, 4.8 Hz, 1H), 4.32 (m, 1H). $^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 14.36, 22.92, 25.12, 25.62, 26.91, 29.35, 29.48, 29.59, 29.68, 29.83, 29.88, 29.92, 32.15, 34.34, 64.74, 66.56, 73.89, 173.90.

1-Acetyl-sn-glycerol (2.10a)

Compound 2.9a (3.3 g, 19 mmol) was dissolved in 15 ml 80% acetic acid solution. The mixture was stirred at room temperature for 16 h and then concentrated under reduced pressure to remove the solvent. 2.4 g pale yellow oil was obtained. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 2.08 (s, 3 H), 3.60 (dd, $J = 11.6$, 6.0 Hz, 1H), 3.70 (dd, $J = 11.6$, 3.6 Hz, 1H), 3.96 (m, 1 H), 4.11-4.19 (2 H). The $^1$H NMR spectrum is identical with that reported previously. The crude product was used directly in the next step without further purification.

1-Palmityl-sn-glycerol (2.10b)

A procedure analogous to that described above for 2.10a was used to get 2.10b. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.87 (t, $J = 6.8$ Hz, 3H), 1.20-1.35 (24H), 1.61 (m, 2H), 2.34 (t, $J = 7.6$ Hz, 2H), 2.39 (br, 1H), 2.78 (br, 1H), 3.57-3.70 (2H), 3.92 (m, 1H), 4.14 (dd, $J = 11.6$, 6.0 Hz, 1H), 4.19 (dd, $J = 11.6$, 4.8 Hz, 1H). $^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$
14.34, 22.92, 25.12, 29.35, 29.48, 29.59, 29.68, 29.83, 29.88, 29.92, 32.15, 34.38, 63.57, 65.36, 70.49, 174.63.

1-Acetyl-3-(tert-butyldimethyl)silyl-sn-glycerol (2.11a)

Compound 2.10a (2.0 g, 14.9 mmol) and triethylamine (1.8 g, 17.9 mmol) were dissolved in 7 ml DMF. To the mixture, tert-butyldimethylsilyl chloride (2.7 g, 17.9 mmol, in 10 ml DMF) was added dropwise within 30 min at room temperature. The mixture was stirred at room temperature for 1 day and then filtered to remove the precipitate. The filtrate was concentrated under reduced pressure to remove the solvent. The obtained residue was purified by flash chromatography on silica gel with hexanes/ethyl acetate (6/1, TLC: Rf = 0.2) to give 2.11a (1.6 g, 44%). $^1$H NMR (CDCl$_3$, 400 MHz): δ 0.0 (s, 6H), 0.82 (s, 9H), 2.02 (s, 3H), 2.49 (br, 1H), 3.54 (dd, $J = 10.0$, 5.2 Hz, 1H), 3.59 (dd, $J = 10.0$, 5.6 Hz, 1H), 3.80 (m, 1H), 4.03 (dd, $J = 11.6$, 6.0 Hz, 1H), 4.07 (dd, $J = 11.2$, 4.4 Hz, 1H). $^{13}$C NMR (CDCl$_3$, 100 MHz): δ -5.24, 18.48, 21.10, 26.04, 63.87, 65.50, 70.11, 171.38.

1-Palmityl-3-(tert-butyldimethyl)-silyl-sn-glycerol (2.11b)

A procedure analogous to that described above for 2.11a was used to get 2.11b (46%). $^1$H NMR (CDCl$_3$, 400 MHz): δ 0.06 (s, 6H), 0.85-0.89 (12H), 1.20-1.35 (24H), 1.61 (m, 2H), 2.32 (t, $J = 7.6$ Hz, 2H), 2.51 (d, $J = 5.2$ Hz, 2H), 3.60 (dd, $J = 10.0$, 5.6 Hz, 1H), 3.65 (dd, $J = 10.0$, 4.4 Hz, 1H), 3.86 (m, 1H), 4.10 (dd, $J = 11.6$, 6.0 Hz, 1H), 4.13 (dd, $J = 11.6$, 4.8 Hz, 1H). $^{13}$C NMR (CDCl$_3$, 100 MHz): δ -5.47, 14.10, 18.25, 22.67,
24.92, 25.81, 29.12, 29.25, 29.35, 29.44, 29.59, 29.64, 29.68, 31.90, 34.18, 63.66, 64.96, 69.97, 173.97.

1-Acetyl-2-(7’-benzyloxy carbonyl)-heptanoyl-3-(tert-butyldimethyl)-silyl-sn-glycerol (2.12a)

Compound 2.11a (0.6 g, 2.4 mmol) and 0.23 g pyridine were dissolved in 6 ml methylene chloride. To the mixture, 7-benzyloxy carbonyl heptanoyl chloride (0.82 g, 2.9 mmol, in 6 ml methylene chloride) was added dropwise at room temperature. The resulting mixture was stirred at room temperature for 6 h and then filtered to remove the precipitate. The filtrate was concentrated under reduced pressure to remove the solvent. The obtained residue was purified by flash chromatography on silica gel with hexanes/ethyl acetate (5/1, TLC: Rf = 0.5) to give 2.12a (pale yellow oil, 1.0 g, 83%).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.05 (s, 6H), 0.88 (s, 9H), 1.31-1.35 (4H), 1.59-1.67 (4H), 2.06 (s, 3H), 2.30 (t, $J = 7.2$ Hz, 2H), 2.35 (t, $J = 7.2$ Hz, 2H), 3.70-3.72 (2H), 4.16 (dd, $J = 12.0$, 6.4 Hz, 1H), 4.32 (dd, $J = 12.0$, 3.6 Hz, 1H), 5.06 (m, 1H), 5.11 (s, 2H), 7.33-7.37 (5H).

$^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 0.0, 23.7, 26.3, 30.2, 31.2, 34.2, 39.7, 66.9, 68.2, 71.6, 133.7, 134.0, 141.5, 176.2, 178.5, 179.0.

1-Palmityl-2-(7’-benzyloxy carbonyl)-heptanoyl-3-(tert-butyldimethyl)-silyl-sn-glycerol (2.12b)

A procedure analogous to that described above for 2.12a was used to get 2.12b (90%).

$^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 0.07 (s, 6H), 0.87-0.92 (12H), 1.20-1.38 (28H),
1.58-1.64 (6H), 2.28-2.32 (4H), 2.35 (t, J = 7.6 Hz, 2H), 3.76 (m, 2H), 4.13 (dd, J = 12.0, 6.4 Hz, 1H), 4.35 (dd, J = 12.0, 3.6 Hz, 1H), 5.05 (m, 1H), 5.10 (s, 2H), 7.30-7.37 (5H).

1-Acetyl-2-(7'-benzyloxy carbonyl)-heptanoyl-sn-glycerol (2.13a)

Compound 2.12a (0.9 g, 1.8 mmol) was mixed with 22 ml DMSO, 2.3 ml THF, and 2.3 ml H2O in a 50 ml flask covered with aluminum foil to protect the reaction from light. Then N-Bromosuccinimide (NBS) (1.5 g, 8.4 mmol) was added to the mixture at room temperature. One hour later, 50 ml 1% sodium thiosulfate solution was added to quench the reaction. The mixture was extracted with ethyl acetate, and the obtained crude product was purified by flash chromatography on silica gel with hexanes/ethyl acetate (1/1, TLC: Rf = 0.4) to give 2.13a (colorless oil, 0.51 g, 74%). ¹H NMR (CDCl₃, 400 MHz): δ 1.30-1.34 (4H), 1.60-1.64 (4H), 2.06 (s, 3H), 2.30-2.36 (4H), 3.72 (m, 2H), 4.22 (dd, J = 12.0, 6.0 Hz, 1H), 4.34 (dd, J = 12.0, 4.4 Hz, 1H), 5.06 (m, 1H), 5.10 (s, 2H), 7.30-7.36 (5H). ¹³C NMR (CDCl₃, 100 MHz): δ 20.97, 24.87, 24.90, 28.81, 28.86, 34.33, 34.38, 61.61, 62.60, 66.37, 72.27, 128.42, 128.77, 136.2, 171.20, 173.53, 173.81.

1-Palmityl-2-(7'-benzyloxy carbonyl)-heptanoyl-sn-glycerol (2.13b)

A procedure analogous to that described above for 2.13a was used to get 2.13b (78%). ¹H NMR (CDCl₃, 400 MHz): δ 0.87 (t, J = 6.8 Hz, 3H), 1.20-1.37 (28H), 1.58-1.65 (6H), 2.10 (t, J = 6.4 Hz, 1H), 2.28-2.39 (6H), 3.72 (m, 2H), 4.24 (dd, J = 12.0, 4.8 Hz, 1H), 4.33 (dd, J = 12.0, 3.6 Hz, 1H), 5.07 (m, 1H), 5.11 (s, 2H), 7.30-7.38 (5H).
1-Acetyl-2-(8'-benzyl oxy-8'-oxooctanoyl)-sn-3-phosphocholine (2.15a)

Phosphorus oxychloride (136 mg, 0.89 mmol) and triethylamine (98 mg, 0.97 mmol) were dissolved in 2.8 ml methylene chloride. To the mixture, compound 2.13a (269 mg, 0.71 mmol, in 2.6 ml methylene chloride) was added dropwise within 10 min at room temperature. After 1.5 h, choline tosylate (311 mg, 1.13 mmol) and 0.9 ml pyridine were added. The mixture was stirred at room temperature for 1 day. Then 0.6 ml water was added. One hour later, the reaction mixture was concentrated under reduced pressure to remove the solvent. The residue was dissolved in THF/H₂O (3/1) and passed through an IWT TMD-8 (H⁺, OH⁻) ion exchange resin column. The crude product was purified further by flash chromatography on silica gel with CH₃CN / H₂O (2/1, TLC: Rf = 0.3) to give 2.15a (colorless oil, 230 mg, 60%). ¹H NMR (CD₃OD, 400 MHz): δ 1.28-1.38 (4H), 1.55-1.65 (4H), 2.02 (s, 3H), 2.32 (t, J = 7.6 Hz, 2H), 2.36 (t, J = 7.6 Hz, 2H), 3.22 (s, 9H), 3.63 (m, 2H), 3.99 (m, 2H), 4.19 (dd, J = 12.0, 6.4 Hz, 1H), 4.27 (m, 2H), 4.37 (dd, J = 12.0, 3.6 Hz, 1H), 5.10 (s, 2H), 5.21 (m, 1H), 7.30-7.35 (5H). ¹³C NMR (CD₃OD, 100 MHz): δ 19.49, 24.57, 24.68, 28.50, 28.53, 33.71, 33.76, 53.50, 59.25, 62.55, 63.67, 65.94, 70.05, 128.03, 128.39, 136.20, 171.20, 173.40, 173.96.

1-Palmityl-2-(8'-benzyl oxy-8'-oxo)-octanoyl-sn-3-phosphatidyl-(N,N,N-trimethylamino)-propanol (2.15b)

A procedure analogous to that described above for 2.15a was used to produce 2.15b (46%). ¹H NMR (CDCl₃, 400 MHz): δ 0.86 (t, J = 6.8 Hz, 3H), 1.19-1.35 (28H), 1.50-1.67 (6H), 2.10 (br, 2H), 2.23-2.28 (4H), 2.33 (t, J = 7.6 Hz, 2H), 3.26 (s, 9H), 3.70 (m, 2H), 3.85-3.98 (4H), 4.08-4.40 (2H), 5.09 (s, 2H), 5.18 (m, 1H), 7.27-7.38 (5H). ¹³C
NMR (CDCl$_3$, 100 MHz): $\delta$ 14.04, 22.60, 24.58, 24.62, 24.80, 28.61, 28.65, 29.08, 29.24, 29.28, 29.45, 29.58, 29.62, 31.83, 34.03, 34.07, 53.24, 61.61, 63.00, 64.00, 65.99, 70.62, 128.04, 128.10, 128.46, 135.98, 172.90, 173.36, 173.47.

**1-Palmityl-2-(8’-benzyloxy-8’-oxooctanoyl)-sn-3-phosphatidyl-(N,N,N-trimethyl-amino)-hexanol (2.15c)**

A procedure analogous to that described above for 2.15a was used to get 2.15c (48%). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.86 (t, $J = 6.8$ Hz, 3H), 1.19-1.38 (28H), 1.43-1.51 (4H), 1.51-1.70 (8H), 1.77 (m, 2H), 2.24-2.29 (4H), 2.33 (t, $J = 7.6$ Hz, 2H), 3.21 (s, 9H), 3.42 (m, 2H), 3.78-3.92 (4H), 4.08-4.30 (5H), 4.37 (dd, $J = 12.0, 2.8$ Hz, 1H), 5.10 (s, 2H), 5.18 (m, 1H), 7.27-7.38 (5H).

**1-Palmityl-2-(8’-benzyloxy-8’-oxo)-octanoyl-sn-3-phosphatidic acid (2.17)**

A procedure analogous to that described above for 2.15a was used to get 2.17 (70%). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.88 (t, $J = 7.2$ Hz, 3H), 1.19-1.40 (28H), 1.49-1.70 (6H), 2.24-2.40 (6H), 4.08-4.40 (4H), 5.10 (s, 2H), 5.24 (m, 1H), 7.27-7.38 (5H).

