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The Lysophosphatidylserines—An Emerging Class of Signalling Lysophospholipids

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Abstract
Lysophospholipids are potent hormone-like signalling biological lipids that regulate many important biological processes in mammals (including humans). Lysophosphatidic acid and sphingosine-1-phosphate represent the best studied examples for this lipid class, and their metabolic enzymes and/or cognate receptors are currently under clinical investigation for treatment of various neurological and autoimmune diseases in humans. Over the past two decades, the lysophsophatidylserines (lyso-PSs) have emerged as yet another biologically important lysophospholipid, and deregulation in its metabolism has been linked to various human pathophysiological conditions. Despite its recent emergence, an exhaustive review summarizing recent advances on lyso-PSs and the biological pathways that this bioactive lysophospholipid regulates has been lacking. To address this, here, we summarize studies that led to the discovery of lyso-PS as a potent signalling biomolecule, and discuss the structure, its detection in biological systems, and the biodistribution of this lysophospholipid in various mammalian systems. Further, we describe in detail the enzymatic pathways that are involved in the biosynthesis and degradation of this lipid and the putative lyso-PS receptors reported in the literature. Finally, we discuss the various biological pathways directly regulated by lyso-PSs in mammals and prospect new questions for this still emerging biomedically important signalling lysophospholipid.

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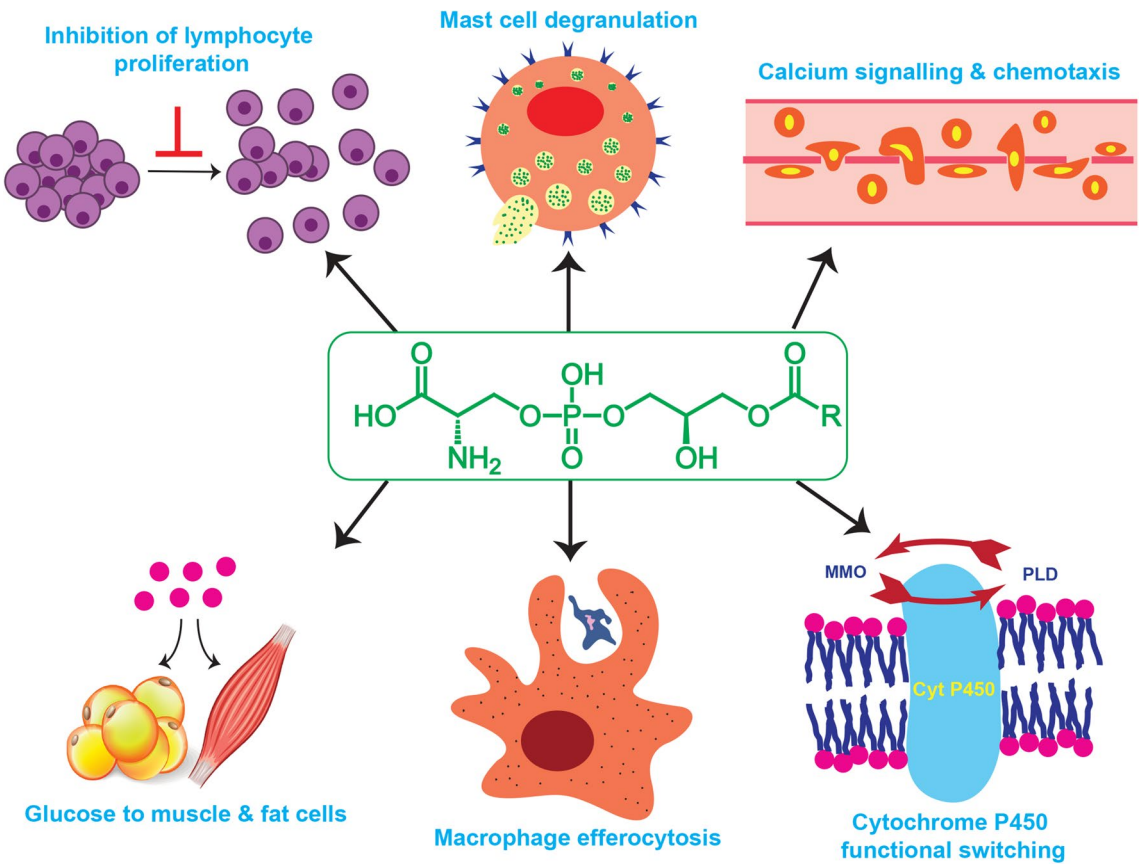
Graphic abstract



Keywords Lysophosphatidylserine · Lipases · GPCR · TLR2 · Mast cell degranulation · Macrophage efferocytosis

Abbreviations

ABHD	α/β-Hydrolase domain
CMC	Critical micellar concentration
CNS	Central nervous system
CoA	Coenzyme-A
ConA	Concanavalin A
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GPCR	G-Protein-coupled Receptors
GPS	Glycero-phospho-*L*-serine
HPLC	High performance liquid chromatography
LC–MS	Liquid chromatography coupled to mass spectrometry
MAG	Monoacylglycerol
MBOAT	Membrane-bound *O*-acyl-transferase
MRM	Multiple reaction monitoring
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PHARC	Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract
PI	Phosphatidylinositol
PS	Phosphatidylserine
S1P	Sphingosine 1-phosphate
TAG	Triacylglycerol
VLC	Very long chain

Discovery

At the turn of the twentieth century, scientists started exploring the potential of lipids to function as hormone-like signalling molecules and began leveraging their biological activity to treat human diseases. This curiosity-led research resulted in a deluge of rigorous studies that conclusively established the role of numerous naturally occurring lipids in regulating important physiological processes in humans. Several of these pioneering studies extracted lipids from bovine or porcine organs, fractionated them

using chromatographic techniques available then, and tested these individual fractions for various biological activities. It was postulated in the early 1950s that a lipid modulates anticoagulation properties of blood, and in a quest to identify this lipid(s), researcher isolated a lipid fraction rich in phosphatidylserine (PS) from the porcine brain that displayed this activity (Silver et al. 1959). Interestingly, on further inspection and subsequent validation, studies showed that a degraded contaminant of PS, lysophosphatidylserine (lyso-PS), was aiding PS solubility, and, in doing so, was facilitating this biological activity. Although unknown at the point, an earlier study had also found something similar. When rabbits were intravenously injected a high dose of a crude PS fraction, it resulted in severe haemolysis and respiratory failure, that eventually caused death of these animals (Goldsmith and Mushett 1954; Mushett et al. 1954). This observation was at that time contrary to the expected role of PS, as in vitro studies had shown PS to aide blood coagulation. This led researchers at that time to hypothesize that "unknown histamine-like substances" which were considered as contaminants in the crude PS fraction (that we now know were lyso-PSs) likely caused the pathophysiological complications in the animal studies. Although the contaminant responsible for the cause of lethality in this experiment was not identified at the time, we now know very well that lyso-PS stimulates mast cells to degranulate and in turn release histamine to produce a similar immunological effect (discussed in later sections). Finally, around the same time, it was found that snake venom also affected the coagulation process, caused haemolysis and bite victims often bled to death. Following up on this initial observation, biochemical studies showed that phospholipases in snake venom preferentially converted PS into lyso-PS, and that lyso-PS enhanced in the haemolytic process (Long and Penny 1957; Rathbone 1962; Rathbone et al. 1962; Rathbone and Maroney 1963). This finding was very exciting, as PS is now known to be a preferential substrate for phospholipases from snake venom, especially those that are present in the circulatory system. Together, these initial observations, and pioneering studies at that time, paved the path for researchers to investigate in mechanistic detail, the role that lyso-PS plays in mammalian physiology.

