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Summary :
Phosphatidylglycerols (PGs) are specific phospholipids bearing negatively charged polar headgroups. Although recognized for long as a major lipid component of membranes in bacteria, it is considered a minor lipid in higher eukaryotes, due to its low abundance in biological fluids or tissues. However, new sensitive lipidomic approaches now provide tools for accurate quantification of PGs in biological samples, and this is likely to uncover new roles for these phospholipids in the near future. This paper reviews our present knowledge in PG function, from studies in microbes and eukaryotic cells, and gathers in one place a diverse range of information spread across many fields. The physical properties of PGs, their biological distribution and molecular functions make them potential actors in host-microbe interaction.

1. Introduction

An assortment of different headgroups are found in phospholipids, mainly choline, ethanolamine, serine, inositols and glycerol, that define 6 distinct structural classes, plus phosphatidic acids in which no head group is added. The diversity of polar head groups is thought to contribute optimal surface charge of membranes or lipid particles so that zwitterionic headgroups balance phospholipids containing acidic headgroups. Besides electrostatic balance, it is likely that some advantage is linked to headgroup diversity versus "one fits all", that can override the cost for stringent regulation synthesis of phospholipids with different headgroup moieties. Especially, integral membrane proteins might require diverse phospholipid environment to adopt correct topology in membranes. Furthermore, specific properties linked to particular phospholipids can exist, such as phosphatidylinositol binding domains in proteins with crucial importance in protein/membrane interaction. With

recently developed high throughput mass-spectrometry-based lipidomics technologies, detailed evaluation of lipid diversity at a molecular scale has become accessible, and is expected to provide new paradigms in understanding lipid diversity. PG levels rise in obesity-associated fatty liver disease (1), type-II diabetes (2), breast cancer (3) and perhaps other still to be defined conditions. In the present paper, we will concentrate on specific features of PGs to review our present knowledge on specific roles and properties of this unique phospholipid class (figure 1).

2. General principles in PGs distribution and abundance

PGs are negatively charged phospholipids found ubiquitously, but a key feature is their differential abundance among organisms. According to biochemistry text books, PGs are found as major phospholipid components of cell membranes in bacteria, representing 20-25% of total lipid phosphorus in most Gram-negative bacteria with a double (outer and inner) phospholipid envelop, where it is restricted within the inner membrane. In Gram-positive strains (with a single phospholipid bilayer coated with peptidoeglycans) it can be as high as 60% and even found as the major phospholipid besides sugar derivatives of diacylglycerol. In plants, PGs are major constituents of the leaves representing approximately 20% of total lipid phosphorus, but is less abundant in fruits, roots and tubers. It is found as the only glycerophospholipid in green alguae. In contrast, PG is a minor phospholipid component in mammals, found at only trace amounts in tissues and fluids except in lung surfactant. In higher eukaryotes it serves mainly as the precursor of mithochondria-specific lipids such as cardiolipin, a diphosphatidyl glycerol molecule structure with 4 fatty acid chains. PG also serves as an intermediate in the synthesis of bis(monoacylglycero)phosphate or BMP, a lipid found enriched in the endolysosomal compartment. Overall, a striking feature of PG biology is its highly variable distribution and abundance in different organisms, from a quasi exclusive glycerophospholipid with structural roles in prokaryotic membranes to trace amounts and highly compartimented distribution in higher organisms.

3. PG analysis using Liquid Chromatography-Electro Spray Ionisation-Mass Spectrometry (LC-ESI-MS/MS)

PGs, as all glycerophospholipids are amphiphilic, and their extraction from biological fluids is therefore achieved by combination of relatively polar (such as methanol) and apolar solvents (such as chloroform) initially introduced by Folch et al., and adapted by Bligh and Dyer to obtain a single extraction phase chloroform/methanol/water (1:2:0.8). At neutral pH, PG is in the anionic form, so that mild acidification of the extraction solvent allows the protonation of PG lipids and a better extraction yield. The modified Bligh and Dyer method developed by Ivanova et al. with mild acidification (0.1N HCl:methanol (1:1)) of the methanol is therefore very efficient for acidic lipids such as PG (4). As for all other glycerolipids, PGs polarity allows their detection using electrospray ionization, but the fast ESI-MS/MS shotgun approach from crude extracts is however not appropriate for minor lipids such as PG whose signal gets easily suppressed by more abundant lipids in the ESI source. PG analysis using MS/MS therefore requires prior separation by liquid chromatography, that can be achieved with hydrophobicity-based separation (chain length and unsaturation) using reversed-phase LC columns (5) or polarity-based separation depending on