**1-Acetyl-2-suberyl-sn-3-phosphocholine (AcSPC, 2.16a)**

Compound 2.15a (73 mg, 0.13 mmol) in 7 ml of methylene chloride was hydrogenolyzed over 10% Pd/C (10 mg) at atmospheric pressure for 1 hour. The catalyst was removed by filtration, and the filtrate was concentrated. The crude product was purified by 0.5 mm preparative silica gel TLC plate with CH$_3$OH/H$_2$O (3/1, TLC: $R_f = 0.3$) to give 2.16a (43 mg, 70%). $^1$H NMR (D$_2$O, 400 MHz): $\delta$ 1.15-1.22 (4H), 1.40-1.50
1-Palmityl-2-suberyl-sn-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-propanol

(PSPP, 2.16b)

A procedure analogous to that described above for 2.16a was used to get 2.16b (61%). 1H NMR (CD3OD, 400 MHz): δ 0.89 (t, J = 6.8 Hz, 3H), 1.25-1.47 (28H), 1.55-1.65 (6H), 2.11 (m, 2H), 2.24 (m, 2H), 2.32 (t, J = 7.6 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 3.15 (s, 9H), 3.48 (m, 2H), 3.93-3.98 (4H), 4.14 (dd, J = 12.0, 6.8 Hz, 1H), 4.43 (dd, J = 12.0, 3.2 Hz, 1H), 5.22 (m, 1H). 13C NMR (CDCl3, 100 MHz): δ 14.36, 18.95, 22.92, 24.77, 25.11, 28.33, 28.62, 29.41, 29.58, 29.60, 29.78, 29.90, 29.94, 32.15, 34.32, 53.55, 62.13, 63.07, 64.00, 64.28, 71.02, 173.54, 173.79. HRMS (FAB): m/z calcd for C33H65NO10P (MH+) 666.4346, found 666.4346.

1-Palmityl-2-suberyl-sn-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-hexanol

(PSPH, 2.16c)

A procedure analogous to that described above for 2.16a was used to get 2.16c (72%). 1H NMR (CD3OD, 400 MHz): δ 0.89 (t, J = 6.4 Hz, 3H), 1.19-1.38 (28H), 1.38-1.55 (4H), 1.56-1.72 (8H), 1.81 (m, 2H), 2.28-2.37 (6H), 3.25 (s, 9H), 3.47 (m, 2H), 3.85 (m, 2H), 3.98 (m, 2H), 4.10-4.35 (2H), 5.21 (m, 1H). 13C NMR (CDCl3, 100 MHz): δ 14.35, 22.45, 22.91, 24.90, 25.10, 25.36, 28.07, 28.48, 29.38, 29.54, 29.59, 29.74, 29.88,
1-Palmityl-2-suberyl-sn-glycerol (PSG, 2.14)

A procedure analogous to that described above for 2.16a was used to get 2.14 (81%).

\[ ^1H \text{ NMR (CDCl}_3, 400 MHz): \delta 0.88 (t, J = 6.8 \text{ Hz}, 3\text{H}), 1.24-1.40 (28\text{H}), 1.58-1.68 (6\text{H}), 2.30-2.40 (6\text{H}), 3.74 (m, 2\text{H}), 4.20 (dd, J = 12.0, 5.6 \text{ Hz}, 1\text{H}), 4.35 (dd, J = 12.0, 4.6 \text{ Hz}, 1\text{H}), 5.09 (m, 1\text{H}). \]

\[ ^1C \text{ NMR (CDCl}_3, 100 MHz): \delta 14.35, 22.91, 24.64, 24.83, 25.10, 28.77, 29.34, 29.49, 29.59, 29.70, 29.84, 29.88, 29.92, 32.14, 34.30, 34.32, 61.68, 62.22, 72.36, 173.54, 174.09. \]

HRMS (FAB): m/z calcd for C\text{27}H\text{51}O\text{7} (MH\text{+}) 487.3635, found 487.3637.

1-Palmityl-2-suberyl-sn-glycerol-3-phosphatidic acid (PSPA, 2.18)

A procedure analogous to that described above for 2.16a was used to get 2.18 (60%).

\[ ^1H \text{ NMR (CD}_3\text{OD, 400 MHz): \delta 0.89 (t, J = 6.8 \text{ Hz}, 3\text{H}), 1.26-1.47 (28\text{H}), 1.55-1.70 (6\text{H}), 2.18-2.40 (6\text{H}), 3.90-4.02 (br, 1\text{H}), 4.15-4.28 (br, 1\text{H}), 4.35-4.60 (2\text{H}), 5.27 (m, 1\text{H}). \]

HRMS (FAB): m/z calcd for C\text{27}H\text{51}NaO\text{10}P (MNa\text{+}) 589.3118, found 589.3122.

1-Palmityl-2-(9'-methoxyl-9'-oxo)-nonanoyl-sn-glycero-3-phosphocholine (P9MNPC, 2.19)

A procedure analogous to that described above for 2.7a was used to get 2.19 (38%).

\[ ^1H \text{ NMR (CD}_3\text{OD, 400 MHz): \delta 0.89 (t, J = 6.8 \text{ Hz}, 3\text{H}), 1.25-1.37 (30\text{H}), 1.55-1.70 (6\text{H}), 2.29-2.36 (6\text{H}), 3.22 (s, 9\text{H}), 3.62-3.66 (5\text{H}), 3.98 (m, 2\text{H}), 4.15 (dd, J = 12.0, 6.8 \text{ Hz}, 1\text{H}). \]
Hz, 1H), 4.27 (m, 2H), 4.45 (dd, J = 12.0, 3.6 Hz, 1H), 5.23 (m, 1H). HRMS (FAB): m/z calcd for C_{34}H_{67}NO_{10}P (MH^+) 680.4502, found 680.4485.

1-Palmityl-2-(8’-amino-8’-oxo)-octanoyl-sn-glycero-3-phosphocholine (P8AOPC, 2.20)

Octanedioyl dichloride (105 mg, 0.5 mmol) was dissolved in 15 ml anhydrous chloroform. To the mixture, 184 mg 4-dimethylaminopyridine was added at room temperature. 10 min later, lysoPC (25 mg, 0.05 mmol, in 1 ml anhydrous chloroform) was added. After 1 hour of stirring, ammonia gas was bubbled into the solution. 25 min later, the mixture was concentrated to remove the solvent. The obtained residue was purified by 0.5 mm preparative silica gel TLC plate with CHCl₃/CH₃OH/NH₃/H₂O (29%) (65/35/8, TLC: Rf = 0.28) to give 2.20 (10.8 mg, 34%). ¹H NMR (CD₃OD, 400 MHz): δ 0.89 (t, J = 6.8 Hz, 3H), 1.25-1.38 (28H), 1.55-1.66 (6H), 2.21 (t, J = 7.6 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 2.35 (t, J = 7.4 Hz, 2H), 3.22 (s, 9H), 3.64 (m, 2H), 4.00 (m, 2H), 4.17 (dd, J = 12.0, 6.8 Hz, 1H), 4.26 (m, 2H), 4.40 (dd, J = 12.0, 3.4 Hz, 1H), 5.23 (m, 2H). HRMS (FAB): m/z calcd for C_{32}H_{64}N_{2}O_{9}P (MH^+) 651.4349, found 651.4347.
2.5 References


7. Jha, V., N.B. Kondekar, and P. Kumar, *Enantioselective Synthesis of syn/anti-1,3-Amino Alcohols via Proline-Catalyzed Sequential alpha-Aminoxylation/alpha-


Chapter 3

Structural basis for the recognition of oxidized phospholipids in oxidized low density lipoproteins by class B scavenger receptors CD36 and SR-BI*

3.1 Background

Atherosclerosis is a principal cause of heart attack and stroke. Substantial evidence suggests that oxLDL contributes to the development of atherosclerosis via various mechanisms.\textsuperscript{1-5} The oxidation of LDL renders it a ligand for several scavenger receptors including class B scavenger receptors CD36 and SR-BI.\textsuperscript{6-8} The oxidized phospholipids in oxLDL are reported to induce the recognition of oxLDL by the CD36 receptor,\textsuperscript{8} which has been shown to play a significant role in atherosclerosis.\textsuperscript{9,10}

Podrez and co-workers earlier demonstrated that a novel family of specific oxidized phospholipids accumulates at sites of oxidative stress \textit{in vivo} such as within atherosclerotic lesions, hyperlipidemic plasma, and plasma with low high-density lipoprotein levels.\textsuperscript{4,11} They then demonstrated that oxPC\textsubscript{CD36} serve as high affinity ligands for both the scavenger receptor CD36 and SR-BI. The structural aspect of oxPC\textsubscript{CD36} essential for high affinity binding to CD36 is a \textit{sn}-2 acyl group incorporating a terminal $\gamma$-hydroxy (or oxo)-$\alpha$,$\beta$-unsaturated carbonyl.\textsuperscript{12} oxPC\textsubscript{CD36} mediate uptake of oxLDL by macrophages via CD36, and promote a pro-thrombotic state via platelet scavenger receptor CD36.\textsuperscript{4} oxPC\textsubscript{CD36} also prevent binding of HDL to SR-BI because of the close proximity of the binding sites for these two ligands on SR-BI. Furthermore, oxPC\textsubscript{CD36} interfere with SR-BI-mediated selective uptake of cholesteryl esters in hepatocytes.\textsuperscript{3} These data clearly demonstrate that oxidative stress and accumulation of specific oxidized phospholipids may have a detrimental effect due to specific interaction with class B scavenger receptors. Elucidation of the structural basis of the recognition of oxidized phospholipids by CD36 and SR-BI is required to better understand their
contribution to cardiovascular pathology. However, the exact molecular mechanism of the recognition of oxPC_{CD36} by class B scavenger receptors is poorly understood.

It has recently been shown that the truncated oxidized sn-2 fatty acid moiety protrudes into the aqueous phase rendering it accessible for recognition.\textsuperscript{13} Dr. Podrez and co-workers previously established that electrophilic reactivity is not a prerequisite for high affinity CD36 binding since the relatively unreactive oxPC_{CD36} with γ-hydroxy-α,β-unsaturated-enoic acid groups at the sn-2 position are excellent ligands.\textsuperscript{14-16} The Podrez group also demonstrated that two lysine groups in CD36 (Lys164/166) are indispensable for the binding of oxPC_{CD36} to CD36. These studies suggested an electrostatic interaction mechanism for the binding,\textsuperscript{17} where the negative carboxylate groups in oxidized phospholipids form salt bridges with the positive lysine groups in the binding domain of CD36.

In the present study, a series of phospholipids having various functional groups at the sn-1, 2 and 3 positions, was used to elucidate the structural basis for the recognition of oxPC_{CD36} by scavenger receptors CD36 and SR-BI.

3.2 Results

3.2.1 A terminal carboxylate group in the sn-2 position of phospholipids engenders high binding affinity for class B scavenger receptors

Kar et al. recently demonstrated that the negative charge at the sn-2 position of the phospholipid is crucial for binding affinity to CD36.\textsuperscript{17} In order to determine if the terminal carboxylate group alone is sufficient to generate high binding affinity to class B scavenger receptors, we designed and synthesized PSPC and PDPC (Fig. 3.1). These
phospholipids lack the \( \gamma \)-\( \alpha \)-\( \beta \)-double bond present in the original \( \text{oxPC}_{\text{CD36}} \) lipids. PSPC and PDPC were incorporated into inert PAPC vesicles (models of the oxLDL phospholipid outer shell) and their affinity was compared to their \( \text{oxPC}_{\text{CD36}} \) analogs the 5-keto-6-octenedioic acid ester of 2-lyso-PC (KodiA-PC) and the 9-keto-10-dodecenedioic acid ester of 2-lyso-PC (KDdiA-PC).\(^\text{11,12}\) Vesicles made of PAPC served as a negative control. \(^{125}\)I-LDL oxidized by the MPO-H\(_2\)O\(_2\)-NO\(_2\)\(^-\) system (\(^{125}\)I-NO\(_2\)-LDL) binds specifically to scavenger receptors CD36 and SR-BI via \( \text{oxPC}_{\text{CD36}} \), therefore we assessed the binding activity of the synthetic phospholipids by their ability to block the binding of \(^{125}\)I-NO\(_2\)-LDL to cells over-expressing CD36 or SR-BI. We found that PSPC and PDPC have \( \text{IC}_{50} \) comparable to their \( \text{oxPC}_{\text{CD36}} \) analogs for both CD36 and SR-BI (Fig. 3.1), while vesicles made of native unoxidized phospholipids had no detectable binding affinity (Fig. 3.1, data for PAPC are shown). Thus, the negative carboxylate group in the \( sn \)-2 position of oxPC suffices to generate high binding affinity to class B scavenger receptors. It should be noted, though, that the presence of \( \gamma \)-\( \alpha \)-\( \beta \)-unsaturation in addition to the carboxylate group as in \( \text{oxPC}_{\text{CD36}} \) was usually associated with notable increases in the lipids' binding affinity (Fig. 3.1).
Fig. 3.1 A negative carboxylate group at the terminus of the \( sn-2 \) acyl group of phospholipids confers significant binding affinity toward CD36 and SR-BI. The synthetic phospholipids were analyzed for their ability to compete with the binding of \( ^{125}\text{I}-\text{NO}_2\text{-LDL} \) (5 \( \mu \)g/ml) to CD36 and SR-BI transfected 293 cells as described under experimental procedures. Binding abilities of the synthetic phospholipids to both receptors were determined by assessing the concentrations of synthetic phospholipids (presented as an equimolar mixture of synthetic phospholipids and PAPC) required to block 50% of \( ^{125}\text{I}-\text{NO}_2\text{-LDL} \) binding (IC\(_{50}\)). Results represent the mean ± SEM of three independent experiments. \# \( P < 0.0001 \) for comparison vs KOdiA-PC, PSPC, KDdiA-PC and PDPC, \(* P < 0.05 \) for comparison vs KOdiA-PC and \( ** P < 0.05 \) for comparison vs KDdiA-PC.

