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Phospholipid and sphingolipid metabolism in *Leishmania*

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Abstract

In many eukaryotes, phospholipids (PLs) and sphingolipids (SLs) are abundant membrane components and reservoirs for important signaling molecules. In *Leishmania*, the composition, metabolism, and function of PLs and SLs differ significantly from those in mammalian cells. Although only a handful of enzymes have been experimentally characterized, available data suggest many steps of PL/SL metabolism are critical for *Leishmania* viability and/or virulence, and could be a source for new drug targets. Further studies of genes involved in the synthesis (*de novo* and salvage) and degradation of PLs and SLs will reveal their diverse effects on *Leishmania* pathogenesis.

Keywords

Leishmania; phospholipid; sphingolipid; plasmalogen; *Trypanosoma*; virulence

1. Introduction

Phospholipids (PLs) are major components of biological membranes with importance extending above and beyond basic structural roles. First, degradation of glycerophospholipids generates a plethora of signaling molecules including inositol 1,4,5-triphosphate, 1,2-diacylglycerol [1,2], arachidonic acid, lyso-phospholipids, and phosphatidic acid [3,4]. In addition, PLs such as phosphatidylinositol (PI) can be modified by a group of PI kinases (PI3K, PI4K, and PIPKs) into bioactive phosphoinositides (PIP, PIP2, and PIP3); PI kinases are activated by small G-proteins and regulate a number of cellular processes including membrane trafficking and cytoskeleton remodeling [5–7]. Furthermore, the exposure of phosphatidylserine (PS) on the outer surface of cells is believed to trigger the removal of apoptotic cells by phagocytes [8–10]. Parasitic protozoans including *Leishmania* have been reported to mimic such PS exposure to avoid destruction and gain entry to host phagocytes [11–13].

Sphingolipids (SLs) constitute another major group of membrane components in eukaryotes. In mammals, virtually every metabolite in SL biosynthesis or degradation has potent activities. These compounds (ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate) mediate many signaling pathways including apoptosis, cell-to-cell recognition, growth, and differentiation [14,15]. In particular, ceramide (generated from *de novo* synthesis or degradation of high order SLs) is a potent secondary messenger capable of activating protein phosphatases PP1 and PP2A [16], protein kinase C ζ [17], and lysosomal protease Cathepsin D [18].

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Trypanosomatid protozoans including *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* species are important human pathogens. While synthesis of the lipid anchor for parasite surface molecules such as lipophosphoglycan (LPG) and glycosylphosphatidylinositol (GPI)-anchored proteins has received considerable attention [19], metabolism of PLs and SLs in *Leishmania* has been relatively understudied. Recent developments in genome sequencing (complete for *L. major*, *L. braziliensis*, and *L. infantum*, ongoing for several other species) and lipidomic tools have forged a powerful platform for the study of lipid metabolism in *Leishmania*. Gaining new knowledge on PL and SL metabolism will not only provide fundamental insight into the molecular bases of *Leishmania* pathogenesis, but also may reveal new targets for selective drugs.

2. Composition of PLs and SLs in *Leishmania*

The analysis of lipid composition and structures requires a combination of chromatography and mass spectrometry/NMR. The use of electrospray ionization mass spectrometry coupled with tandem mass spectrometry (ESI/MS/MS) and collision-induced dissociation, has allowed rapid identification and comprehensive description of individual PLs and SLs [20,21]. While promising, some challenges remain as some lipid species yield useful diagnostic daughter ions less efficiently than others. For example, in some ESI/MS/MS analyses, the cellular level of PS is below the limit of detection in *L. major* promastigotes [22,23]. In these cases, data from alternative analytic methods are needed to give an accurate picture of the overall lipid distribution and abundance. The discussion below reflects a consensus view of PL/SL distribution in *Leishmania*.

Overall, PLs account for ~70% of total cellular lipids in *Leishmania* [24,25] (Fig. 1A). Major PL species classified according to the 'headgroup' include phosphatidylcholine (PC, 30–40%), phosphatidylethanolamine (PE, ~10%), and PI (~10%) (Fig. 1A, left) [22,25,26]. PLs may also be classified based on their lipid anchors, consisting of 1,2-diacyl, 1-alkyl-2-acyl, 1-alkenyl-2-acyl (plasmalogens), or in some cases lyso-alkyl or lyso-acyl groups (Fig. 1A, right). Interestingly, the majority (80–90%) of PE in *L. major* belongs to plasmalogen (PLE) or ethanolamine plasmalogen (1-*O*-1'-alkenyl-2-acyl-phosphatidylethanolamine) [22, 27] (Fig. 1B). PLE is also more abundant than diacyl-PE in the procyclic and blood stream forms of *T. brucei* [28–30]. In contrast, plasmalogen lipids (PLE and choline plasmalogen) only constitute ~20% of total PLs in mammals, although their levels can reach over 50% in heart and nervous tissues [31].

Notably, side chains of *Leishmania* PE can be modified to form cis-9,10-methyleneoctadecanoic acid (4–11%), a cyclopropane fatty acid commonly found in bacteria and other Trypanosomatids [24,32]. These cyclopropane-containing PEs confer acid resistance in *E. coli* [33], although their role is not known in *Leishmania*. In addition to PLE, 1,2-diacyl PE and 1-*O*-1'-alkenyl-2-lyso PE are also present in *Leishmania* at much lower levels [22] (Fig. 1C–D).