Structure, Detection and Biodistribution of Lysophosphatidylserines

Since implicating the role of lyso-PS lipids in various biological process, several studies have elucidated the structure of this bioactive lipid and have made systematic efforts in cataloguing them. Lyso-PSs are classified broadly as a lysophospholipids (or lysoglycerophospholipids), and like all other members of this lipid class, they are structurally made up of a central glycerol backbone (Fahy et al. 2007; Sud et al. 2007). The distinguishing feature of this signalling lysophospholipid compared to other members of this lipid family is the head group, which is phospho-*L*-serine, that forms a phosphoester bond with the *sn*-3 hydroxide of the glycerol backbone, thus forming glycerophospho-*L*-serine (GPS) core (Fig. 1) (Fahy et al. 2007; Sud et al. 2007). Unlike PS lipids, lyso-PSs have only a single fatty acid esterified to either the *sn*-1 or *sn*-2 hydroxide of the GPS head group, and depending on the site of the fatty acid esterification, lyso-PS are sub-classified into 1-(fatty acyl)-2-hydroxy-lysophosphatidylserine (*sn*-1 lyso-PS) and 2-(fatty acyl)-1-hydroxy-lysophosphatidylserine (*sn*-2 lyso-PS) (Fig. 1) (Fahy et al. 2007; Sud et al. 2007). Biologically, the fatty acids found esterified to lyso-PS are very diverse and range from medium chain (C10-C14) to long chain (C18-C20) to very long chain (C22-C24), with varying degrees of unsaturation (e.g.: C18:1, C20:4, C22:6). From a stereochemical perspective, all naturally occurring lyso-PS lipids have two chiral centres: (a) the *sn*-2 carbon atom of the glycerol backbone, which has a (*R*)-configuration; and (b) the Cα-carbon atom of the phospho-*L*-serine head group, thus making all lyso-PS lipids stereoisomers (Fig. 1) (Mallik et al. 2018; Sud et al. 2007). It is now evident from having a better idea of the structure of lyso-PS lipids that it is an intricate mix of hydrophobic (fatty acid tail) and hydrophilic (GPS head group) moieties, which confers lyso-PSs amphiphilic properties, thus enabling them to access various cellular compartments and organelles and making them potent biologically active signalling molecules in mammalian physiology (Vance 2015).

Technological advances in extraction techniques, chromatographic separations and detection have greatly

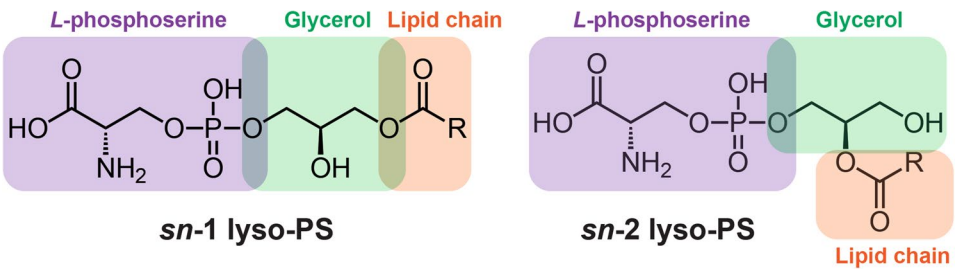


Fig. 1 Chemical structures of sn-1 and sn-2 lyso-PS

facilitated the quantitative estimation of low-abundance signalling lipids like lyso-PSs from various biological systems (e.g. cells, tissues, biological fluids). In particular, liquid chromatography coupled to mass spectrometry (LC–MS), leveraging advances in electrospray ionization-based targeted multiple-reaction monitoring (MRM) techniques have rapidly enabled accurate estimations of lyso-PSs. The first step in detecting lyso-PSs in biological systems is its extraction, and broadly, two methods have been reported for extraction of lyso-PS lipids: (a) a modified Folch extraction protocol using acidified chloroform–methanol as the organic solvent for enriching lyso-PSs (Blankman et al. 2013; Pathak et al. 2018) and (b) an acetonitrile-based protein precipitation method for enriching organic metabolites (Barnes et al. 2015). Following the enrichment of lyso-PSs from the biological system of interest, the organic extracts are chromatographically separated using reverse-phase chromatography typically using a octadecyl-carbon chain bonded silica (C18) matrix, and eluted fractions are injected onto a mass spectrometry system capable of targeted MRM measurements. Thereafter, various mass fragments originating from the parent lyso-PS cleaved using high energy are used for absolute quantitation of lyso-PSs relative to an internal standard (Barnes et al. 2015; Blankman et al. 2013; Kamat et al. 2015; Pathak et al. 2018). Despite these advances, it has been very challenging to differentiate between *sn*-1 and *sn*-2 lyso-PSs extracted from biological samples given that both of these possess the same exact mass. A recent study has shown that the *sn*-1 lyso-PS is significantly more stable, and is the dominant lyso-PS species biologically. It was also shown that at physiological pH, *sn*-2 lyso-PS undergoes an intramolecular acyl migration reaction to convert to *sn*-1 lyso-PS, and thus, *sn*-2 lyso-PSs are rarely detected using standard protocols (Okudaira et al. 2014). The same study also reports that at very low pH (< 4.0), *sn*-2 lyso-PS are substantially more stable than at physiological pH, and suggests that *sn*-2 lyso-PS are likely produced biologically on demand in cellular organelles having low pH (e.g. lysosomes, phagosomes) (Okudaira et al. 2014).

Over the past decade, there is an emerging body of literature that reports the distribution of lyso-PS lipids in various mammalian systems, mostly rodent (mice) models (e.g. primary cells, tissues). A seminal study by Cravatt and co-workers report lyso-PS concentrations in various mouse tissues (e.g. brain, spinal cord, lungs, heart, liver, kidneys), and they show in this study that lyso-PSs are most abundant in the central nervous system (Blankman et al. 2013). Following up on this finding, it was found that lyso-PS lipids are also abundant in primary mouse immune cells (e.g. primary macrophages) and various human cell lines, (including primary human lymphoblast and cancers), where they are present in cells and are also secreted (Chen et al. 2013; Kamat et al. 2015). In another study by Cyster and co-workers, aimed at understanding the role of lyso-PS lipids in mammalian immune system (discussed in a later section), they show that long chain lyso-PSs are also present in the spleen, thymus, lymph nodes and the colon of mice (Barnes et al. 2015). While there are no published reports to the best of our knowledge on reliable tissue lyso-PS measurements in humans, these lipids have been detected along with other lysophospholipids in human plasma samples (Kurano et al. 2015). Taken together, it is clear from all these studies that biologically the long chain lyso-PS lipids, C16:0, C18:0 and C18:1, are physiologically the most abundant, and these lipids, while enriched in the central nervous and immune system, are also found in tissues involved in regulating the immune system (e.g. spleen, thymus, lymph nodes) and energy metabolism (e.g. liver, kidneys, heart) (Barnes et al. 2015; Blankman et al. 2013; Kamat et al. 2015).

Metabolic Pathways Regulating Lyso-PS in Mammals

The last two decades have seen the functional annotation of major enzymatic pathways regulating the biosynthesis or degradation of lyso-PS lipids in mammals. These include the following: (1) the PS-specific phospholipases (PS-PLA1/2 and ABHD16A); (2) the lyso-PS and oxidized PS lipase, ABHD12; and (3) The lyso-PS-specific acyltransferase, MBOAT5.