3.2.2 Neutral polar functional groups at the distal end of the \( sn-2 \) acyl group of phospholipids confer weak binding affinity

In order to see the effects of neutral polar functional groups at the distal end of the \( sn-2 \) position on receptor binding affinity, we designed and synthesized a series of
phospholipids that are similar to PSPC, but possess different functional groups at the sn-2 position (Fig. 3.2). P6HHPC, P8HOPC, P8AOPC and P9MNPC have hydroxyl, amide and methyl ester neutral polar groups at the terminus of sn-2 acyl group (Fig. 3.2). While the negatively charged carboxylate group that can form hydrogen bonds and salt bridges with the amino group of lysines in the binding domain of the receptor, these neutral groups can only form weak hydrogen bonds with the amino group of lysines. In addition, we synthesized PdiOSPC (Fig. 3.2) with two oxygen atoms incorporated into the carbon chain at the sn-2 position which, theoretically, could make the chain more polar, serve as additional hydrogen bond acceptors, and thus increase the binding. The IC₅₀ values (Fig. 3.2) showed that the phospholipids with terminal neutral polar groups have noticeable binding affinity to both receptors, however, the affinity was much weaker compared to phospholipids with negative carboxylate at the distal end of the sn-2 acyl chain (PSPC and PdiOSPC). The affinity of PdiOSPC was similar to that of PSPC, suggesting that in the presence of a negative carboxylate, additional oxygen atoms in the sn-2 chain does not make a significant contribution to binding affinity (Fig. 3.2).
3.2.3 Cooperation of functional groups at the sn-1, 2 and 3 positions of oxidized phospholipids is required for high affinity recognition by CD36 and SR-BI.

Previous experiments demonstrated that a negative group at the sn-2 position of the phospholipid is required for high binding affinity to CD36 and SR-BI. To test whether additional negative charge at the sn-3 position could further increase binding activity, we designed PSPA (Fig. 3.3). At pH 7.2, PSPA has a negatively charged phosphate group at the sn-3 position instead of zwitterionic phosphocholine group in PSPC. PSPA was found to have a higher binding affinity than PSPC (Fig. 3.3), further demonstrating the critical
importance of a negative charge for high-affinity binding to class B scavenger receptors. To check whether a negative group at sn-3 alone is sufficient for the high affinity binding, we tested 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid sodium salt (DPPA) which has a negative phosphate group at the sn-3 position and two long hydrophobic chains at the sn-1 and sn-2 positions (Fig. 3.3). DPPA was found to have very poor binding affinity (Fig. 3.3), demonstrating that a negative group alone at sn-3 is not sufficient to induce high affinity binding. These data suggest that the cooperation of all three parts of oxidized phospholipids may be important for binding to CD36 and SR-BI. To further test this hypothesis, we designed three phospholipids similar to PSPC in which one functional group either at the sn-1, sn-2 or sn-3 positions was significantly shortened or removed, as shown in Fig. 3.3. AcSPC has an acetyl group at the sn-1 position instead of long hydrophobic chain. LysoPC and PSG contain only a hydroxyl group at the sn-2 or sn-3 position, respectively. All three phospholipids (AcSPC, LysoPC and PSG) exhibit a near complete lack of binding affinity to both CD36 and SR-BI (Fig. 3.3). These results strongly suggest that an optimal structure of the sn-1, sn-2 and sn-3 positions of the oxidized phospholipids is important for the recognition by CD36 and SR-BI.
**Fig. 3.3** All three parts of oxidized phospholipids are indispensable for recognition by CD36 and SR-BI. The synthetic phospholipids were analyzed for their ability to compete for the binding of $^{125}$I-NO$_2$-LDL (5μg/ml) to CD36 and SR-BI transfected 293 cells as in **Fig. 3.1**. Results represent the mean ± SEM of three independent experiments. *$P < 0.001$ for comparison vs DPPA, AcSPC, LysoPC and PSG, **$P < 0.05$ for comparison vs PSPC.

3.2.4 The effect of acyl chain length at the $sn$-2 and $sn$-3 position of oxPC on the receptor binding affinity

To assess whether the chain length of the $sn$-2 and $sn$-3 groups is important for optimal binding, we designed, synthesized, and tested a series of phospholipids with
varying chain lengths at these positions, as shown in Fig. 3.4a and 3.4b. The IC$_{50}$ data suggest that the chain length at the $sn$-3 position moderately affects the binding affinity. Elongation of the carbon chain from 2 (choline) to 6 in the $sn$-3 head group resulted in a 20-40% reduction in IC$_{50}$ for the phospholipids (Fig. 3.4a) on both CD36 and SR-BI. A comparison between PSuPC, PGPC, PSPC and PDPC (Fig. 3.4b) shows that an acyl chain length of four carbons at the $sn$-2 position of the phospholipids is sufficient for high affinity binding, with only moderate changes following further elongation. Compared with CD36, SR-BI is more sensitive to the acyl chain length at the $sn$-2 position. For SR-BI, reduction of the chain length to four carbons almost doubles the IC$_{50}$. In addition, SR-BI shows significantly less binding affinity than CD36 for PAcPC that has a very short $sn$-2 acyl chain. For CD36, phospholipids with carbon chain lengths from 4 (e.g. PSuPC) to 12 (e.g. PDPC) have similar binding affinity.
Fig. 3.4 Chain length of the sn-2, 3 substituents modulates the binding affinity of oxidized phospholipids. (a) Longer chain length of the sn-3 head group provides better binding affinity. Results represent the mean ± SEM of three independent experiments. *P < 0.05 for comparison vs PSPC. (b) SR-BI is more sensitive to the acyl chain length at sn-2 position than CD36. The synthetic phospholipids were analyzed for their ability to compete for the binding of $^{125}\text{I}$-NO$_2$-LDL (5μg/ml) to CD36 and SR-BI transfected 293 cells as in Fig. 3.1. Results represent the mean ± SEM of three independent experiments. *P < 0.05 for comparison vs PGPC, PSPC and PDPC.

3.2.5 The role of γ-oxo-α,β-unsaturation in oxPC$_{CD36}$ binding affinity
To test whether it is the electrophilic reactivity of the γ-oxo-α,β-double bond or other properties which contribute to increased affinity of oxPC_{CD36} such as KODiA-PC and KDdiA-PC compared to PSPC and PDPC, we designed PMPC and PPPC, and compared their affinity to that of PSuPC. PMPC and PPPC contain structural moieties at the sn-2 position, which are similar to γ-oxo-α, β-unsaturation of oxPC_{CD36}, but are not electrophilically reactive towards lysine amino groups. The PSuPC used for comparison has an identical chain length to PMPC, but lacks the sn-2 α,β-double bond (Fig. 3.5). The competition assay showed that PPPC and PMPC have higher binding affinity to both receptors compared to PSuPC. This result suggests that electrophilic reactivity is not critical for the binding affinity of phospholipids with the carboxy terminated acyl group at the sn-2 position, and suggest that other properties (e.g. rigidity of the sn-2 chain) may be influential for the binding.

**Fig. 3.5** The rigidity of γ-oxo-α,β unsaturated carbonyl in oxPC_{CD36} increases the binding affinity to both receptors. The synthetic phospholipids were analyzed for their ability to compete for the binding of ^{125}I-NO2-LDL (5 μg/ml) to CD36 and SR-BI transfected 293 cells as in **Fig. 3.1**. Results represent the mean ± SEM of three independent experiments. *P < 0.05 for comparison vs PPPC and PMPC, ** P < 0.05 for comparison vs PMPC.
3.2.6 Model oxidized phospholipid species directly bind to class B scavenger receptors

So far we have employed competition assays to determine the activity of the synthesized lipids. To directly demonstrate the binding of the model synthetic oxPCs to class B scavenger receptors, we performed a parallel series of studies using direct binding assays. Each of a selected number of model synthetic oxPC species covering a wide range of $IC_{50}$ was incorporated into small unilamellar vesicles composed of unoxidized parent lipid as a carrier and a tracer level of $[^3H]$DPPC. Vesicles were then tested for their capacity to specifically bind to CD36$_{118-182}$ or SR-BI$_{183-205}$ GST-fusion proteins that contain the binding domain for oxidized lipoproteins and oxidized phospholipids. Although vesicles composed of PAPC, PLPC or POPC alone failed to demonstrate specific binding (data for POPC are shown), vesicles containing PSPC, PDPC, PSPH or PSPA bound to GST-CD36$_{118-182}$ or GST-SR-BI$_{183-205}$ at significantly greater levels than to GST (Fig. 3.6). At the same time, PSG or P8HOPC, which had weak competitive binding affinity, showed weak but detectable specific direct binding. In general, the ranking order of direct binding affinity among the synthetic oxidized PC species was consistent with their binding inhibitory capacity noted in competition assays. These experiments demonstrate that model oxidized phospholipids directly interact with scavenger receptors. They also strongly suggest that the inhibitory effect of model synthetic oxPC species on the binding of NO$_2$-LDL is not due to indirect interaction with NO$_2$-LDL.

In order to show the role of the interaction between the negative carboxylate group of the phospholipids and the positive lysine amino group in the scavenger receptor
binding domain, two lysine groups (164K, 166K) in GST-CD36\textsubscript{118-182} were replaced by alanine groups (164A, 166A) and the mutated GST-CD36\textsubscript{164A166A} was used in a direct binding assay. The test result demonstrated that replacing the two positive lysine groups with two neutral alanine groups led to a remarkable decrease in the direct binding affinity of model phospholipids with high binding affinity with the native binding domains and a negative carboxylate group at the sn-2 position (Fig. 3.6).

Fig. 3.6 Model synthetic phospholipids bind directly to CD36 and SR-BI peptides containing the binding site for oxidized LDL. Binding of POPC vesicles containing 20 mol\% of synthetic phospholipids and a tracer amount of \[^3\text{H}]\text{DPPC}\) to the indicated glutathione-sepharose-bound GST-fusion proteins (GST-CD36\textsubscript{118-182}, GST-CD36\textsubscript{164A166A} or GST-SR-BI\textsubscript{183-205}) was assessed as described in “Experimental Procedures”. Results represent the mean ± SEM of three independent experiments. *\(P < 0.01\) for comparison vs PSPC, PSPH, PDPC and PSPA, #\(P < 0.05\) and ##\(P < 0.01\).
3.2.7 Model oxidized phospholipids interfere with macrophage foam cell formation induced by oxidized LDL

Our data suggest that synthetic oxidized phospholipids may be able to interfere with foam cell formation by inhibiting the binding and subsequent uptake of oxidized lipoproteins mediated by class B scavenger receptors. Thus, we examined the effects of a select number of model synthetic oxPC species, covering a wide range of IC$_{50}$ (AcSPC, P8HOPC, PSPA, PGPC, PDPC, PSPH and PMPC), on macrophage foam cell formation. Murine peritoneal macrophages were incubated with the indicated oxidized phospholipids, [${}^{14}$C]oleate and LDL modified by the MPO-H$_2$O$_2$-NO$_2^-$ system (NO$_2$-LDL) which is a specific high affinity ligand for class B scavenger receptors, but not for class A scavenger receptors.$^{18}$ Macrophage cholesteryl ester formation was monitored by measuring the incorporation of [${}^{14}$C]oleate into the cholesteryl ester fraction of macrophage lipids. NO$_2$-LDL induced a significant [${}^{14}$C]oleate incorporation into cellular cholesteryl ester pools in SR-BI-deficient macrophages, as anticipated (Fig. 3.7a). In contrast, cells incubated with NO$_2$-LDL in the presence of phospholipids with high binding affinity (PSPA, PSPH and PMPC) had significantly reduced [${}^{14}$C]oleate incorporation into cellular cholesteryl ester pools (Fig. 3.7a). In agreement with binding studies, AcSPC had no effect and P8HOPC had a modest effect on cholesteryl ester accumulation in macrophages exposed to NO$_2$-LDL (Fig. 3.7). Similar results were obtained in CD36-deficient macrophages where uptake of NO$_2$-LDL is mediated by SR-BI (Fig. 3.7b). Receptor binding IC$_{50}$ values of model oxidized phospholipids correlated strongly with the capacity to inhibit cholesteryl ester accumulation (Fig. 3.7c, 3.7d).
Figure 3.7 Model synthetic phospholipids interfere with foam cell formation. 