The most abundant glycerophospholipid in *Leishmania* is PC, consisting of 1,2-diacyl and 1-lyso-2-acyl species with unusually long and unsaturated fatty acid species [22]. The functions of these heterogeneous PC species are not well understood, although their polyunsaturated fatty acid chains could modulate membrane physiology by reducing the melting point and confer resistance to host-derived oxidants. PI is another major group of glycerophospholipids which contains a combination of 1,2-diacyl, 1-alkyl-2-acyl and 1-lyso-2-acyl species [26] (Fig. 1A). In *Leishmania*, diacyl PI mostly exists as the un-conjugated or free form, whereas alkyl-acyl- and lyso-PI are widely found in the anchors of surface glycoconjugates including glycosylinositolphospholipids (GIPLs), GPI-containing membrane proteins, and LPG [34,35]. *Leishmania* parasites also synthesize low levels of phosphatidylglycerol (PG), cardiolipin

(CL), and phosphatidic acid. Functions of these minor classes of phospholipids are not well understood, although PG and CL are known to be important components of mitochondrial membrane in many eukaryotes.

In mammalian cells, PS is asymmetrically distributed across the membrane bilayer and the exposure of PS on the outside surface of cells is an early sign of apoptosis [10]. PS has been detected in *Leishmania* by chromatography-based methods [25,36], or flow cytometry based on reactivity with either annexin V (which binds to PS and other anionic PLs in the presence of Ca^{2+}) or a PS-specific mAb [12,37]. No obvious orthologues for the bacterial CDP-diacylglycerol: serine O-phosphatidyltransferase (PS synthase 1) are evident in the TriTryp genomes (*L. major*, *T. brucei*, and *T. cruzi*). Nonetheless, these parasites contain a putative base exchange enzyme (also called PS synthase 2) and a putative PS decarboxylase, suggesting they possess an active PS metabolism [23](Fig. 2). At present, no data address the question of whether PS is synthesized by *Leishmania* or acquired from the host. In *T. brucei*, recent reports indicate PS can be synthesized by head group exchange with PE [28] and is more abundant in the bloodstream form than in procyclics [30].

PS exposure in *Leishmania* has attracted great interest recently due to its ability to serve as a 'deactivating' signal for host cells, following the paradigm of PS exposure on apoptotic mammalian cells limiting macrophage activation [38,39]. This may occur indirectly through exposure of PS on *Leishmania* infected host cells, which are then engulfed by phagocytic cells. This 'Trojan Horse' model suggests that *Leishmania* co-opts host cells to gain safe entry into macrophages without activation. Other studies raise the possibility that PS exposure on the parasite surface directly interacts with host cells, leading to the release of anti-inflammatory cytokines such as TGF- β and the down-regulation of pro-inflammatory cytokine TNF- α in host phagocytes [12]. Similar apoptotic mimicry has been observed during the internalization of *Leishmania amazonensis* amastigotes [40]. Nonetheless, further studies are needed to determine whether PS exposure on the parasite is actively controlled. Two recent studies suggest that dying parasites with PS exposure may contribute to increased lesion pathology in *Leishmania* infections [12,13]. However since dead antigen co-inoculated with parasites also can result in increased lesion pathology [41,42], further work is needed to establish the role of parasite PS exposure in pathogenicity.

Unlike mammals or plants, *Leishmania* parasites do not synthesize sphingomyelin or complex glyco-SLs. Instead, the majority of SLs in *Leishmania* belong to unglycosylated inositol phosphorylceramide (IPC) and ceramide (Fig. 1E–F)[27, 43, 44]. IPC is absent in mammals but common in fungi, where it exists as free or glycosylated forms [45, 46]. In *L. major*, IPC species include phosphoryl inositol *N*-stearoylhexadecosphing-4-ene (d16:1/18:0-IPC) and phosphoryl inositol *N*-stearoylsphingosine (d18:1/18:0-IPC), with the former being the dominant species (Fig. 1F). Since the long chain base (sphingoid base) of SLs is synthesized through the condensation of serine and fatty acyl-CoA, it is concluded that *Leishmania* enzyme preferentially incorporates myristoyl-CoA (C14) over palmitoyl-CoA (C16) into their long chain base and ceramide (Fig. 1E) [47]. This differs from mammalian cells which mostly use palmitoyl-CoA to synthesize their SLs [48]; or yeasts (*S. cerevisiae*) in which palmitoyl Co-A is the preferred substrate [49] and *in vivo* sphingoid bases arise primarily from C16 and C18 acyl-CoA [50].

In addition to IPC, neutral glycosphingolipids have been isolated from the amastigote forms of *Leishmania amazonensis* in hamster foot lesions [51]. Structure of these surface neutral glycosphingolipids was partially elucidated by negative ion fast atom bombardment mass spectrometry. The glycan moieties presented linear sequences of hexoses and N-acetylhexosamines ranging from four to six sugar residues, and the ceramide moieties consisted of a d18:1 sphingosine and fatty acids of C24:1 or C16:0 [52]. These glycosphingolipids may

play a role in parasite invasion, as monoclonal antibodies specific for their carbohydrate epitopes blocked macrophage invasion by amastigotes [51]. Origin of these neutral glycosphingolipids is not known, as they may be synthesized by the parasites or salvaged from the mammalian host.