The PS-Specific Phospholipases

Phospholipases A1/A2 (PLA1/2) are enzymes that hydrolytically cleave fatty acid ester bonds of phospholipids and are key enzymes involved in the biosynthesis of lysophospholipids (Burke and Dennis 2009a, b; Kalyvas et al. 2009; Richmond and Smith 2011). Having previously shown that the phospholipase(s) from snake venom are PS-specific (Rathbone 1962; Rathbone et al. 1962; Rathbone and Maroney 1963) and that the lyso-PS produced from this enzymatic reaction was responsible for triggering systemic histamine release that caused the hemotoxic effect (Goldsmith and Mushett 1954; Mushett et al. 1954), several groups were interested in identifying mammalian PS-specific phospholipases (PS lipases). Since lyso-PS was shown to robustly elicit histamine release from the degranulation of mast cells, focused screens used this phenotype as an assay to identify enzymes, capable of producing this histamine release from mast cells. Using such phenotypic screens, two lipases namely $sPLA_2$ (Murakami et al. 1991, 1998) and PS-PLA_1 (Hosono et al. 2001; Nagai et al. 1999), were found to have PS lipase activity. Although $sPLA_2$ did induce mast cell degranulation, there was no direct evidence of $sPLA_2$

acting as a PS lipase or its specificity of PS as a substrate (Murakami et al. 1991, 1998). PS-PLA1 on the other hand was found to be a very PS-specific phospholipase, capable of producing *sn*-2 lyso-PS (hence the moniker PS-PLA1) (Fig. 2) (Aoki et al. 2007, 2002). PS-PLA1 had negligible in vitro hydrolase activity against other phospholipids (e.g. phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylinositol (PI)) or their lyso versions or neutral lipids (e.g. triacylglycerol (TAG), diacylglycerol (DAG)). Interestingly, in vitro, PS-PLA1 was also reported to have robust lyso-PS lipase activity against *sn*-2 lyso-PS substrates, though this premise has never been validated in any in vivo setting (Fig. 2) (Aoki et al. 2007, 2002). Bioinformatics analysis of mammalian PS-PLA1 suggests that it belongs to the pancreatic lipase family, and has the conserved canonical catalytic triad (Ser-His-Asp) in the N-terminal domain of the polypeptide sequence (Aoki et al. 2002). Northern blotting analysis shows that this lipase in humans (and rodents) is present mostly in the liver and testis (Aoki et al. 2002). Interestingly,

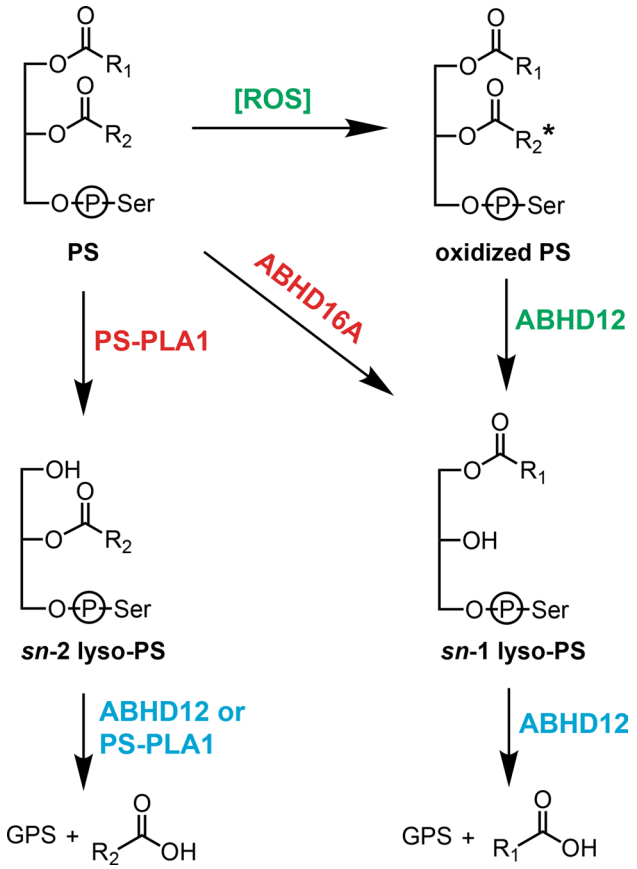


Fig. 2 The major enzymatic pathways regulating lyso-PS metabolism in mammals. The enzymes highlighted in red, green, and blue represent PS-specific phospholipases, oxidized PS lipase and lyso-PS lipases respectively. (*represents oxidatively damaged lipid chain, GPS = glycerophosphoserine)

PS-PLA1 is a secreted lipase, while the bulk of cellular PS is asymmetrically enriched on the inner leaflet of the membrane bilayer. Hence, PS-PLA1 under normal physiological conditions would have very restricted access to bulk cellular PS. It has since been speculated, but not validated, that during oxidative stress, when PS gets oxidized, it flips its membrane orientation, and PS-PLA1 can now access this extracellularly oriented oxidized PS, hydrolyse it, and in doing so prevent cells under oxidative stress from undergoing apoptosis.

Since previous tissue biodistribution studies had shown lyso-PS to be abundant in the brain (Blankman et al. 2013), and because PS-PLA1 was absent in the tissues of the central nervous system (CNS) (Aoki et al. 2002), researchers became interested in finding a cell-resident PS lipase capable of producing lyso-PS from bulk intracellular PS in the mammalian CNS. Using chemoproteomics and biochemical assays in complementation with pharmacological and genetic models, the integral membrane enzyme α/β-hydrolase domain containing protein # 16A (ABHD16A) was identified as a PS lipase, with PLA2-type PS-specific phospholipase activity (Fig. 2) (Kamat et al. 2015). ABHD16A is a member of the metabolic serine hydrolase family and this enzyme is highly conserved in all mammals (> 95% sequence identity) (Long and Cravatt 2011). The polypeptide sequence of this enzyme suggests that it has the canonical α/β-hydrolase domain (ABHD) fold and possesses the invariant catalytic triad (Ser-His-Asp) (Lord et al. 2013; Nardini and Dijkstra 1999; Ollis et al. 1992). Since ABHD16A was initially identified as a one of the major histocompatibility complex genes found as the human lymphocyte antigen B-associated transcript # 5, it is also referred to as BAT5 in the literature (Spies et al. 1989a, 1989b). In vitro, ABHD16A has phospholipase activity against several phospholipids with PS being the most preferred substrate (Savinainen et al. 2014), but not with neutral lipids (e.g. TAG, DAG); however, in vivo, ABHD16A exclusively functions as a PS lipase (Kamat et al. 2015). Biochemical studies have shown that contrary to PS-PLA1, the active site of ABHD16A (ABHD motif) is intracellularly oriented (Kamat et al. 2015), thus giving it access to its preferred substrate PS and enabling it to produce lyso-PS from bulk intracellular PS. ABHD16A is enriched in the mammalian CNS and immune system, where it functions as the principal PS lipase, and its genetic deletion or pharmacological inhibition in these tissues results in decreased lyso-PS biosynthesis (Kamat et al. 2015). There are several inhibitors described in the literature that potently inhibit ABHD16A activity in cells and serve as excellent cellular pharmacological tools; however none of these to the best of our knowledge, have been shown to be active in animal models (Ahonen et al. 2018; Camara et al. 2015; Hoover et al. 2008; Kamat et al. 2015). Given the association of ABHD16A with the CNS

and immune system, its genetic deletion in mice results in a partial degree of embryonic lethality (mice born at sub-Mendelian ratios) and shunted animal growth (Kamat et al. 2015), suggesting that ABHD16A might have a role to play in embryonic development of these tissues as well. Following up on these initial findings, in a follow-up study, we have recently shown using biochemical assays, LC–MS-based lipidomics measurements and immunohistochemical analysis, that in the mammalian brain, ABHD16A is an endoplasmic reticulum (ER)-localized PS lipase, most enriched in the granular/molecular layer of the cerebellum, and disruption of its activity has most profound effects on cerebellar PS and lyso-PS concentrations (Singh et al. 2020). These recent findings provide new insights in our understanding of the neurological disorder PHARC discussed in the next section.