headgroup using normal phase columns (4). The latter chromatography uses however hazardous apolar solvents such as chloroform or hexane and tends to get replaced by hydrophilic interaction chromatography (HILIC), which allows a normal-phase type separation using reversed-phase type solvents at a relatively fast speed with the development of core-shell technology (6). PG lipids are either analysed in positive ESI-MS/MS mode using the specific polar headgroup neutral loss m/z189 (ammonium adduct of the glycerophosphate moiety) or in the complementary negative ESI-MS/MS mode using the headgroup product ion scan (mz 153) or the fatty acyl chains product ion scan. For specific PG quantification, notably in cell and tissue, positive mode should be preferred as it has a distinct fragmentation pattern from its structural isomer bis(monoacylglycero)phosphate (BMP) (7). Lipid chain elucidation of total PG and BMP is obtained in negative mode. Examples of relative fatty acid composition of PGs in mammalian host serum or Lactobacillus pellets are shown in figure 2, highlighting diversity in PG composition.

4. PG synthesis in microbes and hosts.

4.1 Canonical PG synthesis pathway (figure 3)

The pathway for PG synthesis is common in bacteria and higher mammals, elucidated by Kennedy in the 60s (8;9). PG synthesis initiates from the common phospholipid precursor Cytidine Diphosphate Diacylglycerol (CDP-DAG), produced by the CDP-DAG synthase (Cds) also called Phosphatidate Cytidylyltransferase. CDP-DAG is then used by Phosphatidylglycerol synthase (PgsA in *Escherichia coli*), the first committed enzyme in the PG pathway (10;11;11). Bacterial *pgsA* and eukaryotic homologues *PGS1* (12;13) encode the enzymes that displace CDP by glycerol-phosphate to provide PG-phosphate, which is further dephosphorylated by a phosphatase to form PG. There are three *pgp* genes in the *E. coli* genome (*pgpA, pgpB and pgpC*), perhaps not all to serve exclusively for PG phosphate hydrolysis (14). In eukaryotes, multiple PG phosphatase genes also exist (15;16), and enzymatic activity has been detected in mitochondria matrix (17). Whether PG synthesis is essential for bacteria has been debated for some time (18-20). Requirement might depend on the quantitative importance of PG relative to other membrane lipids in microbial species. Indeed, more severe consequence of abrogation of PG synthesis is expected in bacteria with higher abundance of PG in their membranes (21;22). However, it is difficult to decipher the contribution of other PG-derived products, that might also play important roles in growth. In eukaryotes, PG biosynthesis reside predominantly in the mitochondria, PG-phosphate synthase is a component of the inner mitochondrial membrane (23).

4.2 Cardiolipin

Although PG mainly serves a primary structural component of prokaryotic membranes, it is also a required precursor in the synthesis of cardiolipin, which is found at variable amounts in Gram-positive and Gram-negative cell envelops. Three different genes are identified as cardiolipin synthases, named *clsA/B/C*. In *E.coli*, the ClsA protein aggregates two PG molecules for cardiolipin formation (24). Some evidences indicate that the other cardiolipin synthases ClsB/ClsC can use PG but also other phospholipid substrates (25). Further, it was reported recently that ClsB is involved in a new alternative pathway for PG synthesis in *E. coli*, using PE and glycerol-3-phosphate as subtrates (26). Thus, cardiolipin formation is

tightly connected to both PG and PE phospholipids. In eukaryotes, the synthesis of cardiolipin slightly differs from prokaryotes. In *E. coli*, *Staphylococcus aureus* or *Lactobacillus planturum*, two molecules of PG are condensed, while in mitochondria from liver and *Saccharornyces cerevisiae*, the prefered substrates are PG and CDP-DAG (27-29). *S. aureus* express two genes for cardiolipin synthesis, a stress inducible *cls1* and a constitutive *cls2* (30). Their respective contributions in PG/cardiolipin homeostasis is still unknown. Overall, as noticed above, several steps in PG/cardiolipin pathway are assigned multiple genes which may not have redundant functions. Their respective roles in bacterial growth, either in standard laboratory conditions or in "real life" with highly diverse environments still awaits better understanding.