Overall, the composition of PLs and SLs in *Leishmania* is quite different from that in mammals, which may reflect the difference in membrane physiology between parasites and hosts. As major membrane components, these lipids may determine the membrane permeability and fluidity and have profound impact in vesicular trafficking, nutrient acquisition through endocytosis, or cell differentiation which involves extensive membrane remodeling/reorganization and macroautophagy. In addition, the polyunsaturated fatty acid chains found in PC could confer resistance to host-derived oxidants. The difference between *Leishmania* and mammals in lipid composition could be exploited towards selective chemotherapy. For example, lysophospholipid analogs including miltefosine, edelfosine, and ilmofosine have been used successfully as oral drugs to treat cutaneous and visceral leishmaniasis [53,54]. These compounds possess potent leishmanicidal activity, although the molecular mechanism of such activity is still controversial. Amongst their various effects in *Leishmania* parasites, inhibition of membrane lipid synthesis and induction of apoptosis or necrosis have been observed [55–57]. Therefore, understanding the roles of lipid metabolism in *Leishmania* parasites could have important implications in guiding the development of novel PL analogues that possess anti-protozoan activity.

3. Metabolism

3.1 Acquisition and/or synthesis of PL and SL headgroups (EtN, choline, and inositol)

EtN, choline, and inositol are essential nutrients which form the headgroups of most PLs and GPIs. As discussed in 3.3, *Leishmania* can convert serine into EtN via SL metabolism, which is the major pathway for EtN production in promastigotes [23]. It is not clear whether *Leishmania* parasites can synthesize choline *de novo*, although EtN can be incorporated to PC (Fig. 2). In *L. mexicana* and *T. brucei*, inositol is synthesized from glucose 6-phosphate using two sequentially acting enzymes: inositol-3-phosphate synthase which converts glucose 6-phosphate to inositol 3-phosphate, and then inositol monophosphatase which dephosphorylates inositol 3-phosphate to generate inositol [58,59]. In addition to biosynthesis, these head group are also incorporated in *Leishmania* parasites via membrane transporters. The uptake of inositol is mediated by a high-affinity myo-inositol/H⁺ transporter driven by proton gradient [60]. The uptake of EtN and choline has been biochemically characterized in *Leishmania* [61,62], although their respective transporters have yet to be identified.

3.2 Enzymes involved in the synthesis of PE

In eukaryotes, PE may be synthesized via three routes: 1) through the Kennedy pathway: EtN $\Rightarrow$ ethanalamine phosphate (EtN-P) $\Rightarrow$ CDP-EtN $\Rightarrow$ PE (Fig. 2) [63]; 2) through the decarboxylation of PS (by PS decarboxylase) which occurs in mitochondria or Golgi in many eukaryotes [64] (localization is unknown in Trypanosomatids); and 3) via reversible head group exchange (base exchange) between PE, PC, and PS [65]. In *T. brucei*, recent evidence suggests that Kennedy pathway is the dominant route for PE synthesis and essential for survival and mitochondrial function [28,30,66]. In *L. major*, SL and PE metabolism are linked through EtN-P, which can be generated from the degradation of phosphorylated sphingoid bases (Fig. 2) [23]. Most candidate genes for PE synthesis (Fig. 2) have been identified from *Leishmania* genomes, and a handful of them have been experimentally characterized in *Leishmania* or *T. brucei*, as described below.

3.2.1 Glycerol-3-phosphate acyltransferase (GAT) and glycerol-3-phosphate dehydrogenase (G3PD)—Synthesis of diacyl- and ether PLs starts with glycerol-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Fig. 2), which can be generated from the metabolism of sugars. GAT catalyzes the acylation of G3P at the *sn*-1 position to produce 1-acyl-G3P (Fig. 2). 1-acyl-G3P can also be generated from 1-acyl-DHAP by a NADPH-dependent 1-alkyl/1-acyl-DHAP reductase. In mammals and yeast, 1-acyl-G3P is used for the biosynthesis of phosphatidic acid, diacylglycerol, triacylglycerol, and ester PLs (diacyl-PC, PE, PI, and PG) (Fig. 2) [67,68]. *L. major* GAT exhibits a low-affinity for G3P and prefers unsaturated fatty acyl-CoA (C16:1, C18:1, C18:2) as substrates [69]. DHAP, however, is not a preferred substrate for this enzyme. Deletion of *LmGAT* resulted in a major reduction (~50%) in triacylglycerol synthesis but had little effect on diacyl- (PC and PI) or ether PLs [69]. In addition, null mutants of *LmGAT* contained normal levels of LPG and GPI-anchored proteins (which contain ether lipids in their GPI-anchors) and their virulence was not affected in mice. These results suggest that DHAP, not G-3-P, is the primary precursor for PL biosynthesis in *Leishmania*. Alternatively, *L. major* may contain an as yet unidentified GAT that is responsible for the initial step of ester phospholipid synthesis.

L. major contains a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (G3PD) [70] (Fig. 2); in addition, two more putative G3PD genes have been identified from the *L. major* genome. One or more of these enzymes may be involved in the G3P/DHAP shuttle between glycosome and mitochondrion [70].