The Lyso-PS and Oxidized PS Lipase ABHD12

The autosomal recessive human genetic neurological disorder PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract) is caused by deleterious null mutations to the *abhd12* gene that encodes an integral membrane lipase (ABHD12) belonging to the metabolic serine hydrolase family (Fiskerstrand et al. 2010, 2009; Long and Cravatt 2011). Human PHARC subjects have early-onset visual disturbances (cataract, retinal pigmentation), hearing loss, with progressively worsening peripheral neuropathy, sensorimotor functions, and most of them eventually end up with cerebellar atrophy (Chen et al. 2013; Criscuolo et al. 2013; Eisenberger et al. 2012; Fiskerstrand et al. 2010; Nishiguchi et al. 2014; Wortmann et al. 2015; Yoshimura et al. 2015). To study the biochemical basis of this neurodegenerative disease, Cravatt and co-workers generated the ABHD12 knockout mouse, showing that this murine model of PHARC had heightened neuroinflammation, and mimicked the disease phenotypes observed in human PHARC subjects (Blankman et al. 2013). At the time, the lipase ABHD12 lacked a clear in vivo function, and to understand the biological pathways regulated by ABHD12 in the CNS, untargeted lipidomics was performed on the brains of ABHD12 knockout mice (Saghatelian and Cravatt 2005a, b). This study showed that ABHD12 deletion resulted in a massive accumulation of lyso-PS lipids in the brain, with the accumulation of very long chain (VLC) lyso-PS lipids being the most pronounced (~ 50–80 fold) (Blankman et al. 2013). This seminal study provided the first evidence of ABHD12 being a lyso-PS lipase (Fig. 2) and the direct association of lyso-PS lipids with a human disease. Subsequent in vitro substrate profiling studies showed that ABHD12 is indeed a lysophospholipase, with lyso-PS being its preferred substrate (Blankman et al. 2013). ABHD12 is also able to turn over monoacylglycerol (MAG) lipids (Blankman et al. 2007; Navia-Paldanius et al. 2012), but did not have any phospholipase activity and was unable to hydrolyse other neutral lipids (Blankman et al. 2013). Due to the commercial unavailability of *sn*-2 lyso-PS lipids, it has not been possible to ascertain ABHD12's preference for *sn*-1 or *sn*-2 lyso-PS lipids, but given that MAG lipids serve as substrate surrogates for lyso-PSs, based on substrate profiling studies on MAGs, it is likely that ABHD12 can hydrolyse both *sn*-1 and *sn*-2 lyso-PS lipids (Blankman et al. 2007; Navia-Paldanius et al. 2012).

Following up on this seminal finding, we have subsequently shown using immunofluorescence and biochemical studies that ABHD12 is an integral membrane enzyme localized to the ER, and prefers VLC lipids as substrates, thus providing an explanation as to why VLC lyso-PS lipids are elevated in the brains of ABHD12 knockout mice (Joshi et al. 2018). In the same study, we also show that glycosylation is required for optimal ABHD12 activity, and that the active site of ABHD12 (ABHD motif) is luminally (or extracellularly) oriented (Blankman et al. 2007; Joshi et al. 2018). This finding complements the fact that disruption of ABHD12 activity in different mammalian cells results in increased secretion of lyso-PS, while cellular lyso-PS levels remain unchanged (Kamat et al. 2015). ABHD12 also functions as a principal lyso-PS lipase in different immune cells, and its genetic deletion or pharmacological inhibition in these immune cells increases the secreted lyso-PS levels and in turn causes heightened pro-inflammatory cytokine secretion from them (Kamat et al. 2015). In pursuit of lipase(s) capable of metabolizing the pro-apoptotic PS lipids, we first developed a LC–MS assay to quantitatively measure these oxidized PS lipids, and used this as a readout to perform chemical genetic screening in mammalian cells with a focused lipase inhibitor library to identify enzymes capable of turning over oxidized PS lipids (Kelkar et al. 2019). We found from our screens and subsequent biochemical validation studies, that ABHD12 also functioned as a oxidized PS lipase (Fig. 2), and is responsible for regulating the levels of oxidized PS lipids in cells and tissues under oxidative stress (Kelkar et al. 2019). Our studies thus expand the biochemical activities of ABHD12 and suggest that this enzyme hydrolyses oxidized PS to first yield *sn*-1 lyso-PS, which is then further hydrolysed again by ABHD12 to give a free fatty and the GPS head group (Fig. 2). Recently, Cravatt and co-workers have also reported selective in vivo active inhibitors for ABHD12 (Ogasawara et al. 2019, 2018), and using these pharmacological tools, they have shown how ABHD12 intricately regulates various immunological pathways in animal models (Ogasawara et al. 2018) and ferroptosis of human cancer cells (Kathman et al. 2020). From the perspective of the involvement of lyso-PS signalling in PHARC, we show using immunohistochemical analysis that ABHD12 is localized to Purkinje neurons of the cerebellum, and in complementation with lipidomics measurements, we show that its disruption has most profound effects on PS

and lyso-PS levels in the cerebellum, the brain region that undergoes severe atrophy in human PHARC subjects (Singh et al. 2020). Our recent findings though preliminary seem to posit a paracrine mode of signalling for lyso-PS lipids in the mammalian brain (Singh et al. 2020).

The Lyso-PS-Specific Acyltransferase, MBOAT5

Over forty years ago, a PS biosynthetic activity was identified in rats, where an unknown enzyme from liver lysates was found to acylate *sn*-1 lyso-PS lipids using fatty acyl coenzyme-A (CoA) to yield PS, with a strong preference for polyunsaturated fatty acyl CoA substrates especially arachidonoyl-CoA (Holub 1980). Three decades later, lipidomics measurements showed that concomitant to the increased lyso-PS lipids in the ABHD12 knockout brains, there was also a striking increase in arachidonoyl-containing (C20:4) PS lipids (Blankman et al. 2013). Until recently, it was unclear why ABHD12 deletion resulted in the rewiring of PS content, and how C20:4-PS lipids were selectively elevated in the brains of ABHD12-null mice. Members of the membrane-bound O-acyltransferase (MBOAT) family accept lysophospholipid and fatty acyl CoA as substrates and catalyse the formation of phospholipids (Hishikawa et al. 2014, 2008; Shindou et al. 2013). In particular, MBOAT5 (also known as lysophosphatidylcholine acyltransferase 3, LPCAT3) shows very high specificity for polyunsaturated fatty acyl CoA as substrates (e.g. C20:4 CoA) (Gijon et al. 2008; Hashidate-Yoshida et al. 2015; Matsuda et al. 2008; Rong et al. 2015) and is present abundantly in the mammalian brain (Wu et al. 2016, 2009). While the roles of MBOAT5 in PC and PE remodelling are well understood (Rong et al. 2013; Wang et al. 2016), until recently, its role in PS biosynthesis remained cryptic. By generating the ABHD12-MBOAT5 double-knockout mouse model, and by complementing the findings from this genetic model, by pharmacological disruption of ABHD12 activity using selective ABHD12 inhibitors in mice deficient of brain MBOAT5, Cravatt and co-workers have now conclusively shown that MBOAT5 is indeed lysophospholipid acyltransferase responsible for the rapid rewiring of C20:4-PS lipids that is observed in brains of ABHD12 knockout mice (Fig. 3) (Ichu et al. 2020).