4.3 BMP

BMP or bis(monoacylglycero)phosphate is a structural isomer of PG, with one acyl chain linked to each of its glycerol moieties. BMP is thought to be derived from PG since cells deficient in PG synthesis have reduced ability to produce this lipid (31). Remarkably, BMP was reported to date only in eukaryotes, with a strict endosome /lysosomes distribution, and it is still largely unknown how BMP is produced and transported to endo-lysosomes. It remains negatively charged within the acidic environment of lysosomes, and thus might be important in degradative function as well as in preservation of lysosome membrane integrity (32).

4.4 Aminoacyl PGs

Bacterial PG phospholipids can also serve as substrates for amino-acyl-phosphatidylglycerol synthases that will link amino acyl residues on the PG head group. As PG aminoacylation changes envelop properties, these are important genes for bacteria to resist antimicrobial compounds (see review (33)). Aminoacyl-tRNAs are used as aminoacid donors in this reaction, and early studies identified the enzyme MprF (multiple peptide resistance factor) as responsible for the lysylation of membrane PG in *S.aureus* (33). Lysine is most frequently attached to PGs, but alanine and arginine can also be found, which may affect the spectrum of antimicrobial resistance. Further, analysis of the genomic context of amino-acyl-PG synthase genes in bacteria identified additional families of hydrolase-like sequences in many species. One of these, referred to as AhyD in *Enterococcus faecium* encodes an amino-acyl-PG hydrolase, which degrades Ala-PG and Lys-PG (34). Thus, amino-acyl-PG synthases and hydrolases may act in concert to fine tune amino-acyl-PGs levels towards adaptation under changing environmental conditions.

5. Specific roles of PGs among other phospholipids

Relatively high abundance of PGs in bacterial membranes underlines its importance in membrane properties. Since direct electrostatic interactions between charged species are rather strong, it is likely that PGs, as well as other charged lipids, can function both as membrane stabilizers and destabilizers, and thereby play important roles in controlling membrane/protein interactions (for review see (35). Although few systematic studies examined the properties of PE/PG mixtures in the proportion range of that found in microbes to model bacterial membranes, some biophysical approaches indicate that PGs decrease PE headgroup protrusions into the water phase, and motion of PE headgroup along bilayer

normal, due to stronger inter-lipid interactions in the mixed bilayer. Thus PG contribution to membrane lipids enhance interface stability, and likely prevents lipid desorption from the membrane. More compact and less dynamic interface structure can also decrease membrane permeability (36). Few specific roles have yet been assigned to PG phospholipids, and most established ones are summarized below. Elucidation of PG contribution is mainly derived from the study of *pgsA* bacterial mutants with decreased membrane PG abundance, and the controversial question of PG-dependent cell viability (reviewed in (20)) has provided interesting insights into specific PG- regulated process.