3.2.2 Dihydroxyacetone phosphate acyltransferase (DAT) and 1-alkyl-DHAP synthase (ADS1)—In many organisms, the *de novo* synthesis of ether PLs starts with the acylation of DHAP by the enzyme dihydroxyacetone phosphate acyltransferase (DAT) [71] (Fig. 2). The resulting product, 1-acyl-DHAP, is converted to 1-alkyl-DHAP by a FAD-dependent alkyl DHAP synthase (ADS1) [22,72], and further reduced to 1-alkyl-glycerol-3-phosphate (1-alkyl-G3P) by an NADPH-dependent 1-alkyl/acyl-DHAP reductase (Fig. 2). 1-Alkyl-G3P serves as the obligate precursor for all ether PLs, including PLE, alkyl-acyl-PI, and alkyl-lyso-PI species which are incorporated into the GPI-anchors in *Leishmania* (Fig. 2) [19]. *L. major* DAT is unusually large (1436 amino acids) with an N-terminal domain of ~650 amino acids which does not have similarity to any known proteins. This glycosome-localized DAT has a high specificity for DHAP (but not G-3-P) and palmitoyl-CoA. Deletion of the *DAT* gene (*LmjF34.1090*) in *L. major* results in a complete loss of DAT activity [73], suggesting it is the sole functional enzyme. Significantly, *DAT* mutants could not synthesize ether PLs, yet the production of diacyl PLs was not affected [74]. Genetic analysis suggests *LmDAT* is synthetic lethal with *LmGAT* [74]. Together, these data imply both DAT and GAT are involved in the initiation of diacyl PL synthesis and one enzyme can compensate the loss of the other. Finally, null mutants of *DAT* grew slowly as promastigotes with increased cell death during stationary phase and failed to induce pathology in BALB/c mice [73], suggesting ether PLs are important for promastigote virulence.

ADS1 catalyzes the reduction of 1-acyl-DHAP to 1-alkyl-DHAP, the first committed step of ether lipid synthesis. Similar to DAT, *L. major* ADS1 lacks obvious transmembrane domains but contains a tripeptide SKI at the C terminus, which could serve as a targeting signal for the glycosome. Biochemical analysis revealed that *L. major* ADS1 utilizes 1-palmitoyl-DHAP (the main product of DAT) as a preferred substrate. Lipid analysis of *ADS1* mutants demonstrates that this enzyme is required for the synthesis of ether PLs and their derivatives including PLE, LPG, and GIPLs [22] but not diacyl-PC or lyso-PCs. Compared to *DAT*, defects of *ADS1* null mutants were less severe as these cells grew reasonably well as promastigotes, suggesting ether lipids are not essential for survival or *in vitro* growth [22]. Notably, *ADS1* mutants (*adsI*[−]) were sensitive to complement and unable to survive the initial phase of macrophage infection; however, amastigotes of *adsI*[−] were infective in both macrophage and mouse infections [22].

These virulence defects were similar to a LPG-deficient mutant *lpgI*⁻ [75], suggesting PLE or GPIs are not required for *Leishmania* (amastigotes) growth in macrophages or in mouse infections.

3.2.3 Other enzymes involved in PE metabolism—Both *L. major* and *T. brucei* genomes contain two EtN/choline kinases (E/CKs), which catalyze the initial steps of Kennedy pathway to produce EtN/choline phosphate (Fig. 2). TbEK1 (*T. brucei* EtN kinase 1) was shown to be an EtN-specific kinase, whereas TbEK2 was able to phosphorylate both EtN and choline, with choline being the preferred substrate [76]. In addition, recent evidence suggests that in *T. brucei* procyclic forms, the last step in the Kennedy pathway, *i.e.* the conversion from CDP-EtN to PE, is mediated by two separate activities, one leading to PLE and the other leading to diacyl-PE [28]. Furthermore, RNAi or conditional knockout of ethanolamine-phosphate cytidylyltransferase (EPCT) led to disrupted mitochondrial morphology and function, and eventually cell death in *T. brucei* [28,66][30]. Future studies will determine whether the separation of PLE and PE synthesis is due to compartmentalization or substrate specificity. Orthologs of both TbEPT and TbC/EPT are present in *Leishmania* (Fig. 2) and functional characterizations of these genes will unravel their specificities.

Leishmania genomes also contain two genes predicted to encode enzymes that may be involved in the synthesis of PS and/or PE: a based exchange enzyme which swaps headgroups between PS and PE, and a PS decarboxylase which converts PS to PE. Given the importance of PS in parasite-host interaction [38,39], functional studies are needed to reveal the contribution of these enzymes in PL metabolism and *Leishmania* infection.

3.3 Enzymes involved in SL metabolism and EtN production: serine palmitoyltransferase (SPT) and sphingosine-1-phosphate lyase (SPL)

De novo SL biosynthesis starts with the condensation of L-serine and palmitoyl-CoA into 3-ketosphinganine. This is catalyzed by a pyridoxal 5'-phosphate-dependent enzyme serine palmitoyltransferase (SPT, EC 2.3.1.50) consisting of two subunits, SPT1 and SPT2 [77,78] (Fig. 2). Two ORFs with homology to SPT1 and SPT2 have been identified from the *L. major* genome with SPT2 being the putative catalytic subunit. It is noteworthy that the main IPC species in *L. major* contains C16 as the long chain base, suggesting that *L. major* SPT prefers myristoyl-CoA over palmitoyl-CoA as a substrate [27,47]. Deletion of *SPT2* in *L. major* results in a complete loss of IPC and other SLs. Although viable as promastigotes, these *spt2*⁻ mutants failed to differentiate to infective metacyclic parasites and died rapidly. Importantly, *spt2*⁻ parasites maintained 'lipid rafts' as defined by Triton X-100 detergent resistant membrane formation (see more in the next section).