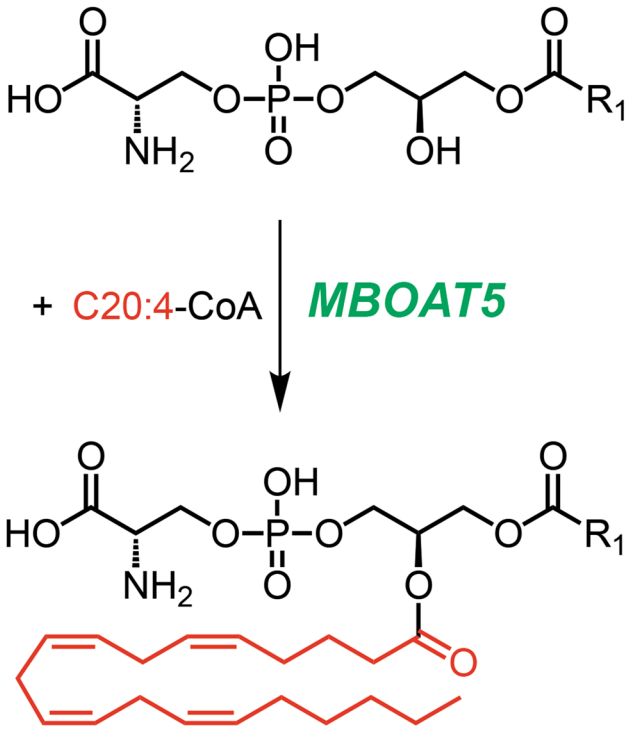


Fig. 3 The enzymatic reaction catalysed by lyso-PS-specific acyltransferase MBOAT5

Lyso-PS Receptors

The G-Protein-Coupled Receptors (GPCRs) are the largest and most versatile family of receptors in eukaryotes (Katritch et al. 2013; Kobilka 2007; Rosenbaum et al. 2009). A survey of known coding sequences in the human genome identified more than 800 distinct members of this family (Fredriksson et al. 2003), of which, several lack known ligands. Since a significant majority of clinically approved drugs target GPCRs (Hauser et al. 2017), numerous studies over the past decade have focused on identifying ligands for the unannotated GPCRs. One such high-throughput study aimed at identifying lipid ligands for GPCRs used a cellular transforming growth factor alpha (TGFα) shedding assay and found that lyso-PSs were putative ligands (agonists) to the unannotated GPCRs: GPR34, GPR174 and P2Y10 (Inoue et al. 2012). Given their association with lyso-PS, GPR34, P2Y10 and GPR174 are also known as $LYPSR_1$ (or LPS1), $LYPSR_2$ (or LPS2) and $LYPSR_3$ (or LPS3), respectively (Inoue et al. 2012; Uwamizu et al. 2015). In this section, we will summarize what is known for the aforementioned GPCRs, along with other putative receptors (TLR2 and G2A) that are speculated to be ligands for lyso-PSs.

GPR34

Several decades of work had indirectly implicated lyso-PSs to be involved in histamine release from the degranulation of primary mast cells. Following on this body of work, it was eventually established by direct in vitro and in situ testing that only lyso-PS, but no other lysophospholipid (lyso-PE, lyso-PC, lyso-PA, sphingosine-1-phosphate (S1P)), was able to robustly release histamine from the degranulation of mast cells in a dose-dependent manner (Lloret and Moreno 1995). This result suggested that there must exist a lyso-PS-specific

receptor on mast cells, that is responsible for histamine release from degranulating mast cells, and in a quest for this, GPR34 was identified as a potential target (Sugo et al. 2006). In this study, human GPR34 was recombinantly expressed in the CHO cell line and it was shown to regulate cyclic AMP production under various pharmacological treatment paradigms involving cellular lyso-PS stimulation (Sugo et al. 2006). Of note, other lysophospholipids (e.g. lyso-PE, lyso-PC, lyso-PA and S1P) failed to elicit any GPR34-dependent response to this phenotype, suggesting that lyso-PS is probably a specific ligand to GPR34 (Sugo et al. 2006). GPR34 is highly expressed in mast cells (Fig. 4) (Wu et al. 2016, 2009), and it has been shown that lyso-PS alone (without the requirement of additional antigens) can induce robust dose-dependent histamine release from mast cell degranulation in the LAD2 cells (a human mast cell line), which highly express GPR34 (Sugo et al. 2006). Further, the expression of GPR34 in HEK293 cells was shown to increase calcium mobilization and its efflux from the ER membrane to the cytosol under the influence of lyso-PS, but not any other lysophospholipid (including lysophosphatidylthreonine) (Iwashita et al. 2009). More recently, the ability to synthesize lyso-PS-like molecules and by leveraging computational docking, more rigorous structure activity relationship studies have been possible towards understanding the ligand specificity of lyso-PS on GPR34 (Sayama et al. 2017, 2020). Interestingly, these studies speculate that GPR34 prefers *sn*-2 lyso-PSs with long chain fatty acids as ligands, though this hypothesis needs to be validated rigorously (Iwashita et al. 2009; Kitamura et al. 2012).

Despite all the aforementioned studies, the role of GPR34 as a lyso-PS receptor is still controversial. A contradicting study found that different mammalian GPR34 expressed in COS7 cells failed to regulate cyclic AMP production upon pharmacological treatment with lyso-PSs (Liebscher et al. 2011). The same study also generated the Gpr34 knockout mouse line, and found that contrary to earlier reports, mast cells derived from wild-type or age-matched GPR34 knockout mice had near identical histamine release profiles upon lyso-PS treatment (Liebscher et al. 2011). These studies suggest that there might be some functional redundancies in lyso-PS signalling in mast cells, since more than one putative lyso-PS receptor is present on mast cells (Fig. 4). Nonetheless, when immunologically challenged, GPR34 knockout mice display a blunted inflammatory response compared to wild-type littermate, thus implicating a role for GPR34 in orchestrating immune responses (Liebscher et al. 2011). From a neuroimmunological perspective, studies have shown that in response to the demyelinating toxin cuprizone, GPR34 was found to be one of a handful of genes whose microglial expression was upregulated in rodents (Bedard et al. 2007), while the deletion of GPR34 resulted in altered morphology and reduced phagocytic ability of microglial cells (Preissler et al. 2015). More recently, animal studies in rodents have shown GPR34 to exacerbate neuropathic pain, by eliciting a microglial pro-inflammatory response, and antagonists to GPR34 have been proposed to alleviate this neuroinflammatory condition (Sayo et al. 2019). From a cancer biology standpoint, cellular pharmacological studies have shown that overexpression of GPR34 (and lyso-PS signalling) promote cancer cell proliferation and pathogenesis, while its downregulation has the opposite effects (Jin et al. 2015; Yu et al. 2013; Zuo et al. 2015). While the role of GPR34 in regulating (neuro)immunological responses