5.1 PGs in extracellular stress sensing and protein secretion across bacterial membranes

5.1.1 Lipoprotein maturation

As initially discovered in eukaryotic cells (37), membrane lipids do not homogenously distribute but can be laterally polarized into domains that produce specific environments for lipid/protein interactions. Cardiolipin, and presumably PGs highly participate in domain formation in *E. coli* and *Bacillus Subtilis* and thereby contribute to cell membrane compartimentation, especially to the attachment of peripheral proteins (38;39). In bacteria, lipoproteins generally refer to a group of membrane-associated proteins linked to the outer membrane layer of gram-negative microbes by lipid anchors. Bacterial lipoproteins attach to membrane through a modified acyl group linked to their N-terminal Cys residue. In *E. coli*, the most abundant lipoprotein, Lpp, is found in the inner leaflet of the outer membrane exposing hydrophilic protein to the periplasm. However, some lipoproteins have been reported to span the outer membrane or expose their protein moiety to the extracellular milieu. Lipoproteins are synthesized in the cytoplasm as precursors and then translocated across the inner membrane mainly through the Sec translocon (40). Overall, studies with *pgsA* mutants enphasized links to PG and cell lipoprotein synthesis. In *E coli* lacking PG synthase but with intact Lpp expression, prolipoprotein maturation and trafficking is hampered, which leads to accumulation within the inner membrane and ultimate cell lysis (41). Therefore, PG is considered crucially important in lipoprotein secretion. Mechanistically, it is believed to provide its non-acylated glyceryl moiety to link the SH group of the N-terminal cysteine residue in the unmodified prolipoprotein, a prerequisite for subsequent acylation of the sn-3 and sn-2 hydroxyls of the glyceryl cysteine to form the diacylglyceryl modified membrane anchored prolipoprotein and later cleavage of the signal peptide (42).

5.1.2 Lipoteichoic acid synthesis by gram positive bacteria.

A comparable role of PG in exchanging glycerol phosphate for the synthesis of lipoteichoic acid (LTA) has also been demonstrated (43). LTAs are composed of poly(sn-glycerol-1-P) attached to a glycolipid anchor and contribute to a protective layer surrounding the bacterium. In the prototypical Gram-positive *B. subtilis*, the length of the polyglycerol-phosphate chain varies from 14 to 33 repeating units. Metabolic labeling experiments identify PG as the source of the glycerol-1-phosphate groups, which means that the biosynthesis of a single LTA molecule requires the utilization of an average of 25 PG molecules.

5.1.3 Extracellular stress sensing in B. Subtilis

In *B. subtilis*, all the genes responsible for the synthesis of the lipid species found within the membrane bilayer are dispensable, except *pgsA*, the first gene committed in PG synthesis (see above). Studies with inducible *pgsA* deletions provided evidence that decreasing membrane PG contents regulated the activity of some Extra Cytoplasmic function (ECF) sigma factors. ECF sigma factors covers a bacterial signalling system specialized in extracellular stress detection, and belong to the large group of sigma factors that are essential components of bacterial RNA polymerase to determine promoter selectivity (reviewed in (44). The substitution of one sigma factor for another can redirect some or all of the RNA polymerase to activate the transcription of genes that would otherwise be silent. Seven ECF sigma factors are found in *B. subtillis*, they are generally cotranscribed with negative regulators including corresponding transmembrane proteins functioning as anti-sigma factors that bind and inhibit the cognate sigma factor. The sigma factor can be released from its inhibitor upon receiving a stimulus from the environment, thereby coupling cytoplasmic transcriptional response to extracellular signals. In *E coli*, acute drop in membrane PG content (by removing a synthetic inducer of a *pgsA* artificial construct) rapidly induced transcriptional activity of sigma M, which is normally known to mediate responses to high salt exposure (45). This might suggest that detection of changes in membrane PG linked to membrane permeability can be an important component in stress response.

5.2 PG in photosynthesis and electron chain transport

A detailed review on this topic is published in (46), only salient observations are briefly summarized below. From determination of crystal structures, not only cardiolipin but also PG are integral components of complexes III and IV of the mitochondria of yeast and mammalian cells (47). In plants, PG can be synthesized in the chloroplast, mitochondria and ER, and is the only major phospholipid present in thylakoid membranes, the site of the light-dependent reactions of photosynthesis. In rice thylakoid membrane, the sn-2 position of PGs were found enriched with a specific delta 3-trans-hexadecenoyl, i.e. a 16:1(3t)PG or palmitoyl PG16:0. Surprisingly, such a 16:1(3t) fatty acid was not abundant in any other thylakoid membrane lipid, suggesting a specific role of PG 16:1(3t)(48). Indeed, 16:1(3t) PG was shown to facilitate dimerization of photosystem II (PSII), the first protein complex in the light-dependent reactions of photosynthesis. Particularly one protein in this complex called intrinsic D1 core peptide has high binding affinity for PG which promotes dimerization of the complex. Thin layer chromatography analysis showed that photosystem II dimers contained four times more PG than their monomeric counterparts but with similar levels of phosphatidylcholine (49). PG depletion from thylakoid membrane in PG mutants causes dissociation of extrinsic proteins and PSII dysfunction (50).