To further examine the potential role of SLs in differentiation and virulence, another mutant which is defective in the degradation of SLs (*spl*⁻) was generated by deleting the gene encoding for sphingosine-1-phosphate lyase (SPL) [23]. SPL is responsible for the breakdown of phosphorylated sphingoid bases, such as sphingosine-1-phosphate (S-1-P) or dihydrosphingosine-1-phosphate (DHS-1-P), to produce EtN-P and hexadecenal/hexadecanal (Fig. 2). Surprisingly, despite their distinct cellular SL levels (*spt2*⁻ promastigotes are SL-null whereas *spl*⁻ mutants show slight increased IPC levels), both mutants contain less PLE compared WT parasites in stationary phase and fail to differentiate to infective metacyclics [23]. More importantly, defects in PLE synthesis and metacyclogenesis can be fully restored in both mutants through the supplementation of ethanolamine (EtN) or EtN-P, the degradative product of SPL (Fig. 2). These data indicate that unlike most other eukaryotes, the primary role of SL metabolism in *Leishmania major* promastigotes is to effectively convert serine into EtN, which is the main source of EtN for promastigotes (Fig. 2).

3.4 Enzymes involved in the metabolism of IPC: IPC synthase and inositol sphingolipidphospholipase C-like protein (ISCL)

IPC, the end product of SL biosynthesis in *Leishmania*, is generated by the transfer of a phosphorylinositol headgroup from PI to ceramide, a process catalyzed by the IPC synthase. In fungi, this enzyme has been established as a target for the development of anti-fungal compounds including aureobasidin A1 [79]. Based on sequence analysis, *L. major* IPC synthase contains six predicted transmembrane regions and two putative luminal motifs (each contains histidine and aspartate residues which mediates nucleophilic attack on lipid phosphate ester bonds) which constitute the catalytic domain [29]. This predicted topology is shared by the sphingolipid synthases in *T. brucei* (a family of four) and is more conserved with respect to mammalian sphingomyelin synthases than to fungal IPC synthases [29,80,81]. Expression of *L. major* IPC synthase in a mammalian cell line (HEK293) or in blood stream trypanosomes leads to the synthesis of an IPC-like species [80][29]. In addition, *L. major* IPC synthase partially complements the pleomorphic defects in cell morphology and division observed in *AURI* mutants of *S. cerevisiae* [80,82]. Further biochemical and genetic analyses are needed to determine whether IPC synthesis is essential for *Leishmania*, especially in the amastigote stage.

The abundance of non-mammalian IPC in *Leishmania* has suggested to many that IPC synthesis may be a good target for chemotherapeutic intervention [80,83], analogous to proposals arising from the toxicity and specificity of the yeast IPC synthase inhibitor aureobasidin A [79,84]. Although several studies point to significant toxicity of aureobasidin A against various *Leishmania* species, that toxicity can occur at concentrations not affecting IPC synthesis [26, 80,85]. For instance, while aureobasidin A inhibits *L. major* promastigote growth with an EC₅₀ of 0.6 μ M, mass spectrometry analysis revealed no effect on IPC synthesis at this concentration, and IPC synthesis inhibition was not seen until >5.0 μ M aureobasidin A [26]. Consistent with these findings, studies of heterologous expressed IPC synthase suggests an enzymatic IC₅₀ on the order of 100 μ M [80]. Similarly, the *T. cruzi* IPC synthase is insensitive to aureobasidin A [86,87]. Lastly, aureobasidin A was similarly toxic to the *spt2*⁻ parasites, which lack IPC [26,44]. These findings suggest that the major inhibitory activity of aureobasidin A against *L. major* *in vivo* may involve a target other than IPC synthase, while not yet excluding this enzyme as a potential amastigote target for more effective inhibitors in the future.

Despite the lack of sphingomyelin synthesis, *L. major* promastigotes possess a potent sphingomyelinase (SMase) activity which is dependent on the ISCL (Inositol phosphoSphingolipid phospholipase C-Like) protein. ISCL is also required for the degradation of IPC, although its activity with sphingomyelin is 10–20 fold greater than that seen with IPC [88]. Such dual activity is also observed from the yeast homolog, ScISC1p, which is both a neutral SMase and an IPC hydrolase [89]. Null mutants of *ISCL* (*isc1*⁻) showed modest accumulation of IPC, but grew and differentiated normally *in vitro*. Interestingly, *isc1*⁻ mutants did not induce lesion pathology in the susceptible BALB/c mice, yet persisted indefinitely at low levels at the site of infection [88]. Notably, the acute virulence of *isc1*⁻ was completely restored by the expression of *ISCL* or heterologous mammalian or fungal SMases, but not by fungal proteins exhibiting only IPCase activity [88]. Since *Leishmania* do not synthesize sphingomyelin, together, these findings strongly suggest the degradation of host-derived sphingomyelin plays a pivotal role in the proliferation of *Leishmania* in mammalian hosts and the manifestation of acute disease pathology.