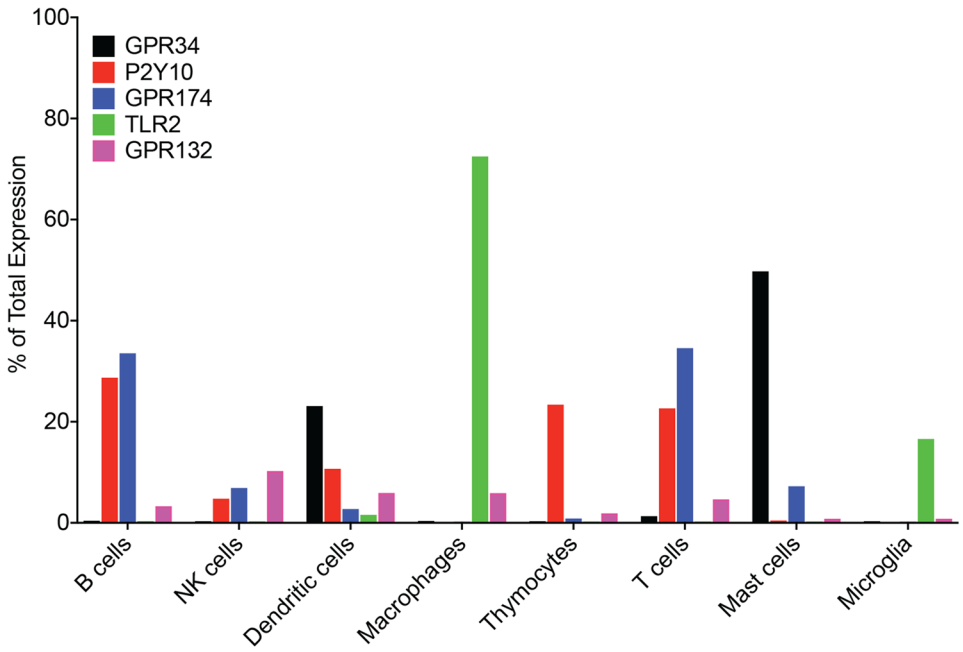


Fig. 4 Expression profile of the different putative lyso-PS in various immune cells from a large scale gene expression database (https://biogps.org)

is fairly well established, how lyso-PSs regulate these processes via GPR34 signalling remains to be explored.

P2Y10

Of the three GPCRs identified as a putative lyso-PS receptors from the TGFα shedding assay (Inoue et al. 2012), P2Y10 is the least characterized (Hwang et al. 2018), and there are no reports, to the best of our knowledge, linking this GPCR to any (patho)physiological conditions in humans (or in other animal models). Like GPR34, the role of P2Y10 as a lyso-PS receptor is also controversial. A study reports that expression of P2Y10 in CHO cells induces intracellular calcium release upon pharmacological treatment with lyso-PA and S1P in a dose-dependent manner, while genetic knockdown of P2Y10 resulted in decreased intracellular calcium release (Murakami et al. 2008). Structure activity relationship studies have shown that P2Y10 agonists (lyso-PS and its analogues) facilitate TGFα-shedding and the formation of actin stress fibres in mammalian cells expressing P2Y10 (Ikubo et al. 2015). P2Y10 is expressed in different immune cells (Fig. 4) (Wu et al. 2016, 2009), and therefore, it has been speculated that the lack of any phenotype observed in GPR34 knockout mast cells upon lyso-PS stimulation might be a consequence of functional compensation by P2Y10.

GPR174

Prior to its association with lyso-PS, it was reported that CHO cells overexpressing GPR174 had an altered morphology and lower cellular proliferation rates compared to mock-transfected cells (Sugita et al. 2013). The basal intracellular cyclic AMP levels were significantly elevated in GPR174-overexpressing CHO cells, and subsequent studies found that these levels increased further, upon pharmacological treatment with lyso-PS in dose-dependent manner while remaining unaltered in mock-transfected CHO cells (Sugita et al. 2013). Following up on this initial result, the TGFα-shedding assay screen found that lyso-PS was in fact an agonist to GPR174 and thus formalized the association between this receptor-ligand pair (Inoue et al. 2012). Hypothesizing that lyso-PS likely suppresses lymphocyte proliferation, in a seminal study, Cyster and co-workers decided to generate and characterize the GPR174 knockout mouse line (Barnes et al. 2015). They found that GPR174 is highly expressed in naïve B cells and T cells (Fig. 4) and that this receptor regulates T cell proliferation and differentiation of immature T cells into regulatory T cells (Tregs) (Barnes et al. 2015). In the same study, it was shown that GPR174 knockout mice showed protection against the progression of disease pathology in an experimental autoimmune encephalomyelitis model, suggesting that antagonists to this receptor might have therapeutic value in treating multiple sclerosis and other autoimmune conditions (Barnes et al. 2015). Next, Cyster and co-workers have also shown that the lyso-PS-dependent suppression of T cell activation occurs via GPR174 and have reported that this phenotype is coupled downstream to the $G\alpha_s$ signalling subunit (Barnes and Cyster 2018; Robert and Mackay 2018). Corroborating these findings, recently, it has been mechanistically delineated in cellular studies that the lyso-PS/GPR174 signalling inhibits the IL-2 production in CD4 T cells (Shinjo et al. 2017), a pathway known to promote differentiation of immature T cells into Tregs, and of CD4 T cells into Th1 and Th2 cells (Konkel et al. 2017). Interestingly, the chemokine CCL21 was also recently reported as a ligand of GPR174, and the GPR174/CCL21 signalling axis has been implicated in conferring sexual dimorphism to humoral immunity (Zhao et al. 2020). The role of the lyso-PS/GPR174 to this immunological process remains to be understood. Given the association of lyso-PS/GPR174 signalling to the immune system, it is not surprising that genetic mutations of this receptors have been linked to various autoimmune conditions in humans. In particular, human genomewide association studies have shown that polymorphisms and/or mutations to GPR174 has a strong causal correlation with the pathology seen in Grave's disease (Chu et al. 2013; Szymanski et al. 2014; Yan et al. 2020; Zhang et al. 2020; Zhao et al. 2013) and Addison's disease (Falorni et al. 2016; Napier et al. 2015, 2014).

Toll-like receptor 2 (TLR2)

The Toll-like receptor (TLR) family members are highly expressed in innate immune cells (e.g. macrophages) (Fig. 4) and play an important role in conferring immunity against extraneous pathogens (Janssens and Beyaert 2003; Kawasaki and Kawai 2014; Parker et al. 2007; Takeda and Akira 2005). To mechanistically understand the pathogenesis caused by schistosomal species, lipids were extracted from *S. mannasoni* (eggs and adult worms), fractionated by column chromatography, and screened for their ability to elicit cytokine production from human peripheral blood mononuclear cells (PBMCs) (van der Kleij et al. 2002). From this screen, a PS-containing lipid fraction induced highest cytokine production via a TLR2-dependent pathway (van der Kleij et al. 2002). Further in the same study, primary macrophages derived from TLR2 knockout mice were found to be unresponsive towards this schistosomal PS fraction. Next, this PS fraction was subjected to HPLC separation, and it was found that lyso-PSs (especially VLC lyso-PSs), but not PS, were responsible for this phenotype, and activation of downstream TLR2-dependent immune activation pathways (van der Kleij et al. 2002). This is the only report to the best of our knowledge showing that lyso-PSs are ligands to TLR2

(van der Kleij et al. 2002), and more studies are needed to verify and mechanistically characterize this pathway.