Murine thioglycollate-elicited peritoneal macrophages were isolated from mice of the indicated phenotype, cultured and incubated with NO2-LDL (25 μg/ml) and [14C] oleate (1.5 μCi/ml) in the presence or absence of the indicated synthetic phospholipids (30 μM). 

(a, b) a [14C] cholesteryl ester synthesis assay was carried out as described under “Experimental Procedures”. (c, d) the IC50 of synthetic phospholipids correlate with the capacity to inhibit cholesteryl ester accumulation in macrophages. *P< 0.05, **P < 0.01 vs NA (no addition).
3.2.8 Synthetic phospholipids with a carboxylate group at the \( sn-2 \) position and high binding affinity to CD36 cannot effectively induce platelet activation

Podrez et al\(^4\) previously demonstrated that NO\(_2\)-LDL and specific oxPC\(_{CD36}\) (e.g., 9-keto-12-oxo-10-dodecenoic acid and 9-hydroxy-12-oxo-10-dodecenoic acid esters of lysoPC, KODA-PC and HODA-PC) can bind to and activate platelets via the CD36 receptor. In the present study, we tested the effects of all of the synthetic phospholipids on the activation of platelets with NO\(_2\)-LDL and KODA-PC as positive controls. Platelet activation was characterized by P-selectin expression on the platelet surface. The test results demonstrated that, although NO\(_2\)-LDL and KODA-PC can induce dramatic expression of P-selectin, most of the synthetic phospholipids in the present study cannot, even if they have high binding affinity to CD36, as shown in Figure 3.8 (phospholipids in 3.8A are good ligands, and phospholipids in 3.8B are weak ligands for CD36). The major difference between KODA-PC and the synthetic phospholipids in the present study is that the former possesses high electrophilic reactivity, and the latter do not. This could be the reason for the difference in their ability to activate platelets via the CD36 receptor.

Although PAcPC is a weak ligand for CD36, it can still induce P-selectin expression comparable to that of the positive control NO\(_2\)-LDL and KODA-PC. Because PAcPC has a very similar structure to platelet activating factor (PAF), the mechanism for its platelet activation effect is probably through the PAF receptor.
Figure 3.8 P-selectin expression. The study was carried out as described under “Experimental Procedures”. Results represent the mean ± SEM of three independent trials. * P < 0.05 vs NA (no addition)

3.3 Discussion

Recognition of oxPC$_{CD36}$ by class B scavenger receptors plays a role in several pathophysiological processes associated with oxidative stress.$^{3, 4}$ Kar et al. recently showed that the mechanism of interaction of oxPC$_{CD36}$ with CD36 is mostly electrostatic and involves two conserved, positively charged amino acids in the binding site of CD36.$^{17}$ Using specifically designed synthetic phospholipids, the present studies systematically investigated the structural basis for the recognition of oxidized phospholipids by two class B scavenger receptors: CD36 and SR-BI.

Recent studies on the conformation and orientation of oxidized phospholipids in cell membranes and oxidized lipoproteins demonstrated that the truncated polar sn-2 residue protrudes into the aqueous phase, making it accessible for receptors.$^{13, 16}$ This exposed sn-
2 fatty acid moiety is very likely a prerequisite for detection of oxidized phospholipids by scavenger receptors. Moreover, the present study clearly demonstrates that all three parts of the oxidized phospholipids play a critical role in the high affinity binding to class B scavenger receptors. Deletion of functional groups appended to any oxygen of the glycerol backbone, as in AcSPC, LysoPC and PSG, leads to the loss of binding affinity to class B scavenger receptors. It is possible that all three side chains on the glycerol backbone of the oxidized phospholipids are required to maintain the proper conformation and orientation of the oxidized phospholipids in the outer shell of oxLDL, or oxidized phospholipids in cell membranes. Our findings are similar to the report of the Berliner group, who reported that all parts of the oxidized phospholipid molecules are required for activation of endothelial cells.¹⁹

The negative carboxylate group at the \( sn \)-2 position of phospholipids can form both salt bridges and hydrogen bonds with the positively charged amino groups of lysines in the receptor binding domain, which can explain the higher binding affinity generated by the carboxylate group. Indeed, the role of positively charged lysines was demonstrated in the direct binding assay with mutated GST-CD36_{164A166A} in which lysines were substituted with neutral alanines. GST-CD36_{164A166A} showed only residual binding of oxidized phospholipids with a terminal negative carboxylate group on the \( sn \)-2 acyl group.

The contribution of a \( \gamma \)-hydroxy group (or oxo)-\( \alpha,\beta \)-unsaturation in the \( sn \)-2 acyl group to binding activity of oxPC_{CD36} was not established before. In this study, we observed that the presence of \( \gamma \)-oxo-\( \alpha,\beta \)-unsaturation is associated with a noticeable increase in binding affinity. The electrophilic reactivity of some of oxPC_{CD36} was
previously ruled out as playing a role in high affinity CD36 binding since the relatively
unreactive oxPC_{CD36} with γ-hydroxy-α,β-unsaturated-enoic acid groups at sn-2 position
are excellent ligands. The analogous saturated carboxylic acid missing the γ-oxo-α,β-
unsaturated double bond is less acidic with a much higher pKa (≈ 5). However, this
probably does not play a significant role since at physiological pH 7.4, more than 99% of
both carboxylic acids with pKa about 5 would dissociate and exist in the form of negative
carboxylate group. Another difference between the γ-oxo-α,β-unsaturated carboxylate
group and the analogous saturated carboxylate group is that the former has more rigidity,
because of its conjugated structure. Our data in Fig. 3.5 support the conclusion that
rigidity could contribute to the higher binding affinity found for the γ-oxo-α,β-
unsaturated carboxylate group. Furthermore, the noticeable reduction in the binding
affinity of γ-hydroxy containing oxPC_{CD36} compared to γ-oxo containing oxPC_{CD36}
can now be explained by the lesser rigidity of the former. Our data in Fig. 3.2 also support the
conclusion that additional polarity of sn-2 position as such does not play significant role
in the binding activity.

In the present studies, CD36 and SR-BI were systematically compared in their
recognition of phospholipids containing different functional groups. Generally speaking,
CD36 and SR-BI show comparable binding affinity to various synthetic phospholipids,
show limited binding affinity to neutral phospholipids, and exhibit much higher binding
affinity to negatively charged oxidized phospholipids (Fig. 3.2). SR-BI has a slightly
lower affinity for the oxidized phospholipids. This difference is especially clear for
phospholipids of relatively low binding affinity. Compared with CD36, SR-BI is more
sensitive to the acyl chain length at the sn-2 position (Fig. 3.4b). While CD36 still
recognizes oxPC with a 1-4 carbon chain, SR-BI rapidly loses its recognition capacity when the sn-2 chain is shortened. Apparently SR-BI is more selective in the recognition of ligands containing oxidized phospholipids compared to CD36. The binding domain of SR-BI for oxidized phospholipids has not yet been identified. This makes it difficult to explain the binding differences of the two receptors.

Many of the lipids tested in the current study are model lipids, and their presence in vivo is either unknown or unlikely. Nevertheless, the information inferred by this work can be applied to lipids that are observed in vivo. Two biologically active oxidized phospholipids - glutaroyl (PGPC) and oxovaleroyl (OV-PC) phospholipids - were previously described by Berliner and coauthors.20 OV-PC has an acyl group at the sn-2 position which has a polarity similar to P6HHPC and P8HOPC; correspondingly, in its free not protein bound form, it has a weak affinity to CD36 as we showed earlier.11,12 In contrast, PGPC possesses a carboxylate group at the sn-2 position, which according to our results, suffices for recognition by Class B scavenger receptors. Indeed, PGPC was found to be a good ligand for CD36 and SR-BI (Fig. 3.4b).

Our present study adds new information useful for the development of phospholipid or small molecule analogs capable of inhibiting the uptake by macrophages of oxLDL and foam cell formation mediated by scavenger receptors and, potentially, suppressing atherogenesis in vivo. According to our study, a phosphatidic acid derivative incorporating a long hydrophobic acyl chain at the sn-1 position and a relatively rigid 6-12 carbon acyl chain, e.g., incorporating a conjugated double bond to enhance the rigidity, with a terminal carboxylate group at the sn-2 position would be a strong inhibitor. Since oxidized phospholipids are hydrolyzed by phospholipase A2, having a sn-2 ether bond is
expected to significantly increase the \textit{in vivo} stability of such a phospholipid. However, several additional considerations should be taken into account. The Podrez group has shown previously that oxPC$_{CD36}$ activates platelets via the scavenger receptor CD36. They have also found that oxPC$_{CD36}$ may potentially inhibit reverse cholesterol transport due to interference with HDL binding to SR-BI. Thus, the effects of such inhibitors on atherosclerosis \textit{in vivo} require further systematic studies that ascertain the potential side effects of inhibitors of oxLDL uptake and foam cell formation.

3.4 Conclusions

The present systematic studies significantly extend the understanding of structural factors governing the recognition of oxidized phospholipids by CD36 and SR-BI receptors. We demonstrated that an intact \textit{sn}-1 hydrophobic acyl chain, a \textit{sn}-3 hydrophilic phosphocholine or phosphatidic acid group and the polar terminus of an \textit{sn}-2 acyl group are absolutely essential for high affinity binding. We further found that a terminal negatively charged carboxylate terminus on the \textit{sn}-2 acyl group suffices to generate high binding affinity to class B scavenger receptors. In addition, factors such as polarity, rigidity, optimal chain length of the \textit{sn}-2 acyl, and \textit{sn}-3 head group and negative charge in the \textit{sn}-3 head group of phospholipids further enhance the binding affinity. We conclude that groups appended to all three hydroxyls of the glycerol backbone of oxidized phospholipids are essential for strong recognition by class B scavenger receptors.

3.5 Experimental procedures

3.5.1 Materials
Tissue culture media and additives were purchased from Gibco BRL (Grand Island, NY). Na\(^{125}\text{I}\) was supplied by ICN Pharmaceutical, Inc. (Costa Mesa, CA). \(^{3}\text{H}\)DPPC were from American Radiolabel Chemicals, Inc., (St. Louis, MO). 1-Hexadecanoyl-2-eicosatetra-5',8',11',14'-enoyl-\(sn\)-glycero-3-phosphocholine (PAPC) and 1,2-dihexadecanoyl-\(sn\)-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

3.5.2 Methods

3.5.2.1 General procedures

All buffers were passed over a column of Chelex-100 resin (Bio-Rad, Hercules, CA) and supplemented with diethylenetriamine pentaacetic acid (DTPA) to remove and/or sequester transition metal ions, which might catalyze oxidation of LDL and phospholipids during incubation. LDL was isolated from fresh plasma by sequential ultracentrifugation,\(^{18}\) and iodination with Na\(^{125}\text{I}\) was performed as previously described.\(^{21}\)

3.5.2.2 Lipoprotein oxidation

LDL was modified by the MPO-H\(_2\)O\(_2\)-NO\(_2^{-}\) system by incubating LDL (0.2 mg protein/ml) at 37 °C in 50 mM sodium phosphate, pH 7.0, 100 \(\mu\)M DTPA, 30 nM myeloperoxidase (MPO), 100 \(\mu\)g/ml glucose, 20 ng/ml glucose oxidase and 0.5 mM NaNO\(_2\) for 8 h \(^{12}\). Oxidation reactions were terminated by addition of 40 \(\mu\)M BHT and 300 nM catalase to the reaction mixture.
Preparation of phospholipid vesicles

PAPC, PLPC, or POPC were mixed with synthetic phospholipids with molar ratios of 1:1 or 4:1 in chloroform in 4 ml glass vials. Chloroform was then evaporated with a stream of nitrogen. Argon-sparged sodium phosphate buffer (1 ml, 100 mM, pH 7.4) was added to the vials, which were then mixed with a vortex mixer. The resulting suspension was then extruded through a 0.1 μm polycarbonate filter 11 times using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL) at 37 °C. For direct binding experiments, [³H] DPPC (25 μCi/mg of phospholipids) was added as a tracer.

Competitive binding experiment

The competitive binding assays were carried out by Dr. Ashraf Zahid from the Podrez group. The ability of synthetic phospholipids to inhibit the binding of ¹²⁵I-NO₂-LDL to class B scavenger receptors CD36 or SR-BI was examined as follows. Synthetic phospholipid vesicles (120 μM, 60 μM, 30 μM, 15 μM, 7.5 μM, 3.75 μM, 1.87 μM, or 0 μM) were mixed with Dulbecco's modified Eagle's medium (DMEM) containing ¹²⁵I-NO₂-LDL (5 μg/ml), 5% fetal bovine serum (FBS), LDL (100 μg/ml), diethylene triamine pentaacetic acid (DTPA, 100 μM) and catalase (300 nM). The resulting mixture was incubated with CD36-over expressing or SR-BI-over expressing HEK-293 cells or control vector cells for 3 h at 4 °C. Then unbound ¹²⁵I-NO₂-LDL was washed away with ice-cold PBS. The cells were lysed by adding 0.1 M NaOH and transferred to scintillation vials. Then the cell-associated radioactivity was quantified with Beckman Coulter Beta/Gamma Counter (LS5000TD). The radioactivity count data are shown in Tables 3.1 and 3.2. Each experiment was performed in triplicate, and results are
expressed as the percentage of control binding and calculated as $100 \times (r/c)$, where $c$ is the radioactivity count in control samples incubated without synthetic phospholipid competitor and $r$ is the radioactivity count in samples incubated with phospholipid competitor. The percentage of control binding vs. Log [synthetic phospholipid] data was plotted using Prism software (GraphPad Inc., San Diego, CA) and the IC$_{50}$ was calculated using the nonlinear regression curve fit with one-site competition. Values are expressed as means ± SEM. Statistical significance was evaluated using a two-tailed unpaired Student’s $t$-test. Results were considered statistically significant with $P$ values less than 0.05.