5.3 PG in lung surfactant

The only biological product with significant PG enrichment in higher eukaryotes is the lung surfactant, with more than 10% of phospholipids as PGs. Lung surfactant occupies the air/water interface of small alveolae where it reduces surface tension and prevents collapse during expiration. Surfactant is produced by type II alveolar cells, stored intracellularly as lamellar bodies, and released extracellularly into alveolar space. Phosphatidyl choline (mainly dipalmitoylphosphatidylcholine, DPPC) is the most abundant phospholipid in surfactant

(80%), followed by PG (15%) and also other lipid components such as cholesterol. Phosphatidylcholine coats the air-water interface of alveoli, with hydrophilic head groups in the water and the hydrophobic tails facing towards the air (51). Surface tension air-water interface tends to make the air bubble smaller by decreasing the surface area of the interface. Surface tension forces also draw fluid from capillaries to the alveolar spaces. By reducing surface tension, surfactant limits fluid accumulation and keeps the airways dry. DPPC has the strongest activity in the pulmonary surfactant mixture, because of high compaction capacity and low bending of the saturated fatty acid chains. However, over pure DPPC, the advantage of a PG/DPPC mixture seems to be linked to lipid monolayer fluidization due to preferentially mono unsaturated fatty acid chains in PG (52). Why PG but not other phospholipids was evolutionary selected still remains elusive. It is estimated that alveolar surfactant has a half life of 5 to 10 hours once secreted. It can be broken down by macrophages and / or reabsorbed into the lamellar structures of type II pneumocytes and recycled back from the alveolar space. This process is believed to occur through specific surfactant proteins especially Surfactant Protein-A (SP-A) which can stimulate receptor mediated, clathrin dependent endocytosis (53).

5.3.1 PG as a marker of foetal pulmonary development

Even if the precise role of PG in lung surfactant is still incompletely understood, its presence is a well recognized marker of surfactant production and pulmonary development in the foetus. Therefore, surfactant PG in amniotic fluid has been used for preterm prediction of lung maturation in infants (54), and risks monitoring of Respiratory Distress Syndrome, a life threatening condition in the newborn.

5.3.2 Surfactant PG in attenuation of pathogen-related inflammatory response

Numerous macrophages reside in the lung, as a first line of defense against inhaled particles or pathogens. Alveolar macrophages are highly phagocytic, and despite continuous stimulation by inhaled particles and pathogens, they display an anti-inflammatory phenotype that includes specific cytokine responses and reduced oxidant production in response to stimuli, a phenomenon referred to as “alternate activation”. Resident alveolar macrophages are bathed in surfactant and have been shown to ingest abundant amounts of it. Therefore, the role of surfactant components in the induction and/or maintenance of an anti-inflammatory phenotype has been of great interest. Some evidence indicate that components of pulmonary surfactant, especially collectins family proteins (to which surfactant proteins belong), might play important roles as modulators of cellular immune activities (55;56). Also, the possibility that phospholipid itself might participate has been less studied. However, some evidence indicate that surfactant is dysfunctional in asthma with diminished PG content, relative loss of PC as well as increasd lysophospholipids (57). It has been suggested that in antigen-challenged patients, secreted phospholipase A2 could participate in surfactant dysfunction, since sPLA2 exhibit preference for PGs over PCs. Regarding a potential role of surfactant PGs in anti-inflammatory response, it was observed that Palmitoyl-Oleoyl-PhosphatidylGlycerol (POPG) alone, added to bronchial epithelial cells in culture inhibited interleukin-6 and -8 production, as well as the cytopathic effects induced by Rous Sarcoma Virus infection. Administration of POPG to mice, concomitant with viral infection, almost completely eliminated the recovery of virus from the lungs at 3 and 5 days after infection