GPR132

Lyso-PS has been implicated in resolution of inflammation, by promoting the macrophage-dependent engulfment of apoptotic cells or pathogens (discussed in next section) (Frasch et al. 2008; Frasch and Bratton 2012), and this led researchers to speculate that there must exist a receptor on macrophages responding to lyso-PS and facilitating this activity. Several lines of study have shown that the orphan GPCR, GPR132 (also known as G2A), is expressed in different immune cells, including macrophages (Fig. 4), activated in response to stress stimuli and prolonged mitogenic signals (Le et al. 2001), and was hypothesized to be a lyso-PS receptor mediating the resolution of inflammation (Frasch et al. 2008, 2013). An antibody-based depletion of GPR132 was found to decrease lyso-PS-mediated phagocytosis by macrophages, while pharmacological treatment with lyso-PS of wild-type macrophages, but not GPR132 knockout macrophages, elevated the Rac1 activity needed for phagocytosis (Frasch and Bratton 2012; Frasch et al. 2013, 2011). Although there are results which suggest that lyso-PS treatment of macrophages increased its phagocytic potential, there has been no direct evidence to the best of our knowledge that supports lyso-PS directly binding to and activating GPR132. The role of GPR132 as a lyso-PS receptor has been more debatable than the other lyso-PS receptors, particularly due to the lack of direct evidence showing its binding to GPR132, and because several reports over the past two decades have shown GPR132 to be a promiscuous lipid receptor, responding similarly to other lysophospholipids (Kabarowski et al. 2000, 2001; Lin and Ye 2003; Murakami et al. 2004; Rikitake et al. 2002; Wang et al. 2005) and oxidized pro-apoptotic lipids (Obinata et al. 2005; Peter et al. 2008).

Biological Pathways Influenced by Lyso-PS

Given their amphiphilic chemical structures, their biodistribution, and presence of their putative receptors on various immune cells, lyso-PSs influence several biological processes (Fig. 5). In this section, we will summarize these immunological pathways, and provide a historical

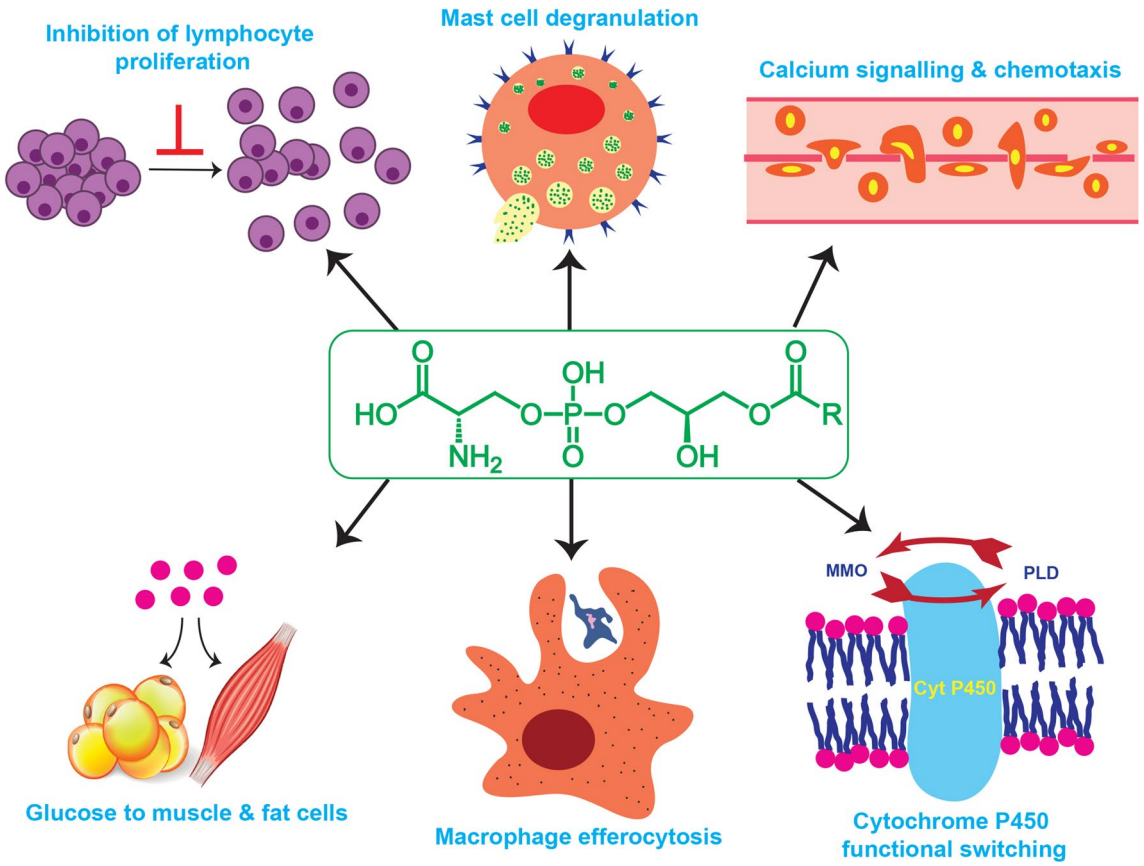


Fig. 5 The physiological processes regulated by lyso-PSs

background on the studies that led to show how lyso-PSs were established as key regulators of these important physiological pathways in mammals.

Facilitation of Histamine Release from Mast cells

Early animal studies showed the injections of brain lipid fraction-containing lyso-PSs in rabbit-caused haemolysis and eventual respiratory failure by systemically triggering a "histamine-like substance" (Goldsmith and Mushett 1954; Mushett et al. 1954). Around the same time as these studies, it was discovered that in response to an inflammatory stimulus, mast cells rapidly release histamine as a signal to other immune cells to clear the allergic antigen (Riley 1953a, b; Riley and West 1953). The first direct evidence of the involvement of lyso-PS on histamine release from mast cells came from researchers studying PS dextran composite liposomes and their effects potentiating this phenotype (Chakravarty et al. 1973). This study suggested that PS needed to be incorporated and/or turned over to a degraded product(s) (that we now know is lyso-PS) by the cell for it to function as a potentiator of histamine release from mast cells. Interestingly, the same effect was not observed with other phospholipids (e.g. PC, PE), suggesting that this process was exquisitely selective for the PS/lyso-PS pair. Following up on this finding, it was subsequently shown that concanavalin A (conA) along with lyso-PS treatment was far more potent in stimulating this phenotype than PS or any other lysophospholipid (Martin and Lagunoff 1979). Interestingly, these studies also suggested that lyso-PS acted as a potentiator of histamine release at concentration well below its critical micellar concentration (CMC), and this fact ruled out the possibility that unlike PS, lyso-PS does not form a vesicle-like structure to induce this effect (Horigome et al. 1986; Martin and Lagunoff 1979; Tamori-Natori et al. 1986). Another study assessing potency of lyso-PS to induce this phenotype showed conclusively that in conjunction with conA, lyso-PS is 1000 times more potent at causing histamine release from mast cells than PS or other phospholipids (Smith et al. 1979), and that at higher concentrations lyso-PS could in fact produce this biological effect in the absence of conA (Iwashita et al. 2009). Quite interestingly, in response to antigens, histamine is released by both mast cells and basophils. However, studies have shown that lyso-PS is incapable of triggering histamine release from basophils and this lyso-PS-mediated process is restricted only to mast cells (Kolster et al. 1987). Few studies in rodents also suggest that the nerve growth factor and lyso-PS synergistically interact to regulate brain glucose metabolism by controlling histamine and adrenaline secretion from mast cells (Bruni et al. 1982). To establish the in vivo role of lyso-PS in histamine release from mast cells, various lysophospholipids were intravenously injected in rodent models, and it was found that lyso-PS, but no other lysophospholipid, increased circulating histamine levels in these animals (Bruni et al. 1984; Mietto et al. 1984). Given the vast body of literature, identification of GPR34 as a putative lyso-PS receptor on mast cells (discussed earlier), and potency of lyso-PS in stimulating this immunological process, lyso-PS-mediated histamine release from mast cells has, over the years, been the most studied biological pathway influenced by this lipid.