Table 3.1 Radioactivity count data (CPM) for cell bound $^{125}$I-NO$_2$-LDL in the competitive binding assay with CD36 expressing HEK 293 cells

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Preparation of GST fusion proteins

All of the GST fusion proteins were made in a Rosetta™(DE3)pLacI strain of Escherichia coli (E. Coli) (EMD Biosciences-Novagen, San Diego, CA). All stock samples of E. coli with expression vectors encoding GST-CD36118-182, GST-CD36164A166A or GST-SR-BI183-205 were provided by Dr. Niladri S. Kar from the Podrez group.

A stock sample of E. coli was added to a 2 L conical flask containing 50 ml LB broth (Miller’s) medium with glucose (1%) and ampicillin (50 µg/ml). The resulting mixture was incubated at 37 °C overnight. Then another 450 ml LB broth (Miller’s) medium with glucose (1%) and ampicillin (50 µg/ml) was added, and the incubation continued at 37 °C until the optical density of the culture medium at 600 nm increased to about 0.6. The E. coli culture medium was cooled to room temperature and isopropyl β-D-thiogalactoside (IPTG) (final concentration 0.5 mM) was added to induce the expression of the GST fusion protein. 3.5 hours later, the solution was cooled with ice, transferred into a centrifuge bottle (500 ml), and centrifuged at 6000 RPM for 10 min at 4 °C. The supernatant was discarded. To the E. coli pellet, 20 ml of cold 1× phosphate
buffered saline (PBS) with lysozyme (1 mg/ml) and protease inhibitor (2 mM AEBSF, 1 µM phosphoramidon, 130 µM bestatin, 14 µM E-64, 1 µM leupeptin, 0.2 µM aprotinin, 10 µM pepstatin A) was added. The suspension of E. coli was transferred to centrifuge tubes (30 ml) and left on ice for 10 min. Then the suspension of E. coli was sonicated for 30 second in an ice bath. Triton X-100 (1%) was added, and the suspension was sonicated for 1 min in an ice bath. The resulting solution was centrifuged at 14,000 RPM for 10 min at 4 ºC. The upper clear supernatant was transferred to a 50 ml conical tube and mixed with 2 ml glutathione agarose resin therein. The mixture was incubated with shaking for 1 hour at 4 ºC. Then the glutathione resin was spun down with a centrifuge at 500 RPM for 5 min, and the supernatant was discarded. The resin was washed with 1× PBS, then with 1× PBS (0.5% Triton X-100). The washing process was repeated, and the resin was then washed with 1× PBS as the last step. The resin was stored at 4 ºC with protease inhibitor and 0.02% sodium azide. The fusion proteins were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 3.8). The molecular weight was found to be close to the predicted value.

Fig. 3.9 SDS-PAGE of GST fusion proteins
Direct Binding Assays

Binding of phospholipid vesicles to GST fusion proteins was assessed by incubating [³H]DPPC-labeled phospholipid vesicles (10 μM) with the glutathione-sepharose-bound proteins (GST-CD36₁₁₈-₁₈₂, GST-CD36₁₆₄A₁₆₆A or GST-SR-BI₁₈₃-₂₀₅) (2.5 μg protein/tube) in PBS for 3 h at 4 °C with gentle rocking. Unbound phospholipid vesicles were removed by repeated washing of the beads with PBS using low-speed centrifugation, and then the beads were transferred to scintillation vials. 5 ml scintillation cocktail was added to the vials, and radioactivity was counted with a Beckman LS 6000 liquid scintillation counter.

The radioactivity count data are shown in Table 3.3.

Table 3.3 Radioactivity count data (CPM) for the direct binding of [³H]DPPC labeled phospholipid vesicles (10 μM) to the GST fusion proteins

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<td>814</td>
<td>972</td>
<td>832</td>
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</tr>
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Cholesteryl ester synthesis assay

Thioglycollate medium (0.5 ml) was injected into the peritoneal cavity of mice to elicit macrophages into the peritoneal cavity. 65 hours later, thioglycollate elicited mouse peritoneal macrophages (MPM) were isolated from wild type, CD36 knockout and SR-BI knockout mice and cultured for 48 hours in RPMI-1640/10% FBS medium\textsuperscript{21} with 12 well cell culture clusters. Confluent MPM was incubated with synthetic phospholipids (30 µM), [\textsuperscript{1-\textsuperscript{14}}C]oleate (1.5 µCi/ml), and NO\textsubscript{2}-LDL (25 µg/ml) at 37 °C. After 24 h, the supernatant was removed, and the cells were washed with PBS three times. 1 ml of hexanes/isopropanol (3/2) solution was added to each well, and the plate was shaken vigorously for 30 min. The resulting extracts were transferred to glass tubes. Then 0.6 ml of hexanes/isopropanol (3/2) solution was added to each well, and the process was repeated. The extracts were dried with nitrogen at room temperature. 100 µl chloroform (containing 1.4 mg/ml cholesteryl oleate as an internal standard) was added to each glass tube to dissolve the dried residue. 50 µl of the extracts in chloroform were applied to a TLC plate, which was developed with hexanes/ethers/acetic acid (70/30/1). The TLC plate was then dried and stained with iodine vapour. The cholesteryl oleate bands were cut and put into scintillation vials. Incorporation of [\textsuperscript{14}C]oleate in cholesteryl esters was quantified by liquid scintillation counting\textsuperscript{22} (Beckman Coulter Limited, UK). The radioactivity count data are shown in Table 3.4.
Table 3.4 Radioactivity count data (CPM) for the cholesteryl [14C]oleate ester that was incorporated into macrophage cells in the cholesteryl ester synthesis assay.

<table>
<thead>
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<th>SR-BI-KO</th>
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<tr>
<td>AcSPC</td>
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</tr>
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</table>

1. Macrophages were incubated with [14C]oleate in the absence of NO2-LDL and phospholipids; 2. Macrophages were incubated with [14C]oleate and NO2-LDL in the absence of phospholipids; 3. Macrophages were incubated with [14C]oleate and NO2-LDL in the presence of various phospholipids

**Preparation of platelets**

Six volumes of fresh human venous blood from healthy volunteers were mixed with one volume of anticoagulant acid-citrate-dextrose (ACD, 85 mM tri sodium citrate, 65 mM citric acid, 111 mM D-glucose, pH 4.6) containing Prostacyclin (PGI2) (0.7 µg/ml). The blood mixture was centrifuged at 800 rpm for 15 min at 22 °C. Platelet rich plasma (PRP, the upper layer) was separated and centrifuged again at 2,000 rpm for 15 min at 22 °C to pellet the platelets. The supernatant was removed, and the isolated platelets were
resuspended in modified Tyrode’s buffer (137 mM NaCl, 12 mM NaHCO₃, 2.5 mM KCl, 10 mM HEPES, 0.1% BSA and 0.1% glucose, pH 7.2). The platelets were further purified by gel filtration on a sepharose 2B column. 2 mM CaCl₂ and 1 mM MgCl₂ were added to the platelet solution immediately after the gel filtration.

**P-selectin expression**

P-selectin expression was assessed by flow cytometric analysis using phycoerythrin-conjugated mouse anti-human CD62P monoclonal antibody. Platelets (1×10⁸ /ml in modified Tyrode’s buffer) were incubated with various agonists (final concentration: 15 µg/ml NO₂-LDL, 20 µM phospholipids, 10 µM ADP) at room temperature for 30 min in the presence of phycoerythrin-conjugated mouse anti-human CD62P monoclonal antibody. Then the incubated mixture was diluted 5 times with 400 µl modified Tyrode’s buffer and analyzed directly by fluorescence-activated cell sorting (FACS) with a FACSCalibur instrument (BD Biosciences). The data obtained were analyzed by FlowJo software and are shown in **Table 3.5**.
**Table 3.5** Data for P-selectin expression on activated platelets (percentage of gated platelets)

<table>
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<td>PSPC 30.59 43.1 18.03</td>
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<tr>
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<td>PMPC 31.03 41.7 23.19</td>
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<td>PdiOSPC 30.64 41.7 16.02</td>
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<tr>
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<td>PSPA 31.92 42.8 17.7</td>
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3.6 References


Chapter 4

Covalent adducts between KOOA/KODA-PC and proteins: pilot study to develop a probe to explore the oxPC binding sites on CD36 and SR-BI receptors
4.1 Background

Podrez and co-workers earlier demonstrated that a novel family of specific oxidized phospholipids accumulates at sites of oxidative stress in vivo such as within atherosclerotic lesions and hyperlipidemic plasma.\textsuperscript{1-3} Among these oxPC\textsubscript{CD36} phospholipids, KODA-PC and KOOA-PC (the 9-keto-12-oxo-10-dodecenoic acid and the 5-keto-8-oxo-6-octenoic acid esters of 2-lysoPC), with a relatively high concentration found in vivo\textsuperscript{1, 3} and high binding affinity to the CD36 receptor,\textsuperscript{2} may play a very important role in promoting the binding of oxLDL to the CD36 receptor. These two phospholipids can also bind to SR-BI with high affinity and inhibit the binding of HDL to SR-BI. Thus, they may have an inhibitory effect on the SR-BI mediated reverse cholesterol transport process.\textsuperscript{4} In addition, they were very potent ligands to activate platelets via CD36.\textsuperscript{1}

Previous studies\textsuperscript{2, 4} showed that KOOA-PC and KODA-PC bind to CD36 and SR-BI with high affinity. However, the binding mechanism is still not clear. Kar and coworkers in the Podrez group demonstrated that the lysine residues 164 and 166 in the CD36 receptor are critical for the binding of oxPC\textsubscript{CD36} and oxLDL to CD36.\textsuperscript{5} In their binding experiment, a bell-shaped pH profile and salt concentration dependence suggest an electrostatic mechanism of the binding.\textsuperscript{5} The study described in Chapter Three also demonstrated the importance of electrostatic interaction. However, for KOOA-PC and KODA-PC, this binding mechanism is expected to be less important because they are neutral molecules. Another binding mechanism could be hydrogen bonding. However, the study described in Chapter Three suggested that hydrogen binding only generates weak binding activity.
With “γ-oxo-α,β-unsaturated-aldehyde” groups at the sn-2 position, KOOA-PC and KODA-PC are highly electrophilic and reactive toward nucleophiles such as the ε-amino group of lysine residues and the imidazole group in histidine residues. Thus, covalent binding is a potential binding mechanism. Studies on the reactivity of 4-oxo-2-nonenal (ONE) by Lin et al. demonstrated that the “γ-oxo-α,β-unsaturated-aldehyde” group forms Schiff base adducts with lysine and Michael adducts with histidine residues within minutes. While the ONE Schiff base and Michael adducts are reversible, the KOOA/KODA-PC Schiff base and Michael adducts with large molecules like proteins could be more stable for several reasons. First, KOOA-PC and KODA-PC exist as part of bulky oxLDL or phospholipid vesicles which could slow the release of KOOA-PC and KODA-PC from the receptors and thus stabilize the Schiff base and Michael adducts by acting as a kinetic trap. Second, the bulky ligands like oxLDL and phospholipid vesicles may shield the Schiff base adduct from water and thus prevent the reverse reaction. Third, cationic protonated Schiff base adducts could be stabilized by forming salt bridges with anionic carboxylate groups in the binding domain.

Previous studies on the binding domain of oxidized phospholipids and oxidized LDL were carried out in an indirect way. Kar and coworkers demonstrated that lysine residues 164 and 166 in CD36 are critical for the binding of oxPC_{CD36} and oxLDL to CD36. In their study, GST fusion proteins and site-directed mutagenesis of CD36 were used to investigate the binding site. Using a similar approach, Pearce et al. reported that CD36 (AA 28-93) contains a binding domain for oxLDL. Ashraf and coworkers, using a fusion protein GST-SR-BI (144-205), demonstrated that SR-BI (AA144–205) contains a binding site for HDL, oxLDL and oxPC_{CD36}. One disadvantage of the above methods is...
that the tertiary structure of the receptors is compromised, and the secondary structure may also be altered to some extent. It is well known that the secondary and tertiary structures are critical for ligand binding. So, it would be much better to find a way of using intact receptor on cells as experimental subjects to explore the binding domains. Since KOOA-PC and KODA-PC can form covalent bonds with lysine and histidine residues in proteins, they can be used to react with intact CD36 or SR-BI expressed on cells. The resulting covalent adducts can be stabilized with reduction and analyzed with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to discover the binding sites.