3.5 Salvage of PLs and SLs

In addition to *de novo* synthesis, *Leishmania* parasites also acquire PLs and SLs through salvage. Promastigotes of *spt2*⁻ and *spl*⁻ (neither can convert SL metabolites to EtN) grow at

near wild type (WT) rates during log phase and contain normal amount of PLE and other *Leishmania*-specific ester PLs [23,27], indicating these parasites can salvage lipid metabolites from the culture medium which contains fetal bovine serum. Amastigotes of *spt2*[−] and *spt*[−] are morphologically normal and fully virulent in both macrophage and mouse infections [26]. Importantly, despite the lack of *de novo* synthesis, amastigotes of *spt2*[−] contain significant amounts of IPC (~10⁸ molecules per cell) [26], a SL not synthesized by mammalian cells; both mutants also make WT-level of PLE (1~2 × 10⁸ molecules per cell) in amastigote stages [26]. Together, these data suggest amastigotes actively acquire and remodel host lipids and convert them into parasite-specific SLs/PLs. So far, amastigotes from all species of *Leishmania* contain host-derived SLs and PLs [90,91], which could be degraded to generate intermediates for lipid synthesis, or serve as precursors for headgroup remodeling. Uptake of PLs may involve membrane-associated ATP-binding cassette (ABC) transporters. Genomes of *L. major* and *L. infantum* contain a family (42 in total) of these transporters. Several of these ABC transporters have been characterized and they possess activity to inwardly translocate PC, PE, PS, alkyl-glycerophosphocholine, and miltefosine [92–95]. Other members of the family, such as the ABCG-like transporters, confer resistance to alkyl-phospholipids when overexpressed [96, 97]. Therefore, these ABC transporters are not only involved in transbilayer lipid movement, but also play a role in the uptake and efflux of drugs.

4. Functions

4.1 Anchors for surface glycoconjugates

In *Leishmania*, surface virulence factors including LPG, GIPLs, and GPI-proteins all contain ether PLs in their anchors. Specifically, the lipid moieties of GIPLs and GPI-anchored proteins are *sn*-1-alkyl-2-acyl-PIs [19,98], whereas that of LPG is *sn*-1-alkyl-2-lyso-PI and the alkyl group is C24–26 [99,100]. The long alkyl chain in LPG has been implicated, biochemically, in the down-regulation of host cell responses, including the inhibition of protein kinase C and nitric oxide (NO) production [101,102]. However, genetic studies with the *ADS1* mutants (null for most GPI-anchored molecules) in *L. major* indicate lyso-alkyl- or alkylacyl-GPI anchors are not solely responsible for the inhibition of macrophage activation or amastigote survival in mammalian hosts [22].

In Trypanosomatids, EtN is a component of the core structure in the GPI-anchored proteins consisting of EtN-phosphate-6-mannose- α 1,2-mannose- α 1,6-mannose- α 1,4-glucosamine- α 1,6-myoinositol-1-phospholipid [19]. The EtN moiety was transferred from PE onto a GPI precursor lipid, followed by the attachment of the precursor to a polypeptide in the endoplasmic reticulum [62,103]. In *T. brucei*, inhibition of PE and PI metabolism has negative impact on the synthesis of GPI-anchored virulence factors [30,104,105].

Finally, IPC is the end product of SL biosynthesis in *Leishmania* and to date, there is no evidence showing that IPC or ceramide can serve as anchors for glycoconjugates. *Trypanosoma cruzi* and *Trichomonas vaginalis*, however, do use IPC as a carrier for LPG-like surface molecules [106,107]. Synthesis of these IPC-anchored glycoconjugates is not well characterized, although current data suggest the ceramide moiety in *T. cruzi* GPI is likely added through a remodeling process [108], whereas *T. vaginalis* ceramide-GPIs is probably made *de novo* since key enzymes in the standard diacyl GPI pathway are missing in the genome.

4.2 Metacyclogenesis

In *L. major*, the exit of replicating promastigotes from the cell cycle into stationary growth phase is accompanied by differentiation to the infective metacyclic form (a process known as metacyclogenesis). Notably, the cellular level of PLE rises significantly (2–3 fold) as promastigotes progress into stationary phase, suggesting this lipid may play critical role(s) as

parasites undergo differentiation. PLE possesses different physical properties compared with diacyl PE [109], including an ability to promote rapid membrane fusions [110], and a propensity to form and/or interact with organized membrane microdomains. These unique membrane properties could play an important role during metacyclogenesis, a time marked by greatly increased membrane trafficking and remodeling. The onset of stationary phase also coincides with an increase in macroautophagy activity, where a key step involves the conjugation of PE to ATG8 proteins [111]; macroautophagy mutants in *L. major* show interesting defects in metacyclogenesis and infectivity, suggesting PE may be involved in promastigote differentiation [112].

4.3 Organization of lipid rafts

As in other organisms, *Leishmania* SLs (mostly IPC) and sterols are enriched in organized membrane microdomains termed lipid rafts, whereas ester PLs such as diacyl PC are usually found in a more fluid and disordered state due to their unsaturated fatty acid chains and low melting temperatures [113,114]. While less extensively studied, ether PLs including PLE are preferentially associated with lipid rafts and may promote the formation of raft-like domains [115–117]. Previous studies have shown that the organization of *Leishmania* membrane differs greatly between procyclics and metacyclics. For example, LPG is not associated with lipid rafts in procyclics but is enriched in detergent resistant membrane (DRM) fraction in metacyclics [113]. Interestingly, it has been suggested that the macroscopically visible alterations in the metacyclic surface arise from the formation of lipid rafts [118], although this must be reconciled with studies in other organisms suggesting that lipid rafts may be considerably smaller [119].

The generation of viable and largely healthy *adsI*[−] and *spt2*[−] parasites provided an excellent opportunity to address an important question: can lipid rafts be formed in the absence of ether PLs or SLs in the context of a living organism? Previous studies employing a limited number of markers suggested that *Leishmania* could maintain lipid rafts despite the complete loss of either of these lipid classes [22,27]. Most studies relied upon the relatively non-stringent method of DRM extraction, as raft proteins are usually found in insoluble fractions after mild treatment with a non-ionic detergent (e. g. Triton X100). Similar results were obtained with the *spt2*[−] mutant with detergent free approaches [27].