Promoting Macrophage Efferocytosis

Efferocytosis ("*efferre*" Latin word meaning "to carry to grave" or "to bury") is an ability of phagocytic cells (e.g. macrophages) to clear out apoptotic cells, invading pathogens and cell debris from an organism (Elliott et al. 2017; Martin et al. 2014). In humans (and mammals), during the event of tissue damage and/or inflammation from an injury, circulating macrophages rely on "find me" and/or "eat me" signals from the damaged and/or apoptotic cells to facilitate their clearance from the site of injury (Doran et al. 2020). While several immune mediators that provide cues to macrophages in locating the site of inflammation and/or apoptosing cells have already been described in the literature, the role of lyso-PS in facilitating this phagocytic activity of macrophages has only been recently elucidated (Frasch and Bratton 2012). The first study on this topic reported that at the site of injury, activated neutrophils upregulate the enzymatic activity of NADPH oxidase that leads to the production of secreted lyso-PS from these cells (Frasch et al. 2008). Subsequent follow-up studies on this initial finding found that NADPH oxidase activity was needed for the secretion of lyso-PS from these cells through the generation of transient oxidized PS lipids, via an unknown phospholipase activity (Frasch et al. 2008, 2011). Lipidomics measurements found that only secretion of lyso-PS, but no other lysophospholipid, was heightened in this process, and the orphan receptor GPR132 (discussed earlier) was implicated in this activity (Frasch et al. 2013, 2011). Recently, it has been shown that pharmacological treatment of primary macrophages with lyso-PS results in increased secretion of pro-inflammatory cytokines (e.g. TNF-α, IL-6), and this response is significantly more in macrophages obtained from ABHD12-null mice that lack lyso-PS lipase activity (Kamat et al. 2015). While several studies now strongly suggest the role of lyso-PS as a mediator of macrophage-mediated efferocytosis, in vivo experiments mechanistically understanding this immunological process are lacking and are much needed to advance our knowledge on this topic.

Stimulates Intracellular Calcium Signalling and Chemotactic Migration in Human Cancer sells

Since lyso-PSs are shown to signal through GPCRs (Inoue et al. 2012), it is not surprising that they regulate calcium signalling and its mobilization within cells. Intracellular

calcium fluxes are associated with signalling cascades downstream of several GPCRs, and calcium is known to act as a secondary messenger in signal transduction pathways that mediate chemical signalling and promote cell proliferation (Fredriksson et al. 2003; Kobilka 2007; Rosenbaum et al. 2009). Several recent reports have shown that lyso-PS induced influx of intracellular calcium, promoted chemotactic (migratory) and stimulatory properties of different mammalian cancer cell lines (Lee et al. 2008; Lloret and Moreno 1995), including mouse fibroblasts (Park et al. 2006), primary human leukemic (Park et al. 2005) and colorectal cell lines (Iida et al. 2014). Most of the aforementioned properties have been attributed to GPR34 (discussed earlier), and GPR34 knockdown or treatment with antagonists/inhibitors to cascades proposed to be downstream of GPR34 in these cell lines have been shown to hamper these properties.

Inhibits Lymphocyte Proliferation

Early studies showed that PS displayed strong inhibitory properties on DNA synthesis in circulating lymphocytes of the peripheral blood mononuclear cells (PBMCs), even in the presence of agents that facilitate DNA synthesis (Caselli et al. 1992a, b). However, when the same pharmacological studies were attempted in cell culture models, they failed in the serum deplete conditions, and PS only displayed very modest inhibitory properties on the DNA synthesis of lymphocytes (Bellini and Bruni 1993). In an effort to explain this discrepancy, subsequent studies showed that the serum-contained phospholipases that converted PS to lyso-PS and that in situ production of lyso-PS was responsible for inhibition of DNA synthesis in the lymphocytes of the PBMCs (Bellini and Bruni 1993; Caselli et al. 1992a). Following up on these initial studies, we now know from recent elegant mechanistic studies by Cyster and co-workers that lyso-PS through GPR174 regulates the proliferation of T cells and their maturation into Tregs (discussed earlier) (Barnes and Cyster 2018; Barnes et al. 2015).

Stimulates Glucose Flux in Adipocytes and Myoblasts

In an effort to understand the physiological properties of lyso-PS, intravenous injection of these lipids in rodents resulted in a hyperglycaemic response and brain glucose accumulation that was found to be independent of insulin (Chang et al. 1988). These initial studies suggested that lyso-PS lipids had hormone-like signalling properties. Subsequent follow-up studies showed that lyso-PS facilitated dose/time-dependent transport and absorption of glucose into muscle fibres and adipocytes through lyso-PS-dependent enhanced GLUT4 expression in these particular cells (Yea et al. 2009). The same study also showed that systemic lyso-PS administration successfully lowered blood glucose levels in diabetic mouse models (Yea et al. 2009). While the association of lyso-PS to this phenotype is robust, mechanisms underlying this biological activity are lacking to the best of our knowledge.

Acts as Functional Switch for Cytochrome P450 Activity

In an effort to understand the functional interactions between charged lysophospholipids, and a sub-set of cytochrome P450s, various lysophospholipids were incubated with cytochrome P450s 1A2 (CYP1A2) and 2E1 (CYP2E1), and their range of enzymatic activities were screened (Cho et al. 2008). It was found from this screen that lyso-PS, but no other lysophospholipid, switched their activities from a mono-oxygenase to functional phospholipase D (PLD) that was specific to PC as a substrate (Cho et al. 2008). Further, the same study showed that this switch-in activity was lyso-PS dose dependent, and lyso-PS, thus, termed as "functional switch" could toggle and tune the biological activities of these ER resident liver enzymes (Cho et al. 2008). Though exciting, more mechanistic studies are needed to understand this enzymatic activity switching and the role of lyso-PSs in regulating such functional redundancies in vivo.

Conclusions

Lysophospholipids regulate several facets of mammalian physiology, and deregulation in metabolic pathways is often associated with human diseases (Rivera and Chun 2008). Lysophosphatidic acid (Contos et al. 2000; Ishii et al. 2004; Rivera and Chun 2008) and sphingosine-1-phosphate (Gonzalez-Cabrera et al. 2014; Rosen and Goetzl 2005; Rosen et al. 2009, 2013; Rosen and Liao 2003) are the best studied lysophospholipids, and their metabolic enzymes and/or receptors are under clinical investigations for the treatment of several human disorders. Even though it was known since the 1950s that lyso-PSs are important bioactive lipids, their precise biological roles, metabolic and signalling pathways have remained cryptic until quite recently. The past two decades have seen a real renaissance in our understanding of these lyso-PS-mediated pathways, particularly because of the discovery of the metabolic (biosynthetic and degradative) enzymes and putative receptors of this signalling lysophospholipid. Despite the recent progress that has tremendously expanded our biological understanding of this emerging lysophospholipid, an exhaustive review summarizing these studies on lyso-PSs has been lacking. In this review, we discuss several important recent findings particularly with regard to the metabolic pathways that regulate lyso-PS concentrations in vivo, its putative receptors and the

biological pathways that this lysophospholipid regulates in mammals. Given that mutations to the metabolic enzymes and/or putative receptors of lyso-PS lead to human neurological and autoimmune diseases, it further emphasizes the critical role that this lysophospholipid plays in human physiology, and in the years to come, detailed mechanistic knowledge of these emerging biological pathways and how lyso-PS regulates them can be leveraged to develop new therapies in treating these as-of-yet incurable human disorders.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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