In this chapter, pilot model studies were done on Schiff base formation between KOOA/KODA-PC and small molecules such as butylamine and short CD36 peptides. Methods to enrich KOOA/KODA-PC modified peptides and proteins were also developed.

4.2 Results and discussion

4.2.1 Pure KOOA-PC and KODA-PC are not stable in aqueous buffer at pH 7.4

Dr. De Lin in the Sayre group reported the dimerization of ONE in his thesis. KOOA-PC and KODA-PC have the same “γ-oxo-α, β unsaturated-aldehyde” group as that in ONE. So, the stability of KOOA-PC in aqueous buffer at pH 7.4 was checked. KOOA-PC (75 µM in 100 mM phosphate buffer) was monitored by ultraviolet (UV) spectroscopy. The UV peak at 226 nm decreased very quickly with time, as shown in Figure 4.1A. KOOA-PC molecules form micelles, in which KOOA-PC molecules are crowded together and stay parallel to each other, making the reaction (e.g. dimerization)
between each other much easier (Figure 4.2). To solve this problem, other inert phospholipids, such as lysoPC, POPC, or PLPC, were mixed with KOOA-PC. These inert phospholipids could insert between KOOA-PC molecules and thus decrease the chances of reactions between adjacent KOOA-PC molecules. The experiments demonstrated that when KOOA-PC was mixed with lysoPC in a 1:20 mole ratio, the KOOA-PC was stabilized dramatically, as shown in Figures 4.1B and 4.1C. Interestingly, when KOOA-PC was mixed with inert phospholipids like POPC and lysoPC, the UV absorbance intensity increased significantly, as shown in Figure 4.1C. This phenomenon is probably due to the addition of 20 times more lysoPC, which could generate more phospholipid micelles that scatter the incident light, especially at short wavelengths.
**Figure 4.1** KOOA-PC is not stable in aqueous buffer. A. KOOA-PC (75 µM in 100 mM phosphate buffer) was monitored by UV spectrosopy. The absorbance intensity at 226 nm dropped dramatically with time. B. The addition of lysoPC stabilizes KOOA-PC. For mixtures of KOOA-PC and lysoPC, the absorbance intensity at 226 nm still dropped with time, but much slower than observed for pure KOOA-PC. C. The time course of absorbance intensity for KOOA-PC (75 µM) and a mixture of KOOA-PC (75 µM) with lysoPC (1.5 mM) at 226 nm.
Figure 4.2 A possible mechanism for reactions between KOOA-PC molecules in aqueous solution. This mechanism was proposed by Dr. De Lin in the Sayre group for the reactions between the analogous ONE molecules.

4.2.2 Inert phospholipids generate high background in UV spectra of KOOA-PC

During the study of KOOA-PC stability, we noticed that the addition of inert phospholipids could lead to increase of absorbance intensity (Figure 4.1C). So, we compared the effects of lysoPC and POPC on the UV absorbance. 1.5 mM lysoPC was dissolved in 100 mM phosphate buffer (pH 7.4) and used directly. The size of a lysoPC micelle is about 6 nm in aqueous solution. POPOC vesicles (30 nm and 100 nm) were made by passing 1.5 mM POPC solution through 30 nm and 100 nm polycarbonate filters 11 times respectively using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL). Their UV spectra were recorded and compared, as shown in Figure 4.3. The UV spectra demonstrated that the larger the vesicle size is, the higher UV absorbance it generates. Part of the reason for high UV absorbance value, especially at short wave lengths, may be due to the scattering of incident light. The above test results suggested
that lysoPC would work better as an additive to KOOA-PC in the UV study because of its low UV absorbance background.

![UV spectrum for lysoPC micelles, 30 nm and 100 nm POPC vesicles.](image)

**Figure 4.3** The UV spectrum for lysoPC micelles, 30 nm and 100 nm POPC vesicles.

### 4.2.3 Schiff base adduct of KOOA-PC and butylamine

A previous study on the reaction between ONE and butylamine by Lin et al. demonstrated that, at early reaction times (e.g., 20 min), the Schiff base is the exclusive product. Their study demonstrated that ONE has an absorbance peak at 226 nm, and there is a red shift in the UV absorption spectrum that accompanies the formation of Schiff base adducts. In the present study, the reaction between KOOA-PC and butylamine was monitored by UV spectroscopy. KOOA-PC was mixed with lysoPC at a molar ratio of 1:10 and dissolved in phosphate buffer (100 mM, pH 7.4). Then 10 mM butylamine was added, and the reaction was monitored by UV spectroscopy. The UV spectrum demonstrated that KOOA-PC also has an absorbance peak around 226 nm, and there is a red shift in the absorption spectrum during the reaction, which suggests the formation of Schiff base adducts within a short time, as shown in **Figure 4.4**.
The UV spectra demonstrated the formation of Schiff base adduct between KOOA-PC and butyl amine in a short time. At the same time, LC-ESI-MS was used to monitor the formation of Schiff base adducts between KOOA-PC and butylamine. KOOA-PC (50 µM) and butylamine (1 mM) were mixed together in phosphate buffer (100 mM, pH 7.4) and incubated at room temperature for 2 h in the presence of NaCNBH$_3$ (100 mM). The resulting samples were analyzed with LC-ESI-MS. The MS spectrum demonstrated the formation of reduced Schiff base adducts between KOOA-PC and butylamine, as shown in Figure 4.5.
Figure 4.5 Mass spectra for reduced KOOA-PC butylamine Schiff base adduct. A. Extracted ion chromatogram (EIC) for reduced KOOA-PC butylamine Schiff base adducts. B. Tandem mass spectrometry for reduced KOOA-PC butylamine Schiff base adducts and tentative structures of fragment ions.

4.2.4 Schiff base adduct between KOOA/KODA-PC and Ac-CD36M

Ac-CD36M is a short CD36 peptide (CD36M, SLINKSKSS) that is acetylated at the N terminus. CD36M was reported to contain the oxLDL binding site and could block the binding of oxLDL to CD36. In the present study, Ac-CD36M was incubated with KOOA-PC or KODA-PC at pH 8.0 for 50 min. The incubation mixtures were reduced by
NaCNBH₃ at pH 6.5 for 10 min, frozen in dry ice and analyzed with LC-MS. The test results demonstrated the formation of Schiff base adducts between KOOA-PC/KODA-PC and Ac-CD36M, as shown in Figure 4.6.

In addition, the LC-MS results suggested that KOOA-PC and KODA-PC may be reduced after incubation with NaCNBH₃ for 10 min at pH 6.5. In the experiment with KOOA-PC, the intensity of the peak at \( m/z \) 650.4 became much higher than the peak at \( m/z \) 648.4 (KOOA-PC), as shown in Figure 4.7. This finding suggests that the aldehyde group at the terminus of the \( sn\)-2 position of KOOA-PC is probably reduced by NaCNBH₃ because the molecular weight for the reduced KOOA-PC is 650.4. The same transformation also happened to KODA-PC after the incubation with NaCNBH₃ at pH 6.5 and even at higher pH (e.g., 7.2 and 8.0, data not shown). It is well known that NaCNBH₃ specifically reduces the Schiff base double bonds in neutral solution, while the carbonyl groups are not affected. The reason for the reduction of KOOA-PC and KODA-PC around neutral pH could be that the conjugated “4-oxo” group has a high electron withdrawing effect and thus makes the aldehyde group at the terminus much more electron deficient and easier to be reduced by NaCNBH₃.
Figure 4.6 Mass spectra for the reduced Schiff base adducts of KOOA-PC/KODA-PC with Ac-CD36M. (A) EIC for the reduced Schiff base adduct of KOOA-PC and Ac-CD36M. (B) EIC for the reduced Schiff base adduct of KODA-PC and Ac-CD36M. (C) Tandem MS of the reduced Schiff base adduct of KOOA-PC with Ac-CD36M and tentative structures of fragment ions. (D) Tandem MS of the reduced Schiff base adduct of KODA-PC with Ac-CD36M and tentative structures of fragment ions.
4.2.5 Study of the effect of sodium cyanoborohydride on the equilibrium of Schiff base formation

It is well known that NaCNBH₃ specifically reduces the Schiff base double bonds in neutral solution, while the carbonyl groups are not affected. Thus, NaCNBH₃ can shift the reaction equilibrium toward the Schiff base formation. However, the present study demonstrated that NaCNBH₃ can reduce KOOA-PC and KODA-PC at neutral pH, and the terminal aldehyde group is the most probable target. So, we were very curious to determine the effect, if any, of NaCNBH₃ on the Schiff base formation equilibrium. Two parallel experiments A and B were designed. In experiment A, KODA-PC and butylamine were mixed and incubated for 30 min at room temperature, followed by the addition of 100 mM NaCNBH₃ and a subsequent 30 min incubation. In experiment B, KODA-PC and butylamine were mixed together and incubated for 30 min at room
temperature in the presence of 100 mM NaCNBH₃. Aliquots were taken, and AcSPC (5 
µM) was added as an internal standard for the LC-ESI-MS/MS test. The levels of reduced 
Schiff base adduct were monitored by selected reaction monitoring (SRM) and compared 
with that of internal standard AcSPC, as shown in **Figure 4.8A**. The peak area of reduced 
Schiff base adducts was divided by that of AcSPC, and the resulting values were 
compared between two experiments A and B, as shown in **Figure 4.8B**. The test results 
demonstrated that the yield of reduced Schiff base in Experiment A (with preincubation 
of KODA-PC with butylamine for 30 min) is about three times of that in Experiment B 
(without preincubation). This finding suggests that the preincubation is necessary to get a 
relatively high yield of Schiff base adducts. The experiments provided no evidence that 
NaCNBH₃ could shift the equilibrium dramatically toward the formation of Schiff base 
formation. The reason could be that NaCNBH₃ can reduce and remove the KODA-PC 
quickly at pH 7.4 before it forms Schiff base adduct with butylamine.

![Figure 4.8](image)

**Figure 4.8** The effect of NaCNBH₃ on the equilibrium of Schiff base formation. (A) LC- 
MS chromatogram for the Schiff base formation. (B) Comparison of levels of reduced 
Schiff base adducts between Experiment A and Experiment B. Data are the average of 
three sets of independent experiments.
4.2.6 Study on the effect of inert phospholipids (e.g., lysoPC, PLPC and POPC) on KODA-PC Schiff base formation

As shown in our above study, the addition of the inert phospholipids to KOOA-PC and KODA-PC can make them more stable in aqueous solution, which, of course, is helpful to get a high level of Schiff base adduct. On the other hand, the addition of inert phospholipids may disperse KOOA-PC and KODA-PC in the aqueous solution, which can probably increase their chances of contacting with amino functional groups, especially in larger molecules. In order to test this hypothesis, various forms of KODA-PC (e.g., pure, mixed with lysoPC or PLPC) were compared in the reactions of Schiff base formation. KODA-PC of various forms were incubated with butylamine or Ac-CD36M at pH 7.4 for about 40 min, followed by the addition of NaCNBH₃ and subsequent incubation for about 10 min at room temperature. AcSPC (1 µM or 2 µM) was then added as an internal standard. Aliquots were taken, frozen in dry ice and used directly in the LC-ESI-MS analysis. The levels of reduced Schiff base adduct were monitored with SRM mode, and the peak area of reduced Schiff base adducts was divided by that of AcSPC. The resulting values were compared between experiments using different forms of KODA-PC, as shown in Figure 4.9. The test results demonstrated that, in the reactions with butylamine, the Schiff base level generated in the experiment using pure KODA-PC is the lowest, as shown in Figure 4.9. The major reason might be that KODA-PC molecules can react with each other quickly when they form small micelles in aqueous buffer, as mentioned above. Similarly, the Schiff base adducts generated may also react with each other or with other adjacent KODA-PC molecules. Another reason could be that KODA-PC molecules form micelles in a similar
way as other oxidized phospholipids, as demonstrated by Pande et al. In micelles, about several hundred KODA-PC molecules are crowded together, reducing the effective concentration of KODA-PC for the steric effect, especially in the reaction with large molecules like peptides or proteins. In comparison, the addition of inert phospholipids (e.g., lysoPC or PLPC) can increase the levels of Schiff base adduct dramatically, as shown in Figure 4.9A. LysoPC is a little more effective than PLPC to promote the reactions. The yields of Schiff base adduct increase with higher ratios of lysoPC, as shown in Figure 4.9B. For the reaction between KODA-PC and Ac-CD36M, the yields of Schiff base adduct also increase with higher ratios of lysoPC. However, the addition of PLPC has a very weak effect on promoting the Schiff base formation between KODA-PC and CD36M, as shown in Figure 4.9C. The Schiff base level from the experiment using PLPC-KODA-PC vesicles is only about 4.7% as high as that from the experiment with lysoPC-KODA-PC vesicles. In the reaction with KODA-PC, the short peptide Ac-CD36M displays strong selectivity for the KODA-PC in lysoPC micelles over that in PLPC vesicles, but butylamine exhibits no such selectivity.