Recently we extended these findings to include other ‘raft markers’. As shown in Fig. 3A, two GPI-anchored proteins, GP63 and GP46, were strongly associated with DRM at 4 °C but not 37 °C in both WT and SL-null parasites (*spt2*[−]), whereas a non-raft membrane protein, glucose transporter [120], was clearly not associated with DRM. In addition, both WT and *spt2*[−] DRMs were rich in ergosterol (the dominant sterol species in *Leishmania*) but not ester PLs (Fig. 3B), suggesting the loss of SLs does not affect the composition of raft lipids in *Leishmania*. To examine whether engineered reporter proteins are targeted to rafts in the absence of SLs, transgenic parasites expressing either a GFP-tagged *L. major* HASPB fusion protein [121] or a HA-tagged *T. cruzi* Calcium-binding protein FCBP [122] were subjected to detergent extraction. Results showed both were associated with DRM as expected, and targeted to the flagellum, which are known to be enriched in lipid rafts (Fig. 3D–E) [123]. Significantly, these behaviors were maintained in the SL-null *spt2*[−] cells (Figs. 3C–E), again pointing to a remarkable ability to maintain lipid raft-like targeting in the absence of SLs.

Furthermore, preliminary results from unbiased proteomic studies of DRMs from WT vs. *spt2*[−] parasites showed a remarkable similarity (>98% of protein spots showed similar abundance between WT and *spt2*[−], Fig. 4). Together, these data suggest that SLs play a relatively minor role in the formation of lipid rafts and the targeting of raft proteins.

Similar to *spt2*⁻, the absence of ether PLs in *ads1*⁻ does not affect the formation of lipid rafts as defined by buoyant DRM criterion [22,74]. Together, these data suggest the rather unique redundancy in lipid composition (the combination of ether PLs, SLs, and ergosterol) may render *Leishmania* parasites more resistant to lipid perturbations that are generally fatal to other organisms.

5. Further characterization of PL and SL metabolism

Leishmania PCs contain relatively long and unsaturated fatty acid species, including C22:5 and C22:6 [22]. Long chain fatty acids are also found in the lyso-PI anchor of LPG [100, 124]. The *L. major* genome contains 13 candidate genes for fatty acid elongases and 12 for fatty acid desaturases. Some elongases are potentially involved in the synthesis of bulk fatty acids using butyl-CoA as a precursor, a process first described in *T. brucei* [125], while others may only elongate palmitoyl-CoA or stearoyl-CoA. Further genetic, biochemical, and lipidomic studies are needed to address the substrate specificity of these enzymes and the roles of long, unsaturated fatty acids in *Leishmania*.

In *Trypanosoma brucei*, PE is a direct precursor of the EtN-phosphoglycerol moiety found on the side chain of Glu362 of eukaryotic elongation factor 1A (eEF1A), as RNAi knock down of enzymes in the Kennedy pathways led to inhibition of eEF1A modification [126]. The function of EtN-phosphoglycerol modification is not clear, although similar eEF1A modifications have been identified in mammalian cells [127,128] and plants [129].

While several studies have focused on SL degradation [23,88], very little is known about the enzymes involved in PL degradation. The activity of phospholipase D has been detected in *L. donovani* and this activity increases in response to acute osmotic stress [130]. *T. brucei* contains a membrane bound glycosyl-phosphatidylinositol phospholipase C (GPI-PLC) which can cleave dimyristoylglycerol from the variant surface glycoprotein and protein-GPI precursors [131,132]. Interestingly, *L. major* cells expressing GPI-PLC have a GPI-deficient phenotype [133]. PL degradation may possess additional functions. For example, living inside the host phagolysosomes, which is rich in lipids and amino acids but poor in sugars [134,135], *Leishmania* amastigotes may depend on salvaged host lipids for carbon sources. Twenty-one putative lipase genes have been identified in the *L. major* gene DB and some could be responsible for the production fatty acids and other nutrients from scavenged host lipids.

6. Comparison of PL and SL metabolism between *L. major* and *T. brucei*

6.1 De novo SL biosynthesis is essential for *T. brucei* but not *L. major*

Unlike *L. major*, which remained fully viable as replicating procyclics in the absence of *de novo* SL synthesis [27,44], inhibition of SPT by myriocin led to aberrant cytokinesis, characterized by delayed kinetoplast segregation and emergence of cells with abnormal DNA content, followed by cell death [136,137]. Similarly, RNAi silencing of SL synthases (which are responsible for the synthesis of high order SLs in *T. brucei* including IPC and sphingomyelin) in bloodstream *T. brucei* led to rapid growth arrest and eventual cell death [29]. Thus unlike *Leishmania* where IPC synthesis is not essential in culture and possibly neither for mammalian infectivity, inhibition of SL synthesis may provide valid drug targets in *T. brucei*.

6.2 Developmental stage-dependent PL and SL composition

As described earlier for *Leishmania* (section 4.2), developmental stage-dependent modulation of lipid metabolism has also been observed in *T. brucei*. For example, procyclic trypanosomes contain IPC and sphingomyelin, while surprisingly bloodstream-stage parasites contain sphingomyelin and ethanolamine phosphorylceramide, but no detectable IPC [29]. As an

extracellular parasite, the lack of IPC may help bloodstream trypanosomes avoid triggering host immune response.