(58). PG was shown also to inhibit inflammatory responses induced by lipopolysaccharide through direct interactions with the Toll-like receptor 4 (TLR4) interacting proteins CD14 and MD-2 (59). These findings highlight potential interest of exogenous administration of surfactant PG phospholipids in pulmonary infections, and identify a specific anti-inflammatory actions of these lipids. In line with this view that PG might be potent signaling modulators, changes in the affinities of Gαi proteins to G-Protein Coupled Receptors (GPCRs) have been recently reported from studies of nanodiscs with various phospholipid compositions (60).

6. Concluding remarks

From knowledge acquired in bacteria and eukaryotes regarding multiple functions of PG phospholipids, salient features in modulation of membrane properties have emerged. Of particular interest, it now appears that PG phospholipids could be membrane modulators of signal transduction, particularly in inflammation and GPCR-associated process. Whether those signal modulation properties could exist outside of the assay tube or specific context of a PG enriched environment such as the lung is an important question. PG is present in the plasma, even if found only at micromolar concentrations, therefore considered a minor component. However, advances in mass spectrometry-based lipidomics technologies now make it possible to detect and quantify changes in circulating PG concentrations in health or disease. Many unanswered questions are raised regarding circulating PGs, related to lipids/proteins associations in serum, origin, biological roles, potential target tissues as well as value as new biological markers in disease conditions.

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Figure legends

Figure 1: Specific roles of PGs across organisms.
The developped formula of PG in the center does not indicate the 2-position of glycerol which is a chiral center. Note that this position is sn-glycerol-1-P in contrast to the backbone, which is sn-glycerol-3-P.

Figure 2: Fatty acid diversity in PGs.
Each circle indicates relative concentrations of PGs (Log scale) quantified by LC/MS in different samples from mammals or microbes as indicated. First number in parenthesis indicates total number of carbons in fatty acid chains, the second is the total number of insaturations in lipid chains. Note that in bacteria odd chains fatty acids corresponding to branched chains are found.

Figure 3: Schematic overview of PG synthesis pathway.
Abbreviations are as follows: CDP-DAG, cytidine diphosphate diacylglycerol. PGP, Phosphatidylglycerol-phosphate. CL, Cardiolipin. PS, Phosphatidylserine. PE Phosphatidylethanolamine. Enzymatic activities (not gene names) are indicated in each step.

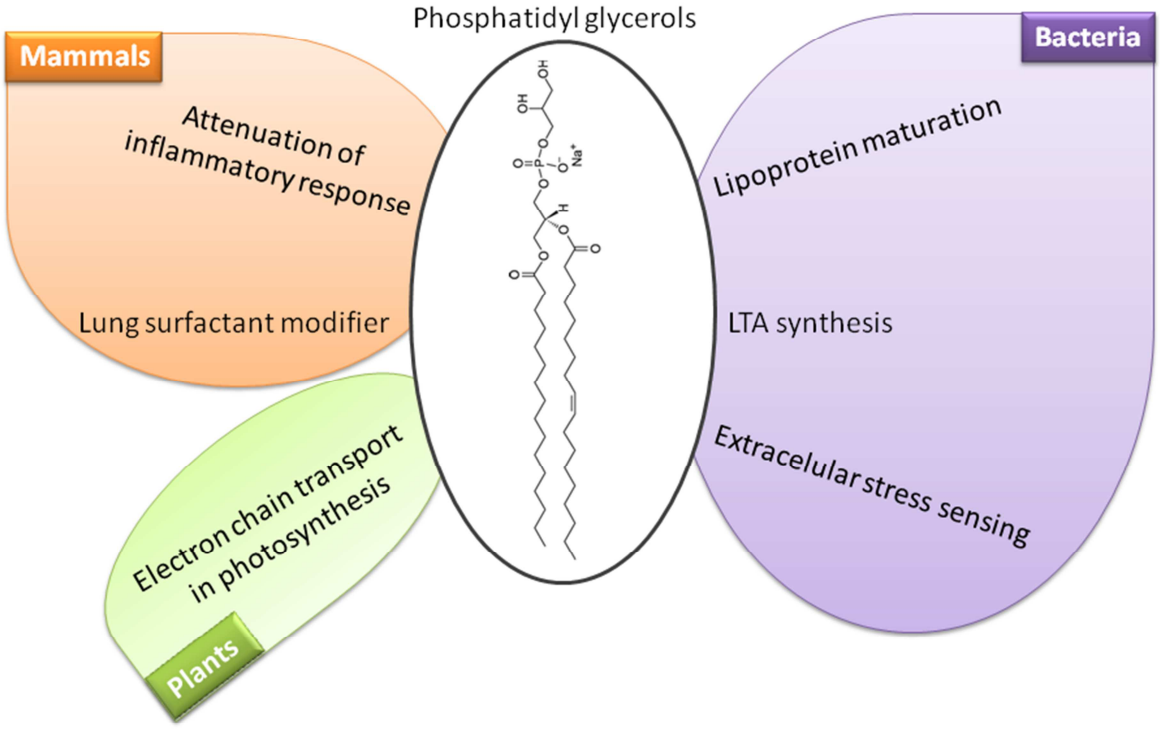
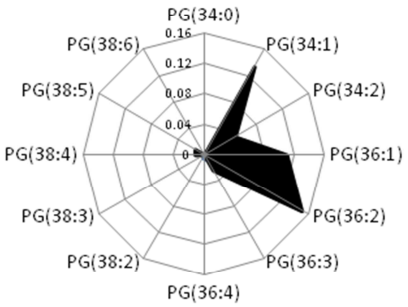