One significant difference between PLPC-KODA-PC vesicles and lysoPC-KODA-PC micelles is their sizes. In the present study, PLPC-KODA-PC vesicles were passed through a 0.1 μm polycarbonate filter 11 times using an Avanti Mini-Extruder Set, and their average diameter is about 100 nm. The size of lysoPC-KODA-PC micelles is about 6-10 nm, according to the study of Mattila et al. As molecular size or particle size increases, diffusion rate and available surface area decrease, slowing the reaction between KODA-PC and Ac-CD36M. Thus, the large size of PLPC-KODA-PC vesicles might be one reason for the low yield of Schiff base adducts between KODA-PC and Ac-
CD36M. To see the effect of KODA-PC vesicle size on the yield of Schiff base formation, we attempted to make PLPC-KODA-PC vesicles with a 30 nm polycarbonate filter, but failed because of some technical problems. It is very difficult to extrude the solution of PLPC-KODA-PC through the 30 nm polycarbonate filter. The reason is uncertain. Then 30 nm POPC-KODA-PC vesicles were successfully made, tested in the reaction with Ac-CD36M and compared with lysoPC-KODA-PC micelles (6-10 nm in diameter) and PLPC-KODA-PC vesicles (100 nm in diameter). The results showed that the yields of Schiff base formation from the experiment using 30 nm POPC-KODA-PC vesicles is about 12% as high as that from the experiment with lysoPC-KODA-PC vesicles. Compared with the 100 nm PLPC-KODA-PC vesicles, the 30 nm POPC-KODA-PC vesicles are more effective for promoting the reaction between KODA-PC and CD36M.

In the present study, although the lysoPC-KODA-PC vesicle (6-10 nm in diameter) is the most effective form of KODA-PC for the Schiff base reaction, it can only be used in model reactions with small molecules and peptides. In the reactions with proteins and cells, lysoPC cannot be used as an additive because of its detergent-like properties, which could lyse cells and denature proteins. The present study suggested that POPC can be used as an additive to facilitate the reaction between KODA-PC and peptides or proteins on cells.
Figure 4.9 Addition of inert phospholipids to KODA-PC can promote Schiff base formation. Figure legends: SB – Schiff base adduct, IS – internal standard.

4.2.7 Labeling of KODA-PC Schiff base adduct with aminoxy biotin derivative

As demonstrated above, NaCNBH₃ was used to reduce the KODA-PC Schiff base adduct specifically, leaving intact the “γ-oxo” group at the sn-2 position of KODA-PC. As reported previously, the “γ-oxo” group at the sn-2 position of KODA-PC can react with an aminoxy-biotin derivative, N-(aminoxyacetyl)-N'(D-biotinoyl) hydrazine (Figure 4.10a). Thus, the aminoxy-biotin can be used to label the reduced Schiff base adduct of KODA-PC and target proteins, which then can be enriched with streptavidin beads. This enrichment step would dramatically enhance the chances to detect KODA-PC.
modified proteins and peptides. In the present study, model reactions between aminooxy-biotin and the reduced Schiff base adduct of KODA-PC with Ac-CD36M were carried out to find the optimal conditions for labeling with aminooxy-biotin. Dr. Podrez and co-workers reported the derivatization of the “γ-oxo” group at the sn-2 position of KDdIA-PC with methoxylamine in phosphate buffer at pH 4.2. Since the aminooxy-biotin also has an alkoxylamine functional group, the aminooxy-biotin labeling reaction should also work well around pH 4. Taking into consideration the possibility of protein precipitation at low pH, I investigated higher pH values at 4.5 and 5.0 for the aminooxy-biotin labeling reaction. In addition, I investigated different temperatures, i.e., 37 °C and 70 °C, for the aminooxy-biotin labeling reaction. LC-ESI-MS/MS was used to monitor the consumption of reduced Schiff base adducts of KODA-PC with Ac-CD36M and the generation of aminooxy-biotin labeled Schiff base. The experiment was carried out as described in the Experimental procedures section.

The test results (Figure 4.10b, c) demonstrated that the aminooxy-biotin labeling reaction works best at pH 4.5, 37 °C. With the same pH but higher temperature (pH 4.5, 70 °C), the yield of aminooxy-biotin labeling reaction decreases substantially. The reason might be that at 70 °C, the reduced Schiff base adducts of KODA-PC and Ac-CD36M are not stable, as shown in Figure 4.10c. Comparison between experiments at pH 4.5 and pH 5.0 showed that the yield of aminooxy-biotin labeling reaction at pH 4.5 is much higher than that at pH 5.0. In summary, the model reactions suggested that the aminooxy-biotin labeling reaction works well at pH 4.5, 37 °C.
**Figure 4.10** Aminooxy-biotin labeling of Schiff base adduct between KODA-PC and Ac-CD36M

### 4.2.8 Enrichment of KODA-PC modified peptides using C18 reversed phase HPLC column

During the study on KODA-PC modified peptides using LC-MS, we noticed that, the KODA-PC modified peptides are usually eluted from a C18 reversed phase HPLC column at much later times than the unmodified peptides. The reason for this phenomenon could be that the long hydrophobic chain at the sn-1 position of KODA-PC makes the KODA-PC modified peptides much more hydrophilic than the unmodified
peptides. This finding suggested that KODA-PC modified peptides can be separated from the unmodified peptides by chromatography on a C18 reversed phase column, and thus can be enriched. To test this hypothesis, we prepared the reduced Schiff base adducts of KODA-PC with two short synthetic CD36 peptides Ac-CD36M and CD36S (SLINKSKSSMF). In addition, we did the chymotryptic digestion of protein mixtures of human serum albumin (HSA), beta-lactoglobulin (β-LG) and alpha-lactalbumin (α-LA).

Then the peptide samples were analyzed by LC-ESI-MS, and the elution chromatograms were compared, as shown in Figure 4.11. The LC-ESI-MS studies demonstrated that most of the peptides from chymotryptic digestion of the protein mixture are eluted before 18 min (Figure 4.11b), and both KODA-PC peptide adducts were eluted after 31 min (Figure 4.11a). This finding suggested that KODA-PC peptide adducts can be easily separated from the unmodified peptides. Thus, it is very promising to enrich the KODA-PC modified peptides using chromatography on a C18 reversed phase HPLC column.

![Figure 4.11](image)

*Figure 4.11* Elution chromatograms of KODA-PC peptide adducts and peptides from chymotryptic digestion of protein mixtures of HSA, β-LG and α-LA.
4.3 Conclusions

Schiff base adducts between KOOA/KODA-PC and small molecules like butylamine and small CD36 peptides were detected by UV and LC-MS/MS. Our studies suggested that pure KOOA-PC and KODA-PC are not stable in aqueous solutions. The addition of excess inert phospholipids (e.g., lysoPC, POPC) could stabilize KOOA/KODA-PC and increase yields of Schiff base adduct. The present studies do not provide any evidence that sodium cyanoborohydride drives the equilibrium toward Schiff base formation. Two enrichment methods have been developed and are being used to explore the oxPC binding sites on CD36 and SR-BI.

4.4 Experimental procedures

4.4.1 Study of the stability of KOOA-PC in aqueous solution with UV spectroscopy

KOOA-PC (50 µg) or a mixture of KOOA-PC and lysoPC (50 µg/744 µg, 1/20, mol/mol) was dissolved in 1 ml of phosphate buffer (100mM, pH 7.4). The resulting solutions were transferred immediately to UV quartz cuvettes (1 ml) and monitored with a UV spectrometer using repetitive spectral scans over the range of 210-400 nm with phosphate buffer (100 mM, pH 7.4) as control. The scan cycle was repeated at 15 min intervals.

Table 4.1 The UV absorbance data at 226 nm

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<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
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<td>KOOA/lysoPC</td>
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<td>0.652</td>
<td>0.638</td>
<td>0.625</td>
<td>0.613</td>
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<td>0.576</td>
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<td>KOOA-PC</td>
<td>0.496</td>
<td>0.435</td>
<td>0.393</td>
<td>0.357</td>
<td>0.328</td>
<td>0.304</td>
<td>0.283</td>
<td>0.264</td>
</tr>
</tbody>
</table>
4.4.2 Study on the Schiff base adduct between KOOA-PC and butylamine in aqueous solution by UV spectroscopy

KOOA-PC (50 µg) and lysoPC (372 µg) in chloroform were mixed in a glass vial at a ratio of 1:10 (mol/mol), and the solvent was evaporated with a stream of nitrogen. The resulting residue was dissolved in 1.5 ml phosphate buffer (100 mM, pH 7.4), and then 10 mM butylamine (final concentration) was added. The reaction mixture was transferred immediately to UV quartz cuvettes (1 ml) and monitored with a UV spectrometer by repetitive spectral scans over the range of 210-400 nm with phosphate buffer (100 mM, pH 7.4) as control. The scan cycle was repeated at 3 min intervals.

4.4.3 Study on the Schiff base adduct between KOOA-PC and butylamine in aqueous solution by LC-ESI-MS

KOOA-PC (50 µg) and POPC (506 µg) were mixed in chloroform. The solvent was evaporated with a stream of nitrogen, and the residue was then dissolved in 1 ml of phosphate buffer (100 mM, pH 7.4). The resulting solution was passed through a 0.1 µm polycarbonate filter 11 times using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL) at room temperature to make 100 nm vesicles. Then, butylamine (final concentration 1mM) and sodium cyanoborohydride (final concentration 100 mM) were added. The resulting mixture was incubated at room temperature for 2 h. Aliquots were taken and analyzed directly by LC-ESI-MS.

In the LC-ESI-MS/MS, isocratic elution with 85% solvent A (methanol/formic acid, 100/0.1, v/v) and 15% solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v) was used. The flow was 0.4 ml/min using an Agilent column (15 cm×4.6 mm ZORBAX SB C8-5 µm).
4.4.4 Study on the Schiff base adducts between KOOA-PC/KODA-PC and Ac-CD36M with LC-ESI-MS

KOOA-PC (50 µg) and lysoPC (765 µg) were mixed together at a ratio of 1:20 in chloroform. Solvent was evaporated under a stream of nitrogen, and the residue was dissolved in 500 µl phosphate buffer (100 mM, pH 8.0). The final concentration of KOOA-PC was 150 µM. To the KOOA-PC solution, Ac-CD36M (2 mM) was added, and the resulting mixture was incubated at 37 °C for 50 min. Then NaCNBH3 was added to a final concentration of 100mM, and HCl was used to adjust the pH to 6.5. The resulting mixture was incubated at room temperature for 10 min. Then aliquots were taken, frozen in dry ice and used directly for LC-ESI-MS analysis. The experiment with KODA-PC was carried out similarly.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H2O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 25% A from 0 to 5 min; increasing to 100% A from 5 to 20 min; isocratic elution with 100% A from 20 to 44 min; decreasing to 25% A from 44 to 45 min; isocratic elution with 25% A from 45 to 50 min. The flow was 0.4 ml/min using an Agilent column (15 cm×4.6 mm ZORBAX SB C8-5 µm).

4.4.5 The effect of NaCNBH3 on the equilibrium of Schiff base formation

Two parallel experiments A and B were carried out. In experiment A, 50 µg KODA-PC and 350 µg lysoPC were mixed at a ratio of 1:10 in chloroform. Solvent was evaporated under a stream of nitrogen, and the residue was then dissolved in 1 ml of phosphate buffer (100 mM, pH 7.4). The final concentration of KODA-PC was 70 µM.
To the KODA-PC solution, butylamine was added to a final concentration of 2 mM, and the resulting mixture was incubated at room temperature for 30 min. Then, 6.3 mg of NaCNBH₃ powder was added, followed by 30 min incubation at room temperature. Experiment B was carried out similarly, except that 6.3 mg of NaCNBH₃ powder was added together with butylamine at the beginning of the incubation, and the entire incubation time was 30 min. Finally, 5 µM AcSPC was added as an internal standard. Aliquots were taken, frozen with dry ice and analyzed directly by LC-ESI-MS/MS.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 65% A from 0 to 4 min; increasing to 100% A from 4 to 5 min; isocratic elution with 100% A from 5 to 26 min; decreasing to 65% A from 26 to 27 min; isocratic elution with 65% A from 27 to 32 min. The flow was 0.4 ml/min using an Agilent column (15 cm×4.6 mm ZORBAX SB C8-5 µm).

4.4.6 The effect of inert phospholipids on the yields of Schiff base adduct

KODA (50µg) or a mixture of KODA-PC (50 µg) with lysoPC, POPC or PLPC in the indicated molar ratios in chloroform was freed of solvent under a stream of nitrogen, and the residue was dissolved in 1 ml of modified Tyrode’s buffer (W/O BSA and dextrose, W/ 50 mM HEPES, pH 7.4). The solution of pure KODA-PC or KODA-PC/lysoPC was used directly in the next step. The suspension of KODA-PC/POPC was passed through a 30 nm polycarbonate filter 11 times, and the suspension of KODA-PC/PLPC was passed through a 100 nm polycarbonate filter 11 times to make small vesicles. To each KODA-PC solution of various forms (micelles or vesicles of different
CD36M was added to a final concentration of 2 mM. Each mixture was incubated at room temperature for 40 min. Then 6.4 mg of NaCNBH₃ was added to a final concentration of 100 mM. After 10 min, AcSPC was added as an internal standard (1 µM for Figure 4.9A, B and C; 2 µM for Figure 4.9D). Aliquots were taken from each incubation mixture, immediately frozen in dry ice, and used directly for LC-ESI-MS/MS analysis.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 35% A from 0 to 3 min; increasing to 85% A from 3 to 4 min; isocratic elution with 85% A from 4 to 6 min; increasing to 100% A from 6 to 7 min; isocratic elution with 100% A from 7 to 22 min; decreasing to 35% A from 22 to 23 min; isocratic elution with 35% A from 23 to 27 min. The flow was 0.4 ml/min using an Agilent column (15 cm×4.6 mm ZORBAX SB C8-5 µm).