Concluding remarks

Lipids are essential components of life and the recent advance of genome sequencing, molecular biology, and lipidomic tools have greatly facilitated the study on lipid metabolism in *Leishmania*. In addition to being membrane components, PLs and SLs play multiple roles in *Leishmania* cell biology: PI serves as anchors for important surface virulence factors; EtN synthesis through SL degradation is essential for the survival and differentiation of stationary phase promastigotes; uptake and turnover of SLs/PLs may be an essential route of nutrient uptake. Compared to other organisms, *Leishmania* membrane is rather unique in that the loss of ether PLs or SLs does not have significant effect on the formation and functions of lipid rafts. Additional roles will be elucidated in the near future and we expect the new knowledge to have important implications in the development of novel drugs.

Abbreviations

PL	phospholipid
SL	sphingolipid
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PI	phosphatidylinositol
PS	phosphatidylserine
IPC	inositol phosphorylceramide
PLE	plasmeneylethanolamine

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Figure 1.

PLs and SLs in *L. major*. **(A)** Lipid composition in *L. major* promastigotes based on the headgroups (left) or anchors (right) of PLs. Structures of each class of lipids were determined by ESI/MS/MS and abundance was estimated as previously described [22,23,26]. Other PLs include phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidic acid. **(B)–(F)** Molecular species of PE and SLs in *L. major*. **(B)** A PLE: 1-*O*-octadec-1'-enyl 2-octadecenoyl *sn*-glycero-3-phosphoethanolamine (*p*18:0/18:1-PE). **(C)** A diacyl PE: 1,2-distearoyl *sn*-glycero-3-phosphoethanolamine (18:0/18:0-PE). **(D)** A lyso-PE: 1-*O*-octadec-1'-enyl 2-lyso *sn*-glycero-3-phosphoethanolamine (*p*18:0/lyso-PE). **(E)** A ceramide: *N*-stearoylhexadecosphing-4-enine (d16:1/18:0-Cer). **(F)** An IPC: phosphoryl inositol *N*-stearoylhexadecosphing-4-enine (d16:1/18:0-IPC).

Figure 2.

Predicted metabolism of SLs and PLs in *Leishmania*. DAG: diacylglycerol; G-3-P: glycerol-3-phosphate; DHAP: dihydroxyacetonephosphate. Enzymes of which candidate genes have been identified in the *L. major* geneDB are indicated. Gray dotted arrows represent pathways where the putative enzymes have yet to be identified. Experimentally characterized enzymes/genes (circled) include DAT (dihydroxyacetone phosphate acyltransferase, system ID: LmjF34.1090), GAT (glycerol-3-phosphate acyltransferase, LmjF03.0080), ADS1 (1-alkyl dihydroxyacetone phosphate synthase 1, LmjF30.0120), G3PD (glycerol-3-phosphate dehydrogenase, LmjF10.0510), SPT (serine palmitoyltransferase, LmjF34.3740 and LmjF35.0320), SPL (sphingosine-1-phosphate lyase, LmjF30.2350), IPCS (IPC synthase, LmjF35.4990), and ISCL (inositol sphingolipidphospholipase C-like, LmjF08.0200). Other enzymes (that have yet to be characterized although their putative genes have been identified from *L. major* genome) include CS (ceramide synthase); CD (ceramidase); SK (sphingosine kinase); E/CK (ethanolamine/choline kinase); EPCT (ethanolamine-phosphate cytidyltransferase); CPCT (cholinephosphate cytidyltransferase); EPT (ethanolamine-specific phosphotransferase); C/EPT (choline/ethanolamine phosphotransferase); BE: (base exchange enzyme or PS synthase); and PSD (PS decarboxylase).

Figure 3.

SL-null *L. major* generates normal DRM rafts. **(A)** TX100 insoluble (I) and soluble (S) fractions from log phase parasites (WT and $spt2^{-}$) were analyzed by western-blot to assess the distribution of gp63, gp46, and glucose transporter (GT). **(B)** Log phase WT and $spt2^{-}$ parasites were labeled with $[1,2]^{14}\text{C}$ -acetate and extracted with 1% TX100 at 4 °C or 37 °C. Detergent insoluble materials were separated from soluble materials by centrifugation. Lipids from both insoluble and soluble fractions were extracted and analyzed by thin layer chromatography. Ergo: ergosterol; PLs: phospholipids. **(C)** WT and $spt2^{-}$ parasites with episomally expressed HASPB-GFP or FCaBP-HA were subjected to detergent extraction and western-blot analysis as described in **(A)**, using anti-GFP and anti-HA antibodies. In addition, these parasites were subjected to fluorescence microscopy, as shown in **D** (GFP epifluorescence) and **E** (primary antibody: anti-HA mAb; secondary antibody: FITC-labeled goat-antimouse IgG). *Leishmania* culture, DRM isolation, and western-blot were performed as previously described [27]. To isolate metacyclics, $spt2^{-}$ mutants were grown to stationary phase in the presence of 500 μM of EtN as previously described [23].

WT log DRM

 $spt2^-$ log DRM **Figure 4.**

2D gel analysis of DRM fraction in *L. major*. DRM fractions were prepared from log phase WT and $spt2^-$ parasites as described [27] and subjected to 2D gel electrophoresis. Among all detectable spots, only ~1.2% showed greater than 2.5-fold variation between WT and $spt2^-$.