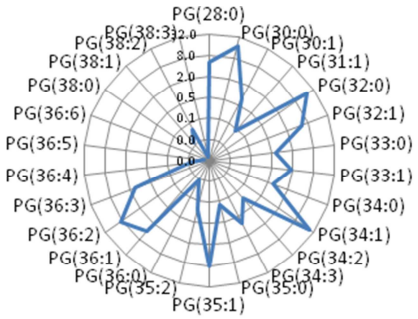
Figure 1

C57Bl6 mice serum
(regular diet)

Human serum
(Obese patients)



Lactobacillus Bifidus



Lactobacillus Casei

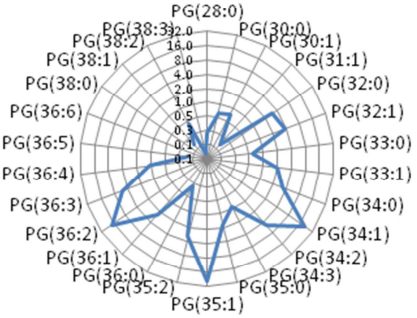


Figure 2

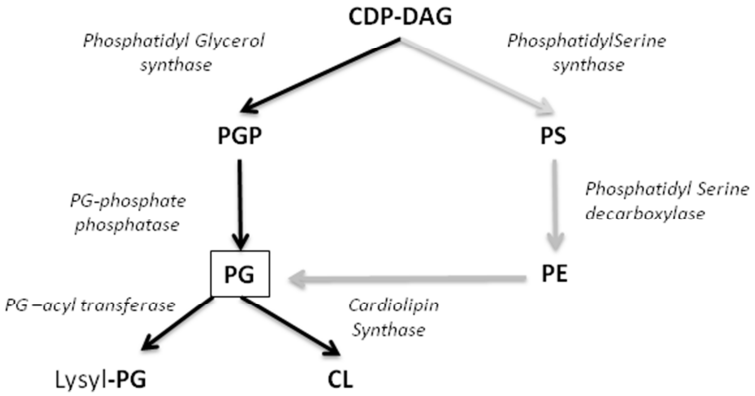


Figure 3

Highlights:

-Common schemes in PG synthesis exist in eukariotes and microbes

-High PG in bacterial membranes is reflected by a mitochondria-specific distribution.

-PG functions cover lipid/protein interactions, electron transport and regulation of bilayer tension

-New roles may emerge due to development of lipidomics by mass spectrometry.