4.4.7 Labeling of the Schiff base adduct of KODA-PC and Ac-CD36M with aminooxy-biotin derivative

KODA-PC (100 µg) and lysoPC (700 µg) were mixed in chloroform, and solvent was evaporated under a stream of nitrogen. The resulting residue was dissolved in 2 ml of phosphate buffer (100 mM, pH 7.4). Ac-CD36M was added to a final concentration of 2 mM. The resulting solution was incubated at room temperature for 40 min. Then 6.4 mg NaCNBH₃ was added to reduce the Schiff base adduct between KODA-PC and Ac-CD36M. 10 min later, the incubation mixture was divided into two equal parts (Part 1 and 2). Part 1 was diluted 10 times with sodium acetate buffer (100 mM, pH 4.5) and
concentrated with centrifugal filter units (Millipore, 10 KDa) to the original volume. The process was repeated two times and the resulting solution was used directly in the ARP labeling reaction. Part 2 was processed in a similar way to that of part 1 except that it was diluted with sodium acetate buffer (100 mM, pH 5.0). To part 1 and part 2, ARP was added to a final concentration of 3 mM. Then part 1 was divided into two equal parts (A and B), and part 2 was divided into another two equal parts (C and D). One set of samples (A and C) was incubated at 37 °C, and the other set (B and D) was incubated at 70 °C. Aliquots were taken from each sample at various times (1.2, 2.3, 4.7, 8.2, 18 hours). To each aliquot, AcSPC was added as an internal standard (1 µM). The resulting aliquots were frozen in dry ice immediately and used directly in the LC-ESI-MS/MS analysis.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 35% A from 0 to 2 min; increasing to 65% A from 2 to 3 min; isocratic elution with 65% A from 3 to 8.5 min; increasing to 100% A from 8.5 to 9.5 min; isocratic elution with 100% A from 9.5 to 23 min; decreasing to 35% A from 23 to 25 min; isocratic elution with 35% A from 25 to 30 min. The flow was 0.4 ml/min using an Agilent column (15 cm×4.6 mm ZORBAX SB C8-5 µm).

4.4.8 Preparation and LC-MS analysis of reduced Schiff base adducts between KODA-PC and two short CD36 peptides Ac-CD36M and CD36S

KODA-PC (100 µg) and lysoPC (700 µg) were mixed in chloroform and solvent was evaporated with a stream of nitrogen. The resulting residue was dissolved in 1 ml phosphate buffer (100 mM, pH 7.4). Ac-CD36M or CD36S was added to a final
concentration of 2 mM. The resulting solution was incubated at room temperature for 40 min. Then NaBH₄ was added to a final concentration of 100 mM to reduce the Schiff base adduct. After 1 h, acetic acid was added to neutralize the solution. The samples obtained were frozen in dry ice and used directly in LC-ESI-MS analysis.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 60% A from 0 to 1 min; increasing to 75% A from 1 to 2 min; increasing to 100% A from 2 to 25 min; isocratic elution with 100% A from 25 to 45 min; decreasing to 60 % A from 45 to 47 min; isocratic elution with 60% A from 47 to 53 min. The flow was 1.2 ml/min using a Higgins column (15 cm×10 mm PROTO 200 C18-5 μm).

The column effluent was split with a flow splitter such that only one small part of the effluent (60 µl/min) was introduced to the mass spectrometer.

4.4.9 Chymotryptic digestion and LC-MS analysis of a protein mixture (HSA, β-LG and α-LA)

HSA (6.6 mg), α-LA (1.5 mg) and β-LG (1.4 mg) were dissolved in 1 ml of guanidine (6 M, with 50 mM Tris, pH 8.0). Dithiothreitol was added to a final concentration of 10 mM, and the resulting mixture was incubated with shaking at 60 °C for 1.5 h. The solution was cooled to room temperature, followed by the addition of 40 µl of 1 M iodoacetamide and 1 h incubation at room temperature. Then, 60 µl of 500 mM dithiothreitol was added, and the incubation continued for another 1 h to consume the excess iodoacetamide. Part of the resulting solution (100 µl) was diluted with Tris buffer (50 mM, containing 10 mM CaCl₂, pH 7.8) to 900 µl. Then 50 µg chymotrypsin was
added, followed by incubation at 37 °C overnight. The resulting solution was frozen in dry ice and used directly for LC-ESI-MS analysis.

For the LC-ESI-MS/MS, a gradient was used by mixing solvent A (methanol/formic acid, 100/0.1, v/v) with solvent B (H₂O/methanol/formic acid, 95/5/0.1, v/v/v), as follows: isocratic elution with 60% A from 0 to 1 min; increasing to 75% A from 1 to 2 min; increasing to 100% A from 2 to 25 min; isocratic elution with 100% A from 25 to 45 min; decreasing to 60 % A from 45 to 47 min; isocratic elution with 60% A from 47 to 53 min. The flow was 1.2 ml/min using a Higgins column (15 cm×10 mm PROTO 200 C18-5 μm). The column effluent was split with a flow splitter such that only one small part of the effluent (60 μl/min) was introduced to the mass spectrometer.

4.4.10 ESI mass spectrometry method

ESI mass spectrometry was performed with a Thermo Finnigan LCQ Deca XP instrument in the positive ion mode using nitrogen as the sheath and auxiliary gas. The heated capillary temperature was 300 °C, the source voltage was 4.50 kV, and the capillary voltage was 13.00V. SRM mode was used for the detection of reduced Schiff base adducts between KODA/KOOA-PC and small molecules such as butylamine or short CD36 peptides. For peptides from chymotryptic digestion of the protein mixture, two scan events were used: (i) m/z 150-2000 full scan MS; (ii) data-dependent full scan MS/MS on the most intense ion from the MS full spectrum. The MS/MS collision energy was set to 35%.
4.5 References


Appendix
Figure A.1 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-maleyl-sn-glycero-3-phosphocholine (PMPC, 2.1a)

Figure A.2 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-phthalyl-sn-glycero-3-phosphocholine (PPPC, 2.1b)
**Figure A.3** $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-succinyl-$sn$-glycerol-3-phosphocholine (PSuPC, 2.1c)

**Figure A.4** $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-succinyl-$sn$-glycerol-3-phosphocholine (PSuPC, 2.1c)
Figure A.5 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-glutaroyl-$sn$-glycero-3-phosphocholine (PGPC, 2.1d)

Figure A.6 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-acetyl-$sn$-glycero-3-phosphocholine (PAcPC, 2.1e)
Figure A.7 $^1$H NMR (CDCl$_3$, 200 MHz) spectrum of 12-benzyloxy-12-oxododecanoic acid (2.3a)

Figure A.8 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 8-benzyloxy-8-oxooctanoic acid (2.3b)
Figure A.9 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(12’-benzyloxy-12’-oxo)-dodecanoyl-$sn$-glycero-3-phosphocholine (2.4a)

Figure A.10 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-(8’-benzyloxy-8’-oxo)octanoyl-$sn$-glycero-3-phosphocholine (2.4b)
Figure A.11 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(3’, 6’-dioxo-8’-benzyloxy)-suberyl-$sn$-glycerol-3-phosphocholine (2.4c)

Figure A.12 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-dodecanedioyl-$sn$-glycerol-3-phosphocholine (PDPC, 2.5a)
Figure A.13 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphocholine (PSPC, 2.5b)

Figure A.14 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphocholine (PSPC, 2.5b)
Figure A.15 $^1$H NMR (CDCl$_3$, 200 MHz) spectrum of 1-palmityl-2-(3’, 6’-dioxo)-suberyl-$sn$-glycero-3-phosphocholine (PdiOSPC, 2.5c)

Figure A.16 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 6-(tert-Butyldimethyl)silyloxy-hexanoic acid (2.6a)
Figure A.17 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 8-(tert-Butyldimethyl)silyloxy-octanoic acid (2.6b)

Figure A.18 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-(6’-tert-butyldimethylsilyloxy)-hexanoyl-sn-glycero-3-phosphocholine (2.7a)
Figure A.19 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-($6'$-hydroxyhexanoyl)-$sn$-glycero-3-phosphocholine (P6HHPC, 2.8a)

Figure A.20 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-(8'$'$-hydroxy)-octanoyl-$sn$-glycero-3-phosphocholine (P8HOPC, 2.8b)
**Figure A.21** $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1, 2-O-isopropylidene glycerol acetate (2.9a)

**Figure A.22** $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1, 2-O-isopropylidene glycerol palmitate (2.9b)
Figure A.23 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1, 2-O-isopropylidene glycerol palmitate (2.9b)

Figure A.24 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-acetyl-$sn$-glycerol (2.10a)
Figure A.25 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-$sn$-glycerol (2.10b)

Figure A.26 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-$sn$-glycerol (2.10b)
Figure A.27 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-acetyl-3-(tert-butylidimethyl)silyl-$sn$-glycerol (2.11a)

Figure A.28 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-acetyl-3-(tert-butylidimethyl)silyl-$sn$-glycerol (2.11a)
Figure A.29 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-3-(tert-butyldimethyl)-silyl-$sn$-glycerol (2.11b)

Figure A.30 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-3-(tert-butyldimethyl)-silyl-$sn$-glycerol (2.11b)
Figure A.31 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-acetyl-2-(7’-benzyloxy carbonyl)-heptanoyl-3-(tert-butyldimethyl)-silyl-sn-glycerol (2.12a)

Figure A.32 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-acetyl-2-(7’-benzyloxy carbonyl)-heptanoyl-3-(tert-butyldimethyl)-silyl-sn-glycerol (2.12a)
Figure A.33 \( ^1 \text{H} \) NMR (CD\textsubscript{3}OD, 400 MHz) spectrum of 1-palmityl-2-(7'-benzyloxycarbonyl)-heptanoyl-3-(\textit{tert}-butyldimethyl)-silyl-\textit{sn}-glycerol (2.12b)

Figure A.34 \( ^1 \text{H} \) NMR (CDCl\textsubscript{3}, 400 MHz) spectrum of 1-acetyl-2-(7'-benzyloxycarbonyl)-heptanoyl-\textit{sn}-glycerol (2.13a)
Figure A.35 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-acetyl-2-(7’-benzyloxycarbonyl)-heptanoyl-$sn$-glycerol (2.13a)

Figure A.36 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(7’-benzyloxycarbonyl)-heptanoyl-$sn$-glycerol (2.13b)
Figure A.37 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycerol (PSG, 2.14)

Figure A.38 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycerol (PSG, 2.14)
Figure A.39 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-acetyl-2-(8’-benzyloxy-8’-oxooctanoyl)-sn-3-phosphocholine (2.15a)

Figure A.40 $^{13}$C NMR (CD$_3$OD, 100 MHz) spectrum of 1-acetyl-2-(8’-benzyloxy-8’-oxooctanoyl)-sn-3-phosphocholine (2.15a)
Figure A.41 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(8’-benzyloxy-8’-oxo)-octanoyl-$sn$-3-phosphatidyl-(N,N,N-trimethyl-amino)-propanol (2.15b)

Figure A.42 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-(8’-benzyloxy-8’-oxo)-octanoyl-$sn$-3-phosphatidyl-(N,N,N-trimethyl-amino)-propanol (2.15b)
Figure A.43 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(8'-benzyloxy-8’-oxooctanoyl)-$sn$-3-phosphatidyl-(N,N,N-trimethyl-amino)-hexanol (2.15c)

Figure A.44 $^1$H NMR (D$_2$O, 400 MHz) spectrum of 1-acetyl-2-suberyl-$sn$-3-phosphocholine (AcSPC, 2.16a)
Figure A.45 $^{13}$C NMR (D$_2$O, 100 MHz) spectrum of 1-acetyl-2-suberyl-$sn$-3-phosphocholine (AcSPC, 2.16a)

Figure A.46 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycerol-3-phosphatidyl-(N,N,N-trimethylamino)-propanol (PSPP, 2.16b)
Figure A.47 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-propanol (PSPP, 2.16b)

Figure A.48 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-hexanol (PSPH, 2.16c)
Figure A.49 $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphatidyl-(N,N,N-trimethylamino)-hexanol (PSPH, 2.16c)

Figure A.50 $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of 1-palmityl-2-(8$^\prime$-benzyloxy-8$^\prime$-oxo)-octanoyl-$sn$-3-phosphatidic acid (2.17)
Figure A.51 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-suberyl-$sn$-glycero-3-phosphatidic acid (PSPA, 2.18)

Figure A.52 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-(9'-methoxyl-9'-oxo)-nonanoyl-$sn$-glycero-3-phosphocholine (P9MNPC, 2.19)
Figure A.53 $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 1-palmityl-2-(8’-amino-8’-oxo)-octanoyl-sn-glycero-3-phosphocholine (P8AOPC, 2.20)
Bibliography


